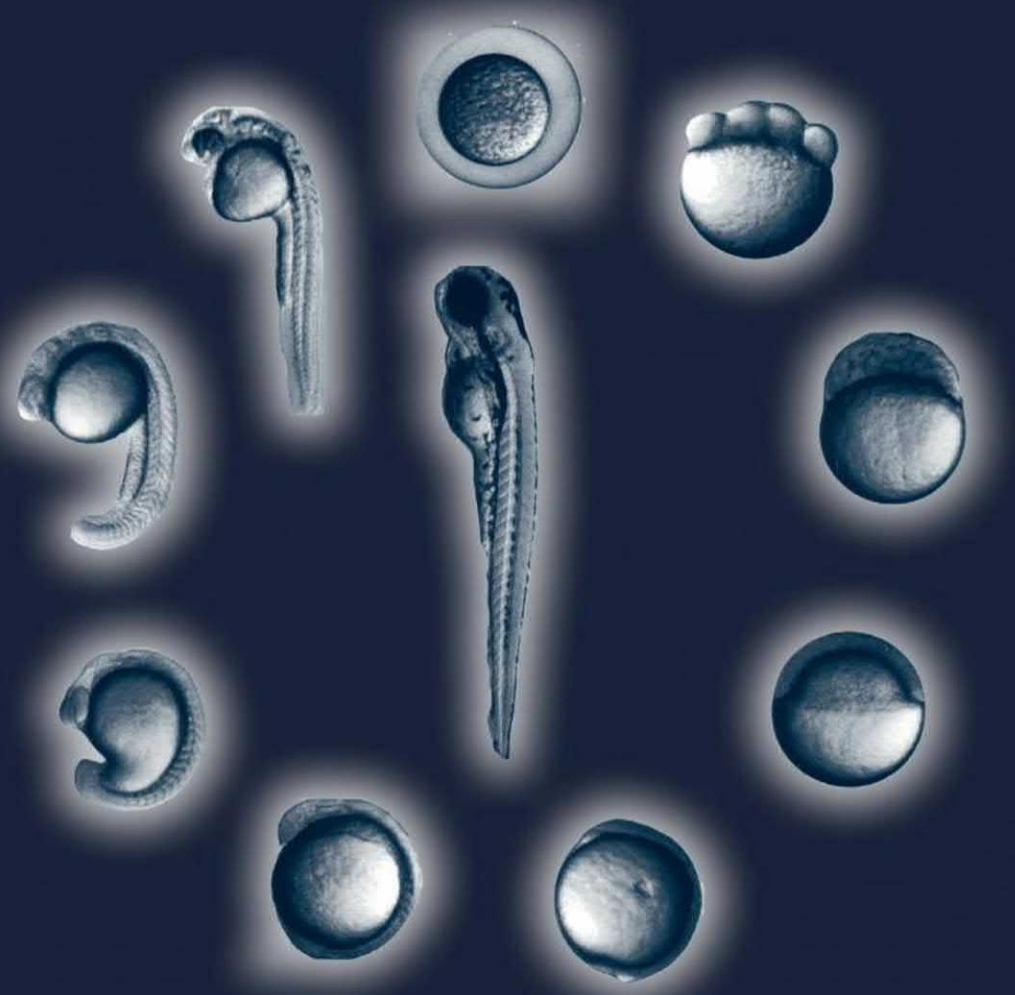
The Zebrafish in Biomedical Research

Biology, Husbandry, Diseases, and Research Applications



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American College of Laboratory Animal Medicine Series

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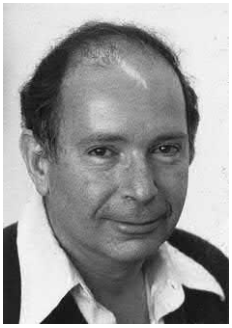
Dedication



We dedicate this book to our late colleague, George Streisinger, PhD, without whom this volume would be unnecessary. Streisinger introduced the world to zebrafish as a genetic model to study development of the vertebrate nervous system. Because of his untimely death in 1984, Streisinger never knew that he founded an entirely new field of research using zebrafish as a genetic model to investigate every aspect of vertebrate development as well as what goes awry in genetic, infectious, and environmental diseases. Streisinger was born in Budapest, Hungary, in 1927 and came to the United States with his family in the late 1930s to escape Nazi persecution. He had a penchant for biology and was the sole author of several publications in the 1940s, while he was still in high school. Streisinger received a Bachelor of Science degree from Cornell University in 1950, received his doctorate in genetics from the University of Illinois in 1953, and conducted postdoctoral research at the California Institute of Technology until 1956, when he joined Cold Spring Harbor. In 1960, Streisinger took a faculty position in the University of Oregon's nascent Institute of Molecular Biology, where he spent the remainder of his scientific career. As a postdoctoral fellow, Streisinger was a pioneering molecular biologist who made major contributions to the genetics of T-even bacteriophages. At the University of Oregon, he turned his attention to more complex systems, using the same types of mutational analysis to understand vertebrate development. Streisinger was elected to the United States National Academy of Sciences in 1975, during the early stages of his zebrafish research. His publication in 1981 of the methodology for producing clonal lines of homozygous zebrafish broke new ground and enabled scientists around the world to envision incorporating zebrafish into their research programs. Streisinger was a master at balancing his scientific and other interests. For example, he was an extraordinary culinarian, a highly sought-after goat show judge, and a serious antiwar activist. Despite not living to see it come to fruition, Streisinger set the tone for a field in which researchers are truly collegial, sharing resources and reagents, and working across local and international boundaries to move science forward. We imagine that he would be delighted and also completely amazed at how this field he started has progressed. With this volume, we salute George Streisinger and his legacy.

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Preface

The American College of Laboratory Animal Medicine (ACLAM) was established in 1957 to support education, training, and research in laboratory animal medicine and to recognize specialists in the veterinary field by providing a route for certification. A mission of the ACLAM is to provide scholarly information to ensure consistent standards for laboratory animal care and improve reproducibility of scientific research. To this end, ACLAM has sponsored a series of reference books on the biology, husbandry, diseases, and use of the most frequently studied animal models of human and animal disease. The first one on the laboratory rabbit was published in 1974. Now with over 15 volumes in the set, they offer state-of-the-art reference materials for biomedical researchers. *The Zebrafish in Biomedical Research* adds to this series and is the first comprehensive publication on this important animal research model.

The contents of this book include 51 chapters divided into five main sections: Introduction, Biology, Husbandry, Diseases, and Scientific Research. The Introduction includes a comprehensive review of the history of the zebrafish in biomedical research, followed by chapters reviewing taxonomy, genetics, natural geographical distribution, and behavior of wild populations. The Biology section includes chapters on the development, anatomy, and physiology of organ systems. The Husbandry section provides extensive coverage of facility design, housing systems and their maintenance, water quality, breeding, colony management, nutrition, and other support activities. There is also a brief review of the regulations, policies, and guidelines pertaining to the use of zebrafish in research in the United States. The section on Diseases provides up-to-date information on the idiopathic, parasitic, bacterial, fungal, and viral diseases of zebrafish. Biomedical researchers and laboratory animal professionals will find the chapter on special procedures for disease diagnosis and treatment very helpful. Scientific Research covers the major uses of the zebrafish in biomedical research as models for developmental, behavioral, and genetic diseases. In addition, there is a chapter reviewing the novel uses of zebrafish in understanding the host−microbe−pathogen interaction. The final two chapters review the zebrafish as a platform for technology development and toxicology and drug screening.

All the contributors are experts in their specialties and the application of the zebrafish model to their research. As with all volumes in the ACLAM series, the authors and editors have donated their time and effort to this publication. Permission fees required for the use of figures, tables, etc., were paid by the University of Alabama at Birmingham. All these efforts were made in the name of fostering continuing education in laboratory animal science and promoting responsible use of animals in biomedical research. A special thanks is extended to reviewers of each chapter and the authors' and editors' home institutions for allowing them to work so diligently on this book. The authors and editors appreciate the helpful support from the Elsevier staff.

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SECTION I

Introduction

CHAPTER

1

History of Zebrafish Research

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Although PubMed (pubmed.gov) lists papers studying zebrafish embryos as early as the 1950s (Fig. 1.1), zebrafish research did not hit its stride until nearly a decade after publication of the pioneering work by George Streisinger in 1981 (Streisinger, Walker, Dower, Knauber, & Singer, 1981). Why has this small tropical fish become an important biomedical model and what has made its popularity soar beginning from the mid 1990s?

Setting the Stage

Scientists have been fascinated with learning how humans and other animals develop since well before the common era. Hippocrates, the renown Greek physician who lived over 2500 years ago and is considered the father of modern medicine, or at least his followers of the Hippocratic school are so considered, provided an overview of human embryology in several volumes including the "Hippocratic Treatises 'On Generation' and 'On the Nature of the Child,'" and noted the similarity to chick embryology (Lonie, 1981; Needham, 1963). Aristotle, the celebrated Greek scientist and philosopher who lived about 200 years later also carried out extensive observations of animal development based on dissections at various developmental stages, which he published in his books "On the Generation of Animals," "The History of Animals," "On Respiration," and "On the Motion of Animals" (Gilbert, Barresi, 2016; Needham, 1963). Fast forward 1700 years to when another historical luminary, Leonardo da Vinci, one of the foremost artists and inventors during the Italian Renaissance, contributed significantly to our understanding of embryonic development through drawings and quantitative measurements of dissected avian and mammalian embryos, including a human fetus (Needham, 1963).

Why the enduring fascination with embryology? Part of it undoubtedly comes from the difficulty of understanding developmental processes that occur deep inside a human fetus's mother, and thus remain mysterious. These invisible processes could be understood if the development of other animals were discovered to mirror that of humans. Finding that this is the case would allow animals to serve as proxies, models that we can investigate in ways in which we cannot study ourselves, and would enable us to discover how human development normally unfolds and how abnormal development might lead to human birth defects. The hypothesis that animal development could serve as a model for human development was established early, for example by comparisons between human and animal embryos made by the Hippocratic school and by Aristotle (Lonie, 1981; Needham, 1963), and reinforced more recently, for example in the late 1800s by Ernst Haeckel whose drawings of developing animals revealed the similarities of their embryonic body plans and how these morphed into related, but distinct, species-specific, adult forms during development (Fig. 1.2).

The idea that studying a variety of animals could reveal the principles underlying development suggests that it is reasonable to choose a model to study based on its experimental tractability for addressing a particular research question. In this regard, teleost fish have been particularly useful for many types of observations (Oppenheimer, 1936; Wourms, 1997; Wourms, Whitt, 1981). For embryology, oviparous teleosts have been particularly advantageous because fertilization, and indeed their entire developmental process, unfolds in the water column, and thus, they do not have to be extracted from the mother for observation or experimentation (Oppenheimer, 1936). In addition, some teleost embryos show "striking lucidity," providing an exceptional platform for microscopic observations of morphogenetic and cellular processes during the unfolding of living embryos (Trinkaus, 1990).

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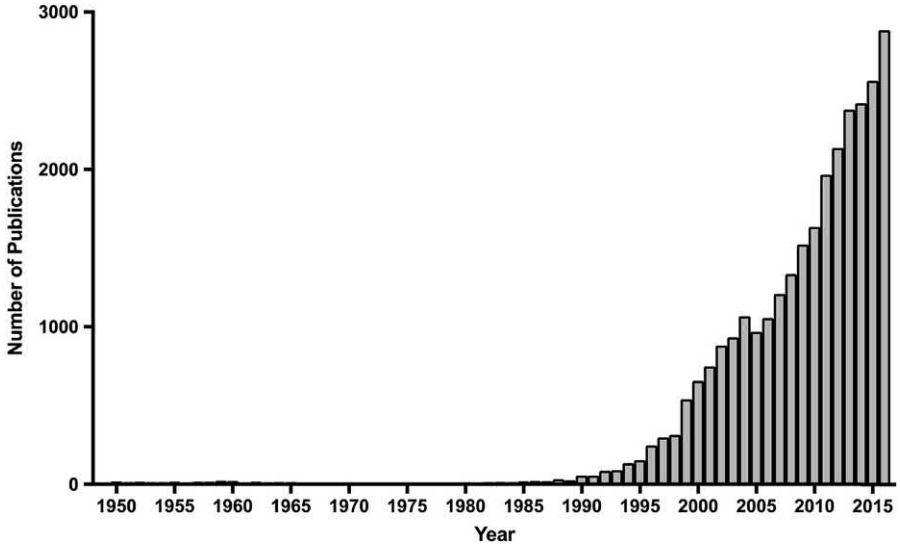

FIGURE 1.1 Since the 1990s there has been significant acceleration in publication of papers concerning zebrafish. *Data from PubMed (https://www.ncbi.nlm.nih.gov/pubmed/?term=zebrafish).*

Interestingly, Aristotle contributed not only to our understanding of embryology of terrestrial vertebrates, but he also appears to have been the first contributor to our recorded knowledge of teleost development, an area to which there was little in the way of additional recorded information until the 1700s (Oppenheimer, 1936; Wourms, 1997). Although he was limited to observations that could be made without magnification, Aristotle was still able to describe aspects of teleost egg development and compare them with the development of avian eggs (Oppenheimer, 1936). From the beginning of the 18th century, a variety of teleost species have been studied to uncover aspects of embryonic development (Wourms, 1997); readers are referred to the excellent reviews cited above to learn more about the breadth of contributions made by these species to understanding embryogenesis.

Notable among the fish that have contributed to understanding vertebrate embryonic development is the killifish, *Fundulus heteroclitus*, commonly known as the mummichog or simply Fundulus (Atz, 1986). This fish played a prominent role in the early days of the now world-renowned Embryology Course at the Marine Biological Laboratories in Wood Hole, Massachusetts (Atz, 1986). In the late 1800s and early 1900s, a number of scientists developed exquisitely detailed descriptions of Fundulus embryology. Their drawings provided a foundation for experimental studies exploring the potential of different parts of the developing embryo to respond to a variety of perturbations, for example, extirpation and grafting. A series of biology luminaries published studies of Fundulus embryos during this time, including Thomas Hunt Morgan, the father of genetics of the fruit fly, *Drosophila melanogaster*, Jacques Loeb, who helped pioneer experimental biology (https://embryo.asu.edu/pages/jacques-loeb-1859-1924) as well as comparative brain physiology and psychology (http://www.thecrimson.com/article/1965/2/11/jacques-loeb-bridging-biology-and-metaphysics/), Philip Armstrong and Julie Swope Child at the Woods Hole Marine Biological Laboratories, Jane Oppenheimer, a pioneering professor at Bryn Mawr, John Paul Trinkaus at Yale, and William Ballard at Dartmouth (Loeb, 1916; Morgan, 1895; Oppenheimer, 1936, 1979; Trinkaus, 1990) (https://embryo.asu.edu/featured/1549). These studies provided important insights into the processes occurring during vertebrate embryogenesis and laid the groundwork for research carried out today.

During this same time frame, the rediscovery of Mendel's genetic experiments ushered in an era of concerted effort to learn the genetic basis of inheritance (Gilbert, 1978). Initially, this work included understanding the relationship between genes and development. However, these disciplines—embryology and genetics—became largely separate in the early 1900s and remained that way for many decades (Gilbert, 1991, 1998). The study of embryology and genetics began to reunite in the 1970s when a number of researchers started to use genetic approaches in fruit flies to dissect the basis of development. For example, as a step toward his dream of building a bridge between developmental genetics and molecular biology, Ernst Hadorn, developmental genetics pioneer of the University of Zurich, organized a conference on this topic in 1972 (Nothinger, 2002). Another developmental genetics pioneer, Edward Lewis at the California Institute of Technology, investigated how genes controlled the development of specialized organs from individual body segments, work for which he

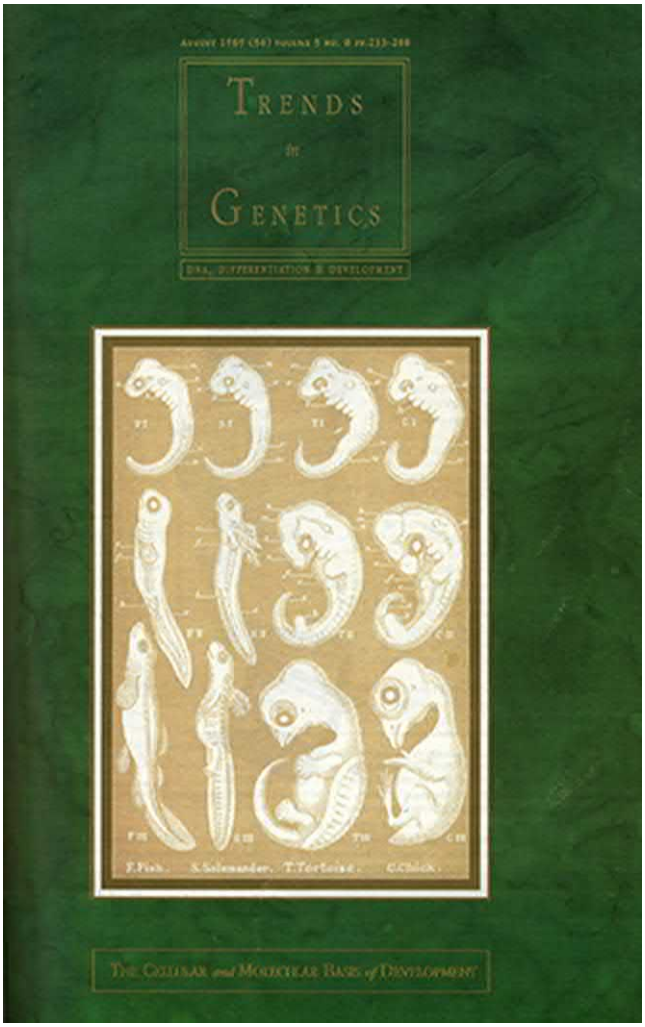

FIGURE 1.2 This cover of the August 1989 edition of *Trends in Genetics* shows the similarity among vertebrate animals in a modified drawing from Ernst Haeckel's 1879 book "The Evolution of Man." The top row shows fish, salamander, tortoise, and chicken embryos at an early stage of embryonic development when they are very similar. The middle and bottom rows show the same animals at increasingly later developmental stages; the last stage shows species-specific characteristics. It is important to note that although the picture shown here has been widely reproduced, it remains controversial because not all features of embryos illustrated in the top row are conserved, as described in more detail by Richardson et al. (1997). *Cover image of* Trends in Genetics *volume 5 issue 56 from August 1989 used with permission from Elsevier.*

was awarded a Nobel Prize in Physiology or Medicine in 1995 (https://www.nobelprize.org/nobel_prizes/medicine/laureates/1995/press.html). Christiane Nusslein-Volhard and Eric Wieschaus conducted a genetic screen in fruit flies to learn the mechanisms underlying body patterning (Nusslein-Volhard & Wieschaus, 1980) and shared the 1995 Nobel Prize with Lewis. Subsequent experiments in the late 1980s and early 1990s uncovered the molecular nature of the mutations isolated in the Nusslein-Volhard/Wieschaus screen (McGinnis & Krumlauf, 1992) and revealed that the basis for the similarities in embryogenesis among disparate animals is that development is orchestrated by orthologs of the same genes in animals as diverse as fruit flies, chickens, mice, and humans (Beddington & Smith, 1993; Carver & Stubbs, 1997; Davidson, 1994; Gellon & McGinnis, 1998; Manak & Scott, 1994). This realization cemented the idea that animals could be investigated not only to unveil the secrets of their development but also as models to reveal the secrets of human development.

Establishing the Zebrafish Model

It is against this backdrop that George Streisinger, a professor in the nascent Institute of Molecular Biology at the University of Oregon, chose zebrafish, *Danio rerio*, a small, tropical, fresh-water fish, as a model in which to explore the genetic basis of vertebrate neural development. Streisinger was a pioneering molecular biologist who contributed significantly to the dawn of the modern molecular era through his historic work on bacteriophage in the 1950s. Among his major contributions were an understanding of the DNA triplet code, including deciphering the first in vivo codon assignments, as well as uncovering the structure of the bacteriophage genome (Grunwald & Eisen, 2002; Stahl, 1995). One of Streisinger's close University of Oregon colleagues, and an avid supporter of his zebrafish endeavors, was Franklin Stahl, another molecular biology pioneer who discovered that DNA replication is semiconservative (Streisinger, 2004). By the mid-1960s, many of the initial questions of molecular biology were nearing resolution and attention was turning toward using genetic approaches to understand the mechanisms underlying more complex systems, such as the "almost mystical" questions of nervous system function and the basis of behavior and cognition (Grunwald & Eisen, 2002). In parallel with Streisinger's choice to use zebrafish to dissect nervous system function and development, two other molecular biology pioneers established other genetic models to investigate development (Grunwald & Eisen, 2002). Sidney Brenner, codiscoverer of messenger RNA, who also showed that the amino acid sequence of a protein is determined by the RNA nucleotide sequence from which it is translated, developed the roundworm, *Caenorhabditis elegans* as a new genetic model, work for which he was awarded a Nobel Prize in Physiology or Medicine in 2002 (http://www.nobelprize.org/nobel_prizes/medicine/laureates/2002/). Seymour Benzer, who used bacteriophage to reveal the structure of genes and how they are aligned within the genome, began studying nervous system function and behavior using fruit flies.

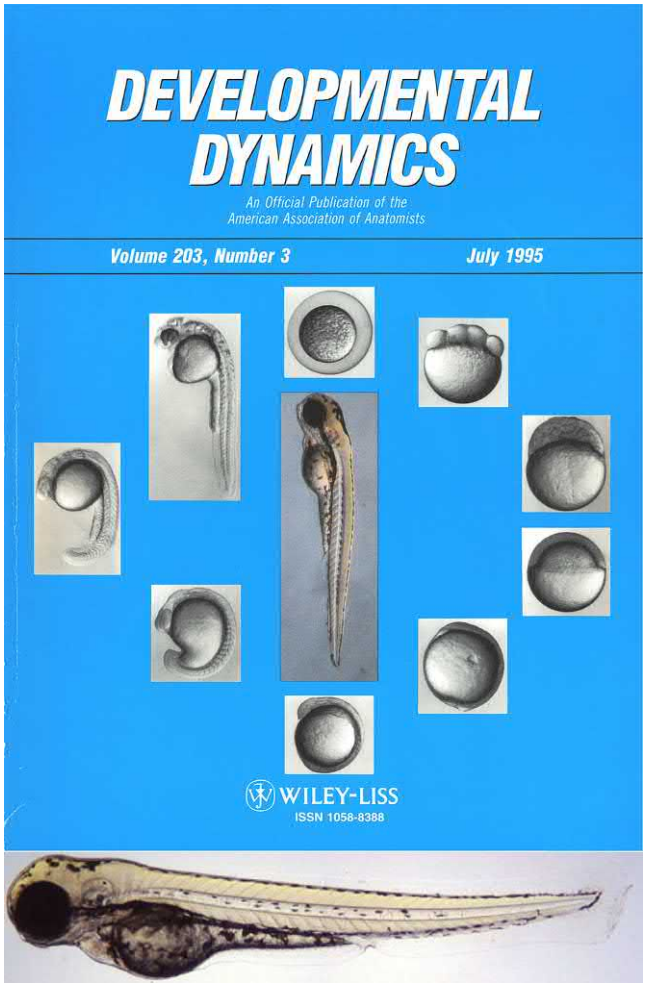

FIGURE 1.3 This cover of the July 1995 issue of *Developmental Dynamics* shows a portion of the zebrafish staging series, a crucial advance for standardizing conditions between different experiments and laboratories. A fertilized egg is shown at the top, and development progresses clockwise. All of the fish were imaged live (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). The fish in the middle is 3 days postfertilization (3 dpf) (an enlarged view of a fish of this stage is also shown separately, below the cover image). By three dpf, zebrafish have hatched and are referred to as larvae, whereas, at earlier stages, before hatching, they are called embryos. The head of the three dpf larva in the cover image is at the top; the head of the larva below the cover image is to the left. The dark region of the head is the eye. The heart appears red because it contains red blood cells. The large structure under the heart is the yolk, which will nourish the larva until it is able to eat, starting about 1 day later than when this photograph was taken. The embryo at the top of the cover image was just fertilized and is shown within its chorion or eggshell. The chorions were removed prior to taking the other photographs. The next embryo, going clockwise, is at the eight-cell stage. All 8 cells sit on top of the large yolk cell, although only four of them are visible; four of them are hidden behind the visible cells. The next embryo is at the 256-cell stage, followed by an embryo at the 50% epiboly stage. During epiboly, the cells move from sitting on top of the yolk to completely surrounding it. The next embryo, at five o'clock in the cover image, is at the bud stage; "bud" refers to the tailbud, the beginning of the tail, which is pointed toward the bottom of the photograph. The embryo at six o'clock in this cover image is at the four-somite stage; somites are the precursors of the segmented body muscles that fish use for swimming. The embryo at seven o'clock is at the fifteen-somite stage, and the embryo at nine o'clock is at the twenty five-somite stage; this embryo is nearly 22 h postfertilization. The embryo at 11 o'clock is about 28 h postfertilization. *Cover image of* Developmental Dynamics *volume 203 number three from July 1995 used with permission from Wiley.*

Why did Streisinger choose zebrafish? Research on Fundulus had already provided many insights into vertebrate development, so Fundulus could have been an obvious choice, despite lacking any previous history of genetic research. There were, however, several other fish species with considerable histories of genetic research, including medaka (Shima & Mitani, 2004), goldfish (Fu, 2016) and Xiphophorus (Kazianis, 2006). Streisinger initially brought a number of tropical fish species, including medaka, into his laboratory; however, no written records describing the basis of his choice have been uncovered (Grunwald & Eisen, 2002). Although it is only speculation, perhaps the difficulty of removing Fundulus embryos from the protective eggshell, or chorion (Atz, 1986; Trinkaus, 1990), or the inclusion within Fundulus and medaka embryos of oil droplets that obscure visibility (Wourms, 1997), contributed to Streisinger's ultimate choice of zebrafish. During the late 1950s and early 1960s, several other laboratories also began investigating zebrafish embryology (Hisaoka, 1958), including muscle and nervous system development (van Raamsdonk, Mos, Smit-Onel, van de Laarse, & Fehres, 1983; van Raamsdonk, Tekronnie, Pool, & van de Laarse, 1980; Weis, 1968a, 1968b), as well as using zebrafish in the aquaculture trade to study toxicology (Laale, 1977). However, none of these studies incorporated the type of genetic analysis that was central to Streisinger's approach to obtaining a mechanistic understanding, and whether any of this work contributed to Streisinger's choice of zebrafish is unknown.

Whatever the reasons for Streisinger's choice of zebrafish, it is now clear that zebrafish is an excellent research model, based on several characteristics of its development. First, in contrast to many fish, zebrafish are not seasonal breeders; thus, embryos can be obtained for study throughout the year. Second, like most fish, fertilization, and all subsequent developmental processes occur within the water column, not inside of the mother; thus, zebrafish are amenable to both observation and manipulation throughout the developmental period. Third, a single female zebrafish can produce hundreds of eggs in a single spawning, making it possible to have statistically significant numbers of closely related embryos for even a single experiment. Fourth, zebrafish embryos and early larvae are nearly transparent, thus using appropriate microscopic methods, it is possible to image essentially every cell during development (Fig. 1.3). Fifth, zebrafish embryos develop rapidly, hatching as predatory larvae in 3 days

(Fig. 1.3), and thus development of individual cells or structures deep inside a zebrafish embryo can be observed in real time, or by time-lapse videography, as they occur.

Streisinger's focus on using genetics to uncover developmental processes necessitated establishing tools appropriate for this purpose. Vertebrate genetics is cumbersome because, after mutagenesis, heterozygous carriers need to be crossed to wild types to establish a stock that will contain both wild types and heterozygotes, and then individuals within that stock need to be crossed to one another to identify those that carry mutations of interest. This identification is accomplished by screening the offspring of the mated heterozygotes, only one-quarter of which will show a phenotype when a mutation is recessive. Streisinger reasoned that he could facilitate the production of homozygous offspring by circumventing the need to breed heterozygous male and female partners (Grunwald & Eisen, 2002). He achieved this goal by adapting methods to activate eggs without fertilization, as well as by methods to produce gynogenotes, homozygous diploid embryos whose entire genetic contribution derives from the mother. These gynogenotes were useful not only for genetic screens, but Cyrus Levinthal at Columbia University obtained some gynogenotes from Streisinger (Charline Walker, personal communication) to investigate axon trajectories of isogenic neurons (http://www.nasonline.org/publications/biographical-memoirs/memoir-pdfs/levinthal-cyrus.pdf). Streisinger's seminal work describing the cloning of zebrafish was published in 1981 in *Nature* (Streisinger et al., 1981) and heralded on the cover (Fig. 1.4), just 7 months after publication in the same journal of the seminal work on fruit flies (Nusslein-Volhard & Wieschaus, 1980) for which Nusslein-Volhard and Wieschaus were awarded the 1995 Nobel Prize in Physiology or Medicine that they shared with Edward Lewis. Streisinger's article received national attention, including an editorial cartoon published in the Chicago Tribune (Fig. 1.5).

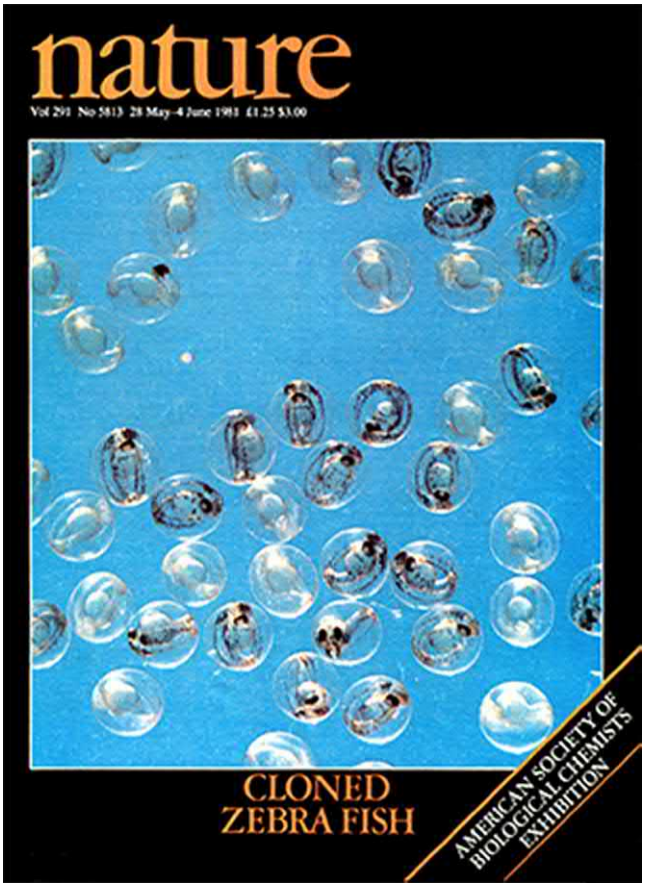

FIGURE 1.4 This issue of *Nature* from May–June 1981 published the groundbreaking paper from George Streisinger showing that zebrafish are amenable to genetic analysis (Grunwald & Eisen, 2002; Streisinger et al., 1981). This cover illustrates zebrafish embryos, enclosed within their chorions. Some of the embryos in this picture have normal, black pigment cells in their skin and eyes, but some lack black pigment cells in their skin and eyes, and thus, appear golden in color. The difference in these embryos is the result of a mutation in a single gene, which is called *golden*, after the appearance of the mutant embryos. The discoverers of the molecular nature of the zebrafish *golden* gene showed that the human ortholog is responsible for 25%–40% of the difference in skin color between Europeans and West Africans (Lamason et al., 2005). *Cover image of* Nature *volume 291 issue 5813 from May–June 1981 used with permission from Springer Nature.*

Synthesizing Genetics and Embryology

Streisinger's ability to develop zebrafish as a model was fostered by the atmosphere of the University of Oregon Institute of Molecular Biology. Founded in 1959, the Institute nurtured a handful of the brightest young molecular biologists, in part by pooling resources for equipment, facilities, and administrative support to help

FIGURE 1.5 This cartoon was published in the Chicago Tribune as a response to George Streisinger's seminal paper describing the cloning of zebrafish (Streisinger et al., 1981). *Wayne Stakyskal cartoon from 1981 used with permission from the Chicago Tribune.*

underwrite long-term projects, including Streisinger's zebrafish research program, which took over 10 years to come to fruition (Grunwald & Eisen, 2002; Streisinger, 2004). Oregon also provided an extremely collegial atmosphere and even before zebrafish was ready for prime time as a genetic model, Charles Kimmel, one of Streisinger's Oregon colleagues who made important early contributions to understanding clonal selection in the immune system (Tauber & Podolsky, 2000), realized the potential of the zebrafish embryo for understanding the cellular basis of neural development and initiated this as a new direction in his research program. Kimmel started his work in this area by describing the development of an identified neuron, the Mauthner cell (Kimmel, Sessions, & Kimmel, 1981), made famous by work in other teleosts and in amphibians (Korn & Faber, 2005). Kimmel's work revealed that morphologically similar neurons were repeated in each hindbrain segment (Metcalfe, Mendelson, & Kimmel, 1986), leading him to ask whether these neurons could be related by cell lineage (Grunwald & Eisen, 2002). The optical clarity and rapid development of the zebrafish embryo make it ideally suited to carry out lineage studies. So in the summer of 1982 Kimmel visited Michael Bennett's laboratory at the Marine Biological Laboratory (Kimmel, personal communication) to learn how to inject individual cells, called blastomeres, in young Fundulus embryos, using a fluorescent dye called Lucifer Yellow, developed to the study passage of molecules between cells via gap junctions (Kimmel, Spray, & Bennett, 1984; Stewart, 1981). Kimmel was later joined in his zebrafish lineage tracing endeavors, by Oregon colleagues Monte Westerfield and Judith Eisen, both of whom had backgrounds using Lucifer Yellow to label and record from neurons, and who worked with Kimmel to establish the use of another fluorescent dye, fluorescein dextran, that did not pass through gap junctions.

Kimmel's lineage studies, many carried out with the partnership of undergraduate students, were completely captivating. No one knew the time course of development, and with the instrumentation available at the time, it was not possible to make the kind of computer-controlled, time-lapse movies that are routine today. Even if it were possible, no forum existed at that time in which such movies could be published. So days were divided up into manageable segments of four or more hours, and teams of people sat during each segment in a completely dark room, except for instrument lights and what would be by today's standards a miniscule video monitor, and watched and took notes, as cell movements and divisions were recorded onto a videocassette recorder, and in many cases traced with different colored markers onto sheets of acetate taped to the video monitor screen. These studies provided unprecedented insights into vertebrate development and enabled Kimmel to describe lineage contributions to essentially all of the major cell types in the embryo (Kimmel & Warga, 1988). During some of these sessions, Eisen and Westerfield watched as the earliest-developing spinal motoneurons extended axons—something never seen before in vertebrate embryos—and were able to classify these neurons into distinct, identifiable subtypes that could later be studied using a genetic approach (Beattie, Raible, Henion, & Eisen, 1999; Eisen, Myers, & Westerfield, 1986).

This was a heady time for those involved. The clarity of the zebrafish embryo was stunning and the application of fluorescent dye technology, previously used only for studies of gap junctional coupling in amphibians and Fundulus (Kimmel et al., 1984; Spray, Harris, & Bennett, 1979), lineage tracing and neuronal morphology in the medicinal leech (Stewart, 1981; Weisblat, Zackson, Blair, & Young, 1980), and laser-ablation in crustaceans (Marder & Eisen, 1984; Selverston & Miller, 1980), meant that researchers learned something new from every gorgeous experiment. And every experiment revealed phenotypes that could be investigated by combining these modern embryological and neurobiological approaches with Streisinger's newly developed genetic tools. Casting a pall over the nascent field of zebrafish developmental genetics, Streisinger died completely unexpectedly in August 1984, and thus, he never saw the blossoming of this new and exciting era.

Streisinger's death could have been the end of the new beginning for zebrafish research. However, his Oregon colleagues, already deeply immersed in their studies of zebrafish lineage and neural development, took up the baton and began promoting zebrafish as an outstanding new model in which to investigate the genetic basis of developmental processes. A critical aspect of support for zebrafish research came from the National Institutes of Health, which allowed Kimmel to become Principal Investigator of Streisinger's grant to study zebrafish genetics. David Grunwald, at that time a postdoctoral fellow in Streisinger's laboratory, and Charline Walker, the research assistant who had worked with Streisinger to develop his genetic tools, were instrumental in helping keep up the momentum of zebrafish research. The Oregon group's studies had already provided important new insights into the commonality of the developmental processes among vertebrates, leading Kimmel to publish an influential article, in the issue of *Trends in Genetics* shown in Fig. 1.2, declaring "the fish is a frog … ….is a chicken … ….is a mouse" that can be studied "for both detailed … ….embryogenesis and … ….genetic analysis" (Grunwald & Eisen, 2002; Kimmel, 1989).

By this time, a number of researchers prominent for their studies of fruit flies, including Jose Campos-

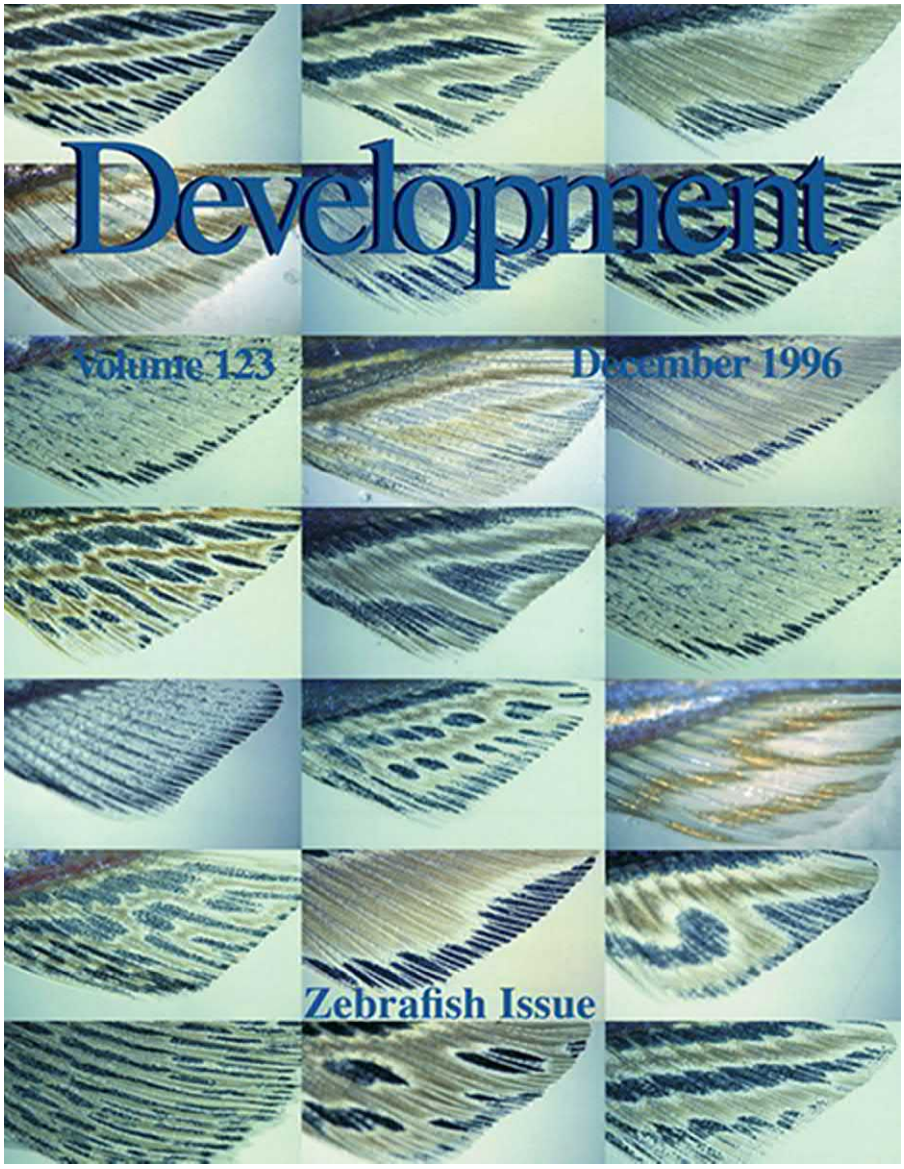

FIGURE 1.6 This issue of *Development* from December 1996 was devoted entirely to papers describing the first large scale genetic screens for zebrafish mutations carried out in the Nusslein-Volhard laboratory at the Max-Planck-Institute fur Entwicklungsbiologie in Tubingen Germany and the Driever and Fishman laboratories at Harvard in Boston Massachusetts. There were 37 papers describing the characterization of about 4000 mutations (Eisen, 1996; Grunwald & Eisen, 2002). *Cover image of* Development *volume 123 from December 1996 used with permission from* Development *and Robert Kelsh.*

Ortega at the University of Cologne, as well as Nusslein-Volhard, had taken note of the work being done in Oregon and had begun to consider adding zebrafish studies to their repertoire (Grunwald & Eisen, 2002). In 1990, the Oregon group facilitated this possibility by hosting a small meeting that brought together researchers from a variety of fields and highlighted the utility of zebrafish as a model. They also established an informal course on zebrafish husbandry, genetics, and embryology, and hosted numerous visiting scientists from around the world who came to learn this new model system (Grunwald & Eisen, 2002). Westerfield created the "Zebrafish Book" (http://zfin.org/zf_info/zfbook/zfbk.html), a primer on zebrafish methodology, and later established the Zebrafish Information Network (zfin; http://zfin.org/), which hosts the Zebrafish Model Organism Database. Westerfield also founded ZIRC, the Zebrafish International Resource Center, the first zebrafish genetic repository. In 1994, Cold Spring Harbor hosted the first open international meeting on zebrafish development and genetics, and in 1998, a course on zebrafish development and genetics was initiated at the Marine Biological Laboratory in Woods Hole, Massachusetts. In the ensuing years, there has been a proliferation of zebrafish meetings, as well as courses held in a variety of venues around the world.

Making a Big Splash

Although the Oregon group continued to use Streisinger's methodology to conduct mutational screens, uncovering genes involved in a variety of different developmental processes, Nusslein-Volhard developed a more ambitious plan, to essentially recapitulate in zebrafish the screen she had carried out to discover embryonic patterning mutants in fruit flies (Mullins, Hammerschmidt, Haffter, & Nusslein-Volhard, 1994; Mullins & Nusslein-Volhard, 1993). As she was developing new mutagenesis and fish-rearing procedures for this endeavor in her laboratory at the Max Planck Institute in Tubingen, Germany, her talented former graduate student, Wolfgang Driever, who had spent a year as a postdoctoral fellow with Westerfield in Oregon, was recruited to Massachusetts General Hospital by Marc Fishman, to establish a parallel screening effort.

In similar fashion to the earlier fruit fly mutational screen, the "Big Screen" in zebrafish revealed mutations that affected nearly every aspect of the embryonic body plan and provided an exceptional window into the genetic processes governing vertebrate development. This was the largest forward mutational screen ever undertaken in a vertebrate and a tour de force by the Nusslein-Volhard and Driever laboratories. The screen involved 65 people, including Nusslein-Volhard's colleague Friedrich Bonhoeffer and Driever's colleague Fishman, as well as many brilliant young students and postdoctoral fellows who went on to establish their own laboratories. Participants examined more than a million and a half embryos over about a 2-year period, resulting in the isolation of over 4000 mutations, about half of which were characterized over the next year (Driever et al., 1996; Eisen, 1996; Haffter et al., 1996).

Rather than publish these characterizations piecemeal, as a series of papers describing mutations affecting specific aspects of embryonic development, the entire set of 37 screen papers was published as a cohesive collection that constituted an entire, ectopic volume of the journal *Development* (December 1996, volume 123; Fig. 1.6), complete with a flipbook that revealed the process of embryogenesis as viewed by time-lapse microscopy (Karlstrom & Kane, 1996). This was a historic decision because it facilitated the assessment of the entire range of mutant phenotypes. The brilliance of both the earlier fruit fly screen and also the zebrafish "Big Screen" was categorizing mutants into phenotypic groups. This organization allowed gene functions to be ascribed to temporal and spatial pathways even before the genes themselves had been identified, and greatly facilitated elucidation of the molecular pathways underlying developmental processes. The "Big Screen" catapulted zebrafish to the forefront as an outstanding new model in which to investigate the genetic underpinnings of essentially every aspect of vertebrate development. Importantly, these mutants were made available so that other laboratories could utilize zebrafish to begin or enhance investigations focused on particular developmental processes.

Developing New Tools for Molecular Analyses

Understanding the mechanisms underlying mutant phenotypes revealed by the "Big Screen" as well as other, smaller screens (for example (Beattie et al., 1999)), required learning which genes were affected and the molecular nature of the defects. Several years earlier, John Postlethwait had anticipated the need for molecular landmarks spread out across the genome and created the first genetic linkage map for zebrafish (Johnson, Midson, Ballinger, & Postlethwait, 1994). Consistent with the Oregon way of carrying out science, much of the mapping was done by a team of exceptional undergraduate students. The techniques used to establish the map (Postlethwait & Talbot, 1997) made it relatively straightforward to begin to clone the genes defined by mutant phenotypes, and thus, to understand not only what had gone awry, but also to learn how these mutated genes and resulting phenotypes corresponded to the genotypes and phenotypes of other organisms. In addition, these studies revealed that the zebrafish lineage had experienced a genome duplication event and that this event was shared by all teleosts, an understanding that allowed accurate connection of the zebrafish genome to the human genome (Amores et al., 1998; Force et al., 1999). Perhaps not surprisingly by this time, the cloning of zebrafish mutations again reinforced the idea that similarities in embryogenesis among different animals are governed by orthologs of the same genes, opening the path to use zebrafish to investigate genome architecture and evolution (Postlethwait et al., 1998), as well as to serve as a model for human genetic diseases (Zon, 1999).

The momentum of zebrafish research was already accelerating, and it was clear that additional resources would enable many more laboratories to embrace this model. The nascent zebrafish research community, represented by John Postlethwait and Marc Fishman, as well as Len Zon from Harvard Medical School and Nancy Hopkins from the Massachusetts Institute of Technology, worked with the then National Institutes of Health Director, Harold Varmus, and in 1998, the NIH established the "Trans-NIH Zebrafish Initiative" in which many participating NIH institutes solicited applications "to increase our support of the zebrafish as an animal model for development, organ formation,

behavior, aging, and disease research" (https://grants.nih.gov/grants/guide/pa-files/PA-01-095.html). It was now evident that zebrafish had arrived as a model!

As more laboratories adopted the zebrafish model, new resources were developed, and following in the tradition of establishing an interactive research community, these resources were readily shared. For example, at one of the early Cold Spring Harbor Zebrafish Development and Genetics Meetings, researchers from Harvard brought and shared reagents, and later Chi-Bin Chien from the University of Utah also shared reagents at a Strategic Conference of Zebrafish Researchers. In a similar fashion, Steve Ekker at the Mayo Clinic developed and shared the antisense methods for knocking out the function of nearly any gene (Nasevicius & Ekker, 2000), and more recently a number of laboratories have developed and shared gene-editing strategies (Varshney, Sood, & Burgess, 2015), as well as new strategies for cloning existing mutations (Hill et al., 2013; Miller, Obholzer, Shah, Megason, & Moens, 2013).

Expanding Zebrafish Research Into New Areas

Zebrafish has continued to increase in popularity as a model to investigate the cellular, molecular, and genetic mechanisms underlying developmental processes (see Chapter 45 by Pathak and Barresi). As of 2019, there were over 1300 laboratories worldwide listed in ZFIN (http://zfin.org/) that utilize zebrafish to investigate many aspects of animal biology, including human disease mechanisms. Below I describe some of the new directions for zebrafish research that extend beyond understanding the types of developmental mechanisms illustrated above.

Learning how the nervous system orchestrates behavior remains a significant challenge that is being addressed by the international "Brain Initiative" (https://www.braininitiative.nih.gov/). Zebrafish are ideally suited for investigating the basic biological mechanisms underlying neural function because of their accessibility for behavioral measurements, in concert with genetic and neural activity manipulation and imaging studies (see Chapter 46 by Mcarthur and colleagues). Zebrafish exhibit a variety of complex behaviors, including, among many others, sleep and social interactions (Orger & de Polavieja, 2017; Stednitz et al., 2018), that should provide new insights into the underlying mechanisms that can then be translated into understanding brain mechanisms and behavior in humans under normal conditions and in disease states.

Understanding the molecular mechanisms of human genetic disorders is a prerequisite for developing new tools to diagnose and treat these diseases. The outstanding experimental attributes of zebrafish are being increasingly leveraged to establish functional models of human genetic diseases and to develop new clinical tools for their diagnosis and treatment (Phillips & Westerfield, 2014) (see Chapter 47 by Phillips and Westerfield and Chapter 49 by Rissone and colleagues). The list of human disease models to which zebrafish is making important contributions includes cancer, tuberculosis, neurodevelopmental, neurodegenerative and psychiatric disorders, kidney diseases, cardiovascular diseases, skeletomuscular diseases, gastrointestinal tract dysfunctions; this list continues to expand at a dizzying pace, as exemplified by many of the chapters within this volume.

Discovering new drugs is critical for developing therapeutic approaches to human diseases. Zebrafish has become an excellent model for drug discovery because it is straightforward to use chemical screens, in which zebrafish, typically embryos or larvae, are exposed to libraries containing many different molecules with pharmaceutical potential, to identify novel therapeutic agents (Peterson, Link, Dowling, & Schreiber, 2000) (see Chapter 51 by Zhang and Peterson). In fact, numerous chemical screens have been carried out and are already providing important insights that have implications for understanding human health (Rennekamp & Peterson, 2015). This effort has been augmented by the recent realization that human cancer cells can be implanted into zebrafish, thus serving as "avatars" that present highly sensitive platforms for developing and testing pharmacological therapies matched to specific tumor behaviors (Fior et al., 2017; Leslie, 2017).

Another exciting new direction for zebrafish research is as a model in which to study host-microbe interactions (see Chapter 48 by Wiles and Guillemin). In many contexts, microbes are thought of as infectious agents that cause disease. The optical transparency and genetic tractability of zebrafish have enabled it to become an outstanding model for two infectious human diseases, tuberculosis (Ramakrishnan, 2013) and leprosy (Madigan, Cameron, & Ramakrishnan, 2017), that have historically been difficult to investigate in mammalian models. These new studies are providing surprising insights that are likely to lead to new therapeutic approaches.

A related aspect of understanding host-microbe relationships is to learn about host interactions with the microbial organisms associated with essentially every plant and animal on the planet. A tenet of animal development is that initial patterning of the body depends on a maternal contribution of molecules packaged into the egg, and then unfolds based on the regulation of zygotic genes and their products (Chan et al., 2009; Li, Lu, & Dean, 2013; Schier, 2007). It is increasingly clear that after an embryo leaves the protective environment either inside its mother or inside its eggshell, it

becomes associated with communities of microbes, often referred to as microbiota that influence many aspects of its subsequent development and physiology. However, the extent to which host-associated microbiota modulate the normal development and/or physiology of any animal species remains unknown. Zebrafish is ideally suited as a model in this burgeoning new field, because it is straightforward to rear zebrafish germ-free, in the absence of any microbes, or gnotobiotically, with exposure to only very specific microbes (Melancon et al., 2017). At the same time, both host and microbial genetics can be manipulated, and the exquisite optical properties of zebrafish larvae enable real-time visualization of microbe biogeography and reciprocal responses of both the host and associated microbes, including normal residents (Wiles et al., 2016), as well as invasive pathogens (Ramakrishnan, 2013).

In addition to the many features conserved with other animals, zebrafish are able to accomplish something that mammals, such as mice and humans, are unable to achieve, which is to regenerate nearly any damaged body part. Thus far, zebrafish have been shown to regenerate damaged limbs, hearts, retinas, brains, and spinal cords (Gemberling, Bailey, Hyde, & Poss, 2013). This feat is not unique to zebrafish, because other nonmammalian vertebrates, such as salamanders and axolotls, can also regenerate limbs (Dall'Agnese & Puri, 2016). However, the ease of studying zebrafish and manipulating its genome has established it as an important model that holds out the promise of discovering mechanisms that might be exploited to aid regeneration in humans (Mokalled & Poss, 2018).

As zebrafish research has expanded, there have been a number of efforts to standardize zebrafish husbandry. Beginning in the early days of zebrafish research, several resources were developed, including "The Zebrafish Book," dedicated to George Streisinger. This book was originally published by Monte Westerfield in 1993 and printed by the University of Oregon Press. This book is now in its fifth edition (Westerfield, 2007); the fourth edition is available online from ZFIN (zfin.org). Since that time, there have been additional books detailing zebrafish husbandry methods for research laboratories (Harper & Lawrence, 2011; Nusslein-Volhard & Dahm, 2002). The importance of standardizing husbandry was made very clear recently when the journal *Zebrafish* devoted an entire issue to health and husbandry (http://online.liebertpub.com/toc/zeb/13/S1). *Zebrafish* also regularly publishes a section on this topic. The many chapters on health and husbandry in this volume complement the chapters on zebrafish research and provide state-of-the-art information for those who rear zebrafish that will enhance the utility of zebrafish as an outstanding research model.

Conclusions

Streisinger's groundbreaking paper describing genetic tools for zebrafish marked a turning point for developmental biology. Since that time, this small fish has become established as a model for uncovering genetic mechanisms underlying many aspects of development, differentiation, physiology, regeneration, and evolution. Zebrafish has also become a model in which to investigate mechanisms by which environmental factors, such as animal-associated microbes, can influence developmental and physiological processes by modulating host genes. Research utilizing the zebrafish model continues to accelerate, making the future bright for even more important discoveries that will expand our understanding of the genetic mechanisms that transform a single cell, the fertilized egg, into a multicellular animal with a backbone, as well as how vertebrate organs function, how these mechanisms contribute to human health, and how they may result in human diseases when they go awry.

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CHAPTER

2

Zebrafish Taxonomy and Phylogeny or Taxonomy and Phylogeny

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Introduction

In 1822, the Scottish physician Francis Hamilton scientifically described the zebrafish as *Danio rerio* along with a few other species of the genus from Eastern India (Hamilton, 1822). *Danio rerio* remains the correct scientific name for zebrafish, despite recurring changes since the description by Hamilton. In 1916, the *Danio* genus was divided into two subgenera: *Danio* and *Brachydanio*, the latter of which included zebrafish (Weber & de Beaufort, 1916). In 1991, *Danio* and *Brachydanio* were synonymized (Barman, 1991) and in 2003 *Danio* was again separated into the genera *Devario* and *Danio*, including zebrafish (Fang, 2003).

The zebrafish is one of more than 20 species within the genus *Danio* and one of more than 4,000 species within the order of Cypriniformes (Parichy, 2015; Stout, Tan, Lemmon, Lemmon, & Armbruster, 2016). In the following, we will briefly describe the phylogenetic position of zebrafish within the broader scale of the vertebrate tree of life and discuss important evolutionary considerations for the use of zebrafish in biomedical research. Along the way, we will discuss zebrafish's relation to other classic and emerging fish model systems for developmental, genomic, evolutionary, and biomedical research to illustrate the diverse evolutionary framework into which zebrafish research is placed.

The Phylogenetic Position of Zebrafish

In everyday language, the term "fish" applies to many different lineages of aquatic vertebrates that have gills and fins (Nelson, 2006). In strict phylogenetic terms, however, all lineages of living vertebrates are fish, with tetrapods, including ourselves, being a specialized type of (lobe-finned) fish capable of living on land (Long, 2011). Evidence for our own piscine evolutionary past is found all over the human body and genome.

Table 2.1 provides a phylogenetic classification of zebrafish. Focusing on living representative of vertebrates, Fig. 2.1 shows the phylogenetic position of zebrafish, a ray-finned fish, within the larger vertebrate tree of life and its relation to our own human species. Fig. 2.2 illustrates zebrafish's phylogenetic relation to other ray-finned fish groups.

The subphylum of vertebrates emerged within the chordate phylum more than 500 million years ago (Pough & Janis, 2019). Their closest living relatives are the subphyla urochordates (tunicates), which include, for example, the developmental research organisms *Ciona* (Kourakis & Smith, 2015) and *Oikopleura* (Marti-Solans et al., 2015) and, more distantly, the cephalochordates with the classic evo-devo model organism amphioxus (*Branchiostoma*) (Escriva, 2018) (Fig. 2.1).

Among the vertebrates, zebrafish belongs to the lineage of jawed vertebrates or gnathostomes. As the name implies, the emergence of the jaw apparatus was a key innovation of gnathostomes that enabled the exploration of novel food sources, thereby, contributing to the evolutionary success of jawed vertebrates (Liem, Bemis, Walker Jr., & Grande, 2001). Another major morphological innovation leading to the gnathostomes was the acquisition of two sets of paired fins, that is, the pectoral and pelvic fins (Liem, Bemis, Walker, & Grande, 2001), as we can find them in a more derived form in zebrafish.

Cyclostomes, that is, lampreys and hagfishes, remain as the only living group of jawless or agnathan

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TABLE 2.1 Phylogenetic classification of zebrafish (*Danio rerio*). Classification below the gnathostome level is following Betancur et al. (2017).

Kingdom	Metazoa (Animalia)
Superphylum	Deuterostomia
Phylum	Chordata
Subphylum	Vertebrata
Infraphylum	Gnathostomata
Megaclass	Osteichthyes (Euteleostome)
Superclass	Actinopterygii
Class	Actinopteri
Subclass	Neopterygii
Infraclass	Teleostei
Supercohort	Clupeocephala
Cohort	Otomorpha
Subcohort	Ostariophysi
Superorder	Cypriniphysae
Order	Cypriniformes
Suborder	Cyprinoidae
Family	Danionidae
Genus	*Danio*
Species	*Danio rerio*

vertebrates as outgroups to gnathostomes. Cyclostomes lack jaws and paired fins and feature many other morphological as well as genomic differences, but also important shared characteristics with the jawed vertebrates. Thus, these jawless fish are central taxa in comparative studies to reconstruct ancestral conditions during the early phase of vertebrate evolution and the evolutionary changes that led to the emergence of jawed vertebrates (Nikitina, Bronner-Fraser, & Sauka-Spengler, 2009; Shimeld & Donoghue, 2012) (Fig. 2.1).

Gnathostomes are further subdivided into two main groups: the cartilaginous fishes (Chondrichthyes), that is, sharks, skates, and chimeras, and the bony vertebrates (Euteleostome or Osteichthyes), to which zebrafish and human belong (Pough & Janis, 2019) (Fig. 2.1). Several cartilaginous fish species are being used in developmental studies (reviewed in Onimaru, Motone, Kiyatake, Nishida, & Kuraku, 2018) and the recent availability of chondrichthyan genomes (Hara et al., 2018; Venkatesh et al., 2014) promises a rich future for cartilaginous fishes as piscine model organisms for the investigation of gnathostome biology and outgroups to bony vertebrates.

Within bony vertebrates, the lineages leading to zebrafish and human then parted around 400–450 million years ago, with the ray-finned fishes (Actinopterygii) that include zebrafish diverging from the lobe-finned fishes (Sarcopterygii) that gave rise to the tetrapods. Only three groups of lobe-finned fishes have survived until today: the coelacanths and the lungfishes, the latter of which are, in turn, the closest living relatives

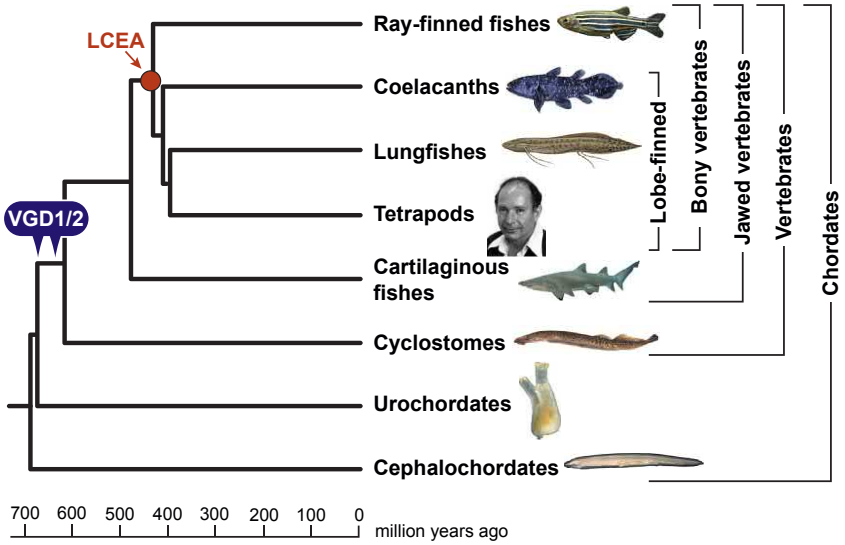

FIGURE 2.1 **Phylogeny of the vertebrate lineage.** Zebrafish belongs to the ray-finned fishes, with their phylogeny further detailed in Fig. 2.2. Reconstructing the last common euteleostome (i.e., bony vertebrate) ancestor (LCEA = *red dot*) is essential for the comparison of ray-finned and lobe-finned vertebrates, especially for the biomedical link of zebrafish to human. VGD1 and VGD2 indicate the likely occurrences of two rounds of vertebrate genome duplication at the base of vertebrates. *The timescales in Figs. 2.1 and 2.2 are based on data obtained from www.timetree.org.*

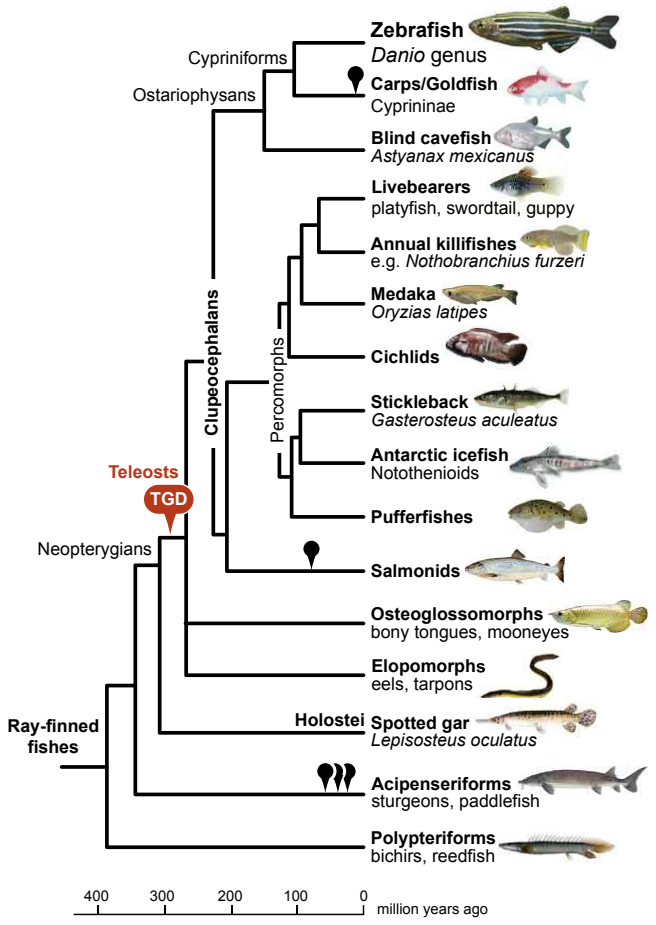

FIGURE 2.2 **Phylogeny of the ray-finned fish lineage** TGD indicates the occurrence of the Teleost Genome Duplication at the base of teleosts. Black pin symbols show the occurrence of additional, lineage-specific genome duplication events.

of the third group, the tetrapods that include us humans (Pough & Janis, 2019) (Fig. 2.1).

With the advent of next-generation sequencing techniques and the acquisition of large-scale genomic data across the tree of life, major strides have been made in phylogenomics (i.e., the use of genome-wide sequence information to infer phylogenetic relationships) to reconstruct the evolution of ray-finned fishes (e.g., Betancur et al., 2013; Hughes et al., 2018; Near et al., 2012). Within the ray-finned fishes, the zebrafish belongs to their most species-rich clade, the teleost fishes (Teleostei) (Fig. 2.2). With more than 25,000 species, teleosts make up almost 50% of all living vertebrates (Helfman, Collette, Facey, & Bowen, 2009; Nelson, 2006). Together with their sister lineage, the holostean fishes that consist of bowfin and gars, teleosts are grouped into the Neopterygii. More distantly, teleosts are related to the acipenseriforms (sturgeons and paddlefishes) and the polypteriforms (bichirs and reedfish), the earliest branching lineage among living ray-finned fishes (Betancur et al., 2013; Near et al., 2012) (Fig. 2.2).

Teleost fishes are divided into three main lineages: the clupeocephalans that include zebrafish and most other fish model systems (see below); the osteoglossomorphs, which include, for example, arowana, African butterflyfish, and mormyrid electric fish; and the elopomorphs to which eels and tarpons belong (Fig. 2.2). The interrelationships of these three major lineages have been difficult to resolve and are a matter of ongoing investigation (Betancur et al., 2017).

Within the clupeocephalans, two main radiations are recognized: the ostariophysans with more than 10,000 species and the percomorphs with more than 14,000 species (Alfaro et al., 2009; Chakrabarty et al., 2017; Near et al., 2013). Ostariophysans include fishes as diverse as catfishes, characins, electric knifefishes, and the cypriniforms with zebrafish (Chakrabarty et al., 2017).

Zebrafish and Related *Danio* Species as an Evolutionary Model System

Cypriniform and Danionid Relationships

Zebrafish is surrounded by cypriniform biodiversity. Cypriniforms represent the largest group of freshwater fishes and besides minnows and suckers also includes species important for aquaculture, such as common carp and grass carp, as well as many popular ornamental species, such as goldfish and rasboras (Stout et al., 2016).

The taxonomy of this diverse group has experienced numerous revisions as more species have been described and phylogenetic inference methods have shifted from morphological to molecular methods. This change is particularly relevant to zebrafish, which is referred to as *Brachydanio rerio* in much of the scientific literature from prior to 1995. Only recently have phylogenomic approaches proved sufficient to provide strong support for relationships between families within Cypriniformes and between species within *Danio* (McCluskey & Postlethwait, 2015; Stout et al., 2016). The most extensive molecular phylogenomic study of *Danio* to date supports *D. aesculapii* as the closest relative of *D. rerio* (Fig. 2.3). Zebrafish is also closely related to *D. kyathit* with evidence of gene flow between those lineages during speciation (McCluskey & Postlethwait, 2015). Relationships within *Danio* will be better resolved as the diversity within this group is further described.

The Emerging Danionid Model System

The dozens of *Danio* species are phenotypically diverse, but all share the major advantages of zebrafish as a model vertebrate making danios ideal for studying interspecific evolution (Irion, Singh, & Nusslein-Volhard, 2016; Parichy, 2006). All danios have

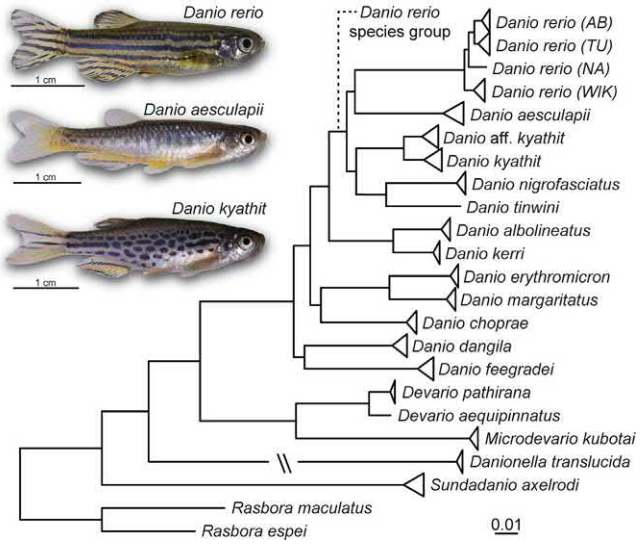

FIGURE 2.3 Phylogenetic tree showing the relation of zebrafish to other *Danio* species. Zebrafish strains include two lab strains (AB and TU), as well as two wild strains (NA and WIK). According to this phylogeny following McCluskey and Postlethwait (2015), *D. rerio* is most closely related to *D. aesculapii* within the larger danionid clade.

externally fertilized, transparent embryos, and high fecundity, making them immediately accessible for developmental studies. In addition, transgenesis methods used for zebrafish also function in other danios, allowing the use of reporter constructs to investigate differences in gene expression across development (Eom, Bain, Patterson, Grout, & Parichy, 2015). Furthermore, interspecific *Danio* hybrids can be readily produced by *in vitro* fertilization and are viable, though many interspecific hybrids are sterile (Parichy & Johnson, 2001). The ability to make hybrids with zebrafish allows for the investigation of phenotypic differences via complementation tests with the huge array of mutants available for *D. rerio* (Quigley et al., 2005; Spiewak et al., 2018). These approaches have been thus far applied to the analysis of pigmentation, the most striking difference between these species, but are also applicable to understanding the evolution of other phenotypes.

Importantly, *Danio* species can be raised under the laboratory conditions used for zebrafish. They benefit, however, from modest accommodations to the care for *Danio rerio*. Environmental enrichment in the form of opaque tank bottoms, substrate, and/or plastic plants is beneficial to many species. Some species may also require larger tanks and high water flow. Dietary enrichment with brine shrimp, bloodworms, or rotifers helps condition fish during development and prior to breeding. Unlike laboratory-adapted zebrafish, danios do not breed well in small, clear crossing tanks. For many danios, breeding in the lab can be induced as described in the home aquarium community. Selecting only conditioned fish, use a static tank with freshwater, a substrate to collect eggs, and plastic plants. Embryos are siphoned from the substrate prior to hatching. If embryos are needed at a specific time, *in vitro* fertilization is often a better option than natural spawning.

The Zebrafish Model in an Evolutionary Context

Evolutionary Considerations for Zebrafish-to-Human Comparisons

For a meaningful comparative approach that makes use of zebrafish as biomedical model for investigating human biology and disease, we need to be able to define those shared characteristics of the zebrafish and human genome and phenotype that are derived from their last common bony vertebrate, that is, euteleostome, ancestor (LCEA in Fig. 2.1) and distinguish them from the lineage-specific, secondary changes that have occurred independently in the ray-finned and lobe-finned lineages.

Clearly, significant evolutionary changes have impacted the zebrafish and human lineages since their last common bony vertebrate ancestor. For example, considering the lobe-finned lineage leading to human, the water-to-land transition and the emergence of tetrapods involved major changes at the morphological level, such as turning fins into limbs, concomitantly with changes at the genomic level, for example the loss of genes encoding some structural components of fins (Amemiya et al., 2013; Wood & Nakamura, 2018).

On the other hand, morphological changes within ray-finned fishes, such as the emergence of a homocercal caudal fin structure in early teleosts or the evolution of the Weberian apparatus within the ostariophysans illustrate important phenotypic changes leading to zebrafish (Metscher & Ahlberg, 1999) that need to be taken into account when aiming to compare zebrafish and human anatomy.

Besides morphological innovations, it might also be secondary reductions or losses of structures, tissues, and cell types that can diversify and thus complicate the connectivity of zebrafish-to-human comparisons. For example, mammals have reduced their repertoire of neural crest cell-derived pigment cell types to the melanocytes, while teleosts like zebrafish possess a whole suite of different chromatophore types (melanophores, xanthophores, iridophores, leucophores, etc.) (Parichy & Aman, 2019), with many of these pigment cell types likely inherited from a bony vertebrate ancestor.

A detailed comparison of zebrafish and human morphology and physiology is thus essential for best practices in the translation of biomedical relevant data across lineages, motivating the development and annotation of phenotypic ontologies as used by The Zebrafish Information Network ZFIN (http://zfin.org/) that can then be applied across species (Van Slyke, Bradford, Westerfield, & Haendel, 2014).

Importance of Genome Duplications for Zebrafish Evolution

At the genomic level, an important aspect of vertebrate evolution that needs to be considered in human-to-zebrafish comparisons is the role of three rounds of whole-genome duplications (or polyploidizations) that have impacted the zebrafish and human lineages in several ways (Figs. 2.1 and 2.2). Polyploidizations are rare events in animals but have occurred comparatively frequently in fishes (Braasch & Postlethwait, 2012) (Fig. 2.2). The amplification and diversification of vertebrate gene families imposed by the three vertebrate genome duplications have led to complex scenarios of gene function evolution that sometimes are difficult to disentangle.

All living teleost species, including zebrafish, are derived from an ancestor that underwent a whole-genome duplication event at the dawn of teleost evolution. This Teleost Genome Duplication (TGD) occurred within the neopterygian lineage after the separation of teleosts from the holostean fishes (gars and bowfin), but before the divergence of the three major teleost lineages of clupeocephalans, osteoglossomorphs, and elopomorphs (Fig. 2.2) (reviewed in Braasch & Postlethwait, 2012). For details on the genomic impacts of the TGD see Postlethwait and Braasch (2019) in this volume.

Following the TGD, the teleost genome originally was tetraploid, but it has secondarily returned to the diploid state through the process of rediploidization. Importantly, depending on methods of TGD gene duplicate inference, it has been estimated that between 1,200 and 3,400 pairs of TGD gene duplicates have remained in the zebrafish genome (Howe et al., 2013; Pasquier et al., 2017; Roux, Liu, & Robinson-Rechavi, 2017). Thus, for a significant portion of human genes, there will be two TGD-derived gene duplicates (also called *co-orthologs* or *paralogs*) in the zebrafish genome. This can complicate comparisons between zebrafish and human as one may have to take two genomic regions in the zebrafish into account, potentially harboring two TGD *co-orthologs* of the gene under investigation. For example, there are two *sonic hedgehog* (*shh*) genes present in the zebrafish genome, *shha* and *shhb*, that are *co-orthologs* to the single *SHH* gene in human.

Since their fixation in the teleost genome, TGD duplicates will have diverged in function in often complex patterns, following evolutionary fates such as subfunctionalization (the distribution of ancestral gene functions among duplicates) and neofunctionalization (emergence of novel gene functions) (Force et al., 1999). Comparing zebrafish to other teleosts, one will further find differences in terms of TGD duplicate retention and loss among species. Although the overall retention rate of TGD duplicates appears to be relatively similar across teleosts, differences in terms of the specific genes retained in duplicate within individual teleost lineages have contributed to the genomic diversification of teleost lineages. Even if different teleost groups have retained both TGD duplicates, lineage-specific divergence in the functional roles among duplicates can be observed (Braasch & Postlethwait, 2012).

In addition to the TGD, the vertebrate lineage is derived from likely two additional, earlier ancient rounds of whole-genome duplication at the base of the vertebrate lineage, that occurred after their separation from nonvertebrate chordates. The evolutionary sequence of these Vertebrate Genome Duplications, VGD1 and VGD2 (Fig. 2.1), remains a matter of ongoing debate (Sacerdot, Louis, Bon, Berthelot, & Roest Crollius, 2018; Smith et al., 2018). Differential loss of VGD1/VGD2 gene duplicates after the divergence of the ray-finned and lobe-finned lineages has led to situations in which no directly orthologous genes remain in the zebrafish and human genomes (Postlethwait, 2007). This can complicate the transition of genetic information from one system to the other. For example, the intensively studied human stem cell factor *POU5F1* (*OCT4*) has no direct ortholog in the zebrafish genome because of secondary loss from the ray-finned genome following VGD1/VGD2; its VGD1/VGD2 duplicate *Pou5f3*, on the other hand, is present in the ray-finned, and thus, zebrafish genome, while having been secondarily eliminated from the eutherian mammal and hence human genome (Frankenberg et al., 2014).

In summary, a careful examination of gene family history evolution is paramount for the transfer of genetic information between zebrafish and human. Gene orthology predictions provided, for example, by the ZFIN and Ensembl (www.ensembl.org) databases are good starting points for in-depth phylogenetic investigations of gene family relationships across vertebrates.

Zebrafish and Its Relation to Other Fish Model Species

Zebrafish is the most commonly used fish species in biomedical research, but it is important to remember that zebrafish is just one of tens of thousands of teleost fish species. It, therefore, provides a snapshot of the tremendous genotypic and phenotypic biodiversity of teleosts, and comparisons to other phylogenetically diverse fish models are essential to inform the evolution of zebrafish in relation to the teleost, ray-finned, and bony vertebrate ancestors.

Medaka

The other main fish species used in biomedical research is the Japanese rice fish, medaka (*Oryzias latipes*), that has been cultivated in Japan for centuries. The medaka is very similar in husbandry and laboratory use to zebrafish (Kinoshita, Murata, Naruse, & Tanaka, 2009), has a sequenced genome (Kasahara et al., 2007), and numerous laboratory and natural strains, mutant and transgenic lines, and other resources that can be received through the NBRP Medaka Resource Center (https://shigen.nig.ac.jp/medaka/). Comparative tables of developmental stages of zebrafish and medaka have been developed (Furutani-Seiki & Wittbrodt, 2004; Tena et al., 2014).

Medaka is embedded in a larger *Orzyias* species complex with largely unexplored potential for evolutionary research (Hilgers & Schwarzer, 2019). Furthermore, medaka is a percomorph teleost, and its lineage diverged from the lineage leading to zebrafish early during clupeocephalan teleost evolution (Fig. 2.2). Thus, from an evolutionary point of view, the use of medaka in biomedical research is highly complementary to that of zebrafish (Furutani-Seiki & Wittbrodt, 2004). Comparison of gene expression and gene functions between the two are useful to evaluate the extent of lineage-specific divergence in either species, which is particularly important given that both lineages diverged relatively soon after the TGD and have hence undergone, for example, separate paths in TGD gene duplicate retention/loss, as well as TGD gene duplicate function divergence (Furutani-Seiki & Wittbrodt, 2004). For example, while the two TGD gene duplicates *shha* and *shhb* are retained in zebrafish, medaka has only kept the *shha* copy of the *sonic hedgehog* gene. In contrast, there are two *sox10* TGD duplicates in the medaka genome, while zebrafish retained only a single *sox10* gene.

"Evolutionary Mutant" Fish Models

Beyond zebrafish and medaka, there are numerous other teleost species utilized in biomedically-focused research, often because they feature specific adaptations that resemble maladaptive conditions and diseases in human. These "evolutionary mutant models" (Albertson, Cresko, Detrich, & Postlethwait, 2009) have become powerful tools for the study of vertebrate development, evolution, and human disease (Braasch et al., 2015; Schartl, 2014). They benefit from recent genome sequencing initiatives, as well as from the transfer of advances in the genetic-developmental analytical toolbox, such as genome-editing, transgenics, and other functional approaches first developed and/or optimized in zebrafish. Below we highlight a few examples of these biomedical fish models, but this list is far from exhaustive.

Goldfish and carp: Within the cypriniforms, goldfish (*Carassius auratus*) and common carp (*Cyprinus carpio*) are important aquaculture species with a centuries-long history of domestication. Particularly goldfish is a promising model for biomedical investigation with its multitude of variants and strains that feature extreme morphologies of eyes, fins, skeleton, body shape, coloration, etc., some of which are resembling human diseases (Omori & Kon, 2019). Importantly, goldfish and carp are both derived from an additional, carp lineage-specific genome duplication event that occurred in their common ancestor within the last few million years (Fig. 2.2). Using zebrafish as "unduplicated" outgroup with respect to the carp genome duplication, analyzing the evolutionary aftermath of the carp genome duplication is becoming an important avenue to understand the genomic and morphological impact of genome duplications in vertebrates (Chen et al., 2019; Xu et al., 2014).

Blind cavefish: Cave populations of the Mexican tetra (*Astyanax mexicanus*), usually referred to as "blind cavefish," have become popular research organism to study traits that evolved in response to the constant darkness and seclusion of cave environments and that have resemblances to human disease phenotypes, including eye regression, albinism, obesity, sleep loss, behavioral changes, and others (Jeffery, 2008; Rohner, 2018). The Mexican tetra is a characiform that belongs to the ostariophysan lineage and thus shares a comparatively close relationship to zebrafish (Fig. 2.2). Hence, zebrafish has been used as surrogate species to study the functional consequences of genetic differences between cave versus surface populations of *Astyanax* (e.g, Gross, Borowsky, & Tabin, 2009; Riddle et al., 2018).

Besides medaka, the percomorph clade of teleosts offers a plethora of fish species gaining popularity in biomedical, evolutionary, and genomic research (Fig. 2.2).

Livebearers: Livebearing poeciliids (guppies, platies, swordtails) are common model species in evolutionary research, and among them, the genus *Xiphophorus* has a long tradition to investigate the genomic basis of skin cancer formation because melanoma formation can be generated by different interspecific crossing schemes (Meierjohann & Schartl, 2006). Poeciliids use internal fertilization and development, and genetic manipulations are thus challenging. Therefore, medaka and zebrafish have been used to study aspects of the *Xiphophorus* oncogenic signaling cascades leading to cancer formation in vivo (Li et al., 2012; Regneri, Klotz, & Schartl, 2016).

Annual killifishes: Annual killifishes are a major model system to investigate rapid aging and developmental arrest (diapause), which are adaptations to their unique life cycle in seasonally desiccating water bodies. The turquoise killifish (*Nothobranchius furzeri*) has

emerged as the most popular annual killifish species in aging research as it represents the most short-lived known vertebrate with a few months of lifespan and sexual maturity within a few weeks after hatching (Cellerino, Valenzano, & Reichard, 2016; Platzer & Englert, 2016).

Cichlids: With several thousand species, cichlids are a prime example for adaptive radiations and phenotypic diversification (Salzburger, 2018) that feature complex social behaviors (Fernald, 2017) and trophic adaptations of the jaw apparatus, which can be used to model human craniofacial diseases (Powder & Albertson, 2016). Again, comparative functional analyses using zebrafish have been instrumental in enlightening the underlying genetic basis of such biomedically relevant phenotypes (Cooper, Wirgau, Sweet, & Albertson, 2013; Powder, Cousin, McLinden, & Albertson, 2014).

Sticklebacks: Three-spined stickleback (*Gasterosteus aculeatus*) and related species are major model organisms for evolutionary and ecological genetics (Peichel & Marques, 2017). Studies on the genetic basis of phenotypic differences among stickleback populations, for example, in the pigmentary and skeletal systems, found parallels to phenotypic diversity among human populations, yet also pointed to human-specific changes during vertebrate evolution, respectively (Indjeian et al., 2016; Miller et al., 2007). Furthermore, sticklebacks often serve as percomorph comparators to zebrafish in developmental-genetic studies (e.g., Askary et al., 2016; Jovelin et al., 2007).

Antarctic icefish: Notothenioid icefish are uniquely adapted to the extreme cold of Antarctic waters: they possess antifreeze proteins, many species are characterized by reduced bone formation, and they lack functional hemoglobin and red blood cells. Thus, they can serve as models for human diseases, such as osteopenia and osteoporosis and anemia (Albertson et al., 2009). Due to their unique ecology, Antarctic icefish are difficult to study in captivity, and thus, zebrafish have been used for example to functionally investigate genes with a role in erythrocyte formation that emerged from investigating the unique blood cell development in icefish (Yergeau, Cornell, Parker, Zhou, & Detrich, 2005).

Nonteleost Fish Provide Connectivity From Zebrafish to Human

Given the derived nature of both teleost phenotypes and the teleost genome as a result of the TGD, zebrafish-to-human comparisons benefit from the inclusion of morphological and genomic data from nonteleost fishes. In recent years, the holostean spotted gar (*Lepisosteus oculatus*; Fig. 2.2) has emerged as a "bridge species" that provides connectivity among bony vertebrate lineages. Due to its "unduplicated" nature with regard to the TGD and its comparatively slow rates of genomic and morphological evolution, gar provides important reference points for the transition of biomedically relevant morphological and genomic information across bony vertebrates and to link zebrafish to human biology (Braasch et al., 2015, 2016). This has helped, for example, to clarify the evolution of joint development in bony vertebrates, enabling to then develop an arthritis model in zebrafish (Askary et al., 2016).

Conclusion and Outlook

Zebrafish is a unique representative of ray-finned teleost fish biodiversity that diverged from human more than 400 million years ago. While being a powerful model system to study vertebrate biology and human disease, it is important to keep in mind that zebrafish neither is a "prototypic" fish that stopped evolving after separation from the human lineage nor that findings in zebrafish are necessarily generalizable across fish lineages.

A meaningful utilization of the zebrafish model in biomedical research, therefore, calls, on the one hand, for the detailed elucidation of shared and divergent characteristics of zebrafish and human at both the phenotypic and genetic levels—as exemplified by the information provided in the chapters of this volume. On the other hand, the inclusion of information from the phylogenetically expanding swarm of fish model systems will put zebrafish research into an enriched evolutionary context. With the relentless technical improvements for its investigation, zebrafish will continue to lead the charge in illuminating the genotype-to-phenotype map in the fish world.

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CHAPTER

3

Zebrafish Genetics

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Introduction

Teleost fish present an amazing variety of forms, display a wondrous diversity of color patterns, vary enormously in size, occupy environments with remarkably different temperatures and pressures, and represent half of all vertebrate species. Additionally, many teleosts thrive with phenotypes that would be pathogenic in a human, including the degenerated eyes of cavefish, the profound anemia of Antarctic icefish, and the reduced bone mineralization of acidic bog-dwelling fish. Understanding the origin of this impressive biodiversity and linking the molecular and cellular basis of phenotypic traits among species—including, importantly, connecting teleost biology to human health and disease—requires an understanding of genetics and genomes. Biologists have focused on the genetics of a few model teleost species in-depth, including medaka (*Oryzias latipes*), stickleback (*Gasterosteus aculeatus*), swordtails and platyfish (*Xiphophorus* species), Mexican tetra cavefish (*Astyanax mexicanus*), the cichlid radiation, and zebrafish (*Danio rerio*). Here, we focus on the genetics of zebrafish, a teleost fish model system for investigating the genetic basis of development, physiology, reproduction, evolution, cell biology, and behavior.

Zebrafish are optically translucent, have externally developing embryos, a rapid life cycle, small adult size, and large clutches; features that make them amenable to large-scale and high-throughput forward genetic investigations, studies that propelled our early understanding of zebrafish genetics. Clear, external embryos provided zebrafish researchers access to the cellular basis of early development that is more difficult to extract from opaque mammalian embryos huddled inside the womb. The connection of zebrafish genetic studies to human biology requires an understanding of a massive genomic event, a whole-genome duplication (WGD), in the ancestors of all living teleosts, the Teleost Genome Duplication (TGD). As a result of this ancient polyploidization event, a significant portion of human genes have two duplicate gene copies in the zebrafish genome; this situation requires researchers to take special care in making human-to-zebrafish gene comparisons. Here, we discuss the zebrafish genome in terms of the TGD and its significance for zebrafish research.

The Zebrafish Karyotype

The zebrafish genome is organized into 25 chromosome pairs (Amores & Postlethwait, 1999; Daga, Thode, & Amores, 1996; Gornung, Gabrielli, Cataudella, & Sola, 1997; Lee & Smith, 2004; Pijnacker & Ferwerda, 1995; Schreeb, Groth, Sachsse, & Freundt, 1993; Sola & Gornung, 2001; Traut & Winking, 2001). The zebrafish karyotype is within the typical range for actinopterygian (ray-finned) fishes, which show remarkable conservation of chromosome numbers: the majority of teleosts have 48 or 50 chromosomes as diploids (Mank & Avise, 2006; Naruse et al., 2004). Zebrafish has predominantly submetacentric and metacentric chromosomes (Amores & Postlethwait, 1999; Phillips, Amores, Morasch, Wilson, & Postlethwait, 2006). Karyotype investigations of laboratory or pet store zebrafish strains have generally found no evidence for sex chromosomes, although one study of wild zebrafish from northwest India reported heteromorphic sex chromosomes with females (WZ) having a submetacentric Z chromosome and a metacentric W chromosome and males (ZZ) having two submetacentric Z chromosomes (Sharma, Sharma, & Tripathi, 1998).

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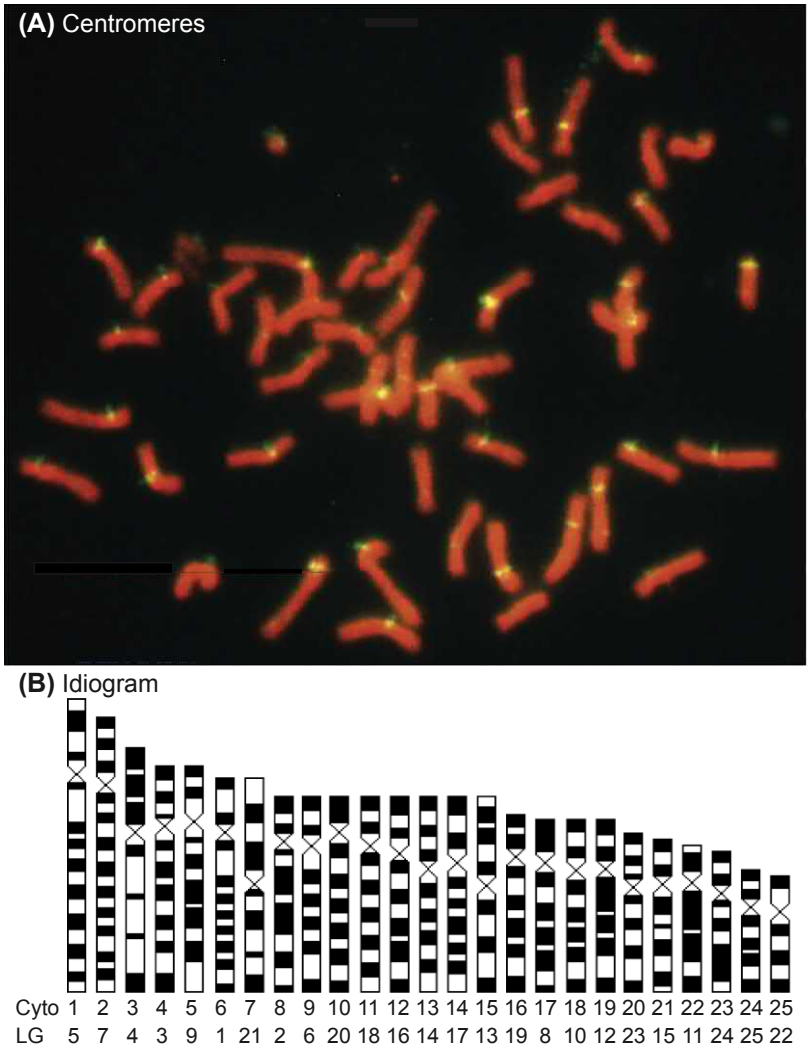

FIGURE 3.1 The karyotype of laboratory strain zebrafish. (A) Centromeres labeled yellow with type I satellite DNA (Phillips et al., 2006). (B) The zebrafish ideogram based on replication banding, with black segments corresponding to early replicating bands (Amores & Postlethwait, 1999). *Cyto*, cytogenetic chromosome number; *LG*, linkage group number.

By convention, cytogenetic studies ordered zebrafish chromosomes by their length (Fig. 3.1), with chromosome 1 substantially larger than the others, chromosome 3 replicating late in the cell cycle, and chromosomes 7 and 15 as the only large chromosomes that are exactly metacentric (Amores & Postlethwait, 1999).

The Zebrafish Genetic Map

In 1974, George Streisinger submitted an application to the US National Science Foundation to make mutations that block early zebrafish development (Varga, 2018). Over the course of the next few years, Streisinger and his colleagues, including Charline Walker and David Grunwald, developed efficient methods for mutagenizing zebrafish and isolating mutants, publishing the first induced mutation in 1988 (Chakrabarti, Streisinger, Singer, & Walker, 1983; Grunwald, Kimmel, Westerfield, Walker, & Streisinger, 1988; Walker & Streisinger, 1983). The induction of other mutations followed (Felsenfeld, Walker, Westerfield, Kimmel, & Streisinger, 1990; Kimmel, Kane, Walker, Warga, & Rothman, 1989), and 1996 saw the publication of hundreds of mutants from two large-scale mutagenesis screens (Driever et al., 1996; Haffter et al., 1996). Although the first induced mutation was cloned in 1994 by a candidate gene approach (*no tail* = *brachyury*, or *T-box transcription factor Ta* (*tbxta*) (Schulte-Merker, van Eeden, Halpern, Kimmel, & Nusslein-Volhard, 1994)), it became clear that genetic mapping would be essential for the molecular identification of most zebrafish mutations.

The first zebrafish genetic map used 401 random amplified polymorphic DNAs (RAPDs) and simple sequence repeats (SSRs) to construct a genetic linkage map and simultaneously position nine induced

mutations on the map (Postlethwait et al., 1994). Subsequent genetic maps used other types of DNA markers, including single-nucleotide polymorphisms (SNPs), to create maps with more markers (Fornzler et al., 1998; Gates et al., 1999; Kelly et al., 2000; Knapik et al., 1996, 1998; Postlethwait et al., 1998; Shimoda et al., 1999; Woods et al., 2000, 2005). The application of Streisinger's techniques to perform half-tetrad genetic analysis with zebrafish (Streisinger, Walker, Dower, Knauber, & Singer, 1981) linked molecular genetic markers to centromeres, and thus, consolidated linkage groups into chromosomal-length constructs (Johnson et al., 1996; Kane, Zon, & Detrich, 1999; Kauffman et al., 1995; Mohideen, Moore, & Cheng, 2000). The use again of Streisinger's zebrafish cloning methods to produce fully homozygous gynogenetic doubled-haploid zebrafish from the AB and the Tübingen (TU) strains, followed by the production and sequencing of 459 of their F2 progeny for 201,917 SNP loci produced the SATmap, with the greatest resolution of any animal genetic mapping panel (Howe et al., 2013; Patowary et al., 2013).

The rate of genetic recombination is not uniform throughout the genome or between the sexes. The recombination rate, in general, is higher near telomeres and lower near centromeres (see, e.g. Howe et al., 2013, Supplementary Fig. 3.3). Sex-specific recombination rates were obtained using androgenetic haploid embryos produced by destroying the egg nucleus with gamma-rays followed by fertilization with normal sperm to provide recombinant offspring derived solely from the male parent (Corley-Smith, Brandhorst, Walker, & Postlethwait, 1999; Singer et al., 2002) to compare with gynogenetic maps based on female meiosis. Results showed that the recombination rate in male meiosis is about half that in female meiosis, especially near the centromere. Consequently, using female meiosis in a positional cloning project gives a greater ratio of genetic map distance in centiMorgans to physical distance in Megabases, but using male meiosis tends to minimize recombination, and thereby, maintain haplotypes intact.

Genetic linkage maps ordered linkage groups according to length, with linkage group (LG) 1 being the longest and LG25 the shortest (Fornzler et al., 1998; Gates et al., 1999; Johnson et al., 1996; Kelly et al., 2000; Knapik et al., 1996, 1998; Postlethwait et al., 1994; Shimoda et al., 1999; Woods et al., 2000, 2005). Due to the variable ways that chromatin coils during metaphase of mitosis and chromosomes recombine during prophase I of meiosis, a chromosome's physical length does not scale one-to-one with its genetic length as determined by recombination rate; thus, the numbering system of cytogenetically defined chromosomes (mitosis) and the numbering convention of linkage groups (meiosis) are not directly synonymous

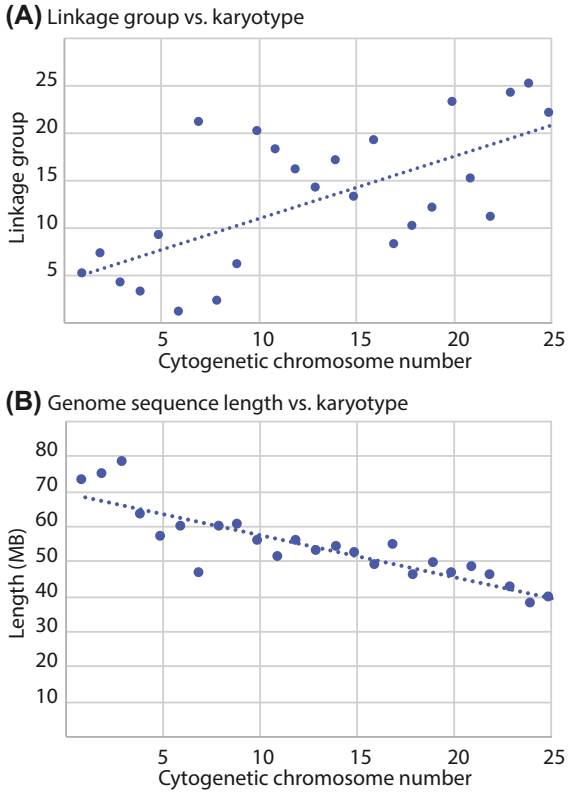

FIGURE 3.2 Lengths of chromosomes, linkage groups, and genome sequences. (A) Linkage group numbers (proportional to lengths in centiMorgans based on recombination rates) plotted versus the cytogenetic chromosome numbers (proportional to physical lengths in metaphase chromosome preparations). (B) Lengths of chromosomes measured in numbers of base pairs in the zebrafish genome assembly (GRCz11, http://uswest.ensembl.org/Danio_rerio/Location/Genome) plotted against cytogenetic chromosome numbers.

(Fig. 3.2). For correcting this problem, genes mapped to LGs were associated with cytogenetic chromosomes by fluorescence in situ hybridization (Phillips et al., 2006). Note that the zebrafish genome assembly (http://uswest.ensembl.org/Danio_rerio/Info/Index?db=core) mixes nomenclature, using the numbers of linkage groups adopted from genetic maps, but the symbol "chr"; thus, cytogeneticists call the longest chromosome "chromosome 1," but gene mappers call it LG 5, and the genome assembly calls it "chr5" or "Chromosome 5" (Figs. 3.1B and 3.2A). Similarly, the late-replicating cytogenetic chromosome 3, which is LG4, becomes "chr4" or "Chromosome 4" in the genome assembly. Note also that this chromosome has more DNA than expected from its physical length, perhaps because of the tight packing of repetitive DNA as chromosomes condense in mitosis (Fig. 3.2B). Current literature uses the genome assembly nomenclature when referring to specific chromosomes or linkage groups. An alternative chromosome nomenclature that minimizes confusion when considering chromosomes

of several species is to use the first letter of the genus and the first two letters of the second term in the binomial name of the species to designate chromosomes, so the zebrafish cytogenetic chromosome 3 (LG4) becomes Dre4 (*Danio rerio* LG4) and human chromosome 7 becomes Hsa7 (*Homo sapiens* chromosome 7).

Zebrafish and the Teleost Genome Duplication

Exploration of the zebrafish genetic map provided an explanation for the early conundrum that "zebrafish has too many genes," for example, three *engrailed*-related genes rather than two as in mammals (Ekker, Wegner, Akimenko, & Westerfield, 1992),: five *msx*-family genes rather than three in mammals (Akimenko, Johnson, Westerfield, & Ekker, 1995; Postlethwait, 2006): and many "extra" *hox*' cluster genes compared to human (Prince, Joly, Ekker, & Ho, 1998). When these "extra" genes were placed on the emerging zebrafish genetic maps, conserved gene content, also known as conserved synteny analyses, showed that they usually resided in pairs of duplicated chromosome segments that represent two fragmented copies of portions of individual human chromosomes (Amores et al., 1998), as would be expected from a genome duplication event. Comparing the seven zebrafish *hox* clusters to the five that had been cloned in fugu pufferfish (Aparicio et al., 1997) showed that each fugu *hox* cluster was orthologous to one zebrafish cluster, including duplicated *hoxa* clusters. The simplest explanation was that the *hox* cluster duplication event occurred before the divergence of zebrafish and pufferfish lineages early in the teleost radiation. Because orthologs of segments of human chromosomes appeared to be present in two copies in both zebrafish and pufferfish, the simplest hypothesis was a whole-genome duplication event shared by clupeocephalan teleosts (Postlethwait, Amores, Force, & Yan, 1998; Taylor, Braasch, Frickey, Meyer, & Van de Peer, 2003; Taylor, Van de Peer, Braasch, & Meyer, 2001), which includes the last common ancestor of zebrafish and fugu and all other living teleosts except the elopomorphs (eels) and osteoglossiforms (bony tongues). Recent data shows that these two basally diverging teleost groups also share the duplication event (Bian et al., 2016; Du et al., 2019; Gallant, Losilla, Tomlinson, & Warren, 2017; Henkel et al., 2012; Jansen et al., 2017; Vialle et al., 2018), which is now called the *teleost genome duplication* (TGD). The TGD occurred after the divergence of teleosts from their sister group, the Holostei (including gars and bowfin), because spotted gar has just one chromosome segment orthologous to each human chromosome segment (Amores, Catchen, Ferrara, Fontenot, & Postlethwait, 2011; Braasch et al., 2014, 2015, 2016).

Hox gene clusters provide a typical example for the evolution of duplicate chromosome segments after genome duplication. The human genome contains four *HOX* gene clusters called *HOXA, HOXB, HOXC*, and *HOXD* on four different chromosomes (Fig. 3.3A). These four paralogons can be explained by the hypothesis of two rounds of whole-genome duplication early in the vertebrate lineage, called the *vertebrate genome duplications* (VGD1 and VGD2), in which a chromosome with one ancestral *hox* cluster duplicated twice to produce four chromosomes, each with one *hox*-cluster and its surrounding genes (Dehal & Boore, 2005; Nakatani, Takeda, Kohara, & Morishita, 2007; Sacerdot, Louis, Bon, Berthelot, & Roest Crollius, 2018, but see Smith et al., 2018). Then the third round of genome duplication, the TGD, resulted in eight *hox* clusters. The eight zebrafish *hox* clusters (*hoxaa, hoxab, hoxba, hoxb, hoxca, hoxcb, hoxda, hoxdb*) reside on eight different chromosomes, but only seven of the zebrafish *hox* clusters contain protein-coding genes (Amores et al., 1998). The eighth cluster, *hoxdb,* lacks any protein-coding gene, but contains a microRNA gene found in the *HOXD* complex of mammals, other vertebrates, and even Drosophila *hox* genes, and is flanked by orthologs of genes that border the human *HOXD* complex (Woltering & Durston, 2006). *Hox* clusters of percomorph fish, including medaka (Kurosawa et al., 2006), generally possess, like zebrafish, seven protein-encoding clusters, but the percomorph *hoxdb* cluster contains protein-coding genes, and reciprocally, percomorphs lack the *hoxcb* cluster.

Zebrafish Gene Nomenclature Conventions

Zebrafish gene nomenclature conventions attempt to reflect gene histories, including evolutionary relationships to human genes and the TGD (see https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Conventions). First, the zebrafish gene name is generally a lower-case version of its human ortholog, reflecting evolution from a single gene in their last common ancestor 450 million years ago. Second, when zebrafish has two genes that are TGD co-orthologs of the human gene, the gene name has an "*a*" or "*b*" appended to it, for example, *hoxa9a* and *hoxa9b* are co-orthologs of the human *HOXA9* gene. Zebrafish genes that are co-orthologs of a single human gene are ohnologs of each other, where ohnologs are paralogs derived from a genome duplication event honoring Susumu Ohno, who suggested the importance of genome duplication in vertebrate evolution (Ohno, 1970). Understanding the TGD and gene map locations showed that, for example, genes formerly called *shh* and *twhh* (Ungar & Moon, 1996) are actually TGD co-orthologs of the human *SHH* (*sonic hedgehog*) gene, and so are

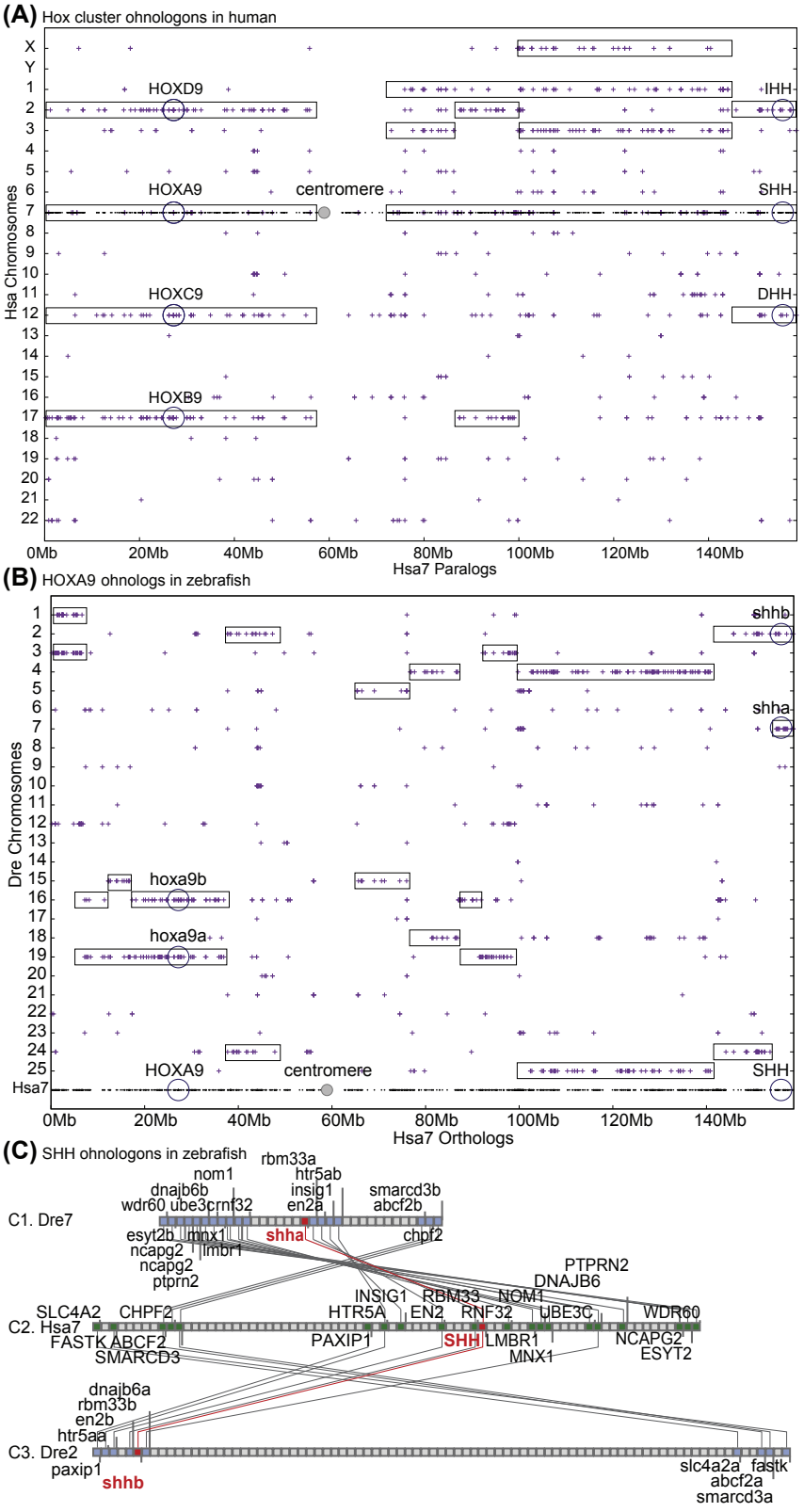

FIGURE 3.3 Evidence for genome duplication. (A) A dot plot of Hsa7 paralogs on other human chromosomes. *Black dots* represent genes along Hsa7; their paralogs on other chromosomes are plotted directly above or below the gene's position on Hsa7. Genes on the left arm of Hsa7 have paralogs, mainly on Hsa2, Hsa12, and Hsa17, including *HOX9* paralogy group genes (*HOXA9, HOXB9, HOXC9,* and *HOXD9*) in the four human *HOX* clusters, as would be expected after two rounds of whole-genome duplication, VGD1 and VGD2. Parts of the right arm of Hsa7 are also present in three or four copies, including three *hedgehog* gene paralogs, *SHH, DHH,* and *IHH.* (B) A dot plot of Hsa7 paralogs on zebrafish chromosomes. *Black dots* represent genes along Hsa7, at the bottom; their orthologs or paralogs on zebrafish chromosomes are plotted directly above the gene's position on Hsa7. Genes surrounding *HOXA9* on Hsa7 have zebrafish orthologs and coorthologs mainly on Dre16 and Dre19, including *hoxa9a* and *hoxa9b* in two of the eight zebrafish *hox* clusters, as would be expected after the TGD. Parts of the rest of Hsa7 also tend to be present in two copies, including two *sonic hedgehog* gene paralogs, *shha* and *shhb.* (C) Double conserved synteny in the zebrafish. C1: The portion of Dre7 containing *shha*. C2: A part of Hsa7 around *SHH.* C3: The neighborhood of *shhb* on Dre2. Lines connect orthologs.

now known as ohnologs, *shha*, and *shhb*, respectively. This nomenclature scheme reflects history and connects zebrafish genetics more appropriately to human biology. Third, genes derived by tandem duplication have a ".1" or ".2" appended to the name, like *alpi.1* and *alpi.2* (Yang, Wandler, Postlethwait, & Guillemin, 2012).

From a practical standpoint, gene names sometimes change over time; for example, the hedgehog genes discussed above; in addition, different authors sometimes use different names for the same gene. Thus, it is essential that, before zebrafish researchers assign a name to a new gene or change a gene name, they contact ZFIN (Zebrafish Information Network) at nomenclature@zfin.org to confirm appropriate usage. In addition, various software applications for RNA-seq or single-cell RNA-seq or whole-genome sequence annotation can use different sources to assign gene names, which can lead to data loss or misapplication. Fortunately, the ZFIN gene search function recognizes and displays gene synonyms. Tracking stable ENSEMBL Gene identifiers (e.g., ENSDARG00000068567 for *shha*) will likely improve a project's long-term data integrity.

Chromosome Evolution After the Teleost Genome Duplication

The chromosome neighborhood that includes the human *SHH* gene illustrates several general principles concerning chromosome evolution in the zebrafish lineage. First, the region shows double conserved synteny with zebrafish: two regions of the zebrafish genome (Fig. 3.1A and C, parts of Dre7 and Dre2) contain genes that are orthologs and co-orthologs of the human genes surrounding *SHH* (Fig. 3.1B). Second, several genes in the human segment are often present in duplicate on both zebrafish ohnologons, for example, besides duplicates of *SHH*, this region has duplicates of *ABCF2*, *SMARCD3*, *HTR5A*, *EN2*, and *DNAJB6*. On the other hand, many genes in the human region are present in a single copy on Dre7 and Dre2 due to gene loss after the TGD (Fig. 3.1). Third, chromosomal rearrangements, especially inversions and transpositions in one or both lineages, have caused gene orders to often differ in human and zebrafish genomes. For example, the orthologs of 11 genes at the left end of the chromosome segment shown in Fig. 3.1A are in the same order in humans but are transposed relative to three genes at the right end of the chromosome segment. Fourth, many human genes in the portion of Hsa7 shown in Fig. 3.1B that do not have orthologs in the portions of Dre2 and Dre7 shown in Fig. 3.1A and C have orthologs on other parts of these two zebrafish chromosomes. Fifth, other genes in the *SHH* region of Hsa7 may be on zebrafish chromosomes other than Dre2 and Dre7 due to chromosomal translocations. Inversions and translocations were essential after the TGD to rediploidize the genome, to return a tetraploid genome to a diploid state. In a tetraploid, four homologous chromosomes (two pairs) form chiasma in prophase I of meiosis and segregate irregularly during anaphase I, thus, giving rise to aneuploid gametes, many of which can lead to lethal aneuploid offspring. Chromosome rearrangements that make the two pairs of original chromosomes fail to pair with each other would reduce recombination between them, and thus, increase the likelihood of forming euploid "diploid" gametes, which can lead to euploid progeny with twice as many chromosomes as before the TGD.

In addition to local rearrangements by inversions and translocations, the zebrafish lineage experienced multichromosomal changes after its divergence from the spotted gar lineage. Spotted gar, whose lineage diverged from teleosts before the TGD, has 58 chromosomes, many of which are conserved intact with some tetrapods for 450 million years. If the last common ancestor of zebrafish and gar had a similar number of chromosomes, and the TGD doubled the number of chromosomes, then teleosts should have far more chromosomes than the 25 possessed by zebrafish and most other teleosts; evidently, chromosome fusions reduced chromosome numbers. A comparison of genes on chromosomes of gar, chicken, and teleosts suggests that these chromosome fusions occurred in the teleost lineage after it diverged from gar but, somewhat surprisingly, before the TGD (Braasch et al., 2016). Genome-wide chromosome fusions like this have occurred recently in some lineages (e.g., the Antarctic bullhead notothen (Amores, Wilson, Allard, Detrich, & Postlethwait, 2017)).

Gene Evolution After the Teleost Genome Duplication

The zebrafish *shh* ohnologons (Fig. 3.3C) reflect the finding that fewer genes were retained as duplicates in the zebrafish genome after the TGD than relapsed to a single copy. Estimates suggest that about 20%–25% of genes in the preduplication ancestor are currently retained in duplicate (Braasch & Postlethwait, 2012). In contrast, the duplicate retention rate for miRNA genes is substantially higher, at about 40% (Braasch et al., 2016). After genome duplication, all regulatory elements and all protein-coding domains are present

in duplicate, causing the two genes to be totally redundant except for dosage issues (Fig. 3.4A and B). Tandem gene duplication events, on the other hand, are not as predictable, with some regulatory elements or coding domains at the ends of duplicated regions perhaps left uncopied. Gene loss, which can occur by mutation to a pseudogene, say by mutation to a premature stop codon (Fig. 3.4C, also called *nonfunctionalization* (Force et al., 1999)), is the most frequent fate of gene duplicates. To explain the retention of both duplicates, Susumu Ohno suggested that one copy preserves the original gene functions while the other copy assumes a new, positively selected function (Ohno, 1970).

Allan Force suggested a revised framework for viewing the preservation of duplicated genes (Force et al., 1999). If one copy, say the "*a*" copy, retained all original functions and in addition evolved a new advantageous function, then the other copy, the "*b*" copy that simply preserved the original functions, would be redundant and would usually be lost. If both copies are to be permanently preserved after the origin of a new function in one of them, then the "*a*" copy would have to both evolve a new beneficial function (the star in Fig. 3.4D) AND lose an original, essential function that the "*b*" copy maintains (the pink regulatory element in Fig. 3.4D) so that the "*a*" copy is preserved by its new advantageous function, and the "*b*" copy persists because it possesses an essential ancestral function that the "*a*" copy lacks, a situation called duplicate gene preservation by neofunctionalization (Force et al., 1999). Alternatively, duplicated genes could be preserved by the reciprocal loss of ancestral essential functions, where the "*a*" copy loses a function that the "*b*" copy maintains (the orange regulatory element in Fig. 3.4E), and reciprocally, the "*b*" copy loses a function that the "*a*" copy retains (the pink regulatory element in Fig. 3.4E), called *duplicate gene preservation* by subfunctionalization (Force et al., 1999). Subfunctionalization is important because it occurs solely by degenerative evolution—the reciprocal knockout of ancestral essential functions—and does not require the "invention" of any previously nonexistent function as required by neofunctionalization (duplication, degeneration, complementation, DDC).

Note that in all three types of duplicate gene evolution, additional functions can be lost or gained over time. For example, after nonfunctionalization, the entire gene with all of its coding domains and regulatory elements could become unrecognizable due to mutation. After neofunctionalization, all of the ancestral functions except the new positively selected function could be lost on one copy while the other copy could retain all ancestral functions. After subfunctionalization, either or both preserved copies could reciprocally lose additional ancestral functions or could gain new functions. Thus, it is important to distinguish between the initial force that preserves both duplicates, and subsequent evolutionary processes that can occur given that both copies still exist (Braasch, Bobe, Guiguen, & Postlethwait, 2018; Sandve, Rohlfs, & Hvidsten, 2018).

Although the relative frequency of duplicate gene preservation by neofunctionalization versus subfunctionalization is not yet known, evidence suggests that

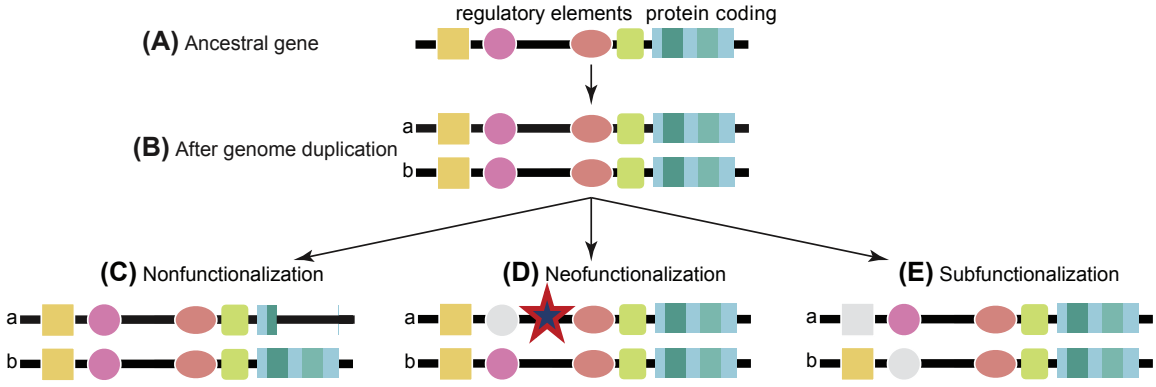

FIGURE 3.4 Duplication, degeneration, complementation model for the preservation of gene duplicates. (A) The ancestral, single-copy gene with regulatory elements and protein-coding domains. (B) Two identical gene copies after genome duplication. (C) Nonfunctionalization destroys gene activity, for example, by an early stop codon mutation. (D) Neofunctionalization involves the gain of a beneficial function (*star*) and loss of an ancestral essential function in one gene copy (gray circle) and the maintenance of that lost gene function in the other gene copy (pink circle) so that both copies are essential. (E) Subfunctionalization involves the reciprocal loss of ancestral functions (gray square and gray circle) so that the two gene copies complement each other, and both are essential for survival. Once preserved, gene copies can further evolve new functions or reciprocally lose additional functions. Functions can be regulatory elements or protein-coding domains.

both mechanisms play an important role in zebrafish genetics. Some examples include *en2a* and *en2b* (Fig. 3.3C) (Force et al., 1999; Postlethwait, Amores, Cresko, Singer, & Yan, 2004) and *atp1a1* paralogs (Serluca, Sidow, Mably, & Fishman, 2001), where TGD duplicates are expressed in alternative ancestral expression domains; *mitfa* and *mitfb*, where the "*a*" and "*b*" copies use different promotors (Lister, Close, & Raible, 2001); hematopoietic growth factor genes (Pazhakh & Lieschke, 2018); *tlr5a* and *tlr5b* (Voogdt, Wagenaar, & van Putten, 2018); fatty acid-binding protein genes (Laprairie, Denovan-Wright, & Wright, 2017), and many others (Pasquier et al., 2017).

For zebrafish genetics, the consequence of the TGD followed by subfunctionalization is that researchers must examine both duplicates to draw valid inferences regarding the functions of the ancestral preduplication gene, and thus, to connect zebrafish research to human biology. The consequence of neofunctionalization as a mechanism for duplicate gene preservation is that one or both of the zebrafish gene ohnologs may possess novel roles not already present in the ancestral preduplication gene, and hence in humans.

The Zebrafish Genome Sequence Assembly

With the accumulation of mutated genes to clone and the growing utility of zebrafish as an experimental model for vertebrate gene function, sequencing the zebrafish genome became a priority (Howe et al., 2013). Efforts to sequence the zebrafish genome from the TU strain began at the Wellcome Trust Sanger Institute (UK) in 2001 and culminated in the formal genome article published in 2013 (Howe et al., 2013). Initial forays sequenced DNAs extracted from about a 1000 TU individuals, a strategy intended to distinguish SNPs that were preexisting in the population from mutations chemically induced in this strain. The published genome assembly combined sequences from high-quality finished clone sequences and whole-genome shotgun sequences to assemble contigs and scaffolds that were then anchored onto the Sanger AB-TU meiotic map (SATmap) to achieve a chromosome-level assembly (Howe et al., 2013). The zebrafish genome assembly, currently in version GRCz11, is updated by the Genome Reference Consortium (https://www.ncbi.nlm.nih.gov/grc/zebrafish) and the Alliance for Genome Resources (https://www.alliancegenome.org/). The zebrafish genome assembly can be accessed through multiple web-based browsers, such as ZFIN (http://zfin.org/action/gbrowse/), Ensembl (http://www.ensembl.org/Danio_rerio/Info/Index), NCBI (https://www.ncbi.nlm.nih.gov/genome/gdv/?org=danio-rerio), or UCSC (https://genome.ucsc.edu).

Major features of the zebrafish genome include its size, burden of repetitive sequences, genetic similarity to human genes, high rate of heterozygosity, and frequency of evolutionarily fixed chromosome rearrangements. With 1.674 GB (giga base pairs) in the assembly version GRCz11, the zebrafish genome is substantially larger than that of many teleosts, four times the fugu pufferfish genome (0.391 GB, version FUGU5) (Elgar, 1996), more than twice as large as medaka (0.734 GB, ASM223467v1) (Kasahara et al., 2007), and it reaches about half the size of the human genome (3.609 GB, GRCh38.p12) (Freeman et al., 2007).

The bloating of the zebrafish genome is due to zebrafish-specific amplification of certain transposable elements, which occupy about 55% of the zebrafish genome (Chalopin, Naville, Plard, Galiana, & Volff, 2015; Howe et al., 2013). This huge content of repetitive DNA frustrated early zebrafish genome sequencing and assembly efforts.

The current zebrafish genome annotation contains 25,592 coding genes and an additional 6599 noncoding genes, including 1046 microRNA genes (Desvignes, Beam, Batzel, Sydes, & Postlethwait, 2014). The GRCh38.p12 version of the human genome sequence contains just 20,465 annotated protein-coding genes, 20% fewer than zebrafish (25,592). The larger gene catalog in zebrafish might reflect the legacy of the teleost genome duplication, because many other well-studied teleost fish also have more annotated protein-coding genes than human (e.g., Mexican tetra, 26,698 (McGaugh et al., 2014) and medaka, 23,622 (Kasahara et al., 2007)), but spotted gar, which diverged from the teleost lineage before the TGD, has just 18,341 genes (LepOcu1 (Braasch et al., 2016)). About 70% of human genes have at least one ortholog in zebrafish, and reciprocally, about 70% of zebrafish genes have at least one ortholog in the human genome (Howe et al., 2013). About 47% of the human/zebrafish orthologs are one-to-one pairs. The finding that 70%–80% of human genes that have been associated with a disease or specific human phenotypes have an ortholog in zebrafish enhances the value of zebrafish as a model for human biology.

Zebrafish has an exceedingly high rate of genetic polymorphisms. The sequencing of the two individual founders of the SATmap, each of which was completely homozygous at all loci, revealed about seven million SNPs, which is far greater than that seen between any two humans (Genomes Project et al., 2012; Howe et al., 2013). The sequence of a single zebrafish taken from the wild in northeastern India also had about seven million single-nucleotide and structural polymorphisms with respect to the reference genome sequence (Patowary et al., 2013).

Chromosome rearrangements also impact zebrafish genetics. Some analysts had suggested that the zebrafish

genome experienced more intrachromosomal rearrangements, such as inversions or transpositions, after the TGD than other fish lineages (Semon & Wolfe, 2007). After building synteny blocks using only genes with orthologs among all species tested (zebrafish, stickleback, medaka, green spotted pufferfish, and chicken, which have gene orders that are well-conserved from the ancestral stem bony vertebrate), zebrafish appears to have about the same intrachromosomal rearrangement rate as other teleosts: about 40% of genes that are neighbors in chicken have orthologs that are also neighbors in zebrafish, stickleback, medaka, and pufferfish (Howe et al., 2013).

In contrast to intrachromosomal rearrangements, interchromosomal rearrangements, such as translocations and chromosome fissions and fusions, are more frequent in the zebrafish lineage than in some other teleost lineages. If no interchromosomal rearrangements had occurred after the TGD, then ohnolog pairs would occupy the same total number of chromosomes as twice the preduplication chromosome count. Each translocation or chromosome fission after the TGD, however, would increase the number of chromosomes with ohnolog pairs. Chromosome fusions, on the other hand, might decrease the number of chromosomes with ohnolog pairs, and the comparator species have fewer chromosomes than zebrafish, whose haploid number is 25 (stickleback, 21; medaka, 24; green spotted pufferfish, 21). Nevertheless, it takes more chromosomes in zebrafish to reach 1% of the total number of ohnologs than it does for any of the other species, consistent with more translocations and/or chromosome fusions in the zebrafish lineage than in the three percomorph fish tested (Howe et al., 2013). Despite this finding, most of the interchromosomal rearrangements observed comparing zebrafish to human are shared among teleosts; for example, contrast breaks in syntenies comparing Hsa7 to the zebrafish genome with breaks comparing Hsa7 to the medaka genome (Fig. 3.5). This result shows that these synteny breaks occurred before the divergence of the zebrafish and medaka lineages over 250 million years ago (Near et al., 2012).

Genetics of Zebrafish Sex Determination

Zebrafish geneticists "using standard lines of zebrafish that have long been maintained in laboratories are often plagued by severe sex ratio distortions" (Lawrence, Ebersole, & Kesseli, 2008). This situation produces problems for researchers trying to mate specific rare genotypes and poses the interesting question of what causes an individual zebrafish to develop ovaries or testes. Laboratory strain zebrafish pass through a "juvenile ovary" phase, in which gonads form meiotic oocytes that die in individuals that become males, and survive in fish that become females (Rodriguez-Mari et al., 2010; Takahashi, 1977; Uchida, Yamashita, Kitano, & Iguchi, 2002; Wang, Bartfai, Sleptsova-Freidrich, & Orban, 2007). Animals containing gonads developing without germ cells become males (Siegfried & Nusslein-Volhard, 2008; Slanchev, Stebler, de la Cueva-Mendez, & Raz, 2005), and specifically, meiotic oocytes are required to maintain ovary differentiation (Dranow, Tucker, & Draper, 2013; Draper, McCallum, & Moens, 2007; Rodriguez-Mari et al., 2010, 2011), leading to the hypothesis that meiotic oocytes signal the gonadal support cells to maintain expression of aromatase (Kossack & Draper, 2019; Rodríguez-Marí & Postlethwait, 2011), the enzyme that converts testosterone to estrogen. Candidate signaling mechanisms include the FGF, BMP, and WNT signaling pathways (Dranow et al., 2016; Kossack et al., 2019; Leerberg, Sano, & Draper, 2017).

Environmental factors can strongly affect the sex ratio in zebrafish, with harmful factors like gamma rays, hypoxia, high density, high temperature, altered thermocycles, and poor nutrition favoring male development (Abozaid, Wessels, & Horstgen-Schwark, 2012; Delomas & Dabrowski, 2018a, 2018b; Hosseini, Brenig, Tetens, & Sharifi, 2019; Liew et al., 2012; Ribas, Liew, et al., 2017; Ribas, Valdivieso, Diaz, & Piferrer, 2017; Santos, Luzio, & Coimbra, 2017; Shang, Yu, & Wu, 2006; Valdivieso, Ribas, & Piferrer, 2019; Villamizar, Ribas, Piferrer, Vera, & Sanchez-Vazquez, 2012; Walker-Durchanek, 1980). However, it is unlikely that zebrafish has an established environmental sex determination mechanism (ESD) like some fish and some sauropsids (reptiles) (Charnier, 1966; Conover, 1984; Lang & Andrews, 1994; Merchant-Larios & Diaz-Hernandez, 2013; Mork, Czerwinski, & Capel, 2014; Ospina-Alvarez & Piferrer, 2008) because male and female zebrafish develop in tanks maintained at constant temperatures, and single pair matings show that zebrafish sex determination has a strong genetic basis (Liew et al., 2012). Nevertheless, the offspring of a single fully homozygous doubled haploid AB fish mated to a completely homozygous doubled haploid TU fish made genetically identical males and females, showing that genetic differences are not essential to make the two sexes in zebrafish (Howe et al., 2013).

Several studies have attempted to isolate genetic markers linked to sex determination in zebrafish strains adapted to laboratories, but each identified different regions of the genome, often several regions that appear to be important in the same study (Anderson et al., 2012; Bradley et al., 2011; Howe et al., 2013; Liew et al., 2012; Liew & Orban, 2014; Orban, Sreenivasan, & Olsson, 2009; Siegfried, 2010; Tong, Hsu, & Chung, 2010; Wilson et al., 2014), suggesting a polygenic sex-determination system.

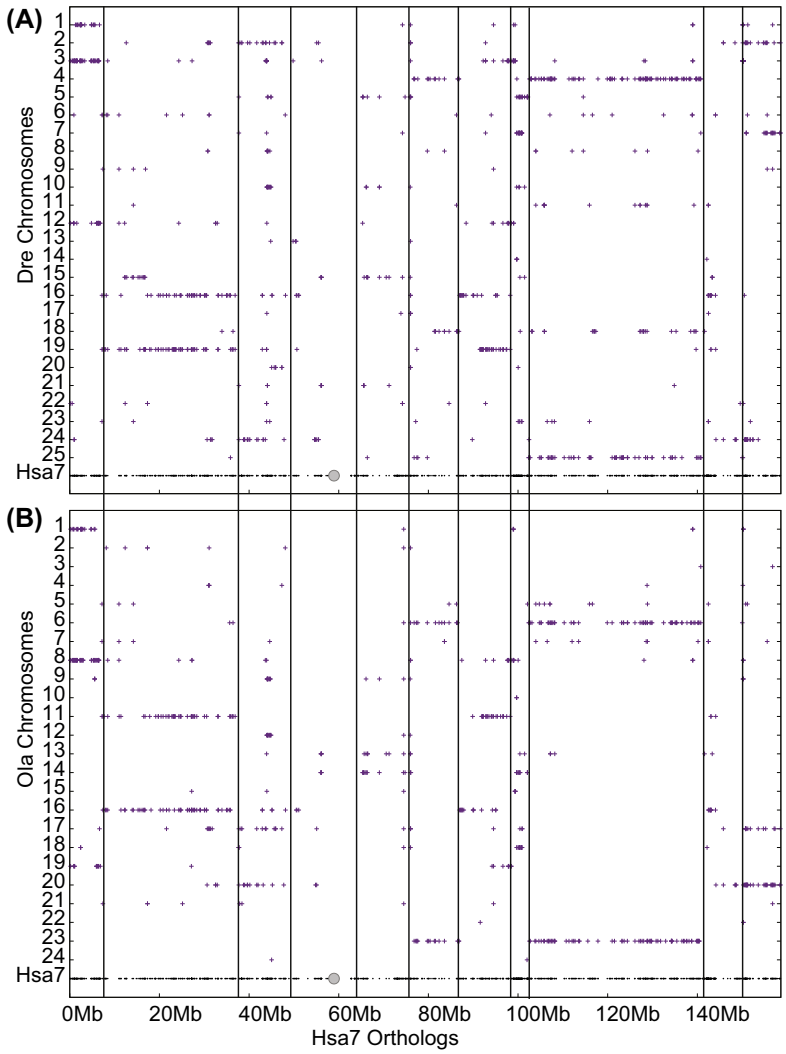

FIGURE 3.5 Most chromosome rearrangements occurred before the diversification of the largest group of teleosts. (A) A dot plot comparing human chromosome 7 to the zebrafish genome, with gene orders as in Hsa7. (B) A dot plot comparing Hsa7 to the medaka genome. Most breaks in synteny mapped onto the human chromosome are in the same place for both teleosts, as expected if most chromosome rearrangements occurred after the TGD but before the divergence of the medaka and zebrafish lineages more than 250 million years ago.

Two studies included zebrafish strains that had not been adapted to laboratory life using tens of thousands of DNA polymorphisms identified by sequencing the ends of restriction enzyme fragments (RAD-tags). These investigations identified a Sex-Associated Region on Dre4 (*sar4*) about 2 MB long at the distal tip of the long arm of chromosome Dre4 (Anderson et al., 2012; Wilson et al., 2014). Recall from Figs. 3.1 and 3.2 that Dre4 is a special chromosome, the only one with nearly an entire arm appearing as heterochromatic and late replicating, properties shared by many sex chromosomes, for example, the human Y chromosome. Identified polymorphisms showed that female zebrafish are heterogametic and male zebrafish tended to be homogametic, as expected for a WZ female/ZZ male chromosomal sex determination mechanism. Chromosomally, WZ individuals sometimes developed as phenotypic males, but ZZ individuals never developed as females. This finding suggests that (1) the W chromosome contains a gene or allele that is necessary but not sufficient to cause a gonad to develop into an ovary, or (2) that two doses of the Z chromosome actively extinguish ovary development.

The molecular genetic sex determining factor in *sar4* has not yet been identified, and so we do not yet know how it works. Identifying the factor is stymied by two factors: the nature of sex chromosomes and the animals used for the zebrafish reference genome sequence. The long right arm of chromosome Dre4 is late replicating and largely heterochromatic (Fig. 3.1) and has a plethora of repetitive elements (Howe et al., 2013). These repetitive regions have thwarted the assembly of the

sequenced genome around *sar4*. In addition, the reference genome sequence comes from the AB and TU strains of fish (Howe et al., 2013), which appear to have lost sex-specific genetic polymorphisms. The AB strain was produced from multiple rounds of gynogenesis (Walker-Durchanek, 1980), where offspring retain chromosomes only from the mother, each individual is homozygous for all loci, and gynogens tend to develop as males, presumably due to the deleterious effects of inbreeding (Delomas & Dabrowski, 2018a). The TU strain was developed to be lethal free by multiple single-pair matings (Mullins, Hammerschmidt, Haffter, & Nusslein-Volhard, 1994). In both cases, the accidental use of WZ females mated to sex-reversed WZ males could produce WW individuals, which can develop into either females or males (Wilson et al., 2014). Thus, by chance, both AB and TU strains could have drifted to become WW and still produce both males and females. If the sequenced genome comes from chromosomally WW fish, then it will lack a polymorphism for the primary genetic sex-determining mechanism. Over the more than two decades of laboratory domestication, the AB and TU strains, due to "natural" selection imposed by zebrafish geneticists, apparently evolved alternative environmental or genetic factors that increase the likelihood that males can develop, and these alleles are likely the weak factors identified by some genetic studies (Anderson et al., 2012; Bradley et al., 2011; Howe et al., 2013; Liew et al., 2012). Identifying the molecular genetic nature of the sex determinant in *sar4* should be a research priority of zebrafish geneticists.

Conclusions

Zebrafish provides a remarkably helpful system to investigate the molecular, cellular, and genetic mechanisms underlying a wide variety of processes common to all vertebrates. To fully leverage the zebrafish system, we must understand its genome, what it contains, and how it got here. The legacy of the teleost genome duplication and the partitioning of ancestral vertebrate gene functions between retained ohnologs provides a natural experiment useful for dissecting how the genome functions. Coupled with modern ways to study chromatin modifications and gene expression in single cells, zebrafish genetics will continue to contribute to understanding how animals with backbones develop, function, and evolve.

Acknowledgments

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CHAPTER

4

Geographic Range and Natural Distribution

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Geographic range and natural distribution are two of the fundamental characteristics of a species (Gaston, 2003). This chapter provides a broad overview of the range and distribution of zebrafish. It describes the typical habitats of wild zebrafish, the biotic and abiotic factors that determine range limits, and the potential implications of environmental change and human interference. Knowledge on wild zebrafish populations and the environment in which they live is vital for understanding the natural genetic and phenotypic variation, interpreting observations on their morphology, physiology, and behavior in the laboratory, and in designing and refining optimum housing systems and husbandry practices.

Geographic Range

The geographic range of a species comprises the regions in which that species can be found. The geographic ranges of fish species vary in size enormously. At one end of the spectrum are those whose distribution extends the entire world across tropical or temperate waters, such as the swordfish (*Xiphias gladius*) and the basking shark (*Cetorhinus maximus*) (Gaither, Bowen, Rocha & Briggs, 2016). At the other end of the spectrum are endemic species confined to a restricted area, such as the gizani (*Ladigesocypris ghigii*), which lives only in streams on the Greek island of Rhodes (Stoumboudi, Barbieri, Mamuris, Corsini-Foka & Economou, 2002), and the Quitobaquito pupfish (*Cyprinodon macularius eremus*), which is found in a single spring outflow in the Arizona desert (Douglas, Douglas & Brunner, 2001).

The factors that limit geographic ranges are so varied and the interactions among them so complex that they are not fully understood for any single species (Gaston, 2009). Climate (especially temperature) and water chemistry (including levels of dissolved oxygen and acidity) play an important role in determining the geographic range of freshwater fish species (Jackson, Peres-Neto & Olden, 2001). Biotic factors, such as predation and competition, are also influential (Gaston, 2003).

Determining the geographic range of a species can be tricky. In fact, it is usually impractical, in terms of time and budget, for a single researcher to conduct a complete survey across a large geographical area, and so data from a variety of sources, such as biological surveys, scientific reports, and museum specimens, are often relied on. The accuracy of this information can vary and without careful scrutiny errors in identification or taxonomy may perpetuate over time (Kottelat & Freyhof, 2007).

Geographic Range of the Zebrafish

The geographic range of the zebrafish extends across much of India, Bangladesh, and Nepal, from the Pakistan border in the west to the Myanmar border in the east, and from the foothills of the Himalaya in the north to the paddy fields of Karnataka in the south (Fig. 4.1; Engeszer, Patterson, Rao & Parichy, 2007). Geographically, this area comprises three major regions: the northern mountains, the Indo-Gangetic Plain, and the Indian peninsula. The northern mountains comprise the Himalaya and Hindu Kush ranges, which run from east to west in an arc across the entire northern boundary of India. South of the mountains, the Indo-Gangetic Plain encompasses the vast, fertile floodplains of the Indus, Ganges, and Brahmaputra rivers. These floodplains comprise most of northern India and almost all of Bangladesh. To the west lies the desert of Rajasthan, while to the south is the Indian peninsula, an immense plateau flanked by the coastal mountains of the Western and Eastern Ghats, which projects into the Indian Ocean (Thapar, 2004).

As with most species, the zebrafish is not evenly distributed throughout its range. Reliable first-hand reports document populations in the streams and

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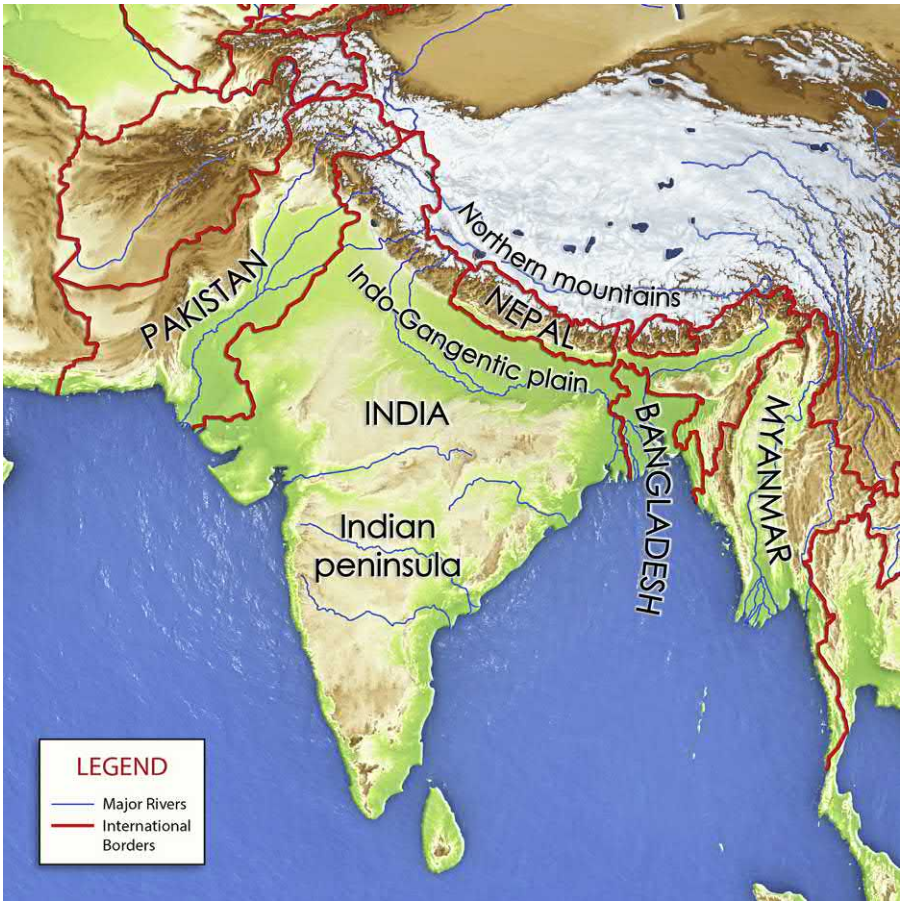

FIGURE 4.1 The geographic range of the zebrafish comprises the foothills of the northern mountains, the Indo-Gangetic Plain, and the Indian peninsula.

FIGURE 4.2 Wild zebrafish found in Bangladesh. *Photo by Gregory Paull.*

tributaries of rivers in the Himalaya drainage system of Nepal (Dhital & Jha, 2002), the floodplains of the Ganges and Brahmaputra rivers in India and Bangladesh (Fig. 4.2; Spence et al., 2006; Engeszer et al., 2007; Arunachalam, Raja, Vijayakumar, Malaiammal & Mayden, 2013), and along the Western Ghats (Dahanukar, Raghavan, Ali, Abraham & Shaji, 2011). Compilations of older records report specimens found in Rajasthan (Datta & Majumdar, 1970) and in the peninsula's southeast region (Menon, 1999). No systematic field studies have mapped the occurrence of the zebrafish throughout its range, and it may be missed from species surveys due to its small size and lack of commercial value (Spence et al., 2008). As a result, zebrafish may be more widely distributed than previously thought.

It should be recognized that the geographic range of any species is dynamic. It expands, contracts, or shifts over time in response to changes in climate or physical geography (Thomas, 2010). The ability of the zebrafish to expand or move its range boundaries in response to environmental change is constrained by the physical barriers of the northern mountain ranges to the north, east and west, and by the Indian Ocean to the south. Within its range boundaries, the dispersal of zebrafish to new areas in response to changing conditions is limited to the floodplains and drainage basins that it inhabits and is influenced by interactions with other species (predators and competitors) whose abundance

and diversity are also affected by climate (Thomas, 2010). Human activities, such as farming, flooding arable land to create paddy fields, and connecting previously unconnected water bodies with drainage ditches, may also influence the dispersal of zebrafish.

Home Range

Most animals live within a home range—an area that contains the resources that they need to avoid predation, find food, and reproduce (Welsh, Goatley & Bellwood, 2013). Usually, an animal's resource requirements change as it grows and matures, and therefore the size of its home range changes with ontogeny. With fish, the smaller the animal, the higher the risk of predation (Miller, Crowder, Rice & Marschall, 1988). A young fish may restrict its home range and avoid risky locations to avoid predators. As it grows, the young fish's vulnerability to predation decreases and its food requirement increases, allowing it to expand its home range. Finally, as the fish matures, its home range must be big enough to allow it find a mate and reproduce (Welsh et al., 2013). For example, gold-spot mullet (*Liza argentea*) spawn in coastal waters and the lower reaches of estuaries in tropical and temperate regions (Kendall & Gray, 2008). Larval mullet migrate into the estuaries and seek protection in dense vegetation or in mangrove forests where a labyrinth of tangled roots hampers the movements of large predators (Laegdsgaard & Johnson, 2001). As they grow, juvenile mullet develop greater mobility and can more easily escape from predators. This enables them to move to adjacent habitats, such as seagrass beds or mudflats, and then to open estuarine habitats where they feed on larger prey items before joining adult populations in deep waters (Laegdsgaard & Johnson, 2001).

Little is known about the average size of a zebrafish home range. Studying the home range of fish in the low visibility of vegetated habitats or turbid waters is challenging, especially with a diminutive, highly mobile species such as the zebrafish. Tagging individual fish to radiotrack their movements has been used successfully to study larger species (Cooke et al., 2013) but has not been attempted with wild zebrafish. However, recent advances in technology have resulted in smaller, more efficient tags and passive integrated transponders that increase the potential to track smaller fish species and juvenile life stages (Cooke et al., 2016). Innovations continue, and the home range of the zebrafish should soon be testable.

As water levels rise at the start of the monsoon, zebrafish are thought to migrate laterally from rivers, streams, and irrigation channels where they spend the dry months to flooded areas such as paddy fields where they spawn in the nutrient-rich waters. When water levels recede at the end of the monsoon, adults and young-of-the-year migrate out of the floodplains and into rivers, streams, and channels (Engeszer et al., 2007). Little is known about this lateral migration, and the distances covered have not been measured. Zebrafish home range size therefore varies seasonally and may differ in streams versus floodplains due to the correlation of home range size with food supply (Minns, 1995). The spatial behavior of zebrafish at all life stages and throughout the seasons needs to be assessed to understand the extent of their home-ranging behavior.

Natural Distribution

The terms geographic range and natural distribution are sometimes used interchangeably, but here we define natural distribution as "the specific areas, within its geographic range, in which a species occurs." For example, the *geographic range* of the zebrafish includes the Western Ghats of India. Within the Western Ghats, the *natural distribution* of the species includes the Thunga River in the state of Karnataka and the Kabini River in the state of Kerala where populations of zebrafish have been found (Arunachalam et al., 2013). A species' natural distribution may change over time, regardless of whether its geographic range moves, as habitats become more or less favorable due to changes in the physical or biological environment (Lawton, 1996).

Much of the zebrafish's natural distribution lies within the extensive floodplains of the Ganges and Brahmaputra rivers. Indeed, the zebrafish has been described as a "floodplain species" (Spence et al., 2008) and seems well adapted to constantly changing environmental conditions in the floodplains. Typical habitats of zebrafish include streams, drainage ditches, and ponds, often adjacent to rice paddies (McClure, McIntyre & McCune, 2006; Spence et al., 2006). In addition, Engeszer et al. (2007) and Arunachalam et al. (2013) found zebrafish in secondary and tertiary channels of large alluvial rivers. Together, these field studies report a range of habitat features in the specific locations where zebrafish were found. Some areas have still waters while others have slow to medium water flow. Water clarity ranges from clear to muddy, and substrates are recorded from silt at one end of the spectrum, through sand, gravel, pebbles, and boulders, to bedrock at the other end of the spectrum. Vegetation ranges from none at all to an abundance of submerged plants, with or without overhanging canopy. Some of these habitats are permanent while others are transitory and form during the monsoon. In summary, the zebrafish can be found in a diverse array of habitats throughout its huge geographic range; however, the geographic, climatic, and temporal factors that influence the distribution of zebrafish and the endogenous and exogenous pressures that drive them to occupy certain habitats are not well understood.

Floodplains

Floodplains are areas that are periodically inundated by the lateral overflow of rivers or lakes and/or by direct precipitation (Junk, Bayley & Sparks, 1989). They contain a medley of habitats and support diverse biotic communities. The floodplains of northern India and Bangladesh flood seasonally from June to October in response to monsoon rains and snow melt (Craig, Halls, Barr & Bean, 2004). Most of this vast region has been converted to agricultural land for the production of rice and other crops while fishing, by professional and subsistence fishers, contributes to floodplain livelihoods (Hoggarth et al., 1999). During monsoon rains, the floodplains are inundated with nutrient-rich waters, causing rapid growth of macrophytes and periphyton, increased concentrations of invertebrates, and an accumulation of detritus from vegetation and phytoplankton. Fish follow the advancing water's edge and flooded areas become important food-rich nurseries for larval and juvenile fish. When water levels drop, vegetation becomes stranded, the food supply decreases, and surviving adult and young-of-the-year fish of many species migrate from the floodplains to channels, rivers, and other permanent water bodies (Bayley, 1988).

Zebrafish are believed to spawn in shallow waters during the monsoon, and their opportunistic life history strategy of small body size, rapid growth, early maturity, and high fecundity allows them to reproduce and disperse while conditions are favorable and food is plentiful. Floodplain fishes rarely survive for more than a year (De Graaf, 2003), and field observations of wild zebrafish support this assumption (Spence, Fatema, Ellis, Ahmed & Smith,2007). Within the floodplains are a mosaic of habitat types, including major rivers and their secondary channels, canals, permanent lakes (*baors*), floodplain depressions (*beels*), excavated fish pits (*kuas*), and household ponds (*mathels*) (Hoggarth et al., 1999). Field studies have reported populations of zebrafish in most of these habitats.

Rivers

The two major floodplain rivers within the zebrafish's natural distribution are the Ganges and the Brahmaputra. Bedload sediments of sand and silt roll along the bottom of the main channels of both rivers, forming a sequence of dunes up to 6 m high, which slowly move along with the current (Garzanti et al., 2010). These shifting substrates are unfavorable for aquatic plants and invertebrates. As a result, vegetation tends to fringe the main channel borders and side channels where substrates are more stable (De Graaf, 2003). Fish abundance is highest at the channel borders where plant beds create diverse habitats and food is more plentiful (Junk, Bayley & Sparks, 1989).

FIGURE 4.3 Zebrafish have been found in tributaries of major rivers such as the Brahmaputra but have not been reported in the main rivers themselves. *Photo by Gregory Paull.*

Zebrafish have been captured in the tributaries and drainages of the Ganges and Brahmaputra, but none have been reported in the main rivers themselves (Fig. 4.3). Elsewhere in India, Arunachalam et al. (2013) collected zebrafish in a side channel of the Dikrong River, a major tributary of the Brahmaputra, and in a secondary channel of the Thunga River in the Western Ghats. During a field trip to investigate the habitats of wild zebrafish, Engeszer et al. (2007) traveled to northeast India and found populations in the Ghotimari River and in a tributary of the Jorai River, both in West Bengal, and further populations in the Dukan River in the hills of Meghalaya. The absence of zebrafish in major rivers is likely due to the effects of predation and competition or a lack of suitable food resources or spawning areas. Predators in rivers and streams affect habitat choice of prey species such as zebrafish, which tend to move into shallow waters or complex habitats or leave the site to avoid predators (Jackson, Peres-Neto & Olden, 2001). The effects of interspecific competition on river fish communities may also play a role in the absence of zebrafish from major rivers, although this has not been tested in the field.

Streams

Stream types range from perennial snow- or monsoon-fed hill streams that drain the northern mountains and the Western Ghats to seasonal *nalas* that develop during the monsoon. Zebrafish are absent from fast-flowing hill streams but have been found in slower streams with shallow waters (Fig. 4.4). They appear to have restricted ranges of current velocity and water depth but tolerate variety in other habitat

FIGURE 4.4 Zebrafish have been found in slow streams with shallow waters and abundant vegetation. *Photo by Gregory Paull.*

FIGURE 4.5 Sampling zebrafish in a shallow pond in Bangladesh. *Photo by Gregory Paull.*

features, such as substrate type and the presence or absence of submerged vegetation. A detailed account of the stream habitat preferences of zebrafish is given in a field report by Arunachalam et al. (2013) who collected individuals from 11 streams in the Western Ghats and northern and northeastern states of India. The team found zebrafish in stream areas with overhanging vegetation or undercut banks and in places where alcoves or shallow pools had been created by bedrock or large boulders. Substrates varied from silt and sand to cobbles and bedrock, but all the habitats had low water flow (3.5−13.9 cm/s) and shallow depth (<110 cm). These observations are consistent with other field studies (Engeszer et al., 2007; McClure, McIntyre & McCune, 2006; Spence et al., 2006).

Natural Still Bodies of Water

Natural wetlands comprise a variety of lakes, ponds, pools, and marshes. Permanent lakes, or *baors*, are often large and deep, fed by rivers, streams, or groundwater discharges, supplemented by monsoon rains, and have relatively small changes in water level. Most baors are fringed by marginal plants but contain little aquatic vegetation. In contrast, shallow lakes, or *beels*, are seasonal with large changes in water level. They form in natural depressions that flood during the monsoon. During the dry season, waters recede leaving small shallow lakes that eventually dry up. Beels support abundant aquatic vegetation and diverse communities of birds, fish, and invertebrates. Oxbow lakes, or *jheels*, formed by dead arms of rivers, are also seasonal, flooding deeply during the monsoon and drying partially or completely in winter. Isolated ponds, marshes, and swamps also contribute to the extensive wetlands of the Indian subcontinent (Gopal, Sengupta, Dalwani & Srivastava, 2010; Hoggarth et al., 1999; Khondoker, Hossain & Moni, 2014).

Zebrafish have been found in many wetland habitats. Populations have been recorded in beels and jheels (Arunachalam et al., 2013), in lakes and ponds (Fig. 4.5; Pritchard, 2001; Spence et al., 2006; Spence, Fatema, et al., set link 2007), and in shallow pools and swamps (Engeszer et al., 2007). All the recorded occurrences of zebrafish in wetlands have been in shallow areas, usually with abundant vegetation.

Man-Made Habitats

In addition to natural wetlands, zebrafish inhabit man-made habitats such as canals, fish pits, household ponds, and, famously, rice fields (Spence et al., 2008). Rice is grown throughout much of the Indian subcontinent. During the rainy season, rice fields flood and become connected to neighboring water courses and wetlands. The floodwater carries aquatic organisms, including fish, into the fields where invertebrates, plants, and microorganisms are a rich food source and rice plants provide hiding places and spawning sites. During the dry season, the water level drops and the fields stagnate and eventually dry, forcing fish to return to the water courses or become trapped in shallow water. Rice fields can be considered as agriculturally managed marshes, and fish harvested from rice fields are an additional source of income for farmers (Das, 2002; Fernando, 1993).

Zebrafish are well adapted to conditions in rice fields (Fig. 4.6) and adjacent irrigation and drainage ditches where shallow waters, abundant food, and lack of large predatory fish are ideal for them. They are eurythermal

FIGURE 4.6 Zebrafish are commonly found in rice fields. *Photo by Gregory Paull.*

(Cortemeglia & Beitinger, 2005) and tolerate the wide-ranging temperatures and fluctuating levels of oxygen that result from shallow water depth. Although zebrafish have little commercial value and are caught for food only in rural areas of Bangladesh (Hossain & Afroze, 1991), their presence in rice fields may benefit farmers by reducing the need for pesticides and fertilizers without lowering rice productivity (Lansing & Kremer, 2011).

Connectivity Between Habitats

Connectivity refers to the pathways that link habitats and the relative ease with which individuals can move through the landscape between habitat patches (Joint Nature Conservation Committee, 2017). It also refers to linkages between adjacent and distant habitats. The interconnected waterways, drainage ditches, and canals that crisscross much of the zebrafish's area of natural distribution, together with large-scale flooding during monsoons, create connectivity between populations, despite the movements of individuals being limited by their diminutive size (Gratton et al., 2004). One way to assess connectivity is to measure gene flow (Hedgecock, Barber & Edmands, 2007). A genetic analysis of four zebrafish populations in eastern India from sites 50 or more miles apart suggests that connectivity between the four habitats is continuous and that significant mixing of populations occur (Gratton et al., 2004). Another study found that populations in the Western Ghats, southern Bangladesh, and western and central Nepal are genetically distinct from those in the Ganges and Brahmaputra river basins, indicating that physical barriers exist between regions within the species' geographic range (Whiteley et al., 2011).

Temporal Distribution

The spacial ecology of zebrafish and how this changes over time is not well understood. Zebrafish are believed to migrate, at the onset of monsoon rains, from rivers, streams, and channels to flooded areas where they breed (Engeszer et al., 2007). However, the factors that influence this temporal distribution and the amount of time that populations spend in these different habitats have not been tested in the field. Similar questions have been addressed in mosquitofish, *Gambusia affinis*, and other small fishes by using minnow traps to determine their temporal dispersal in rice fields in the United States (Blaustein, 1989; Davey & Meisch, 1977). This simple method could be tested on zebrafish in rice fields and natural habitats. A deeper understanding of the ecology of zebrafish at a variety of spatial and temporal scales can be used to identify critical habitats and inform decisions regarding housing and husbandry methods for the generation and maintenance of laboratory zebrafish.

Climate

The Indian subcontinent is a region of extreme climates. In the far north is alpine tundra, in the far south are tropical rainforests, and in between is the wettest place on earth (Mawsynram, in the state of Meghalaya; Guinness World Records, 2018). The climate is influenced by the northern mountains, which prevent frigid air flowing down from central Asia, and by the western desert, which draws moisture-laden winds over the oceans. These monsoon winds dominate the region's climate and provide most of its rainfall (Fig. 4.7; Krishnamurthy, 2017). The zebrafish's areas of natural distribution fall within "subtropical humid zones" (the foothills of the northern mountains and the floodplains of the Ganges and Brahmaputra) and "tropical wet and dry zones" (the Western Ghats), according to the Koppen system of climatic classification (Köppen, 1884).

Seasons

The Indian subcontinent has four seasons. January and February, the coldest months, are considered the winter season. From March until May is the summer, or premonsoon season, when temperatures rise and remain high until the start of the monsoon rains in June. The monsoon season lasts until October and is followed by a postmonsoon, or "retreating monsoon" season which continues until the end of the year (Krishnamurthy, 2017). The life cycle and growth of tropical floodplain fish is highly seasonal, with maximum feeding and growth occurring in the

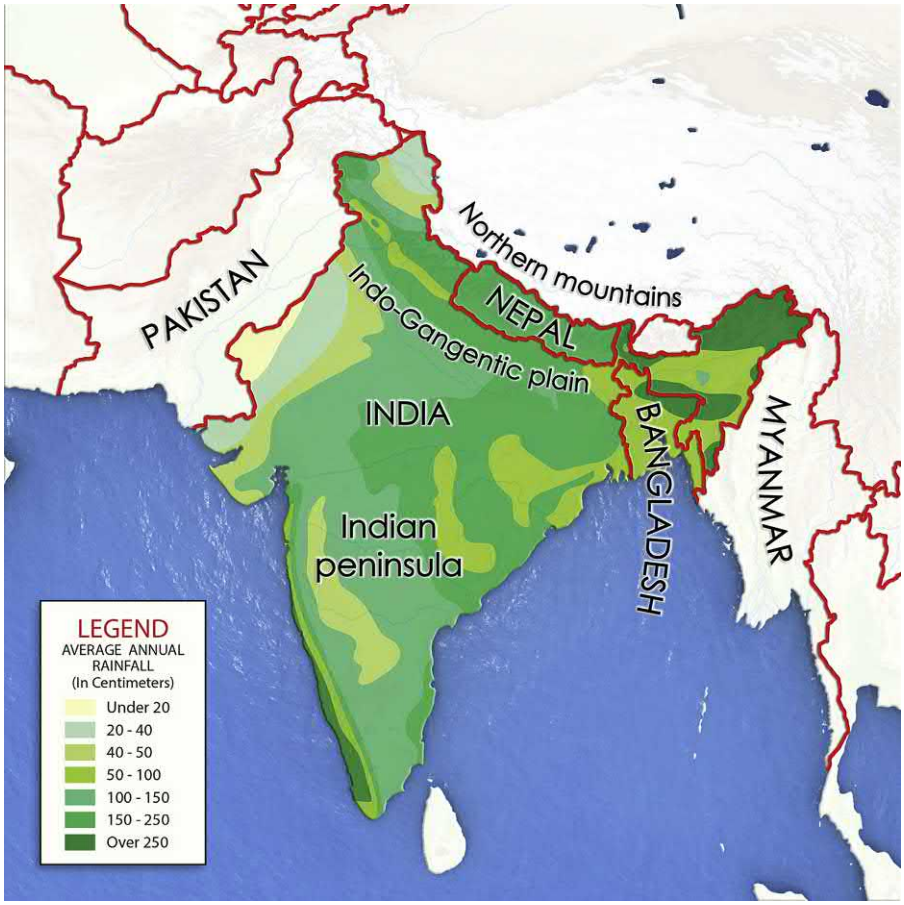

FIGURE 4.7 Annual rainfall across the Indian subcontinent.

monsoon and postmonsoon seasons (Hoggarth et al., 1999), and this is likely also true of zebrafish.

Winter

The relatively cold winter months of January and February are also the driest months throughout much of the subcontinent although there are a few exceptions. Snowfall is common in the northern mountains, and some areas, such as the Coromandel Coast in the east of the peninsula, receive heavy winter rainfall (Krishnamurthy, 2017). Zebrafish adults and young-of-the-year are believed to spend the winter in perennial streams and lakes (Engeszer et al., 2007) as many rice fields and other seasonally flooded areas are partially or completely dry at this time of year (Das, 2002).

Summer

The summer months of March, April, and May, also known as the "premonsoon" months, are characterized by rising temperatures and low humidity. Hot, dry winds originate in the western desert and blow over the Gangetic Plain, creating dust storms and drying vegetation. Violent storms, accompanied by heavy showers of rain or hail, occur in eastern and northeastern parts of the subcontinent, and thunderstorms develop in the far southern peninsula and Western Ghats (Jain, 2017). Summer storms are most intense in Bangladesh, where southerly cyclonic winds may reach speeds of 160 km per hour and cause severe flooding in coastal areas (Karim & Mimura, 2008).

Monsoon

The hot air that built up during the summer months creates low-pressure areas in the Gangetic Plains into which moisture-bearing southwestern monsoon winds flow from the Indian Ocean. The monsoon breaks on the west coast of India at the beginning of June, bringing heavy rains and strong winds, and reaches most parts of the subcontinent by the end of June. During July and August, monsoon rains are interspersed with dry spells. This pulsating of the monsoon is irregular and varies across the subcontinent, causing flooding in some areas and droughts in others (Jain, 2017). More than 12% of the

land surface of India is prone to annual flooding (Sharma & Priya, 2001), and in Bangladesh, annual floods submerge 20%–70% of the country (Mirza, 2002). As floodplains are inundated by monsoon rains, the biomass and production of periphyton and invertebrates increase and many fish species migrate from river channels to floodplains to breed and give their offspring the advantage of this timely opportunity to feed and grow (Hoggarth et al., 1999).

Postmonsoon

From the middle of September, the southwestern monsoon winds begin to weaken as the trough of low pressure over the Indo-Gangetic plains gradually moves to the Bay of Bengal. The winds then change direction and begin to blow from the northwest, causing the southwestern monsoon to "retreat." This change from monsoon to postmonsoon or "retreating monsoon" season is gradual and continues until the end of the year. The beginning of the postmonsoon is marked by a return of the intense storms that affect Bangladesh and the southern coast of India during the summer, but eventually, the monsoon recedes, clear skies replace dense clouds, temperatures drop, and the transition to the cooler, drier winter season is complete (Jain, 2017).

Regional Microclimates

Individual animals experience a series of fine-scale microclimates rather than the average conditions of their home range (Suggitt et al., 2011). Riparian and aquatic vegetation, water depth and air temperature all affect freshwater microclimates (Welch, Jacoby & May 1998). Increasing temperatures stimulate production of microorganisms and invertebrates and so benefit fish, but effects are negative if temperatures exceed the maximum for optimum fish growth (Welch, Jacoby & May 1998). Riparian vegetation shades the edges of streams and lakes, creating microclimates that provide cooler refuges for fish. This cooling effect decreases with increased water body size (Brazier & Brown, 1973). Small streams and ponds with low flow rates, shallow depth, and overhanging vegetation, as favored by zebrafish, are generally cooler in summer and warmer in winter than similar waters with no vegetative cover (Welch, Jacoby & May 1998). Interestingly, fish may move among habitats to take advantage of thermal diversity. For example, lake-dwelling juvenile sculpin (*Cottus extensus*) feed at the bottom of the lake during the day and spend the night at warmer temperatures higher in the water column, a behavior which is believed to aid digestion and therefore increase growth (Neverman & Wurtsbaugh, 1994). Similarly, stream-dwelling juvenile coho salmon (*Oncorhynchus kisutch*) feed in cold habitats where food is abundant and the move to warmer habitats to assimilate their food (Armstrong et al., 2013). Systematic fine-scale sampling of water temperature, depth, and vegetation could help identify environmental relationships, investigate microclimate use by zebrafish, and study changes in microclimates over the seasons.

Altitudes

Altitude and temperature are strongly correlated. Altitude creates gradients in temperature and precipitation and affects levels of dissolved oxygen in water, all of which impact fish diversity (Boll et al., 2016). Generally, species diversity decreases as altitude increases (Zhao, Fang, Peng, Tang & Piao, 2006). Zebrafish have been recorded at a wide range of altitudes, from 14 m in the coastal region of Orissa province in eastern India (Engeszer et al., 2007) to 1576 m in the hills of Arunachal Pradesh in northeastern India (Arunachalam et al., 2013). Most field reports, however, do not include altitude data, and it is possible that zebrafish occur at higher or lower altitudes than previously reported. In addition, likely differences in abundance and diversity of zebrafish prey and predators at different altitudes remain to be investigated.

Temperatures

Temperatures across India vary widely between seasons and across regions (Fig. 4.8). Northern and central areas experience summer temperatures in excess of 45°C, while the west coast and southern parts of the peninsula are generally 5–10°C cooler and temperatures in the far northern mountains rarely exceed 25°C (Jain, 2017). In Bangladesh, summer temperatures range from 38 to 41°C with only slight variations between regions, and daytime winter temperatures are typically 16–20°C (Siddik & Rahman, 2014). Northern parts of India have a mean daytime winter temperature of 20°C, but this may fall to below freezing point at night. Temperatures increase from north to south, with the west coast and southern India having daytime winter temperatures of around 30°C, falling to 20°C at night. In the Western Ghats, night-time winter temperatures may drop to freezing point (Jain, 2017; Jain, Agarwal & Singh, 2007).

Zebrafish are eurythermal (Cortemeglia & Beitinger, 2005). Their temperature tolerances in the wild are unknown, but field studies at sites where zebrafish were sampled report temperatures ranging from 12°C in Arunachal Pradesh (Arunachalam et al., 2013) to 39°C in

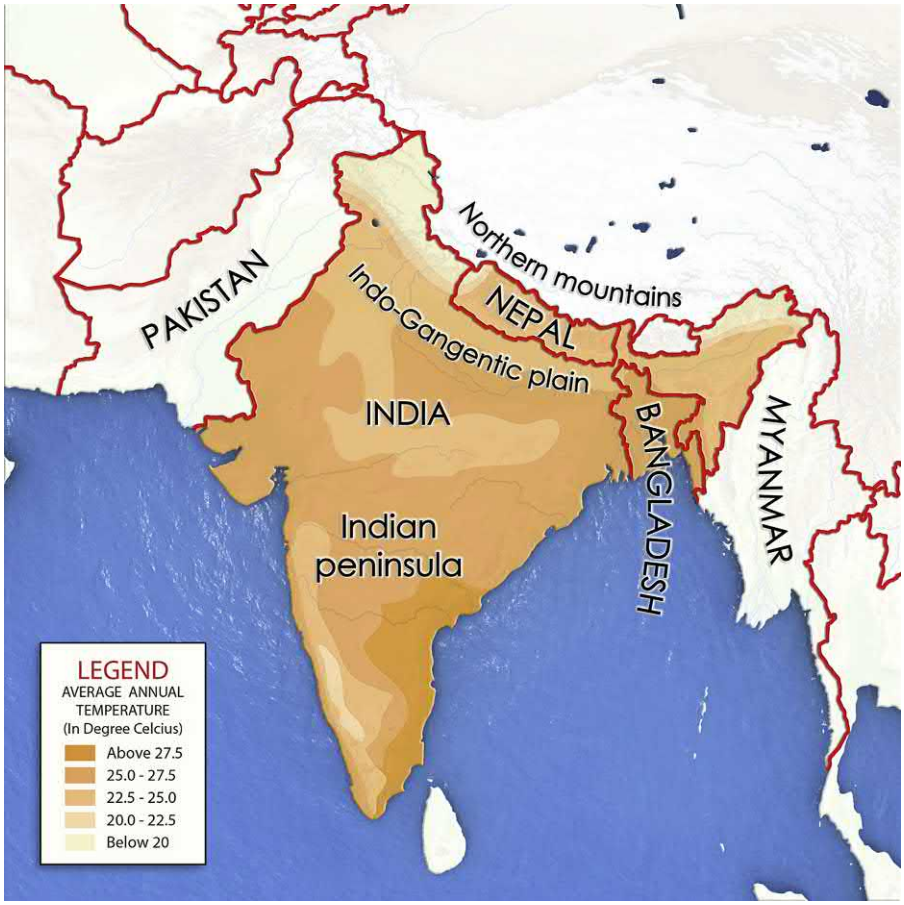

FIGURE 4.8 Mean annual temperatures across the Indian subcontinent.

Orissa (Engeszer et al., 2007). Laboratory studies suggest that wild-type zebrafish have a lower lethal temperature of $6.2 \pm 0.28°C$ and an upper lethal temperature of $41.7 \pm 0.35°C$ (Cortemeglia & Beitinger, 2005).

Water Conditions

Water conditions, including depth, flow, turbidity, pH and salinity, change drastically between wet and dry seasons in floodplains, rice fields, and streams. These variables contribute to the organization of the communities of organisms that occupy these areas. As flood waters rise, seasonally dry areas become colonized by invertebrates and fish that enter the floodplains from the river, forming a complex community in the nutrient-enriched waters. As flood waters recede, microbes in decomposing plant biomass consume large volumes of oxygen and releases carbon dioxide into the water, creating hypoxic conditions (Junk, Bayley & Sparks, 1989) that are intolerable to many species that must return to the rivers or become stranded in increasingly shallow and eutrophic water. Most of these environmental variables are regulated by water depth (Miranda, 2011). Overall, the ever-changing quality and mixing of waters creates a diverse floodplain ecosystem (Moyle, Crain & Whitener, 2007).

Depth

Zebrafish have been found in water depths from 5 cm (in an irrigation channel; Suriyampola Shelton, Shukla, Roy & Bhat, et al., 2015) to around 1 meter (in a small pond; Spence et al., 2006). Such shallow waters tend to have stronger fluctuations of temperature and dissolved oxygen levels than deeper waters (Miranda, 2011). They have reduced habitat complexity and may be more turbid and eutrophic due to agitation of the benthos by bottom-feeding organisms (Miranda, 2011). For small species such as zebrafish, shallow waters offer protection against larger-bodied fish and piscivores that tend to be intolerant of these conditions (Shields & Knight, 2013), but many avian predators, such as the common kingfisher (*Alcedo atthis*) and the Indian pond heron (*Ardeola grayii*), are attracted to shallow waters (Spence et al., 2008).

Flow

Water flow rate is an important component of microhabitat use by zebrafish. As water velocity increases, the metabolic cost to a fish of holding its position and swimming increases while foraging success decreases (Hill & Grossman, 1993). Zooplankton and insects that form a large part of zebrafish diet (McClure, McIntyre & McCune, 2006) may be harder to locate and catch in swiftly flowing water, increasing the energetic cost of foraging. Water flow also affects the survival of eggs and juveniles. Eggs may be swept away, and young fish may have insufficient swimming ability to counteract high velocities (Sukhodolov, Bertoldi, Wolter, Surian & Tubino, 2009). Zebrafish have been found in still waters (Spence et al., 2006) and in waters with flow rates of up to 18 cm per second (Suriyampola et al., 2015). This variation is likely due to the use of different habitats in different seasons, with the high flow rate recorded in a fast-moving river during the postmonsoon season (Suriyampola et al., 2015) when the fish were likely not breeding. Zebrafish have only rarely been found in large river systems where the flow is high. Individuals that respond to the fluctuating benefits and costs of different microhabitats and flow rates may have a survival advantage.

Turbidity

Turbidity is a measure of water clarity (Voichick, Topping & Griffiths, 2017). Dissolved solids and suspended particles in water scatter and absorb light, decreasing the amount of sunlight that penetrates the water column and reducing visibility (Voichick, Topping & Griffiths, 2017). Low visibility reduces the ability of visually foraging fish to detect their prey, but the level of effect depends on the type of prey. In clear waters, piscivorous fish can detect their prey (other fish) at greater distances than planktivorous fish can detect their prey (zooplankton). However, in turbid waters, the hunting ability of piscivores is impaired more than the hunting ability of planktivores (Voichick, Topping & Griffiths, 2017). For a prey species such as zebrafish whose diet consists largely of zooplankton, increasing turbidity reduces not only the hunting ability but also the risk of predation. Studies show that the decrease in predation risk is greater than the decrease in feeding opportunity (Voichick, Topping & Griffiths, 2017), so turbid waters may offer an advantage to zebrafish.

Turbidity levels vary among zebrafish habitats (Fig. 4.9) and seasonal changes in turbidity may also occur due to extreme weather patterns, such as monsoon winds and rains. Spence et al. (2006) used a Secchi disk

FIGURE 4.9 (A) Measuring water clarity with a Secchi disk; (B–D) examples of waters with different turbidity levels. *Photos by Gregory Paull.*

(Hou, Lee & Weidemann, 2007, Fig. 4.9A) to measure water clarity at several locations where they found zebrafish. The highest clarity was 50 cm in a ditch and the lowest was 15 cm in several ponds. The combination of shallow waters and moderate turbidity may allow zebrafish to decrease predator avoidance behavior and increase feeding opportunities. Turbidity may also lower the predation risk for larval zebrafish.

pH

The acidity of water is measured on a potential hydrogen (pH) scale of 0–14. The lower the pH value, the more acidic the water, with 7 representing neutral—neither acidic nor alkaline (Dodds, 2002). Factors that affect the pH in natural freshwaters include the mineral composition of the surrounding rock, acidic precipitation (acid rain), wastewater and other discharges from anthropogenic sources, and photosynthesis by aquatic plants (Alabaster & Lloyd, 1982).

The pH of water affects the ability of fish to regulate basic physiological functions such as respiration and the exchange of gasses and salts with the water (Alabaster & Lloyd, 1982). The pH of most natural freshwaters in which fish are found range from about 6 to 9 (Ellis, 1937). Wild zebrafish have been caught in waters with pH values from 5.9 (Engeszer et al., 2007) to 9.8 (Arunachalam et al., 2013), while laboratory zebrafish survive in acidic waters as low as pH 4.0 (Kwong, Kumai & Perry, 2014) although their ability to breed at such low pH values was not tested.

Salinity

As with other water parameters, salinity levels (quantified as mass of salt per unit volume) vary among zebrafish habitats. Spence et al. (2006) measured salinity at nine sites in Bangladesh where zebrafish were present. Their highest recording was 0.6 g per liter in ponds of brackish water in the north of the country, while in the south, salinity levels in a ditch and in an isolated river channel were ~0.01 g per liter. Although the zebrafish is generally considered a stenohaline species, tolerant of very low salinities (Craig, Wood & McClelland, 2007), these measurements uphold the suggestion that it may be adapted to osmoregulate under higher salinities (Best, Adatto, Cockington, James & Lawrence, 2010). Some laboratories keep larval zebrafish at salinities of 5 g per liter and culture them together with a food source of live saltwater rotifers (Lawrence et al., 2016).

Substrates

Substrates are an important component of freshwater habitats. Large diameter substrates provide foraging opportunities, refuge from predators, and shelter from strong currents (Rankin, 1986). Substrate size influences habitat selection by some freshwater fish species (Johnson & Kucera, 1985; Rankin, 1986), and substrate stability affects the diversity and densities of benthic insects (Cobb, Galloway, & Flannagan, 1992) on which fish may prey.

Zebrafish are found over substrates ranging from silt, sand, and gravel to pebbles, boulders, and even bedrock (Arunachalam et al., 2013; Engeszer et al., 2007; McClure, McIntyre & McCune, 2006; Spence et al., 2006). Some of this variance may be explained by diel differences in behavior. Zebrafish usually spawn in the early morning, scattering their eggs over the substrate. Although they provide no parental care and may eat their own as well as others' eggs, zebrafish are selective about oviposition site and prefer to spawn on gravel (Spence, Ashton & Smith, 2007). Egg survival is greatest in gravel—the interstitial spaces within gravel aid oxygenation and protect eggs from predation—and the use of gravel as spawning substrate may be an adaptive strategy (Spence, Ashton et.al., 2007). At other times, when fish are not spawning, choice of substrate may be less important and fish may move to microhabitats that provide better opportunities to forage, shelter, or avoid predation.

Vegetation

Submerged, floating, and emergent plants, together with riparian vegetation, increase habitat complexity and play an important role in the ecology of freshwaters. Fish densities are higher in vegetated than in unvegetated areas due mainly to increased food availability and shelter from predation (Rozas & Odum, 1988). Submerged leaves, stems, and roots form large surface areas to which algae can attach and complex structures in which zooplankton and invertebrates can feed and hide (Thomaz & da Cunha, 2010). As a result, these organisms are more diverse and abundant in vegetated habitats (Ganesan & Khan, 2008; Gregg & Rose, 1985). In addition, terrestrial insects falling from riparian vegetation are an important food source for many species (Kawaguchi, Taniguchi & Nakano, 2003), including zebrafish (Arunachalam et al., 2013).

In the wild, zebrafish are most often found in habitats associated with aquatic vegetation, such as floating fern (*Salvinia natans*), swampweed (*Hygrophila* sp.) and

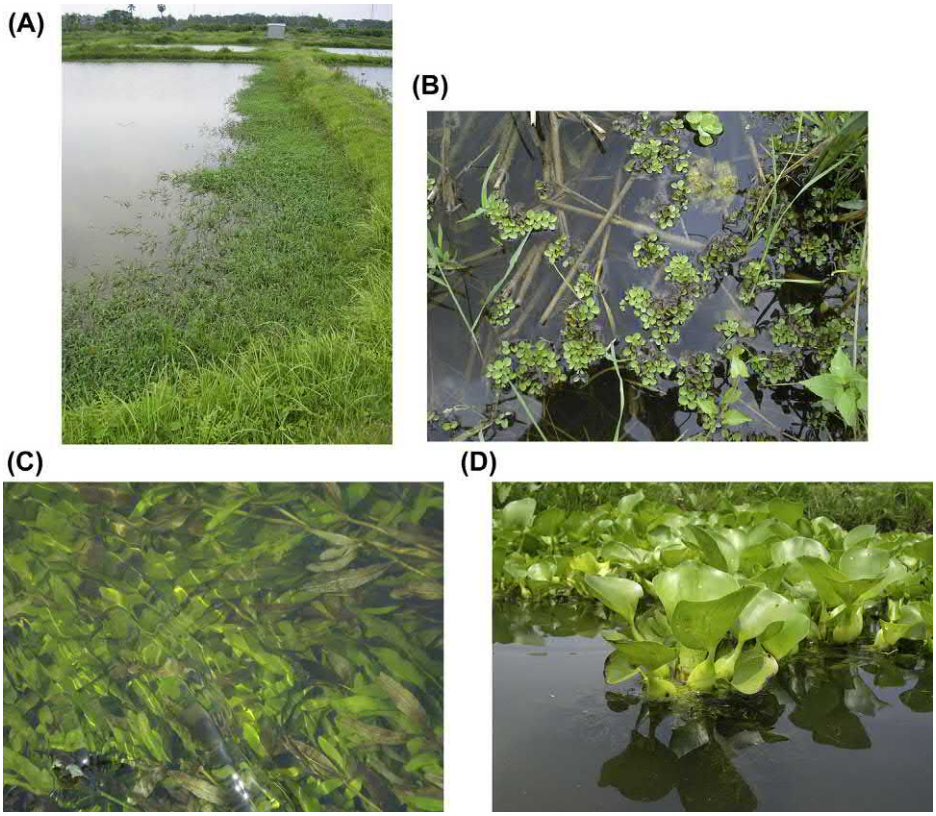

FIGURE 4.10 Vegetation typically found in zebrafish habitats (A) include *Salvinia natans* (B), *Hygrophila sp.* (C), and invasive plants such as *Eichhornia crassipes* (D). *Photos by Gregory Paull.*

duckweed (*Lemna sp.*), and invasive plants such as water hyacinth (*Eichhornia crassipes*) (Fig. 4.10). Aquatic vegetation may play a role in the survival of larval zebrafish. Laboratory studies show that newly hatched larvae move along the substrate until they encounter a hard surface, such as a rock or a plant stem or leaf, to which they adhere by means of small secretory cells on their heads (Laale, 1977). Over several hours, the larvae repeatedly release, propel themselves upwards, and reattach at a shallower depth, until they reach the water surface where they inflate their swim bladders to attain buoyancy and become free-swimming (Lindsey, Smith & Croll, 2010). By laying eggs in shallow waters close to vegetation to which larvae may attach, female zebrafish give their offspring the best chance of survival, and thereby increase their own fitness. Thereafter, plants provide shelter and a source of food for larvae and juveniles.

Human Impact

Humans have manipulated zebrafish habitat for millennia. The spread of rice cultivation in the Indian subcontinent, since its origins in the Gangetic floodplains over 4000 years ago, is associated with the development of urbanism, the expansion of labor-intensive agriculture, and the creation of major irrigation works (Fuller & Qin, 2009). The subcontinent is now one of the most densely populated regions in the world. It comprises 4% of the world's landmass and is home to 23% of the world's population (United Nations, 2017). The increase in population and industrialization has created a demand for water that threatens to outstrip supply and has resulted in a substantial decline of wetland resources in the region (Vass et al., 2011).

Major threats to river systems throughout the subcontinent include flow diversion and the alteration of habitats, degradation and siltation of waterways, deforestation of catchments areas, excessive abstraction of groundwater, discharge of untreated domestic and industrial wastes, overexploitation of water resources for hydroelectric purposes, indiscriminate fishing, and the introduction of invasive exotic species (Hoggarth et al., 1999; Prasad et al., 2002; Vass et al., 2011). In addition, the retreat of Himalayan glaciers and a decline in the region's rainfall have resulted in a reduction in flow of the Ganges, further reducing the supply of water for drinking and irrigation (Vass et al., 2011).

To fulfill the growing demand for water and mitigate supply problems, the Indian government has initiated a

huge scheme to connect the major rivers of India and the Himalayas through a system of 30 interlinking canals and tunnels and 3000 dams and reservoirs, to redirect water from areas with "surplus" water to regions that experience a water shortfall (Lakra, Sarkar, Dubey, Sani & Pandey, 2011). The National River Linking Project will move water from Himalayan-fed rivers to drought-prone western states, with further links planned to bring river water to needy areas in the southern peninsula (Bagla, 2014). Such major alterations to water quantity and seasonal flows are likely to have negative consequences for freshwater fish biodiversity due to the loss of wetlands and floodplain habitats, destruction of migration routes, and changes to the physical features of feeding and spawning grounds (Sarkar et al., 2012).

Climate change poses an additional threat to freshwater biodiversity. Although the causes of the observed changes in global climate are debated, there is growing consensus that anthropogenic emissions of greenhouse gasses are partially responsible (Stern & Kaufmann, 2014). In the Indian subcontinent, an increase in temperature, the occurrence and severity of storms and droughts, regional variation in monsoons, and the retreat of Himalayan glaciers have been observed (Vass et al., 2011), all of which may directly or indirectly affect the distribution of freshwater fish. When increased water temperature is combined with chemical pollution, the effects can profoundly impact fish populations. For example, the endocrine-disrupting chemical clotrimazole induces male-skewed sex ratios in zebrafish, and this effect is greater at elevated water temperatures (Brown et al., 2015). Such a change can reduce population viability and growth, especially in small populations, leading to an increased risk of extinction (Brown et al., 2015).

Chemical pollution is a growing environmental problem in India. Around 70% of all available freshwater in India is polluted, due in part to the manufacture of pharmaceuticals outsourced to India by western countries (Mathew & Unnikrishnan, 2012). Production costs in India are around 50% lower than in industrialized countries, and India is now the world's biggest exporter of generic prescription drugs, accounting for 40% of the world's needs (Altstedter, 2017; Mathew & Unnikrishnan, 2012). In addition, wastewater effluents from municipal, industrial, and hospital sources and the disposal of solid wastes without proper treatment have degraded waterways throughout much of the Indian subcontinent (Mathew & Unnikrishnan, 2012; Srivastava, Ismail, Singh & Singh, 2015).

Conclusions

The geographic range of the zebrafish includes much of the Indian subcontinent where it occupies a diverse range of habitats from hill streams to floodplains and from natural wetlands to man-made ditches. Zebrafish tolerate an equally diverse range of altitudes, temperatures, and water conditions and have been found over different substrates, with or without associated vegetation. Despite the importance of zebrafish to science, surprisingly little is known of the biology and behavior of wild populations. The zebrafish's natural range boundaries and average home range size are still to be determined, as are its temporal distribution and microhabitat use. Also unknown are the distances traveled by zebrafish when migrating between habitats and the spatial behavior of the species at different life stages.

Detailed knowledge of the natural habitat of zebrafish, including water conditions, habitat structure, temperature ranges, and seasonal changes, can be used to inform decisions about how fish can best be maintained and bred in the laboratory and how standards of welfare might be improved. In turn, providing optimum housing conditions for laboratory zebrafish will likely improve the reliability of research data.

Wild zebrafish may prove invaluable for studies of adaptive evolution, wild traits, and associations between genotype and phenotype (Whiteley et al., 2011) and for interpreting responses of laboratory zebrafish to experimental manipulation (Suriyampola et al., 2015). However, the survival of natural populations is threatened by habitat degradation and destruction due to the drainage of wetlands for urban or industrial development, pollution of waterways, and an increasingly dry climate. The planned large-scale extraction of water from rivers and its subsequent transfer between regions will likely change the physical characteristics and ecosystems of habitats that zebrafish currently occupy. Geographic barriers prevent zebrafish from extending their range to evade environmental stressors, but within this restricted range, the zebrafish's natural distribution will likely reduce or change.

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CHAPTER

5

Behavior of Wild Populations

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Introduction

The environment in which wild zebrafish live is complex, highly transitory, and intrinsically linked to patterns of rainfall driven by the monsoon climate of the Indian subcontinent. Dramatic changes in, for example, water chemistry, flow, turbidity, and habitat structure can occur very rapidly (even within minutes), thus exposing zebrafish to behavioral and physiological challenges. Equally, however, these environmental fluxes allow populations to colonize new habitats after heavy rainfall (Paull, 2008). Despite the growing body of evidence describing the habitat types occupied by zebrafish in the wild, there is limited information regarding the behavior of wild populations, their movements between locations, or how zebrafish use different habitats during the various stages of their life cycle. In fact, to date, only one study has reported zebrafish behavior in-situ (Suriyampola, Shelton, Shukla, Roy, Bhat, & Martins, 2015).

Most observations of wild zebrafish have been from studies where captured fish have been placed in field-based mesocosms (Fig. 5.1; Spence & Smith, 2006; Spence, Fatema, Ellis, Ahmed, & Smith, 2007) or through controlled laboratory-based studies targeting specific behaviors (Bhat, Greulich, & Martins, 2015; Hutter, Penn, Magee, & Zala, 2010; Pritchard, 2001).

The paucity of behavioral studies of zebrafish in the wild is in part due to poor water clarity, heavily vegetated habitats, and the remote locations in which they live; and this combined with their small size and rapid swimming speed make field observations difficult. In fact, sudden bursts of rapid movement make monitoring zebrafish behavior challenging even in controlled laboratory settings. Most published studies on zebrafish behavior track individual fish or small groups of two to six fish as the tracking software available is limited and not readily applicable to monitoring larger group sizes (Lucon-Xiccato & Dadda, 2014; Paull, Filby, Giddins, Coe, Hamilton, & Tyler, 2010; Tran & Gerlai, 2013).

Life History of the Zebrafish

The patterns of behavior seen in zebrafish emerge gradually during development, from initial muscle twitches in 1-day old embryos, through early social interactions of juveniles, to the full repertoire of complex behaviors seen in adult fish. A fish's social relationships and responses to its environment differ at various ages (Dreosti, Lopes, Kampff, & Wilson, 2015). Briefly, the life history of the zebrafish comprises four broad stages (Fig. 5.2).

Embryo: The fertilized eggs develop rapidly. Within 24 h all of the major organs and tissues have formed within the transparent embryos, and within three days the embryos have hatched into free-swimming larvae (Wilson, 2012). Locomotor behavior begins with spontaneous muscle contractions from 18 h postfertilization (hpf) and by 48 hpf the embryos are responsive to touch that induces a rapid flipping movement (Colwill & Creton, 2011).

Larva: After hatching, the larvae associate with hard surfaces by means of small secretory cells in the epidermis of the head (Laale, 1977). During the next 24–48 h they move to the surface of the water column where they inflate their swim bladders with air (Goolish & Okutake, 1999). Initially, zebrafish larvae rely on yolk sac nutrients for nourishment; but by seven days postfertilization (dpf), the yolk sac is completely absorbed, and the larvae feed exogenously (Wilson, 2012). Larvae quickly develop distinct patterns of movement, including slow and burst swimming, routine and escape turns, and complex behaviors that allow them to capture their prey (Budick & O'Malley, 2000).

Juvenile: From around 14 to 29 dpf, larvae undergo a metamorphosis into the juvenile form during which

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FIGURE 5.1 Dr. Rowena Spence observing wild zebrafish spawning at sunrise in Bangladesh in outdoor aquaria. *Photo by Carl Smith.*

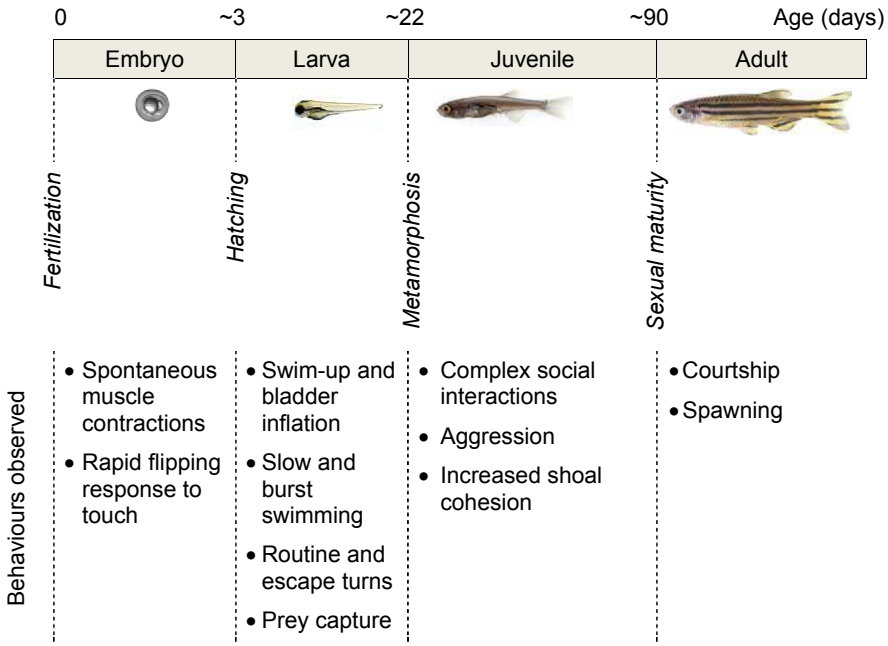

FIGURE 5.2 The life history stages of laboratory zebrafish. Representative events separating different stages are shown. At ~3 days post-fertilization, the embryo hatches and emerges as a free-swimming larva. The larva grows and undergoes a period of metamorphosis during which larval features are lost and juvenile features are acquired. The juvenile stage lasts until sexual maturity which marks the start of the adult stage and after which spawning occurs (Parichy et al., 2009.). *Adapted from Levraud, J.-P., Palha, N., Langevin, C., & Boudinot, P. (2014).*

time larval features (such as fin-fold) are lost, and juvenile features (such as scales) are acquired (Parichy, Elizondo, Mills, Gordon, & Engeszer, 2009). As the young fish develop, their social interactions become increasingly complex. Aggressive encounters occur, and shoal cohesion increases (Buske & Gerlai, 2011).

Adult: At around three months, the juveniles become sexually mature adults and spawning occurs (Harper & Lawrence, 2012). Courtship behavior is displayed from around 40 dpf when males begin to chase females. Females respond to this behavior by swimming alongside males or by halting and presenting their

flanks to them (Darrow & Harris, 2004). By 90 dpf, most females spawn eggs and most males attempt to mate (Darrow & Harris, 2004).

The duration of each life stage is variable and dependent upon factors such as temperature and food availability (Parichy et al., 2009). Wild populations generally begin to spawn shortly before the start of the monsoon, in the month of June and grow fastest during the monsoon season (Spence, Gerlach, Lawrence, & Smith, 2008) although females with mature ova have been caught in January. Both wild zebrafish and laboratory stock start to reproduce when they reach a standard length of around 23 mm (mm). Wild fish though have a slower growth rate and mature at a later age (ten months) compared to laboratory fish (three months) (Spence et al., 2008). This difference in growth rate is likely due to laboratory fish receiving regular rations of high-quality food and being maintained at constantly high temperatures.

Spence, Ashton, et al. (2007) sampled wild zebrafish in Bangladesh and determined that the species is annual in nature. In contrast, a lifespan study of laboratory zebrafish by Gerhard et al. (2002) reported a mean lifespan of 42 months, with the oldest individual living for 66 months. The subjects in the latter study were outbred wild-type zebrafish. A similar investigation by Herrera and Jagadeeswaran (2004) tested an inbred strain and reported a mean lifespan of 31 months with the oldest fish surviving for 45 months. The variance between these studies suggests that life expectancy of laboratory zebrafish may be influenced by strain.

Social Behavior

Social behavior may be defined as "a set of interactions among individuals of the same species" (McGlynn, 2010). Social behavior may increase an animal's fitness. For example, fish that shoal with conspecifics reduce the risk of predation by interfering with a predator's ability to target individual prey (Hasenjager & Dugatkin, 2017). Other advantages of social behavior include increased foraging efficiency and improved access to potential mates, but these advantages are offset by increased competition and by the increased risk of pathogen transmission (Sueur et al., 2011). The compromise between predation pressure and foraging opportunities is thought to cause individuals to constantly assess and re-assess the benefits of joining, staying with, or leaving conspecifics (Croft et al., 2003).

Field studies indicate that freshwater fish, in general, prefer to shoal with phenotypically similar individuals, and shoals are generally composed of unrelated individuals of the same species and similar body length (Hoare, Ruxton, Godin, & Krause, 2000). The tendency of fish of similar appearance to shoal together may be explained in part by the "selfish herd effect" by which individuals hide behind group members to reduce the chance of being caught by a predator, and also by the "oddity effect," whereby an individual that is phenotypically different from the group is conspicuous, and therefore, more vulnerable to predation (Landeau & Terborgh, 1986).

The social organization of free-ranging zebrafish shoals has not been investigated in the field, and so our knowledge of shoal-mate characteristics preferred by zebrafish is limited to predictions from laboratory studies. Engeszer, Ryan, and Parichy (2004) demonstrated that zebrafish can visually distinguish between different pigment patterns and prefer to shoal with fish of their own phenotype. However, no such preference was reported in a similar study for the effects of coloration on the choice of social partners (Snekser, Mcrobert, Murphy, & Clotfelter, 2006). No differences in shoaling tendency were found among zebrafish derived from four wild-caught populations reared in different environments (Wright, Rimmer, Pritchard, Krause, & Butlin, 2003), but tendency to shoal was reduced in a laboratory strain compared to the F_2 offspring of wild-caught fish (Wright, Nakamichi, Krause, & Butlin, 2006). Furthermore, when given the choice of swimming with a group of males or a group of females, male zebrafish spent more time with the female shoal, whereas females showed no preference, suggesting that shoaling behavior in males may be influenced by sexual selection (Ruhl & McRobert, 2005). The artificial nature of laboratory studies, where a test fish may be held in isolation with only visual contact with its shoal, means that test results may not reflect the behavior of fish under natural conditions (Miller & Gerlai, 2007). Despite this, a review of predictions made from laboratory studies of the social organization of several fish species compared to wild populations has shown that laboratory results are largely consistent with field reports (Krause, Butlin, Peuhkuri, & Pritchard, 2000).

In addition to conspecifics, wild zebrafish are known to shoal with flying barb (Esomus danricus) and to share macrohabitats with potential competitors, such as blue panchax (*Aplocheilus panchax*) and several Puntius and danionin species, as well as with juvenile Jerdon's carp

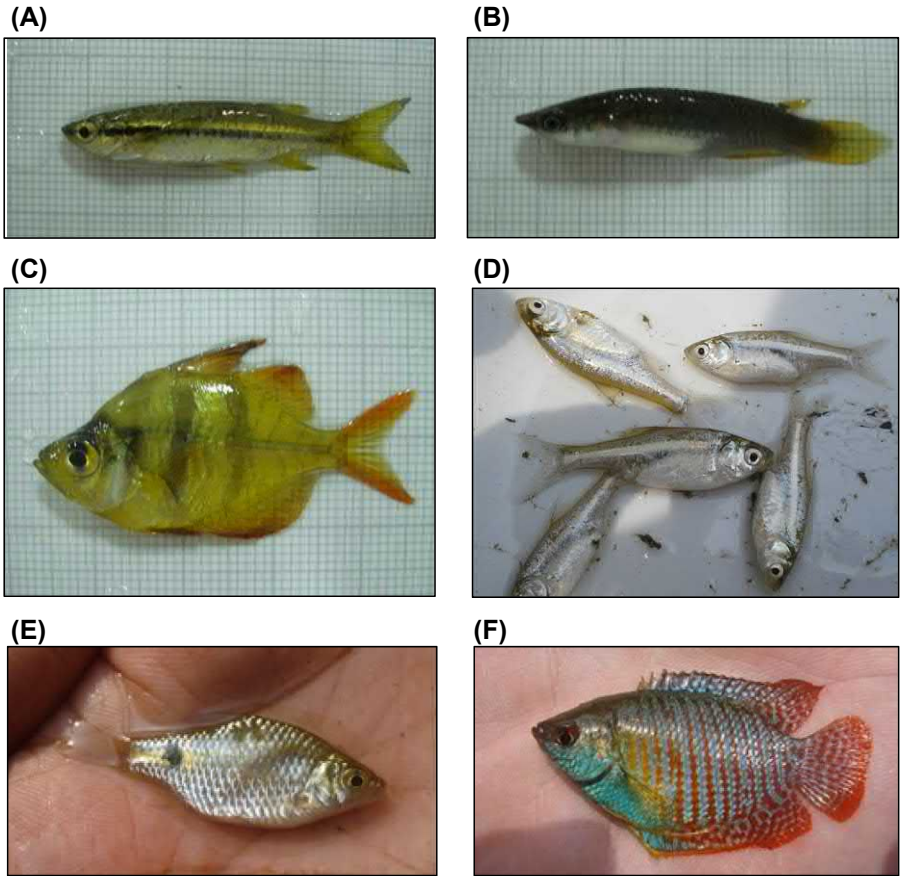

FIGURE 5.3 Competitor species that coexist with wild zebrafish include (A) flying barb (*Esomus danricus*), (B) blue panchax (*Aplocheilus panchax*), (C) highfin glassy perchlet (*Parambassis lala*), (D) Mola carplet (*Amblypharyngodon mola*), (E) ticto barb (*Pethia ticto*), and (F) dwarf gourami (*Colisa lalia*). *Photos by Gregory Paull.*

(*Hypselobarbus jerdoni*) and ghat barb (*Barilius gatensis*) (Fig. 5.3; Spence et al., 2008; Arunachalam, Raja, Vijayakumar, Malaiammal, & Mayden, 2013; Paull, 2008).

Group Size

A group of fish that stays together for social reasons is termed a *shoal*, and a shoal that swims in a synchronized and polarized way is termed a *school* (Pitcher & Parrish, 1986). Any sudden stimulus can cause a shoal to rapidly shift into a tight school, with fright being a principal cause, and with the school reverting to an unstructured shoal when the threat has passed (Breder, 1959; Hamilton, 1971). Shoaling behavior in zebrafish develops during ontogeny. Larvae and juveniles less than two months of age do not shoal and scatter on fright instead of schooling; in contrast, older juveniles and adults typically shoal as a general situation and school as a fright response (Breder, 1959).

Shoal fidelity and site fidelity in zebrafish have not been reported, and it remains to be investigated how often wild shoals meet, how often individuals transfer between shoals, and whether shoals range over a large area. Shoal size can influence foraging success and predation risk for individual group members (Hoare et al., 2000). For example, fish in larger shoals locate food faster than when in small shoals (Pitcher, Magurran, & Winfield, 1982), but fish in smaller shoals find food more quickly when the food is hidden (Day, Macdonald, Brown, Laland, & Reader, 2001). The latter is attributed to the reluctance of fish to leave a shoal increasing with shoal size.

In wild zebrafish, shoaling behavior varies among populations in different habitats. For example, wild fish in a slow-moving stream have been observed shoaling in groups of four to twelve individuals, whereas those in still waters were seen to swim in tight-knit groups of up to 22 fish (Suriyampola et al., 2015). Populations have been seen in a fast-moving stream in groups of up to 300 fish (Suriyampola et al., 2015). Such differences reflect the ability of zebrafish to adapt to different environments. More research is needed to understand how the behavior of zebrafish is influenced by habitat complexity.

There is evidence that shoaling behavior in zebrafish is also affected by individual social history. For example, Breder and Halpern (1946) found that zebrafish reared in isolation for six months immediately joined a shoal when introduced to other fish. In contrast, six-month old group-reared fish held in isolation for six months and then returned to the group took up to one week before joining a shoal.

Aggression

Scientists disagree on a comprehensive definition for the term aggression, but it may be described as "the forceful and deliberate attempt to inflict harm on another individual" (Huntingford & Turner, 1987). Aggressive encounters, including physical attacks and threat displays, are typically used to gain access to or defend resources, such as food, territory, mates, spawning sites, and shelter (Archer, 1988).

In the only published study on wild zebrafish behavior in the wild, Suriyampola et al. (2015) found that fish in slow-moving streams were more aggressive than those in still or fast-flowing water. Mesocosm and lab-based studies have observed aggressive behaviors in wild-caught zebrafish, with aggression heightened during spawning (Spence, Fatema, et al., 2007), when vegetation was provided (Bhat et al., 2015), and with increasing duration of confinement (Martins & Bhat, 2014). Aggressive behavior in laboratory zebrafish has been observed during feeding (Grant & Kramer, 1992), spawning (Spence & Smith, 2005), when fish were kept at low stocking densities (Paull et al., 2008), and when fish were provided with environmental enrichment (Woodward, Winder, & Watt, 2019). Aggression occurs in both males and females and is often associated with greater body size (Paull et al., 2010).

Patterns of Aggression in Zebrafish

In laboratory zebrafish, levels of aggression are generally seen to be highest in the morning and greater in males than in females (Paull et al., 2010). Because spawning occurs at dawn, this suggests that the function of aggression in zebrafish is predominantly to facilitate spawning or to control spawning activities between individuals. However, in females, levels of aggression do not significantly differ between the morning and the afternoon, which suggests that aggression may play a more general role in maintaining social structure or hierarchy in females. Aggression in zebrafish has also been related to foraging when dominant individuals attempt to monopolize a food source (reviewed in Spence et al. 2008).

Aggression is costly in terms of energy expenditure and risk of injury, and rivals may first assess each other through ritualized displays (Reebs, 2001). In such displays, two fish approach each other with raised fins and undulating body movements, often leading into an elaborate circling motion between the fish that can last from a few seconds to several minutes. If neither fish retreats, the contest may escalate into direct physical contact in which fish charge, bite, and chase each other until one flees and adopts a submissive posture, often "freezing" at the bottom of the tank (Oliveira, Silva, & Simoes, 2011).

Fish of some social species are able to assess the competitive ability of others by watching fights between rivals and then may adjust their behavior in subsequent interactions with the fish they have observed (Grosenick, Clement, & Fernald, 2007; Oliveira, Mcgregor, & Latru, 1998). It seems likely that zebrafish share this ability. Zebrafish have been shown to pay attention to social interactions between conspecifics, although the subsequent use of information gained during such "social eavesdropping" has yet to be determined (Abril-de-Abreu, Cruz, & Oliveira, 2015).

Dominance Hierarchies

Aggression is central to the establishment and maintenance of dominance hierarchies, and rank may be determined by the ability to control resources and by relative differences in size, strength, or fighting ability (Broom, Koenig, & Borries, 2009). Some of the most familiar dominance hierarchies are observed in pack animals, such as wolves and wild dogs (Mech, 1999), but perhaps less familiar are the dominance hierarchies that occur in fish species, including laboratory-based zebrafish (Paull et al., 2010). Currently there is no information on dominance hierarchies obtained from studies in wild zebrafish populations, but they are highly likely to occur based on experiments with other species of wild fish held under simulated natural conditions (brown trout (*Salmo trutta*): Sloman, Gilmour, Taylor, & Metcalfe, 2000; Pacific salmon (*Oncorhynchus* spp.): Van Leeuwen, Rosenfeld, & Richards, 2011).

In laboratory zebrafish, dominant males are nearly always larger in size (Hamilton & Dill, 2002; Paull et al., 2010; Spence & Smith, 2005), and this is also the case for dominant females. Studies by Paull et al. (2010) have shown that the dominant female ranked as the most aggressive fish after the dominant male and was, therefore, deemed a major player in the social dynamics of the group. Dominance hierarchies in female zebrafish have been documented in two other studies. Delaney, Follet, Ryan, Hanney, Lusk-Yablick, and Gerlach (2002) noted that female zebrafish dispersed to different parts

of the aquarium and spent only five percent of their time together. Studies by Gerlach (2006) found that competition between females was often displayed without much physical interaction and that dominance was instead exerted using waterborne pheromones. In contrast, some observations in our laboratory (G. Paull, personal observation) have noted that when very large females are present (much larger than the males), they often are the most aggressive and dominant fish in the entire group. Similarly, Grant and Kramer (1992) noted that sex was not an important factor in determining which fish became the dominant fish when zebrafish compete for food.

Effects on Lifetime Fitness

Aggression can influence reproductive success and, consequently, lifetime fitness of laboratory zebrafish when it is used to maintain dominance and prevent subordinates from acquiring a territory or mate (Ariyomo & Watt, 2012). Dominant males generally sire a greater number of offspring than subordinate males and, although dominant females do not spawn more eggs than subordinate females, more of the eggs from the dominant female are fertilized by the territorially dominant male (Paull et al., 2010). However, aggression is costly for both winners and losers. In addition to the risk of physical injury and other effects on health status, there are metabolic costs of fighting, such as elevated oxygen consumption, energy depletion, and the accumulation of lactate (Filby, Paull, Bartlett, Van Look, & Tyler, 2010; Neat, Taylor, & Huntingford, 1998).

Reproductive Behavior

Zebrafish, like most fish species, are oviparous. Females scatter large numbers of small eggs that are fertilized externally and left to develop, hatch, and grow with no parental care. The zebrafish reproductive strategy, featuring high growth rate, short generation time, and the production of many offspring, enables the rapid colonization of new niches and fits with the transitory nature of the species' natural habitat; but each offspring produced has a relatively low probability of surviving to adulthood.

Laboratory zebrafish are typically maintained as a tropical species at around 28°C and, as a result, can be induced to spawn all year round, provided they are kept in breeding condition with suitable food and water parameters. In the wild, spawning is more seasonal as temperatures in the zebrafish's natural range may fall as low as 6°C in the winter and climb to over 38°C in summer (Spence et al., 2008).

There are no observational data of zebrafish spawning in the wild, but fish collected by Spence et al. (2008) over a 12 month period displayed an increase in growth and maturation that coincided with the monsoon season, which suggests that reproduction in wild zebrafish is cued by the arrival of the rains (Munro, 1990; Talwar & Jhingran, 1991). Changes brought about by the rains, such as increased water current speed, altered water chemistry, and a concomitant increase in food availability, provide chemical cues or proximate factors that stimulate the onset of reproduction. Zebrafish typically spawn at dawn in both the laboratory and the wild; and under controlled laboratory conditions, mature females are capable of spawning on an almost daily basis (Eaton & Farley, 1974; Selman, Wallace, Sarka, & Qi, 1993; Spence, Fatema, et al., 2007; Spence & Smith, 2006).

Female zebrafish with mature ova have been collected in the wild during the dry season, suggesting that reproduction may not be cued solely by season but also by food availability, which is generally positively correlated with rainfall (Spence, Fatema, Reichard, Huq, Wahab, Ahmed, et al., 2006). In the wild, zebrafish spawn after heavy rainfall, and adding a dash of cold water to aquaria could encourage spawning in captive fish (Breder & Rosen, 1966; Spence et al., 2008). Thus, changes in water parameters, such as temperature or water level, may provide additional cues that stimulate zebrafish to spawn. It is also highly likely that local variations in weather patterns, habitat disturbance, and availability of resources (food, spawning sites, and presence of mature mates) may influence spawning outside of the main monsoon period. Such mechanisms are worth considering when attempting to encourage spawning in laboratory-maintained zebrafish.

Finding a Mate

Zebrafish have a promiscuous mating system; both males and females repeatedly spawn with multiple partners. However, females are selective about the sites where they scatter their eggs, and some males try to monopolize access to preferred spawning sites, using aggression to repel rivals (Spence & Smith, 2005). Laboratory experiments have shown that males prefer large females and that females prefer certain males, but female preference does not appear to be influenced by male size or dominance (Hutter et al., 2010; Spence & Smith, 2006).

Zebrafish lack strong sexual dimorphism. It may be difficult for the casual observer to distinguish between males and females. There are subtle differences between the sexes in body shape (females are more rounded and

less streamlined than males) and coloration (the light stripes of females appear silvery, whereas those of males are more golden). These differences become pronounced during courtship and spawning (Hutter, Zala, & Penn, 2011, 2012, 2010; Nasiadka & Clark, 2012). Studies have shown that zebrafish use color to discriminate between the sexes and that individual males with brighter colored light stripes perform courtship behaviors more often than their less conspicuous counterparts (Hutter, Hettyey, Penn, & Zala, 2012).

Waterborne sex pheromones (odors released by an individual to attract a conspecific to mate) play an important role in fish reproduction, enabling males to locate receptive females and influencing courtship and mating behavior (Corkum & Cogliati, 2015). Evidence suggests that zebrafish males release a primer pheromone to trigger ovulation in females and that exposure to this pheromone can increase both egg output and egg viability (Gerlach, 2006; Hontela & Stacey, 1990; Stacey & Sorensen, 1991). After initial ovulation, females release pheromones that attract males and stimulate them to begin courtship (van den Hurk & Lambert, 1983). Pheromones may also account for some of the variations observed in female reproductive success in laboratory zebrafish as dominant females have been shown to use pheromones to suppress reproduction in subordinate females (Gerlach, 2006).

Courtship and Spawning

Courtship is "the suite of behaviors displayed by an individual to attract and eventually reproduce with an individual of the opposite sex" (Mitoyen, Quigley, & Fusani, 2019). Although the courtship behavior of zebrafish in the wild has not been studied, courtship in laboratory fish is well described (Darrow & Harris, 2004; Delaney et al., 2002; Hutter et al., 2010; Nasiadka & Clark, 2012). The male swims alongside or slightly behind the female and repeatedly nudges her side or tail with his nose or head. He circles around or in front of the female, or sweeps her body with his tail while circling her in a figure-eight pattern. The female may swim alongside the male or stop in front of him and expose the side of her body to him. She may return repeatedly to the same location in the tank. Finally, the male and female swim side by side, the male oscillates his tail rapidly against the female's side, prompting her to release eggs as he simultaneously releases sperm (Nasiadka & Clark, 2012). Females typically scatter five to twenty eggs at a time in a series of releases, producing up to several hundred eggs during a single spawning event (Skinner & Watt, 2007).

In the laboratory, courtship of females by territorial males is confined to within a few body lengths of the males' chosen spawning site, which they aggressively defend against rivals. Territorial males hover, quivering, over their spawning site, trying to attract females down to spawn. They repeatedly swim rapidly up to the females and back to their territory, with an occasional foray to chase off other males from the females or from the spawning site. This activity can be frenetic. In contrast, some males are nonterritorial and pursue females all around the tank (Spence, Fatema, et al., 2006). Females too may be territorial around spawning sites or feeding sites (Hutter et al., 2010).

Spawning Strategies

Zebrafish are commonly believed to be asynchronous, batch spawners that breed in small groups (Lawrence, 2007; Nasiadka & Clark, 2012; Spence et al., 2008). When wild-caught zebrafish from India were housed in large 1100 L tanks, researchers discovered that the fish spawned in pairs rather than in groups; and similar behavior was seen in laboratory zebrafish housed in 600 L tanks (Delaney et al., 2002; Hutter et al., 2010). In another study in which wild fish were housed in 450 L mesocosms, courtship chases involved groups of three to seven fish; and when captive-bred offspring of wild fish were housed in conventional 17 L tanks at higher stocking densities and with limited spawning sites, group spawning occurred in 72% of spawning events (Hutter et al., 2010; Spence, Fatema, et al., 2007). These studies suggest that group spawning may be a consequence of artificial conditions, where small aquaria result in mating pairs being unable to escape from conspecifics (Hutter et al., 2010).

Nonterritorial "sneaker males" mate with females but do not guard a territory. Sneaker males benefit from fertilization success without the energetic costs of territory defense but often have a lower reproductive success than territorial males (Bleeker, de Jong, van Kessel, Hinde, & Nagelkerke, 2017). Female zebrafish do not discriminate between dominant and subordinate males (Spence & Smith, 2006) and may benefit from mating with multiple males through higher sperm numbers, increased fertilization rate, and greater genetic variability of the clutch (Reichard, Le Comber, & Smith, 2007). In an experiment with laboratory zebrafish, egg production decreased at high stocking densities, which the researchers attributed to increased aggression by territorial males toward sneaker males, which interrupted female spawning attempts (Spence & Smith, 2005). By observing the reproductive behavior of wild zebrafish in large tanks, Hutter et al. (2010) concluded that the active pursuit of females, rather than territoriality, is likely to be the primary mating tactic in the wild.

Female zebrafish are choosy with respect to the suitability of oviposition sites, preferring to release their

eggs over gravel substrates in vegetated areas (Spence, Fatema, et al., 2007). Wild zebrafish have been seen spawning in shallow waters along the margins of ponds, where zooplankton and organic matter are abundant (Spence et al., 2008). The choice of oviposition site is one of the few ways in which oviparous species with no parental care might be able to maximize offspring survival. Therefore, males that adopt territoriality as a mating tactic may increase their reproductive success by guarding such sites (Paull et al., 2010; Spence et al., 2008).

Given the variation in habitat type that zebrafish occupy and the variation in group size that they may find themselves in when conditions are ripe for spawning, it is highly likely that they can adopt a broad range of spawning strategies to meet the conditions at that time.

Foraging Behavior

Foraging is an almost constant activity for zebrafish. Fish in planted tanks can be seen swimming around, searching for prey items amongst the plants, plucking at leaves and winnowing food particles buried in the substrate. Wild fish must allocate time for foraging and reproduction in order to maximize fitness. They must decide when and where to look for food, whether to forage alone or with others, what food items to select, and how long to stay at a particular food patch (Hart, 1986). Fish must balance the costs and benefits of their foraging strategies, taking into account the presence of conspecifics and predators (Hart, 1986).

Searching for Food

Wild zebrafish are highly social and have been reported in groups of up to 300 individuals (Suriyampola et al., 2015). Foraging in a large shoal allows fish to spend more time searching for prey and less time being actively vigilant for predators (Hart, Webster, & Ward, 2008). Groups of fish use social information, cues generated by other foragers about the location or quality of a food patch, to find food faster than individuals, and large shoals tend to find food faster than small shoals (Brown & Laland, 2003; Krause & Ruxton, 2002). Another benefit of joining a larger shoal is that gaining access to a defended food patch becomes easier with increasing shoal size. For example, when individual giant danios (*Danio aequipinnatus*) defended a food source from shoals of smaller zebrafish, their ability to defend decreased as zebrafish shoal size increased (Chapman & Kramer, 1996).

Individuals within a shoal also compete for food and defend food from conspecifics (Hamilton & Dill, 2002; Lee, Paull, & Tyler, 2018). Food sources are often discovered by a few individuals at the front edge of the shoal, where predation risk is greatest, who are then joined by the rest of the shoal after food is found, but when food items are small, peripheral individuals may try to consume all of the food before conspecifics arrive, creating a within-group scramble (Hirsch, 2007). Dominant zebrafish prefer to forage behind the peripheral fish to take advantage of food discoveries while reducing the risk of predation (Hirsch, 2007). Laboratory zebrafish are able to assess the nutritional status of conspecifics; hungry zebrafish prefer to shoal with well-fed conspecifics and forage more successfully in shoals of well-fed fish than in shoals of food-deprived fish (Krause, Hartmann, & Pritchard, 1999).

The effects of predation risk on foraging behavior in wild zebrafish remain unexplored. Field studies of other social pond-dwelling species (including bluegill sunfish, crucian carp, Eurasian perch and several species of minnow) suggest that in the absence of predators, foraging fish occupy all habitats, from shallow vegetated areas to open water. When predators are present, foragers remain in vegetated areas which provide shelter from predation but where food is less plentiful, leading to increased competition and slower growth (He & Kitchell, 1990; Jacobsen & Berg, 1998; Reebs, 2001; Tonn, Paszkowski & Holopainen, 1992; Werner, Gilliam, Hall, & Mittelbach, 1983). Some wild fish change prey type in response to increased predation pressure (Ibrahim & Huntingford, 1989), but whether this occurs in zebrafish is not known.

Many freshwater fish species have flexible feeding habits and share habitats with other species of fish (Larkin, 1956). However, when competition between species exists, mutual predation and cannibalism often occur (Larkin, 1956). Spence, Jordan, and Smith (2006) conducted an experiment under semi-natural conditions to assess the behavior of zebrafish housed with four species with which they are commonly found (blue panchax, *Aplocheilus panchax*; dwarf gourami, *Colisa lalia*; glassfish, *Parambassis lala*, and flying barb, *Esomus danricus*) plus one introduced species (Nile tilapia, *Oreochromis niloticus*). Habitat overlap was found among all of the tested species, but no between-species aggression was observed. These results are consistent with field observations by Arunachalam et al. (2013). Whether foraging zebrafish face competition from conspecifics and from other fish species remains to be tested.

Prey Selection

After a fish has found a suitable foraging patch, it must decide what to eat and, with motile prey, how to catch it. The choice of food items may depend on the fish's level of hunger and the type of habitat in which it is foraging. Zebrafish are omnivorous. Zooplankton, aquatic and terrestrial insects, phytoplankton, algae,

plant material, and the eggs of microcrustaceans have all been found in the guts of wild zebrafish (Dutta, 1993; McClure, McIntyre, & McCune, 2006; Spence et al., 2006). This broad diet suggests that zebrafish feed from all parts of the water column, including the surface and substratum, and that their foraging behavior is flexible, an important adaptive feature in a spatially and temporally varied environment (Dill, 1983). There may be individual variability in foraging behavior, and not all individuals in a population will eat the same range of available food items (Gerking, 1994).

Zebrafish foraging strategies remain poorly understood. There are no field reports of the foraging techniques of wild zebrafish, but a controlled experiment to determine the sensory modalities and predation techniques of laboratory zebrafish found that adults rely on vision, rather than olfaction or mechanosensory cues, to hunt larval conspecifics and that they are active hunters rather than sit-and-wait strategists (Howe, Mcintyre, & Wolman, 2018). Wild zebrafish must be able to cope with fluctuating availability of resources and seasonal changes in prey items (Spence et al., 2008); but how they locate, select, and handle different food types and how their foraging behavior changes during ontogeny is not well understood.

Macro and Microhabitats

Different zebrafish populations are likely to experience contrasting environmental conditions and to display different behaviors in order to adapt to their surroundings, albeit we have no evidence of regional behavioral variations in zebrafish foraging behavior. Habitats frequented by wild zebrafish often support a rich array of flora and fauna that varies with habitat type and geographical location. Arunachalam et al. (2013) reported the preference of zebrafish for habitats with riparian vegetation, and Spence, Jordan, et al. (2006) observed that, even in open water habitats which made up most of their collection sites, there was almost always abundant vegetation at the margins, suggesting the importance of aquatic and emergent plants for shelter, foraging, and spawning. Paull (personal observation) noted that after the rice has been harvested in the paddy fields, the remaining stubble provides significant structure for fish. Microhabitat complexity affects the ability of fish to forage and escape predation and so influences fish abundance and distribution (Gotceitas & Colgan, 1989).

Antipredatory Behavior

Fish use many strategies to avoid being detected or caught by predators (Keenleyside, 1979). Some species hide under rocks or leaves or in dense vegetation while others use camouflage in order to resemble their surroundings or countershading to appear inconspicuous when viewed from above or from below (Keenleyside, 1979). Solitary fish may flee along an erratic path when approached by a predator while shoaling species group closer together and swim in a polarized school (Keenleyside, 1979). If attacked, a school may expand outwards in a "flash expansion" before reforming into a tight group (Keenleyside, 1979) or "skitter" in rapid boomerang movements to prevent the predator from focusing on a target (Pitcher, 1998). Fish in shoals may detect an approaching predator sooner due to the "many eyes" effect (Krause & Ruxton, 2002) and then use a variety of evasion tactics (reviewed by Pitcher & Parrish, 1986), depending on the actions of the predator.

One way in which shoaling fish respond to the approach of a stalking predator is by predator inspection, whereby individuals break away from the shoal, approach the predator, and then turn and return to the group (Pitcher & Parrish, 1986). Inspectors gain information about the predator, such as its hunger level and readiness to attack, which is transmitted to shoal members, allowing the group to move to a safer location if necessary (Light, 1989; Pitcher, 1992). Predator inspection has been recorded in many fish species, including laboratory-housed zebrafish (Dugatkin, McCall, Gregg, Cavanaugh, Christensen, & Unseld, 2005).

Wild zebrafish coexist with a variety of predators, such as the snakehead (*Channa* spp.), freshwater needlefish (*Xenentodon cancila*), catfish (*Mystus bleekeri*), knifefish (*Notopterus notopterus*), and leaffish (Nandus nandus) and with avian piscivores, such as the Indian pond heron (*Ardeola grayii*) and common kingfisher (*Alcedo atthis*) (Fig. 5.4; Arunachalam et al., 2013; Engeszer, Patterson, Rao, & Parichy, 2007; Paull, 2008; Spence et al., 2006). It is likely that some or all of these species prey upon zebrafish but this assumption has not been tested by gut content analyses.

In addition to shoaling, schooling, and predator inspection, zebrafish defense against predation may include phenotype. A zebrafish's stripes may serve an antipredator function. A shoal of striped fish may be difficult for a predator to focus on as the stripes may disrupt the outline of an individual fish or create an optical illusion that impairs the predator's ability to judge an individual's speed (Price, Weadick, Shim, & Rodd, 2008).

Water turbidity reduces visibility and impairs the ability of predators and prey to detect each other (Abrahams & Kattenfeld, 1997; Snickars, Sandstrom, & Mattila, 2004). Predator-prey encounters occur at reduced distances with increased turbidity, and the effectiveness of antipredator behavior reduces as the predator gets closer to the prey. In turbid water,

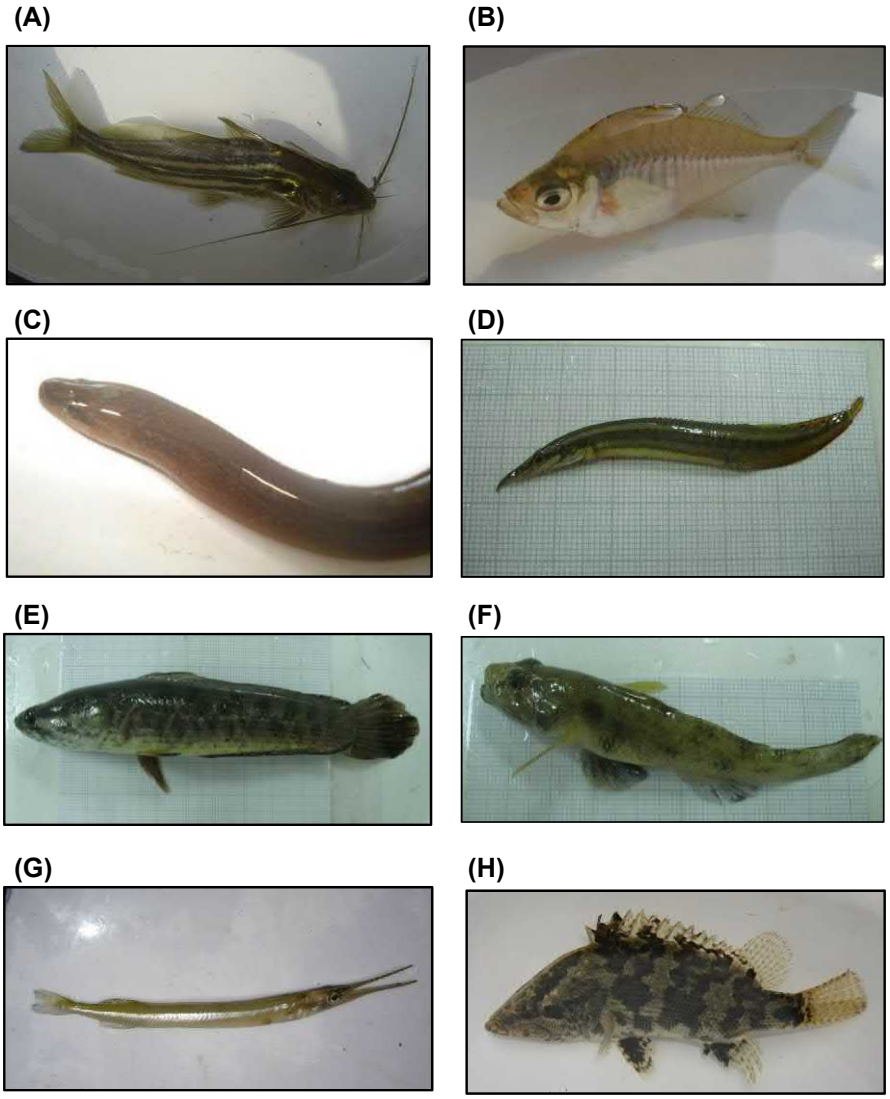

FIGURE 5.4 Piscivorous species that coexist with wild zebrafish include predators, such as (A) Day's mystus (*Mystus bleekeri*), (B) Indian glassy fish (*Pseudambassis ranga*), (C) mud eel (*Monopterus cuchia*), (D) lesser spiny eel (*Macrognathus aculeatus*), (E) spotted snakehead (*Channa punctata*), (F) tank goby (*Glossogobius giuris*), (G) needlefish (*Xenentodon cancila*) and (H) Gangetic leaffish (*Nandus nandus*). *Photos (A) to (F) by Gregory Paull, (G) and (H) by Mostafa Hossain.*

the probability of escape by prey is determined not by behavior but by the probability of predator and prey encountering each other (Abrahams & Kattenfeld, 1997). Many prey species reduce antipredator behavior in turbid waters (pike larvae (*Esox lucius*): Lehtiniemi, Engstrom-Ost, & Viitasalo, 2005; three-spined sticklebacks (*Gasterosteus aculeatus*): Fischer & Frommen, 2013; guppies (Poecilia reticulate): Borner, Krause, Mehner, Uusi-heikkilä, Ramnarine, & Krause, 2015). As turbidity affects activity levels, aggression, and shoaling tendency in zebrafish (Wojtas, Pokorny, & Mucha, 2015), it seems probable that turbidity also affects antipredator behavior.

Vegetation provides refuge for prey species while impairing the visual ability of predators to detect prey (Snickars et al., 2004). However, high-density vegetation impairs the movements of prey fish and reduces foraging efficiency. Fish may, therefore, choose to forage in open habitats close to vegetation where they can quickly hide, and trade-off between predation risk and improved foraging in a simple habitat (Snickars et al., 2004). Interactions between zebrafish and predators at different plant densities have yet to be investigated.

Laboratory zebrafish react with alarm behaviors in response to real or artificial predators, to a pheromone released when the skin of a conspecific is injured, and to a synthetic alarm substance (Dill, 1974a, 1974b; Parra, Adrian, & Gerlai, 2009; Speedie & Gerlai, 2008). Reported reactions include fleeing, increased shoal cohesion, erratic movements, "freezing" on the

substratum, and jumping. Laboratory zebrafish also responded in different ways to the sight of four stimulus fish: a sympatric and an allopatric predator and a sympatric and allopatric harmless fish (Bass & Gerlai, 2008). A sympatric predator, the leaffish (*Nandus nandus*), has been shown to elicit a significant fear response in the zebrafish, whereas an allopatric predator, the cichlid (*Nimbochromis compressiceps*), and two harmless fish, a sympatric giant danio (*Danio malabaricus*) and an allopatric swordtail (*Xiphophorus helleri*), did not evoke a fear response. The zebrafish used in this study had no previous exposure to the stimulus fish, and the researchers attributed their different responses to the four species to the genetically driven instinct that formed during evolution (Bass & Gerlai, 2008).

There is evidence that zebrafish use social learning to assess risk. Social learning refers to "any incident in which individuals acquire new behavior or information about their environment via observation of, or interaction with, other animals or their products" (Brown & Laland, 2003). When Suboski, Bain, Carty, McQuoid, Seelen, and Seifert (1990) exposed zebrafish concurrently to an alarm pheromone from an injured conspecific and to morpholine, a novel odorant, the test fish reacted to these substances with alarm behavior (moving to the substrate). Later, the same test fish showed an alarm response to morpholine alone. When the test fish were joined by naïve fish and tested for reaction to morpholine, the naïve fish demonstrated both acquired predator recognition and the ability to communicate this information to a new group of naïve observers (Suboski et al., 1990). This experiment demonstrates visual communication, learned predator recognition, and social learning of predator recognition in laboratory zebrafish (Suboski et al., 1990).

Variations in Behavior

Behavior is shaped by the combined effects of genes and the environment (Foster & Endler, 1999). Natural selection, gene flow, and genetic drift all play a part in the geographical variation in behaviors seen across many fish species (Foster & Endler, 1999). Differences in behavior occur among individuals and among populations. They may reflect predation pressure, food availability, competition, or a variable environment; and they may be influenced by a single environmental factor or by multiple factors that create a cascade effect (Foster & Endler, 1999; Magurran, 2013).

Behavioral Differences Among Individuals

Consistent individual differences in behavior, such as sociability, boldness, aggressiveness, activity, and exploration, are common across a wide variety of species. Such personality traits are often heritable and have fitness consequences that vary according to environmental conditions (Bell, Hankison, & Laskowski, 2009; Smith & Blumstein, 2008). For example, bold individuals are more likely to become dominant, to defend territories, and to have higher reproductive success than shy individuals (Ariyomo & Watt, 2013). However, bold fish take more risks and may have lower survival than their shy counterparts in areas of high predation (Smith & Blumstein, 2008). Behavioral traits have associated fitness costs depending on the particular context, and selective forces that promote timidity toward predators, for instance, are different from the selective forces in play during social interactions with conspecifics (Wilson, 1998).

Laboratory zebrafish show consistent individual differences in activity levels (Tran & Gerlai, 2013), risk-taking (Dugatkin et al., 2005), aggression, boldness, fear, and exploration (Toms & Echevarria, 2014). Zebrafish decision-making strategies are also consistent over time. For example, some individuals typically make slow, accurate decisions while others make fast, imprecise choices, and pairs of fish with individual personality traits may make consensus decisions (Wang, Brennan, Lachlan, & Chittka, 2015). Consistent individual differences in social fish may also drive collective behaviors, including movement dynamics and foraging performance (Jolles, Boogert, Sridhar, Couzin, & Manica, 2017).

Behavioral Differences Among Populations

Variations in body shape and behavior have been found among populations of wild zebrafish from diverse habitats in northern, southern, and eastern India (Shukla & Bhat, 2017; Suriyampola et al., 2015). Fish in slow-moving waters tend to have deeper bodies and caudal peduncles and longer fins than fish in still waters, and fish from waters with the highest dissolved oxygen content have relatively smaller heads (Shukla & Bhat, 2017). Body shapes are influenced by the physical, biological, and hydraulic characteristics of the waters that fish inhabit and by environmental pressures, such as foraging and predator avoidance (Gaston & Lauer, 2015; Webb, 1984). Wild zebrafish in habitats that differ in water flow and vegetation cover exhibit differences in shoaling behavior and aggression. The largest groups were seen in algal patches on a fast-flowing river (up to 300 fish in algal patches compared to 200 in open water in the same river). Zebrafish in slow-flowing water were more aggressive than those in still or faster-flowing water (Suriyampola et al., 2015).

FIGURE 5.5 (A) The complex and highly transitory natural habitat in which wild zebrafish are found contrasts with (B) the limited space and featureless environment of most laboratory tanks. *Photos by Gregory Paull.*

When fish from four wild populations were brought into the laboratory, variations in behavior were observed among the groups (Roy & Bhat, 2018). Fish from a high-predation habitat were bolder (performed more predator-inspections) than fish from low-predation habitats; males had a higher shoaling tendency than females in the presence of a predator; and larger fish were more cautious and active than smaller fish (Roy & Bhat, 2018). Another study found that levels of aggression and boldness were consistent within populations and varied between populations (Martins & Bhat, 2014). Zebrafish from streams and irrigation canals in the north of India were bold and aggressive whereas fish from irrigation canals and a lake in the south of the country were nonaggressive and shy (Martins & Bhat, 2014). A third study measured behavioral plasticity among two wild and one laboratory populations of zebrafish in four environments of differing water flow and vegetation (Bhat et al., 2015). The researchers found that plasticity varied among populations and that vegetation tended to increase aggression while water flow decreased latency to feed after a disturbance (Bhat et al., 2015). These studies provide evidence of variations in zebrafish morphology and behavior across populations. Further experiments with wild-caught fish could assess the extent of genetic and plastic influences on phenotypes. Interactions between environmental conditions, such as water flow, predator regimes, and vegetation, could be explored further in order to disentangle the role of selection on behavioral traits in wild zebrafish.

Conclusions

The lives of zebrafish are dictated by the need to eat, the need to avoid predation, and the need to reproduce. The complex and highly transitory habitats in which wild zebrafish are found and the impact of competition and predation on survival and reproduction mean that wild fish face constant decisions about what to do at any given time and must weigh the pros and cons of such decisions and accept inevitable trade-offs. Laboratory fish, in contrast, receive food ad libitum, protection from disease, and face no predators. Limited space, often a featureless environment, high stocking density, and little variability in the age and size of individuals in most laboratory tanks mean that social relations between fish are likely to differ from those occurring in the wild (Fig. 5.5).

Behavior is the basis for the relationship between a zebrafish and its environment. Despite the use of emerging technologies, such as underwater video cameras, telemetry, and acoustic tags, we still know little about the movements and behavior of wild zebrafish in their natural environment and about the effects of domestication and differences in behavior between wild fish and laboratory stocks. Knowledge of wild fish behavior could be usefully acquired to better inform decisions on how best to maintain zebrafish in the laboratory. Understanding the macro and microhabitat choices of wild fish could benefit water quality management decisions; knowledge of prey selection and diet choice by wild zebrafish could be used to develop improved feeding practices and larval rearing techniques; understanding social interactions among fish could inform decisions about optimal tank sizes, group composition and stocking densities; and understanding the reproductive behavior of wild zebrafish could help improve spawning practices. Further observations in the field and targeted laboratory experiments are needed to have a more complete understanding of the behavior of this model species and this, in turn, will lead to improved health and wellbeing of laboratory zebrafish, and increased research quality and productivity for studies using this model.

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SECTION II

Biology

CHAPTER

6

Zebrafish in Biomedical Research: Head and Body: Anatomy

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- External Anatomy
  - Body Configuration (fusiform)
    - Mouth
    - Operculum
    - Vent
    - Coelomic cavity and internal organ systems
  - Sensory organs
    - Nostril
    - Barbels
    - Eyes
    - Lateral Line
  - Skin
    - General coloration
    - Scales
      - General pattern and type
    - Mucous (slime) layer
  - Fins
    - Pectoral
    - Dorsal
    - Pelvic
    - Anal
    - Caudal

Introduction

This chapter will describe the external anatomy of the zebrafish (*Danio rerio*). Additional descriptions of organ systems indicated in this chapter are covered in more detail in later chapters. Together, these features contribute to the significant use of zebrafish as a biomedical research animal model.

The external anatomy of the zebrafish can be divided by body sections, the head, the trunk, and the tail. Zebrafish are fusiform in shape, which refers to the overall shape in which the head is smaller than the trunk, with a taper from the mouth to a point behind the pelvic fins that is the deepest (dorsal to ventral) point of the fish (Stoskoff, 1993). The body tapers from the head, including the operculum, to the trunk and from the trunk to the caudle peduncle and tail. There are other types of fish morphology; however, this configuration is very common and similar to other species of fish that are used in research, commercial aquaculture, and sport fisheries worldwide (Stoskoff, 1993). The mouth (entrance to gastrointestinal tract), nostrils (entrance into the nasal passages), and vent (containing the combined urogenital openings) are externally located portals into and out of the fish, and their location and configuration are similar to other cypriniform fishes (Figs. 6.1 and 6.2).

Because fish do not have a diaphragm physically separating a thorax from an abdomen, the coelomic cavity holds all of the internal organ of the fish. This cavity is lined by a peritoneal layer, similar to mammals; however, unlike mammals, this layer is pigmented (Ferguson, 2006). The organ systems include musculoskeletal, cardiovascular, digestive, hematopoietic, endocrine, renal, and reproductive (ovary or testes). The respiratory system is physically located outside of and anterior to the coelomic cavity within the opercular chamber (Stoskoff, 1993). Organ systems indicated are described in subsequent chapters of this volume.

The sensory organs associated with the head are the nostrils, barbels, and eyes; all are paired structures associated with chemoreception and vision. The nostrils contain the olfactory organs that are separate from the respiratory system (Hara, 2000). Barbels are accessory feeding structures that carry sensory organs for olfaction/gustation and are one characterization of Cypriniformes of which zebrafish are a representative (Elliott, 2000; Hara, 2000). The lateral line system is incorporated into the skin of the zebrafish and is located from the head of the fish to the caudle peduncle, includes all of the fins,

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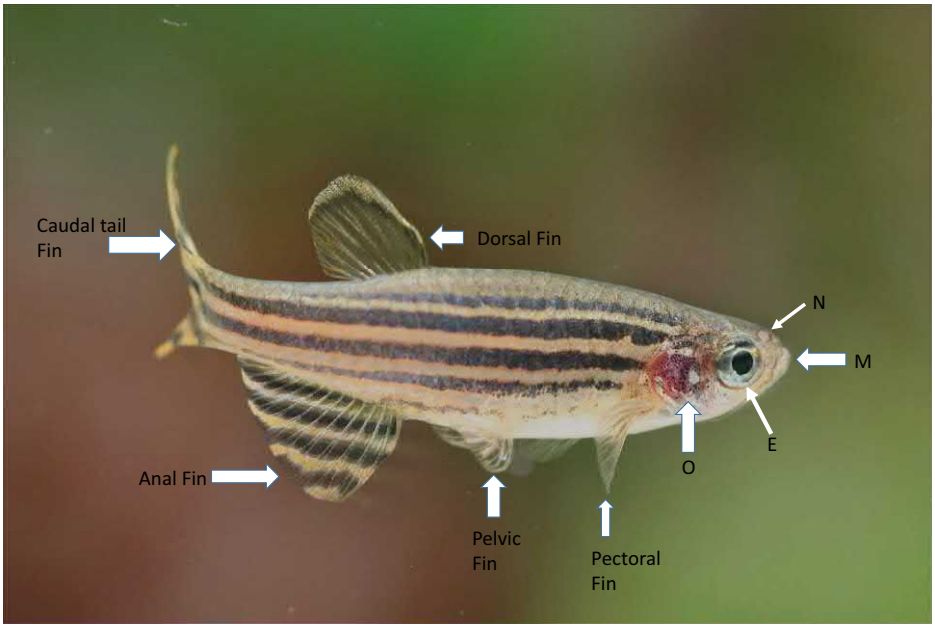

FIGURE 6.1 Digital photograph of a male zebrafish. The following anatomical features are indicated by *arrows*: mouth (M), nostril (N), eye (E), operculum (O), pectoral, dorsal, pelvic, anal, and caudal tail fins. Note the fusiform body shape of the head, body, and tail with the horizontal dark and light stripes. *Photo provided by William Trevarrow, Ph.D.*

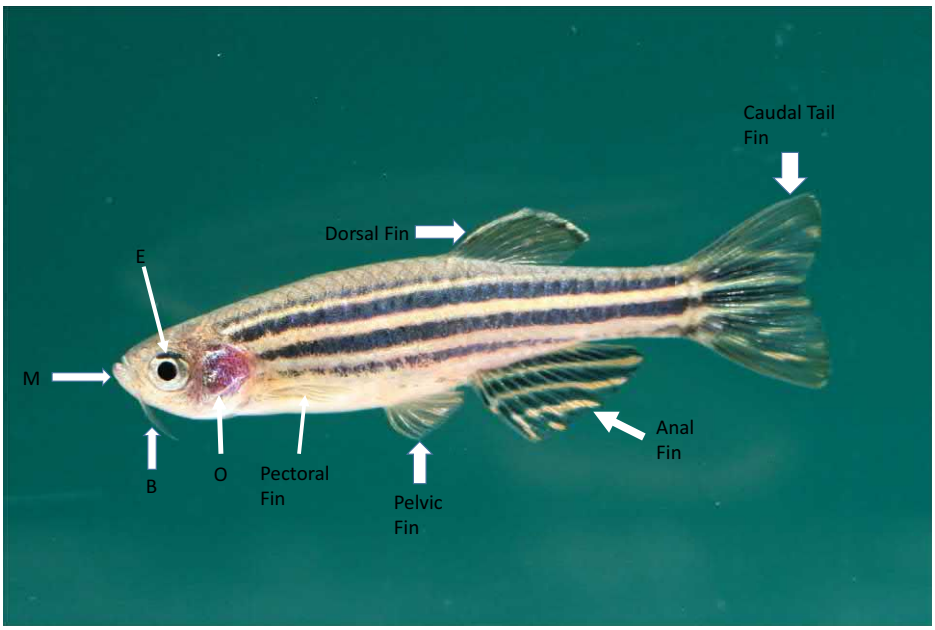

FIGURE 6.2 Digital photograph of a male zebrafish. The following anatomical features are indicated by *arrows*: mouth (M), eye (E), operculum (O), barbel (B), pectoral, dorsal, pelvic, anal, and caudal tail fins. Note the fusiform body shape of the head, body, and tail with the horizontal dark and light stripes. *Photo provided by William Trevarrow, Ph.D.*

and are associated with pressure wave detection and gustation (Hara, 2000). Fish do not have external pinnae but do have internal ears. This portion of the sensory system is associated with the semicircular canals and endolymph-filled ampullae/maculae each with its own calcareous otolith (Stoskoff, 1993).

As in mammals, the skin is the largest organ system of the fish. The integument is a protective physical barrier that also assists in osmoregulation and is inclusive of the characteristic horizontal stripes on the fish (Elliott, 2000; Harper and Lawrence 2011). Several types of pigment cells, and their final combinations, are necessary and

associated with the general coloration of the striped zebrafish. Scales are overlapping, bony, protective and flexible structures associated with the integument of the zebrafish. These are classified as cycloid, which is described as being a round, thin, flat scale type (Elliott, 2000; Harper and Lawrence 2011). The entire fish is covered with mucous secretions that keep the skin surface free of pathogens by means of constant sloughing, renewal, and the presence of antimicrobial substances (Elliott, 2000).

The fins of the fish consist of paired pectorals and pelvic with unpaired dorsal, anal, and caudal tail fins and are associated with propulsion and steering (Figs. 6.1 and 6.2). The pectoral fins are ventrally located on the cranial trunk and originate from a musculoskeletal girdle associated with the skull. The pelvic fins are also ventrally located but are located cranial to the common urogenital and anal openings, also known as the vent, (Stoskoff, 1993; Stiassny, 2000) but are associated with a different musculoskeletal girdle. The anal fin is located ventrally caudal to the vent and the dorsal fin is located in the caudal one third of dorsal aspect of the fish.

Conclusion

The external anatomy of zebrafish *(Danio rerio)* and the varying components briefly described above are important to the use of this fish as an established and growing research animal model. Understanding these components, how they develop, function, and can be modified is critical to its continued and future incorporation in the search for knowledge about other aquatic animal models and terrestrial organisms on the phylogenetic scale of complexity.

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CHAPTER

7

Establishing The Body Plan: The First 24 Hours of Zebrafish Development

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The Early-stage Embryo

Fertilization and Cleavage

Fertilization of a zebrafish egg occurs *ex utero*, which is one of the reasons zebrafish has become an important system for the study of early embryonic development. Embryogenesis of the transparent zebrafish embryo can be easily studied from fertilization onwards, whereas studying the embryogenesis of placental animals is much more difficult because the embryo develops inside the mother's body. The sperm enters through a structure called the *micropyle*, which acts as a guidance system to direct the sperm to the egg (Amanze & Iyengar, 1990). The micropyle is established during oogenesis on what will be the future *animal pole* of the egg, which is the side where the embryo itself will form. The opposite pole is called the *vegetal pole*, and the animal-vegetal axis will eventually correspond to the anterior-posterior axis of the embryo once gastrulation has finished (Fig 7.1A).

The first cellular division occurs 75 min after sperm entry, with following divisions occurring synchronously every 15 min for the first 3 h after fertilization. The first 12 divisions of the *blastomeres*, or the actively dividing cells in the animal pole, are synchronous and the cells have only 2 cell cycle phases, M-phase (mitosis) and S-phase (DNA replication), and so are essentially dividing as fast as they possibly can (Kimmel & Law, 1985). The cellular divisions in the animal pole are *mesoblastic*, meaning that only the embryonic cells divide while the yolk stays intact as one large cell. The long-time window before the first cleavage is very useful for experimental embryology since molecules, such as mRNA, DNA, gRNAs and Cas9 for CRISPR, etc., can be injected during this time and they will be incorporated in all the tissues of the embryo, including the future germ cells. This has made it relatively easy to produce transgenic zebrafish lines by introducing foreign DNA using transposase systems (Kawakami, 2007), as well as to edit the genome using CRISPR (Li, Zhao, Page-McCaw, & Chen, 2016).

Blastula Stage

At 2 ¼ h after fertilization, the blastula period begins. This stage begins as the accumulating blastomeres start to look like a ball of cells mounded upon the yolk cell. The 10th cell division marks the first cell movements, as three distinct cell populations begin to form. The *yolk syncytial layer* (YSL) forms when the most vegetal cells, which have kept a common cytoplasmic connection with the yolk during the blastomere cleavages, form into a multinucleate network that has an essential role in signaling to the overlying cells (Kimelman & Griffin, 1998). The *enveloping layer* (EVL) is comprised of the most superficial cells covering the animal pole, which is an epithelial layer one cell thick. All of the cells between the EVL and YSL are called *deep cells*, and they are the cells that will form the embryo proper (Fig. 7.2).

Three hours postfertilization (denoted as hpf) at the 10th cell division, which is often called the *midblastula transition* (MBT), a number of dramatic changes happen, including the start of asynchronous cell divisions, with the introduction of an interphase period in the cell cycle, and the start of zygotic gene transcription (Fig. 7.1B) (Kane & Kimmel, 1993). Thus, this period is also called the *maternal-to-zygotic transition* (MZT). Up until this time, the embryo relies on maternal proteins and mRNAs, but at the MBT/MZT, an increasing number of zygotic genes are transcribed as the embryonic cells change from a pluripotent state to cells with increasingly restricted cell fates (Langdon & Mullins, 2011). This is

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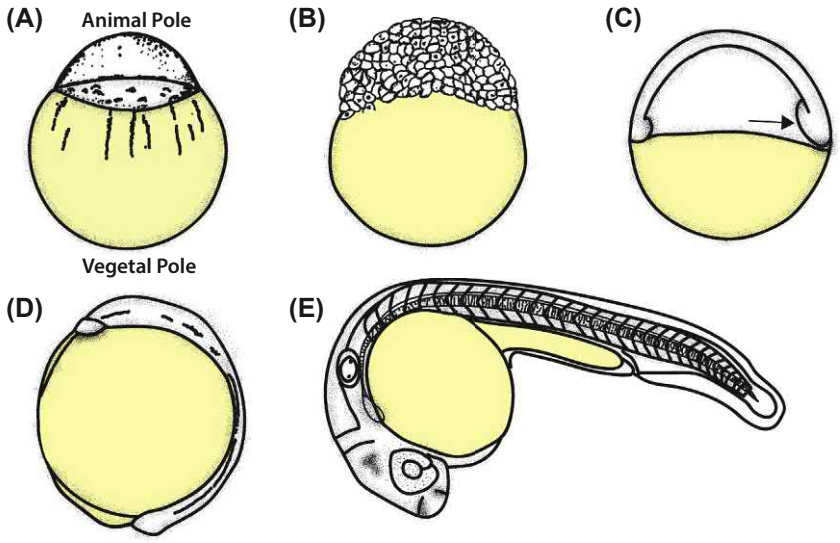

FIGURE 7.1 Sample images from the staging series, or a defined set of developmental stages by which researchers describe the age and developmental point of the zebrafish embryo. (A) The single-cell embryo just proceeding fertilization. The animal and vegetal poles are labeled. (B) The midblastula embryo at three hpf, with a mound of dividing blastomeres atop the yolk. The blastomeres are initiating the MBT/MZT. (C) The early gastrula embryo at six hpf, during which the cells are at 50% epiboly and the shield (*arrow*) has formed along one side of the embryo. (D) The end of gastrulation at 10 hpf, marked by the completion of epiboly around the entire yolk. The future head and tail are distinct at the animal and vegetal poles, respectively, which are in the same orientation as shown in panel A. (E) The 24 hpf embryo has a distinct body plan with a head, trunk, and tail somites, and developing organs. If the embryo in panel E was rotated 90 degrees clockwise, it would be in the same orientation as the embryos in panels A–D.

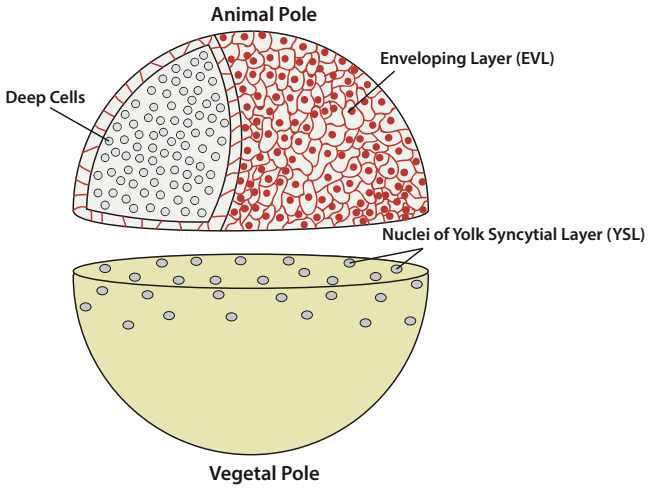

FIGURE 7.2 A detailed view of the midblastula embryo. The actively dividing blastomeres are mounded atop the yolk cell. The most external layer of cells is called the enveloping layer (EVL), and the cells along the yolk surface are the yolk syncytial nuclei (YSL). All the cells between are the deep cells, which will give rise to all of the structures in the mature embryonic body.

due to a variety of intercellular signaling factors expressed within specific domains of the blastoderm, which instruct cells as to their particular identity (Langdon & Mullins, 2011). Among the earliest divisions of the embryonic cells made by these factors are what are called the *three germ layers*: the ectoderm (skin and nervous system), mesoderm (e.g., muscle, vasculature, and dermis) and endoderm (e.g., gut, liver, and pancreas).

Gastrula Stage

As the blastula stage ends, the embryo becomes highly spherical, and then the embryonic cells begin to spread around the yolk with the yolk bulging up toward the animal pole. This process is called *epiboly*, and it will continue throughout the gastrula stages until all of the embryonic cells, with the EVL atop, have enveloped the yolk. A useful analogy is to think of putting a stretchy woolen ski cap on top of your head, and grabbing the brim and pulling it down all around your head until the hat completely covers your skull. The characteristic appearance of the embryo at each stage from 50% epiboly to the end of gastrulation is very useful for determining exactly how far into gastrulation the embryo is. Gastrulation begins at 50% epiboly (5 ¼ hpf) with the formation of a thickened ring of tissue all around the equator of the embryo called the *germ ring*, and it continues until epiboly completes at 10 hpf (Fig. 7.1D). The germ ring is comprised of the future mesodermal and endodermal tissues.

Soon after 50% epiboly, a small thickening within the germ ring is observed on what is called the dorsal side of the embryo at six hpf (Fig. 7.1C, arrow). This structure, which is named the *shield*, will become the mesoderm

of the head and represents the first cells to move into the deeper layers of the embryo in a process called *involution*. The shield also plays a critical role as a source of essential signaling molecules that will define the fate of much of the early embryo and so it is often called the *organizer* (Shih & Fraser, 1996). Once the shield becomes visible, one can predict with a fairly high degree of accuracy, as to what cells in the different regions of the embryo will become, often shown in a fate map (Fig. 7.3A).

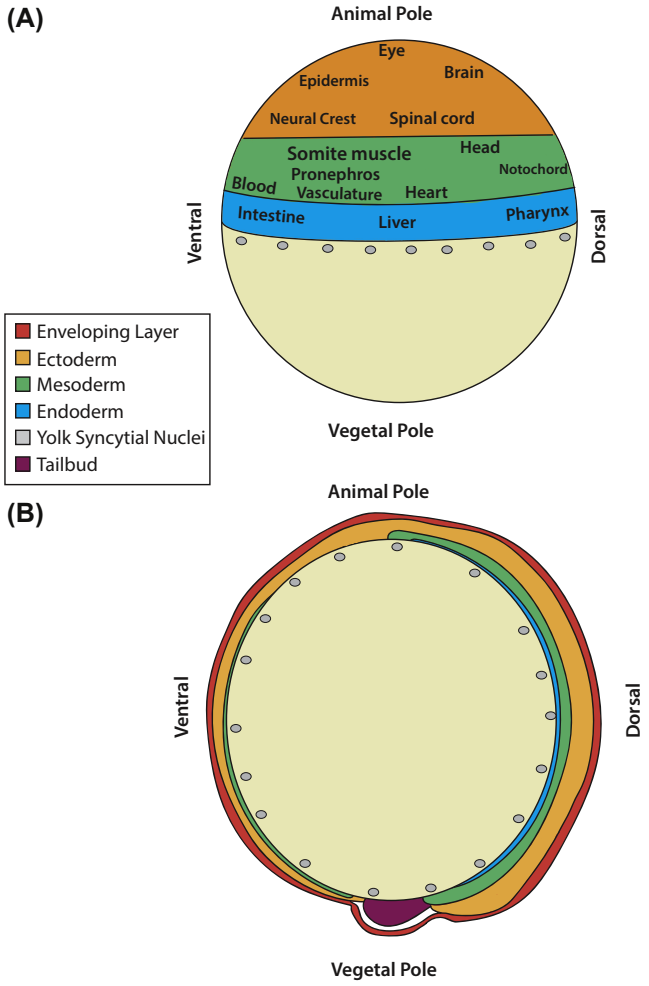

FIGURE 7.3 (A) A fate map of the early gastrula embryo, which can reliably predict the eventual fates of the deep cells. Color coding represents the location within the mature body structure, with the ectodermal fates nearest the animal pole and the endodermal fates along the surface of the yolk. (B) A detailed view of the end of gastrulation, showing the completion of epiboly around the yolk cell and the presumptive head and tail forming at the animal and vegetal poles, respectively. The tailbud, located at the vegetal pole, is the structure from which the bipotential neuromuscular progenitors will originate. *Figure adapted from Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. Developmental Dynamics,* 203*(3), 253–310. http://10.1002/aja.1002030302.*

The process of involution begins with the shield, then proceeds around the equator until all of the cells of the germ ring have begun the involution process, which involves cells migrating underneath the surface layer of the cells to create multiple layers. This will continue throughout gastrulation, moving the mesoderm underneath the ectoderm and the endoderm underneath the mesoderm. As this is happening, a variety of other cell movements are also happening. The shield cells are moving toward the animal pole, the cells in the middle of the germ ring are moving toward the dorsal side of the embryo, and cells on the most ventral side move downward toward the vegetal pole. The net result of all these movements is that the embryo is now formed along the dorsal side of the embryo, with the anterior (future head) at the animal pole, and the posterior (future tail) at the vegetal pole (Fig. 7.3B).

Somitogenesis

As gastrulation comes to a close, the process of somitogenesis begins, as groups of mesodermal cells called *somites* form into morphologically distinct units, which develop primarily into fast and slow muscle. This is called the segmentation period of development and is marked by the continual extension of the tail as the anterior-posterior axis elongates. Somites are formed consecutively from anterior to posterior until approximately 32 somites have formed at 24 hpf (Fig. 7.1E). The somites anterior to the anus are called the *trunk somites*, and those posterior to the anus are the tail somites. Many of the trunk somites arise from cells that moved toward the dorsal side during gastrulation, whereas the tail somites form from a vegetal structure formed at the end of gastrulation called the *tailbud* (Fig. 7.3B).

The tailbud is an important structure that not only contributes to the somites but also produces the spinal cord. Both of these cell populations originate in a bipotential neuro-mesodermal progenitor (NMP) population, located in the tailbud at the very posterior end of the embryo (Fig.7.4A). Whereas most germ layer decisions are made very early in embryogenesis, the NMPs are unusual as they give rise to both neural (a subset of ectoderm) and mesodermal cell fates. Cells continuously leave the NMP population as somitogenesis continues, forming an ordered sequence of somites as well as the future spinal cord until the population of NMPs is exhausted and somitogenesis ends (Kimelman, 2016). The exit of the mesodermal cells from the NMPs requires a mesenchymal to epithelial transition (EMT) as the stationary NMP cells are converted to migratory mesenchymal cells.

During the process of somitogenesis, the mesodermal cells enter a region called the *paraxial mesoderm*, where

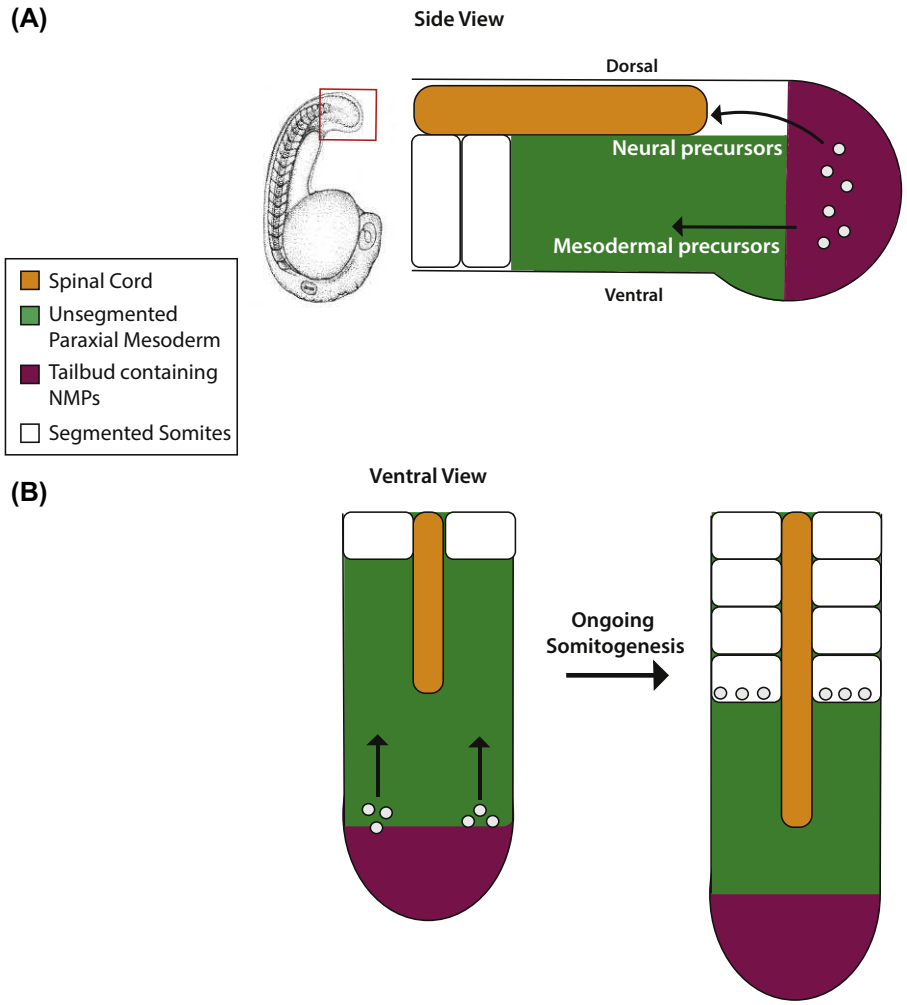

FIGURE 7.4 (A) A side view of the tailbud and developing posterior end of the embryo. The tailbud contains the bipotential neuromuscular progenitor cells (NMPs), which allocate between neural and muscle tissues as the tail develops throughout the somitogenesis stage. The red box on the embryo picture (left side of the panel) shows the location of the tailbud during the somitogenesis stages. (B) A ventral view of the incorporation of newly fated mesodermal cells into developing somites. As cells in the tailbud acquire the mesodermal fate, they begin to migrate anteriorly through the unsegmented paraxial mesoderm.

cells are allocated into the physically distinct blocks that form the visible somites (Fig. 7.4B). A "clock and wave" mechanism controls the periodic segmentation of cells into groups, which involves both a repeating oscillator and a gradient of a signaling molecule emanating from the posterior end that operates with remarkable fidelity (Saga & Takeda, 2001). Intercellular signaling between cells at the somite boundaries defined by the clock and wave system initially helps to form the borders, which are then maintained by the deposition of extracellular matrix (Saga & Takeda, 2001).

Early Head Formation

The patterning of the zebrafish brain begins around 9 h postfertilization before the gastrulation period has come to a close, and many of the structures for the mature brain are developed by the end of 24 h (Kimmel, 1993). The first steps of brain development in any vertebrate is the formation of the neural tube. In the case of zebrafish, this process of neurulation begins with a section of ectoderm called the *neural plate*. The neural plate is formed as epithelial cells on the dorsal side compact to form a thick column (Lowery & Sive, 2004). These epithelial cells continue to thicken and sink deeper into the mesodermal layer of the embryo, as well as extending posteriorly down the anterior-posterior axis. At this point, the columnar structure is separated from the epithelial layer that lies above and is called the *neural rod*. It is here that a lumen begins to open, starting on the ventral side of the neural rod, thus creating a true neural tube (Lowery & Sive, 2004).

The next hallmark of vertebrate brain formation is the formation of vesicles from the neural tube, a process that involves the swelling of the lumen within the tube to

create distinct cavities. In zebrafish, these periodic constrictions of the tube are called *neuromeres* (Kimmel, 1993). Neuromeres begin to form after 12 hpf. By 18 hpf, 10 distinct neuromeres have segmented. Three of these represent the forebrain and midbrain, and seven cavities form the hindbrain (Kimmel, 1993). This initial pattern of 10 neuromeres develops quickly into a mature brain by 24 hpf, which has distinct functional regions, such as the epiphysis and cerebellum. At this time, the brain has many different neuronal cells, all arising from the primary neurons, which begin in the center of each neuromere and send out long axonal projections, which interconnect the neurons in the mature brain (Kimmel, 1993). Some of these primary neurons will become motor or sensory neurons by sending their long projections outside of the neural tube into the periphery (Schilling & Kimmel, 1994). As early as 16 hpf, some sensory neurons have already sent projections along the ectoderm, allowing the embryo tactile sense (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). *Neural crest cells* migrate into the brain from the dorsal side of the neural tube and populate it with various kinds of neurons (Schilling & Kimmel, 1994).

The other parts of the zebrafish head, such as the eye and jaw, fully form later into development. The zebrafish eye begins to develop around 11 hpf as the diencephalon neuromere begins to open its lateral walls (Kimmel et al., 1995). By 24 hpf, this invagination is covered by a bilayer of retinal cells that is enclosing what will become the eye lens (Dahm, Schonthaler, Soehn, van Marle, & Vrensen, 2007). Meanwhile, the *pharyngeal arches*, or seven bands of cartilaginous tissue that give rise to the mature structures of the face such as the jaw and throat, are being formed from all three germ layers. Primarily, the neural crest cells form cartilage, neurons, and glia, and the underlying mesodermal cells generate muscle cells (Schilling & Kimmel, 1994).

Initial Formation of Differentiated Cell Types: Ectoderm

The ectodermal layer has two principle derivatives, the skin and nervous system, which form from the animal pole of the embryo (Fig. 7.3A). The skin in zebrafish is initially a single layer called the *periderm* that derives from the EVL, and then a second layer forms underneath called the *epidermal basal layer* derived from the deep cells during gastrulation (Chang & Hwang, 2011). During these early stages, the embryo does not have a dermis, which only forms much later. On the dorsal and ventral surfaces of the trunk area, the ectodermal cells begin to move outward, forming what is called the *median fin fold*, which will continue to develop into the dorsal and ventral fins.

The neural tube undergoes a variety of patterning events along the dorsal-ventral axis regulated by intercellular signaling. The ventral aspect of the neural tube is patterned by the underlying notochord and the ventral most tissue of the neural tube called the *floor plate*, whereas the dorsal aspect of the neural tube is patterned by the overlying ectoderm and the dorsal most tissue of the neural tube called the *roof plate*. Above the neural tube and below the ectoderm are the neural crest cells (Fig. 7.5), which will migrate ventrally around the somites to produce the glia and peripheral nerves.

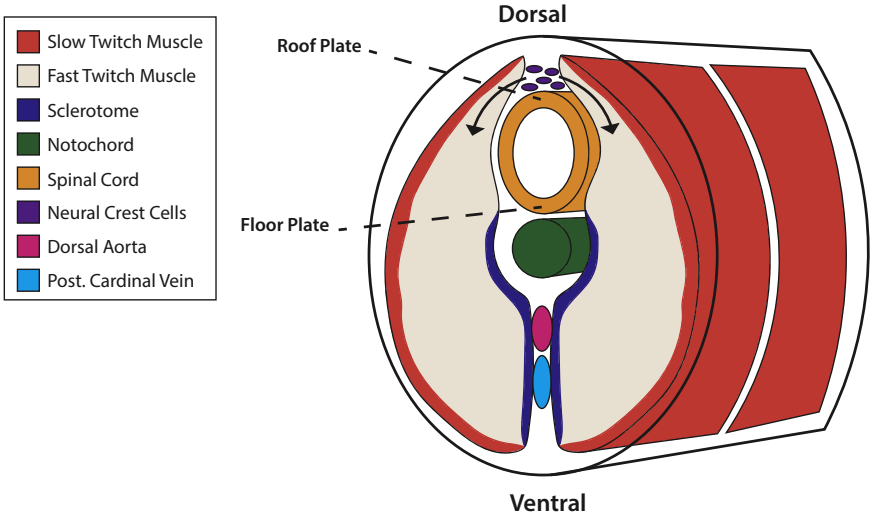

FIGURE 7.5 A cross-sectional view of the differentiating tail of the zebrafish embryo. The somites have differentiated into multiple tissue types, including slow- and fast-twitch muscle. The spinal cord runs lengthwise along the body axis and is being patterned by adjacent tissues along the roof and floor plates. The dorsal aorta and posterior cardinal vein precursors have migrated toward the midline of the embryo. The trunk is similar except that the gut tube is beneath the blood vessels, and the pronephric ducts are lateral to the vessels.

These neural crest cells form crucial structures in both the cranium and trunk of the embryo and are fated based on where they end after migrating from the dorsal surface of the neural tube (Klymkowsky, Rossi, & Artinger, 2010).

Initial Formation of Differentiated Cell Types: Mesoderm

The mesoderm comprises a wide variety of tissues in the early embryo, including the muscle, the vasculature, blood and heart, the cartilage, and the kidneys. Along the trunk, the cells along the borders of each somite undergo a mesenchymal to epithelial transition (MET), compacting to form an epithelial sheath. Most of the cells within the somite will become fast-twitch muscle, but a small population of cells located next to the notochord will migrate from the center of the body toward the outside of the somites to produce slow-twitch muscle (Fig. 7.5) (Goody, Carter, Kilroy, Maves, & Henry, 2017). As the functional units of muscle form, muscle fibers anchor themselves to the anterior and posterior boundaries of the somite, elongating it to create a distinct chevron shape (Kimmel et al., 1995). At 19 hpf, most of the trunk somites exhibit this shape and are able to start spontaneous twitching of muscular contractions (Kimmel et al., 1995). A small population of cells located initially ventrally in the somites will produce the sclerotome, which will migrate around the notochord and neural tube and give rise to the cartilage and vertebrae (Stickney, Barresi, & Devoto, 2000).

The *notochord*, a cartilaginous rod running from the first trunk somite to the very posterior of the tail directly down the midline of the embryo (Fig. 7.5), originates in the dorsal mesoderm of the early gastrula embryo (Fig. 7.3A). It plays key roles as a signaling center and is also essential for the early morphogenesis of the embryonic body as it elongates along the anterior-posterior axis. The notochord cells become ensheathed in the extracellular matrix, and then expand with fluid-filled vacuoles, forming a semi-rigid structure running down the middle of the embryo, allowing the embryos to locomote as their muscles become active (Stemple, 2005).

The vasculature originates in the ventrolateral mesoderm of the early gastrula embryo (Fig. 7.3A), and after gastrulation, it lies ventrally on either side of the midline in the intermediate cell mass (Gore, Monzo, Cha, Pan, & Weinstein, 2012). The cells then migrate to the midline forming the dorsal aorta and posterior cardinal vein below the notochord during the somitogenesis stages (Fig. 7.5). Additional vascular cells at the most posterior end of the embryo derive from the neuromesodermal population described above (Row, Tsotras, Goto, & Martin, 2016). The vessels form an open lumen by 23 hpf, and blood circulation begins soon thereafter. Around this time, sprouts emerge from the dorsal aorta and migrate dorsally, forming additional vessels throughout the body (D). A half-day later, sprouts will emerge from the posterior cardinal vein, completing the early embryonic vasculature.

The "primitive" blood also originates in the ventrolateral mesoderm and subsequently, the intermediate cell mass (Davidson & Zon, 2004). Like the cells of the vasculature, the blood migrates to the center of the body during the segmentation stage, where it is incorporated into the blood vessels. The mature blood originates around 24 hpf from within the dorsal aorta (Davidson & Zon, 2004).

The early zebrafish kidneys, or pronephros, are comprised of a relatively simple pair of nephrons, which are the basic unit of epithelial cells that function to filter the blood for the first few weeks of life (Gerlach & Wingert, 2013). The pronephros progenitors arise initially from the ventrolateral mesoderm located near and partly intermingled with the blood and vascular progenitors (Fig. 7.3A). During gastrulation, these cells migrate to the lateral boundaries of the embryo, but unlike the blood and vascular cells, they remain lateral. During the segmentation period, the pronephros differentiates into an anterior-posterior series of different domains with podocytes forming at the most anterior end and a pronephric duct which connects to the cloaca at the most posterior (Gerlach & Wingert, 2013). However, functional blood filtration does not begin until the 24–48 hpf stage.

The heart is the first organ to develop and function during organogenesis. During the early gastrula stage, heart progenitors can be identified along the ventral lateral region of the blastoderm (Fig. 7.3A) (Stainier, 2001). By the beginning of somitogenesis, these progenitors lie in the lateral plate mesoderm below the hindbrain and comprise both endocardial and myocardial precursors. At 19.5 hpf, these precursors have migrated to form a traffic cone-shaped structure with the base comprised of atrial cells and the interior surface of ventricular cells (Stainier, 2001). At 24 hpf, this cone telescopes to form the heart tube, which then lies along the anterior-posterior axis. The functional atrial and ventricular chambers will develop from this tube over the next 24 h.

Initial Formation of Differentiated Cell Types: Endoderm

The endoderm is the deepest tissue layer in the zebrafish embryo, which will give rise to the epithelial cell lining of the gut, respiratory tract, the thyroid and

thymus, and associated gut organs, such as the liver and pancreas (Tam, Kanai-Azuma, & Kanai, 2003). The endodermal precursors are found on the lateral margin of the late blastula embryo intermixed with mesodermal precursors (Fig. 7.3A) (Tam et al., 2003). Although a majority of the endodermal organs do not fully develop until later in development, by 24 h the zebrafish has a defined anterior-posterior patterning of precursors for most of these organs (Chu & Sadler, 2009).

By the end of gastrulation (10 hpf), the endoderm precursors have moved toward the midline of the embryo and arranged themselves along the anterior-posterior axis in accordance to their eventual tissue fates. The cells that will comprise the pharynx and thyroid are most anterior, while the tissues of the foregut, midgut, and hindgut align more posteriorly (Kinkel & Prince, 2009). By 20 hpf, this line of cells condenses to form a rod down the center of the embryo, much like the neural tube (Ober, Field, & Stainier, 2003). Later in development, this rod will hollow out to form the gut tube. The further differentiation of the endoderm along this linear gut tube occurs in steps: the region of the foregut then goes through organogenesis to develop further organs, such as the liver and pancreas (Kinkel & Prince, 2009).

The hepatocyte precursors that give rise to the liver are first distinguishable in mid-somitogenesis and lie beneath the first somite (Ober et al., 2003). These cells are integrated into the rod of gut precursors so by the end of 24 hpf, a thickening in the rod signals the beginning of a projection of hepatocytes that will branch out to form the mature liver (Ober et al., 2003). The pancreas develops in the trunk region from cells generally associated with the foregut, posterior to the liver bud. A developing pancreas is comprised of two buds, ventral and dorsal, which later merge to form the mature organ (Kinkel & Prince, 2009). At the end of 24 h, the dorsal pancreatic bud is beginning to form off of the gut tube, with associated endocrine and exocrine cells already differentiating (Kinkel & Prince, 2009). Although this bud is not distinct until 24 h, the associated endocrine cells start to secrete insulin at 15 hpf (Ober et al., 2003). The dorsal bud initially forms deep to somites three and four along the midline and moves more posteriorly as the body axis elongates (Kinkel & Prince, 2009). This dorsal pancreatic bud gives rise to the majority of the hormone-producing tissue in the mature pancreas (Tiso, Moro, & Argenton, 2009).

The development of the zebrafish embryo over the first 24 h of life encompasses a remarkable program of cell rearrangements and differentiation, all of which take place in a transparent embryo, easily visible to our eye. This patterning occurs in an extremely predictable fashion, making the zebrafish a powerful tool for studying the early development and organogenesis of higher vertebrates.

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CHAPTER

8

Zebrafish Integumentary System

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Introduction

The zebrafish, like all animals, is covered with sophisticated skin that acts as both a wall and a window to the external environment. The skin is traditionally divided into three layers. A superficial epidermis—comprising a stratified epithelium—provides barrier and sensory function. The underlying dermis is dominated by bony elasmoid scales and a deep collagenous stroma that together armor and support the muscles and organs. The relatively thin hypodermis lies deeper, at the boundary between skin and muscle, and harbors adipocytes and pigment cells. Below, we give a brief primer on the development and anatomy of zebrafish skin.

Epidermal Development and Anatomy

The epidermis represents the ultimate boundary between the fish and its environment. It forms a semiimpermeable barrier while also allowing sensory functions. The primary epidermis, known as the enveloping layer, first develops before gastrulation (Kimmel, Warga, & Schilling, 1990). Following gastrulation, the enveloping layer becomes the outermost skin cell layer, the periderm, and basal cells arise from nonneural ectoderm, thereby generating a bilayered epidermis (Bakkers, Hild, Kramer, Furutani-Seiki, & Hammerschmidt, 2002; Lee & Kimelman, 2002). Periderm cells are connected by tight junctions forming a solute barrier and are decorated with elaborate, wavy microridges that increase epidermal surface area and likely aid in mucous retention (Hawkes, 1974; Kiener, Selptsova-Friedrich, & Hunziker, 2008; Lam, Mangos, Green, Reiser, & Huttenlocher, 2015; Whitear 1970). This bilayered state persists through the early larval period until 6–7 standardized standard length [SSL (Parichy et al. 2009); ~6–7 mm standard length], when descendants of basal cells generate the intermediate suprabasal cell layers (Guzman, Ramos-Balderas, Carrillo-Rosas, & Maldonado, 2013; Lee, Asharani, & Carney, 2014).

In adult skin, there are two to eight layers of suprabasal cells over the surface of the fish, whereas superficial periderm and deep basal cells remain as monolayers (Fig. 8.1). Similar to mammalian epidermis, basal cells serve as epidermal stem cells, and their proliferation as well as suprabasal cell proliferation is regulated by ΔN-p63. Intermediate suprabasal cells are the most proliferative cells in the epidermis and most appear undifferentiated. It is possible they serve as a transient amplifying population, though precise lineages and stem cell kinetics of the epidermis remain unresolved (Guzman et al. 2013; Le Guellec, Morvan-Dubois, & Sire, 2004; Lee & Kimelman, 2002; Quilhac and Sire 1999; Richardson et al. 2013).

The epidermis harbors several additional cell types. Goblet cells secrete mucous and club cells produce alarm substances (Jevtov, Samuelsson, Yao, Amsterdam, & Ribbeck, 2014). Ionocytes and chemosensory cells aid in maintaining chemical homeostasis (Coccimiglio & Jonz, 2012; Cruz, Chao, & Hwang, 2013; Hwang & Chou, 2013). Somatosensory cells send their nerve endings into the epidermis enabling a sense of touch (Rasmussen, Vo, Sagasti, 2018). Moreover, the mechanosensory neuromasts of the lateral line are hosted by the epidermis and enable detection of water motions against the surface of the fish (Lee, Asharani, Carney, 2014; Metcalfe, 1985).

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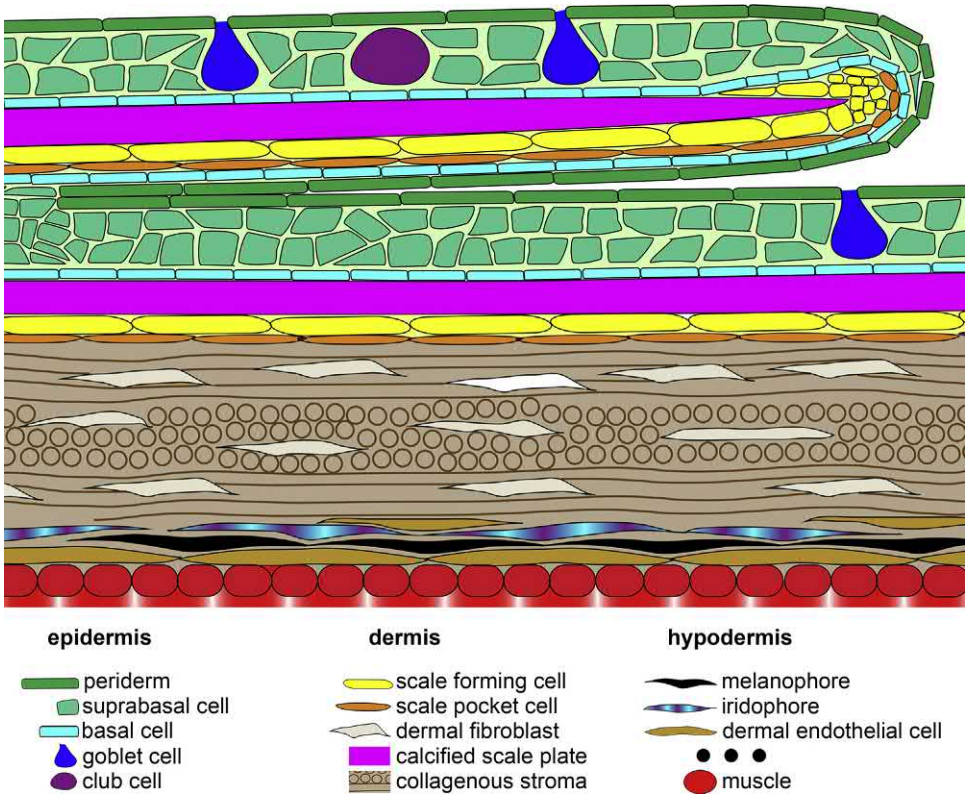

FIGURE 8.1 **Schematic section through adult zebrafish skin.** Depicted is a simplified coronal skin section at the level of a dark stripe in the trunk. For clarity, adipocytes, blood vessels, nerves, chemosensory cells, ionocytes, immune cells, and other cells are omitted, and those cell types shown are rendered with exaggerated thickness. The outermost periderm (green) and suprabasal cells (teal) overlie the basal cell monolayer (cyan). Epidermis wraps around the posterior margin of the calcified scale plate (magenta) and the scale-forming cells (yellow) and is closely associated with the scale pocket cells (orange). The epidermis contains specialized cell types including goblet cells (blue) and club cells (purple), secreting mucous and alarm substance, respectively. The surface of the fish is protected by at least two scales. Beneath the scales lies a collagenous stroma, the stratum compactum (tan), harboring dermal fibroblasts (beige). Dermal endothelial cells of the hypodermis (light brown) line the muscles (red) at the deep limit of the skin. Pigment cells, including melanophores (black) and iridophores (cyan/purple), reside in close proximity to hypodermal cells.

Development and Anatomy of the Hypodermis and Collagenous Dermal Stroma

The dermis contains a dense collagenous stroma—the stratum compactum—that imbues the skin with mechanical strength. Basal epidermal cells begin producing a primary collagenous stroma by 24 h postfertilization (hpf). Once initiated, the stroma thickens throughout the life of the fish. By 72 hpf, thin layer of dermal endothelial cells begins to accumulate along the surface of the muscles to constitute the hypodermis. These cells also contribute to the growth of the primary stroma. Basal epidermal and hypodermal cells continue to produce collagen through the early larval period, building an ever thicker collagenous stroma. Hypodermal cells remain a sparse layer in adult fish, where they likely provide trophic and other support to pigment cells comprising the stripe pattern and to dermal adipocytes (Fig. 8.1) (Hirata, Nakamura, & Kondo, 2005; Lang, Patterson, Gordon, Johnson, & Parichy, 2009; Le Guellec, Morvan-Dubois, Sire 2004; Minchin and Rawls 2017).

Coincident with stratification of the epidermis, the primary collagenous stroma of the dermis becomes organized into a plywood-like arrangement of orthogonally aligned collagen fibers. This stroma remains devoid of cells until ~8 mm SSL (Parichy, Elizondo, Mills, Gordon, Engeszer, 2009) when it is populated by dermal fibroblasts to generate the stratum compactum, a tough yet transparent structural component of the skin that resists tearing and may provide elastic recoil to the swim stroke (Fig. 8.1) (Szewciw and Barthelat 2017).

Development and Anatomy of Elasmoid Scales

Hundreds of arrowhead-shaped, calcified scales are embedded in the dermis of adult zebrafish (Fig. 8.2A). These overlap precisely like roof tiles so that every position along the body is covered by at least two scales, providing flexible armor that likely protects against puncture injuries that might be inflicted by cooccurring

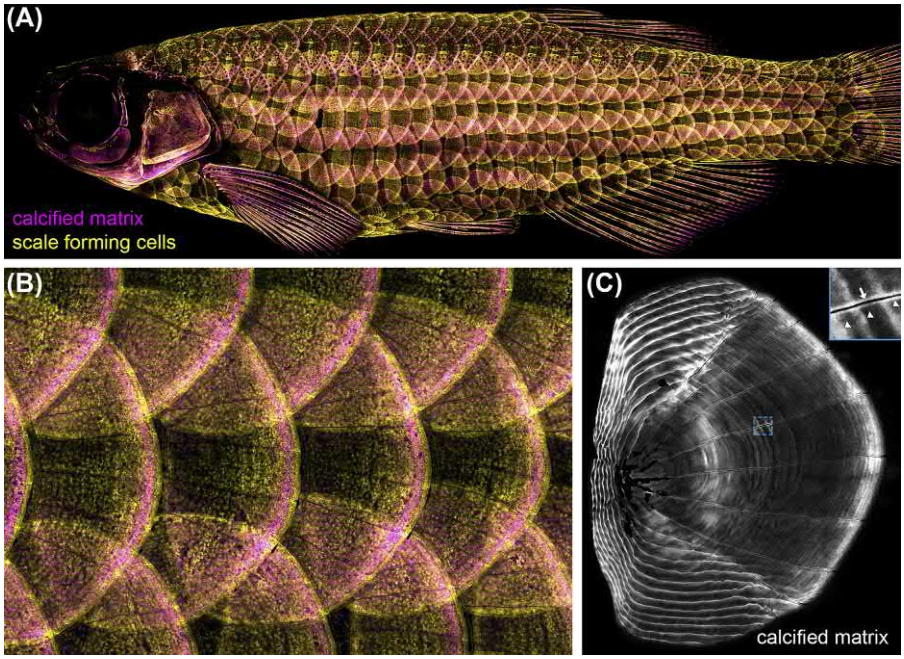

FIGURE 8.2 Distribution and morphology of zebrafish scales. (A) Scales cover the postcranial surface of the fish, here revealed by expression of *sp7:EGFP* in scale-forming cells (yellow) as well as Alizarin Red-S labeled calcified matrix (magenta). (B) Overlapping scales are arranged in a half-offset, hexagonal grid. (C) A freshly plucked, Alizarin Red-S stained scale shows a relatively heavily calcified limiting layer at the posterior margin and concentric circuli (arrowheads in inset). Note the different arrangement of circuli in the protruding posterior and embedded anterior portion of the scale. The protruding portion of the scale contains matrix-free radii that organize and house neurons and vasculature of the skin (arrow in inset).

predators or other types of trauma (Figs. 8.1, 8.2A,B) (Engeszer, Patterson, Rao, & Parichy, 2007; Sire, Allizard, Babiar, Bourguignon, Quilhac, 1997; Zhu, Szewciw, Vernerey, Barthelat, 2013).

After the stratum compactum forms, in 8–10 SSL larvae, dermal cells accumulate at the superficial limit of the dermis, in close proximity to the epidermis. These superficial cells aggregate to form scale papillae (Le Guellec et al. 2004; Mongera and Nüsslein-Volhard 2013; Shimada et al. 2013; Sire et al. 1997). Dermal papillae form first in the caudal peduncle and above the ribs. Additional papillae are added in rows and columns to generate a half-offset hexagonal grid of scales (Fig. 8.2A,B) (Aman, Fulbright, & Parichy, 2018; Sire et al. 1997).

Initiation of scale papillae relies on interactions between epidermis and dermis involving ectodysplasin, Wnt/β-catenin, and fibroblast growth factor (Fgf) signaling pathways (Aman et al. 2018; Daane, Rohner, Konstantinidis, Djuranovic, & Harris, 2015; Harris et al. 2008; Rohner et al. 2009). Remarkably, interactions between signaling pathways and the cell behaviors they govern during zebrafish scale development resemble those governing formation and patterning of skin appendage like feathers and hair in terrestrial vertebrates. This implies that, despite profound differences in matrix composition—dermal calcified matrix of zebrafish scales and epidermal keratin of terrestrial appendages—all skin appendages likely share a common ancestry, and mechanisms that govern their early development are similar across vertebrates.

Following aggregation, dermal papillae produce the initial calcified matrix of the scale plate (Aman, Fulbright, Parichy, 2018; Sire et al. 1997). Developing scales are oriented toward the posterior by the planar cell polarity machinery, and their growth is driven by Sonic hedgehog (Shh) and Fgf-dependent proliferation and growth of osteoblast-like scale-forming cells accompanied by expansion of the calcified matrix (Aman et al. 2018; Cox et al. 2018; Iwasaki, Kuroda, Kawakami, & Wada, 2018; Rasmussen et al. 2018; Sire et al. 1997).

The structure of the scale plate consists of a basal layer of weakly ossified collagen, called isopedine, capped by the more strongly ossified external layer. At the posterior margin of the scale, a highly calcified, collagen-poor limiting layer is present (Fig. 8.2C). The scale-forming cells line the deep aspect of the scale and loop around the limiting layer at the posterior scale margin (Fig. 8.1). Gene expression in these posterior margin scale-forming cells is distinct from other scale-forming cells. It is this population that proliferates to add scale-forming cells during growth (Aman et al. 2018; Cox et al. 2018; Iwasaki et al. 2018). As growth proceeds, limiting layer matrix is deposited in waves, leading to concentric arcs of more heavily calcified circuli in the mature scale (Fig. 8.2C) (Sire et al. 1997). The scale

plate is also punctuated by matrix-free channels, the radii, that organize and house neuronal processes and vasculature of the skin (Rasmussen et al. 2018; Sire et al. 1997). During scale outgrowth, epidermal Shh triggers accumulation of a thin layer of scale pocket cells underneath the scale-forming cells (Fig. 8.1). Epidermal cells invaginate along these cells yielding the final partially protruding organization of the adult scale (Aman et al. 2018; Sire et al. 1997).

The evolutionary relationships between elasmoid scale extracellular matrix and calcified matrices in mammals—enamel, dentin, cartilage, and bone—remain uncertain. The precise homologies of cells secreting these matrices are similarly ambiguous. Paleontological and other evidence indicate that thin, flexible scales of zebrafish and other teleosts descended from heavy, enameled rhomboid scales of ancient vertebrates. Such rhomboid scales contain clear examples of matrix resembling enamel, dentine, and bone, a state preserved in teleost sister groups, the elasmobranchs (sharks/rays), polypterids (bichirs/reedfish), and holosteans (bowfin/gars) (Janvier, 1996; Märss, 2006; Sire 1990; Sire and Huysseune 2003). In this respect, scales of zebrafish resemble mammalian teeth perhaps more than mammalian bone.

The cells that produce the ossified matrix of the zebrafish scale plate have been called osteoblasts because the matrix they secrete is calcified like bone and because they express *sp7/osterix*, encoding a transcription factor utilized by mammalian osteoblasts (Cox et al. 2018; Iwasaki et al. 2018; Metz, de Vrieze, Lock, Schulten, & Flik, 2012; Rasmussen et al. 2018). It is important to note, however, that mammalian *sp7* is a general regulator of multiple cell types that produce calcified matrices, including enamel-producing ameloblasts and dentin-expressing odontoblasts (Bae et al. 2018).

To acknowledge the presently ambiguous homology of zebrafish-calcified matrix-producing cells, and to honor the prior nomenclatural recommendations of Jean-Yves Sire, we recommend referring to these Sp7+ cells simply as scale-forming cells, rather than osteoblasts per se (Sire et al. 1997). Deeper investigation of gene expression and its regulatory network should allow a more precise understanding of scale-forming cell evolution and will enable rigorous comparisons of biology between these and other matrix-secreting populations across vertebrates.

Zebrafish Skin as a Model for Skin Disease, Wound Healing and Regeneration

The optical transparency and superficial location of zebrafish skin make it exceptionally accessible to manipulation and imaging. These advantages, coupled with histological and molecular similarities to human skin, make zebrafish an attractive system for understanding cellular and molecular basis of skin disease, wound healing, and regeneration. Indeed, zebrafish with mutations in genes associated with human cutaneous disease often recapitulate aspects of these diseases and can provide insights into pathology (Feitosa, Richardson, Bloch, & Hammerschmidt, 2011; Hatzold et al. 2016; Li et al. 2011a, 2011b). Zebrafish skin is also highly regenerative and is proving to be an excellent system for uncovering mechanisms that underlie injury response, wound healing, and tissue regeneration (Armstrong, Henner, Stewart, & Stankunas, 2017; Chen et al. 2016; Cox et al. 2018; Gault, Enyedi, & Niethammer, 2014; Kang, Nachtrab, & Poss, 2013; Richardson et al. 2013, 2016).

Outlook

Zebrafish skin affords the biomedical research community a powerful system to address fundamental questions of stem cell biology, patterning, morphogenesis, regeneration, and disease. Emerging genetic reagents, as well as imaging and analytical methods, will further enable the study of zebrafish skin and will likely contribute novel insights into these important areas of biology and pathology.

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CHAPTER

9

Zebrafish Pigmentation

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Introduction

The pigmentation of adult zebrafish consists of several different elements and depends on several distinct classes of pigment cells. By far the most prominent elements are the dark stripes and light "interstripes" that run horizontally across the body. Pigment cells—in ectotherms, sometimes referred to as chromatophores—are also present on the scales (conferring a darker cast dorsally than ventrally), on the head, and on the fins, where they form stripes and other arrangements.

Chromatophores are derived embryologically from the neural crest, which also contributes to the craniofacial skeleton, peripheral nervous system, and other tissues and organs (Dupin et al., 2018; Hörstadius, 1950). In zebrafish, the most abundant chromatophores are the black melanophores, yellow/orange xanthophores, and iridescent iridophores (Fujii, 1993; Johnson et al., 1995; Mort et al., 2015; Schartl et al., 2016). Melanophores are homologous to melanocytes of birds and mammals but differ from melanocytes in retaining melanin granules rather than transferring them to keratinocytes. Xanthophores contain pteridines, carotenoids, or both pigments, which are detectable by their autofluorescence. Iridophores, by contrast, depend for their iridescence on crystalline guanine, held within stacked reflecting platelets. Additional cell types—white, or white and yellow leucophores—occur in the fins (Lewis et al., 2019). As for melanophores, pigments and platelets of xanthophores, iridophores, and leucophores, are retained intracellularly. So the pigment pattern is a direct indication of chromatophore distributions.

Adult stripes consist of melanophores and sparse iridophores, whereas interstripes have densely packed iridophores and xanthophores (Fig. 9.1(Patterson and Parichy, 2009)). Such a distinctive pattern suggests behavioral or ecological significance, and indeed, pigment patterns of other fishes can have many roles: helping individuals to avoid predators, to recognize others of their own species, and to choose their mates (Marshall et al., 2018; Price et al., 2008). Laboratory studies of zebrafish suggest that pigmentation, and stripes in particular, facilitate social aggregation, or shoaling (Engeszer et al., 2008; Parichy, 2015; Rosenthal et al., 2005). However, the natural history of zebrafish remains poorly understood, and the specific functions of pigmentation have yet to be addressed in the wild.

Because stripe pattern formation is understood best, its events are described in some detail below. Other tissues are mentioned briefly, as are physiological and pathological changes that can affect pigmentation. In recent years, transgenic models of zebrafish also have been used to understand the origins and progression of melanoma and to identify potential therapies for this deadly cancer of the melanocyte lineage (Kaufman, 2016). This important but distinct topic is not reviewed here.

Stripes and Their Development

The pigmentation of adult zebrafish differs markedly from the earliest expression of this trait, in late embryos and early larvae (EL) (Fig. 9.1) (Dutton et al., 2001; Kimmel et al., 1995; Parichy et al., 2009). At these stages, melanophores occur on the head and extend posteriorly along the dorsal myotomes, wrapping around to the ventral myotomes. Melanophores also line the dorsal and ventral edges of the yolk and swim bladder. A few melanophores are found in the middle of the flank at the horizontal myoseptum. Iridophores occur sparsely in the regions occupied by melanophores. By contrast, xanthophores are scattered widely over the flank and dorsum. These cells gradually fade and are no longer apparent by ~5.0 standardized standard length (SSL, approximating 5.0 mm or ~10-day postfertilization) (McMenamin et al., 2014; Parichy et al., 2009). What, if any, function the EL pattern serves, remains unclear,

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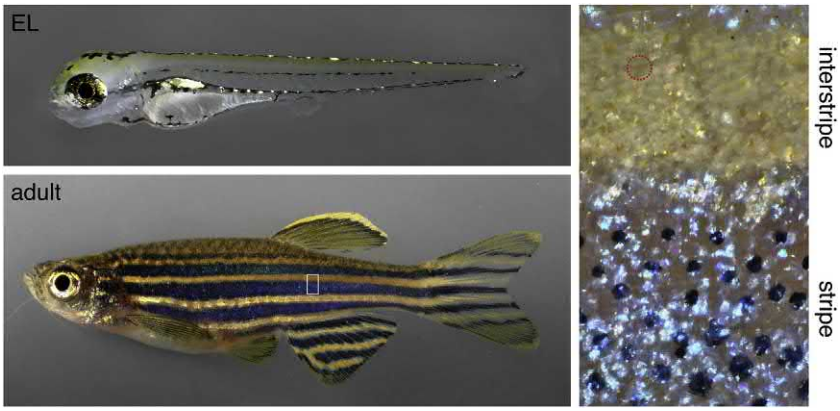

FIGURE 9.1 Embryo/early larva (EL) and adult pigmentation of zebrafish. Closeup of adult stripe and interstripe corresponds to approximate position of boxed regions. Pigment cells have been treated with epinephrine, which contracts pigment of melanophores and xanthophores toward cell centers. Melanophores are black, xanthophores appear as pale orange (e.g., red outline), and iridophores are iridescent, forming a yellowish mat in the interstripe and dispersed blue cells in the stripe.

but given the locations of melanophores and iridophores, one might imagine they help to protect stem cell populations (brain, spinal cord, gonads) or other tissues from ultraviolet light in the shallow waters where zebrafish breed (Mueller et al., 2014; Parichy, 2015).

Numerous mutants have defects in EL pigmentation and the genes corresponding to many of these have been identified (Arduini et al., 2008; Cornell et al., 2004; Elizondo et al., 2005; Kelsh et al., 1996; Odenthal et al., 1996). Often, defects arise from failures to synthesize cell-type-specific pigments or to localize them in specialized organelles (Dooley et al., 2013b; Lamason et al., 2005; Lister, 2019). Pigmentation defects in other mutants arise because of failures to specify or maintain one or more chromatophore lineages (Dutton et al., 2001; Lister et al., 1999; Lopes et al., 2008; Nord et al., 2016; Parichy et al., 2000; Petratou et al., 2018). Only a few mutants have been identified that affect the patterning or localization of chromatophores (Camargo-Sosa et al., 2019; Parichy et al., 1999; Svetic et al., 2007; Zhang et al., 2018), suggesting a robustness to these processes, or a dependence on genes having essential functions prior to EL pattern formation.

The EL arrangement of chromatophores persists with few changes until 4.5–5.0 SSL, when new chromatophores start to appear, and the adult pattern begins to form (Parichy et al., 2009) (Fig. 9.2). Although overt morphological changes are not manifest until the larva-to-adult transformation—along with other changes to the skin and other organs—remodeling of pigmentation depends on multipotent, neural-crest derived pigment cell progenitors established within the peripheral nervous system during early embryonic development (Budi et al., 2011, 2008; Dooley et al., 2013a; Hultman et al., 2009; Saunders et al., 2019; Singh et al., 2014, 2016; Tryon et al., 2011). During later adult pattern formation, some progenitors migrate to the hypodermis of the skin (Aman & Parichy, this volume) where they differentiate as iridophores that will form a "primary" interstripe in the middle of the flank. Iridophores first appear anteriorly, then far posteriorly, and then in between, until the interstripe is continuous (Parichy et al., 2009). The positioning of iridophores requires normal myotome development, as mutants with defects in myoseptal boundaries have disrupted interstripes (Frohnhofer et al., 2013; Parichy et al., 2015). Additional signals required for iridophore differentiation, proliferation, and survival come from fibroblasts or other cells of the skin, or superficial cells of the myotomes (Fadeev et al., 2018; Krauss et al., 2014; Lang et al., 2009; Mo et al., 2017; Spiewak et al., 2018).

Shortly after adult iridophores begin to develop, pigment cell progenitors contribute new melanophores as well (5.9 SSL) (Parichy et al., 2003b, 2009). Newly melanizing cells are evident in prospective stripe and interstripe regions. As pattern implementation continues, additional, morphologically distinct iridophores appear within the prospective stripes.

Subsequently, the initial pattern of an interstripe and two stripes becomes more distinctive. This depends on a consolidation of the melanophores into stripe regions: melanophores differentiating outside of these regions—and a few EL melanophores at the horizontal myoseptum—either migrate short distances to join stripes, or they die or are obscured by iridophores (Parichy et al., 2000, 2003b; Patterson et al., 2013; Takahashi et al., 2008). Several studies have revealed the importance of iridophores in promoting melanophore localization to stripe regions, and excluding these cells from the interstripe itself (Fadeev et al., 2015; Frohnhofer et al., 2013; Krauss et al., 2013; Patterson et al., 2013, 2014).

Two events occur nearly simultaneously with stripe consolidation and are important to this process. First, new iridophores appear and become increasingly dense

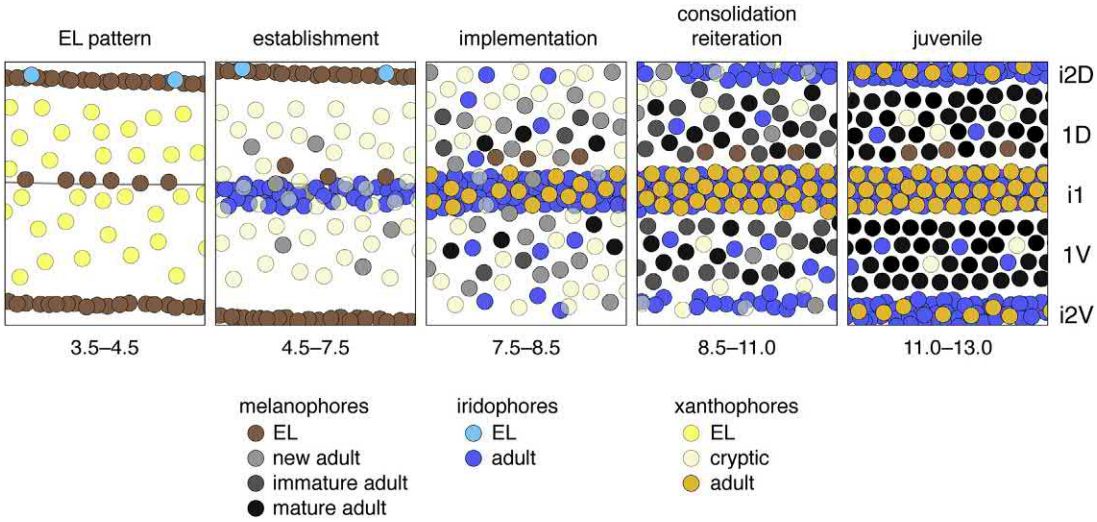

FIGURE 9.2 Events of adult pattern formation. In the EL pattern, melanophores, xanthophores, and a few iridophores are present. *Gray line* indicates horizontal myoseptum. By the establishment of the adult pattern, the fish is growing rapidly, and EL xanthophores have faded, entering a cryptic state. Some EL melanophores move, and some are lost. New adult iridophores start to differentiate, and new adult melanophores begin to appear. During a period of pattern implementation, adult melanophores increase in number, xanthophores acquire new pigment, and iridophores appear within the prospective stripe regions. Consolidation of stripes occurs with the onset of pattern reiteration. Ultimately, a juvenile pattern is formed, which persists with some additional reiteration in the adult. i1: primary interstripe; 1D, 1V: primary stripes; i2D, i2V, secondary interstripes. Ranges below panels are SSL (Parichy et al., 2009).

within new, "secondary" interstripes that develop adjacent to the primary stripes and serve to bound their "distal" edges (Patterson et al., 2013, 2014; Singh et al., 2014; Spiewak et al., 2018). Second, xanthophores differentiate in association with iridophores of the primary interstripe (~8.0 SSL) owing to a xanthophore-maturation factor produced by these iridophores (Patterson et al., 2013). Iridophores, therefore, contribute to the arrangement of pigmented xanthophores, in addition to their effects on melanophores. Xanthophores, in turn, influence the localization and survival of melanophores and are essential for normal consolidation and subsequent maintenance of the stripes (Hamada et al., 2014; Nakamasu et al., 2009; Parichy et al., 2000, 2003a). Both xanthophores and melanophores additionally depend on permissive factors provided by other integumentary cell types (Hultman et al., 2007; Patterson et al., 2013).

The origin of many xanthophores differs from adult melanophores and adult iridophores. Rather than arising from a postembryonic pigment cell progenitor, many pigmented xanthophores of the adult come directly from xanthophores of the EL pattern. As noted above, these cells fade from view, but they also proliferate and persist during subsequent stages, and it is some of these cells that reacquire pigmentation, when in association with interstripe iridophores (McMenamin et al., 2014). The color of xanthophores at EL stages is due to yellow pteridines (Lister, 2019; Odenthal et al., 1996). Their color in the adult results from the thyroid-hormone dependent processing and accumulation of dietarily derived yellow/orange carotenoids (Granneman et al., 2017; Saunders et al., 2019). Besides xanthophores that develop directly from the neural crest and EL xanthophores, at least some adult xanthophores develop from postembryonic pigment cell progenitors (McMenamin et al., 2014; Singh et al., 2016).

Together, these events are responsible for a primary pattern consisting of an interstripe bordered by two stripes. As the fish grow, this pattern is reiterated with increasingly well-defined secondary interstripes and stripes, added dorsally and ventrally. Just as interactions between pigment cells are required for patterning the primary pattern elements, interactions between pigment cells, and between pigment cells and other cells in their environment, are required for patterning the secondary elements (Parichy et al., 2003a; Patterson et al., 2013, 2014; Spiewak et al., 2018). The overall dynamics of stripe development resemble those predicted by Turing models of pattern formation (Watanabe et al., 2015); grounding such similarities in discrete biological mechanisms remains a substantial and important challenge.

By late juvenile stages (~16 SSL), a flank pattern of stripes and interstripes has formed that will persist into the adult (≥26 SSL). Also by juvenile and adult stages, pigment cells comprising this pattern have become stratified: xanthophores are outermost, and iridophores, then melanophores, are found inwardly. An additional, less studied, population of spindle-shaped iridophores with large reflecting platelets occurs in smaller numbers even deeper in the hypodermis (Hirata et al., 2003, 2005).

Scales, Fins, and Other Sites of Pigmentation

As zebrafish enter the juvenile stages of development, they have transparent scales covering their bodies ((Parichy et al., 2009); Aman & Parichy, this volume). The same multipotent pigment cell progenitors that give rise to many chromatophores of the hypodermal stripes and interstripes are responsible for populating scales with chromatophores; indeed, individual progenitors can contribute to both locations (Singh et al., 2016). Melanophores differentiate prominently on the dorsal scales, but these cells are repressed from differentiating ventrally by the agouti signaling pathway, which has a similar function in repressing the differentiation of melanocytes on the ventrum of many mammals (Cal et al., 2019).

Pigmentation of adult fins begins as soon as they develop; pattern outcomes differ between anatomical locations (Parichy et al., 2009). In the caudal and anal fins, stripes and interstripes develop, but their arrangements are independent of iridophores, which occur in relatively small numbers at these sites. The dorsal and paired fins have similar complements of chromatophores, but the cells remain largely intermingled and so do not generate distinct patterns.

Fins also harbor leucophores (Lewis et al., 2019). Distal regions of the dorsal fin and the most distal portions of the caudal fin lobes develop leucophores that arise by transdifferentiation of melanophore progenitors. These cells lose melanin, and in its place, acquire crystalline deposits of guanine. These "melanoleucophores" are white owing to a disordered arrangement of irregularly shaped organelles containing guanine crystals. This contrasts with the iridescence conferred by stacked, regularly shaped reflecting platelets of guanine crystals in iridophores. Melanoleucophores reflect light of all wavelengths: they are bright in visible light, and the reflections from these cells can be mistaken for the fluorescence of transgenic reporters. Due to their prominent locations, these cells may contribute to species recognition, or aggressive or courtship displays. In the laboratory, zebrafish prefer to shoal with fish that have intact complements of melanoleucophores. The second class of leucophores, "xantholeucophores," is found in the interstripes of the anal fin, and contains yellow/orange carotenoids, similar to xanthophores. Crystalline guanine is not detectable in xantholeucophores.

Finally, zebrafish also have chromatophores in other locations, including iridophores that line the peritoneum and cover the operculum, and cells of the choroid and iris of the eye (retinal pigmented epithelium derives from the central nervous system) (Hirata et al., 2005; Spiewak et al., 2018). Little is known about the development or functional significance of these features.

Physiological and Pathological Effects on Pigmentation

Pigmentation changes ontogenetically, but pigment cells also respond physiologically to alterations of environment or health status. A normal physiological response occurs in background adaptation. When fish are placed on a light background, melanophores contract melanin-containing melanosomes toward their centers, resulting in an overall paler appearance to the fish. On a dark background, the opposite response occurs. Such behaviors depend on endocrine and neuroendocrine effectors and have been studied extensively in zebrafish and other species (Counts et al., 2009; Fujii, 1993; Iwashita et al., 2006; Lewis et al., 2019; Oshima et al., 2002; Sheets et al., 2007). Although responses are physiological, long-term stimulation can lead to morphological alterations resulting from cell death or overproduction (Sugimoto, 2002; Sugimoto et al., 2005).

Pigmentation can also change in response to stress or pathology, sometimes mimicking the blanching response of healthy fish adapted to a light background. Other pathologies can yield dark phenotypes. Additionally, injuries sometimes result in pigmentary "scars." Zebrafish have a remarkable ability to heal integumentary wounds, but deep wounds, trauma, or inflammatory responses can generate ectopic accumulations of chromatophores (Levesque et al., 2013; Richardson et al., 2013) Bilateral asymmetry typically distinguishes such marks from pattern phenotypes arising through genetic alterations.

Conclusions

Studies of zebrafish pigmentation have provided insights into mechanisms of pattern formation, specification, and differentiation of neural crest lineages, cellular physiology, and individual behavior. Pigmentation can also provide clues to fish health and physiology. Due to the superficial location of chromatophores, their accessibility to visualization, and their cell-autonomous markers of differentiation state, this system should continue to be useful for understanding the basic and applied aspects of organismal form and function.

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CHAPTER

10

Respiratory System

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Introduction

This chapter describes the general appearance and morphology of the respiratory structures in zebrafish and how they work together to form a functional system. The structure and function of the respiratory system in zebrafish is not particularly unique among other cyprinid fishes. Therefore, where information was not available in zebrafish, some of the information contained within this chapter may be derived from studies on other species.

For further information relating to the physiology of gas exchange and respiratory physiology, the reader is referred to the reviews cited in this chapter.

The Mechanics of Respiration

During ventilation, water first enters through the mouth and into the buccal cavity, and then moves to the opercular chambers across the gills and out through an opening formed by each of the opercula (Fig. 10.1). The operculum is a thin, bony flap of tissue that covers and protects the entire gill complex on both sides. The movement of water through the buccal and opercular cavities and across the gills during ventilation is orchestrated by a sequence of changes in volume and pressure driven by a muscular pump (Moyes & Schulte, 2008). The fish first creates a negative pressure within the buccal cavity, drawing water in through the mouth and oral valve. With the opercula closed, the opercular cavities then begin to expand while positive pressure is created in the buccal cavity by the closure of the oral valve and buccal contraction. These forces lead to movement of water in a caudal direction toward the gills. Finally, the opening of the opercula and compression of the opercular cavities project water through the gill complex, and across the respiratory surface for gas exchange, and out to the external environment. Debris, such as food or other particulate matter, may obstruct the ventilatory cycle. However, the pharyngeal (or gill) arches form gill rakers. These are small paired structures that arise from the arches and are oriented toward the buccal cavity, and help to filter out particles and prevent them from becoming lodged within the gills.

The Gills

Morphology and Blood Flow

The gills are multifunctional organs that perform many roles, such as osmoregulation, acid-base regulation, excretion of nitrogenous waste, and gas exchange (Evans, Piermarini, & Choe, 2005). For an exceptional and unparalleled account of gill anatomy in fish, the reader is referred to Hughes (1984). In teleost fish, such as zebrafish, four gill arches are present on either side of the animal and are oriented along the dorsoventral axis. The gill arches are supportive structures and are additionally important in respiration because they deliver deoxygenated blood from the heart and ventral aorta to the gill filaments via afferent branchial arteries; they carry oxygenated blood from the gill filaments to the dorsal aortae and systemic circulation via the efferent branchial arteries.

The gill arches give rise to two rows of numerous gill filaments that run perpendicular to the orientation of the gill arches. Between each pair of filaments lies a small interbranchial septum that extends from the gill arch and attaches near the base of each filament. The septum is the site of attachment of the adductor muscles that join with the gill filaments. Contraction of the adductor muscles draws together pairs of filaments and allows for the rhythmic movement of the filaments (Hughes, 1984). The number and length of the gill filaments in all teleosts is variable and continues to increase with age. In mature zebrafish, the length of a single filament

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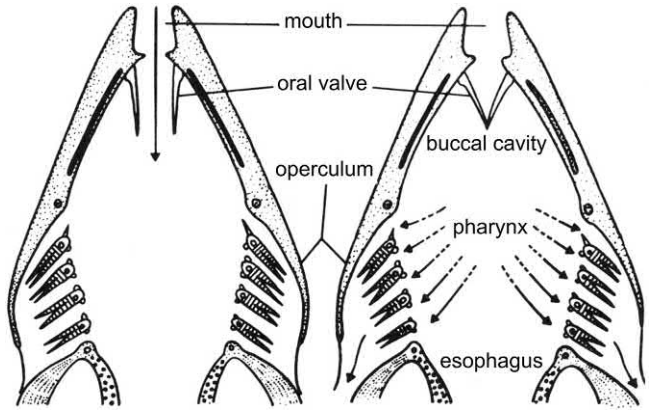

FIGURE 10.1 The mechanics of respiration in teleost fishes, including zebrafish. Schema of the frontal section. Oral valves are actually dorsal and ventral but are shown laterally for simplicity. *Arrows* show the direction of water flow. *Left*: During inhalation, water enters through the mouth and oral valve into the buccal cavity while the opercula are closed. *Right*: The oral valve closes, the buccal cavity contracts, and water passes into the opercular chambers, where the gills are located, and through the open opercula. The opercular chambers are located between the pharynx and opercula. *After Weichert, C. K. (1967). Elements of chordate anatomy. (3rd ed.). New York: McGraw-Hill with permission from McGraw-Hill Education.*

may typically measure 1−2 mm. The gill filaments may sustain injury or infection in fish in the wild or captivity, but recent evidence indicates that resected gill filaments in zebrafish regenerate during the course of days or weeks (Jonz, Zachar, Da Fonte, & Mierzwa, 2015). Deoxygenated blood from the gill arches enters the afferent filament arteries of each filament, and the efferent filament arteries return oxygenated blood to the gill arches (Olson, 2002).

Each gill filament gives rise to numerous secondary lamellae, which generally project in a dorsoventral direction, perpendicular to the filaments. The lamellae are evenly spaced, flattened structures that form small channels between them, thus allowing for the flow of water across their surface. The lamellae are thin-walled and highly vascularized and are the site for gas exchange in all water-breathing fish, including zebrafish. Blood flow throughout the lamellae runs in the direction opposite to that of the flow of water over the gills. This is called *countercurrent flow*, and efficiently maximizes the exchange of O_2 and CO_2 across the lamellar surface (Moyes & Schulte, 2008).

Many teleosts, including zebrafish, have a reduced gill-like structure, called the *pseudobranch*, located anterior to the first gill arch and embedded within the opercular tissue (Jonz & Nurse, 2006; Laurent & Dunel-Erb, 1984). In zebrafish, the pseudobranch appears as a small gill with fused filaments and lamellar-like structures. It is only visible upon anterior dissection or removal of the operculum. Since it lacks access to the external environment and receives blood from the gills that has already been oxygenated, the pseudobranch is thus not believed to be involved in respiration. Although speculative, the pseudobranch may act as a gland, sense hypoxia, supply O_2 to the retina, or participate in the regulation of blood pH (Bridges, Berenbrink, Müller, & Waser, 1998; Kern, Bösch, Unterhuber, & Pelster, 2002; Laurent & Dunel-Erb, 1984).

Gill Development

In developing zebrafish, the pharyngeal arches first produce gill filament primordia at 3 days postfertilization (dpf; Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995; Jonz & Nurse, 2005). The filaments of the gills in zebrafish develop primarily from the pharyngeal ectoderm (Hogan et al., 2004), although some structures internal to the gills may arise from the neural crest (Mongera et al., 2013) and endoderm (Hockman et al., 2017). At these early stages, the gills lack secondary lamellae, and therefore, may not contribute significantly to the gas exchange. The gills first become functional as a respiratory organ at approximately 14 dpf (Rombough, 2002), and around this time begin to take on an adult-like morphology, including the addition of secondary lamellae (Shakarchi, Zachar, & Jonz, 2013). Before this time, zebrafish larvae rely primarily on cutaneous respiration for gas exchange (see below).

Internal Morphology

Gill filaments are supported by a cartilaginous rod in order to provide structure or stiffness. Within the lamellae lie the pillar cells, which extend lateral processes toward one another to create a vascular cavity for blood to flow (Wilson & Laurent, 2002). Pillar cells may have a contractile function to control or redistribute lamellar blood flow during respiration (Laurent, 1984). The gill filaments and lamellae are covered by a thin epithelium that lies atop a basal lamina and within which reside numerous cell types that are critical for the functionality of the gills (Laurent, 1984; Wilson & Laurent, 2002; Evans et al., 2005). The filament epithelium is nonrespiratory and is covered predominantly by cuboidal and squamous pavement cells and mucous (goblet) cells. Neuroepithelial cells (NECs) of the gill filaments are O_2 chemoreceptors that detect changes in the environmental or blood O_2 and CO_2/H^+ (Jonz, Fearon, & Nurse, 2004; Qin, Lewis, & Perry, 2010), and thus lead to autonomic responses, such as hyperventilation or changes in heart rate, in order to maintain homeostasis.

The lamellar epithelium is particularly specialized for respiration and gas exchange. It overlays the arterioarterial circulation (Olson, 2002), and is generally two to 3 cell layers thick in most fish species (Wilson &

Laurent, 2002). The cell types found in the lamellar epithelium include the cuboidal and squamous pavement cells, undifferentiated cells that contact the basal lamina, and some mucous cells (Evans et al., 2005; Wilson & Laurent, 2002). Neuroepithelial cells have also been found in the lamellae of zebrafish (Jonz & Nurse, 2003), although a role in O_2 sensing for these cells has still not been established.

Neurobiology

The gill is a highly perfused organ and is a major site of circulatory and vasomotor control (Nilsson & Sundin, 1998). Such control is mediated by the nervous system and is critically important in regulating respiratory function. Innervation of the gills by the cranial and spinal nerves has been described in teleost fish, including zebrafish (Nilsson, 1984; Sundin & Nilsson, 2002; Jonz & Nurse, 2008; Jonz & Zaccone, 2009). As in other species, the gills are innervated by the nerve fibers of the cranial nerves IX (the glossopharyngeal nerve) and X (the vagus nerve), and by fibers originating from the sympathetic chain and entering the cranial nerve trunk via gray rami communicantes (Fig. 10.2). Each gill arch is innervated by two nerve rami through which all nerve fibers are delivered: a pretrematic ramus that lies anterior to the gill slit (the space between gill arches), and a posttrematic ramus that lies posterior to the gill slit. Pretrematic rami carry the sensory (afferent) fibers, while posttrematic rami carry the sensory and motor (efferent) fibers. Chemoreceptive NECs of the gill filaments receive sensory innervation via the cranial nerves IX and X and convey information, such as low-O_2 status (hypoxia) of the environment, to the central nervous system (Jonz & Nurse, 2003).

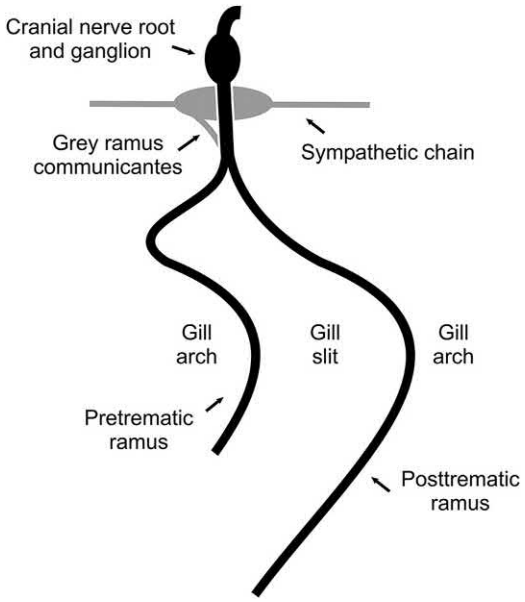

FIGURE 10.2 Simplified scheme of innervation to the gill region in teleost fish. The gills are innervated by nerve fibers from the cranial nerves, and by fibers from the sympathetic chain that enter the cranial nerve trunk via gray rami communicantes. Each gill arch is innervated by a pretrematic ramus (anterior to the gill slit) and a posttrematic ramus (posterior to the gill slit) Pretrematic rami carry sensory fibers, while posttrematic rami carry sensory and motor fibers. *Reprinted from Jonz M. G., Zaccone, G. (2009). Nervous control of the gills. Acta Histochemica, 111 207–216 with permission from Elsevier.*

Cutaneous Respiration

In many anamniotic vertebrates, such as fish, the skin is an important site of gas exchange during early developmental stages (Rombough, 1988). In developing zebrafish, cutaneous respiration accounts for nearly all gas exchange and does not become limiting until approximately 10 dpf (Rombough, 2007), when the gills are fully functional. Moreover, there is evidence that the pectoral fins in developing zebrafish contribute to respiration by actively moving O_2-depleted water away from the body surface and pulling in O_2-rich water toward the body surface, thereby enhancing gas exchange (Hale, 2014). Cells with chemoreceptor activity have also been described in the skin of zebrafish that detect changes in O_2 and elicit hyperventilatory responses to hypoxia (Coccimiglio & Jonz, 2012).

Indicators of Stress to the Respiratory System

Hyperventilation

All fish hyperventilate in response to reduced O_2 availability (Perry, Jonz, & Gilmour, 2009). Hyperventilation in fish is described as an increase in breathing frequency, as can be visually observed by an increased rate of movement of the mouth and opercula. Fish may also produce a hyperventilatory response to high levels of water CO_2 or H^+ (Gilmour, 2001). In zebrafish, these responses have been shown to be mediated by O_2-chemoreceptive NECs of the gill filaments (Abdallah, Jonz, & Perry, 2015a; Jonz et al., 2004; Porteus et al., 2014; Qin et al., 2010). A typical resting ventilatory rate for adult zebrafish is approximately 160 breaths min^{-1}; whereas hypoxia (P_{O_2} of 35–40 mmHg) may elevate ventilation frequency to above 300 breaths min^{-1} (Jonz & Nurse, 2005; Vulesevic, McNeill, & Perry, 2006). Adult zebrafish will begin to display an increase in ventilation frequency under mild hypoxia at P_{O_2} of approximately 110 mmHg (Vulesevic et al., 2006), where normal P_{O_2} would be approximately 150 mmHg.

In developing zebrafish, the hyperventilatory response to hypoxia is not mature but begins to appear

at approximately 7 dpf, when the gill chemoreceptors become innervated (Jonz & Nurse, 2005). Before this time, zebrafish display an early behavioral response to hypoxia that involves an increase in whole-body movement (Coccimiglio & Jonz, 2012). Compared to adults, zebrafish larvae display lower ventilation frequencies. Although variable, resting ventilation frequency is typically 50–100 breaths min^{-1} in zebrafish larvae, with responses to hypoxia measuring at about 150 breaths min^{-1} (Rahbar, Pan, & Jonz, 2016).

Aquatic Surface Respiration

Under severe hypoxic stress, in addition to hyperventilation, fish display aquatic surface respiration (ASR)—a behavior in which the fish seeks well-oxygenated water by moving up to the air-water interface (Chapman & McKenzie, 2009; Perry et al., 2009). Zebrafish may begin to engage in ASR as P_{O_2} reaches 50 mmHg or lower, although a threshold P_{O_2} of 30 mmHg was calculated as the level below which zebrafish will rely on ASR for survival (Abdallah, Thomas, & Jonz, 2015b). Decreasing P_{O_2} results in an increased amount of time that zebrafish will spend performing ASR. Evidence also indicates that as zebrafish are acclimated to hypoxia, the ASR threshold will decrease, meaning that zebrafish acclimated to hypoxia are able to postpone performing ASR until more severe levels of hypoxia. ASR is evident in zebrafish as early as 5 dpf, and larvae display an ASR threshold of 16 mmHg.

Conclusion

This chapter has outlined the important structures of the respiratory system of zebrafish for the researcher or student interested in using zebrafish as a model organism in biological or biomedical research. The mechanisms of the buccal and opercular cavities, together with the structure of the gills, were described to provide an anatomical overview of the important structures of the respiratory system. In zebrafish, in particular, information regarding the role of chemoreceptors and the nervous system in mediating responses to hypoxia, and identification of behavioral indicators of stress, were described in order to help researchers and animal care personnel recognize when the zebrafish respiratory system is under stress. The zebrafish respiratory system is very much like that of other related fish species, and so the interested reader will have access to a vast literature base for further reading.

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CHAPTER

11

Skeletal System Morphophysiology

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The zebrafish skeleton is a spatially distributed organ that extends to many parts of the body. The bony tissues of the skeleton, together with other connective tissues, including muscle, cartilage, ligaments, tendons, and intermediate subtypes of these tissues, comprise the zebrafish musculoskeletal system. This primer introduces the functions of the zebrafish skeleton and compares these functions to those in the mammalian skeleton. Next, follows a survey of the hierarchical structure of the adult zebrafish skeleton, starting from the nanoscale (molecules), and ending with the macroscale (bone as an organ). Finally, this section concludes with a discussion of assays for the interrogation of skeletal function and form in zebrafish. Because most of the skeleton ossifies postembryonically, the focus is primarily on the adult skeleton. This chapter is adapted from (Kwon, Watson, & Karasik, 2019), a manuscript originally published by the authors. Aspects of this chapter are reproduced or modified with permission (copyright 2019 by Elsevier) from (Kwon et al., 2019).

The Functions of the Skeleton are Diverse

The functions of the skeleton can generally be separated into two areas; mechanical and nonstructural. Within these two areas, skeletal functions in the zebrafish can be further divided into those that do or do not maintain a resemblance to terrestrial organisms. Mechanically, the zebrafish skeleton performs similarly to the mammalian skeleton in several ways. It facilitates movement by providing a rigid structure that helps to transmit muscle-generated forces. This includes movements during locomotion, articulation of the jaw, and movements of the opercula during respiration. A number of features facilitate swimming, including skeletal structures that support the fins, as well as elongated body shape. The skeleton also provides structural support for some tissues and organs, such as the gill tissues and pharyngeal teeth, as well as mechanical protection for internal organs. The Weberian apparatus helps to transmit vibrations from the swim bladder to the inner ear.

The skeleton also possesses nonstructural functions with a varying degree of conservation between mammals and fish. A nonstructural function traditionally ascribed to the skeleton is to facilitate mineral homeostasis. This is achieved in at least two ways: by regulating phosphate reabsorption in the kidney through bone-kidney crosstalk, and by serving as a reservoir for the storage and release of calcium and phosphorus. For the latter, since calcium regulation can occur through the gills in fish, the coupling of the skeleton to calcium homeostasis in fish may differ from that in mammals (Apschner, Schulte-Merker, & Witten, 2011). Fat storage is another function attributed to the mammalian skeleton, as bone adiposity responds to metabolic demands (de Paula & Rosen, 2013). In this context, a number of zebrafish bones possess adipocytes within their marrow spaces; however, whether such adiposity responds to metabolic demands remains to be seen.

Two other nonstructural skeletal functions that have unknown or lack of conservation in mammals are participation in the endocrine and hematopoietic systems, respectively. Bone regulates a number of metabolic processes independent of mineral metabolism. For example, the bone-derived hormone osteocalcin has been shown to regulate glucose homeostasis, cognition, and male fertility (Moser & van der Eerden, 2018). These functions highlight the role of the skeleton as an endocrine organ that regulates the function of distant cells and tissues through hormone secretion. Whether such

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endocrine functions are conserved in the zebrafish skeleton requires further investigation. Finally, a function traditionally ascribed to the skeleton is the maintenance of the hematopoietic niche. However, the bone marrow spaces in fish lack hematopoietic tissue. Thus, unlike in mammals, the zebrafish skeleton is not a site of hematopoiesis (Apschner et al., 2011).

Bone Develops Through Different Modes of Ossification

In zebrafish, bones develops through three major modes of ossification: (1) ossification of a connective tissue membrane (intramembranous ossification), (2) around a cartilage template (perichordal ossification), (3) or within a cartilage template (endochondral ossification). Following the convention of (Cubbage & Mabee, 1996), bones formed via a cartilage model (either through perichordal or endochondral ossification) are termed *cartilage bones*. Dermal bones are those that form via direct ossification of a connective tissue membrane (intramembranous ossification), and which are homologous with bones formed through the same process in primitive vertebrates; membrane bones are those that are homologous with cartilage bones in primitive vertebrates.

The developmental morphology of the zebrafish craniofacial skeleton has been well-described, both during early larval development (Piotrowski et al., 1996; Schilling et al., 1996; Schilling & Kimmel, 1997) and during the larval-to-adult transition (Cubbage & Mabee, 1996). In total, there are 74 bones in the adult zebrafish craniofacial skeleton; of these bones, 29 are dermal bones, 43 are cartilage bones, and one is a membrane bone (Cubbage & Mabee, 1996). The developmental morphology of the axial skeleton has also been well-described (Bird & Mabee, 2003). A number of elements comprising the vertebral column form via intramembranous ossification. Some exceptions include the parapophyses, some elements of the Weberian apparatus, as well as the neural arch on vertebra 5, which form via endochondral ossification. Another exception is the mineralization of the cartilage-like notochordal sheath, in a process called *perichordalossification* (Inohaya, Takano, & Kudo, 2007). In this process, centrum formation is initiated by mineralization of the notochordal sheath. Centrum formation then proceeds by bone apposition onto the mineralized surface via intramembranous ossification. The bone mineral can also be stained to facilitate visualization. A common approach is to use alizarin red or calcein to stain mineralizing surfaces. These fluorochromes can be administered ex vivo or in vivo by immersing live fish in fluorochrome solution (Du, Frenkel, Kindschi, & Zohar, 2001). Labels can be monitored in vivo in bones that are optically accessible, such as early developing vertebrae, and adult fin rays. Double fluorochrome labeling has been used to demarcate bone formation between labeling periods (Inohaya et al., 2007; Kimmel, DeLaurier, Ullmann, Dowd, & McFadden, 2010; Recidoro et al., 2014; Suniaga et al., 2018), similar to dynamic histomorphometric approaches in mammals. Labels are often readily observed in whole-mount, alleviating the time and resources required for tissue sectioning. Additionally, cartilage tissue can be visualized in fixed specimens by staining with Alcian blue, which stains the proteoglycan components of the cartilage extracellular matrix (Piotrowski et al., 1996; Schilling et al., 1996).

Bone Exhibits a Hierarchical Structure

Bone is distinct as a biological tissue in regard to its combination of mechanical stiffness, strength, and lightness; such properties arise through a hierarchical structure spanning several different length scales.

Nanoscale: Matrix and Mineral

At the nanoscale, bone tissue consists of a mineralized organic matrix. The organic matrix is primarily composed of collagen type I. In humans, collagen type I is a heterotrimer composed by two alpha chains, α1(I) and α2(I), encoded for by the *COL1A1* and *COL1A2* genes, respectively. The α1 and α2 chains trimerize in a 2:1 ratio, respectively, to form a fibril with a triple helix structure. In zebrafish, the collagen type I triple helix is composed of three α chains, α1(I), α2(I), and α3(I), which are encoded for by the genes *col1a1a*, *col1a2*, and *col1a1b*, respectively (Gistelinck et al., 2016a). In zebrafish, alpha chain stoichiometry within collagen type I fibrils may vary depending on anatomical site (Gistelinck et al., 2016a). Transmission electron microscopy (TEM) images of adult zebrafish bones have shown collagen fibrils arranged in laterally aligned bundles (Mahamid, Sharir, Addadi, & Weiner, 2008). TEM studies have also shown plate-shaped mineral crystals residing within the fibrils, with their c-axes aligned with the fibril axes, similar to other vertebrates. Thus, at the nanoscale, zebrafish bone is reminiscent of mammalian bone up to the level of aligned mineralized collagen fibrils (Mahamid et al., 2008).

In mammals, the primary inorganic phase in bone is carbonated hydroxyapatite (HA). Recent studies (Fiedler et al., 2018; Suniaga et al., 2018) showed the potential to adapt assays of bone quality in mammals to zebrafish. Quantitative backscattered electron microscopy and Fourier-transform infrared spectroscopy were used to show changes in vertebral tissue

composition, including changes in mean calcium content, matrix porosity, mineral crystallinity, and collagen maturity (Fiedler et al., 2018). Raman and Fourier-transform infrared (FTIR) spectroscopy (Mahamid et al., 2008) imaging in zebrafish has shown that zebrafish bone contains carbonated HA, as well as other mineral phases, similar to mammalian bone. Tissue mineral density (TMD) is a commonly used measure of mineralization that reflects the amount of mineral per unit volume of bone tissue. TMD values of 450–600 mg HA/cm^3 have been reported in the vertebrae of adult zebrafish (Hur et al., 2017). Observed zebrafish measures are noticeably less than the TMD values of 800–1000 mg HA/cm^3 in the cortical bone of adult mice (Main, Lynch, & van der Meulen, 2014), but only slightly less than the 600–700 mgHA/cm^3 observed in the cancellous bone of the same animals. In human cancellous bone, TMD values of 800–1000 mgHA/cm^3 have been reported (Wang, Kazakia, Zhou, Shi, & Guo, 2015). Differences in mineral density have been attributed as a possible reflection of adaptation to mechanical loading and bi- or quadrupedalism in terrestrial mammals (Doherty, Ghalambor, & Donahue, 2015).

Microscale: Cellular Composition and Related Microstructure

At the cellular level, zebrafish bone comprises several cell types that are distinct in both their morphology and function. Osteoblasts are bone-forming cells. A large fraction of the zebrafish skeleton is covered in a single monolayer of osteoblasts (Weigele & Franz-Odendaal, 2016). This is similar to mature osteoblasts in rats, which have been reported to appear as a single layer of cuboidal cells (Florencio-Silva, Sasso, Sasso-Cerri, Simoes, & Cerri, 2015). Osteoclasts are bone-resorbing cells. There are at least two morphologically identifiable types: mononucleated and multinucleated (Witten, Hansen, & Hall, 2001). Mononucleated osteoclasts appear during early development and have been found in bones of the craniofacial skeleton. The resorptive function of these mononucleated cells is suggested by their presence at sites of resorption pits, as well as positive staining for tartrate-resistant acid phosphatase (TRAP). In zebrafish, multinucleated osteoclasts appear during the larval-to-adult transition (Witten et al., 2001). By adulthood, multinucleated osteoclasts are the most predominant, although mononucleated osteoclasts do exist (Witten et al., 2001). By contrast, in mammals, mononucleated cells are thought to primarily be a precursor to multinucleated cells, which carry out the resorptive function. Osteocytes are osteoblasts that have become entrapped into the matrix during bone formation. Osteocytes are present in zebrafish, although not in all bones. In this context, two types of bone are identifiable in zebrafish: cellular bones with osteocytes, and acellular bones that lack osteocytes. Cellular and acellular bones are present in both the cranial and post-cranial skeletons (Weigele & Franz-Odendaal, 2016). Cellular bones have also been identified in zebrafish vertebrae; these bones contain osteocytes with a poorly developed lacunocanalicular system (Cao et al., 2011).

Histological stains enable visualization of osteoblasts, osteoclasts, and osteocytes. Osteoblasts can be demarcated by staining for mRNA highly expressed in osteoblasts in zebrafish, such as osterix, osteocalcin, or by assessing protein activity in alkaline phosphatase (ALP) (Edsall & Franz-Odendaal, 2010). The uncharacterized antigen ZNS-5 is an antibody that stains cell membranes and can help in identifying bone lining osteoblasts (Johnson & Weston, 1995). In zebrafish, osteocytes can be visualized in hematoxylin-eosin (H&E) stained sections, as cells embedded in the bone matrix (Cao et al., 2011). Osteoclasts can be visualized based on the expression of mRNA for CTSK (Sharif, de Bakker, & Richardson, 2014), or by TRAP activity (Witten et al., 2001). Osteoblasts can be demarcated in vivo using fluorescent reporters driven by regulatory sequences for genes highly expressed in osteoblasts. Examples of transgenic lines include *sp7:EGFP* (DeLaurier et al., 2010; Singh, Holdway, & Poss, 2012) and *ocn:EGFP* (Singh et al., 2012), which demarcate early and more mature osteoblasts, respectively. A double transgenic reporter line expressing fluorescent markers for *sp7* and *bglap* (*ocn*) was used to gain insights into the effects of calcitriol and cobalt in zebrafish operculum development, with osteoblast maturation shown to be stimulated and inhibited, respectively, by these two agents (Tarasco, Laize, Cardeira, Cancela, & Gavaia, 2017). Osteoclasts can be demarcated using the zebrafish transgenic line *ctskb:YFP* (Sharif et al., 2014). In medaka, a similar transgenic line has been used in parallel with heat shock-inducible Receptor Activator Of Nuclear Factor-Kappa B (RANK) to screen for drugs that inhibit bone resorption (To et al., 2012; Yu et al., 2016), as well as to examine osteoclast-osteoblast interactions (Yu & Winkler, 2017).

Bone develops first as woven bone, after which, parallel-fibered bone develops in more mature individuals (Apschner et al., 2011). Similar to mice, zebrafish do not (or rarely) form osteons—concentric layers of compact bone tissue surrounding a Haversian canal—although such structures have been observed in larger teleosts (Witten & Hall, 2002). In this context, some zebrafish bones have been reported to be avascular (Paul et al., 2016) as blood vessels do not (or rarely) become embedded within the bone matrix. However, blood vessels often reside in close proximity to bone tissue, including within bone marrow spaces.

Mesoscale and Macroscale: Identifiable Bone Types and Functional Groupings

Mammalian bone is traditionally classified as either cortical (compact) or cancellous (trabecular or spongy) bone. Because most zebrafish bones are dense and homogenous, they are referred to as compact bones (Weigele & Franz-Odendaal, 2016). However, zebrafish do not possess cortical bone in a traditional sense, in that their bones do not encapsulate a hematopoietic bone marrow cavity. While much more limited compared to compact bone, zebrafish also possess spongy, trabecular-like bones. For instance, spongy bone has been found in several bones of the skull (Weigele & Franz-Odendaal, 2016). A number of these spongy bones form via endochondral ossification (Weigele & Franz-Odendaal, 2016).

Aside from compact and spongy bone, other macroscopically identifiable bone types have been defined in zebrafish. One such bone type is tubular bone, a type of endochondral ossifying bone that is filled with adipose tissue (Weigele & Franz-Odendaal, 2016). Another bone type is the chondroid bone, which is comprised of chondrocyte-like cells embedded with a mineralized matrix (Apschner et al., 2011). Chondroid bone is an example of an intermediate tissue that shows the characteristics of multiple connective tissue types (Apschner et al., 2011). There is evidence that chondroid bone can grow rapidly (Huysseune & Verraes, 1986; Weigele & Franz-Odendaal, 2016), a property which may be important for bone repair. For instance, the chondroid bone formation has been observed during zebrafish jawbone regeneration, as well as fracture repair in other vertebrates (Paul et al., 2016).

At the organ level, the zebrafish skeleton can be regionalized into functional groups, including the craniofacial skeleton, the vertebral column, the median fins, and the paired fins. The developmental morphology of the craniofacial skeleton has been well-described (Cubbage & Mabee, 1996; Piotrowski et al., 1996; Schilling et al., 1996), and there is a large literature on the genetics of craniofacial skeletogenesis in zebrafish (Mork & Crump, 2015; Willems et al., 2015). Notably, the number of bones in the adult zebrafish skull greatly exceeds that in adult mammals (Cubbage & Mabee, 1996). The zebrafish vertebral column can be regionalized into three main groups (anterior-posteriorly): Weberian vertebrae, precaudal vertebrae, and caudal vertebrae (Bird & Mabee, 2003). The total number of vertebrae in zebrafish can vary slightly but seems to be around 31 (Bird & Mabee, 2003), similar to that in mammals. Vertebrae one to four contribute to the Weberian apparatus. Vertebrae 5–14 and 15–31 are the precaudal and caudal vertebrae, respectively. Each vertebra possesses a centrum, a neural arch, and a neural spine that extends from the neural arch. Many elements are comprising the vertebral column form via intramembranous ossification, while parapophyses, as well as the neural arches on vertebrae one to five, form via endochondral ossification (Bird & Mabee, 2003).

The fins include the dorsal, pectoral, anal, and caudal fins. Radial bones, such as those at the base of the pectoral fin, adjacent to the dermal fin rays, share a developmental similarity with mammalian long bones in that they form via endochondral ossification. The dermal fin rays consist of segmented bone rays, surrounded by nerves, blood vessels, pigment cells, and fibroblastic/mesenchymal cells residing within the intra- and inter-ray spaces (Watson & Kwon, 2015). Each fin ray is composed of multiple segments joined by fibrous ligaments and lined by a monolayer of osteoblasts. Like other teleosts, following fin amputation, zebrafish can regenerate their fin bone rays through epimorphic regeneration. In adults, newly synthesized bone appears within two to 3 days, and subsequent nerve, joint, circulatory, and mature bone tissue are largely restored within 2 weeks (Watson & Kwon, 2015).

Radiography has been shown to be a practical tool to screen for skeletal abnormalities in adult zebrafish (Fisher, Jagadeeswaran, & Halpern, 2003). Severe skeletal defects can be readily visualized in a noninvasive manner. Moreover, the speed of acquisition facilitates in vivo imaging. Several recent studies have demonstrated the utility of microCT as a practical tool for the detection of skeletal abnormalities in zebrafish (Charles et al., 2017; Fiedler et al., 2018; Hur et al., 2017; Khajuria, Kumar, Gigi, Gedanken, & Karasik, 2018). Examples include differences in skeletal shape and density in the developing C1 and C2 vertebrae (Charles et al., 2017), as well as centrum, neural arch, and hemal arch tissue mineral density (TMD), length, and thickness in almost the whole vertebral column (24 individual vertebrae) (Gistelinck et al., 2016b, 2018a; Hur et al., 2017, 2018). High-resolution (1um voxel size) microCT can be used to resolve the aspects of osteocyte lacunar orientation, shape, and size in the centrum (Suniaga et al., 2018). Other measurements include the neural arch area (Charles et al., 2017) and angle (Charles et al., 2017). In zebrafish and medaka, the neural arch area is influenced by bone modeling activity, due to the fact that the neural arch undergoes continual modeling during growth, with osteoclasts on the inner surface, and osteoblasts on the outer surface (Chatani, Takano, & Kudo, 2011). Thus, altered neural arch phenotypes may indicate changes in bone modeling mediated by coupling between osteoblasts and osteoclasts. MicroCT has been used to analyze

aspects of zebrafish skull morphology in zebrafish both qualitatively (Gistelinck et al., 2018b; Paul et al., 2016), as well as quantitatively (Charles et al., 2017). In regard to the latter, volume and TMD can be readily measured in the parasphenoid bone, which resides at the skull base, and forms through endochondral ossification (Charles et al., 2017). Semiautomated methods for 3D segmentation of bones in the fish skull remain nascent.

One limitation of microCT is that in the absence of contrast agents, the zebrafish skeleton is insufficiently ossified at early developmental stages for mineralized tissue to be resolved. Generally, when sexual maturity is achieved by 2–3 months postfertilization, the skeleton is sufficiently mineralized in wildtype zebrafish to be imaged via microCT. Because experimental throughput and scalability is generally increased in younger fish, the identification of experimental "windows" in which animals are still relatively young, still their skeletons are sufficiently mineralized to be detected by quantitative approaches, may be beneficial. It has been shown that contrast staining using silver nitrate ($AgNO_3$) can allow for microCT analysis of the zebrafish skeleton at early stages of development (Buchan et al., 2014).

Summary

In conclusion, the zebrafish skeleton possesses a hierarchical structure that enables it to carry out diverse functions. While a number of functions are shared with the mammalian skeleton, others, such as participation in mineral homeostasis, endocrine regulation, and hematopoiesis have been unknown or lack of conservation. Advances in histology, transgenic reporters, microCT, and bone quality assays have enabled a better understanding of the interrelationships between form and function in the zebrafish skeleton at different scales.

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CHAPTER

12

Zebrafish Myology

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Embryonic Origins

The myofibers that comprise skeletal muscle can vary in their embryonic origin, molecular patterning, and physiology, depending on the location and function. Most craniofacial muscles develop from unsegmented head mesoderm using genetic programs distinct from trunk myomeres, which form from postcranial spheres of cells termed *somites* (Bryson-Richardson & Currie, 2008). Both head and trunk muscles contain physiologically distinct fast and slow myofibers, the proportions of which can change over time (Hernandez, Patterson, & Devoto, 2005). Slow-twitch muscle fibers are fatigue resistant, metabolically oxidative, heavily vascularized, and are the first fiber type to arise in zebrafish. Fast-twitch fibers function in rapid, powerful actions and are metabolically glycolytic. Studies of postcranial myogenesis have shown that during development, fast and slow fiber types segregate through complex cellular movements within each somite. Slow myofibers arise along the midline and migrate laterally to a superficial position along the myotome. Fast myofibers, in contrast, form from two distinct intrasomitic populations. Cells of the posterior somite differentiate into fast myofibers, whereas cells of the anterior somitic border migrate laterally to form a thin monolayer superficial to the slow fibers. These "external cells" then secondarily ingress between slow myofibers to form additional fast fibers (Bryson-Richardson & Currie, 2008; Stellabotte & Devoto, 2007). Ultimately, the slow muscle fibers form a wedge-like stripe that is positioned lateral to the horizontal septum, running outside and parallel to the fast fibers, which comprise the bulk of the trunk musculature.

Head Muscles

Extraocular Muscles

Similar to other vertebrates, zebrafish rotate their eyes using six extraocular muscles: the *superior* and *inferior obliques*, and the *medial, lateral, superior,* and *inferior rectus* muscles all of which form by around 72 hpf (Easter & Nicola, 1996) (Fig. 12.1). In 96 hpf fish, both the *superior* and *inferior obliques* originate along the rostral orbit and insert on the dorsal and ventral surfaces of the eye, respectively. The *superior*, *inferior*, and *medial rectus* muscles arise from a similar site along the posterior orbit. Whereas the *superior* and *inferior* rectus insert just caudal to the superior and inferior *obliques*, the *medial rectus* extends between the *obliques*, inserting on the anteromedial surface of the eye. Unlike the other rectus muscles, the *lateral rectus* originates outside of the orbit posterior to the diencephalon and inserts on the posterior sclera (Kasprick et al., 2011). Notably, the insertion sites of the *superior oblique* and *rectus* muscles overlap in adults (Fig. 12.1B). A similar late overlap has been shown for the *inferior oblique* and *rectus* (Kasprick et al., 2011) (Fig. 12.1C). Three cranial nerves (CN) innervate the extraocular muscles: CNIII innervates the *inferior oblique, superior rectus, inferior rectus,* and *medial rectus;* CNIV innervates the *superior oblique*; and CNVI innervates the *lateral rectus* (Schilling & Kimmel, 1997). Interestingly, a visually evoked startle response develops about 20 h later than the touch startle response, roughly coincident with early maturation of the extraocular muscles (Easter & Nicola, 1996).

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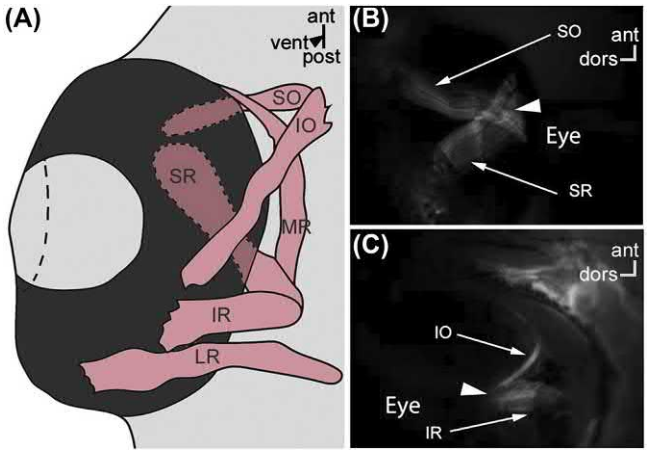

FIGURE 12.1 **Extraocular muscles.** (A). Ventral view of the six extraocular muscles of a 96 hpf larvae (modified from the camera lucida drawing of Easter and Nicola, 1996:fig11). *Dashed lines* indicate deeper structures. (B,C). Adult transgenic [Tg(α-actin:EGFP)] with muscle GFP (Kasprick et al., 2011:fig2). Arrowhead marks muscle overlap. *ant*, anterior; *dors*, dorsal; *IO*, inferior oblique; *IR*, inferior rectus; *LR*, lateral rectus; *MR*, medial rectus; *post*, posterior; *SO*, superior oblique; *SR*, superior rectus; *vent*, ventral.

Arch-associated Muscles

In jawed vertebrates, craniofacial muscles develop in association with segmentally arranged arches surrounding the pharynx, an embryological feature reflected in adult patterns of innervation. Zebrafish possess seven pairs of pharyngeal arches: anterior-to-posterior, these are the mandibular arch, hyoid arch, and five gill-associated branchial arches, each with distinct skeletal elements and muscles (Cubbage & Mabee, 1996; Schilling & Kimmel, 1997).

Mandibular Arch Muscles

Muscles associated with the first arch (mandibular arch) include the *intermandibularis*, *adductor mandibulae*, and a dorsal group consisting of the *levator arcus palatini* and *dilatator operculi*, all of which are innervated by CNV (Fig.12.2) (Schilling & Kimmel, 1997). The *intermandibularis anterior* joins the left and right dentary bones (mandible) and remains largely unchanged between larval and adult stages. The *intermandibularis posterior* becomes associated with the *interhyoideus* (a second arch muscle) in larvae (Fig.12.2B), forming a continuous muscle, the *protractor hyoideus*. In adults, the *protractor hyoideus* is longitudinally split into dorsal and ventral portions connecting the anterior ceratohyal and ventral hypohyal bones with the dentary (Fig.12.2D) (Diogo, Hinits, & Hughes, 2008). The *protractor hyoideus* depresses the mandible and is innervated by both CNV and VII, reflecting its complex ontogeny (Diogo et al., 2008).

In early larvae, the *adductor mandibulae* initiate as a single mass (Fig.12.2A,B), but in adults subdivides into four bundles: *A0*, *A1*, *A2*, and *Aω* (Fig.12.2B) (Diogo et al. 2008). Collectively, *A0*, *A1*, and *A2* primarily extend below the eye, connecting the preopercle (*A0*, *A1*, *A2*), quadrate (*A0*, *A1*), hyomandibula *(A2)*, and metapterygoid *(A2)* with the mandible and maxilla (Diogo et al., 2008). Aω originates along the medial surface of the mandible (angulo-articular and dentary) and inserts on the tendon of *A2*. Whereas the *adductor mandibulae* complex primarily closes the mouth, the maxillary component (specific to *A0*) functions in mouth protrusion (Diogo et al., 2008).

Larval zebrafish possess a single dorsal mandibular premuscle mass, which segments to form the *levator arcus palatini* and the *dilatator operculi* in adults (Fig.12.2A,C) (Schilling & Kimmel, 1997). The *levator arcus palatini* originates along the sphenotic bone of the neurocranium and extends to the metapterygoid and hyomandibula to abduct/elevate the jaw suspensorium. The *dilatator operculi* neighbors the *levator arcus palatini* and lies superficial to the *adductor hyomandibulae* (a second arch muscle), joining the frontal and pterotic bones, hyomandibula, and opercle for opercular abduction (Diogo et al., 2008).

Hyoid Arch Muscles

Muscles associated with the second arch, the hyoid arch, include the *interhyoideus*, *hyohyoideus*, *adductor hyomandibulae*, *adductor operculi*, *levator operculi*, and *adductor arcus palatini*, all innervated by cranial nerve VII (Schilling & Kimmel, 1997; Diogo et al., 2008) (Fig.12.2). As mentioned above, the *interhyoideus* together with the *intermandibularis posterior* comprises the *protractor hyoideus*. By 4 dpf, the *hyohyoideus* consists of a well-formed *hyohyoideus inferior*, as well as posterior myofibers [*hyohyoideus superior*: Diogo et al. (2008)] that segregate to form the *hyohyoideus abductor* and *adductors* following ossification of the branchiostegal rays (Fig.12.2). In adults, the *hyohyoideus inferior* and *abductor* originate from the anterior ceratohyals and first branchiostegal ray, respectively, and meet their opposite partner along the jaw medial aponeurosis. The medialmost branchiostegal ray separates the *hyohyoideus abductor* from the laterally positioned *adductors*, which extend between the branchiostegal rays, opercle and subopercle. The *hyohyoideus abductor* and *adductors* expand and constrict the branchiostegal membranes, respectively (Diogo et al., 2008).

The *adductor operculi* extends from the pterotic bone to the opercle for opercular adduction. The *levator operculi* originates along the ventrolateral margin of the pterotic, runs lateral to the *adductor operculi*, and inserts on the

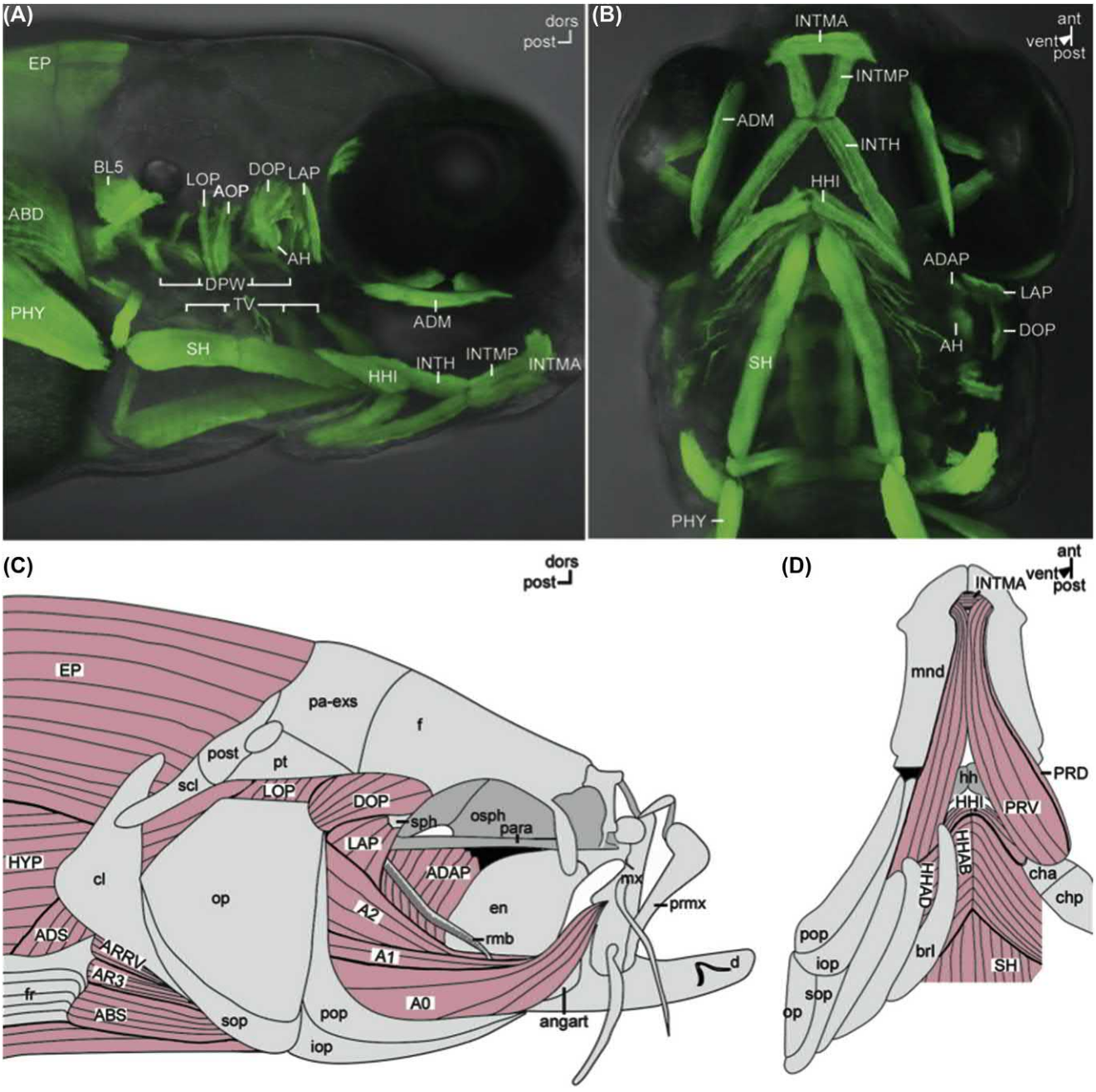

FIGURE 12.2 **Cephalic muscles.** Arch associated muscles in larval (A,B) and adult (C,D) zebrafish in lateral (A,C) and ventral (B,D) view. (A,B). Confocal maximum projections of 4 dpf Tg(Acta1:EGFP) larvae. Skeletal muscle is green. (C,D). Adult illustrations modified from Diogo et al (2008:fig5). In (D), part of the *hyohyoideus adductors* (HHAD), as well as the opercle (op), interopercle (iop), subopercle (sop), and preopercle (pop) are removed from the right side. *A0, A1, A2*, bodies of the *adductor mandibulae* (ADM); *ABD*, fin abductor; *ABS*, abductor superficialis; *ADAP*, adductor arcus palatini; *ADS*, adductor superficialis; *AH*, adductor hyomandibulae; *angart*, anguloarticular; *AOP*, adductor operculi; *ARRV*, arrector ventralis; *BL5*, fifth branchial levator; *brI*, branchiostegal ray I; *cha*, anterior ceratohyal; *chp*, posterior ceratohyal; *cl*, cleithrum; *den*, dentary; *DPW*, dorsal pharyngeal wall muscle; *DOP*, dilatator operculi; *en*, entopterygoid; *EP*, epaxialis; *f*, frontal; *fr*, fin rays; *hh*, ventral hypohyal; *HHAB*, hyohyoideus abductor; *HHI*, hyohyoideus inferior; *HYP*, hypaxial muscle; *INTMA*, intermandibularis anterior; *LAP*, levator arcus palatini; *LOP*, levator operculi; *mnd*, mandible; *mx*, maxilla; *osph*, orbitosphenoid; *pa-exs*, parieto-extrascapular; *para*, parasphenoid; *post*, posttemporal; *PRD*, dorsal protractor hyoideus; *prmx*, premaxilla; *PRV*, ventral protractor hyoideus; *pt*, pterotic; *rmb*, medial branch ramus mandibularis; *scl*, supracleithrum; *SH*, sternohyoideus; *sph*, sphenotic; *TV*, transversalis ventralis.

opercle (Fig.12.2C). In zebrafish, as in other teleosts, the *levator operculi* is thought to function through the opercular series and the interoperculo-mandibular ligament in lower jaw depression (Diogo et al., 2008). The *adductor hyomandibulae* extends from the neurocranium to the medial margin of the hyomandibula. The *adductor arcus palatini* extends from the neurocranium to the hyomandibula, metapterygoid, and entopterygoid, antagonizing the function of the *levator arcus palatini* by adducting the jaw suspensorium (Diogo et al. 2008).

Posterior Branchial Arch Muscles

At 96 hpf, the muscles associated with the posterior branchial arches include the *dorsal pharyngeal wall muscles* [*branchial levatores*: Hernandez et al. (2005)], *rectus ventralis*, *transversus ventralis*, and *rectus communis* [Schilling & Kimmel, 1997; see also Winterbottom (1974) for review] (Fig.12.2A,B). The *dorsal pharyngeal wall muscles* are positioned between the dorsal branchial cartilages and neurocranium (Hernandez et al., 2005). In both larvae and adults, the anterior-most levatores are relatively small, whereas the fifth is larger for its role in feeding and consists exclusively of fast fibers (Hernandez et al., 2005). The *transversus ventralis* muscles function in respiration and extend from the ceratobranchials to the median raphe of the ventral midline (Schilling & Kimmel, 1997). The *rectus ventralis* muscles are positioned between the aforementioned branchial arch muscles, joining adjacent ceratobranchials. In contrast, the *rectus communis* forms a band that extends across the third through fifth branchial arches (Schilling & Kimmel, 1997). Branches of CNIX and X innervate the muscles of the posterior branchial arches (Schilling & Kimmel, 1997).

Hypobranchial Muscles

Zebrafish possess a single hypobranchial muscle, the *sternohyoideus*. Unlike the cranial mesoderm-derived pharyngeal muscles described above, the sternohyoideus arises from postcranial somitic mesoderm that becomes secondarily anteriorized during development, a process likely mediated by the growth dynamics of the circumpharyngeal tissues (Lours-Calet et al., 2014). Interestingly, the anterior somites that give rise to the sternohyoideus also contribute striated myofibers to the esophagus for ingestion (Minchin et al., 2013). Whereas the sternohyoideus muscles are paired in larvae (Fig.12.2A,B), they form a single sheet in adults, extending from the cleithrum to the urohyal (Fig.12.2C,D). Anterior branches of the occipito-spinal nerves innervate the sternohyoideus, which functions in hyoid depression, suspensorium abduction, and mouth opening (Diogo et al., 2008).

Trunk Muscles

The trunk musculature of zebrafish consists of approximately 32 muscle blocks (myomeres), which reflect somitic segmentation. Vertical connective tissue myosepta separate the myomeres and transmit their contractions to the skeleton for locomotion. In addition to vertical myosepta, zebrafish possess horizontal myosepta that split each myomere into dorsal epaxial and ventral hypaxial regions (Figs.12.2C and 12.3A). In adult teleosts, the epaxial myomeres collectively comprise the *epaxialis* muscle (Fig.12.2C). In addition to the epaxial and hypaxial myomeres, a transient muscle, the *hypaxialis posterior*, contributes to the ventral body wall of early larvae (Fig. 12.3A) and is thought to function with the *sternohyoideus* in suction feeding (Hernandez, Barresi, & Devoto, 2002). The *hypaxialis posterior* arises using a developmental program similar to the appendicular muscles (Haines et al., 2004), and in adult pearlfish, it is incorporated into the *obliquus inferior* (hypaxial body wall muscle) (Windner et al., 2011).

Pectoral Fin Muscles

In zebrafish, the pectoral fins develop in two continuous phases. The first starts with the onset of fin bud outgrowth around 26 hpf and is marked by the formation of a fin skeleton consisting of an endoskeletal disc proximally and actinotrichia distally. The second phase begins during the third week of development and involves fin rotation, remodeling of the endoskeleton to form distinct radials, and differentiation of lepidotrichia distally (Grandel & Schulte-Merker, 1998).

The myofibers of the pectoral fins appear early during the first phase of fin development, forming two simple muscle groups, an abductor and an adductor, each positioned on opposite sides of the endoskeletal disk (Fig. 12.3A,B). Like tetrapod limbs, the fin muscles of zebrafish derive from migratory somitic myoblasts (Neyt et al., 2000), and therefore, can be classified as *abaxial* using the terminology of Burke and Nowicki (2003). During the juvenile stage, the abductors and adductors increase in thickness and split to form superficial and deep groups (the *abductor superficialis*; *abductor profundus*; *adductor superficialis* and *adductor profundus*), plus two ventral *arrectors* (*arrector ventralis*, *arrector 3*) and one dorsal *arrector* (*arrector dorsalis*) along the leading margin of the fin (Thorsen & Hale, 2005; Siomava & Diogo, 2017) (Fig.12.3B–D). The pectoral fin muscles originate from the shoulder girdle and extend toward the fin rays. Whereas the *arrectors* insert exclusively on the first ray, the *abductor profundus*, *adductor superficialis*, and *adductor profundus* insert

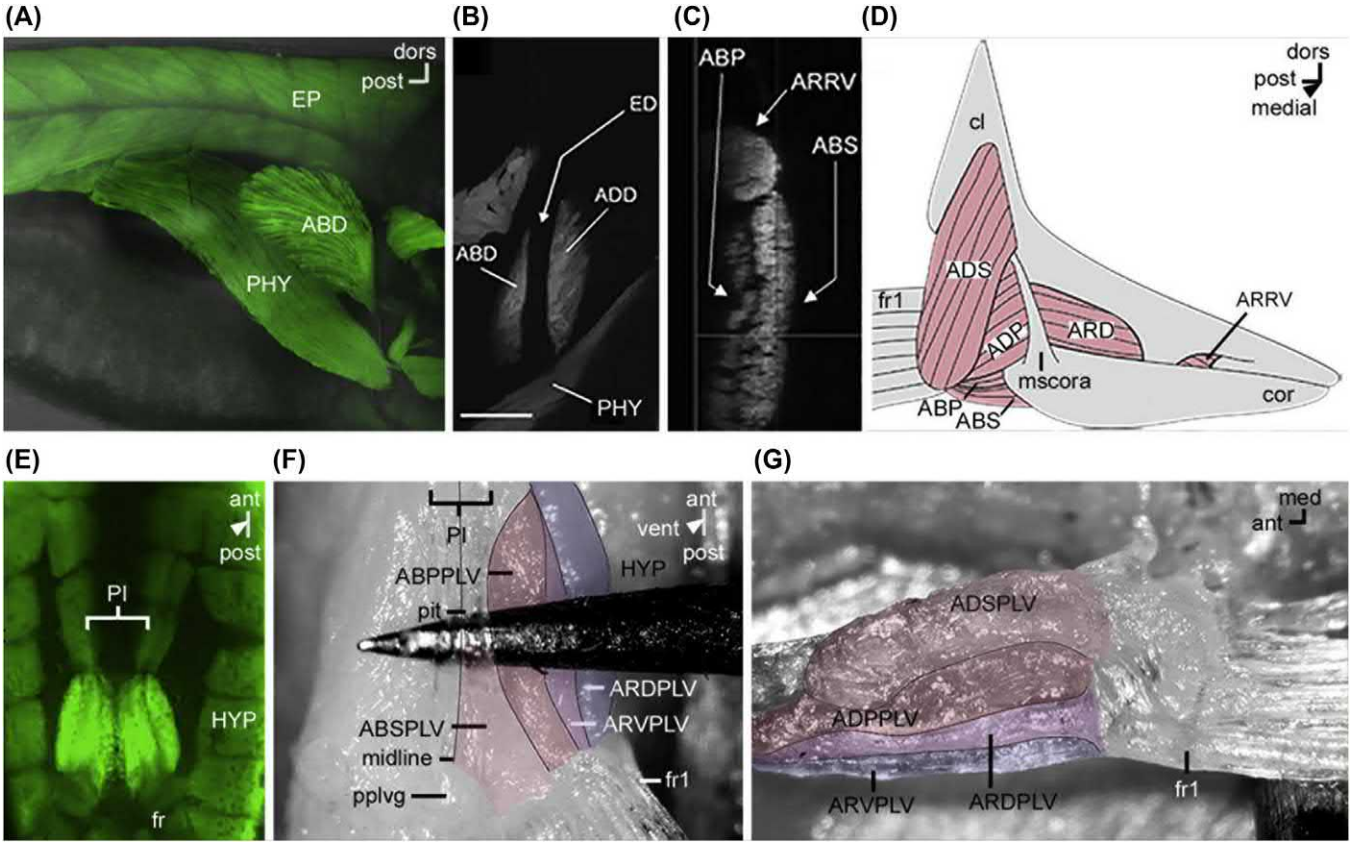

FIGURE 12.3 **Appendicular muscles.** (A). Lateral view of a 5 dpf Tg(Acta1:EGFP) larvae. Muscle is green. (B,C). Confocal cross-sections through the pectoral fin of a 5 dpf larvae (B) and 8.9 mm juvenile (C) Tg(α-actin:EGFP) from Thorsen and Hale (2005:figs2 and 6). In larvae, the endoskeletal disk (ED) separates the abductor (ABD) and adductor (ADD) muscles. By 8.9 mm, the *abductor superficialis* (ABS), *abductor profundus* (ABP), and *arrector ventralis* (ARRV) are segmented. (D). Illustrated medial view of adult pectoral fin muscles modified from Diogo and Abdala (2007:fig8). (E–G). Pelvic fin muscles shown by fluorescence [Tg(Acta1:EGFP] (E) and gross dissection (F–G) in ventral (E,F) and dorsal (G) views. (F) and (G) are modified from Siomava and Diogo (2017:fig2) with pseudocoloring based on original tracings and personal communications (N.S.). *ABPPLV*, abductor profundus pelvicus; *ABSPLV*, abductor superficialis pelvicus; *ADP*, adductor profundus; *ADPPLV*, adductor profundus pelvicus; *ADS*, adductor superficialis; *ADSPLV*, adductor superficialis pelvicus; *ARD*, arrector dorsalis; *ARDPLV*, arrector dorsalis pelvicus; *ARRV*, arrector ventralis; *ARVPLV*, arrector ventralis pelvicus; *cor*, coracoid; *EP*, epaxialis; *fr1*, first fin ray; *HYP*, hypaxial muscle; *mscora*, mesocoracoid arch; *PHY*, posterior hypaxial muscle; *PI*, *pit*, protractor ischii and tendon; *pplvg*, posterior process pelvic girdle.

distally on all rays except the first (Siomava & Diogo, 2017). In addition to splitting, the majority of the muscles of the pectoral fin secondarily shift into the body wall, such that in adults, only a small portion remains within the fin. Pectoral nerves 1–4 (the spino-occipital nerve and spinal nerves 1–3) innervate the pectoral fin muscles (Thorsen & Hale, 2007). Based on studies of teleosts, the *arrector ventralis* is thought to initiate down-stroke of the fin's leading edge, which is further powered by the superficial and deep abductors. The *arrector dorsalis* contracts following peak down-stroke, and along with the superficial and deep adductors antagonize abduction to retract the fins (Thorsen & Hale, 2005). Notably, zebrafish are reported to possess a transient *protractor pectoralis* muscle connecting the skull and pectoral girdle. This muscle derives from the cardiopharyngeal field and is branchiomeric rather than intrinsically appendicular (Siomava & Diogo, 2017).

Pelvic Fin Muscles

In zebrafish, the pelvic fins form approximately 4 weeks after initiation of the pectoral fins (and are present by 8 mm standard length) (Parichy, Elizondo, Mills, Gordon, & Engeszer, 2009). Two muscles anchor the pelvic girdle to the trunk. The *protractor ischii* extends from the cleithrum and ventral trunk myomeres on either side of the body, forming a single tendon that attaches to the posterior process of the pelvic girdle (Fig. 12.3E,F). Caudally, the *retractor ischii* extends from the pelvic girdle to the anal fin. Like the pectoral musculature, the muscles of the pelvic fin are organized into superficial and deep layers of abductors (*abductor superficialis pelvicus* and *abductor profundus pelvicus*) and adductors (*adductor superficialis pelvicus* and *adductor profundus pelvicus*) that extend to most rays, as well as arrectors (*arrector ventralis pelvicus* and *arrector dorsalis pelvicus*)

that insert on the first ray (Siomava & Diogo, 2017) (Fig. 12.3F,G). Whereas pectoral myoblasts migrate long distances, pelvic myoblasts reach the base of the fin via direct epithelial extension before delaminating to form the dorsal and ventral muscle groups (Cole et al., 2011).

Median Fin Muscles

Zebrafish have three median fins: the dorsal, anal, and caudal fins. The dorsal fin bears paired sets of *inclinator*, *erector*, and *depressor dorsalis* muscles arranged in series on either side of the fin blade (Fig.12.4A) (Schneider and Sulner, 2006; Siomava and Diogo, 2017). The *erector* and *depressor* muscles connect the proximal fin radials with the base of individual fin rays to raise and lower the fins, respectively. The *inclinator* muscles extend from the skin and epaxial musculature to the lateral edge of each ray to bend the fin sideways. In addition to the muscles described above, two longitudinal muscles, the *protractor* and the *retractor dorsalis*, raise and lower the fin, respectively. Muscles associated with the dorsal fin are innervated by a nerve plexus formed by the dorsal rami of spinal nerves between the ninth and 17th vertebrae (Schneider & Sulner, 2006). The musculature of the anal fin is similar to that of the dorsal fin (Siomava & Diogo, 2017).

The musculature of the caudal fin consists of superficial and deep sets of muscles on either side of the lateral midline (Fig.12.4B,C). The superficial muscles include dorsal and ventral bodies of three primary groups, the *lateralis profundus*, *lateralis superficialis*, and the *interfilamenti caudalis*. The *lateralis profundus dorsalis* and *ventralis* extend from the caudal vertical myosepta and proximal caudal fin bones to the base of several dorsally and ventrally positioned fin rays (6–8 and 21–24, respectively) (Schneider & Sulner, 2006; Siomava & Diogo, 2017). The *lateralis superficalis dorsalis* and *ventralis* lie between the *lateralis profundus* muscles. These triangular muscles have a broad base of attachment along the lateral midline and narrow distally at their single ray insertion sites. The *interfilamenti caudalis dorsalis* and *ventralis* neighbor the *lateralis superficialis*. These muscles arise from a narrow base (along the proximal dorsomedial and ventromedial fin rays), and fan out to insert along several medially positioned rays (Siomava & Diogo, 2017). Notably, an *interradialis caudalis* primarily extends along the fifth ray and is unique to the dorsal side of the fin. Deeper within the caudal fin, a series of flexors (*flexor caudalis dorsalis inferioris* and *ventralis inferioris; flexor caudalis dorsalis superioris* and *ventralis superioris)* and an adductor *(adductor caudalis ventralis)* originate from the posterior-most vertebrae and proximal caudal fin bones and insert along the proximal caudal fin rays (Siomava & Diogo, 2017). The nerve roots of spinal segments 27–31 innervate the muscles of the caudal fin (Schneider & Sulner, 2006).

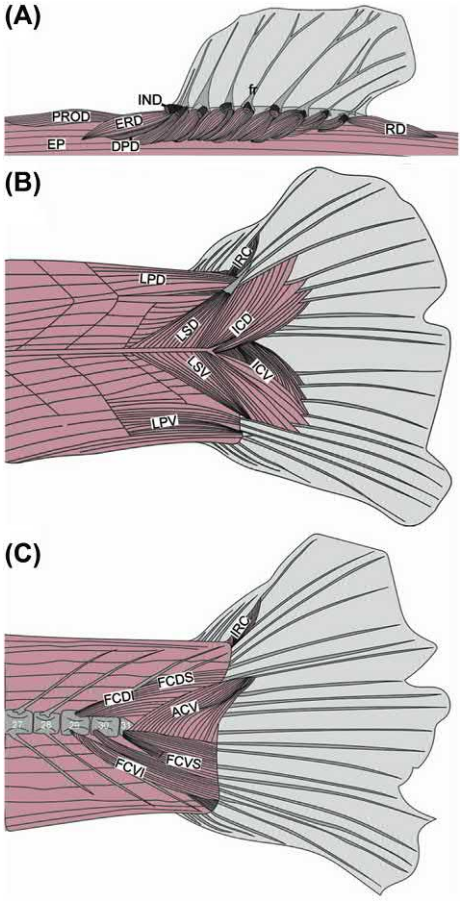

FIGURE 12.4 **Median fin muscles.** Left-lateral view of the muscles of the dorsal (A) and caudal (B,C) fins. (A). Dorsal fin illustration modified from Schneider and Sulner (2006:fig1a). Skin and axial muscles are removed. (B,C). Illustrations of superficial (A) and deep (B) caudal fin muscles modified from Schneider and Sulner (2006:fig6). In (C) the axial muscles and superficial caudal fin muscles have been removed. *ACV*, adductor caudalis ventralis; *DPD*, depressor dorsalis; *EP*, epaxialis; *ERD*, erector dorsalis; *FCDI*, flexor caudalis dorsalis inferioris; *FCDS*, flexor caudalis dorsalis superioris; *FCVI*, flexor caudalis ventralis inferior; *FCVS*, flexor caudalis ventralis superior; *ICD*, interfilamenti caudalis dorsalis; *ICV*, interfilamenti caudalis ventralis; *IND*, inclinator dorsalis; *IRC*, Interradialis caudalis; *LPD*, lateralis profundus dorsalis; *LPV*, lateralis profundus ventralis; *LSD*, lateralis superficialis dorsalis; *LSV*, lateralis superficialis ventralis; *PROD*, protractor dorsalis; *RD*, retractor dorsalis.

Conclusions

Zebrafish provide an excellent model for investigating the genetic basis of form and are particularly amenable to imaging morphogenesis in vivo. Moreover, as representative actinopterygians, zebrafish occupy a key phylogenetic position for anchoring comparisons with amniotes. Here, we have summarized aspects of

zebrafish myology, including descriptions of the muscles of the head, trunk, and appendages. These descriptions, together with the excellent works by the authors summarized above, provide anatomical context for studies of muscle development, regeneration, physiology, disease modeling, and evolution.

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CHAPTER

13

It Takes Guts: Development of the Embryonic and Juvenile Zebrafish Digestive System

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Introduction

Zebrafish have increasingly become a model organism used to investigate vertebrate digestive development. Although the zebrafish intestine has some differences when compared to mammalian organs (Fig. 13.1), many studies have highlighted the high conservation in developmental pathways, cellular composition, and function of the digestive organs (Zhao & Pack, 2017). Additionally, much of the molecular pathways that initiate and promote development of the digestive organs but also regulation of repair following injury of the mature system have been conserved (Brugman, 2016; Carten & Farber, 2009; Sadler, Rawls, & Farber, 2013; Seth, Stemple, & Barroso, 2013; Zhao & Pack, 2017). Therefore, zebrafish has become a vital model vertebrate in which to study digestive development, physiology, and disease.

Endoderm Formation and Migration to Midline

Digestive development begins with the specification of the three germ layers before gastrulation. By 50% epiboly, fate-mapping experiments demonstrate endodermal progenitors arise from the marginal cells of the developing blastula (Warga & Nusslein-Volhard, 1999). The dorsal–ventral organization of endodermal progenitors at the blastoderm margin gives rise to anterior–posterior portions of the future digestive tract. The dorsal most endodermal progenitors give rise to the most anterior portion of the digestive tract (Warga & Nusslein-Volhard, 1999). Proceeding further ventrallyare the endodermal progenitors that give rise to more posterior portions of the digestive tract.

Following specification at the margin of the blastula, endodermal cells migrate to the midline and aggregate as a thin rod of cells along the anterior to the posterior axis (Warga & Nusslein-Volhard, 1999). These endodermal cells undergo high rates of proliferation up to the third day of embryogenesis, to give rise to the lining of the digestive tract, as well as major components of the swim bladder, pancreas, and liver (Fig. 13.2).

Organization of the Embryonic Endoderm

Formation of the digestive tract in many vertebrate species begins with the formation of a common tube from which all of the digestive organs differentiate (Zorn & Wells, 2009). In zebrafish, a common tube also forms but does not occur until later in development as digestive organs have begun to differentiate (Wallace & Pack, 2003). The first appearance of organization in the zebrafish digestive system begins in the intestinal endoderm with the radial organization of endodermal progenitors. The radial organization first occurs at the most anterior region of the future intestine at 21 h postfertilization (hpf) followed by endodermal cells in at the posterior region at 26 hpf (Wallace & Pack, 2003). By 34 hpf epithelium throughout the anterior to the posterior intestinal region has radial organization and displays characteristic markers of polarized epithelial cells with a histologically visible lumen. The intestinal lumen matures over the course of the next 2 days as epithelial cells develop from a cuboidal shape with few microvilli to columnar shape with numerous microvilli (Wallace & Pack, 2003). The epithelial cells also develop increased apical/basal polarization. Between 34 to 74 hpf it has also been reported that the lumen is still not patent throughout the length of the intestine (Alvers et al., 2014).

The Zebrafish in Biomedical Research
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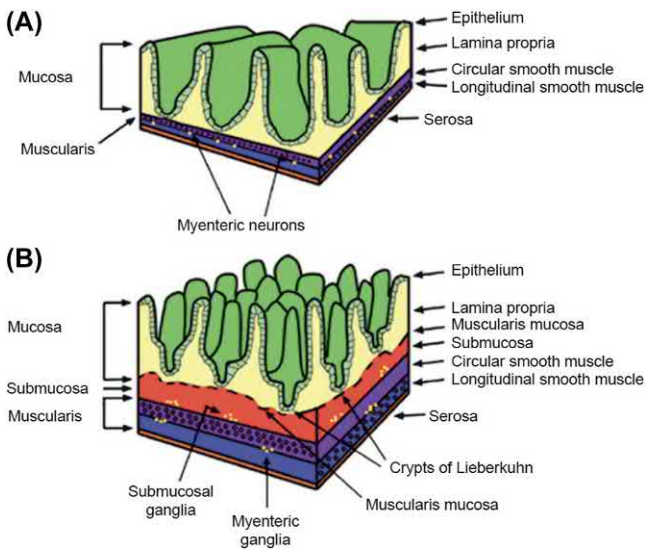

FIGURE 13.1 Comparison of the teleost intestine to the mammalian intestine. The teleost intestine (A) has broad irregular folds rather than individual villi as in mammals (B) and lacks crypts. The teleost has no muscular mucosa and only a thin layer of connective tissue separates the base of the folds from the circular and smooth muscle layers. Enteric neuronal cell bodies are located between the circular and longitudinal smooth muscle but do not form ganglia as in mammals. *Adapted from Wallace, K. N., Akhter, S., Smith, E. M., Pack, M. (2005). Intestinal growth and differentiation in zebrafish.* Mechanisms of Development, 122 *157–173.*

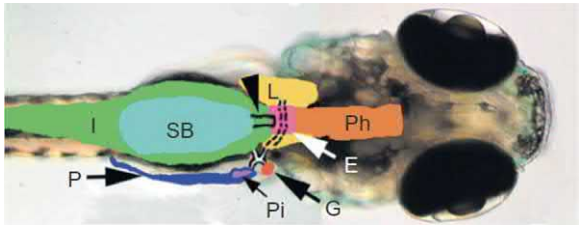

FIGURE 13.2 Zebrafish digestive anatomy. The pharynx (Ph) connects to a short, muscular esophagus (E) which connects to the intestine (I). The liver (L) straddles the esophagus. The hepatopancreatic duct enters the anterior intestine connecting the liver, gall bladder (G), and pancreas (P). The pancreas has a single islet (Pi) with the tail containing exocrine tissue. *Reproduced from Wallace, K. N., Pack, M. (2003). Unique and conserved aspects of gut development in zebrafish.* Developmental Biology, 255*(1), 12–29.*

While the intestinal endoderm is the first to begin organizing, the pharynx and esophageal endoderm begin organizing at different times. The pharyngeal endoderm has begun to polarize at 34 hpf, but there is no histological evidence of lumen formation (Wallace & Pack, 2003). By 50 hpf the pharyngeal endoderm has formed a wide bilayer tube. The esophageal endoderm also begins polarizing during this period. Both the pharynx and the esophagus have a lumen later during the second day. By 58 hpf the esophagus is patent with the anterior intestine, but the posterior pharynx is still not patent with the esophagus (Wallace & Pack, 2003). The entire tract is patent by 74 hpf; however, the anus does not open to the exterior until 96 hpf (Wallace & Pack, 2003).

Although zebrafish do not develop a stomach at any time during their life cycle, there is gene expression of markers that are indicative of stomach differentiation in other vertebrates (*sox2*, *barx1*, *gata5*, and *gata6*) (Muncan et al., 2007). While markers of a stomach are present, no stomach related structures develop and the esophagus transitions directly into the intestine. Some early stomach development markers may be expressed, however, it has been proposed that lack of or inactivation of genes involved in gastric function (proton pumps and pepsinogens) results in lack of a stomach phenotype in groups as diverse as teleosts, holocephali, dipnoids, and monotremes (Castro et al., 2014). As a result, species within these groups (including zebrafish) are likely to be no longer able to develop a functional stomach.

Both Sonic hedgehog a (Shha) and Indian hedgehog a (Ihha) are expressed throughout much of the endoderm, and act on Ptc1 and Ptc2 in the surrounding mesoderm (Korzh et al., 2011). Loss of *ihha* reduces the proliferation of both the endoderm and surrounding mesoderm (Korzh et al., 2011), which differentiates into circular and longitudinal smooth muscle. Migration and development of enteric neurons is also severely reduced with the loss of *ihha* (Korzh et al., 2011). Failure of enteric neurons to differentiate with loss of *ihha* may be due to defects in smooth muscle development. Large increases in numbers of differentiating enteric neuron are associated with the differentiation and maturation of longitudinal smooth muscle (Olden et al., 2008). Loss of smooth muscle differentiation and/or functionality may remove the necessary signals for enteric neuron differentiation.

Within the region of the esophagus and the swim bladder (which buds from the posterior esophagus and is a lung homolog (Perry et al., 2001)), Ihha and Shha in the endoderm also induce Fgf10 in the mesoderm to act on Fgfr2 in the endoderm (Korzh et al., 2011). The feedback loop between the two germ layers is critical for the development of both the esophagus and the swim bladder (Korzh et al., 2011). These defects are similar to the mouse model, where either loss of Hh or Fgf10 during the critical period of the trachea and esophagus separation results in tracheoesophageal malformation (Diez-Pardo et al., 1996; Hajduk, Murphy, & Puri, 2010).

Proliferation of the Intestinal Endoderm

Following the arrival of cells at the embryonic midline, the endoderm begins proliferating at a high rate. During this period, proliferation appears to occur throughout the entire endoderm. The rate of endodermal proliferation is reduced between 34 and 74 hpf,

dropping from over 40% to a little over 25%, respectively (Wallace et al., 2005). The last 2 days of embryonic development have significantly reduced proliferation with rates lower than 10% on the fourth day and less than 5% of the intestinal epithelium on the fifth day (Wallace et al., 2005). The lower endodermal proliferation rates correspond to increased epithelial differentiation and fold formation. As within the amniote intestine (Shyer et al., 2015), zebrafish intestinal fold formation also appears to initiate the process of restricting proliferating cells to the base of the developing folds with the majority of proliferating cells becoming localized to the base of the developing folds during the fourth and fifth days of embryogenesis (Wallace et al., 2005).

During the postembryonic period at 12 days post fertilization (dpf), 5-bromo-2′-deoxyuridine (BrdU) nucleotide incorporation reveals proliferative epithelial cells solely restricted to the folded base (Ng et al., 2005). By 33 dpf (first month after embryogenesis) the intestinal epithelium has matured to the adult state of proliferation. Unlike amniotes that develop crypts just before or slightly after birth (Trier & Moxey, 1979), the zebrafish intestinal epithelium does not develop crypts (Wallace et al., 2005). At 33 dpf, numerous proliferative stem cells are present at the folded base, which produce progeny that migrate up the folds and undergo apoptosis at the tips (Crosnier et al., 2005), similar to what is observed in adult fish (Wallace et al., 2005).

The canonical Wnt signaling pathway has been shown to play a role in the proliferation of the zebrafish intestinal epithelium. Increases in Wnt signaling due to inhibition of the GSK-3 β-catenin destruction complex by LiCl or an *axin1* mutant during embryogenesis have been demonstrated to increase the proliferation rates in six and eight dpf postembryonic individuals, as well as increase the Wnt target genes (Cheesman et al., 2011). Furthermore, the introduction of the *H. pylori* gene CagA, which increases the intestinal epithelial proliferation, also increases the nuclear accumulation of β-catenin (Neal et al., 2013). Increases in epithelial proliferation can be prevented with the introduction of a mutant *tcf4* gene, demonstrating that Wnt signaling plays a role in regulating the epithelial proliferation during this period (Neal et al., 2013). While Wnt signaling is active in the regulation of epithelial proliferation during the postembryonic period, loss of signaling does not result in complete loss of proliferation, suggesting that other signaling pathways also play a role in promoting proliferation during this period.

Later, 2 days after maturation of the intestine into the adult form, Wnt signaling has been demonstrated to be critical for maintenance of intestinal epithelium proliferation. In juveniles with mutant *tcf4*, just after the intestinal epithelium has converted to the adult form, proliferation is lost in the mid and distal portions of the intestine (Muncan et al., 2007). Loss of proliferation during the intestinal maturation process suggests that Wnt signaling becomes much more prominent in the promotion of epithelial proliferation in the adult form. This is similar to other vertebrates, where the loss of Wnt signaling also results in loss of intestinal epithelial proliferation (Stange & Clevers, 2013).

During resection of the adult intestine, for use as a model for short bowel syndrome, there is a compensatory three-fold increase in epithelial proliferation localized to the intervillous stem cell zone for 2 weeks following the procedure (Schall et al., 2015). During the 2 weeks of increased epithelial proliferation, there was also a statistically significant increase in the transcription of *egf* receptor and two EGF ligands (*egf* and *btc*), as well as the transcription of two IGF1 ligands (*igf1a* and *1gf2a*) (Schall et al., 2015). These results demonstrate that in addition to Wnt signaling, zebrafish epithelial proliferation can also be modulated by both the EGF and IGF pathways.

Maturation of the Embryonic Intestinal and Esophageal Endoderm

Intestinal endodermal cells are initially cuboidal on the first and second days of embryogenesis. During this period, epithelial cells gradually acquire more and higher expression of markers of polarized cells (laminin at basal, cadherin at lateral, and β actin/alkaline phosphatase at the apical surfaces) (Wallace et al., 2005). The epithelial cells also acquire full microvilli complement at the apical surface by 74 hpf (Wallace et al., 2005). At the beginning of the third day, the epithelial cells transition to a more columnar appearance and markers of differentiated cell types begin to appear. During the third day, each of the intestinal epithelial cells (enterocytes, goblet cells, and secretory cells) begin to differentiate (Wallace et al., 2005).

During this period, not only do individual cell types begin differentiating, but regionalization of the intestine along the anterior to posterior axis is also occurring. Goblet cells are restricted to the posterior while other secretory cells are distributed only in the anterior or posterior region with the dividing line near the posterior end of the developing swim bladder (Wallace et al., 2005) (Fig. 13.3). Toward the posterior end of the intestine, there is a short region that lacks the enterocyte marker, NaPi cotransporter (Wallace et al., 2005). This region may be equivalent in function to the large intestine in mammals. By the end of embryogenesis, the intestine is divided into three regions, anterior, middle and posterior (Wallace et al., 2005). Within the posterior end of the middle region, there is a group of cells that are able to sample the contents of the lumen

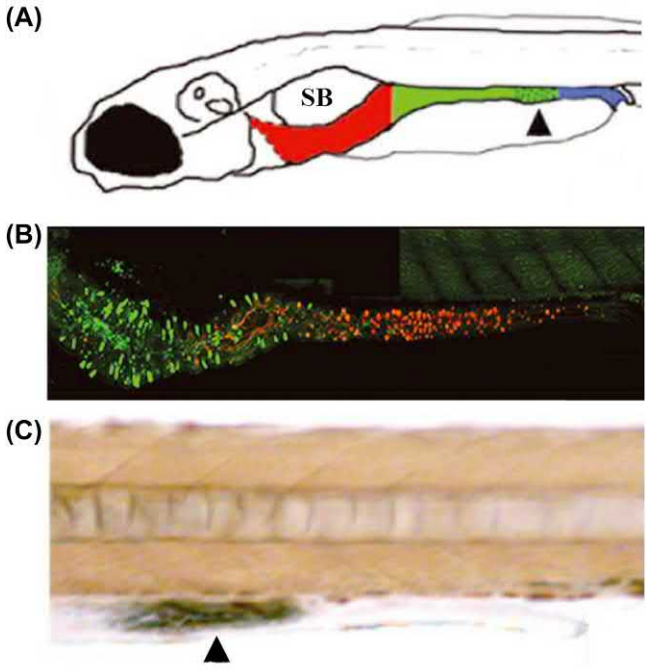

FIGURE 13.3 Regionalization of the intestine. (A). Anterior (red), mid (green) and posterior (blue) segments of the 5 dpf larval intestine. The distal region of the middle intestine that contains specialized enterocytes is stippled (arrowhead). (B). Lateral view of the 120 hpf intestine showing the regionalized distribution of differentiated epithelial cells. Pancreatic polypeptide containing enteroendocrine cells (green) are restricted to the anterior intestine (intestinal bulb), whereas goblet cells identified with WGA (red) are restricted to the middle intestine. NaPiC enterocytes are present throughout the anterior and middle intestine, but not the posterior intestine (not shown). (C). Lateral view of a portion of the middle intestine at 96 hpf that has ingested horseradish peroxidase protein (HRP). Following pinocytosis, HRP can be detected histochemically (*arrowhead*) within the apical cytoplasm of specialized enterocytes of the middle intestine (segment 2). *Adapted from Wallace, K. N., Akhter, S., Smith, E. M., Pack, M. (2005). Intestinal growth and differentiation in zebrafish.* Mechanisms of Development, 122 *157–173.*

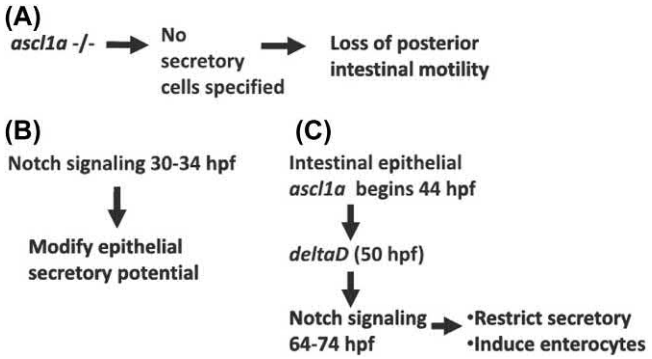

FIGURE 13.4 *(A) ascl1a* expression is critical for the specification of secretory cells. Loss of secretory cells results in loss of distal but not proximal intestinal motility. Notch signaling is active at two points in specification secretory or enterocytes.(B) Notch signaling between 30 and 34 hpf is involved in the potentiation of the intestinal epithelium to become secretory. (C) Later, *ascl1a* is expressed in cells with secretory potential, inducing *deltaD* to activate Notch signaling in surrounding cells. This is likely to involve lateral inhibition to restrict the number of secretory cells. *Adapted from Roach, G., et al. (2013). Loss of ascl1a prevents secretory cell differentiation within the zebrafish intestinal epithelium resulting in a loss of distal intestinal motility.* Developmental Biology, *376(2), 171–186.*

(Wallace et al., 2005). These cells may be the functional equivalent of M cells in the mammalian intestine.

One of the early choices in the differentiation of epithelial cells is the distinction of whether a cell will become an enterocyte or a secretory cell (Fig. 13.4). The choice is initiated by lateral inhibition utilizing the canonical Notch signaling pathway (Crosnier et al., 2005). During early development, there are two periods during which Notch signaling is critical to the proper distinction between secretory cells and enterocytes. The first period is early during the 30 to 34 hpf period and may play a role in establishing epithelial regions that will have the potential to become secretory (Flasse et al., 2013; Roach et al., 2013). The second begins with the expression of *ascl1a* beginning at 44 hpf in groups of epithelial cells (Flasse et al., 2013; Roach et al., 2013). Loss of *ascl1a* prevents differentiation of all secretory cells with all epithelial cells becoming enterocytes (Flasse et al., 2013; Roach et al., 2013). Loss of *ascl1a* also results in loss of intestinal motility posterior to the position of the swim bladder (Roach et al., 2013). *ascl1a* expression later appears to initiate expression of the Notch ligand *deltaD* beginning at 50 hpf also in the individual epithelial cells. DeltaD activates Notch signaling and limits the number of epithelial cells that become secretory cells between 64 and 74 hpf (Roach et al., 2013). Following specification as secretory, these cells then receive subsequent cues to differentiate into goblet cells and all types of enteroendocrine cells.

The esophagus initially develops as a columnar epithelium but at the end of embryogenesis and continuing to seven dpf, a portion of the dorsal endodermal region of the esophagus develops a nonkeratinized stratified squamous epithelial appearance, similar to the structure of adult human esophageal endoderm (Chen et al., 2015). This region has homology to the human homolog as demonstrated with the expression of common genes necessary for esophageal development (squamous epithelial genes *krt5* and *p63* and esophageal transcription factors *sox2* and *pax9*) (Chen et al., 2015).

Development of Smooth Muscle and Enteric Neurons

Smooth muscle precursors originate from *hand2* expressing lateral plate mesodermal (LPM) cells on the bilateral sides of the endoderm at 24 hpf

(Gays et al., 2017). Between 30 and 48 hpf *hand2* expressing LPM cells migrate and surround the endoderm (Gays et al., 2017). By 60 hpf, the *hand2* cells express *acta2* (α-smooth muscle actin) and *tagln* (Gays et al., 2017). These are the earliest markers of committed smooth muscle progenitors, which continue to be expressed in mature muscle (Georgijevic et al., 2007; Santoro, Pesce, & Stainier, 2009; Solway et al., 1995). TGFβ/Alk5 signaling has been shown to be required for the migration of smooth muscle precursors to surround the endoderm (Gays et al., 2017). Alk5 induction initiates *zeb 1a* expression to drive migration and *foxo 1a* to drive differentiation to smooth muscle precursors (Gays et al., 2017). The micro RNA miR-145 is also induced by Alk5 to negatively regulate *zeb 1a* and *foxo 1a* expression (Gays et al., 2017).

Enteric neuronal precursors migrate through the digestive system by first entering the pharynx near the posterior of the ear from the vagal neural crest at 33 hpf (Shepherd et al., 2004). Enteric precursors then migrate along the lateral aspects of the developing intestine and proliferate at high rates of 55% at 50 hpf and 44% at 58 hpf, reaching the posterior by 66 hpf (Olden et al., 2008). During the third day, the enteric precursor proliferation drops to 18% as more enteric neurons continue to differentiate (Olden et al., 2008).

During enteric precursor migration, expression of *sox10*, *phox2bb*, and *ret* are critical for migration, proliferation, and differentiation into enteric neurons (Shepherd & Eisen, 2011). By using these three markers, the enteric precursors have been shown to be heterogeneous, suggesting different levels of differentiation, migratory potential, and fate (Taylor et al., 2016). Enteric precursors expressing all three markers are proposed to remain as precursors while *phox2bb/sox10* may be fated to glial cells (Taylor et al., 2016). At least two types of neurons may differentiate from *phox2bb/ret* and *phox2bb* only cells (Taylor et al., 2016).

Enteric neurons begin differentiating at 50 hpf in the esophagus and anterior intestine, which continues to the posterior during the second day (Olden et al., 2008). As enteric precursors are still only on the lateral aspects of the intestine, enteric neurons only differentiate in these lateral aspects initially (Olden et al., 2008). At 74 hpf enteric neurons begin differentiating circumferentially around the anterior intestine but remain on lateral aspects in the posterior (Olden et al., 2008). A large proportion of the enteric neurons contain nNOS (40%), while calbindin, calretinin, and serotonin are expressed in a number of neurons at 72 hpf but increase significantly at later stages (Uyttebroek et al., 2010). Each group has regional variations from anterior to posterior (Uyttebroek et al., 2010). Vasoactive intestinal peptide and pituitary adenylate cyclase-activating peptide are expressed in varicosity-like structures along nerve fibers (Uyttebroek et al., 2010). No ChAT expression is detected during embryogenesis among enteric neurons (Uyttebroek et al., 2010).

Differentiation of intestinal smooth muscle has been correlated with milestones in enteric neural development. As smooth muscle cells begin differentiating, circular smooth muscle first forms between 63 and 69 hpf and is associated with the growth of enteric axons and an increase in enteric neurons (Olden et al., 2008). When longitudinal smooth muscle differentiates by 89 hpf there is a second increase in the number of enteric neurons (Olden et al., 2008). As smooth muscle matures between 93 and 98 hpf there is a final rapid increase with approximately 44% of the enteric neurons differentiating at this phase (Olden et al., 2008). The observed increases suggest that cues are provided by the smooth muscle to induce enteric neuron differentiation. When the smooth muscle is prevented from fully differentiating with *shha*, *ihha*, or *hand2* downregulation, there are lower numbers of enteric neurons differentiating (Korzh et al., 2011; Reichenbach et al., 2008) suggesting that smooth muscle provides a functional substrate and cues for enteric neural precursors to migrate, proliferate, and differentiate within.

Liver Development

Liver development in zebrafish has been extensively investigated. Genes involved are generally conserved in mammalian liver development, and a number of comprehensive reviews have been written on the subject (Goessling & Stainier, 2016; Wang et al., 2017; Wilkins & Pack, 2013). Here, we will provide an outline of liver development as it proceeds through each of the steps first with the specification, followed by the differentiation of specific cell types and then expansion.

As the endoderm is completing migration to the embryonic midline, fate mapping identifies cells within the anterior endoderm as hepatoblasts between 11 and 14 hpf with their fate regulated by Bmp2b and Wnt2bb signaling from the lateral plate mesoderm, as well as FGF (Chung, Shin, & Stainier, 2008; Ober et al., 2006; Shin et al., 2007). Hepatoblasts can be identified at 22 hpf with the expression of *hhex* and *prox1* (Wallace et al., 2001; Wallace & Pack, 2003).

Following the specification of hepatoblasts, markers of differentiation begin to be expressed from 32 to 50 hpf. The first marker of differentiation is the expression of the ferroxidase enzyme, ceruloplasmin at 32 hpf (Korzh, Emelyanov, & Korzh, 2001). Additional hepatocyte markers *liver-type fatty acid-binding protein (L-FABP)* and *transferrin* are expressed later at 48 hpf (Her et al., 2003; Mudumana et al., 2004). Biliary epithelial progenitors (marked with 2F11 antibody) are first observed at

36 hpf (Lorent et al., 2010), which begin maturing (expressing cytokeratin 18) between 50 and 60 hpf with a branching network of ducts formed between 70 and 80 hpf (Lorent et al., 2004). The biliary network is functional between 4 and 5 dpf, as there is flow of a fluorescent marker to the gallbladder and anterior intestine during these times (Farber et al., 2001).

The hepatic sinusoids originate from endothelial cells in the nearby region of the cardinal vein, migrate around the organ at 54 hpf and invade by 72 hpf (Hen et al., 2015). By 80 hpf endothelial cells form a highly branched network within the liver and aid in polarizing adjacent hepatoblasts (Sakaguchi et al., 2008). From 80 hpf through postembryonic phase cells of the liver continue to grow and expand the size of the organ.

Pancreas Development

As with the liver, fate mapping identifies both endocrine and exocrine pancreatic progenitors in the anterior endoderm, as they complete migration to the midline with their fate is in part regulated by levels of Bmp2b and FGF expressed in the lateral plate mesoderm (Chung et al., 2008; Manfroid et al., 2007). Pancreatic progenitors later migrate into two slightly different positions, one larger group of precursors that gives rise to most of the endocrine pancreas (and begins differentiating early at 14 hpf-see below) and a smaller group of precursors just anterior and slightly ventral to the other group (and relative to the developing intestine), which contains exocrine, pancreatic duct precursors, and endocrine precursors. The endocrine precursors of the anterior-ventral group give rise to some cells in the initial primary islet which forms during embryogenesis but primarily give rise to secondary islets that begin developing in the postembryonic fish (Field et al., 2003; Wallace & Pack, 2003).

The exocrine cells of the smaller anterior group are recognized with the expression of *ptf1a* and surround a group of Notch responsive progenitors (lacking *ptf1a* expression) that have been fate mapped to form secondary islets during postembryonic and adult phases (Parsons et al., 2009; Wang et al., 2011). As the anterior-ventral group of pancreatic cells migrates to the larger posterior-dorsal group between 44 and 48 hpf, the pancreas develops three layers of cells with the endocrine in the center followed by the Notch responsive progenitors, and finally, the *ptf1a* cells on the outside (Field et al., 2003; Lin et al., 2004; Wang et al., 2011; Wendik, Maier, & Meyer, 2004; Zecchin et al., 2004). The Notch responsive progenitors later reside along the ventral pancreatic duct and give rise to ducts, centroacinar, and endocrine cells during the formation of secondary islets later in the postembryonic and adult phase (Wang et al., 2011). *ptf1a* cells can later give rise to acinar, endocrine, and Notch responsive cells (Wang et al., 2015).

Endocrine cells begin to differentiate early between 14 and 24 hpf with the appearance of insulin, somatostatin, and glucagon expressing cells (Biemar et al., 2001). During this period, endocrine cells begin as diffuse groups (Biemar et al., 2001) but coalesce into a group that is recognized with *pdx1* and *hhex* expression between 21 and 26 hpf (Wallace & Pack, 2003). The final arrangement of endocrine cells at five dpf contains a central core of insulin-producing cells, surrounded by glucagon, somatostatin, and ghrelin-positive cells, which is similar to the mammalian islet organization. Secondary islets form through the tail of the pancreas by 33 dpf (Prince, Anderson, & Dalgin, 2017).

Exocrine tissue begins expanding around the islet and growing posteriorly after 50 hpf until it reaches its final embryonic size at 96 hpf (Yee, Lorent, & Pack, 2005). At 50 hpf both acinar (identified with carboxypeptidase A expression) and ductular (identified with cytokeratin) cells begin differentiating but do not connect to the extrapancreatic duct (Yee et al., 2005). By 96 hpf acinar cells are columnar and polarized forming first-order branches of pancreatic ducts. Second-order branches of pancreatic ducts and fully polarized acinar cells form at 120 hpf, and small, centrally located centroacinar cells develop (Yee et al., 2005). The duct system appears to organize and join other units rather than grow by reiterative branching. The pancreas continues to grow in size and develops into a diffuse organ that resides between multiple loops of the intestine (Yee et al., 2005).

Maturation of the Postembryonic Digestive System

While the digestive system is functional at the end of embryogenesis, it has not matured to the final form and function. Maturation of the digestive system occurs over the next 4 weeks (6–33 dpf) after embryogenesis encompassing a postembryonic phase (Crosnier et al., 2005). After embryogenesis, the intestine remains as a straight tube until around 26 dpf when it begins bending to develop the S loop that is present later in the adult intestine (Crosnier et al., 2005). During the fourth week of postembryonic phase (27–33 dpf), multiple cells at the folded base enter into mitosis. Similar to the adult form, progeny of these cells begin migrating up the fold and undergo apoptosis at the tips (Crosnier et al., 2005). The adult proliferating region at the folded base also expresses intestinal stem cell marker *sox9b* (Cheesman et al., 2011).

The dorsal region of the esophagus continues to elaborate the nonkeratinized stratified squamous

epithelium that began developing at the end of embryogenesis into the 7dpf. At 90 dpf the nonkeratinized stratified squamous epithelium develops high levels of *pax9* and *krt5* expression is histologically similar to the human esophageal epithelium (Chen et al., 2015).

Conclusions

Milestones in the development of the embryonic zebrafish digestive system have been well characterized. The use of forward mutant screens and transgenic tools have allowed for the identification of a number of genes and signaling pathways involved in the formation of the organs. With the ongoing use of Crispr/Cas9, increasing numbers of mutants and conditional transgenics are being generated. As a number of events in digestive development occur later in embryonic development and into post embryonic phase, transgenics that conditionally delete or modify gene expression will further refine knowledge of digestive development.

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CHAPTER

14

The Zebrafish Cardiovascular System

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Introduction

The zebrafish has recently emerged as a key vertebrate model organism for studying the cardiovascular system. The successful formation of the vascular network is one of the first and most critical events for successful organogenesis in the embryo, and the cardiovascular system features prominently in a variety of different pathological conditions in humans including heart disease, ischemia, and cancer. Building on the basic genetic and experimental advantages of the zebrafish, a variety of tools and methods have been developed that further amplify these advantages for studying heart and vessel growth and development and its molecular regulation.

The Cardiovascular System

During organogenesis, organs develop a vascular supply to modulate the delivery of nutrients and oxygen and promote the clearance of waste. Because of the critical nature of these functions to the viability of the embryo, formation, and maintenance of the vasculature during development and postnatal adult life is tightly regulated, and any significant disruption to this organ system leads to embryonic lethality. Many of the pathways that are critical regulators of early vascular development are also required for postnatal neo-angiogenic processes, including wound healing, tissue regeneration, and disease progression (e.g., the formation of a vascular bed to support tumor growth). Elucidating the mechanisms and signaling pathways controlling vascular development continues to be an essential step in the identification of therapeutic targets for adult pathologies, including stroke, cancer, diabetes, and heart disease.

The Zebrafish as a Cardiovascular Model Organism

The zebrafish provides many unique features as a model organism to study vascular development (Gore, Monzo, Cha, Pan, & Weinstein, 2012; Isogai, Horiguchi, & Weinstein, 2001; Isogai, Lawson, Torrealday, Horiguchi, & Weinstein, 2003; Lawson & Weinstein, 2002b; Yaniv et al., 2006). First, the embryos are small and externally fertilized, making them easily accessible for observation and for experimental manipulation, including DNA/RNA overexpression, gene suppression methodologies (including morpholino administration and chemical inhibitor treatments), and reverse genetic technologies, such as TALEN and CRISPR (Auer & Del Bene, 2014; Blackburn, Campbell, Clark, & Ekker, 2013; Chang et al., 2013; Hruscha et al., 2013; Hwang, Fu, Reyon, Maeder, Kaini, et al., 2013; Hwang, Fu, Reyon, Maeder, Tsai, et al., 2013; Jao, Wente, & Chen, 2013; Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995; Meng, Noyes, Zhu, Lawson, & Wolfe, 2008; Reade et al., 2017; Villefranc, Amigo, & Lawson, 2007). Additionally, zebrafish embryos receive sufficient oxygen through passive diffusion to survive and develop in the absence of a functioning cardiovascular system vasculature for several days. This makes it possible to assess vascular-specific phenotypes without confounding secondary defects caused by sick or dead embryos.

Second, zebrafish embryos are optically clear, allowing for high-resolution imaging of the vasculature at all sites within the embryo over long time courses (Fig. 14.1). Confocal, multiphoton, and single plane illumination microscopy (SPIM) imaging techniques have all been optimized to assess vessel formation, vessel patency, and vessel stabilization over both short

[a] These authors contributed equally to this work.

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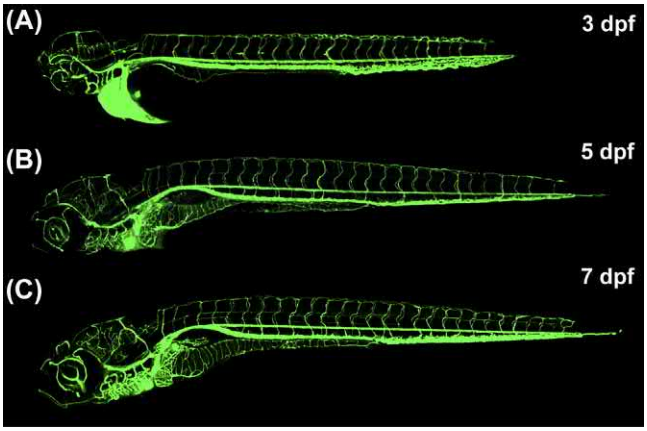

FIGURE 14.1 **The zebrafish vascular system.** (A–C) Confocal micrographs of green fluorescent fluorescein dextran injected into the circulation of three dpf (A), 5 dpf (B) or 7 dpf (C) developing wild type zebrafish embryos, highlighting the intricate network of blood vessels found in these animals at each stage. Images are lateral views, rostral to the left. *Figures (A–C) modified after Isogai et al., 2017; Isogai, S., Horiguchi, M., Weinstein, B. M. (2001). The vascular anatomy of the developing zebrafish: An atlas of embryonic and early larval development.* Developmental Biology, 230(2), 278–301. https://doi.org/10.1006/dbio.2000.9995.

and multiple daytime courses. Fish are also amenable to studies involving real-time angiography, permeability, and solute uptake techniques (Choi et al., 2013; Isogai et al., 2001; Jung et al., 2016; Kamei, Isogai, Pan, & Weinstein, 2010; Lawson & Weinstein, 2002b; Stratman et al., 2017; Venero Galanternik et al., 2017; Yaniv et al., 2006).

Third, the zebrafish is ideal for transgenesis and large-scale forward genetic screens, thanks to the features noted above, as well as a large number of adult animals that can be kept in a modest space, and a large number of embryos that can be obtained from a single mating. Recently, many new transgenic lines labeling varying components of the circulatory system with fluorescent tags have been developed. Besides being useful for imaging and experimental analysis of the developing vasculature, these lines have been used extensively in unbiased forward-genetic screens to identify new genes regulating heart and vessel formation (Butler, 2000; Driever et al., 1996; Mullins, Hammerschmidt, Haffter, & Nusslein-Volhard, 1994; Stainier et al., 1996; Weinstein & Fishman, 1996; Weinstein et al., 1996; Westerfield, 1995).

All these features, taken together, have brought zebrafish to the forefront as a premier model organism to study the development of the cardiovascular system.

Anatomy of the Adult Cardiovascular System

Adult Vascular Anatomy

The vertebrate cardiovascular system is a closed-loop system consisting of three main components: the heart, the blood vascular system, and the lymphatic vascular system. Blood is pumped away from the heart through arteries, perfused through body tissues via the capillaries, and is returned to the heart through veins (Cleaver & Krieg, 1998; Isogai et al., 2001). Modifications to this basic plan have evolved to meet the requirements of individual vertebrate species. Fish, as an example, utilize a system in which blood is oxygenated at the gill capillaries, sent directly throughout the body tissues, and then returned to the heart. Because of this more streamlined system, the fish heart contains only a single atrium and ventricle, unlike mammals and avians with two atria and two ventricles incorporating the pulmonary circulation (Bagatto & Burggren, 2006; Isogai et al., 2001; Lee, Stainier, Weinstein, & Fishman, 1994). In fish, blood enters the heart through the atrium and exits through the ventricle, while in air-breathing vertebrates an additional pulmonary circulatory loop to the lungs is utilized for blood oxygenation. In most respects, however, the structure and function of the circulatory system in teleost fish (including zebrafish) is remarkably similar to that of other higher order vertebrates (Drake & Fleming, 2000; Hu, Yost, & Clark, 2001; Weinstein & Fishman, 1996).

In all vertebrates, blood exits the heart through the ventricle to be delivered to the surrounding tissues. The aorta is the major axial artery from which blood circulates through a series of hierarchical branching arteries to all parts of the body, first through smaller arterioles and then through capillaries, where the majority of nutrient and waste exchange happens. Once through the capillary plexus, blood is carried back to the heart by way of increasingly larger venules and veins before returning to the heart. Despite their differing functions, adult arteries and veins have a similar basic architecture, with a single endothelial cell layer lining the lumen of the blood vessel (called the *tunica intima*) surrounded by connective tissue and multiple layers of vascular smooth muscle cells/pericytes embedded in an extracellular matrix (called the *tunica media*). The tunica media is further encompassed by the tunica adventitia, an outer layer of connective tissue (Fig. 14.2A). Although arteries and veins both have associated smooth muscle cells, the layers of these cells are much more developed on arteries, as this is the side of the vascular tree that primarily regulates vascular tone and blood pressure (Fig. 14.2B). Additionally, nerves run throughout the adventitia to innervate both the smooth muscle and endothelial cells. This entire basic structure is maintained in the zebrafish, with a highly stereotyped hierarchy of vessels that become ensheathed by smooth muscle cells over time (Ando et al., 2016; Butler, Gore, & Weinstein, 2011; Cleaver & Krieg, 1998; Santoro, Pesce, & Stainier, 2009; Seiler, Abrams, & Pack, 2010; Stratman et al., 2017; Venero Galanternik et al., 2017; Wang, Pan, Moens, & Appel, 2014).

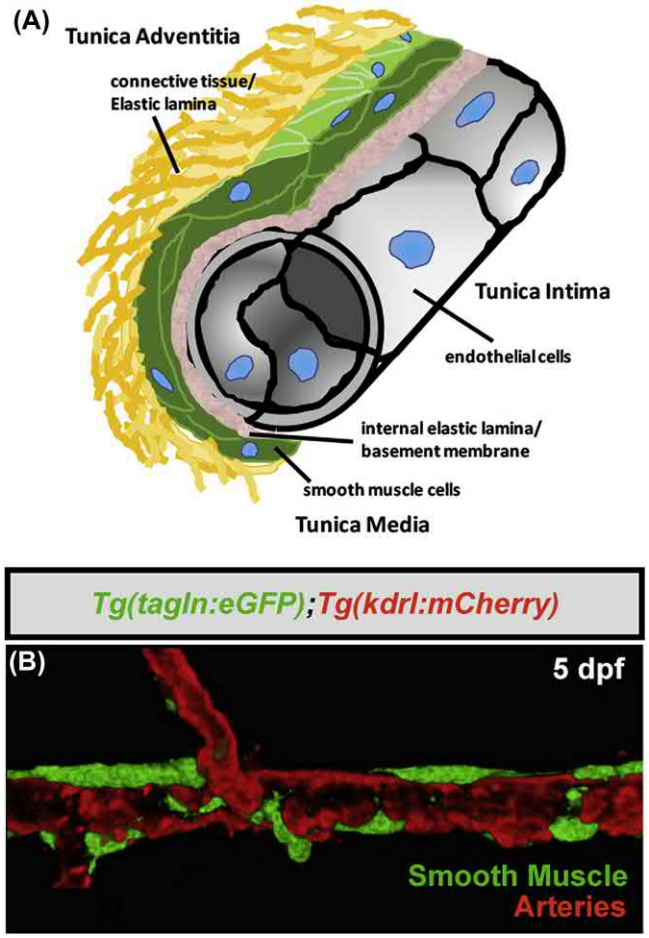

FIGURE 14.2 **Smooth Muscle associated with blood vasculature in the zebrafish.** (A) Diagram of the basic architecture of the blood vessel wall. (B) Confocal micrograph of the dorsal aorta in the mid-trunk of a 5 dpf *Tg(tagln:eGFP);Tg(kdrl:mCherry)* double transgenic zebrafish embryo, showing GFP-labeled mural cells (smooth muscle/pericytes) in green and the mCherry-labeled dorsal aorta endothelium in red. Lateral view, rostral to the left.

In addition to the blood vasculature, vertebrates including fish have a separate parallel endothelial vessel network called the *lymphatic system*. Unlike the circulatory system described above, which forms a continuous loop, the lymphatic system is a blind-ended vascular tree. Lymphatic vessels are also lined by a single layer of endothelial cells with extremely "loose" intracellular junctions in lymphatic capillaries that permit easy entry of fluids, macromolecules, and cells. The primary function of the lymphatic system is, in fact, to maintain organ and tissue homeostasis by taking up and draining interstitial fluid and by transporting immune cells (Fig. 14.3). The fluid within the lymphatic vessels, called *lymph*, is a combination of water, fat, dissolved molecules, triglycerides, and white blood cells/immune cells (Astin & Crosier, 2016; Butler, Isogai, & Weinstein, 2009; Cha et al., 2012; Hogan & Black, 2015; Jung et al., 2017; Oliver, 2004; Petrova et al., 2002; Sabin, 1902; Venero Galanternik et al., 2017; Venero Galanternik, Stratman, Jung, Butler, & Weinstein, 2016; Yaniv et al., 2006, 2007). Because this system is not connected to a single centralized pump, it has evolved to contain a number of valves that help to drive unidirectional fluid transport. Low pressure generated by the surrounding skeletal muscle helps squeeze fluid through the lymphatic vasculature into progressively larger lymphatic structures, most of which terminate at either the right lymphatic duct or the thoracic duct, depending on where the lymph originated. These two ducts then return the lymph back into the blood circulatory system by way of the right and left subclavian veins. Postnatally, impairment in the function of the lymphatic vasculature can result in the formation of lymphedema, while the excessive formation of new lymphatic vessels in tumors has been shown to facilitate the spread of metastatic cancer. Interestingly, for both the lymphatic and blood vascular systems, reactivation or impairment of developmental pathways that govern the formation and stabilization of either system (covered in later sections of this review) during adulthood can substantially contribute to disease progression including stroke, diabetes, cancer, and heart disease.

Adult Heart Anatomy

The zebrafish adult heart is located anteroventrally in the thoracic cavity between the operculum and the pectoral fin's bone (Hu et al., 2001). The heart is composed of a pear-shaped bulbous arteriosus, a pyramidal shaped ventricle, an atrium, and a sinus venosus. The ventricle and atrium of the adult heart are single chambers formed by a tri-layered tissue composed of epicardium, myocardium, and endocardium. The atrium possesses only a thin layer of the myocardium, while the ventricular myocardium is composed of thick muscular trabeculae that give the heart its typical internal spongy mesh texture and is required for blood pumping. The sinus venosus lacks the muscular myocardium and the bulbous arteriosus consists of an external elastic lamina layer, a medial thick multilayer of smooth muscle enclosed in collagen and reticular fibers and a central elastic subendothelial layer (Bartman et al., 2004; Hu et al., 2001; Poon & Brand, 2013; Stainier, Beis, Jungblut, & Bartman, 2002). The atrium connects to the sinus venosus by the AV-valve, and the ventricle is connected to the bulbous arteriosus by a BV-valve, both formed mostly of endocardium. Prior to entering the atrium, blood from the common cardinal vein collects in the sinus venosus (Hu et al., 2001). The blood will pass from the atrium to the ventricle and then into the bulbous arteriosus. This last chamber is connected to the ventral aorta, a large vessel connected to the branchial arterial vasculature that branches into the gills (Hu et al., 2001).

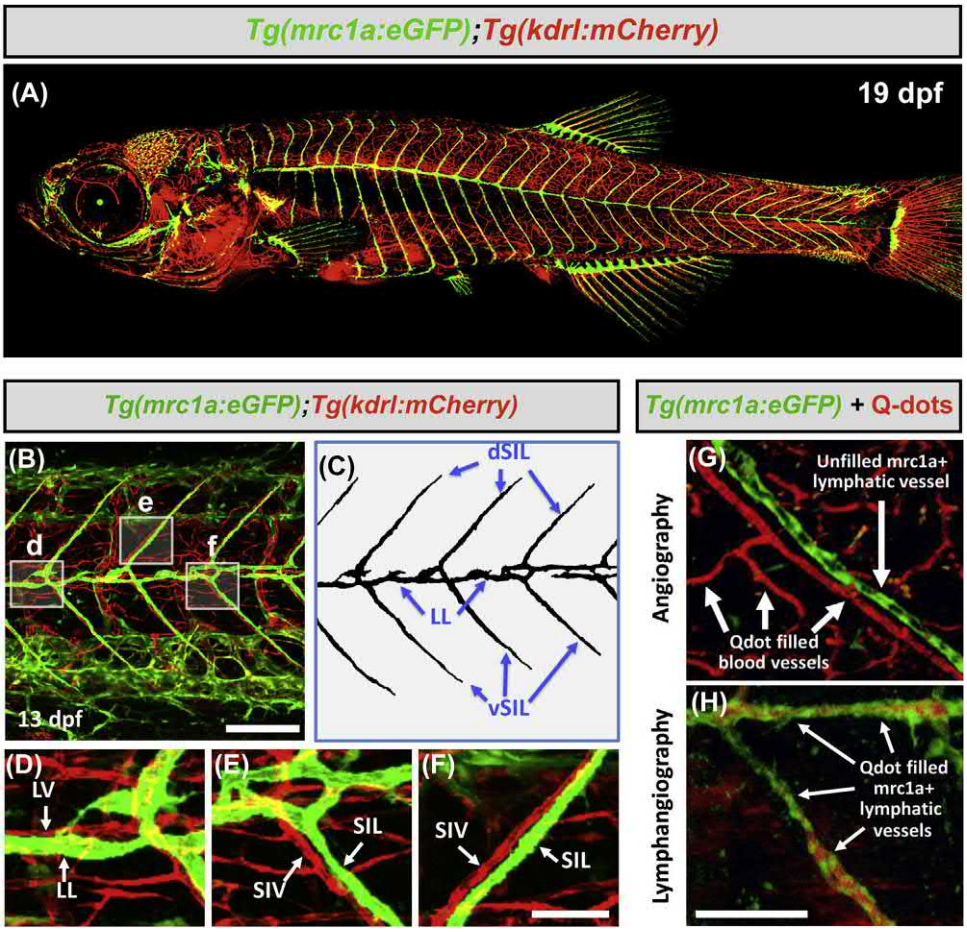

FIGURE 14.3 **Zebrafish possesses a well-developed lymphatic system.** (A) Composite tiled confocal image of a 19 dpf *Tg(mrc1a:egfp);Tg(kdrl:mCherry)* double transgenic zebrafish showing superficial blood (red) and lymphatic (green) vessels. (B) Confocal micrograph of the mid-trunk of a 13 dpf *Tg(mrc1a:egfp)*^{y251}, *Tg(kdrl:mcherry)*^{y171} double-transgenic zebrafish (lymphatics, green; blood vessels, red). The boxes labeled d, e, f note the positions of the magnified images shown in panels D, E, F. (C) Schematic diagram showing the superficial lymphatic vessels imaged in panel B. (D-F) Magnified portions of the confocal micrograph in panel B showing Mrc1a:eGFP-positive lymphatic vessels aligned with adjacent Kdrl:mCherry−positive blood vessels. (G) Confocal microangiogram of superficial vessels in the mid-trunk of a 28 dpf *Tg(mrc1a:egfp)*^{y251} transgenic zebrafish after intravascular injection of Qdot705 quantum angiography to label the blood vascular system (lymphatics, green; blood vessels labeled by intravascular Qdot705, red). (H) Confocal lymphangiomicrograph of superficial vessels in the mid-trunk of a 28 dpf *Tg(mrc1a:egfp)*^{y251} transgenic zebrafish after intramuscular injection Qdot705 dots into the tail that drain into and label the trunk lymphatic system (lymphatics, green; intralymphatic Qdot705, red). LV: Parachordal lymphatic vessel, LL: Lateral Lymphatics, dSIV/dSIL and vSIV/vSIL: Dorsal or ventral Superficial Intersegmental Vessels and Lymphatics. *Figures E−H modified after Jung, H. M., Castranova, D., Swift, M. R., Pham, V. N., Venero Galanternik, M. V., Isogai, S., et al (2017). Development of the larval lymphatic system in the zebrafish.* Development, https://doi.org/10.1242/dev.145755.

The Developing Cardiovascular System

Tools and Methods for Visualizing Heart and Vessels in Developing Zebrafish

From the beginning of the establishment of the first genetic tools and methods in the early 1980s, the zebrafish has rapidly become one of the most highly utilized vertebrate developmental biology model organisms. With the more recent invention of a variety of powerful new specialized tools and methods specifically for visualization and study of the developing heart, blood vessels, and lymphatic vessels, the fish has also become a preeminent model for studying cardiovascular development. We briefly describe some of these tools and methods below.

Microangiography, Dye Fills, Resin Casting

Classic vascular embryologists, like Florence Sabin, used dye injection to understand the blood and lymphatic vessel anatomical patterns and interactions (Sabin, 1902). Intravascular injection of India ink, Berlin blue or Evans blue dyes, or more modern-day fluorescent Dextran dyes and quantum dots are still widely used for the same purpose in mammalian, avian, amphibian, and piscine models. Microangiography and lymphangiography provide common and powerful methods to detect

not only vascular anatomical integrity but flow directionality, permeability, drainage, and solute/fluid uptake (Figs. 14.1 and 14.3G,H). The transparency and small size of zebrafish embryos and larvae make this a superb model in which to observe these biological processes in vivo, in an intact developing organism (Isogai, Hitomi, Yaniv, & Weinstein, 2009; Jung et al., 2017; Venero Galanternik et al., 2017; Yaniv et al., 2006).

Plastic resin-based casting provides another method to visualize both major and micro-vessels. Blood or lymphatic vessels are injected with resin that subsequently polymerizes in situ. After the plastic resin hardens, the surrounding tissues are digested away to reveal a cast of the vascular tree. When combined with electron microscopy, resin casts can detect even the smallest vessels in embryonic and adult tissues (Murakami, 1972), although proper preparation of resin casts is technically challenging and requires skill and care (refer to Jung et al., 2016). Although the wide availability of vascular-specific transgenic lines has reduced the need for these traditional methods, they still represent fast, robust, and cost-effective methods for visualizing the vasculature, particularly in nontransgenic animals or at later stages of development.

Particle Image Velocimetry (PIV)

Blood flow and cardiac hemodynamics can be measured noninvasively by injecting tracer particles into the bloodstream and then tracking their speed through different vessels (Poelma, Kloosterman, Hierck, & Westerweel, 2012). PIV has been used in various animal and culture settings, where the flow speed is determined by calculating the displacement of tracer particles over time (Vennemann et al., 2006). Due to the depth and/or the lack of transparency of many tissues and vessels in animal models in vivo, PIV has mainly been used during embryonic stages or in cell culture devices, although recent advances in confocal microscopy, ultrasound, or tomography tools and methods have facilitated deeper imaging in more opaque tissues.

Transgenic Zebrafish and Time-lapse Imaging

The optical clarity of zebrafish embryos and larvae has made them a favorite animal model for noninvasive, real-time high-resolution optical imaging, especially in conjunction with transgenic zebrafish lines highlighting the circulatory system. In recent years, advances in molecular cloning and zebrafish transgenesis methods have led to the generation myriad transgenic lines utilizing tissue-specific promoters to drive cytoplasmic, nuclear, or membrane-localized fluorescent proteins (Kawakami et al., 2010; Kwan et al., 2007; Zhang, Werling, & Edelmann, 2012).

Countless cardiovascular-related tissues or cell types have been marked and studied using transgenic animals, including the heart endocardium and myocardium, blood and lymphatic vessel endothelium, hematopoietic derived cells, perivascular cells like pericytes, and very recently, fluorescent granular perithelial cells (also named Mural Lymphatic cells or muLECS) (Figs. 14.2B,14.3, and 14.4B–D) (Bower et al., 2017; Ellett,

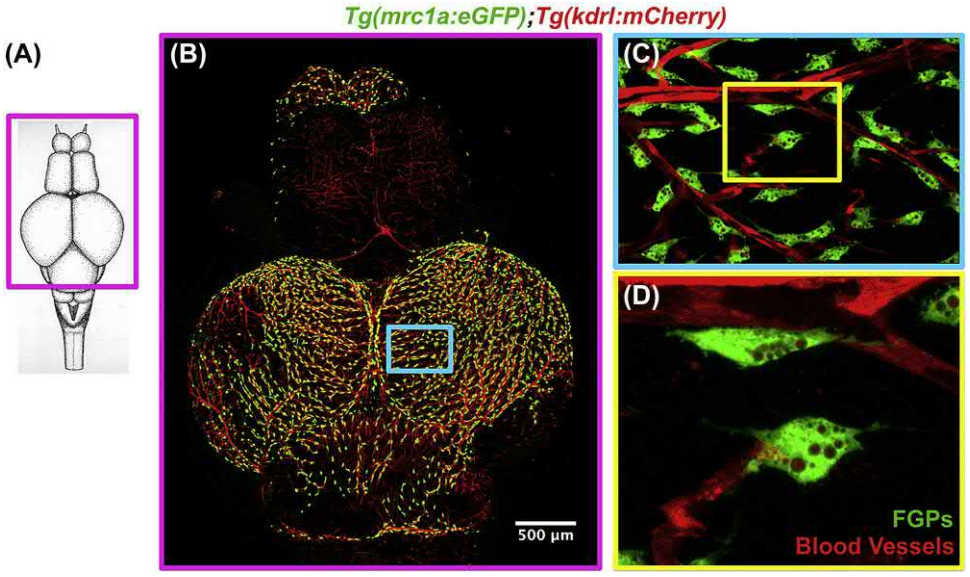

FIGURE 14.4 **Newly characterized lymphovenous perivascular cells (FGPss) cover the zebrafish brain.** (A) Diagram of the adult zebrafish brain showing the area imaged in panel B. (B) Confocal micrograph of an adult dissected zebrafish brain of a *Tg(mrc1a:eGFP);Tg(kdrl:mCherry)* double-transgenic zebrafish (EGFP and mCherry are shown in green and red, respectively). Dorsal view, rostral at top. (C) Higher magnification confocal micrograph of approximately the blue boxed region in panel B, showing GFP-labeled fluorescent granular perivascular cells (FGPs; green) adjacent to mCherry-labeled blood vessels (red) on the surface of the adult zebrafish brain. (D) Higher magnification of confocal micrograph of the yellow boxed region in panel C, showing the distinctive internal vesicles found inside FGPs. *Modified after Venero Galanternik, M., Castranova, D., Gore, A. V., Blewett, N. H., Jung, H. M., Stratman, A. N., et al (2017). A novel perivascular cell population in the zebrafish brain.* Elife, *6*. *https://doi.org/10.7554/eLife.24369*.

Pase, Hayman, Andrianopoulos, & Lieschke, 2011; Hall, Flores, Storm, Crosier, & Crosier, 2007; Jung et al., 2016, 2017; Kamei et al., 2010; Kawakami et al., 2010; Kikuchi et al., 2010; Lawson & Weinstein, 2002b; Okuda et al., 2012; Renshaw et al., 2006; Rhodes et al., 2005; Sood et al., 2010; Stratman et al., 2017; Venero Galanternik et al., 2016, 2017; Yaniv et al., 2006). Long-term time-lapse methods have also been developed that permit continuous, real-time imaging of heart, blood vessel, and lymphatic vessel development using these transgenic lines (Jung et al., 2016; Kamei et al., 2006, 2010). Together, the availability of fluorescent transgenic reporter lines and sophisticated tools and methods for high-resolution, dynamic imaging of these lines, has revolutionized the study of cardiovascular development.

Heart Development and Regeneration

Heart disease affects millions of individuals worldwide and is one of the main causes of human mortality. Cardiac injury or tissue scarring is a common side effect of myocardial infarction in humans. These persistent scars greatly impair ongoing cardiac function and are a consequence of the poor regenerative properties of adult mammalian cardiac muscle In comparison, many other vertebrates including some teleost fishes have retained a powerful regenerative capacity for their cardiac muscle (Jopling et al., 2010; Major & Poss, 2007; Poss, 2007; Poss, Wilson, & Keating, 2002; Sleep et al., 2010). Notwithstanding their superior regenerative properties, the early developmental steps and signaling pathways regulating zebrafish heart formation are conserved among vertebrates, including humans (Auman & Yelon, 2004; Yelon, 2012; R.; Zhang et al., 2013).

Heart development starts during gastrulation, where vertebrate cardiac progenitor cells appear bilaterally at the most anterior lateral plate mesoderm, from where they migrate medially and form the tubular epithelial primordia on either side of the midline, finally fusing to form the definitive heart tube, composed of an outer myocardium and an inner endocardium. Around, 36 hpf, small indentations in the heart tube begin establishing the heart's chamber boundaries and providing the initial morphological evidence of chamber formation, and by 48 hpf, these chambers can be discerned clearly. As the fish develops and blood circulation begins, venous blood enters the heart at the sinus venosus, and flows in through the atrium, ventricle, and bulbous arteriosus, before exiting into the ventral aorta. In air-breathing amniotes, the atria and/or ventricle become divided later in development to accommodate the pulmonary vasculature, but zebrafish and other teleost fishes circulation lack a pulmonary dependent oxygen exchange. Therefore, the heart outflow that empties into the ventral aorta feeds into a series of aortic arches, which in teleosts, are retained as the gill capillary network responsible for oxygen exchange.

The zebrafish heart has remarkable regenerative capabilities. Following chemical-, cryo-dependent or surgically induced cardiac insults, the zebrafish heart can completely regenerate its tissues in a period of approximately 30 days (Choi et al., 2013; Jopling et al., 2010; Kikuchi et al., 2010; Lepilina et al., 2006; Major & Poss, 2007; Sleep et al., 2010; Zhang et al., 2013). Zebrafish heart regeneration includes cardiomyocyte replenishment by proliferation, epicardium and endocardium rearrangement, neovascularization of the regenerative tissues, and complete recovery of cardiac function, all without the formation of scarring. Current methods of inducing injury include surgical resection, chemical ablation (Cre- and Diphtheria-dependent), and cryo-induced injury. All these methods cause an average of 20%–25% ventricular damage but so far only cryo-affects the final shape of the heart, which is otherwise scarless and completely functional (Gonzalez-Rosa, Martin, Peralta, Torres, & Mercader, 2011; Kikuchi & Poss, 2012; Lai et al., 2017). Cardiomyocyte proliferation and fast immune response and have been two of the most studied features responsible for the regenerative response in zebrafish regeneration. In surgically resected heart ventricles, robust cardiomyocyte proliferation at the apex of injury seems to be a key feature during heart regeneration, and mutants affecting the cell cycle show poor regenerative properties and scarring (Poss et al., 2002). Mammalian cardiac tissue is only able to proliferate in utero or at birth (P0) after which cardiomyocytes leave the cell cycle (Porrello, Johnson, et al., 2011; Porrello, Mahmoud, et al., 2011). It has been hypothesized that the capacity of zebrafish cardiac muscle to reenter the cell cycle is favored by their mono-nucleated cardiomyocytes (Kikuchi & Poss, 2012). Recent evidence regarding the role of the immune system in heart regeneration comes from comparative transcriptomic analysis of zebrafish versus the nonregenerative teleost Medaka (*Oryzias lapides*). Zebrafish display quicker and stronger macrophage recruitment and neutrophil clearance to the injured cardiac region (Lai et al., 2017), while the response is delayed and less robust in Medaka. Clodronate-dependent depletion of macrophages in cryo-injured zebrafish hearts leads to compromised regeneration and neovascularization of the damaged tissue, recapitulating the response in Medaka and leading to scar tissue formation (Lai et al., 2017).

Blood Vessel Development

The blood vascular system is required for the dispersal of blood, oxygen, and nutrients throughout the body. The earliest vessels that appear are formed through the process of vasculogenesis, the de novo generation of new blood vessels from individual mesoderm-derived cells (Flamme, Frolich, & Risau, 1997). These mesoderm cells differentiate into endothelial precursors called *angioblasts*, which acquire endothelial identity before coalescing and lumenizing to form the earliest vascular plexus. In fish, angioblasts emerge from the lateral plate mesoderm, and they are dependent on vascular endothelial growth factor receptor (VEGFR2) mediated signaling for their survival, proliferation, and motility (Fouquet, Weinstein, Serluca, & Fishman, 1997; Liao et al., 1997; Risau & Flamme, 1995; Thompson et al., 1998; Zhong, Rosenberg, Mohideen, Weinstein, & Fishman, 2000). Following initial primitive plexus formation, new vessels that develop to support growth of the embryo are derived via a process called *angiogenesis* (Carmeliet & Collen, 2000; Childs, Chen, Garrity, & Fishman, 2002; Ferrara, 1999; Flamme et al., 1997; Gore et al., 2011; Isogai et al., 2003). Angiogenesis is classified into two types: (i) nonsprouting or intussusceptive, where transvascular pillars form within a vessel to subdivide the lumen into multiple smaller structures, and (ii) sprouting angiogenesis where endothelial cells in a vessel wall reactivate and "sprout," generating a new structure from the preexisting ones. Angiogenesis is essential to remodel the primitive vascular plexus formed via vasculogenesis into a mature network that grows in parallel with the overall embryo. Following establishment of an endothelial tubular network, vessels secrete factors that drive recruitment and differentiation of smooth muscle cells/or pericytes that promote vascular stability and quiescence (Ando et al., 2016; Santoro et al., 2009; Seiler et al., 2010; Stratman & Davis, 2012; Stratman et al., 2017; Wang et al., 2014; Whitesell et al., 2014). The generation of a detailed anatomical atlas of the developing zebrafish circulatory system had shown that the initial circuitry of vessels formed in fish is remarkably similar to that found in developing birds and mammals, making the zebrafish a reliable tool for a comparative study of vascular development (Isogai et al., 2001).

One of the earliest steps in the formation of the vasculature is the establishment of arterial versus venous identity. Originally, it was assumed that hemodynamic forces against the vessel wall drove arterial-venous (AV) specification; however, it has now been appreciated that genetic programs likely partially dictate endothelial AV identity prior to the onset of flow. EphrinB2 expressed by arteries and its receptor EphB4 expressed by veins, the Notch signaling pathway, and VEGF signaling have all been shown to be critical for the establishment of initial AV identity prior to or concurrent with initiation of circulatory flow (Abtahian et al., 2003; Adams et al., 1999; Eichmann et al., 2005; Gerety, Wang, Chen, & Anderson, 1999; Moyon, Pardanaud, Yuan, Breant, & Eichmann, 2001; Torres-Vazquez, Kamei, & Weinstein, 2003). Zebrafish studies have been central in demonstrating that initial AV identity is established early during embryogenesis, and for elucidating the molecular pathways regulating this (Ando et al., 2016; Geudens et al., 2010; Hen et al., 2015; Herbert et al., 2009; Herzog, Kalcheim, Kahane, Reshef, & Neufeld, 2001; Hogan et al., 2009; Isogai et al., 2001; Lawson et al., 2001; Lawson & Weinstein, 2002a; Santoro et al., 2009; Seiler et al., 2010; Swift et al., 2014; Wang et al., 2014; Weinstein & Lawson, 2002; Whitesell et al., 2014; Williams et al., 2010; Zhong et al., 2000).

At a molecular level, vascular development is coordinated by a number of different signaling inputs that are processed by the endothelial cells to guide vascular differentiation and patterning. The most well-studied and prominent among these is the vascular endothelial growth factor (VEGF) pathway. The VEGF ligand and its associated receptor VEGFR2 have been shown to be absolutely critical during both early vasculogenic and later developmental and adult angiogenic vessel formation processes for migration, permeability, proliferation, and survival of endothelial cells (Carmeliet & Collen, 2000; Carmeliet et al., 1996; Ferrara, 1999; Ferrara, Gerber, & LeCouter, 2003; Gerber et al., 1999). VEGF ligands are produced by a large variety of different cell types and tissues closely associated with the vasculature, including endothelial cells themselves, somites, macrophages, T-cells, smooth muscle cells, and pathologically, tumor cells (Jakeman, Armanini, Phillips, & Ferrara, 1993; Liang et al., 1998, 2001). The VEGFR2 receptor is expressed almost exclusively by endothelial cells and hematopoietic progenitor cells, thus establishing the specificity of the pathway. A number of mutants in the VEGF signaling pathway have been identified in the zebrafish, including mutants in the VEGFR2 receptor and VEGF ligands, and downstream signaling mediators and transcriptional regulators (Astin et al., 2014; Cermenati et al., 2013; Covassin et al., 2009; Covassin, Villefranc, Kacergis, Weinstein, & Lawson, 2006; Gore et al., 2011; Habeck et al., 2002; Hogan et al., 2009; Jin et al., 2007; Koltowska, Paterson, et al., 2015; Lawson, Mugford, Diamond, & Weinstein, 2003; Lawson, Vogel, & Weinstein, 2002; Lee et al., 2002; Liang et al., 2001; Liang et al., 1998; Pan et al., 2012; Pham et al., 2007). Ongoing studies have also implicated the critical nature of endocytic vesicle trafficking for VEGFR internalization, recycling, and signal activation (Lanahan et al., 2010; Nakayama et al., 2013; Pitulescu & Adams, 2014;

Simons, 2012). Although a significant amount of research in the vascular research field has focused on the VEGF signaling pathway, there is still much to uncover about additional pathways and VEGF signaling regulators that dictate and direct blood vessel morphogenesis. A major challenge currently facing the field is to understand how inputs from the many different signaling pathways implicated in vascular development are integrated within endothelial cells.

Lymphatic Vessel Development

Maintenance of fluid homeostasis and the dissemination and absorption of nutrients, macromolecules, and fatty acids relies on the lymphatic system, a distinct, blind-ended open endothelial network of vascular tubes often found in close proximity to blood vessels. This complementary system is also only found in vertebrates, and in recent years, lymphatic research has also benefited enormously from the use of the experimentally and genetically accessible zebrafish. The same features that make the fish a valuable model organism for studying blood vessels—rapid embryonic development, optical clarity, and the increasing availability of transgenic reporter lines—have made the zebrafish a powerful tool for studying lymphatic development (Alders et al., 2009; Cermenati et al., 2013; Jung et al., 2017; Koltowska, Lagendijk, et al., 2015; Okuda et al., 2012; Venero Galanternik et al., 2016; Yaniv et al., 2006). The shared anatomical, morphological, and developmental features of lymphatics in zebrafish and mammals were first highlighted only about a decade ago (Yaniv et al., 2006). Similarities between higher mammals and zebrafish lymphatics can be found at different levels. (1) The appearance of lymphedema in zebrafish with impaired lymphatics has been widely reported. In humans, lymphatic vessel impairment due to surgery, genetic disorders, or parasitic infections manifests as lymphedema, a severe fluid accumulation in tissues that makes affected patients more prone to infections, metastatic tumor invasion, and potential amputations. (2) Like mammals, zebrafish lymphatic endothelial cells emerge from preexisting veins, take up interstitial fluid, transport immune cells, and lack circulating erythrocytes (Jung et al., 2017; Kuchler et al., 2006; Venero Galanternik et al., 2016; Yaniv et al., 2006). (3) Moreover, it is known now that molecular genetic players such as *prox1*/Prox1, *vegfr3/vegfc*, *ang2*, *sox18*, *and nrp2* are all conserved between zebrafish and other vertebrates (Cermenati et al., 2013; Hermans et al., 2010; Koltowska, Lagendijk, et al., 2015; Kuchler et al., 2006; Nicenboim et al., 2015; Yaniv et al., 2006).

The initial steps of embryonic lymphangiogenesis have been characterized in the fish. Angioblasts located in the ventral side of the trunk posterior cardinal vein (PCV) acquire a lymphatic identity as they begin expressing Prox1. This is followed by asymmetric division where new lymphatic endothelial cells (LECs) exit the PCV and migrate dorsally to form the bilateral cords of lymphatic progenitor cells known as the parachordal lines (PACs) at the superficial trunk midline. Wnt5b from the endoderm induces LEC specification in the adjacent PCV (Nicenboim et al., 2015). Zebrafish mutants for *prox1a/prox1b* or *vegfc/vegfr3* are defective lymphatic vessel development, confirming the conserved role of these genes. In addition, *sox32/Casanova* mutants, which lack endoderm-derived tissues, show a considerable reduction in PACs (Koltowska, Lagendijk, et al., 2015; Koltowska, Paterson, et al., 2015; Le Guen et al., 2014; Nicenboim et al., 2015). Once the PAC is established, PAC lymphatic progenitors migrate dorsally to form the dorsal longitudinal lymphatic vessel and ventrally to assemble the main embryonic trunk lymphatic vessel of the developing zebrafish, the thoracic duct, right between the dorsal aorta and the PCV (Yaniv et al., 2006). The thoracic duct and other lymphatic vessels in the embryo share morphological features of lymphatic vessels noted in other vertebrates, such as a thin-walled endothelium with overlapping cells, reduced tight junctions, and the presence of anchoring filaments (Yaniv et al., 2006).

The thoracic duct and other earlier-developing lymphatic vessels have been studied in the most detail, with relatively less known about later-forming lymphatic vessels. However, the recent generation of new specific transgenic reporter lines has made it possible to study the assembly of juvenile and adult fish lymphatics in detail (Fig. 14.3) (Jung et al., 2017; Okuda et al., 2012). The facial lymphatic network (FL) forms rostrally, comprised of the lateral and medial facial lymphatic, the otolithic lymphatic vessel, and the four branchial arch lymphatics form from sprouting LECs coming from the common cardinal vein and extends to the more anterior primary head sinus, branching toward the eyes, the otic vesicle, and the jaw (Jung et al., 2017; Okuda et al., 2012; Yaniv et al., 2006). More caudally, additional later-developing trunk lymphatic structures that include the collateral cardinal lymphatics, superficial lateral lymphatics, superficial intersegmental lymphatics, and spinal lymphatics (Fig. 14.3B and F) (Jung et al., 2017). A recent report from our laboratory also documented that zebrafish lymphatics mediate the transport of immune cells at later stages, further confirming the important functional parallels between fish and human lymphatics, and the usefulness of this comparative model (Jung et al., 2017).

Lymphatic Perivascular Cells

The classical notion that lymphatic vessels are excluded from the central nervous system was challenged in 2015 when two separate studies in mice and humans described a set of lymphatic vessels aligning with the dural sinuses in the brain meninges (Aspelund et al., 2015; Louveau et al., 2015). Shortly after, reports of a novel perivascular lymphatic endothelial-related cell type were described on the zebrafish brain (Bower et al., 2017; Venero Galanternik et al., 2017). Specific lymphovenous transgenic lines revealed that the optic tectum, cerebellum, olfactory bulbs, and the dorsal side of the telencephalon of the zebrafish brain are covered by a perivascular cell population that aligns to the meningeal vessels (Fig. 14.4). These cells share some key features of lymphatic endothelial cells: (1) they express lymphatic markers, such as Lyve1, Mrc1a, and Prox1. (2) They sprout from the Choroidal Vascular Plexus (CVP), a venous plexus located right behind the eye inside the brain, (3) their specification depends on *vegfc/vegfr3/ccbe1* signaling and (4) they have endocytic properties, taking up macromolecules like lipids and toxic waste that likely leaks from adjacent blood vessels into the environment (Bower et al., 2017; Venero Galanternik et al., 2017). However, unlike lymphatic vessels, these perivascular cells do not form vascular tubes, nor do they drain the lymph back into the blood circulation. Furthermore, their morphology, location, function, and characteristic internal autofluorescent vesicles resemble a specialized type of cell in mammals known as *Fluorescent Granular Perithelial cells* (Fig. 14.4) (FGPs, aka "Mato Cells") (Mato & Mato, 1983; Mato, Ookawara, Aikawa, & Kawasaki, 1981; Mato, Ookawara, Sano, & Fukuda, 1982; Mato, Ookawara, Sano, Fukuda, & Sokabe, 1983; Mato, Ookawara, Sano, & Kurihara, 1982; Mato, Ookawara, Sugamata, & Aikawa, 1984; Venero Galanternik et al., 2017). Although the precise function of these perivascular lymphatic cells is still under debate, they may be important for proper angiogenesis of the brain vasculature, maintenance of the brain-blood barrier, toxic waste clearance from the brain, and act as potential viral reservoirs, which could have important repercussions in the development of neurodegenerative conditions, such as Alzheimer's and virus-dependent infectious diseases like AIDS (Bower et al., 2017; Filipowicz et al., 2016; Mato et al., 1981; Venero Galanternik et al., 2017).

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CHAPTER

15

Development of The Zebrafish Pronephric and Mesonephric Kidneys

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Introduction

The kidney is essential for filtering blood, expelling waste, and maintaining fluid homeostasis. These functions are performed by nephrons, which comprise tubules attached to glomeruli (blood filters) that integrate with the vasculature. Waste is excreted from the nephron into a duct system prior to leaving the body as urine. The filtrate generated at the glomerulus gets modified as it passes along different tubule segments via the reabsorption and secretion of solutes (Naylor, Qubisi, & Davidson, 2017).

Mammals form three kidney structures progressively during the embryonic development: the pronephros, mesonephros, and metanephros. The first two kidneys provide little to no renal function and degenerate, whereas the metanephros persists as the permanent adult kidney (Dressler, 2006).

In zebrafish, the pronephros is fully functional and is essential to sustain embryonic and larval life. It comprises a glomerulus and two tubules that are divided into proximal and distal segments, with each segment carrying out specific tasks that can be distinguished by the expression of specific cohorts of solute transporters. Importantly, there is considerable structural and genetic conservation between the zebrafish pronephros and mammalian nephrons, making it a useful model to study the human kidney (Naylor, Qubisi, et al., 2017).

A postembryonic metamorphosis occurs in zebrafish at around 2 weeks of age and is characterized by the gain, loss, and reorganization of many tissues (Parichy, Elizondo, Mills, Gordon, & Engeszer, 2009). The mesonephros is also formed during this phase, presumably due to increased body mass, causing a higher demand on the renal function that cannot be met by the pronephros. This mesonephros persists as the permanent adult kidney in fish, and no metanephros is formed.

Unlike mammals, where nephrogenesis ceases around birth when kidney progenitor cells become exhausted, nephrogenesis in zebrafish is continuous throughout the adult life (Zhou, Boucher, Bollig, Englert, & Hildebrandt, 2010). As fish grow, their kidneys also increase in size by the addition of new nephrons. In response to kidney injury from nephrotoxins, such as gentamicin, the rate of de novo mesonephrogenesis increases as part of a regenerative response. In addition, the damaged nephrons are also capable of undergoing self-repair, a process that appears similar to mammalian nephron repair (Johnson, Holzemer, & Wingert, 2011). With features that are unique and in common with the mammalian kidney, the zebrafish kidney serves as an alternative model for understanding kidney development, disease, and repair (Jerman & Sun, 2017).

Structure of The Pronephros and Mesonephros

The pronephros comes from the mesoderm germ layer that is created during gastrulation. The mesoderm becomes divided into the paraxial mesoderm, lateral plate mesoderm, and intermediate mesoderm (IM). The IM, located between the other mesoderm layers, is the source of the pronephros. At the end of gastrulation, the IM appears as bilateral stripes on either side of the embryonic trunk and proliferates during early somitogenesis stages (10–16 h post fertilization; hpf) in response to Wnt8a, a member of the Wnt family of secreted factors. This early wave of growth expands

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the pool of pronephric progenitors prior to their differentiation into renal epithelial cells (Naylor, Han, Hukriede, & Davidson, 2017).

At around 48 hpf, the pronephros becomes functional (albeit not yet fully mature) and comprises bilateral nephrons with fused glomeruli in the anterior trunk and tubules that run to the cloaca at the posterior. The glomeruli (G) are connected to the tubules via a short neck segment containing motile cilia. The tubules are divided into four functionally distinct segments: proximal convoluted tubule (PCT), proximal straight tubule (PST), distal early tubule (DE), and distal late tubule (DL) (Fig. 15.1). Recent data suggest that the DL segment serves a dual tubule/duct function (Naylor, Qubisi, et al., 2017) and while the posterior terminus of the DL segment has been considered to be solely ductal in nature (Wingert et al., 2007), further work is needed to clarify the identity of this region.

The pronephros likely gets overwhelmed in larger juveniles with a higher body mass. For coping with this, the mesonephros is formed by the addition of new nephrons onto the existing pronephros. Mesonephrogenesis during this larva-to-juvenile transition occurs during metamorphosis at around 14 days post fertilization (dpf) (Diep et al., 2011; Zhou et al., 2010). The first mesonephric nephron appears at the ~5.2 mm total length stage (13–14 dpf), based on detection of *cdh17* and *podocin* transcripts. It is located at the level of the sixth somite on top of the distal portion of the pronephric DE segment (Fig. 15.2A). The second and third mesonephric nephrons form level with the seventh and eighth somites, respectively. This stereotypical pattern is short-lived, and from the 6.5 mm stage onwards nephrons appear randomly near the most proximal portion of the DE segment in the position of the future head kidney and on top of both the DE and DL segments (Fig. 15.2B). By 9 mm, the nascent mesonephros morphologically resembles the adult kidney and has the head, trunk, and tail regions (Fig. 15.3) (Diep et al., 2011, 2015; Zhou et al., 2010). Successive waves of nephrogenesis result in a complex network of interconnected nephrons that plumb into pronephric and mesonephric DE and DL segments. It is hypothesized that the pronephric DE and DL segments eventually transform into the two major collecting ducts that run down the midline of the adult mesonephros (Fig. 15.2C) (Diep et al., 2015).

Formation of The Pronephros

Early pronephric progenitors can be subdivided into rostral and caudal populations based on the expression of Notch receptor ligands (*deltaC* and *jagged2a*) and the *mecom* transcription factor gene, respectively (Li, Cheng, Verdun, & Wingert, 2014). The rostral domain corresponds to podocyte, neck, PCT, PST, and DE progenitors, while the caudal domain corresponds to DL progenitors (Naylor, Dodd, & Davidson, 2016). The relative sizes of the rostral and caudal domains are established by retinoic acid (RA) signaling during gastrulation and early somitogenesis stages (Naylor, Skvarca, et al., 2016; Wingert et al., 2007). RA signaling promotes rostral gene expression but is believed to be degraded in more caudal portions of the IM by the Cyp26a1 enzyme (Naylor, Skvarca, et al., 2016; Wingert et al., 2007).

The effect of RA likely occurs through the expression of downstream transcription factors. In the most anterior IM (but also in the surrounding mesoderm), RA appears to directly induce the expression of the *Wilms' tumor suppressor-1a* (*wt1a*) transcription factor gene. *Wt1a* plays a central role in directing IM cells to differentiate into podocytes, which are key filtration cells in the glomerulus (Tomar, Mudumana, Pathak, Hukriede, & Drummond, 2014). The Notch ligands *jagged1b* and *jagged2b* are also dependent on RA signaling for their early expression in the anterior IM and have been implicated in podocyte formation (O'Brien et al., 2011).

The partially redundant transcription factor genes *pax2a* and *pax8* are the earliest markers of the IM and are expressed along its entire length, without

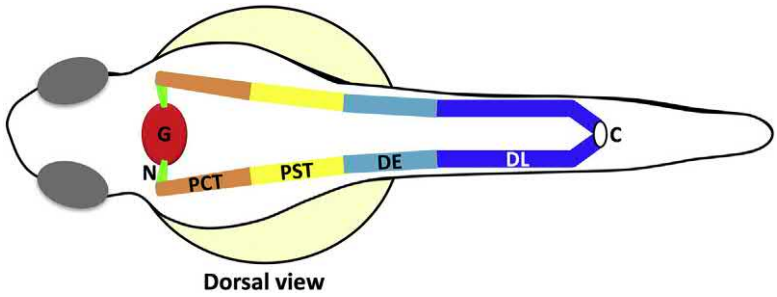

FIGURE 15.1 **Pronephros structure.** The pronephros at 48 hpf comprises a central fused glomerulus, and this is connected to the bilateral tubules by short neck segments. The tubule is divided into four functionally distinct segments that express specific solute transporters. (G, glomerulus; N, neck segment; PCT, proximal convoluted tubule; PST, proximal straight tubule; DE, distal early tubule; DL, distal late tubule; C, cloaca).

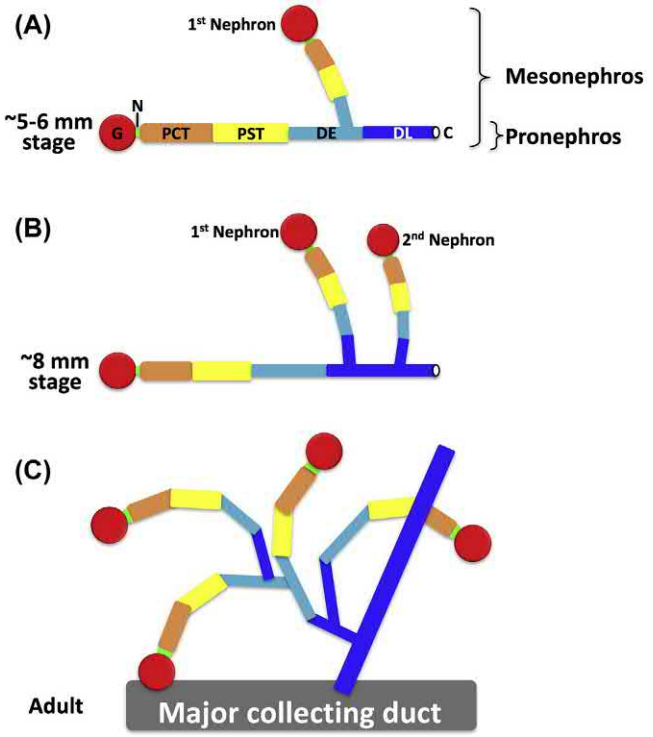

FIGURE 15.2 **Mesonephros structure.** (A) The first mesonephric nephron appears in ~5.2 mm larvae at the distal portion of the pronephric DE segment. (B) At the ~8mm stage, more mesonephric nephrons are present on top of the pronephric DE (not shown) and DL segments. (C) The adult mesonephros has an interconnected network of nephrons, where branching occurs at the DE and DL segments and waste is drained into the bilateral major collecting ducts.

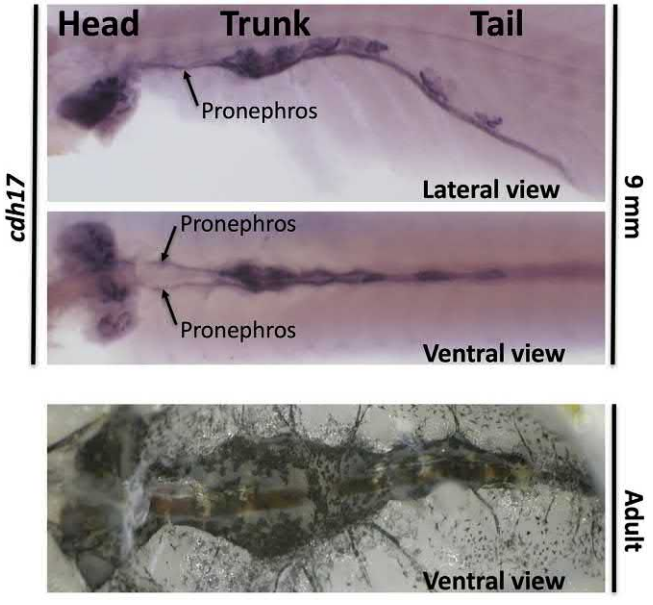

FIGURE 15.3 **Mesonephros morphology.** The nascent mesonephros at 9 mm morphologically resembles the adult mesonephros, where both consist of a head, trunk, and tail region. The pronephros (bilateral tubules running the length of the trunk) serves as a scaffold for the addition of mesonephric nephrons. The top and middle images (of the same animal) are from in-situ hybridization with probes for *cdh17*. The bottom image is of an adult kidney inside the body cavity.

dependency on RA signaling. Double knockdown of *pax2a* and *pax8* has revealed their requirement for tubule differentiation and as likely upstream inducers of the *hnf1ba/b* paralogs (herein referred to as *hnf1b*) encoding homeobox-containing transcription factors (Naylor, Przepiorski, Ren, Yu, & Davidson, 2013). Hnf1b is essential for pronephric tubule maturation into the four segments and the activation of segment-specific marker genes, such as the solute transporter genes *slc20a1a* (PCT), *trpm7* (PST), *slc12a1* (DE), and *slc12a3* (DL) (Naylor et al., 2013) (Fig. 15.1). How different segment identities are induced downstream of the Pax2/8 → Hnf1b pathway remains uncertain but additional transcription factors, such as Sim1 (PST), Irx3b (DE), and Mecom (DL) may contribute to specific segmental fates and/or their proliferation (Cheng & Wingert, 2015; Li et al., 2014; Wingert & Davidson, 2011).

There is also an intrasegmental diversification in cell phenotype with some cells in the PCT, PST, and DE segments developing bundles of motile cilia that aid in the movement of the filtrate along the pronephros to the cloaca. These multiciliated cells are present in a "salt and pepper" pattern and are thought to be regulated by the Notch pathway, via a classic lateral inhibition mechanism, and the Etv4/Etv5a transcription factors (Marra & Wingert, 2016).

During tubulogenesis, the IM undergoes a mesenchymal-to-epithelial transition beginning around the 12–14 somite stage (~14 hpf) and forms a lumenized tubule by the 20 somite stage (21 hpf). There is also evidence for tight junctions between the tubular epithelial cells. Based on the expression of *claudin* genes (encoding components of the tight junction that control the paracellular flow of molecules) it can be inferred that the PCT and PST segments are more "leaky" than the DE and DL segments, similar to what is found in the mammalian nephron (McKee, Gerlach, Jou, Cheng, & Wingert, 2014; Naylor & Davidson, 2014).

The tubule cells also contain adherens junctions that maintain cell-to-cell adhesion via homotypic interactions between cadherins. The cadherin genes of importance in the zebrafish pronephros include *cadherin-17*, which is expressed in all tubule segments and its knockdown results in a general loss in tubule cell adhesion (Horsfield et al., 2002), *cadherin-6* in podocyte and neck precursors (Kubota, Murakami, Mogi, & Yorifuji, 2007), and *cadherin-1* (E-cadherin) in the DL segment (Naylor et al., 2013).

Around the time that tubule epithelialization is occurring (12–20 somite stage), and cells become adhered to one another, the tubular cells also undergo dynamic morphological changes. Current evidence indicates that the DL segment compacts as it migrates and fuses with the cloaca, leading to a pulling and stretching of the PST and DE segments (Naylor, Dodd, et al., 2016). Thus, as tubulogenesis proceeds, the rostral domain

(comprising PCT, PST, and DE progenitors) becomes progressively elongated while the caudal domain (DL progenitors) shortens. The tension applied to the DE segment, and possibly also the PST segment, during this process is believed to drive stretch-induced proliferation and contribute to a second (post-Wnt8a) wave of tubule growth (Naylor, Han, et al., 2017).

Starting around the 18 somite stage and progressing to 48 hpf, the podocyte precursors migrate to the midline, fuse, and recruit a blood supply from the overlying dorsal aorta (Drummond et al., 1998). While filtration starts around this time, the glomerular filter is not fully mature until 96 hpf when it shows size-selectivity and fully differentiated podocytes (Kramer-Zucker, Wiessner, Jensen, & Drummond, 2005).

Once flow initiates in the tubule, a collective cell migration process initiates in the PCT, resulting in its compaction and convolution (Vasilyev et al., 2009). The proximal compaction here exerts a new mechanical tension on the tubule but this time in the opposite direction to that seen at the earlier stage. This leads to the stretching and elongation of the more distal segments (particularly the DE) and stretch-induced proliferation (comprising the third wave of tubule growth) (Naylor, Dodd, et al., 2016). As a result, the DE segment increases significantly in length until the 5 mm stage at which point it reaches its maximal length and extends over the swim bladder just caudal of the glomerulus (Diep et al., 2015).

Formation of The Mesonephros

Mesonephrogenesis is initiated when a few single mesenchymal progenitor cells coalesce into a basophilic cluster. The progenitors are labeled in the *Tg(lhx1a:EGFP)* transgenic line. Single *lhx1a:EGFP*$^{+}$ cells are seen attached to the top of the pronephric DE segment at the ~4 mm (~10 dpf) stage. More progenitors appear later along the length of the pronephric tubules together with *lhx1a:EGFP*$^{+}$ clusters. Single progenitors have filopodia-like projections that may be involved in their migration toward each other and to distant sites of nephrogenesis.

lhx1a:EGFP^{+} clusters express nephron progenitor markers (*wt1b*, *pax2a*, *lhx1a*) and proliferate. They epithelialize into "renal vesicles" and then elongate into nascent nephrons with restricted marker gene expression. For instance, transcripts for *wt1b* (a paralog of *wt1a*) are detected in the future glomerulus while *lhx1a* and *pax8* are expressed in more distal nephron regions. In the mammalian metanephros, Wnt9b induces nephrogenesis (Carroll, Park, Hayashi, Majumdar, & McMahon, 2005) and it is interesting to note that *wnt9a* is expressed in the pronephric distal tubules after 4 dpf, making it a potential nephron-inducing candidate (Curtin, Hickey, Kamel, Davidson, & Liao, 2011). Indeed, recent findings show that *wnt9a/b* are expressed in the distal tubules and collecting ducts during regeneration and that the canonical Wnt signaling pathway regulates the proliferation of nephron progenitor cells and morphogenesis of nascent nephrons (Kamei, Gallegos, Liu, Hukriede, & Drummond, 2019). Further research is needed to determine whether similar events occur during mesonephros development.

The first mesonephric nephron becomes functional at the ~6 mm stage and initially comprises a nephron with a glomerulus-neck-PCT-PST-DE structure. It becomes fused to the pronephric DE segment, resulting in a DE-to-DE junction (Fig. 15.2A). However, at later stages, this nascent mesonephric nephron acquires a DL segment that expresses *slc12a3*, and a DL-to-DL junction is seen with the pronephros (Fig. 15.2B). Whether this phenomenon is the result of transdifferentiation events occurring in these mesonephric and pronephric segments is unclear and requires more investigation.

By the ~8 mm stage, numerous mesonephric nephrons are formed with their DL segments fusing to the pronephric DL segment, and thus, draining into a common pronephric tubule (Fig. 15.2B). Eventually, the pronephric glomerulus is lost, supporting the idea that the pronephros eventually loses kidney function to become a scaffold for the mesonephros. In this hypothesis, the bilateral pronephric tubules become the two major collecting ducts where all the mesonephric nephrons drain their waste. The first wave of mesonephrogenesis provides primary nephrons that fuse directly onto the pronephric DE and DL segments (Fig. 15.2B). Later waves of nephrogenesis produce nephrons that fuse onto primary nephrons, resulting in branching predominately at the DL segment, as revealed by the expression of the *slc12a3* (DL) in adult kidneys, but in some cases, fusion also occurs with the DE segment (Diep et al., 2011). Overall, common genes (*lhx1a*, *pax2a*, *wt1b*) are associated with the development of both pronephros and mesonephros, and the nephrons in both kidneys share a conserved segmentation pattern (Figs. 15.1 and 15.2).

Mesonephrogenesis continues during juvenile life at a linear rate with respect to body mass or length (Diep et al., 2011; Zhou et al., 2010). In adults, nephrogenesis is decreased to a basal rate with approximately two renal vesicles (*lhx1a*$^{+}$, *wt1b*$^{+}$) in a normal kidney at any one time, and there are approximately 300 total nephrons

in the mesonephros of 1-year old fish (Diep et al., 2011; Zhou et al., 2010).

The rate of nephrogenesis is increased in response to acute kidney injury, such as that induced by intraperitoneal injection of the nephrotoxin gentamicin. Within 8 h, all nephrons lose expression of the PCT marker *slc20a1a* and cannot absorb filtered dextran. Around 4–6 days later, some damaged nephrons reexpress *slc20a1a* and begin reabsorbing dextran again, consistent with the damaged nephrons undergoing self-repair, a feature shared with mammals (Diep et al., 2011). Notable at this time is the appearance of numerous nascent nephrons. These new nephrons are presumed to have arisen from basophilic clusters that were either pre-existing, perhaps in a "poised" state, or induced to form from single nephron progenitors. By four to 6 weeks after injury, the kidney is largely repaired and repopulated with nephrons. These are assumed to be a mix of repaired nephrons and newly generated nephrons. However, it is challenging to assess the proportion of nephrons that have repaired from those arising de novo.

New nephrons successfully integrate with the vasculature and the existing tubule/collecting duct network. They are capable of absorbing filtered dextran, and histological sections confirm that new basophilic tubules fuse with mature existing nephrons to share a common lumen (Fig. 15.4). The fusion of new nephrons to an existing kidney architecture is an essential process when considering future cell-based therapies that seek to replace nephrons in damaged human kidneys. In this respect, larval mesonephrogenesis may be a good model for furthering our understanding of the steps involved.

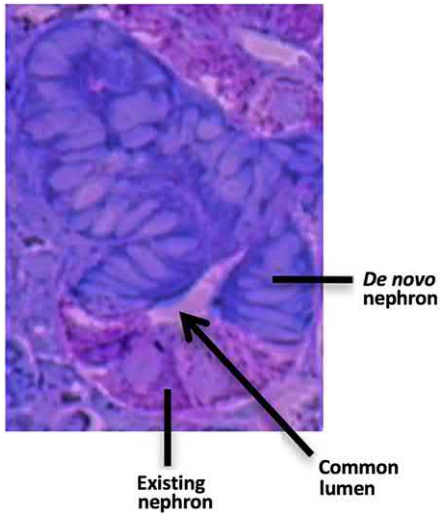

FIGURE 15.4 **Integration of new and existing nephrons.** De novo nephrons fuse with existing nephrons to share a common lumen.

Concluding Remarks

The zebrafish has been an important model for understanding kidney development, regeneration, and disease. The pronephros is simple with two mostly linear nephrons, making it ideal for studying the developmental processes that govern kidney formation such as specification from mesoderm, progenitor proliferation, the transcription factor networks that drive differentiation, and the morphological changes, such as collective cell migration and compaction that influence the final form and function of the kidney. The mesonephros is emerging as an attractive model for understanding how transplanted nephron progenitors can migrate and self-organize into nephrons, and how new nephrons integrate into an existing kidney—processes that are at the heart of future regenerative therapies in humans.

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CHAPTER

16

The Reproductive System

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Introduction

One of the main advantages of zebrafish as a model organism is that they are asynchronous breeders, and thus, capable of spawning year-round. As such, the ovaries and testes of adults contain germ cells that are at all stages of oogenesis and spermatogenesis, respectively. This feature makes the zebrafish an ideal model for the study of gonad development and function. Gonads (i.e., ovaries and testes) are composed of both germ cells and specialized somatic gonad cells. The germ cells are derived from the embryonic primordial germ cells (Yoon et al., 1997). It is likely that the somatic gonad cells originate from the coelomic epithelium, a derivative of the lateral plate mesoderm, as this is their origin in mice and medaka (Karl & Capel, 1998; Nakamura et al., 2006), although this remains to be determined for zebrafish. In this chapter, we will review what is known about the developmental origin and structure of zebrafish gonads and how reproductive functions are regulated by hormones produced by the hypothalamus-pituitary-gonad axis.

Primordial Germ Cell Specification and Migration (0–24 h Postfertilization, hpf)

Primordial germ cells (PCG) in zebrafish are the first cell type to be set aside in the embryo and are specified, and then maintained, by gene products encoded by maternally supplied mRNAs that are asymmetrically localized during early embryonic cell divisions (Hashimoto et al., 2004; Koprunner et al., 2001; Yoon et al., 1997). The first several cell divisions of the zebrafish zygote are incomplete divisions that do not bisect the large yolk cell. Several maternally supplied mRNA's that are involved in PGC specification or maintenance [e.g., *DEAD box helicase 4*, *nanos3* (formerly called *nanos1)*, *dead end*] become initially localized to the lateral apexes of the cleavage furrows during the first 2 cell divisions, which results in two and four foci of localized mRNA at the 2- and 4-cell stages, respectively (Draper et al., 2007; Gross-Thebing et al., 2017; Koprunner et al., 2001; Weidinger et al., 2003; Yoon et al., 1997). Localization of these mRNAs appears to require the maternally supplied kinesin Kif5Ba and the germ granule protein Buckyball (Campbell et al., 2015). Between the 4- and 32-cell stage, only four PGC mRNA foci are present. At the 32-cell stage, the PCG mRNAs become localized within four individual cells(Yoon et al., 1997). From the 32- to the 1000-cell stage, the PGC mRNA's continue to be localized to only 4 cells, indicating that they are asymmetrically localized to only one of the daughter cells during each division (Knaut et al., 2000). Beginning around the 1000-cell stage (~3 hpf), the PCG mRNA's become evenly distributed between the daughter cells and therefor PGC numbers begin to increase (Knaut et al., 2000). At the beginning of gastrulation (shield stage, 6 hpf), there are four clusters of ~4 cells each that are evenly arranged around the margin of the embryo. Shortly after gastrulation initiates, PGCs begin to display migratory behavior that will eventually result in their migration to the site where the future gonad forms (Raz & Reichman-Fried, 2006; Reichman-Fried et al., 2004). This migration is regulated by the cytokine ligand Cxcl12a (formerly called Sdf1a), which is expressed dynamically in various tissues along the migratory route, and the PCG-expressed cytokine receptor Cxcr4b (Doitsidou et al., 2002; Knaut et al., 2003). Migration initiates toward the beginning of gastrulation and is complete by 24 hpf,

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when there is an average of 30 PGCs per embryo arranged in two bilateral clusters located lateral to somite 5 (Koprunner et al., 2001; Raz, 2003; Ye et al., 2019; Yoon et al., 1997). At this stage, the PGCs appear to enter a quiescent phase as their numbers do not further increase until around 7–8 days postfertilization, dpf (Leerberg et al., 2017).

The Larval Bipotential/Undifferentiated Gonad (5–25 Dpf)

The primordial germ cells have reached the location, where the gonad will eventually form by 24 hpf (Braat et al., 1999; Yoon et al., 1997), but it is not known with certainty when the first cells of the somatic gonad are produced. Histological analysis has identified somatic cells associated with germ cells as early as 5 dpf (Braat et al., 1999). By 10 dpf, the early somatic gonad has organized into a discrete two-layered structure, where an outer epithelial layer surrounds an inner mesenchymal core (Leerberg et al., 2017). The mesenchymal cells, in turn, surround the developing germ cells and will give rise to the germ cell-supporting and steroid hormone-producing cell lineages of the gonad (see below). At this point in development, the gonads are located in a position that is ventrolateral to the posterior end of the swim bladder, and dorsal to the posterior end of the intestine. The location of the gonad is most easily determined using transgenic reporter lines that express a fluorescent protein in all germ cells [e.g., Tg(*ddx4:egfp*) and Tg(*piwil1:egfp*) (Krovel & Olsen, 2004; Leu & Draper, 2010; Ye et al., 2019)].

The mechanism of sex determination in zebrafish is not well understood, but it is clear that it initiates in the gonad, which then produces the sex appropriate hormones that regulate the secondary sexual phenotypes and behaviors of adults (Kossack & Draper, 2019; Liew & Orban, 2014). Based on gene expression analysis, the zebrafish gonad is likely bipotential prior to 18–20 dpf. For example, while the genes encoding the anti-Müllerian hormone, *Amh*, and the aromatase, *Cyp19a1a*, will eventually be expressed predominantly in testes and ovaries, respectively, and cells expressing *amh* and *cyp19a1a* can be detected in all larval gonads during the bipotential stage (Leerberg et al., 2017; Rodriguez-Mari et al., 2005). This indicates that prior to 18–20 dpf, zebrafish larvae appear undetermined. Interestingly, differences in germ cell abundance as early as 1 dpf have been correlated with eventual sexual fate: more germ cells correlate with female development and less with male (Tzung et al., 2015; Ye et al., 2019). However, at what stage germ cell abundance might influence sex determination is not known.

Zebrafish are classified as undifferentiated gonochoristic because the gonads of all larvae, regardless of their eventual sex, initially pass through an ovary-like stage and produce some early-stage oocytes (Yamamoto, 1969). The first wave of germ cells entering meiosis in zebrafish can be detected as early as 13 dpf, during the early part of the bipotential gonad phase. In animals that will become females, the early oocytes continue to mature as the animal transitions from larval to juvenile females (~20–25 dpf). In animals fated to become males, the gonad goes through a transient intersex phase during which the oocytes do not progress past the early diplotene stage and instead begin to undergo apoptosis around 18–20 dpf (Uchida et al., 2002). In transitioning males, the majority of oocytes are cleared from the gonads by 30 dpf (Uchida et al., 2002). After this point, the remaining premeiotic germ cells begin to initiate a spermatogenic program. Because germ cell number is correlated with the eventual female versus male sex determination, with the appropriate germline-expressed transgene (e.g., *ddx4:egfp* or *piwil1:egfp*), gonad size at 20–30 dpf can be a reliable predictor of male versus female sex before overt morphological differences between males and females, such as sex-specific pigmentation or behavior, are evident (Dranow et al., 2016; Krovel & Olsen, 2004; Wang & Orban, 2007).

Sex Determination and Differentiation (20–30 Dpf)

The domesticated zebrafish lines that are most commonly used in the laboratory (e.g., AB and Tu) do not have sex chromosomes, but instead utilize a polygenic sex determination mechanism that varies between strains (Anderson et al., 2012; Bradley et al., 2011; Howe et al., 2013; Liew et al., 2012). By contrast, zebrafish that have been isolated more recently from the wild were found to use a ZZ/ZW chromosomal sex-determining system, where the heterogametic sex (i.e., ZW) was female (Wilson et al., 2014). The W-associated sex determinant has been mapped to the end of the long arm of Chromosome 4, but the gene encoding the determinant has yet to be identified.

Regardless of the genetic mechanisms that regulate zebrafish sexual development, germ cells have been shown to be necessary for female sex determination and for maintenance of the adult female sexual phenotype. If PGCs are either eliminated or their numbers greatly reduced, all animals develop as phenotypic males (Siegfried & Nusslein-Volhard, 2008; Slanchev et al., 2005; Tzung et al., 2015). Moreover, mutations that affect only the ability of animals to produce oocytes during the bipotential stage, but do not affect overall PGC numbers, result also in all-male development

(Rodriguez-Mari et al., 2010; Shive et al., 2010). These studies have led to the model that the oocytes formed during larval stages produce an unknown factor that acts on the somatic gonad to promote and/or stabilize female-specific gene expression, and thus, female sex determination. If a threshold amount of this signal is required for female development, then only those animals that produce the requisite number of oocytes during the bipotential phase can become female, and all others differentiate as males. Although it is not known how oocyte production during this period is regulated, there is a strong correlation between the numbers of PGCs present in 1 dpf animals and the eventual sex of the animal (Tzung et al., 2015; Ye et al., 2019). Thus, PGC survival and/or proliferation during embryogenesis may be a key determinant of sex in domesticated zebrafish. Because equivalent studies have not been performed with zebrafish lines more recently derived from wild populations, the role of germ cells in wild zebrafish sex determination remains to be determined.

The Adult Gonads

Ovary: The ovaries of zebrafish are classified as a cystovarian type because mature oocytes are ovulated into an ovarian cavity that forms from epithelial cells on the dorsal side of the ovary. Ovarian cavity formation becomes evident around 17–24 dpf (Takahashi, 1977). At the posterior of the ovary, the ovarian cavity transitions to the oviduct, which then connects to the genital papilla.

The zebrafish ovary is composed of developing follicles embedded in a vascularized stroma of interstitial tissue all surrounded by the ovarian epithelium. The major functional somatic cell types of the ovary are the granulosa cells, which are in direct contact with the oocytes and the steroid producing theca cells. The major structural cell types are the interstitial stromal cells and the ovarian epithelial cells.

At 30–40 dpf zebrafish ovaries are relatively flat ovoid-shaped structures that are elongated along the anterior-posterior axis. By adult stages (>90 dpf) the ovary has increased greatly in size due to the increase in oocyte numbers and to the increase in oocyte volume as they progress through oogenesis (see below). In adults, the ovary fills the space bounded on its lateral side by the body wall, and medially by the swim bladder and intestine (Fig 16.1A,B).

Stages of Oogenesis

Zebrafish spawn year-round and are capable of producing new oocytes throughout their life span. Thus, their ovaries contain all stages of germ cells, from premeiotic oogonial stem cells to fully mature eggs (Fig. 16.2A). The basic characteristics of each stage are detailed below:

Premeiotic Germ Cells

Oogonial stem cells (OSC): OSCs are self-renewing single cells, measuring between 7 and 10 μm in diameter, and have a large nucleus to cytoplasmic ratio (Fig. 16.2A,B). In addition, the nuclei of OSC containing a single, large, and centrally localized nucleolus

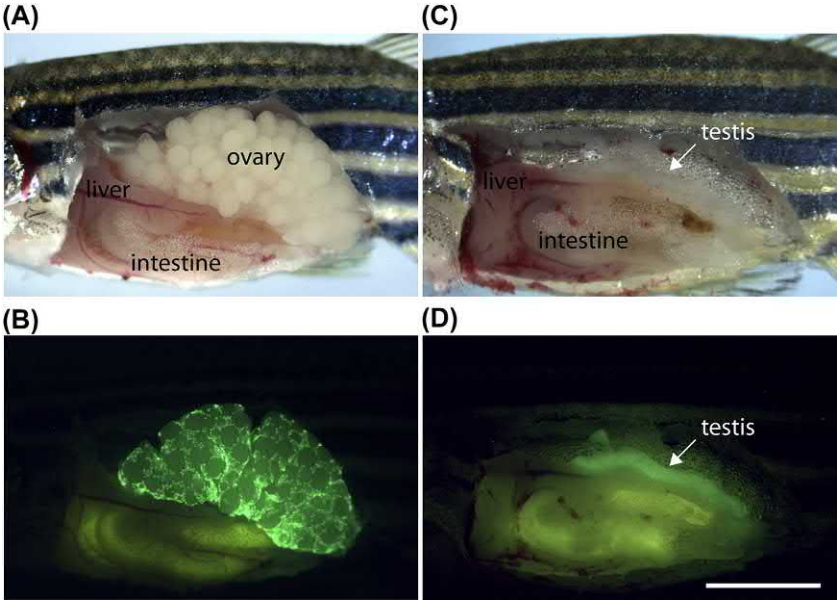

FIGURE 16.1 Location of ovaries and testes in adult zebrafish. The body wall has been removed to reveal the locations of the ovary (A and B) or testis (C and D) in adult Tg(*piwil1:egfp*) zebrafish, as visualized by either light (A and C) or fluorescent microscopy (B and D). The *piwil1* promoter drives GFP expression in all germ cells. The fish are oriented anterior to the left, dorsal up. Scale bar in (D), for (A–D), 5 mm.

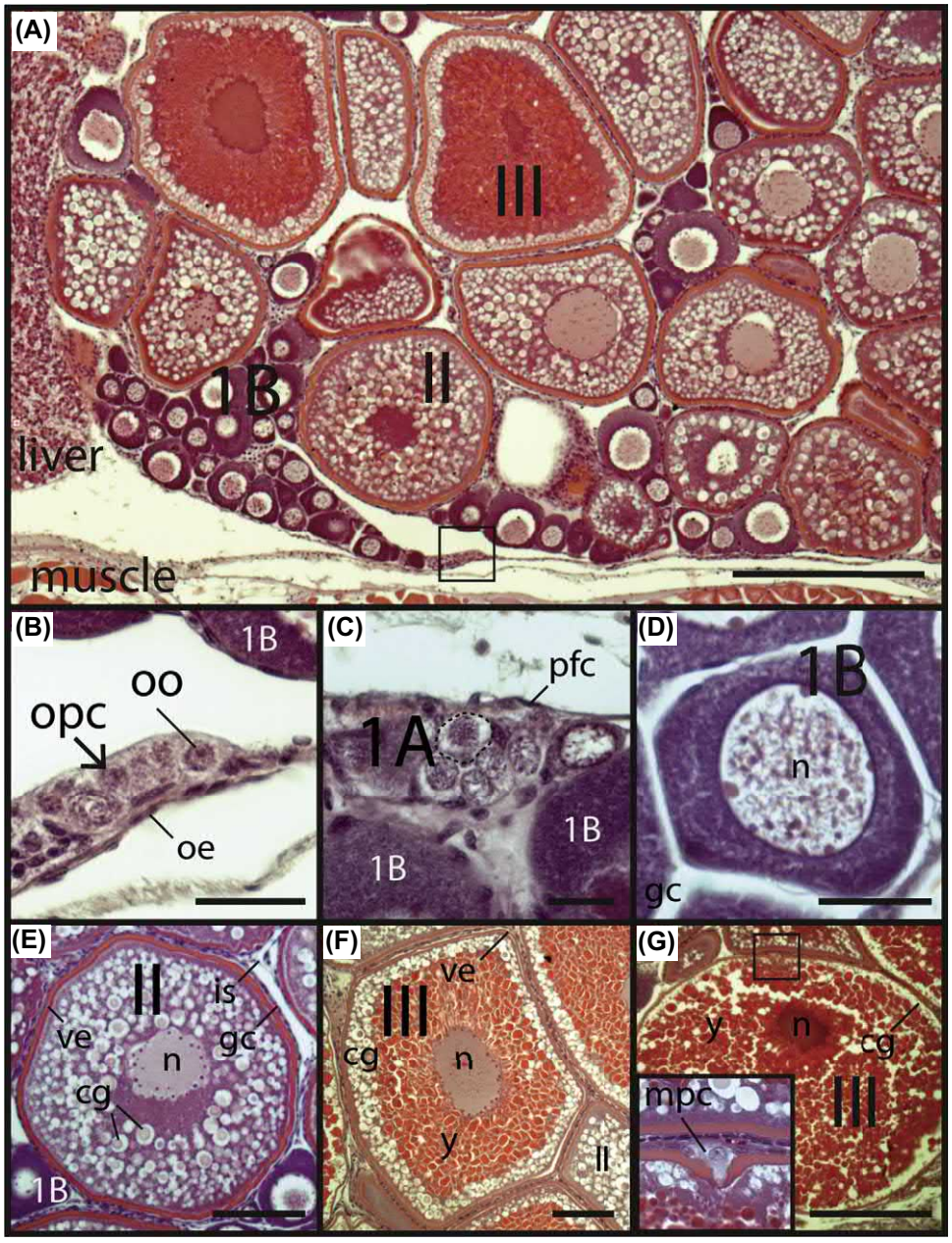

FIGURE 16.2 Representative histological images of zebrafish early germ cells and oocytes present in an ovary (60 dpf). Orientation in (A), anterior to left, medial up. (A) Low magnification image of a horizontal section through the left juvenile ovary shows oocytes at multiple stages of development. (B) High magnification view of area boxed in (A) showing premeiotic oogonia in the germinal zone. (C) A cyst of Stage 1A oocytes surrounded by a layer of prefollicle cells. Dotted outline defines one oocyte. Note that individual chromosomes can be identified in the nucleus. (D) Stage IB oocytes have a large nucleus to cytoplasm ration and their cytoplasm stains uniformly. (E) Stage II oocytes accumulate cortical granules in their cytoplasm, and a prominent vitellin envelope becomes apparent. (F) A mid-Stage III oocyte has eosinophilic yolk granules that accumulate around the nucleus, restricting the cortical granules to the periphery (G) Yolk granules fill the cytoplasm of a late Stage III oocyte, and a micropylar cell can be identified (inset shows high magnification view of boxed area in G). Panels (D–G) are not taken from image in (A). *cg*, cortical granules; *gc*, granulosa cell; *is*, interstitial cells; *mpc*, micropylar cell; *n*, nucleus; *oe*, ovarian epithelium; *oo*, oogonia; *opc*, oocyte progenitor cell; *pfc*, prefollicle cell; *ve*, vitellin envelop; *y*, yolk. Scale bars in (A), 150 μm; (B) and (C), 10 μm; (D), 25 μm; (E) and (F), 100 μm; (G), 250 μm.

(Beer & Draper, 2013). OSCs are a small fraction of the premeiotic germ cells in the ovary, and can be identified by *nanos2* expression (Beer & Draper, 2013; Cao, Mao, & Luo, 2019; Draper, 2017), a conserved marker of germline stem cells in vertebrates (Nakamura et al., 2010; Tsuda et al., 2003). The OSCs localize to discrete zones on the lateral and medial surfaces of the ovary, called the germinal zone (boxed area in Fig. 16.2A), that runs parallel to the anterior-posterior axis (Beer & Draper, 2013; Draper et al., 2007).

Oocyte progenitor cells (OPC): OPCs are the most abundant premeiotic germ cells in the ovary (Beer & Draper, 2013; Draper, 2012). In most stem cell systems, the stem cells are relatively slow dividing but give rise to progenitor cells (often referred to as transient amplifying cells) that undergo several more rapid rounds of division before differentiating. In the case of germ cells, differentiation is synonymous with meiotic entry. While both OSCs and OPCs undergo mitotic division, OPCs are distinct because they downregulate the expression of *nanos2* and they divide with incomplete cytokinesis where the daughter cells (cystocytes) remain connected by intercellular bridges called ring canals (Beer & Draper, 2013; Marlow & Mullins, 2008). These progenitor cells, therefore, have synchronized cell division dynamics and developmental progression (Beer & Draper, 2013; Elkouby & Mullins, 2017a; Marlow & Mullins, 2008). In zebrafish, it is not known how many divisions the oocyte progenitor cells undergo before entering meiosis, but it is likely between four to six as clusters of 16- to 32-cells expressing early meiotic markers, such as *dmc1* and *sycp3*, are common (Beer & Draper, 2013; BW Draper unpublished observations). Thus far, no markers have been identified that specifically mark oocyte progenitor cells, but these cells label well with anti-VasaDdx4 antibodies, are generally 7–10 μm in diameter, have a large nucleus to cytoplasm ratio, and nuclei containing two small nucleoli (Draper, 2012).

Meiotic Germ Cells

Once oocytes enter meiosis, they progress through to the diplotene stage of prophase I, where meiosis arrests until maturation and the first meiotic division occurs in late-stage oocytes. Oocytes are classified into five major stages based on cell appearance, physiology, and biochemistry (Selman et al., 1993). Below is a brief summary of these stages together with their defining characteristics (Selman et al., 1993).

Stage IA: Prefollicle stage (cell diameter ca. 7–20 μm). During this stage, the germ cells are present within clonal clusters of interconnected cells (also referred to as "cyst" or "nest") surrounded by prefollicle cells, where they synchronously enter and progress through prophase of meiosis I (leptotene to early diakinesis; Fig. 16.2C). Stages of meiosis can also be further refined by assaying for the localization of telomeres using the TeloFISH technique (Blokhina et al., 2019; Elkouby et al., 2016; Elkouby & Mullins, 2017a). During late leptotene, the telomeres become associated with the nuclear envelope but are randomly distributed around the periphery, and the chromatin is poorly condensed. During zygotene, the telomeres become asymmetrically localized to the side of the nucleus adjacent to the centrosome as the chromosomes assume the bouquet arrangement that is thought to aid in homolog pairing and synapsis (Elkouby et al., 2016; Scherthan, 2001). At this stage, chromosomes have become sufficiently compacted that individual chromosomes can be distinguished. During pachytene, telomeres dissociate from the nuclear envelope and are once again randomly distributed. The chromosomes remain condensed but appear thinner as homologs become dissociated in regions other than the crossover chiasmata. At the diakinesis stage, the cyst is surrounded by a layer of prefollicle cells (Fig. 16.2C). At this stage, germ cells arrest meiotic progression until oocyte maturation. It is likely that at this time many of the oocytes undergo apoptosis, as has been previously observed in mice, where an estimated 2/3rds of oocytes die soon after entering meiosis (Pepling & Spradling, 2001; BW Draper, unpublished observation). Several markers of Stage IA oocytes have been identified, including the early meiosis-specific genes *dmc1* and *sycp3* (Beer & Draper, 2013; Rodriguez-Mari et al., 2013).

Stage IB: Follicle phase of primary growth (oocyte diameter ca. 20–140 μm). Once oocytes have arrested in diakinesis, they become separated from the cyst and are enveloped by a single layer of granulosa cells (Fig. 16.2D). At this stage, they have entered the follicle phase of development or Stage 1B. The cytoplasm of the Stage 1B oocyte is relatively translucent when viewed in the light microscope, as these cells have not yet begun yolk uptake. The chromosomes begin to decondense into the characteristic lamp-brush chromosome morphology.

Stage II: Cortical alveolus stage (oocyte diameter ca. 140–340 μm). Cortical alveoli (also known as Cortical vesicles) form subjacent to the oocyte plasma membrane (Fig. 16.2E). The components of the cortical vesicles are synthesized by the oocyte and will play important roles in fertilization, primarily as the slow block to polyspermy. During stage II the vitelline envelope begins to develop between the oocyte plasma membrane and the overlying granulosa cells, reaching a thickness of 6.0 μm by the end of Stage II.

Stage III: Vitellogenesis stage (oocyte diameter ca. 340–690 μm). During stage III the oocyte begins to endocytose vitellogenin, a female-specific yolk protein that is synthesized in the liver and transported to the oocytes via the circulatory system. As this occurs, the oocyte cytoplasm becomes increasingly opaque. The yolk accumulates in small membrane-bound bodies that accumulate toward the center of the oocyte, causing the cortical alveoli to become displaced toward the cortex (Fig. 16.2F,G). During Stage III a specialized follicle cell, the micropylar cell, forms that will organize the formation of the micropyle, an opening in the chorion of a spawned egg that allows sperm entry during fertilization (Fig. 16.2G and inset Hart & Donovan, 1983). Recent

results have shown the importance of the Hippo pathway for specification of the micropylar cell fate (Dingare et al., 2018, p. 145; Yi et al., 2019).

Stage IV: Oocyte maturation (oocyte diameter ca. 0.69–0.73 mm). During this stage, the nucleus begins to migrate toward the future animal pole of the egg. The nuclear envelope breaks down, as meiosis reinitiates. The oocyte completes meiosis I and continues to metaphase of meiosis II where it will arrest until after fertilization.

Stage V: Mature egg (egg diameter ca. 0.73–0.75 mm).

The Testis

The zebrafish testes are paired elongated organs located dorsal-laterally in the body cavity and are attached to the body wall by the mesorchium (Leal et al., 2009). The two testes convene caudally and join to a single spermatic duct, which attaches to the urogenital papilla. The testis is comprised of two main compartments, germinal and interstitial, which are separated by a basement membrane (Fig. 16.3). The germ cells and Sertoli cells are housed within the

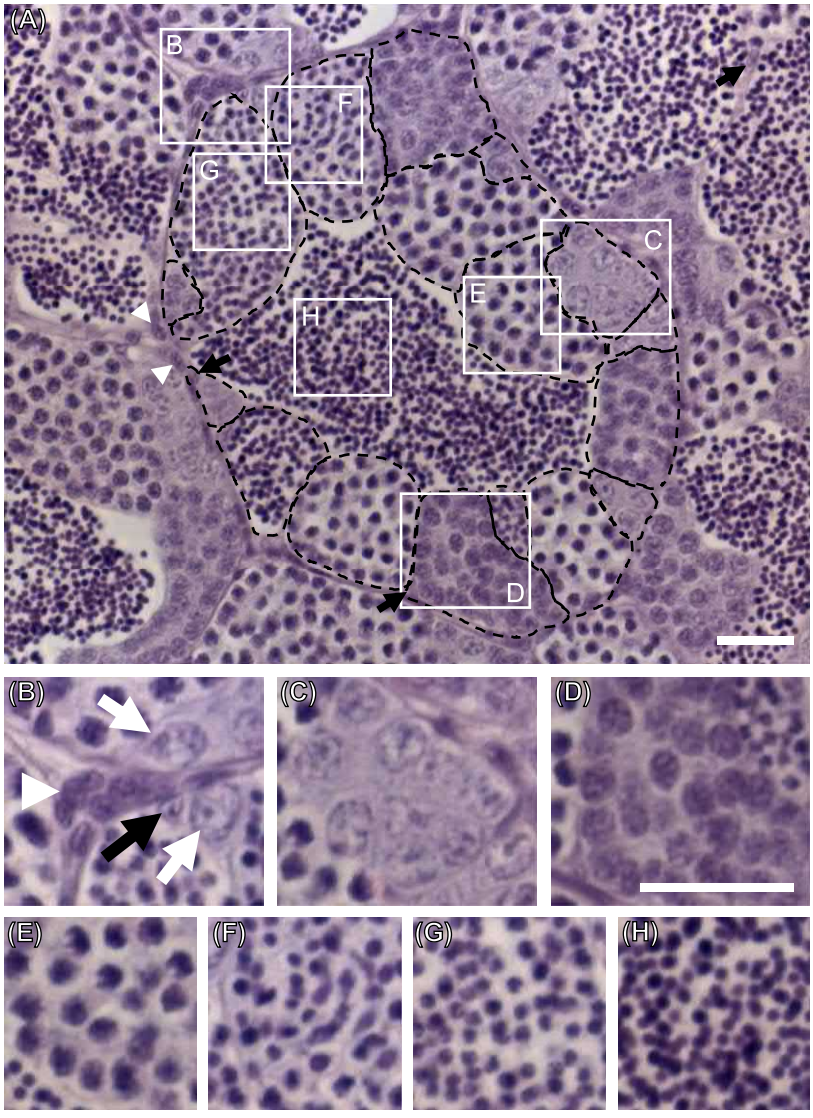

FIGURE 16.3 Representative histological images of an adult zebrafish testis. (A) Low magnification image showing the tubular organization of the testis. Within each tubule germ cells reside in cysts of synchronously developing germ cells surrounded by Sertoli cells (*black arrows*). Germ cell cysts within one testis tubule are outlined (*black dashed lines*). Sertoli cells are thinly stretched around cysts; therefore, nuclei are not readily observed around all cysts. Leydig cells (*white arrowheads*) reside in interstitial regions, between tubules. (B–H) Higher magnification images of various stages of spermatogenesis. (B) Spermatogonia A_{undiff} (*white arrows*) are often found adjacent to the interstitial compartment near clusters of Leydig cells (*white arrowhead*). An individual Sertoli cell nucleus (*black arrow*) can be seen associated with the lower A_{undiff} cell. (C) A cyst containing Spermatogonia B germ cells. (D) Primary spermatocytes in the leptotene stage. (E) Primary spermatocytes in the late zygotene stage. (F) Secondary spermatocytes. (G) Spermatids. (H) Spermatozoa. Scale bars are 20 μm, (B–H) are the same magnification.

germinal compartment whereas Leydig cells, blood vessels, myoid cells, and nerve fibers are located in the interstitial compartment (Uribe et al., 2014). The zebrafish testis has an unrestricted tubular organization, characterized by having a germinal epithelium organized into anastomosed tubules with spermatogonia cells located throughout the tubules rather than only at one end (Grier et al., 1980; Leal et al., 2009). Within the testis tubules, germ cells are housed in cysts of synchronously developing germ cells surrounded by Sertoli cells. Each cyst begins as a single spermatogonium cell surrounded by one or two Sertoli cells, both of which proliferate to generate germ cell cysts (Leal et al., 2009). Thus, cysts are comprised of a clone of germ cells surrounded by Sertoli cells. The Sertoli cell population expands approximately 9-fold per cyst, primarily during spermatogonial stages (Leal et al., 2009). The germ cells within a cyst, progress synchronously through spermatogenesis. As the sperms mature, they are released into the central lumen of the tubule, which connects to the testicular efferent ducts on the dorsal side of the testis (Leal et al., 2009). In the interstitial compartment, myoid cells are located around the tubules but do not completely surround tubules in teleost fish (Grier et al., 1980). The Leydig cells are the steroid producing cells of the testis and typically are observed in clusters, sometimes surrounding blood vessels (Leal et al., 2009). It has been proposed that these clusters of Leydig cells and blood vessels are part of the spermatogonial stem cell niche (Nobrega et al., 2015).

Premeiotic Germ Cells (Spermatogonial Cells)

Spermatogonial cells (SG) are the mitotically dividing population of testicular germ cells, including the stem cells. The SGs divide within the germ cell cysts with incomplete cytokinesis leaving cytoplasmic bridges between cells (Leal et al., 2009). There are two major stages of SGs, spermatogonia A and B type, which can be further broken down into several substages (Leal et al., 2009). Overall, there are 8 SG cell divisions; however, the average number of germ cells per cyst after the final division was 208, on average. Therefore, approximately 20% of germ cells are lost during this phase.

Spermatogonia A: The type A spermatogonia (SGA) cells comprise cysts of up to eight germ cells (Leal et al., 2009). These can be further subdivided into type A_{undiff} and A_{diff}.

Type A_{undiff} are single SGs with a large nucleus with poorly condensed chromatin and one or two prominent nucleoli (Fig. 16.3B) (Leal et al., 2009). A_{undiff} cells have slow cell cycles, as demonstrated by bromodeoxyuridine (BrdU) label retention, and therefore, are likely the stem cell population (Nobrega et al., 2010). They are most abundant adjacent to the interstitium near the vasculature and Leydig cell clusters, which points to these cell types as potentially important components of the stem cell niche (Nobrega et al., 2010). Interestingly, *amh* mutants have reduced Leydig cell populations but overproliferation of spermatogonial cells, indicating that having abundant Leydig cells is not necessary for robust SG proliferation (Lin et al., 2017; Yan et al., 2019). Therefore, the Leydig cells may be important for regulating the proper balance of proliferation and differentiation of the spermatogonial stem cells.

Two apparent subtypes of A_{undiff} exist in the adult zebrafish testis, $A_{undiff*}$ and A_{undiff}. The former is distinguished by a more irregular shaped nuclear envelope (Leal 2009). BrdU label retention studies demonstrated that the $A_{undiff*}$ population of cells is stable in the testis for at least 25 days, whereas the A_{undiff} population declines by 18 days after BrdU exposure suggesting that the $A_{undiff*}$ cells are largely quiescent while the A_{undiff} population has a relatively higher proliferation rate (Nobrega et al., 2010). Furthermore, stem cell replacement experiments using donor cells enriched for A_{undiff} (A_{undiff} and $A_{undiff*}$) demonstrate that this cell population contains the germline stem cells of the testis (Nobrega et al., 2010). To date, there are no molecular markers that specifically label the A_{undiff} population or distinguish $A_{undiff*}$ from A_{undiff} in zebrafish.

Type A_{diff} spermatogonia are in cysts of two to eight germ cells. They have denser nuclei than A_{undiff} with some heterochromatin forming around the nuclear envelop and two to three nucleoli (Leal et al., 2009).

Spermatogonia B: Spermatogonia B (SGB) cells can be divided into B early (16–32 cells/cyst) and B late (64–256 cells/cyst) (Leal et al., 2009). SGB cells have denser nuclei than SGA cells, with nuclear density increasing in SGB late relative to SGB early. SGB early have a round/elongated nucleus with one to two small nucleoli. SGB late have a round nucleus with increased heterochromatin (Leal et al., 2009).

Meiotic Germ Cells (Spermatocytes)

Once male germ cells enter meiosis, they are referred to as spermatocytes. As germ cells initiate meiosis, they replicate their DNA then undergo two divisions without an intermediate DNA replication event. This gives the reductionist divisions of meiosis that produce the haploid gametes. Cells in meiosis I, are primary spermatocytes, which have a prolonged prophase, whereby the homologous chromosomes pair and undergo recombination. The appearance of the chromatin indicates the stage of meiotic prophase (Fig. 16.3A,D,E). During Leptotene, the chromosomes condense displaying a

thread-like appearance which becomes more pronounced in zygotene (Leal et al., 2009; Saito et al., 2011). During zygotene the "bouquet" structure of the chromosomes forms, where chromosome ends cluster to one side of the nuclear envelope, which facilitates pairing and synapsis (Blokhina et al., 2019; Saito et al., 2014; Zeng et al., 2017). Meiotic crossovers form in the pachytene stage, which holds the chromosomes together during diplotene. Secondary spermatocytes are undergoing the second meiotic division, which is quicker than meiosis I and separates the sister chromatids. During meiosis, there is an approximately 10% loss of germ cells (Leal et al., 2009).

Postmeiotic Germ Cells (Spermatids and Spermatozoa)

Upon completion of meiosis, the haploid spermatids undergo differentiation to form the spermatozoa or mature sperm. The sperm nuclei continue to condense, and cytoplasm is expelled and phagocytosed by the Sertoli cells (Grier et al., 1980). Interestingly, zebrafish sperm lack protamines, and instead, the DNA is compacted by histones (Wu et al., 2011). The sperm flagella also form during spermiogenesis. Finally, the mature spermatozoa are released from the cyst into the central lumen of the seminiferous tubule and then travel to the efferent ducts.

Molecular Markers for Specific Spermatogenic Stages (Antibody and in Situ)

It can be difficult to discern specific spermatogenic stages by histological methods alone, and different fixatives and stains may yield slightly different nuclear appearances. There are a handful of molecular markers that have been carefully characterized to label specific spermatogenic stages (Table 16.1 Beer & Draper, 2013; Ozaki et al., 2011). Fluorescent proteins expressed from transgenes are often expressed in stages beyond where the endogenous protein or RNA is expressed (e.g., Tg(*sycp1:GFP*) Gautier et al., 2013), and are, therefore, not included. In addition, meiotic prophase stages in spermatocytes can be distinguished through telomere labeling in a similar fashion as described above for female germ cells (Blokhina et al., 2019; Ozaki et al., 2011; Saito et al., 2014).

Hormonal Regulation of Reproduction

Sex differentiation and reproduction are regulated at many levels by various hormones. Most notably are the sex-specific hormones testosterone (or its derivatives) and estrogen. The sex hormones, which are produced predominantly by specialized cells in the gonads, regulate differentiation of the ovaries and testes and regulate the development of the secondary sexual phenotypes and behavior. In adults, reproductive function is also regulated by the hypothalamus-pituitary-gonad axis (HPG) through the hypothalamus-regulated production of the gonadotropins, luteinizing hormone (Lh) and follicle-stimulating hormone (Fsh), by cells in the anterior pituitary.

Steroid Hormone Synthesis

The major bioactive sex hormone in females is the E2 form of estrogen, 17β-Estradiol, while in male fish it is the testosterone derivative 11-Ketotestosterone (11-KT) (for review, see Devlin & Nagahama, 2002). Synthesis

TABLE 16.1 Molecular Markers of zebrafish spermatogenesis.

Expression pattern	Gene name	Method	References
Single SG and SG in cyst up to 4 cells	nanos2	RNA in situ	Beer & Draper, 2013
Single SG and SG in cyst up to 8 cells	Plzf	Antibody	Ozaki et al., 2011
SG and SC up to preleptotene	Cyclin B	Antibody	Ozaki et al., 2011
Primary spermatocytes- specific prophase stages can be discerned	Sycp1	Antibody	Ozaki et al., 2011 Blokhina et al., 2019
Primary spermatocytes- specific prophase stages can be discerned	Sycp3	Antibody	Ozaki et al., 2011 Saito et al., 2011 Blokhina et al., 2019

of the major precursors to both E2 and 11-KT occur in the theca and Leydig cells in the ovary and testis, respectively, in a step-wise pathway that begins with cholesterol. Fig. 16.4 summarizes a likely pathway for the synthesis of E2 and 11-KT (For a more complete review of steroid hormone synthesis, see (Tokarz et al., 2013). It is likely that the synthesis pathway from cholesterol to testosterone is similar in both males and females because similar steroidogenic enzymes are expressed in both sexes. The pathway bifurcates after testosterone, as the steps that lead to E2 and 11-KT are catalyzed by sex-specific enzymes. In males, Cyp11c1 (formerly called Cyp11b1) and Hsd11b2 act sequentially to convert testosterone to 11-KT. In females, the ovary-specific aromatase Cyp19a1a catalyzes the direct conversion of testosterone to E2. While all steroid synthesis steps in males appear to occur in Leydig cells, in female zebrafish there is evidence that both theca and granulosa cells contribute to the production of E2 as *cyp19a1a* is expressed in theca cells, as well as in a subset of granulosa cells (Dranow et al., 2016). *cyp19a1* is similarly expressed in Medaka, suggesting that expression of *cyp19a1* orthologs in theca cells is common in teleost fishes (Nakamura et al., 2009).

Mutant analysis has shown that the ability to produce of E2 is critical for larval zebrafish to differentiate into females, as all *cyp19a1a* mutant animals differentiate into males that are fertile as adults (Dranow et al., 2016; Lau et al., 2016; Yin et al., 2017). By contrast, the gonads of animals that cannot produce androgens due to mutations in *cyp17a1* differentiate into testes that can produce functional sperm, but these animals do not develop male-specific secondary sexual characteristics (e.g., dark yellow pigmentation) or spawning behavior (Zhai et al., 2018). Thus, unlike the requirement of E2 for female development, 11-KT production is not required for all aspects of male development.

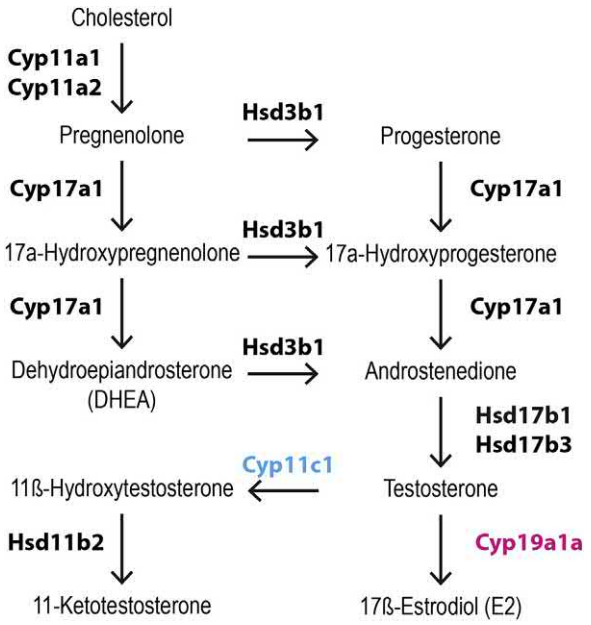

FIGURE 16.4 Steroid synthesis pathway showing intermediates (normal font) and enzymes that catalyze the indicated conversions (bold font). Enzymes in black are expressed in both testes and ovaries, while those in blue and pink are expressed primarily in testes or ovaries, respectively.

Steroid Hormone Receptors

E2 and 11-KT exert their action through binding to the Estrogen and Androgen nuclear hormone receptors (*ER* and *AR*), respectively. While mammals have two ERs, called *Esr1* and *Esr2*, three Er-encoding genes have been identified in zebrafish owing to the duplication of *esr2* (Bardet et al., 2002; Menuet et al., 2002). As in mammals, zebrafish have a single Ar (de Waal et al., 2008; Hossain et al., 2008).

ar is expressed in Sertoli cells in the testis, and its expression in the ovary has been detected by reverse transcriptase-polymerase chain reaction (RT-PCR de Waal et al., 2008; Hossain et al., 2008). Mutations in *ar* result in a strongly female-biased sex ratio, but some animals can develop as males, albeit somewhat abnormal: mutant males do not have the normal pigmentation, and although they form testes that produce functional sperm, the testes were disorganized and lacked seminiferous tubules. By contrast, *ar* mutant females have normal secondary sexual characteristics but decreased fecundity, apparently due to oocyte maturation defects (Crowder et al., 2018).

RT-PCR assays have shown that *esr1*, *esr2a*, and *esr2b* are expressed in follicle cells in the ovary, but the specific cell-type has not been identified (Liu et al., 2017). In addition, expression of *ers2a*, but not *esr1* or *esr2b*, was detected in oocytes by RT-PCR (Liu et al., 2017). Mutational analysis has shown that all single mutants have normal sex ratios and fecundity; however, triple mutants were all male as adults (Lu et al., 2017), consistent with the phenotype of *cyp19a1a* mutants (Dranow et al., 2016; Lau et al., 2016; Yin et al., 2017).

Regulation of Reproduction by the HPG Axis

The hypothalamus-pituitary-gonad axis regulates aspects of reproduction in vertebrates. In mammals, neurons within the hypothalamus release gonadotropin-releasing hormone (Gnrh) that then stimulates gonadotropic cells located within the anterior pituitary to synthesis and secrete the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH; for review, see Plant, 2015). LH and FSH are heterodimeric glycoproteins composed of a shared

alpha subunit that is encoded by the *Glycoprotein hormone alpha* gene, *Cga*, and a hormone-specific beta subunit encoded by the genes *Fshb* and *Lhb*, respectively (Gharib et al., 1990). LH and FSH are carried to the gonad by the circulatory system where they bind their respective G-protein coupled transmembrane receptors called Luteinizing Hormone/Choriogonadotropin Receptor (LHCGR) and Follicle Stimulating Hormone Receptor (FSHR), respectively (for more detailed review, see (Ascoli et al., 2002; Simoni et al., 1997). In mammals, FSHR is expressed by granulosa cells in the ovary and Sertoli cells in the testis, whereas LHCGR is expressed by Theca and granulosa cells in the ovary and Leydig cells in the testis. In mammalian females, FSH promotes the growth of the oocyte-follicle complex while LH functions to promote maturation of the fully grown oocyte and ovulation. Both LH and FSH promote the production of estrogen by the ovary. In mammalian males, FSH acts on the Sertoli cells to stimulate sperm production, while LH stimulates Leydig cells to produce testosterone. There are several differences between the reproductive strategies of eutherian mammals and fish that spawn their eggs. Nonetheless, recent genetic studies in zebrafish have uncovered both similarities and apparent differences in the roles the HPG axis plays in reproduction in teleost versus mammals. The phenotypes of zebrafish mutants in HPG axis components are summarized in Table 16.2.

Follicle-stimulating hormone (Fsh): Fsh appears to play a more prominent role in the ovary than the testis in zebrafish. Analysis of loss-of-function *fshb* mutants indicates that Fsh plays an essential role in promoting oocyte development (Chu et al., 2015; Zhang et al., 2015b). *fshb* mutant females have delayed ovary development during the juvenile stages (30–60 dpf), and oocyte development appears to be delayed at the transition from stage IA to stage IB (primary growth to follicle stage). However, mutant females are fertile at 90 dpf, suggesting that once the transition occurs, Fsh is not required for further oocyte development. Paradoxically, zebrafish mutant for the Fsh receptor, *fshr*, have a more severe phenotype then *fshb* mutants. Although male *fshr* mutants are fertile, the oocytes of female mutants arrest development at stage 1A, indicating that signaling through Fshr is necessary to promote the transition from stage 1A to stage IB. *fshr* mutant animals have a normal sex ratio during early juvenile stages (25–50 dpf), but the sex ratios become skewed toward male in

TABLE 16.2 Summary of HPG axis mutant phenotypes.

Gene mutation	Phenotype	References
gnrh2 *gnrh3* *gnrh2;gnrh3*	Single and double mutants are fertile with normal sex ratios	Marvel et al., 2018
Ihb	Males fertile Females do not spawn due to oocyte maturation and ovulations defects	Chu et al., 2014 Zhang et al., 2015a Shang et al., 2019
Ihcgr	Fertile with normal sex ratios ovaries and testis development delayed	Chu et al., 2014 Zhang et al., 2015b
fshb	Males fertile Females have delayed ovary development, but fertile at 90 dpf Some females sex revert to male	Zhang et al., 2015a Chu et al., 2015
fshr	Males fertile with delayed testis development Females infertile with oocyte arrested at transition from stage 1A to stage IB (PG to follicle phase). Some females sex revert to fertile males.	Zhang et al., 2015b Chu et al., 2015
fshb; Ihb	All male Testis development delayed	Zhang et al., 2015a
fshr; Ihcgr	All male Sterile. Testis development arrested	Zhang et al., 2015b Chu et al., 2015

late juvenile and adult stages (Zhang et al., 2015a). This suggests that mutant females are sex reverting to males. This latter result is consistent with the known role of oocytes in maintaining female sex determination in zebrafish (Dranow et al., 2013). Fsh is apparently dispensable for spermatogenesis in zebrafish. Mutant *fshb* and *fshr* males have a modest delay in testis development, but adults are fertile with a normal-sized testis (Chu et al., 2015; Zhang et al., 2015b).

Luteinizing hormone (Lh): Current evidence strongly argues that Lh plays an essential role in regulating oocyte maturation and ovulation in zebrafish but is not required for spermatogenesis. Wild-type zebrafish spawn soon after the vivarium lights come on (artificial sunrise). Under normal *in vivo* conditions oocyte maturation has been shown to initiate around 1.5 h before sunrise (i.e., lights on), as measured by assessing the transparency of fully grown oocytes: immature oocytes are opaque, while mature oocytes are more translucent (Shang et al., 2019). Correlated with visible indicators of oocyte maturation, expression levels of *lhb* peak at 2.5 h before dawn, as measured by RT-PCR (Shang et al., 2019). Finally, mutational analysis confirms that *lhb* function is required for oocyte maturation and ovulation, as *lhb* mutant ovaries have all stages of oocytes, from oogonia to Stage IV fully grown (FG) oocytes, but FG oocytes never undergo maturation or ovulation (Chu et al., 2014; Shang et al., 2019; Zhang et al., 2015b). As such, *lhb* mutant females are sterile, but their gonadal-somatic index gradually increases as their ovaries fill with FG oocytes. In contrast to *lhb* mutants, *lhcgr* mutant females are fertile (Chu et al., 2014; Zhang et al., 2015a). This result has led to the hypothesis that Lh can also signal through the Fsh receptor, a hypothesis consistent with the more severe phenotype of *fshr* versus *fshb* mutants described above. Finally, mutant analysis suggests that Lh signaling has a less important role in the testis than in the ovary. *lhb* and *lhcgr* mutant males are fertile with normal histology (Chu et al., 2014; Zhang et al., 2015a, 2015b). Furthermore, *lhb* mutants have wild-type levels of testosterone, suggesting that Lh is not necessary for the production of this hormone by Leydig cells, as it is in mammals (Shang et al., 2019).

Disrupting both Fsh and Lh signaling more severely disrupts gonad development than disrupting only one pathway. Double mutants for either *fshb;lhb* or *fshr;lhcrg* are all-male indicating failure of ovary development (Chu 2015; Zheng 2015a). A curious result is that *fshb;lhb* double mutants have a less severe testis phenotype then *fshr;lhcrg* double mutants. The ligand double mutant males have delayed testis development, and although their testes contain germ cells at all stages of spermatogenesis, including mature sperm, they have an overaccumulation of apparent spermatogonia (Chu et al., 2015; Zhang et al., 2015a). By contrast, the double receptor mutants are sterile males with testes that contain mostly apparent spermatogonia, few spermatozoa, and no mature sperm. It has been proposed that the differences between the ligand and receptor double mutant phenotypes are due to weak ligand-independent constitutive activity of the receptors, as has been demonstrated in heterologous tissue culture assays (Chu et al., 2015).

In summary, gonadotropin signaling in zebrafish plays an essential role in regulating germ cell development in both males and females, but in contrasts to mammals, current evidence supports more overlap in the expression and function of Fshr and Lhcrg in zebrafish than has been found in mammals. In addition, there is evidence that in fish, these receptors are not as specific for the Fsh and Lh ligands as are their mammalian counterparts. This apparent cross-talk may be explained by the evolution of this signaling pathway. For example, lamprey, a basal vertebrate, have only a single gonadotropin ligand that signals though probable homologs of Lhcgr and Fshr (Sower et al., 2009). In addition, Lhb and Fshb in teleost and mammals appear to have arisen by duplication of the beta subunit of this ancestral gonadotropin, which in fish appear to have retained their ability to activate both Lhcrg and Fshr receptors, but in mammals have further evolved to become receptor-specific.

Summary

Given the progress that has been made over the past several years defining the genes involved in development and function of the zebrafish reproductive system, researchers are in an excellent position to continue making significant contributions to our understanding of vertebrate reproductive biology. The ease with which genes now can be manipulated opens up the possibility to develop conditional knockout tools that will be necessary to study the actions of genes required at earlier stages of development. Finally, new developments in cell and organ culture systems open up exciting possibilities for in vivo imaging of reproductive processes (Elkouby & Mullins, 2017b).

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CHAPTER

17

Endocrine Systems

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Introduction

The endocrine system is composed of glands throughout the body that secrete and respond to hormones. Historically, all endocrine glands were thought to secrete hormones into the bloodstream, rather than into ducts or onto adjacent cells as occurring in exocrine glands, such as the salivary glands. We now appreciate that there are cases where hormones are secreted directly onto their target tissue, instead of secreting into the bloodstream (for example, neurons in the hypothalamus that deposit hormones directly to the pituitary gland without first entering the blood). However, endocrine glands are still referred to as ductless glands by many textbooks.

Endocrine glands produce structurally diverse small molecule and peptide hormones, such as steroids, prostaglandins, and neuropeptides. A single hormone may have different effects on multiple target tissues, while multiple hormones may have different effects on the same target tissue. Because hormones synthesized by endocrine glands are often secreted into the blood, endocrine hormones have the opportunity to target all tissues into which blood flows.

All organs produce and respond to hormones. This chapter focuses on glands, whose primary function is to produce and/or secrete hormones. The major endocrine glands are the hypothalamus, pituitary, pineal, thyroid, parathyroid, adrenal, pancreas, ovary and testes. In this chapter, we will discuss the development and function of zebrafish endocrine glands except for the gonads, which are covered in a separate chapter. We will compare and contrast the anatomy and function of the endocrine system between zebrafish and humans and commonly used research animals, such as mice.

The endocrine glands of zebrafish and humans are structurally different. Endocrine function, however, is similar between zebrafish and humans. Zebrafish and humans produce similar, and in many cases, identical hormones. Endocrine disorders that occur in humans, such as diabetes and Cushing's disease, can be induced in zebrafish, allowing scientists to study endocrine disorders in zebrafish (Liu et al., 2011; Mullapudi et al., 2018).

Zebrafish hormones of the hypothalamus, pituitary, and gonad have similar actions as their mammalian equivalents. Enzymes that synthesize and metabolize hormones, such as aromatase and deiodinases, are present in the zebrafish genome (Orozco & Valverde, 2005; Tokarz, Moller, de Angelis, & Adamski, 2013; Trant, Gavasso, Ackers, Chung, & Place, 2001; Walpita, Crawford, Janssens, Van der Geyten, & Darras, 2009). Crucial signaling molecules, such as estradiol, testosterone, cortisol, and thyroid hormones are identical in zebrafish and humans (Brown, 1997; Gorelick & Halpern, 2014; Liu et al., 2009; Lohr & Hammerschmidt, 2011; McGonnell & Fowkes, 2006; Tokarz et al., 2013; Tonyushkina, Shen, Ortiz-Toro, & Karlstrom, 2014). Zebrafish and humans have orthologous nuclear receptor genes (Bardet, Horard, Robinson-Rechavi, Laudet, & Vanacker, 2002; Bertrand et al., 2007; Essner, Breuer, Essner, Fahrenkrug, & Hackett, 1997; Lassiter, Kelley, & Linney, 2002; Liu, Lo, & Chan, 2000; Marchand et al., 2001; McGonnell & Fowkes, 2006; Menuet et al., 2002), with the caveat that, due to a whole-genome duplication in the teleost lineage, zebrafish have multiple copies of some receptor genes (Amores et al., 1998; Gates et al., 1999; Postlethwait et al., 1998). For example, zebrafish have two copies of the estrogen receptor beta (ERβ) gene designated *esr2a* and *esr2b* (Bardet et al., 2002; Menuet et al., 2002). Functionally, they are similar

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to each other and to mammalian ERβ: they both bind estrogens and activate transcription via identical estrogen response element DNA sequences (Bardet et al., 2002; Costache, Pullela, Kasha, Tomasiewicz, & Sem, 2005; Menuet et al., 2002, 2004). The same is true for thyroid hormone receptor alpha, where zebrafish have two genes, *thraa* and *thrab*, that function similarly to human *THRA* (Bertrand et al., 2007; Essner et al., 1997; Marchand et al., 2001). Furthermore, disease-causing mutations in human thyroid hormone receptors exert similar effects in zebrafish (Marelli et al., 2016, 2017), underscoring the similarity between endocrine system function in zebrafish and humans.

To investigate hormone function, scientists use tools to block hormone action, such as small molecules that prevent a hormone binding to its target receptor, or genetic modifications that reduce the activity of a hormone or its receptor. In zebrafish, it is possible to mutate or knockout genes using CRISPR-Cas, TALEN, or zinc finger nuclease technology (Bedell & Ekker, 2015; Cade et al., 2012; Gupta et al., 2012; Hwang et al., 2013; Jao, Wente, & Chen, 2013). It is also possible to reduce, or knockdown, gene expression by injecting embryos with morpholino antisense oligonucleotides (Nasevicius & Ekker, 2000). Morpholinos can be nonspecific, reducing expression of both intended and unintended targets (Kok et al., 2014). This leads to mistakenly concluding that a phenotype is caused by knocking down of the target gene, whereas, the phenotype is actually caused by knocking down a gene that was not the intended target. It is difficult, and sometimes impossible, to control for the nonspecific effects of morpholinos (Blum, De Robertis, Wallingford, & Niehrs, 2015; Schulte-Merker & Stainier, 2014; Stainier, Kontarakis, & Rossi, 2015; Zimmer, Pan, Chandrapalan, Kwong, & Perry, 2019). In this chapter, we discuss results from both gene editing and morpholino studies. Where studies have been performed targeting the same gene or signaling pathway using morpholino and gene editing approaches, we defer to the gene-editing approach. In some cases, only morpholino results are available, and we caution the reader to interpret these results carefully.

Hypothalamus and Pituitary

The hypothalamus in the brain is the primary homeostatic regulator for multiple physiologic systems, including metabolism and food intake, the stress response, circadian regulation, growth, electrolyte balance, thermoregulation, and reproduction (Fig. 17.1) (Saper & Lowell, 2014). In mammals, neurons in the hypothalamus project directly to the posterior pituitary (neurohypophysis) or they project to blood vessels (hypophyseal portal veins) that travel directly to the anterior pituitary (adenohypophysis). These hypothalamic neurons release hormones onto their targets. Magnocellular neurons release oxytocin and arginine vasopressin into the posterior pituitary, from where they can enter general vascular circulation. Parvocellular neurons release thyrotropin-releasing hormone (TRH), growth hormone-releasing hormone (GHRH), somatostatin (SST), gonadotropin-releasing hormone (GnRH) and dopamine (DA) into hypophyseal portal veins that travel to the anterior pituitary. Orthologues for these hormones are present in zebrafish (Lohr & Hammerschmidt, 2011); however, the anatomy of this neuroendocrine system is different between zebrafish and mammals. Zebrafish lack hypophyseal portal veins. Instead, parvocellular neurons project directly to the anterior pituitary (Lohr & Hammerschmidt, 2011).

Anatomical correlation between hypothalamic neurons in zebrafish and mammals is not always clear. In mammals, the paraventricular nucleus (PVN, an anatomic designation), contains both magnocellular and parvocellular neurosecretory cells and is integral to hypothalamic function. However, the corresponding anatomic location in the zebrafish brain does not appear to contain equivalent neurosecretory cells. Rather, the neurosecretory preoptic area (NPO) of the zebrafish brain is a possible functional homolog of the mammalian PVN based on gene expression and cellular projection patterns (Herget & Ryu, 2015; Nagpal, Herget, Choi, & Ryu, 2019). The function of neuroendocrine cells is conserved between zebrafish and mammals, although their anatomical organization may be different.

In response to hormones secreted by neurons in the hypothalamus, the anterior pituitary produces hormones and releases them into the blood. Cell types in the pituitary can be distinguished based on anatomy and gene expression. For example, TRH released by the hypothalamus stimulates cells in the anterior pituitary to release thyroid-stimulating hormone (TSH) into the blood.

Similar to mammals, activation and inhibition of pituitary hormone release are controlled by positive and negative feedback loops. For example, the hypothalamus secretes CRH into the anterior pituitary, which responds by increasing expression of proopiomelanocortin (POMC, a prohormone that is cleaved to become ACTH, which stimulates adrenal gland/interrenal cells to produce cortisol). Exposing zebrafish embryos to dexamethasone, a potent synthetic glucocorticoid, induces a negative feedback loop that causes downregulation of *pomc* gene expression (Liu et al., 2003; To et al., 2007).

By 5 dpf (days post fertilization), the zebrafish pituitary contains anatomically and genetically distinct cell types: corticotropes that produce ACTH, melanotropes

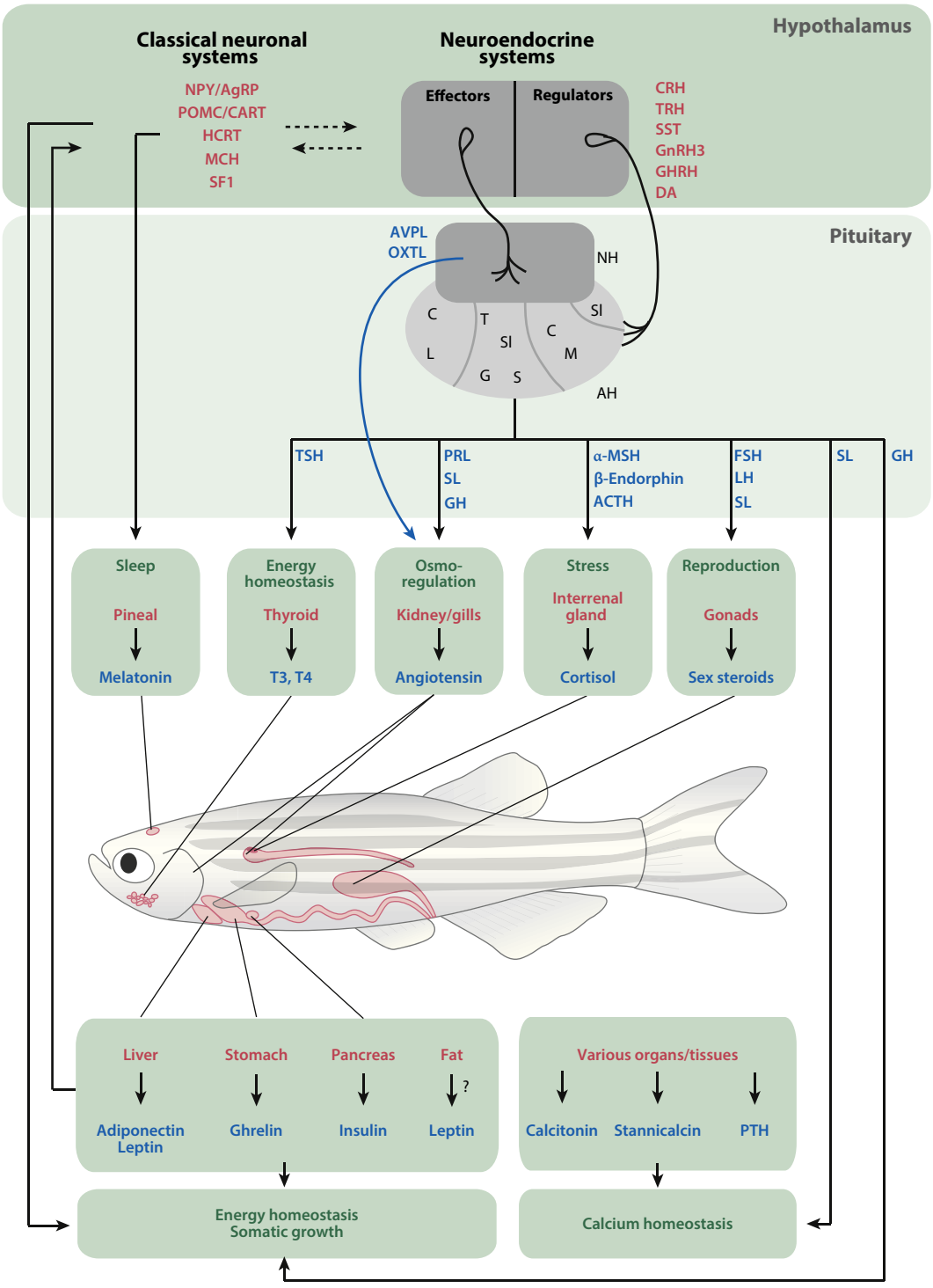

FIGURE 17.1 **Overview of the zebrafish endocrine system.** Hormones of the hypothalamus (red), hormones of the pituitary and other tissues (blue), sites of hormone production (pink) and the physiologic processes regulated by hormones (green). *ACTH*, adrenocorticotropic hormone; *AgRP*, agouti-related peptide; *AH*, adenohypophysis; *AVPL*, arginine vasopressin like; *C*, corticotropes; *CART*, cocaine- and amphetamine-regulated transcript; *CRH*, corticotropin-releasing hormone; *DA*, dopamine; *FSH*, follicle-stimulating hormone; *G*, gonadotropes; *GH*, growth hormone; *GHRH*, growth hormone-releasing hormone; *GnRH*, gonadotropin-releasing hormone; *HRCT*, hypocretin; *IGF*, insulin-like growth factor; *L*, lactotropes; *LH*, luteinizing hormone; *M*, melanotropes; *MCH*, melanin-concentrating hormone; *MSH*, melanocyte-stimulating hormone; *NH*, neurohypophysis; *NPY*, neuropeptide Y; *OXTL*, oxytocin like; *POMC*, proopiomelanocortin; *PRL*, prolactin; *PTH*, parathyroid hormone; *PTHrP*, parathyroid hormone-related peptide; *S*, somatotropes; *SF1*, steroidogenic factor; *SL*, somatolactin; *SI*, somatolactotropes; *SST*, somatostatin; *T*, thyrotropes; *TRH*, thyrotropin-releasing hormone; *TSH*, thyroid-stimulating hormone. *Modified from Lohr, H., & Hammerschmidt, M. (2011). Zebrafish in endocrine systems: Recent advances and implications for human disease.* Annual Review of Physiology, 73, *183–211, with permission of Annual Reviews.*

that produce melanocyte-stimulating hormone, lactotropes that produce prolactin, somatotropes that produce growth hormone, and thyrotropes that produce thyroid-stimulating hormone (Herzog et al., 2003).

Thyroid

Control of thyroid hormone levels in zebrafish is similar to the thyroid axis of mammals and amphibians. Similar to mammals, TRH is produced by the zebrafish hypothalamus and stimulates the pituitary to release thyroid-stimulating hormone (TSH), which then stimulates the thyroid gland to produce thyroid hormones thyroxine (T4) and triiodothyronine (T3) (Lohr & Hammerschmidt, 2011; Nica, Herzog, Sonntag, & Hammerschmidt, 2004; Opitz et al., 2011, 2012; Tonyushkina et al., 2014).

Similar to other vertebrates, the thyroid axis in zebrafish mediates development and metabolism. Addition of exogenous thyroid hormone during development causes accelerated growth of pectoral and pelvic fins (Brown, 1997). Thyroid inhibition during development, using thyroid hormone receptor antagonists, delays development of the GI tract, swim bladder, and cartilage of the lower jaw (Liu & Chan, 2002). T3 increases heart rate in zebrafish embryos (Romano et al., 2017) as it does in humans (Klein & Ojamaa, 2001). Thyroid-stimulating hormone and its receptor have been found in gonadal tissue of other teleosts, suggesting that thyroid may play a role in reproduction (MacKenzie, Jones, & Miller, 2009). Indeed, thyroid hormone has been found to regulate Sertoli cell development in the zebrafish testis (Schulz et al., 2015).

Before the thyroid gland is functional, the human fetus is exposed to thyroid hormones via transplacental transfer (Chan, Vasilopoulou, & Kilby, 2009). Similarly, zebrafish embryos are exposed to thyroid hormones maternally deposited in their yolk (Campinho, Saraiva, Florindo, & Power, 2014), at developmental stages before the thyroid gland becomes functional. Maternal thyroid hormone and receptor mRNA is present within the oocyte, detectable within hours of fertilization. During the midblastula stage (by 2.5 hours post fertilization (hpf)), the zebrafish embryo begins expressing its own thyroid hormone receptor (Essner et al., 1997; Liu & Chan, 2002). Thyroid follicle development begins at 60 hpf, earlier in development relative to most mammals. Like mice, the thyroid-stimulating hormone is not required to initiate thyroid follicle development. By 72 hpf, the thyroid gland is functional, creating thyroglobulin and T4 (Alt et al., 2006). In adulthood, the thyroid gland becomes a loose chain of thyroid follicles rather than a distinct organ. Thus, zebrafish embryos are exposed to thyroid hormones before the embryos themselves are capable of synthesizing thyroid hormones. The same is likely true for other endocrine hormones, such as estrogens and androgens.

Interrenal Cells (Adrenal Equivalent)

In mammals, the adrenal glands, located above the kidneys, produce androgens, glucocorticoids, mineralocorticoids, and catecholamines. Zebrafish lack a distinct adrenal gland. Instead, specialized cells in the kidney, termed *interrenal cells*, produce adrenal hormones. Zebrafish interrenal cells are considered the functional equivalent of the mammalian adrenal gland.

The physiologic stress response in zebrafish is similar to the stress response in mammals. The hypothalamic-pituitary-interrenal axis in zebrafish functions similarly to the hypothalamic-pituitary-adrenal axis in mammals. In response to stress, corticotropin-releasing hormone (CRH) is released by hypothalamic neurons into the zebrafish pituitary (Alderman & Bernier, 2009). CRH stimulates the production of proopiomelanocortin (POMC), which is posttranslationally modified to become adrenocorticotropic hormone (ACTH) (Liu et al., 2003). ACTH is released by the pituitary into the blood, where it travels to interrenal cells, binds to type 2 melanocortin receptors (MC2R), and induces the synthesis of cortisol and other hormones (To et al., 2007).

Cortisol is the primary corticosteroid in zebrafish (Alsop & Vijayan, 2008, 2009; Tsachaki et al., 2017). Cortisol is released in response to acute stressors. Cortisol levels were increased in adult zebrafish following acute overcrowding or isolation, net handling, contact with a predator, or air exposure (Barcellos et al., 2007; Grzelak et al., 2017; Pagnussat et al., 2013; Ramsay et al., 2006, 2009). Additionally, chronic stress can result in a reduced increase in cortisol in response to acute stressors (Giacomini et al., 2015). Cortisol levels are typically measured in whole-body homogenates. Cortisol levels can also be measured in the blood on the order of μg/dL, although the assay requires zebrafish euthanasia for blood collection (Grzelak et al., 2017). There is currently no method to nonlethally collect sufficient amounts of blood for measuring levels of cortisol or other steroids in zebrafish.

Like mammals, zebrafish express both glucocorticoid and mineralocorticoid receptors (Pippal, Cheung, Yao, Brennan, & Fuller, 2011). Unlike mammals, zebrafish are thought to lack the enzyme aldosterone synthase, and therefore, cannot synthesize aldosterone, a mineralocorticoid receptor ligand. Thus, glucocorticoids likely act by activating both glucocorticoid and mineralocorticoid receptors in zebrafish (Katsu, Oka, & Baker, 2018).

Both humans and zebrafish have one glucocorticoid receptor gene. Like the human glucocorticoid receptor,

the zebrafish homolog can be spliced into two isoforms, alpha and beta. The beta isoform inhibits expression of the alpha isoform, and this balance is thought to influence the development of immune-related diseases in humans. This similarity makes zebrafish a powerful system in which to study glucocorticoid signaling in physiology and disease (Schaaf, Chatzopoulou, & Spaink, 2009).

Development of Interrenal Cells and Corticosteroid Stress Response

It is not clear at what developmental stage zebrafish are first able to modulate cortisol levels in response to stress. Cortisol was detected in zebrafish embryos at 1 day postfertilization (dpf), although depending on the study the amount detected varied between 0.8 pg cortisol per embryo (or 120 fg cortisol/μg protein) (Alderman & Bernier, 2009) and 1.1 pg per embryo (or 70 fg cortisol/μg protein) (Alsop & Vijayan, 2008). In one study, cortisol levels remained static in zebrafish through 6 dpf (Alderman & Bernier, 2009) while a different study reported that at 6 dpf, cortisol levels rose to 21.5 pg per larva (or 1.87 pg cortisol/μg protein) (Alsop & Vijayan, 2008). This study also reported cortisol present in embryos at 1.5 hpf (4 pg cortisol per embryo or 0.24 pg cortisol/μg protein), suggesting that cortisol is maternally deposited into oocytes (Alsop & Vijayan, 2008). A third study found that at 10 hpf, cortisol levels were 1.66 ± 0.18 pg cortisol per embryo, dropping to 0.20 ± 0.04 at 22 hpf, then rising to 1.53 ± 0.36 at 3 dpf, 5.35 ± 0.54 at 4 dpf and 6 ± 4.24 at 5 dpf (Parajes et al., 2013).

What might account for the discrepancy in reported cortisol levels in these studies? One possibility is that cortisol levels are variable between zebrafish strains or between age-matched fish of the same strain housed in different fish facilities, suggesting that cortisol levels are sensitive to the environment. Another possibility is that some assays used to measure cortisol in zebrafish embryos and larvae are more accurate than others. Parajes et al. (2013) used liquid chromatography/tandem mass spectrometry, whereas the other studies used immunoassays. The most accurate and precise approach for detecting steroids in tissue or plasma uses chromatography and mass spectrometry (Auchus, 2014); thus, studies detecting low levels of cortisol in zebrafish embryos using immunoassays should be interpreted with caution. Studies in zebrafish at later stages of development and in adulthood are likely more accurate due to the higher levels of endogenous circulating cortisol. We have much to learn about corticosteroid levels and their role in zebrafish embryos. It is still not clear at what developmental stage zebrafish can first respond to stress by changing cortisol levels.

Parathyroid Hormones

In mammals, parathyroid glands secrete parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHrP), proteins that increase calcium and phosphate levels in the blood. Zebrafish lack a distinct parathyroid gland. However, zebrafish produce PTH and PTHrP and express PTH receptors (PTH and PTHrP bind to the same receptors) (Gensure et al., 2004; Hogan et al., 2005; Okabe & Graham, 2004; Rubin et al., 1999; Rubin & Juppner, 1999).

The zebrafish genome contains two PTH paralogues, *pth1a*, and *pth1b* (also known as *pth1* and *pth2*) (Gensure et al., 2004; Hogan et al., 2005; Okabe & Graham, 2004). Transcripts of *pth1a*, *pth1b* and PTH receptors were detected by RT-PCR in most tissues in adult zebrafish, including the gills (Kwong & Perry, 2015; Lin, Su, & Hwang, 2014; Okabe & Graham, 2004).

One idea is that the mammalian parathyroid gland and fish gills share a common evolutionary history (Okabe & Graham, 2004). In mice and humans, the transcription factor glial cells missing 2 (*Gcm2*) is expressed in the parathyroid primordium and is required for normal development of the parathyroid gland. Morpholino knockdown of *gcm2* in zebrafish embryos caused reduced or absent budding of gill filaments at 4–5 dpf (Hogan et al., 2004; Okabe & Graham, 2004). Gills are important for calcium uptake (Hunn, 1985), suggesting a functional connection between the parathyroid gland and fish gills. However, the widespread expression of *pth* genes in zebrafish suggests that the function of a distinct parathyroid gland is distributed among multiple cell types in adult zebrafish.

In zebrafish embryos and larvae, *pth* genes are expressed along the lateral line and in the ventral neural tube (Hogan et al., 2005). Morpholino knockdown of *pth1* caused reduced calcium uptake at 4 dpf (Kwong & Perry, 2015). Consistent with a role for PTH in calcium homeostasis in zebrafish embryos, housing zebrafish from 1-4 dpf in low calcium water (25 μM vs. standard 250 μM) caused a doubling of *pth1* mRNA levels (Kwong & Perry, 2015). The cell types in which PTH and its receptors act to regulate calcium homeostasis in zebrafish remain unknown.

Pineal

The pineal gland is an endocrine gland present in most vertebrate brains. The pineal produces the hormone melatonin, a small molecule that synchronizes circadian rhythms, such as the cycle of sleeping and waking. Melatonin promotes sleep. In humans, melatonin production begins in the evening as natural light

diminishes. Melatonin acts by binding to melatonin receptors 1 and 2.

Similar to other vertebrates, zebrafish possess a light-sensitive pineal gland and synthesize melatonin. Genes encoding enzymes that synthesize melatonin are expressed in the adult zebrafish pineal (Begay, Falcon, Cahill, Klein, & Coon, 1998; Gothilf et al., 1999). Expression of these genes is regulated by a circadian clock (Begay et al., 1998). More broadly, light regulates mRNA and microRNA expression in the zebrafish pineal gland (Ben-Moshe et al., 2014). The zebrafish genome contains several melatonin receptor genes that encode proteins activated by melatonin (Reppert, Weaver, Cassone, Godson, & Kolakowski, 1995; Shang & Zhdanova, 2007; Zhdanova, Wang, Leclair, & Danilova, 2001). Thus, zebrafish possess the ability to synthesize and respond to melatonin.

Adult zebrafish sleep at night (Sorribes et al., 2013; Yokogawa et al., 2007). Genetic ablation of the pineal clock (by expression of a dominant negative Clock protein in the pineal gland) disturbed the normal rhythm of melatonin production in adults (Ben-Moshe Livne et al., 2016). Studies using zebrafish with mutations in the hypocretin (orexin) signaling genes revealed a circuit of hypocretin neurons and the pineal gland that regulate melatonin synthesis and sleep (Appelbaum et al., 2009; Yokogawa et al., 2007).

In light of these findings, it is common practice to house adult zebrafish under conditions that alternate light and dark on a 24-h cycle (e.g., 14 h of light followed by 10 h of darkness, beginning at 8 a.m. daily) to reduce stress and promote health and fertility.

Development of the Pineal Gland in Zebrafish Embryos and Larvae

Zebrafish embryos and larvae also possess circadian rhythms regulated by the pineal gland. Gene expression studies suggest that the pineal gland forms by 1 dpf (Gamse et al., 2002). At 20–26 hpf, a circadian oscillator that regulates melatonin synthesis is likely functional (Kazimi & Cahill, 1999). Expression of serotonin N-acetyltransferase, the penultimate enzyme in melatonin synthesis, was first detected in the pineal at 22 hpf. When zebrafish embryos were housed in a 14 h light, 10 h dark cycle, a nocturnal increase in melatonin was detected beginning at the second night postfertilization (Kazimi & Cahill, 1999). Between 2 and 5 dpf, mRNA levels exhibit a 24 h rhythm, with high levels at night and lower levels during the day (Gothilf et al., 1999; Ziv & Gothilf, 2006).

Melatonin promotes a sleep-like state in larval zebrafish 7–14 dpf (Zhdanova et al., 2001). Genetic evidence suggests melatonin promotes sleep directly in 5–7 dpf larvae, separate from its effects on the circadian clock (Gandhi, Mosser, Oikonomou, & Prober, 2015). Genetic ablation of the pineal clock disturbed normal rhythms of locomotor activity in larvae (Ben-Moshe Livne et al., 2016).

Is there a health benefit to zebrafish if embryos and larvae are housed under a daily light-dark cycle, rather than in constant darkness or constant light? Embryos housed in constant darkness exhibited melatonin-dependent delayed neurogenesis and formation of neuronal processes in the habenula (de Borsetti et al., 2011). However, the effects of these delays on health and fertility of juvenile and adult zebrafish are not known. Housing zebrafish embryos in constant darkness is not lethal and does not cause gross changes in morphology or prevent synchronization of melatonin rhythms later in life at juvenile and adult stages (Kazimi & Cahill, 1999). One study housed zebrafish under constant light from fertilization through 48 dpf. The fish exhibited low mortality and were low to moderately fecund, with the average breeding pair producing 30–50 embryos (Dabrowski & Miller, 2018). Thus, it is not clear when (or even if) laboratory zebrafish should be exposed to regular light-dark cycles to improve zebrafish health and welfare. Whether the onset of the external light-dark cycle occurs at 1 h postfertilization or 5 days postfertilization may not matter. On the other hand, it is low cost and low effort to include a light and timer in a standard laboratory incubator that houses plates of zebrafish embryos.

Endocrine Pancreas

Similar to mammals, the zebrafish pancreas consists of exocrine and endocrine tissue. The endocrine pancreas is organized as a single, principal islet with a variable number of smaller and irregularly sized secondary islets (Li, Wen, Peng, Korzh, & Gong, 2009). As in mammals, the islets contain α-cells which produce glucagon, β-cells which produce insulin, and δ-cells which produce somatostatin. In adult zebrafish, the endocrine pancreas also includes γ-cells, which secrete pancreatic polypeptide (Argenton, Zecchin, & Bortolussi, 1999). The mammalian "mantle-core" structure of the islets is also preserved in zebrafish, with a core of β-cells surrounded by α-, δ-, and a small percentage of γ-cells in the mantle (Chen, Li, Yuan, & Xie, 2007; Li, Wen, et al., 2009). Because the endocrine pancreas of zebrafish closely resembles that of humans, zebrafish have become an appealing model for studying development and disease of this system.

α-Cells

The function of zebrafish α-cells is only partially homologous to humans and other mammals. In teleosts and mammals, the precursor to glucagon, proglucagon, also encodes glucagon-like peptide (GLP). These related hormones are spliced differently in mammalian pancreas and intestine. In the pancreas, proglucagon is converted to glucagon. In the intestine, proglucagon is converted to GLP1 and GLP2. In mammals, proglucagon is encoded by a single gene. In contrast, the zebrafish genome contains a mutated duplicate copy of proglucagon which does not encode a Glp2 sequence (Irwin & Mojsov, 2018; Roch, Wu, & Sherwood, 2009). In zebrafish, both glucagon and Glp1 are made in the pancreas, and the receptors for both hormones share a high degree of homology (Irwin & Wong, 2005). Unlike mammals, the function of zebrafish Glp1 is similar to glucagon, stimulating gluconeogenesis in the liver rather than the release of insulin.

β-Cells

Zebrafish have two insulin genes and two insulin receptor genes (Irwin, 2004; Milewski, Duguay, Chan, & Steiner, 1998; Toyoshima et al., 2008). The two insulin genes, *insa* and *insb*, are highly conserved, suggesting similar functionality. Both insulin receptor isoforms contribute to glucose level control, with single mutant fish exhibiting increased blood glucose concentrations, increased food consumption and increased body weight (Mullapudi et al., 2018; Yang et al., 2018).

Adult zebrafish have also increasingly been used to study the disease process and develop cures for diabetes. As in mammals, treating adult zebrafish with β-cell toxin streptozotocin results in islet cell destruction and secondary hyperglycemia. Unlike mammals, zebrafish are able to spontaneously regenerate lost β-cells without insulin therapy, making them valuable for studying how to replace or expand functional β-cells (Moss et al., 2009). Zebrafish are used in chemical screens to identify small molecules that increase the number of β -cells or increase insulin expression (Helker et al., 2019; Matsuda et al., 2018; Rovira et al., 2011; Wang et al., 2015). Zebrafish models of type 2 diabetes have also been generated using diet-induced obesity, which demonstrate glucose intolerance similar to type 2 diabetes in mammals. In these zebrafish, antidiabetic drugs metformin and glimepiride reduced blood glucose levels (Zang, Shimada, & Nishimura, 2017), suggesting that zebrafish can be used to study type 2 diabetes.

δ-Cells

Somatostatin is produced in the pancreas, nervous system, and gastrointestinal tract of zebrafish (Li, Wen, et al., 2009). Its primary function in the pancreas is to inhibit insulin and glucagon release from α- and β-cells. Five somatostatin genes (*sst*) were identified in the zebrafish (Tostivint, Gaillard, Mazan, & Pezeron, 2019). Somatostatin 1 (*sst1.1*) is thought to be the ortholog to mammalian somatostatin and is expressed in the tissues mentioned above (Tostivint, Lihrmann, & Vaudry, 2008). *sst2* (previously referred to as *sst4*) is orthologous to mammalian cortistatin, expressed exclusively in the pancreas except during early development (Devos et al., 2002; Tostivint, Joly, Lihrmann, Ekker, & Vaudry, 2004). *sst2* mutants grow faster than wild-type zebrafish, indicating that *sst2* is an important regulator of growth (Sui et al., 2019). *sst1.2* (previously referred to as *sst3*) is a paralog to *sst1.1*, identified in several teleost species (Tostivint et al., 2008). *sst5* is present in all teleosts, and the predicted protein product is nearly identical to Sst1.1, but the *sst5* gene appears to have been lost in the tetrapod lineage (Liu et al., 2010). Based on homology, *sst6* is likely a paralog to *sst2* (Tostivint et al., 2019). The unique and overlapping functions of the different zebrafish somatostatins have not been fully characterized.

Development of the Endocrine Pancreas

Similar to mammals, the zebrafish pancreas arises from the posterior foregut endoderm as a dorsal bud and a ventral bud, dependent on gradients of Pdx1. These buds form in zebrafish at 24 hpf and 32 hpf, but their cellular constituents begin to differentiate before the buds are visible. The dorsal bud only develops into cells of the endocrine pancreas in zebrafish, whereas in mammals this bud becomes both exocrine and endocrine pancreatic cells (Li, Wen, et al., 2009; Wang et al., 2011, 2015). Commitment to an endocrine fate requires inhibition of Notch signaling in mammals and zebrafish (Zecchin et al., 2007). Many early transcription factors expressed in islet cell differentiation are also expressed in the early central nervous system, including *neuroD* (14 hpf), which later is expressed in β-cells only (Korzh, Sleptsova, Liao, He, & Gong, 1998). Expression of insulin begins at 15 hpf, somatostatin at 17 hpf, and glucagon at 21 hpf (Biemar et al., 2001; Kinkel & Prince, 2009). Cells from both the dorsal and ventral buds contribute to the zebrafish primary β-cell islet, which starts to form around 18 hpf. Ventral bud proliferation and differentiation is roughly equivalent to the secondary transition in

mammalian pancreas formation (Tehrani & Lin, 2011). The dorsal and ventral buds fuse about 48–50 hpf to form the mature endocrine pancreas. At 72 hpf, the exocrine pancreas begins to express acinar cell markers and digestive enzymes and has surrounded the endocrine islets (Field, Dong, Beis, & Stainier, 2003). As the embryo develops and the gut rotates, the pancreas is located on the right side of the coelom. After the first week of zebrafish development, multiple secondary islets have formed (Parsons et al., 2009). Studying signaling pathways in the zebrafish pancreas has enhanced our understanding of human pancreas development and function and advanced the potential for regenerative treatments for diabetes and other diseases of the endocrine pancreas.

Growth

In zebrafish, as in other vertebrates, growth hormone-releasing hormone (Ghrh) released from the hypothalamus binds to somatotroph cells in the anterior pituitary to stimulate the release of growth hormone (Gh). The cDNA sequence of zebrafish Ghrh is identical to that of goldfish (*Carassius auratus*) and over 80% homologous to human GHRH (Lee et al., 2007). The high degree of homology among diverse vertebrates suggests a strong selection pressure to preserve growth hormone function. The growth hormone-releasing hormone receptor (Ghrhr) is a G protein-coupled 7-transmembrane receptor that mediates intracellular cAMP levels. Two growth hormone-like peptides (Ghrh-lp) have been identified in zebrafish, along with at least one receptor. Although similar in structure to Ghrh, Ghrh-lp does not efficiently activate zebrafish Ghrhr (summarized in Busby, Roch, & Sherwood, 2010). Based on the expression patterns of Ghrh-lp in the gustatory and visceral nuclei of the hindbrain, it may have functions in controlling feeding and appetite (Castro et al., 2009). The synteny of Ghrh-lp with human GHRH-LP (also referred to as PRP or pituitary adenylate cyclase-activating polypeptide-related protein) suggests a similar evolutionary origin, though humans and other mammals do not have similar receptors for these peptides (Lee et al., 2007; Wang, Li, Wang, Kwok, & Leung, 2007).

In mammals, pituitary adenylate cyclase-activating polypeptide (PACAP) affects the release of several hormones, including insulin and epinephrine. PACAP regulates carbohydrate and lipid metabolism and ovarian maturation (summarized in Busby et al., 2010). Two forms of Pacap were cloned in zebrafish (Fradinger & Sherwood, 2000; Wang, Wong, & Ge, 2003). Treatment of zebrafish pituitary cells with Pacap1 results in increased expression of *gh* transcripts (Lin & Ge, 2009). mRNA encoding receptors for Pacap1 and Pacap2, including *adcap1a*, *adcap1b* and *pac1r1* transcripts, are maternally deposited into oocytes, suggesting that Pacap may affect growth hormone signaling in zebrafish embryos (Herzog et al., 2003).

Growth hormone itself has direct and indirect effects in zebrafish. In muscle tissue, Gh acts directly to increase DNA and protein synthesis as well as increase lipolysis. Indirect effects of Gh occur via insulin-like growth factor 1 (Igf1). Transcription and secretion of Igf1 from the liver are increased in response to Gh signaling. The zebrafish genome contains one *gh* gene and two Gh receptor genes, *ghra* and *ghrb* (Liongue & Ward, 2007). In zebrafish, as in humans, mutations in the growth hormone gene (*gh1*) cause retardation of body growth (Hu, Ai, Chen, Wong, & Ge, 2019; McMenamin, Minchin, Gordon, Rawls, & Parichy, 2013).

Insulin-like growth factor is a key regulator of growth in vertebrates. Igf1, Igf2, and insulin proteins are structurally similar, and in zebrafish are encoded by a group of six related genes: *igf1, igf2a, igf2b, igf3, ins* (previously known as *insa*, *inas1*) and *insb* (previously known as *ins2*) (Argenton et al., 1999; Ayaso, Nolan, & Byrnes, 2002; Chen et al., 2001; Irwin, 2004; Maures et al., 2002; Papasani, Robison, Hardy, & Hill, 2006). There is high functional conservation among Igf1 proteins from different species, as zebrafish embryonic cells will proliferate equally after exposure to human, fish, or chicken Igf1 (Maures et al., 2002). Through knockout, knockdown, and transgenic dominant negative and overexpression studies, *igf1* has been found to affect patterning of anterior development, the nervous system, and migration of primordial germ cells to the gonadal ridge (Eivers, McCarthy, Glynn, Nolan, & Byrnes, 2004; Onuma et al., 2011; Sang, Curran, & Wood, 2008; Schlueter, Peng, et al., 2007, Schlueter, Sang, et al., 2007').

Insulin-like growth factor 2 (Igf2) is a major regulator of prenatal growth in mammals and embryonic development in zebrafish. The two *igf2* genes in zebrafish have distinct expression patterns: *igf2a* is primarily found in the notochord and *igf2b* along the dorsal midline, forebrain, and nephron primordia. Knockdown of genes for these hormones and their receptors indicate overlapping roles in dorsal patterning, with unique roles for Igf2b in the developing zebrafish brain and kidney (White, Kyle, & Wood, 2009).

Igf1 and both forms of Igf2 bind to two receptors, Igf1ra and Igf1rb, which are homologous to the human IGF1R gene. These receptors are ubiquitously expressed in zebrafish embryos (Ayaso et al., 2002; Li, Wu, Liu, Wang, & Cheng, 2014; Maures et al., 2002; Schlueter, Sang, et al., 2007). Knockdown of these receptors results in altered patterning and reduced survival in zebrafish embryos less than 30 hpf (Schlueter et al., 2006, Schlueter, Sang, et al., 2007).

Insulin and somatostatin also affect growth. *insb* is expressed in the blastomere and head during early embryo development. The insulin receptors Insra and Insrb are also required for normal growth and cell differentiation of the embryonic zebrafish brain and heart, respectively (Toyoshima et al., 2008). Additional details on insulin and somatostatin regulation are provided in the endocrine pancreas section earlier in this chapter.

Ghrelin, a peptide hormone, stimulates the release of growth hormone, but ghrelin does not appear to be essential for development in mice (Pfluger et al., 2008; Wortley et al., 2004). In zebrafish, however, knockdown of ghrelin results in reduced expression of growth hormone, delayed development, multi-organ abnormalities, and death within 10 dpf (Li, He, Hu, & Yin, 2009).

Regulation of zebrafish growth involves many of the same critical endocrine factors as in other vertebrates, including Gh, Igf, insulin, somatostatin, and ghrelin. Modern genetic techniques have identified overlapping and unique roles for these proteins in zebrafish development, facilitating comparison with mammalian models and other species. As in many organisms, control of zebrafish growth is closely tied to the body's regulation of feeding and metabolism, which are discussed in the following section.

Energy & Metabolism

In general, the mechanisms regulating energy expenditure and storage are conserved among mammals and fish. As for many other endocrine systems, zebrafish have become an important model for studying the genetics, development, and regulation of energy homeostasis.

Many of the important hormones regulating appetite can be grouped into the categories of orexigenic or central appetite-stimulating hormones, anorexic or central appetite-inhibiting hormones, and peripheral appetite hormones. The first category is comprised of neuropeptide Y (Npy), agouti-related protein (Agrp), orexin, and melanin-concentrating hormone (Mch). Npy is a potent appetite stimulant in mammals and also has been shown to regulate food intake in teleosts (Narnaware, Peyon, Lin, & Peter, 2000; Silverstein, Breininger, Baskin, & Plisetskaya, 1998). Npy is expressed in the brain of zebrafish throughout development and adulthood (Soderberg et al., 2000). Npy receptors also called *Y receptors*, are a family of G-protein coupled receptors. Zebrafish express orthologs to mammalian Y1, Y2, and Y4 receptors, as well as unique Y7, Y8a, and Y8b isoforms (Fredriksson, Sjodin, Larson, Conlon, & Larhammar, 2006; Mathieu, Trombino, Argenton, Larhammar, & Vallarino, 2005; Salaneck, Larsson, Larson, & Larhammar, 2008). The effects of signaling through many of these receptor types are still a topic of study. Npy-related peptide YY (with paralogs Pyya and Pyyb in zebrafish) is expressed in the GI tract and brain of zebrafish and also stimulates appetite (Soderberg et al., 2000; Sundstrom, Larsson, Brenner, Venkatesh, & Larhammar, 2008).

Agouti related neuropeptide (Agrp) is an antagonist for the anorexigenic melanocortin receptors. Overexpression of Agrp in zebrafish has been used as a genetic model for obesity (Song & Cone, 2007). Orexin A and B, also known as hypocretin, is expressed in the zebrafish hypothalamus (Kaslin, Nystedt, Ostergard, Peitsaro, & Panula, 2004; Song, Golling, Thacker, & Cone, 2003). In addition to regulating energy homeostasis, overexpression of the orexins leads to insomnia-like behavior in zebrafish, suggesting an important role in setting circadian cycles (Prober, Rihel, Onah, Sung, & Schier, 2006; Yokogawa et al., 2007).

Melanin-concentrating hormone (Mch, encoded by the *pmch* gene) was first discovered in fish and named for its role in regulating skin pigment (Enami, 1955; Imai, 1958; Pickford & Atz, 1957). Zebrafish contain two Mch genes, *pmch* (formerly known as *pmch2*) and *pmchl* (formerly known as *pmch1*) (Berman, Skariah, Maro, Mignot, & Mourrain, 2009). Zebrafish, humans, and rodents all have different numbers of Mch receptors, which may relate to the variable effects on appetite reported in different species (Logan et al., 2003).

Central inhibition of appetite is regulated by leptin, melatonin, and several Pomc cleavage products, such as melanocyte-stimulating hormone (Piccinetti et al., 2010). The melanocortin receptor family, especially Mc4r, is important for appetite regulation in humans, mice and zebrafish (Huszar et al., 1997; Song & Cone, 2007; Vaisse, Clement, Guy-Grand, & Froguel, 1998; Yeo et al., 1998; Zhang, Forlano, & Cone, 2012). Leptin from adipocytes, the GI tract, muscle, and brain stimulates Mc4r and melanocyte-stimulating hormone to reduce food intake. Zebrafish have two leptin sequences, though Lepa and Lepb have low protein sequence homology and are thought to have different functional roles, with Lepa as the primary appetite regulator (Gorissen, Bernier, Nabuurs, Flik, & Huising, 2009). Chronic exposure of adult zebrafish to melatonin results in decreased appetite, dose-dependent increases in *lepa* and *mc4r* transcripts, and a reduction in orexigenic peptides Ghrelin and Npy (Piccinetti et al., 2010).

Nesfatin-1 is another anorexigenic factor, one of three nesfatin proteins produced by cleavage from nucleobindin2 (Nucb2) proteins, encoded by *nucb2a* and *nucb2b* genes in zebrafish (Hatef, Shajan, & Unniappan, 2015). The function of nesfatin-2 and -3 are unknown (Schalla & Stengel, 2018a). *nucb2* genes are expressed in multiple tissues in many species, with highest levels in the liver, brain, and GI tract of zebrafish (Gonzalez, Kerbel,

Chun, & Unniappan, 2010; Hatef et al., 2015; Lin, Zhou, et al., 2014). In goldfish, injection of nesfatin-1 resulted in a reduction in food intake, consistent with anorexigenic roles reported in rodents, but similar studies in zebrafish have not been reported (Gonzalez et al., 2010). In zebrafish, expression of *nucb2*a and *nucb2b* mRNA in the brain is decreased 3 h after feeding, as well as after 7 days of fasting (Hatef et al., 2015). Zebrafish fed a probiotic (*Lactobacillus rhamnosus*) in conjunction with a high-fat diet had reduced Nucb2/nesfatin-1 immunoreactivity in the GI tract and decreased food intake, suggesting a link between nesfatin-1 and microbiome-driven regulation of appetite (Falcinelli et al., 2017). Though the full mechanism of action has not been elucidated in zebrafish, studies of nesfatin-1 in rats and chicks indicate that the protein may act through melanocortin and corticotropin-releasing factor pathways to inhibit food intake (Schalla & Stengel, 2018a).

Phoenixins (Pnx), a recently discovered family of peptide hormones, regulate energy and metabolism in several species (Schalla & Stengel, 2018b; Stein, Haddock, Samson, Kolar, & Yosten, 2018; Wang et al., 2018). Phoenixin is co-expressed with nesfatin-1 in the hypothalamus of rats and in spotted scat (*Scatophagus argus*), a euryhaline subtropical fish from the Indo-Pacific (Wang et al., 2018). In addition to gene expression patterns, increased expression of Pnx following a period of fasting and subsequently decreased expression following re-feeding suggest an anorexigenic role, but more research is needed to confirm this in zebrafish and explore the proposed link between Pnx and Gh signaling (Wang et al., 2018). How phoenixins influence appetite and feeding behavior in zebrafish has yet to be fully explored.

In the last two decades, zebrafish have become a popular animal in which to study obesity and other metabolic diseases. Despite significant differences in lifestyle and size from humans, zebrafish share many of the endocrine pathways controlling feeding and metabolism. Zebrafish, through manipulation of gene expression and function, altered diets, and/or microbiota, are proving to be an important tool in understanding the molecular and cellular physiology of energy homeostasis (Faillaci et al., 2018).

Conclusion

Endocrine systems in humans and zebrafish are structurally different but functionally similar. For example, zebrafish lack anatomically distinct parathyroid and adrenal glands, and the zebrafish thyroid is a loose chain of follicles rather than a discrete organ as in humans. However, the hormones secreted by the human parathyroid, thyroid, and adrenal glands are present in zebrafish and function similarly in both species.

Another feature of the zebrafish endocrine system is that zebrafish embryos are competent to respond to hormones at developmental stages before hormone synthesis begins. For example, female zebrafish deposit thyroid hormones and thyroid hormone receptor mRNA into oocytes. Thus, following oocyte fertilization, the embryo is competent to respond to thyroid hormone signaling before the embryo produces its own thyroid hormones and receptors. The same is likely true for other hormones, such as estrogens (Romano et al., 2017). Similarly, many mammalian embryos are exposed to endocrine hormones via transplacental transfer. Thus, while structurally different (zebrafish lack a placenta), the functional outcome of transplacental transfer occurs in zebrafish via maternal deposition of hormones into oocytes.

There is much we do not know about how endocrine function influences health and welfare in zebrafish. For many hormones, especially steroids, we lack the ability to reliably and reproducibly measure hormone levels in zebrafish embryos and larvae. There are no diagnostic assays that can be used to assess hormone levels as a reliable biomarker for health or disease in zebrafish. It is not known at what developmental stage zebrafish can first respond to stress by changing cortisol levels. Regarding energy and metabolism, there is a paucity of data on how dietary and microbiota composition influence growth, sex ratios, or fecundity. It is not clear how exposure to light and darkness during embryonic and larval stages influences metabolism, or whether such metabolic changes are retained through adulthood. With so many interesting questions to be answered, the zebrafish will be a valuable animal in which to study endocrinology for years to come.

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CHAPTER

18

Zebrafish Nervous Systems

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The Central Nervous System

Development of the central nervous system

Cells of the prospective central nervous system (CNS) can be identified by expression of *sox* genes and traced back to specific cells at the early blastula stage (16-cell stage). The neural plate is detectable by the end of gastrulation at 10 h postfertilization (hpf) with distinct anterior-posterior domains corresponding to the major CNS divisions in the adult (Helde, Wilson, Cretekos, & Grunwald, 1994; Woo & Fraser, 1995). In teleost fish, the neural tube forms via secondary neurulation, which involves the cavitation of an initially compact mass of cells, the neural rod (Lowery & Sive, 2004; Miyayama & Fujimoto, 1977; Reichenbach, Schaaf, & Schneider, 1990). At early somite stages, the neural plate is formed into a solid rod via complex cell movements and intermediate structures (Fig. 18.1A) (Clarke, 2009; Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). The rod undergoes cavitation to form a lumen and transforms into a neural tube around 24 hpf by aligning mirror-symmetric dividing neural progenitors and establishing apicobasal polarity along the midline (Tawk et al., 2007). The first neuronal clusters and pioneering axon tracts are established prior to the formation of the neural tube. The first postmitotic neurons are detected at early somite stages, and first neuronal nuclei and axon tracts are detected around 18 hpf (Kim et al., 1996; Wilson, Ross, Parrett, & Easter, 1990). Patterning of the neural tissue into region-specific territories already starts during gastrulation and the major brain regions, such as forebrain (telencephalon, diencephalon), midbrain (mesencephalon), hindbrain (metencephalon), and spinal cord are morphologically discernible around 1 day postfertilization (dpf) (Fig. 18.1B–D (Grinblat, Gamse, Patel, & Sive, 1998; Hauptmann, Soll, & Gerster, 2002; Wilson & Rubenstein, 2000). Neural development proceeds quickly in zebrafish and the majority of the adult neuronal cell types, nuclei, and circuits are formed and functional around the time of hatching at 3 dpf and continue to grow at further larval stages (Fig. 18.1E). At the time of hatching the zebrafish larva displays relatively complex behaviors and is able to react to different sensory stimuli, for example, the escape response, where larvae rapidly swim away from a threat, which can be elicited already in the 2 dpf larva. However, more complex behaviors, such as social interactions and shoaling, gradually develop over many weeks in the juvenile zebrafish (for a recent review see (Orger & de Polavieja, 2017).

Organization and Function of the Main Brain Regions and Spinal Cord

The zebrafish brain has a relatively typical morphology and shape for a teleost fish without overt enlargement of any brain region (Fig. 18.2). The olfactory bulbs, starting rostrally, extend directly from the telencephalic main body. The forebrain is comprised of the telencephalon and diencephalon. The telencephalon consists of a dorsal structure that forms two well discernible hemispheres that entirely covers the ventral part. At the telencephalic and diencephalic junction, the habenula and pineal gland are readily visible from the dorsal side and the optic nerves and chiasm at the ventral side. The midbrain is dominated by the dorsally located paired optic tectal lobes. Ventrally four paired lobes form the hypothalamus. A prominent pituitary is found in close proximity beneath the main body of the hypothalamus. Posterior to the optic tectum a nonfoliated round body forms the cerebellum and associated cerebellar structures. Posterior to the cerebellum lies the medulla oblongata that contains two paired

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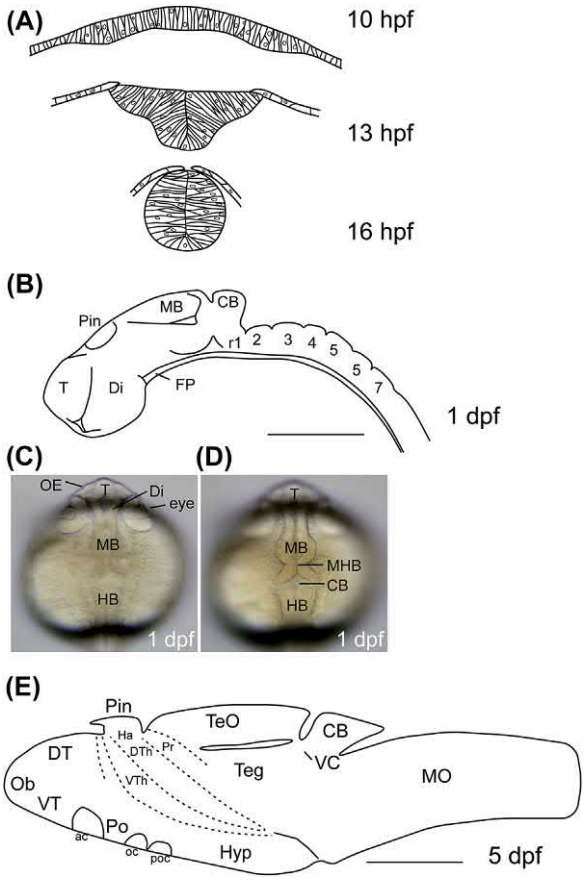

FIGURE 18.1 **Early development of the neural tube and schemata of the major brain regions at 1 and 5 days postfertilization.** (A) The neural plate (top, 10 h postfertilization (hpf) folds inwards at the midline to form the neural keel (middle, 13 hpf). The neural keel rounds to develop into the neural rod (bottom, 16 hpf). (B) The major brain regions, such as forebrain (telencephalon, diencephalon), midbrain (mesencephalon), hindbrain (metencephalon), and spinal cord, are morphologically discernible around 1 day postfertilization (dpf). (C) Dorsal view of the forebrain including the forming eye and olfactory epithelium of a 1-day old live zebrafish embryo. (D) Dorsal view of the midbrain, hindbrain and the prominent midbrain-hindbrain boundary of a 1-day old live zebrafish embryo. (E) At 5 dpf, the major brain regions are further subdivided into distinct nuclei. *ac*, anterior commissure; *CB*, cerebellum; *Di*, diencephalon; *DT*, dorsal telencephalon; *DTh*, dorsal thalamus; *Pin*, pineal (epiphysis); *FP*, floor plate; *Ha*, habenula; *HB*, Hindbrain; *Hyp*, hypothalamus; *hpf*, hours postfertilization; *MO*, medulla oblongata; *MB*, midbrain; *MHB*, midbrain-hindbrain boundary; *Ob*, olfactory bulb; *oc*, optic chiasm; *OE*, olfactory epithelium; *Po*, preoptic area; *poc*, postoptic commissure; *Pr*, pretectum; *r*, rhombomeres; *Teg*, tegmentum; *T*, telencephalon; *TeO*, optic tectum; *VC*, valvula cerebelli; *VT*, ventral telencephalon; *VTh*, ventral thalamus. Scale bar = 200 um in B, = 100 um in E. *(A,B) Adapted from Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., Schilling, T. F. (1995). Stages of embryonic development of the zebrafish.* Developmental Dynamics: An Official Publication of the American Association of Anatomists, 203*(3) 253–310 doi:10.1002/aja.1002030302*; *(E) Adapted from Mueller, T., Wullimann, M. F. (2002). BrdU-, neuroD (nrd)- and Hu-studies reveal unusual non-ventricular neurogenesis in the postembryonic zebrafish forebrain.* Mechanisms of Development, 117*(1–2), 123–135.*

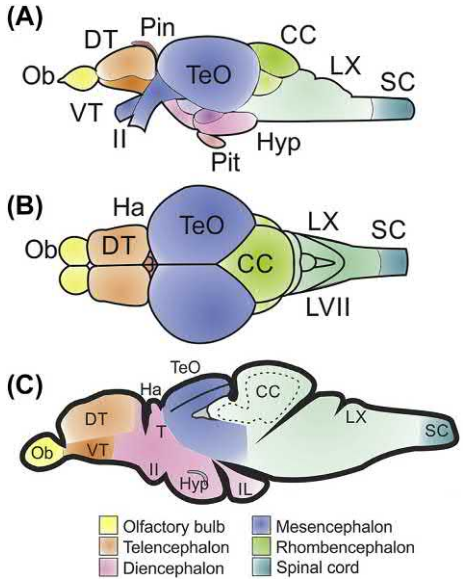

FIGURE 18.2 **Overview of the main brain parts.** Whole-mount side (A) and dorsal (B) view of the adult zebrafish brain. (C) Sagittal section through the adult zebrafish brain. *CC*, corpus cerebelli; *DT*, dorsal telencephalon; *Ha*, habenula; *Hyp*, hypothalamus; *IL*, inferior lobe; *LX*, vagal lobe; *Ob*, olfactory bulb; *Pin*, pineal (epiphysis); *SC*, spinal cord; *TeO*, optic tectum; *T*, thalamus; *VT*, ventral telencephalon; *II*, second cranial nerve (optic nerve); *LVII*, facial lobe.

smaller lobes, the facial and vagal lobes—difficult to discern well macroscopically—that taper off toward the spinal cord. In the next part, we provide an overview of the main brain parts, their morphology, and function.

The Telencephalon and Olfactory Bulb

The rayfin teleost zebrafish telencephalon is an enigmatic and debated brain part because of the challenges in comparing its anatomy and function with other vertebrate lineages. In rayfin fish, it develops through eversion of the neural tube and complex morphogenetic movements in contrast to the better-understood evagination process of the telencephalon in all other vertebrates (Folgueira et al., 2012). This has resulted in difficulties to pinpoint relevant counterparts in zebrafish for nuclei and circuits that are important for mammalian key behaviors and human diseases, such as cortex, hippocampus, striatum, and amygdala. However, recent works studying molecular markers, imaging of telencephalic development and functional analysis of behavior have rapidly improved the understanding of the teleost telencephalon (Folgueira et al., 2012; Ganz et al., 2012, 2014; Mueller & Guo, 2009; Mueller, Wullimann, & Guo, 2008; Perathoner, Cordero-Maldonado, & Crawford, 2016).

The olfactory bulb is composed of four layers, olfactory nerve, glomerular, mixed mitral cell/plexiform, and granule cell layers (Byrd & Brunjes, 1995). The zebrafish telencephalon consists of two major parts, a dorsal pallial and a ventral subpallial part (Figs 18.1, 18.2, 18.3A–C). Each of these parts can be further subdivided into smaller subregions based on morphological, molecular, and functional differences (Butler & Hodos, 2005; Northcutt, 1995). Two major axons tracts, the medial and lateral forebrain bundle, run through the telencephalon into the diencephalon and prominent axon tracts and cross the hemispheres via the anterior commissure that lies approximately at the midpoint along the anterior-posterior axis of the telencephalon. The dorsal telencephalon consists of several continuous neural nuclei mapped around the perimeter of the hemisphere and named accordingly: medial (Dm), dorsal (Dd), central (Dc), lateral (Dl), and posterior (Dp) part. Equivalents of mammalian neocortex and hippocampus are thought to be located in the dorsal telencephalon based on molecular markers, developmental origin, input/output, and role in modulating behaviors (Ganz et al., 2014; Mueller et al., 2008; O'Connell & Hofmann, 2011; Yamamoto & Oda, 2015). The zebrafish pallium, like other teleost fish, lacks a clear layered structure similar to the neocortex of mammals. However, teleost fish display distinct nuclear masses that on the circuit level may share similar architecture with some of

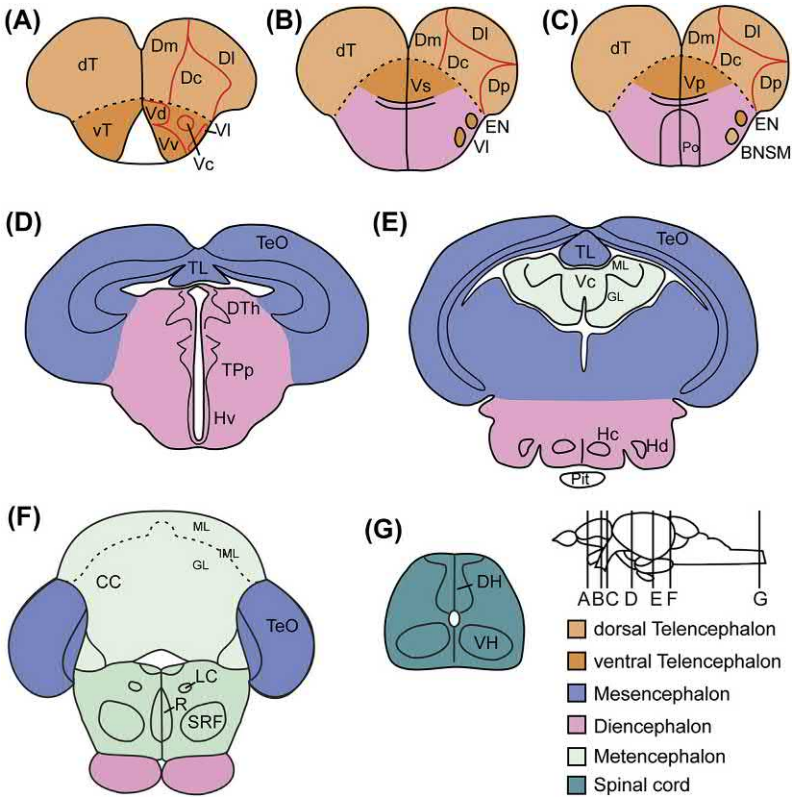

FIGURE 18.3 **Main nuclei in the telencephalon, diencephalon, and mesencephalon.** (A) Cross section at a rostral level; the ventral telencephalon (vT) is subdivided in a ventral (Vv), dorsal (Vd), central (Vc), and lateral (Vl) nucleus, the dorsal telencephalon (dT) is subdivided in a medial (Dm), central (Dc) and lateral (Dl) nucleus. (B) At the level of the anterior commissure, Vd is contiguous with the supracommissural nucleus (Vs), dT is subdivided in Dm, Dc, Dl, and posterior (Dp) nucleus. (C) More caudally, Vs is contiguous with the postcommissural nucleus (Vp), dT is subdivided in Dm, Dc, Dl, and Dp. Ventral to Vp is the preoptic area (Po), part of the diencephalon. Ventral to dT, the entopeduncular nucleus (EN), and the bed nucleus of the stria medullaris (BNSM) are found. (D) The two lobes of the optic tectum (TeO) overlay different diencephalic nuclei including the dorsal thalamus (DTh), the posterior tubercle nucleus (TPp), and the ventral hypothalamus (Hv). (E) The two lobes of the TeO overlay the torus longitudinalis (TL) and the valvula cerebelli (Vc). The Vc consist of a molecular layer (ML) and granule cell layer (GL). Ventrally, the four paired lobes that form the hypothalamus include the caudal hypothalamus (Hc) and dorsal hypothalamus (Hd). The pituitary gland (Pit) is located ventral to the hypothalamic nuclei. (F) The cerebellum (CC) consists of a three-layered cortex: a molecular layer (ML), an intermediate layer (IML) and the granule cell layer (GL). Lateral to the CC lies the TeO. Ventral to CC, the locus coeruleus (LC), the raphe (R) and the superior reticular formation (SRF) are located. (G) In the spinal cord, sensory neurons are located in the dorsal nucleus or dorsal horn (DH). The motor neurons are located in the ventral nucleus or ventral horn (VH). A-E Cross sections at the level indicated in the schema. *Schema modified from Ganz, J., Kroehne, V., Freudenreich, D., Machate, A., Geffarth, M., Braasch, I., et al. (2014). Subdivisions of the adult zebrafish pallium based on molecular marker analysis.* F1000Res, 3 *308 doi:10.12688/f1000research.5595.2; Wullimann, M. F., Rupp, B., Reichert, H. (1996). Neuroanatomy of the zebrafish brain: A topological atlas. Basel; Boston: Birkhäuser Verlag.*

the basal circuits of the mammalian cortex (Ito and Yamamoto, 2009) and the interplay between several of the dorsal nuclei is thought to serve neocortical like functions. The lateral part of the dorsal pallium has been proposed to serve hippocampal like functions because of its involvement in memory and spatial location (Broglio et al., 2005; O'Connell & Hofmann, 2011).

The subpallium consists of two major components, the striatopallidal complex and the septum (Butler & Hodos, 2005; Northcutt, 1995). The striatopallidal complex, consisting of the striatum and the pallidum, is a part of the basal ganglia, which play a key role in the control of movement (Butler & Hodos, 2005). In zebrafish, the rostral part of the ventral telencephalon is comprised of four main nuclei: a dorsal (Vd), ventral (Vv), central (Vc), and lateral (Vl) part (Fig. 18.3A–C). At the level of the anterior commissure, the Vd is contiguous with the supracommissural nucleus (Vs) and more caudally with the postcommissural nucleus (Vp). At this level, two additional nuclei can be identified, the entopeduncular nucleus (EN), and the bed nucleus of the stria medullaris (BNSM) that based on molecular markers also originate from the telencephalon (Ganz et al., 2012; Mueller & Guo, 2009). Based on molecular markers, neurochemical, and connectional data, Vd and Vv have been suggested to be the equivalent to the basal ganglia (striatum-pallidum) with the ventral most part of Vv being equivalent to the septum. The dorsal part of Vs has been proposed to be homologous to the central amygdala, whereas the ventral most part has been suggested to be homologous to the bed nucleus of the stria terminalis (Ganz et al., 2012; Mueller et al., 2008; Mueller & Guo, 2009). Although our understanding of the teleost telencephalon has progressed, it is still not equal to mammalian systems on cell, circuit, physiology, and behavior levels. This makes comparisons of brain parts of the rayfin teleost telencephalon and areas in the telencephalon of all other vertebrates challenging.

Diencephalon

The diencephalon consists of the preoptic region, habenula, thalamic nuclei, posterior tuberculum, pretectum, and hypothalamus (Figs. 18.1, 18.2 and 18.3D,E). The habenula is located dorsal to the ventral thalamus and consist of a dorsal and ventral nucleus. Based on connections and molecular markers, the zebrafish dorsal habenula has been proposed to be analogous to the medial habenula of mammals (Aizawa et al., 2005; Gamse et al., 2005) and the ventral habenula to the mammalian lateral habenula (Amo et al., 2010). The zebrafish thalamic nuclei are situated dorsally in proximity to the junction between the telencephalon and the optic tectum. Although there are similarities in layout and function between the zebrafish and the mammalian thalamus, there are also differences; for example, thalamocortical connections are lacking in zebrafish (Mueller, 2012). The pretectum is located in the caudal part of the diencephalon and relays signals from the optic tectum to various brain centers (Yanez, Suarez, Quelle, Folgueira, & Anadon, 2018). The largest part of the diencephalon is the hypothalamus, which is subdivided into ventral, dorsal, and caudal parts (Wullimann, Rupp, & Reichert, 1996). The hypothalamus controls central aspects of physiological homeostasis and behavior. It regulates the endocrine hormone released through the pituitary gland that controls stress response and growth (Gutnick & Levkowitz, 2012). It also connects to the autonomic nervous system and other brain parts to control body temperature, feeding, and sleep (Biran, Tahor, Wircer, & Levkowitz, 2015; Machluf, Gutnick, & Levkowitz, 2011; Xie & Dorsky, 2017).

Mesencephalon

The midbrain (mesencephalon) is dominated by the large dorsally located lobes of the optic tectum (Figs. 18.2 and 18.3D,E), which process visual information. The optic tectum sits on top of the diencephalic nuclei, and is thus, accessible for circuit analysis (Marachlian, Avitan, Goodhill, & Sumbre, 2018; Nevin, Robles, Baier, & Scott, 2010). It mainly receives sensory inputs from the retina, and its main function is to detect and process sensory stimuli and generate appropriate motor responses, for example, prey capture and visual escape responses (Marachlian et al., 2018). Other midbrain regions, such as the dopaminergic substantia nigra or ventral tegmental area are difficult to pinpoint because of the close proximity to diencephalic nuclei and lack of markers for early embryonic midbrain and diencephalic regions (Panula et al., 2010; Vaz, Outeiro, & Ferreira, 2018; Xie & Dorsky, 2017).

Metencephalon

Dorsally, the cerebellum and its associated structures form a macroscopically easily distinguishable, nonfoliated smooth lobe posterior to the paired lobes of the optic tectum (Figs. 18.2 and 18.3). The general organization and cellular architecture of the cerebellum are highly conserved in vertebrates. The cerebellum of all jawed vertebrates consists of a major lobe, the corpus cerebelli (cerebellar corpus) and two bilateral lobes, the auricle (also known as the vestibulocerebellum). The architecture of the cerebellum in zebrafish is highly similar to other vertebrates, but there are some notable differences (for review see Kaslin & Brand, 2012). The most striking difference is the lack of deep cerebellar nuclei and of a well-defined white matter. The cerebellar corpus in zebrafish consists of a single folium, and it has an anterior extension, the valvula cerebelli, which extends into the tectal ventricle below the optic tectum

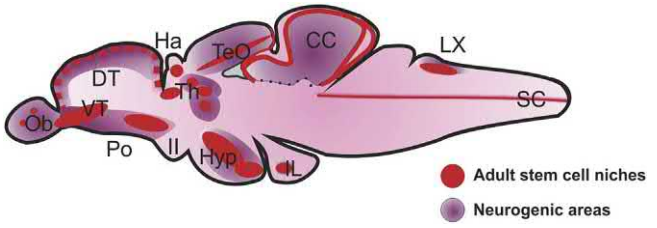

FIGURE 18.4 **Zones of proliferation and neurogenesis in the adult zebrafish brain.** Sagittal view of the zebrafish brain; proliferation and neurogenesis occurs along the entire rostrocaudal axis of the brain. Proliferation and neurogenesis also continue into the adult spinal cord. *DT*, dorsal telencephalon; *CC*, cerebellum; *Ha*, habenula; *Hyp*, hypothalamus; *IL*, inferior lobe; *LX*, vagal lobe; *Ob*, olfactory bulb; *Po*, preoptic area; *SC*, spinal cord; *TeO*, optic tectum; *Th*, thalamus; *VT*, ventral telencephalon; *II*, second cranial nerve (optic nerve). *Modified from Kaslin, J., Ganz, J., Brand, M. (2008). Proliferation, neurogenesis and regeneration in the non-mammalian vertebrate brain.* Philosophical Transactions of the Royal Society of London B Biological Sciences, *363(1489), 101–122 doi:10.1098/rstb.2006.2015*

(Fig. 18.3E,F). The zebrafish cerebellum consists of a three-layered cortex: a molecular layer, an intermediate layer, and the granule cell layer (Fig. 18.3E,F). Zebrafish display obvious equivalents in cell types to mammalian counterparts based on developmental origin, morphology, marker expression and circuitry, including stellate, Golgi, Purkinje, and granule cells, as well as Bergmann glia (Kaslin & Brand, 2012; Kaslin et al., 2009). In addition, zebrafish and other teleost fish have an additional cell type within the cerebellar cortex, the eurydendriod cell that may be equivalent to the deep cerebellar nuclei of other vertebrates. The cerebellum is important for integrating sensory information and motor control for precise motor behavior, as well as cognitive functions (Hibi & Shimizu, 2012; Kaslin & Brand, 2012).

Posterior to the cerebellum, two less pronounced lobes, the vagal and facial lobe, are located. The vagal and facial lobe process gustatory information and receive sensory projections from both the facial and vagal nerves that provide the extrinsic innervation of the gut (Yanez, Souto, Pineiro, Folgueira, & Anadon, 2017). Several specific neuronal clusters are of notable interest in the hindbrain, because of their roles in controlling diverse functions and behaviors, for example, the serotonergic neurons of the raphe nuclei and the noradrenergic locus coeruleus (Fig. 18.3F) (Kaslin & Panula, 2001; Vaz et al., 2018). In addition, the hindbrain contains several large primary sensory and motor nuclei from the V-Xth cranial nerves, as well as prominent columns of cells in the reticular formation.

In addition, the very large and distinct Mauthner neuron is part of the reticular formation. The Mauthner neuron and reticulospinal neurons play an important role in the escape response that has been very well studied at circuit level (O'Malley, Kao, & Fetcho, 1996; Orger & de Polavieja, 2017).

Spinal Cord

The zebrafish spinal cord has a similar layout, as found in all vertebrate classes. It has dorsal and ventral gray matter areas equivalent to dorsal and ventral horns in mammals (Fig. 18.3G). The spinal cord contains sensory neurons, motor neurons, and a high diversity of interneurons, including central canal contacting neurons, such as GABAergic Kolmer-Agduhr cells that are located in the ependymal layer (Djenoune & Wyart, 2017; Lewis & Eisen, 2003). The interplay of the different neuron types controls locomotor behaviors (Berg, Bjornfors, Pallucchi, Picton, & El Manira, 2018). In the embryo, primary motor neurons have a stereotypical position and projection pattern within each segment. They are individually identifiable by their cell body position and their axon projection (Eisen, Myers, & Westerfield, 1986; Myers, Eisen, & Westerfield, 1986). The later-developing secondary neurons are smaller and more numerous (Eisen et al., 1986; Lewis & Eisen, 2003).

Adult Neurogenesis and Regeneration

Adult neurogenesis—the generation of new neurons in the adult—occurs to a different extent in divergent vertebrate lineages. In contrast to mammals that have limited neurogenesis in their adult brains, zebrafish display widespread adult neurogenesis along the entire rostrocaudal axis of the brain and spinal cord (Fig. 18.4) (Adolf et al., 2006; Grandel, Kaslin, Ganz, Wenzel, & Brand, 2006). This constitutive generation of new neurons relies on the presence of stem cells located in stem cell niches at ventricular zones of the brain and spinal cord (Kaslin, Ganz, & Brand, 2008; Lindsey et al., 2018). The ability to continuously produce new neurons in the adult has been linked to the remarkable ability of the zebrafish to regenerate injuries to their nervous system (Kaslin et al., 2008; Kizil, Kaslin, Kroehne, & Brand, 2012).

The Peripheral Nervous System

The peripheral nervous system (PNS) consists of the neurons and ganglia outside the brain and the spinal cord and provides the connection between the CNS and the organs in the body. The neural crest is a transient, multipotent cell population, which arises at the dorsal border of the developing nervous system (Le Douarin, 1982). Among the multiple cell types that the neural crest generates are the neurons and glial cells of the PNS (Le Douarin, 1982). The PNS is subdivided into the somatic PNS and the autonomic nervous system (ANS). The somatic PNS consist of sensory and motor nerves, such as sensory ganglia that transmit information from peripheral targets to the CNS. Sensory ganglia include the dorsal root ganglia (DRGs) that transmit

somatosensory information to the CNS. The ANS is divided into the enteric nervous system, the sympathetic nervous system, and the parasympathetic nervous system. The ANS innervates the smooth muscles, skin, exocrine glands, and internal organs (Le Douarin, 1982).

Development and Function of Dorsal Root Ganglia

The dorsal root ganglia (DRG) consists of segmentally arranged bilateral sensory neurons and glial cells. The DRGs are located adjacent to the ventrolateral part of the neural tube (Fig. 18.5B) (An, Luo, & Henion, 2002). The DRG sensory neurons transmit different stimuli from the body, such as touch, temperature, perception of pain (nociception), and sense of limb movement and position (proprioception) to the CNS. Around 2 dpf, only one to three neurons are present in each DRG, but this number postembryonically increases until over 100 neurons are found in 28 dpf fish (An et al., 2002; McGraw, Snelson, Prendergast, Suli, & Raible, 2012).

Development and Function of the Enteric Nervous System

The enteric nervous system (ENS) is the largest part of the peripheral nervous system and provides the intrinsic innervation of the intestinal tract or gut. The ENS forms a complex network of diverse neuronal and glial subtypes that regulate all gut functions, including motility, secretion, blood flow, nutrient uptake, and homeostasis (Furness, 2006). In zebrafish, as in other vertebrates, most ENS cells are derived from vagal neural crest progenitor cells that migrate from the postotic hindbrain, enter the foregut, migrate caudally to colonize the entire gut, and then differentiate into neurons and glia (Ganz, 2018; Olden, Akhtar, Beckman, & Wallace, 2008). Enteric progenitor cells enter the anterior part of the developing gut at around 32 hpf, and then migrate into two bilateral streams until they reach the end of the gut (Olden, Akhtar, Beckman, & Wallace, 2008) (Elworthy, Pinto, Pettifer, Cancela, & Kelsh, 2005; Olden, Akhtar, Beckman, & Wallace, 2008; Shepherd, Pietsch, Elworthy, Kelsh, & Raible, 2004). Enteric neurons differentiate anteriorly, while enteric progenitor cells are still migrating in the posterior part of the gut (Olden, Akhtar, Beckman, & Wallace, 2008). Enteric neurons are found as scattered cells or small neuronal clusters located in a single layer, the myenteric plexus, sandwiched between the circumferential and longitudinal smooth muscle cells (Fig. 18.5A,C) (Wallace, Akhter, Smith, Lorent, & Pack, 2005). ENS neurons innervate the smooth muscle cells and interact with the interstitial cells of Cajal (Furness, 2006). At least 10 subpopulations of ENS neurons have been identified in zebrafish based on different neurochemical markers,

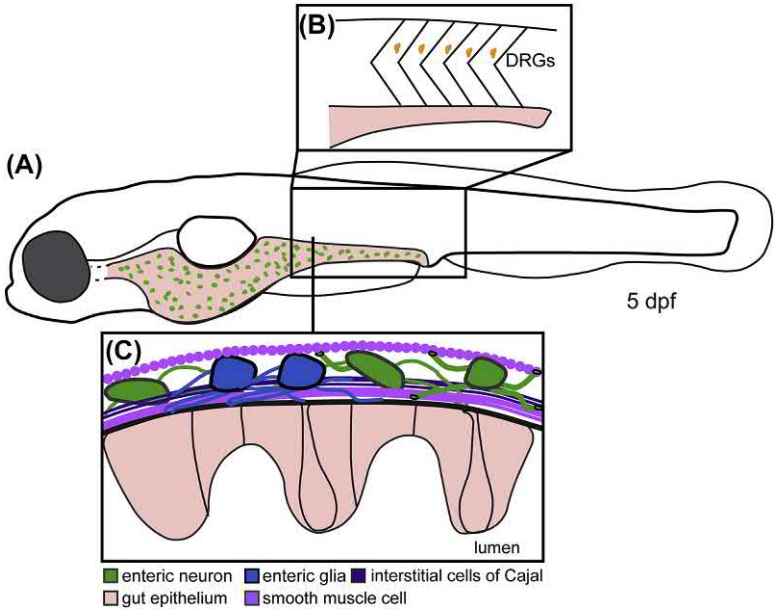

FIGURE 18.5 Overview of the enteric nervous system and dorsal root ganglia. (A) Schema of a 5 dpf zebrafish larvae showing the location of the enteric nervous system (*green dots*) innervating the gut (light pink). (B) Dorsal root ganglia (DRGs, orange) consist of segmentally arranged bilateral sensory neurons and are located adjacent to the ventrolateral part of the neural tube. Boxed area in (A) shows location of close-up. (C) Cross-section through the gut at the level indicated in (A) shows that ENS neurons (green) and glial cells (blue) are sandwiched between the smooth muscle cells (purple) that sit on top of the gut epithelium (light pink) and adjacent to the interstitial cells of Cajal (dark blue). *Modified from Ganz, J. (2018). Gut feelings: Studying enteric nervous system development, function, and disease in the zebrafish model system.* Developmental Dynamics: An Official Publication of the American Association of Anatomists, 247*(2), 268–278 doi:10.1002/dvdy.24597.*

including neurotransmitters and neuropeptides (Uyttebroek et al., 2010; Uyttebroek, Shepherd, Hubens, Timmermans, & Van Nassauw, 2013).

Development and Function of Sympathetic Neurons

The sympathetic neurons are divided into a rostral cervical and a trunk population. These neurons are located ventral to the notochord and adjacent to the dorsal aorta as two rows of neurons (An et al., 2002). Sympathetic neurons regulate different aspects of body function and homeostasis, for instance, vascular tone, exocrine glands, and gut function (Le Douarin, 1982). In zebrafish, sympathetic neurons initially are present as single neurons at larval stages, then they aggregate into small ganglia and continue to increase in numbers to reach ganglia size of around 50 neurons at 28 dpf (An et al., 2002).

Conclusions

As parts and function of the zebrafish nervous system continue to be investigated, we are developing a better idea of homologies between brain regions of zebrafish and other vertebrate model species. The unique experimental advantages of the zebrafish model system, such as the ability to perform whole-brain activity studies in combination with genetic manipulations, will advance our understanding of neuronal development, function, and what goes awry in human nervous system diseases.

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CHAPTER

19

Immunology

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Introduction

Zebrafish have been used to study the pathogenicity of bacterial, viral, and eukaryotic pathogens, as well as the immune response to such pathogens [see Chapter 51 and (Lieschke & Trede, 2009; Masud, Torraca, & Meijer, 2017; Traver et al., 2003a; Trede, Langenau, Traver, Look, & Zon, 2004; Yoder, Nielsen, Amemiya, & Litman, 2002)]. The transparent nature of the zebrafish embryo allows the observation of immune cells interacting with pathogens in real time in vivo (Herbomel & Levraud, 2005; Torraca, Masud, Spaink, & Meijer, 2014). The high quality annotated zebrafish reference genome (Howe et al., 2013) permits thorough and accurate analyses of transcriptional changes in response to infection (Stockhammer, Zakrzewska, Hegedûs, Spaink, & Meijer, 2009). As few antibodies are available for labeling and sorting different hematopoietic lineages, transgenic zebrafish have been generated in which specific immune cell lineages are labeled by expression of fluorescent proteins. These transgenic lines have been employed to investigate immune cell function and in-vivo behavior in response to injury and infection (Masud et al., 2017). Zebrafish, in which immune genes are either knocked-down or -out, have been used to study the roles of various genes in hematopoiesis, immune cell function, or host defense.

The zebrafish adaptive immune system is mediated by B and T lymphocytes whose receptors undergo V(D)J recombination (Castro et al., 2011; Fillatreau et al., 2013; Seelye, Chen, Deiss, & Criscitiello, 2016; Wienholds, Schulte-Merker, Walderich, & Plasterk, 2002; Willett, Cherry, & Steiner, 1997a). Zebrafish B cells produce three immunoglobulin isotypes IgM, IgD, and IgZ (aka IgT) (Fillatreau et al., 2013). Although the adaptive immune system provides immunological memory, it is not fully functional in the embryonic and larval stages (Willett, Cortes, Zuasti, & Zapata, 1999). During these early life stages, individual zebrafish rely on the innate immune system (e.g., macrophages and neutrophils) for defense against pathogens, which are augmented by maternally derived immune molecules (Wang, Ji, Shao, & Zhang, 2012, 2009).

Innate immunity in zebrafish is mediated by monocytes/macrophages, neutrophils, dendritic cells, mast cells, and there is growing support for NK cell lineages. Transgenic zebrafish expressing a fluorescent protein in macrophages or neutrophils, have contributed significantly to the understanding of how these cells migrate to and interact with sites of infection or injury (Ellett, Pase, Hayman, Andrianopoulos, & Lieschke, 2011; Mathias et al., 2006; Renshaw et al., 2006; Sanderson et al., 2015; Walton, Cronan, Beerman, & Tobin, 2015). Although the zebrafish genome encodes Toll-like receptors (TLRs) and cytokines that share orthology with mammalian genes, its genome also encodes a number of genes within these families that are "fish-specific." In addition, a number of "fish-specific" receptor families that are predicted to function in immunity have been described (Wcisel & Yoder, 2016). This chapter will highlight the major features of the cellular and molecular components of the zebrafish immune system, including those that are shared with mammals and those that are fish-specific.

Cellular Components of the Immune System and Their Lineage Markers

With the zebrafish being a relatively new model for the study of hematopoiesis and immunity, the availability of tools and reagents to study specific cell types has only recently emerged. Over the past decade, many transgenic

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TABLE 19.1 Representative transgenic zebrafish lines for hematopoietic studies.

Promoter	Reporter	Tissue	References
lmo2	GFP, DsRed	Pre-hematopoietic, vasculature	(Lin et al., 2005; Zhu et al., 2005)
kdrl	EGFP, DsRed	Pre-hematopoietic, vasculature	(Cross et al., 2003; Jin et al., 2007)
fli1a	EGFP, DsRed	Pre-hematopoietic, vasculature	(Jin et al., 2007; Lawson & Weinstein, 2002)
itga2b	GFP	EMPs, HSCs, thromobocytes	(Lin et al., 2005)
$runx1^{promoter\ 1}$	GFP	EMPs	(Lam et al., 2009)
$runx1^{promoter\ 2}$	GFP	HSCs	(Lam et al., 2009)
myb	GFP	HSCs, neural	(Bertrand, Kim, Teng, & Traver, 2008)
ptprc	DsRed	Pan-leukocyte	(Bertrand et al., 2008)
gata1a	GFP, DsRed	Red blood cells	(Long et al., 1997; Traver et al., 2003b)
spi1b	EGFP	Myeloid and immature hematopoietic cell	(Hsu et al., 2004)
mpeg1.1	GAL4, mCherry	Embryonic macrophages	(Ellett et al., 2011; Tokarz et al., 2017)
csf1ra	mCherry	Macrophages	(Gray et al., 2011)
mfap4	TdTomato, Turquoise2,YFP	Macrophages	(Walton et al., 2015)
mpx	EGFP	Adult neutrophils	(Mathias et al., 2006; Renshaw et al., 2006)
lyz	EGFP, DsRed	Adult neutrophils	(Hall et al., 2007)
gata2a	EGFP	Eosinophils	(Balla et al., 2010)
cd4-1	EGFP	T lymphocytes, macrophages	(Dee et al., 2016)
cd4-2	EGFP	T lymphocytes	(Dee et al., 2016)
fox3pa	EGFP	Regulatory T lymphocytes	(Kasheta et al., 2017)
rag2	EGFP, DsRed	Immature B and T cells	(Langenau et al., 2003, 2007)
lck	EGFP	Mature T cells	(Langenau et al., 2004)
ighm	EGFP	B Cells	(Page et al., 2013)
ighz	mCherry	B Cells	(Page et al., 2013)
mhc2dab	GFP, AmCyan	B cells, macrophages, dendritic cells	(Wittamer et al., 2011)

lines have been created to dissect the cellular components of the immune system, which are compiled in Table 19.1. We will reference many of these resources as each of the major immune cell types are described below.

Hematopoiesis and Immune-Related Tissues

In all animals studied, from flies to humans, developmental hematopoiesis during embryogenesis occurs in multiple waves and within shifting anatomical sites of blood cell production (Cumano & Godin, 2007; Martinez-Agosto, Mikkola, Hartenstein, & Banerjee, 2007). Developmental hematopoiesis in mammals and teleosts occurs in four sequential waves. The first two are termed "primitive," with each generating transient precursors that respectively give rise to embryonic myeloid cells and erythrocytes (Cumano & Godin, 2007; Davidson & Zon, 2004; Palis & Yoder, 2001). In zebrafish, primitive red cells form within the developing cardinal vein to rapidly oxygenate embryonic tissues once circulation ensues at around 26 h post-fertilization (hpf). Primitive myeloid cells consist of both macrophages and neutrophils. The former distributes throughout the animal, providing a first wave of phagocytes to act in host defense (Herbomel, Thisse, & Thisse, 1999; Herbomel & Levraud, 2005). Primitive macrophages have also been observed to directly colonize the brain to generate embryonic microglia, the tissue-resident macrophages (TRMs) of the central nervous system (Herbomel, Thisse, & Thisse, 2001). Ongoing studies suggest that this primitive population of microglia is later replaced by permanent, definitive precursors, raising the possibility that each microglial subset differs in its ability to provide immune protection

(Ferrero et al., 2018; Xu et al., 2015). The next two waves of developmental hematopoiesis consist of "definitive" hematopoietic precursors, defined as multipotent progenitors that generate adult cell types. The first to arise are erythromyeloid progenitors (EMPs) that give rise to erythroid and myeloid lineages (Bertrand et al., 2007; Frame, McGrath, & Palis, 2013; Palis, 2016). Recent lineage-tracing studies in the mouse have suggested that the murine EMP is the founder of most TRMs (Gomez Perdiguero et al., 2015; Kierdorf, Prinz, Geissmann, & Gomez Perdiguero, 2015; Mass et al., 2016), possibly providing an important component of immunity to each major organ system. These studies, however, are controversial due to the difficulty of interpreting complex lineage tracing data. The fourth and final wave of developmental hematopoiesis is the generation of multipotent hematopoietic stem cells (HSCs), which have the potential to both self-renew and generate all adult hematopoietic cell types (Dzierzak & Speck, 2008; Medvinsky & Dzierzak, 1998; Morrison, Uchida, & Weissman, 1995). The HSC is the keystone cell of the adult hematopoietic system, giving rise to the vast majority of leukocytes that act as the primary defense against pathogens.

Whereas HSCs are born from a specialized population of endothelial cells comprising the floor of the dorsal aorta (Bertrand et al., 2010; Bollerot, Pouget, & Jaffredo, 2005; Dzierzak & Speck, 2008; Kissa & Herbomel, 2010), they rapidly leave this site to colonize subsequent hematopoietic organs. In the zebrafish, the first site of HSC colonization is the venous plexus in the ventral tail that has been termed the *caudal hematopoietic tissue* (CHT) (Murayama et al., 2006). Here the first HSC-derived leukocytes are born, which distribute rapidly throughout the developing larva. By 3 days post-fertilization (dpf), HSC daughters colonize the thymic rudiment to initiate the formation of T lymphocytes (Trede et al., 2004). Thymic education appears to occur within 4–5 days, as the first circulating T lymphocytes are observed at 7–8 dpf (Trede et al., 2004). The CHT remains active for several weeks (Murayama et al., 2006), and it is here that the first generation of multilineage hematopoiesis occurs. During this time of robust production of adult hematopoietic cells, the pronephros is colonized. Hematopoietic stem and progenitor cells (HSPCs) can be visualized within the developing kidney by 4–5 dpf. This site gradually increases hematopoietic output until it becomes the predominant site of hematopoietic production in the zebrafish.

Blood production in adult zebrafish, like other teleosts, occurs in the kidney, which supports both renal functions and multilineage hematopoiesis (Zapata, 1979). Similar to mammals, T lymphocytes develop in the thymus (Trede & Zon, 1998; Willett et al., 1999) (Fig. 19.1), which exists in two bilateral sites in zebrafish (Hansen & Zapata, 1998; Willett, Zapata, Hopkins, & Steiner, 1997b). The teleostean kidney is a sheath of tissue that runs along the spine (Fig. 19.1); the anterior portion, or head kidney, shows a higher ratio of blood cells to renal tubules than does the posterior portion (Zapata, 1979), termed the *trunk kidney* (Fig. 19.1B,C). All mature blood cell types are found in the kidney and morphologically resemble their mammalian counterparts (Fig. 19.1G), with the exceptions that erythrocytes remain nucleated, and thrombocytes perform the clotting functions of platelets (Jagadeeswaran, Sheehan, Craig, & Troyer, 1999). Histologically, the zebrafish spleen (Fig. 19.1D) has a simpler structure than its mammalian counterpart in that germinal centers have not been observed (Zapata & Amemiya, 2000), although relatively small clusters of B lymphocytes have been observed in the channel catfish spleen (Saunders, Oko, Scott, Fan, & Magor, 2010). The absence of immature precursors in the spleen, or any other adult tissue, suggests that the kidney is likely the sole hematopoietic site in adult zebrafish. The cellular composition of whole kidney marrow (WKM), spleen, and blood are shown in Fig. 19.1F–H. Morphological examples of all hematopoietic cell types are presented in Fig. 19.2.

B Lymphocytes

Unlike T cells, which are rapidly generated from HSPCs in the CHT, the process of B cell generation takes much longer, with IgM^+ cells arising between 12 and 18 dpf along the posterior aorta (Page et al., 2013). These B cell precursors then appear to migrate to the pronephros, which continues as the site of B cell production for the remainder of life. These results suggest that B cell development shifts from the CHT to the kidney, as the fish age, similar to a developmental shift in mammals, whereby B cell precursors in the fetal liver home to and engraft in the bone marrow for the remainder of life (reviewed in Douagi, Vieira, & Cumano, 2002). These findings in zebrafish suggest that shifting sites of B cell development is a feature of all jawed vertebrates. The late generation of B lymphocytes, relative to other leukocytes and other vertebrate organisms, indicates that antibody-mediated immune responses are not utilized during zebrafish embryonic and larval stages. Rather, the innate immune system appears sufficient to protect juvenile animals from the immune challenge.

In the adult animal, different classes of B lymphocyte are present, each defined by the isotype of immunoglobulin presented as their B cell receptor and secreted as antibodies upon activation (see Molecular components of the adaptive immune system section for details on immunoglobulins). The isotype of the immunoglobulins

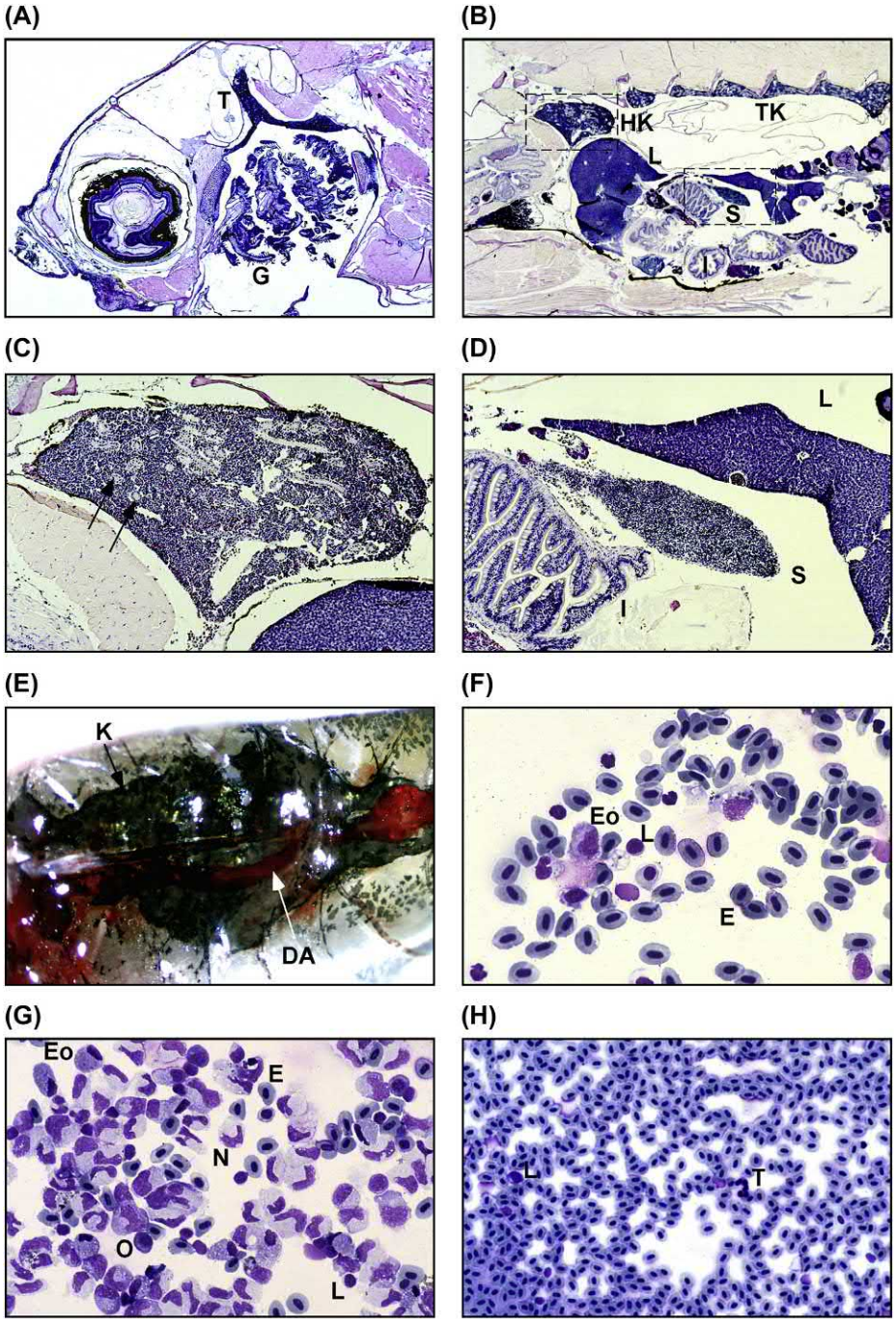

FIGURE 19.1 **Histological analyses of adult hematopoietic sites.** (A) Sagittal section showing the location of the thymus [T], which is dorsal to the gills [G]. (B) Midline sagittal section showing the location of the kidney, which is divided into the head kidney [HK], and trunk kidney [TK], and spleen [S]. (C) The head kidney shows a higher ratio of blood cells to renal tubules (*black arrows*), as shown in a close-up view of the HK. (D) Close up view of the spleen [S], which is positioned between the liver [L] and the intestine [I]. (E) Light microscopic view of the kidney [K], over which passes the dorsal aorta [DA, white arrow]. (F) Cytospin preparation of splenic cells, showing erythrocytes [E], lymphocytes [L], and an eosinophil [Eo]. (G) Cytospin preparation of kidney cells showing cell types as noted above, plus neutrophils [N] and erythroid precursors [O, orthochromatic erythroblast]. (H) Peripheral blood smear showing occasional lymphocytes [L] and thrombocyte [T] clusters among mature erythrocytes. (A–D) Hematoxylin and Eosin stains, (F–H) May-Grünwald/Giemsa stains. *Figure reproduced with permission from Stachura, D. L., Traver, D. (2016). Cellular dissection of zebrafish hematopoiesis.* Methods in Cell Biology, *133 11–53.*

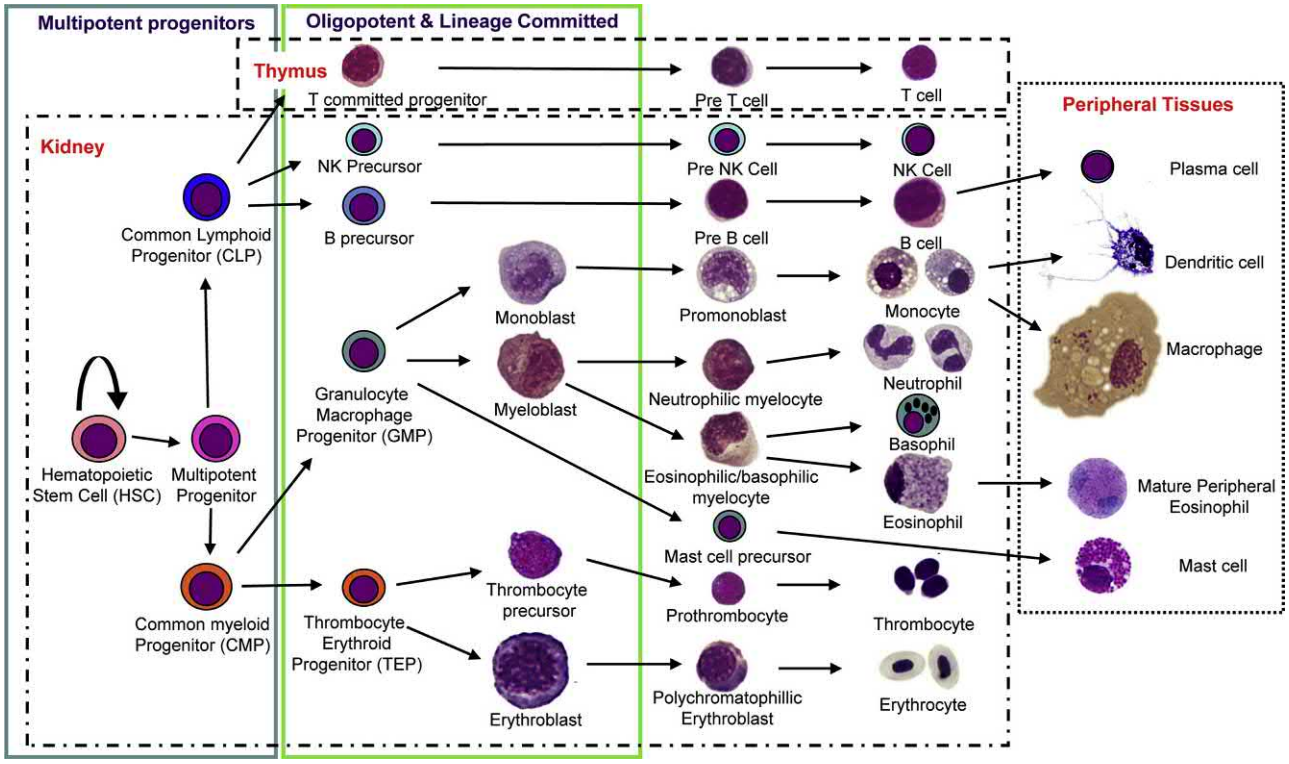

FIGURE 19.2 **Proposed model of zebrafish definitive hematopoietic differentiation.** Isolated, cytospun, and stained blood cells from the zebrafish kidney, thymus, and peripheral tissues and their proposed upstream progenitors. Proposed lineage relationships are based on those demonstrated in clonogenic murine studies. *Figure reproduced with permission from Stachura, D. L., Traver, D. (2016). Cellular dissection of zebrafish hematopoiesis.* Methods in Cell Biology, *133 11–53.*

varies among jawed vertebrates. Most teleosts have three immunoglobulin isotypes, IgM, IgD, and IgZ (IgZ is named IgT in some teleosts) and two types of B cells: IgM/IgD-expressing B cells and IgZ-expressing B cells (Danilova, Bussmann, Jekosch, & Steiner, 2005; Schorpp et al., 2006). In contrast, mammals have five immunoglobulin isotypes (IgM, IgD, IgG, IgE, IgA), and birds, amphibians, and reptiles have three to five isotypes (Danilova & Amemiya, 2009). One commonality is that all jawed vertebrates possess IgM, as well as an isotype that appears to provide mucosal immunity: IgA in mammals and birds, IgX in amphibians, and IgZ/IgT in most teleosts (Danilova & Amemiya, 2009; Zhang et al., 2010). Thus, jawed vertebrates have evolved different ways of producing a diversified B cell population that can respond to both systemic and mucosal insults.

A variety of fluorescent transgenic lines, including some that employ promoters from the IgM and IgZ heavy chain genes (*ighm* and *ighz* which encode IgHμ and IgHζ, respectively), have been utilized to prospectively isolate and study B lymphocytes and their precursors in the zebrafish (Table 19.1). In particular, double transgenic *Tg(ighm:EGFP)* x *Tg(rag2:DsRed)* animals have been used to define pro-, pre-, and immature/mature B cell populations via their differential expression of *IgM*, *rag2*, and *pax5* (Page et al., 2013). The recent description of transgenic zebrafish expressing GFP under control of CD79 gene regulatory sequences provide another means to specifically identify B lymphocytes. Mammalian CD79 proteins associate in the plasma membrane with the B cell receptor to transduce signals received by these membrane immunoglobulin receptors (Chu & Arber, 2001; Neuberger et al., 1993). In the zebrafish, the CD79 gene has been duplicated to yield CD79a and CD79b proteins (Liu et al., 2017a). Examination of animals carrying both a *Tg(CD79:EGFP)* transgene and a *Tg(rag2:mCherry)* transgene revealed that, unlike in mammals, B cell development in zebrafish appears to occur without passing through a requisite pre-B cell stage, defined phenotypically as $Rag2^{hi}$ $CD79^{+}$ $IgH\mu^{+}$ (Liu et al., 2017a). In addition, crossing *Tg(ighm1:EGFP)* fish with *Tg(cd45:DsRed)* fish can be used to visualize and isolate B cells, T cells, and monocytes. This is due to a unique feature of the CD45-transgenic line, where expression of DsRed is expressed in all major leukocyte subjects except B cells (Wittamer, Bertrand, Gutschow, & Traver, 2011), in contrast to the endogenous cd45 expression in all leukocytes. Thus, flow cytometric analysis of *Tg(ighm1:EGFP)* x *Tg(cd45:DsRed)* animals yields $GFP^{+}IgM^{+}$ B cells and $DsRed^{+}$ T cells. In addition,

Tg(cd45:DsRed) x *Tg(blimp1:GFP)* double transgenic animals have been utilized to visualize and isolate presumptive plasma B cells based upon their $CD45^{-}Blimp1^{+}$ phenotype. Thus, some basic tools exist to further studies of B cell ontogeny and maturation in the zebrafish.

These tools have also provided the means to study the B lymphoid immune response in zebrafish. Relative to mammals, the teleost immune response is slower. Whereas activated, germinal center B cells appear in murine lymphoid tissue 2–4 days after immunization and plasma B cells appear in bone marrow by 7–10 days (reviewed in Tarlinton & Smith, 2000), significant numbers of total B cells or antigen-binding B cells do not appear until 10–14 days post-immunization in the zebrafish. These results are consistent with IgM secretion not being detected until 4 weeks after intraperitoneal (i.p.) immunization of zebrafish and trout (Gong, Xiang, & Shao, 2009; Swan, Lindstrom, & Cain, 2008). In the zebrafish, i.p. immunization led to increased B cell numbers in the kidney, spleen, gut, and peritoneal exudate, with the most dramatic increases in antigen-binding B cells in the spleen and gut (Page et al., 2013). Correspondingly, these sites were reported to be likely sites of immune responses to bacterial and viral infections in zebrafish (Pereiro et al., 2015; Pressley, Phelan, Witten, Mellon, & Kim, 2005; Vojtech, Sanders, Conway, Ostland, & Hansen, 2009). The spleen has also been reported to be a major secondary lymphoid organ in other bony fish (reviewed in Zwollo, 2011). Although neither classical germinal centers nor lymph nodes have been described in fish, germinal center-like structures were found in the spleen of catfish (Saunders et al., 2010). With improved transgenic animals, appropriate reagents now exist to more precisely address these issues in the zebrafish.

T Lymphocytes

Early studies identified several genes involved in the developmental specification of T lymphocytes, including *ikaros*, *gata3*, *rag1*, *rag2* and *lck* (Trede, Zapata, & Zon, 2001; Willett et al., 1997a, 2001), enabling the study of T cell formation in the zebrafish. By 3 dpf, the bilateral thymic rudiments are seeded by early hematopoietic precursors to generate thymocytes. While it was initially assumed that these first thymic emigrants were the descendants of HSCs formed from hemogenic endothelium comprising the aortic floor, recent lineage tracing studies have suggested that a dedicated population of T cell precursors arises before HSCs during zebrafish development (Tian et al., 2017). Activation of an indelible lineage label via targeted confocal microscopy showed that both cells in the posterior blood island (PBI) and in the aortic floor generated T cell precursors. Interestingly, descendants of the PBI generated $CD4^{+}$ thymocytes only whereas the progeny of the aorta generated both $CD4^{+}$ and $CD8^{+}$ thymocytes. Temporal labeling suggested that the precursors seeding the thymus before 8 dpf are HSC-independent, and only after 8 dpf do HSC-derived precursors generate thymocytes. These are compelling results, and may shed light upon earlier studies in the mouse showing that T cell precursors are present in the extraembryonic yolk sac before HSC emergence within the embryo proper (Eren, Zharhary, Abel, & Globerson, 1987; Godin, Dieterlen-Lièvre, & Cumano, 1995; Liu & Auerbach, 1991; Palacios & Imhof, 1993; Yoshimoto et al., 2012). These findings nicely illustrate the types of experiments possible in the zebrafish that are not feasible in other vertebrate models. The first thymic emigrants can be observed to enter circulation by 7–8 dpf, when they initiate their role as immune sentries. It remains to be determined if this first wave of T cells, derived independently of HSCs, display different functions from later waves, including the generation of γδ T cells that are known to arise early in mammals (Allison, 1993; Cheng et al., 1991; Hedrick & Sharp, 1998; Ikuta et al., 1992; Rothenberg, 1992; Tonegawa et al., 1989).

γδ T cells represent one of the two major types of T lymphocytes, the other defined by expression of αβ T cell receptor (TCR) genes (see Molecular components of the adaptive immune system section for information on TCR genes). γδ T cells show a more limited repertoire of γδ TCR gene segments and possess a variety of different functions from αβ T cells, most notably in their ability to respond to so-called *unconventional antigens*. Unlike αβ T cells, which are restricted to recognize peptide antigens presented by Major Histocompatibility Complex (MHC) class I (MHCI) or class II (MHCII) complexes (via $CD8^{+}$ "cytotoxic" T cells or $CD4^{+}$"helper" T cells, respectively), γδ T cells respond more broadly to antigens derived from bacterial lipids and metabolites (Cheng et al., 1991; Tonegawa et al., 1989). The TCRs of γδ T cells often serve as pattern recognition receptors, and are, thus, thought to serve as "innate" T cells able to respond to common antigenic stimuli following colonization by pathogens. γδ T cells are rare relative to αβ T cells, representing only 1%–10% of the total T cell pool in human peripheral blood (Hayday, 2000). However, their abundance is higher at sites of mucosal immunity, including the skin, intestines, lung, and reproductive tract (Nielsen, Witherden, & Havran, 2017; Steele, Oppenheim, & Hayday, 2000). The γδ TCR loci were recently studied in the zebrafish, and antibodies were raised against the constant regions of each of the four TCR subunits, α, β, γ, and δ (Wan et al., 2016). Isolation of γδ T cells showed them to be $CD4^{-}$ and $CD8^{+}$, similar to γδ T cells in mammals, and tissue

localization studies showed the highest proportions to exist in the skin, gills, and gut. These authors also demonstrated that zebrafish γδ T cells possessed the functional attributes of murine γδ T cells, including phagocytosis of particulates and the ability to stimulate the production of antigen-specific IgZ molecules from B cells lining the intestinal mucosa (Wan et al., 2016). Thus, these studies demonstrate that zebrafish possess both major subtypes of T cells, indicating that these T cell subsets had been specialized before the split of the bony fishes from tetrapods during vertebrate evolution.

Two main types of αβ T cells have been identified in fish based on the expression of CD4 or CD8 (Castro et al., 2011). Although fish genomes frequently contain a duplicated CD4 gene termed CD4-2, this variant shows a non-canonical immunoglobulin structure and appears to only be expressed in a minor subset of T cells (Takizawa et al., 2016). It thus remains to be determined what functions this unusual CD4 molecule encode. Antibodies have been generated that specifically recognize zebrafish CD4-1, and CD8α (Miyazawa, Matsuura, Shibasaki, Imamura, & Nakanishi, 2016). CD4 transgenic zebrafish have been recently described, where a BAC transgene faithfully recapitulates the endogenous expression of zebrafish *cd4-1* (Dee et al., 2016). This line has been exceptionally useful in elucidating the anatomical localization of $CD4^+$ T cells in all tissues and has enabled the study of the diverse subsets of T cells present within this population. Isolation of $CD4^+$ T cells by flow cytometry has demonstrated that a *fox3pa*$^+$ population is present within the gut mucosa, suggesting these cells may include regulatory T (Treg) cells that suppress the activation of autoreactive T cell clones that escape negative selection in the thymus. Similarly, $CD4^+$ T cells within the gills express *interleukin-4* and *interleukin-13*, markers of resident Th2-type cells in mammals. A more recent study by Kasheta et al. (2017) has made use of a *Tg(fox3pa:EGFP)* transgenic animal to further study the biology of Tregs. Combined expression with a *Tg(rag2:mCherry)* animal demonstrated that expression of *Tg(fox3pa:EGFP)* initiates during thymic development and that Tg(*fox3pa:EGFP*)$^+$ cells expressed only *cd4-1* and not *cd8a* (Kasheta et al., 2017). Interestingly, *cd4-2* was expressed in peripheral *Tg(fox3pa:EGFP)*$^+$ cells, and at higher levels than within bulk *Tg(lck:EGFP)*$^+$ T cells, suggesting a possible role for this understudied CD4 orthologue. A comparison of the whole genome transcriptome between zebrafish *Tg(fox3pa:EGFP)*$^+$ cells and an established murine dataset showed a strong correlation in gene signatures, suggesting the two populations have an evolutionarily conserved gene expression pattern. Also consistent with the premise that *Tg(fox3pa:EGFP)*$^+$ cells are Tregs are the findings in *fox3pa* mutant animals (Kasheta et al., 2017; Sugimoto, Hui, Sheng, Nakayama, & Kikuchi, 2017). Here, profound expansion of lymphocytes occurred at the expense of myeloid cell development, which correlated with chronic hyperinflammation, increased expression of inflammatory cytokines, and death. These findings are consistent with a normal suppressive role of this newly described *Tg(fox3pa:EGFP)*$^+$ Treg-like population. Taken together, the development of new transgenic lines and antibodies that mark each of the major T cell subsets has enabled the study of diverse T cell subsets and assays for their unique functions. Collectively, these results also indicate that each of the major T cell subsets identified and studied extensively in the murine model was present during evolution in the shared ancestor of teleosts and tetrapods.

Natural Killer (NK) Cells

Mammalian natural killer (NK) cells were first characterized in 1975 for having the ability to recognize and kill tumor cells without any prior induction period (Herberman, Nunn, Holden, & Lavrin, 1975). Like cytotoxic T lymphocytes, NK cells directly lyse target cells to maintain normal homeostasis of the host. Unlike T cells, NK cells are not MHC restricted but rather are activated to kill via recognition of "nonself" molecules (e.g., stress-related or virally encoded surface markers) or by the loss of endogenous markers of self (e.g., "missing self"). NK-like lymphocytes (large and granular) were observed from carp 30 years ago (Bielek, 1988). Although clonal NK-like cell lines have been derived from alloantigen stimulated catfish peripheral blood leukocytes (PBLs) (Shen et al., 2002; Yoder, 2004), the tools for isolating NK cells from any fish species are limited.

Although surface markers for identifying NK cells are not well conserved among vertebrate lineages, candidate NK receptors that would differentiate between self and nonself, have been described from teleosts including zebrafish (see Fish-specific families of innate immune receptors section). Antibodies have been developed against a putative zebrafish NK receptor, novel immune-type receptor 9 (Nitr9), which have proven useful for molecular analysis, but less effective for cell purification techniques (Shah et al., 2012). The field, thus, needs improved techniques for isolation of zebrafish NK cells to further their study. One recent paper described single-cell transcriptomics (Moore et al., 2016), where a hematopoietic cell population expressing an NK-lysin gene, *nkl.4*, was also found to coexpress the *lck*, *tcra*, *tcrb*, and *interleukin-7 receptor*. Interestingly, this population did not express *cd4* or *cd8*, thus, likely representing a type of NKT cell. In the absence of T lymphocytes in *rag2* mutant animals, this population expanded over 10-fold, consistent with previous reports in the mouse where *rag2*-deficient animals show

expansions of NK cells (Shinkai et al., 1992; Wang et al., 1996). Another similar report using single-cell transcriptomics also describes gene expression sets indicative of zebrafish T lymphocytes and NK cells within splenic *Tg(lck:EGFP)*$^{+}$ cells (Carmona et al., 2017), and proposes a series of NK-specific markers. Finally, a similar single-cell transcriptomics paper revealed another cell population, in this case from a *Tg(lck:EGFP)*$^{-}$ fraction of kidney hematopoietic cells, that also showed expression of NK-associated markers (Tang et al., 2017). Further work will be necessary to develop the tools to prospectively identify each of these NK-related cell subsets for functional studies. Interestingly, all reports demonstrate less conservation between zebrafish and murine NK cells than between similar T cell subsets, reflecting the fact that NK cells in different taxa have developed different means of allorecognition and self-recognition via relatively rapid evolution of different receptor subtypes (Yoder & Litman, 2011).

The rejection of allogeneic and xenogeneic tissue grafts is known to be mediated by NK cells (Manilay & Sykes, 1998; Vilches & Parham, 2006). Recent efforts in the zebrafish to generate mutant lines better able to tolerate foreign tissue grafts suggest that the cell types discussed above include NK-like cells. Langenau and colleagues have generated a number of mutant lines where key components of T lymphocytes and NK cells have been targeted to ablate each lineage (Moore et al., 2016). Targeted mutagenesis of the *prkdc* gene led to the near-complete loss of B and T lymphocytes. Cells expressing the NK-lysin genes, however, appeared to be spared. While this mutant line could engraft zebrafish muscle cells having MHC mismatches, it could not accept xenografts using the mouse or human donor cells. That these cells cannot engraft in the absence of T lymphocytes suggests that NK cells are present in the *prkdc* mutants and responsible for graft rejection. This notion is consistent with NK cells being unaffected in mouse *prkdc* mutants (Dorshkind, Pollack, Bosma, & Phillips, 1985). Improved precision in understanding these effects awaits the development of transgenic lines utilizing gene regulatory sequences from each specific NK-like cellular subset.

Mononuclear Phagocytes

Teleosts, in general, possess the major myeloid lineages observed in mammals. Monocytes and macrophages appear similar to those of mammals in terms of ultrastructure and cytochemistry, and phagocytic and secretory abilities (Hanington et al., 2009; Hodgkinson, Grayfer, & Belosevic, 2015; Neumann, Stafford, Barreda, Ainsworth, & Belosevic, 2001; Secombes et al., 2001). The mononuclear phagocyte system (MPS) comprises monocytes, tissue macrophages, and dendritic cells, as well as their lineage-committed progenitor cells (Hume, 2006), with its primary function being the clearance of pathogens via phagocytosis. Mononuclear phagocytes are also key in the removal of apoptotic cell corpses during embryonic development and in adults to maintain tissue homeostasis. In mice, embryonic macrophages colonize several structures bound to be removed during development, including interdigital tissues, the hyaloid vasculature and pupillary membranes (Lang & Bishop, 1993; Sasmono et al., 2003). In addition, macrophages residing in hematopoietic tissues support erythroblast proliferation and differentiation and engulf dying erythrocytes in the spleen (Kawane et al., 2001; Yoshida et al., 2005). The capacity to engulf a wide array of particles relies upon the existence of different surface receptors such as scavenger receptors (e.g., Marco and CD163), or Toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns (PAMPs) (Barton & Medzhitov, 2002; Plüddemann, Neyen, & Gordon, 2007). Following phagocytosis, macrophages and dendritic cells can activate antigen-specific T lymphocytes, a process dependent upon MHCII molecules.

The first macrophages in the zebrafish embryo originate from the anterior lateral plate mesoderm (ALPM) at approximately 20 hpf, and migrate over the yolk before colonizing other tissues (Herbomel et al., 1999, 2001), including the brain parenchyma to generate the first microglia (Herbomel et al., 2001). These primitive macrophages can be visualized in vivo using a variety of transgenic lines in which fluorescent protein expression is driven by myeloid-specific promoter sequences (Table 19.1), including *Tg(spi1b:EGFP)*, *Tg(lyz:EGFP)*, or *Tg(mpx:EGFP)* (Hall, Flores, Storm, Crosier, & Crosier, 2007; Hsu et al., 2004; Mathias et al., 2006; Renshaw et al., 2006; Ward et al., 2003). However, in the embryo and larva, these promoters are not specific to macrophages as each is also expressed in primitive granulocytes which also arise from the ALPM just after primitive macrophage emergence (Le Guyader et al., 2008). More recently, macrophage-specific reporter lines have been generated using the *mpeg1.1* promoter (Ellett et al., 2011), the *csf1ra* promoter (Gray et al., 2011) and the *mfap4* promoter (Walton et al., 2015). Crossing these lines to others, specifically marking neutrophils, for example, has allowed the direct imaging of how these first-line defenders respond in real time against microbial infection. These studies have demonstrated that neutrophils home rapidly to a site of infection and exit the wound site after only a period of hours, whereas macrophages enter the site much more slowly and persist there for days (Gray et al., 2011). Similar live imaging approaches have demonstrated distinct modes of migration for each cell type through interstitial tissues (Barros-Becker, Lam, Fisher, & Huttenlocher, 2017). Generation of macrophage-specific reporter lines have also

led to the exploration of previously unknown roles in developmental events, including their assistance in HSC emergence (Travnickova et al., 2015), stripe formation (Parichy, Ransom, Paw, Zon, & Johnson, 2000; Parichy & Turner, 2003; Patterson & Parichy, 2013), and vasculogenesis (Fantin et al., 2010).

When the term MPS was coined, it was believed that all populations of macrophages ultimately derived from HSCs, with each subset being replenished from circulating monocytic precursors as needed (Hume, 2006). Work in recent years, however, indicates that macrophage ontogeny is more complex, with many populations of TRMs being seeded during embryogenesis by precursors independent from HSCs (Ginhoux & Guilliams, 2016; Hoeffel & Ginhoux, 2018; Kierdorf et al., 2015, 2013; Mass et al., 2016). Interestingly, these TRM populations appear to self-renew for life and are not replenished by bone marrow-derived precursors unless required through loss by wounding or disease. This system appears to have evolved early, as recent studies in the zebrafish have demonstrated that multiple waves of microglia are present. Xu et al. (2015) demonstrated via targeted, Cre-based transgenic reporter recombination that the first wave of microglia develops from ALPM-derived primitive macrophages, followed by generation of adult microglia derived from HSPCs emerging from the aortic floor. Further studies are needed to determine if similar waves of TRMs are seeded during embryonic or larval development.

Another branch of the MPS generates dendritic cells (DCs), which act as immune sentries throughout most tissues. In the mouse, it has been demonstrated that DCs are by far the most potent antigen-presenting cells (APCs), able to activate antigen-specific T lymphocytes and bridge the innate and adaptive immune systems to orchestrate an immune response (Steinman, 2007). In fish, however, DCs have not received much attention. By utilizing an in-vivo phagocytosis assay in zebrafish, it was demonstrated that a subset of phagocytes morphologically resembles the DCs of higher vertebrates (Lugo-Villarino et al., 2010). This subset, however, was relatively rare, so was enriched via affinity to the lectin PNA to enable assays for APC function in the zebrafish. Compared to unfractionated WKM, DCs could be enriched over 500-fold via prospective isolation utilizing light scatter characteristics, PNA positivity, and neutrophil depletion using an *Tg(mpx:EGFP)* transgenic animal. A subset of PNA^{hi} myeloid cells displayed the classic morphological, histochemical, and ultrastructural features of mammalian DCs, and engulfed labeled bacterial preparations in vivo. In addition, purified PNA^{hi} myeloid cells robustly upregulated gene products following LPS stimulation associated with mammalian DC function, including *IL-12, MHCII invariant chain* and *mcsfr.* Importantly, PNA^{hi} cells could activate T lymphocytes in an antigen-dependent manner. Collectively, these results suggest that the cellular constituents responsible for antigen presentation were present in the ancestors common to teleosts and mammals and that their characteristics and functions have been highly conserved over evolutionary time.

Granulocytes

Histochemical analyses of various zebrafish tissues have identified several distinct subsets of granulocytes, including neutrophils, eosinophils, and mast cells. Due to their abundance and the availability of lineage-specific transgenic lines noted above, neutrophils have received the widest attention. This ancient cell type appears to be remarkably well-conserved among vertebrate animals, patrolling tissues for danger. When encountering pathogens, neutrophils work to neutralize the threat via phagocytosis, the release of noxious compounds, recruitment of other leukocyte populations, and via formation of neutrophil nets that result upon the expulsion of their nuclei to the extracellular milieu (Hong, 2017; Yin & Heit, 2018). With the zebrafish becoming a preeminent model for host-microbe studies, the roles of neutrophils in bacterial and fungal colonization have been well-characterized (Harvie & Huttenlocher, 2015). A study by Huttenlocher and colleagues took advantage of the clarity of the zebrafish larva and photoconverted a fluorescent Dendra2 molecule in neutrophils that had homed to a wound site (Yoo & Huttenlocher, 2011). These neutrophils were then observed to repeat forward and reverse migration between the wound and nearby vasculature before dispersing throughout the body as the wound healed. These studies set the stage for further work on neutrophil reverse migration, where a compound screen identified a small molecule able to enhance the resolution of the neutrophil inflammatory response in a sterile wounding assay (Robertson et al., 2014). Of note, this compound, tanshinone IIA, was likewise able to block the inflammatory response in human neutrophils, highlighting the utility of the zebrafish to identify new drugs targeting these key mediators of inflammation.

Zebrafish infection models have shown neutrophils to play a variety of roles, including protection against bacterial invasion following intravenous administration (Harvie, Green, Neely, & Huttenlocher, 2013), clearance of mycobacteria within tuberculous granulomas (Yang et al., 2012), and formation of protective granulomas following *Mycobacterium abscessus* infection (Bernut et al., 2016). In addition, zebrafish neutrophils have been demonstrated to play a variety of lesser-known roles. While the specific branch of the vertebrate

immune system is known to be critical in the response against viral infection, recent work from Levraud and colleagues has shown neutrophils to play a critical role in the containment of Chikungunya Virus, an arbovirus that has re-emerged as an expanding mosquito-borne disease vector. Zebrafish infected with Chikungunya Virus largely clear the virus over several days in a type I interferon-dependent manner (Palha et al., 2013). Interestingly, neutrophils were not infected by the virus and were the critical cell type in producing type I interferons. Ablation of neutrophils led to increases in disease severity and mortality, demonstrating their key protective role. In addition to producing interferons, zebrafish neutrophils also generate TNFα, another important mediator of immune defense against infection. In another surprising role for neutrophils, a recent study demonstrated that the production of TNFα by neutrophils was necessary for the proper emergence of HSCs during zebrafish development (Espín-Palazón et al., 2014). Primitive neutrophils that emerge from the ALPM were shown to produce TNFα that was necessary for reception via TNFR2 present on aortic endothelium. TNFR2 ligation was required for the expression of Jagged1a, a ligand for the Notch receptor Notch1a that is required for the emergence of HSCs from hemogenic endothelium. Thus, the unique strengths of the zebrafish system have led to a variety of novel discoveries regarding this ancient cell type that is required for survival in all vertebrate animals.

In addition to neutrophils, zebrafish also possess eosinophilic granulocytes. Eosinophils are present within the five classes of vertebrates and at least three major phyla of invertebrates (Lee, Jacobsen, McGarry, Schleimer, & Lee, 2010). This remarkable conservation suggests selective importance for the roles of eosinophils in host defense and immune homeostasis. Understanding these roles in terms of human health and disease has been a significant concern in research for many decades https://paperpile.com/c/vFIzha/pMHm, and to this end, animal models have been utilized for nearly as long (Bittner, Halberg, & Vermund, 1956). Together, these efforts have delineated the diverse activities of eosinophils (Hogan et al., 2008), the bulk of which have implicated them in host defense against parasitic helminth infection and exacerbation of allergic pathologies (Cadman & Lawrence, 2010; Walsh, Stokes, & August, 2010). Eosinophils, to definitively establish their role(s) in health and disease, have been selectively ablated in different mouse models. Despite these efforts, the specific roles of eosinophils remain unclear, as mice lacking eosinophils are generally healthy, display no obvious defects in mounting a response against helminth infection, and do not appear to be prone to allergic disease (Humbles et al., 2004; Lee et al., 2004; Swartz et al., 2006). Therefore, a general reassessment of the functions of eosinophils may be necessary to understand their contributions to health. Eosinophils in the zebrafish, like in other teleosts, have been identified among other leukocytes by their affinity for histochemical stains and by electron microscopy. Specifically, zebrafish eosinophils stain positively with periodic acid-Schiff (PAS) but lack myeloperoxidase (MPO) activity and toluidine blue (TB) affinity, thus distinguishing them from neutrophils, which are negative for PAS but positive for MPO, and mast cells, which are positive for TB (Balla et al., 2010; Bennett et al., 2001; Dobson et al., 2008; Lieschke, Oates, Crowhurst, Ward, & Layton, 2001). It has also been demonstrated that expression of a *Tg(gata2:EGFP)* transgene, in combination with light scattering characteristics, can be used to isolate a pure population of zebrafish eosinophils (Table 19.1). Within WKM, approximately 5% of leukocytes are $gata2^{hi}$, whereas over 50% of peritoneal leukocytes are $gata2^{hi}$ eosinophils under steady-state conditions. By contrast, there exist very few neutrophils in the peritoneal cavity, suggesting that eosinophils serve as a key mediator of innate immune defense in this peripheral site in the zebrafish. Functional experiments demonstrated that zebrafish eosinophils degranulated in response to *Heligmosomoides polygyrus* extract and that injection of papain, which mimics a helminth protease, resulted in the development of profound eosinophilia in the peripheral blood. Together, these results suggest that innate immune protection against helminth infection may be a shared feature of vertebrate eosinophils. It will be of interest to determine the effects of eosinophil ablation in zebrafish.

Finally, a few studies have supported the existence of mast cells in the zebrafish. The *carboxypeptidase A (cpa5)* gene has been reported to specifically mark mast cells (Da'as et al., 2012; Dobson et al., 2008). It will be of interest to determine if *cpa5* regulatory elements can be incorporated into a fluorescent transgene in order to study mast cells in vivo and further our understanding of mast cell function in teleosts.

Molecular Components of the Adaptive Immune System

As a jawed vertebrate, zebrafish encode immunoglobulin and T-cell receptor (TCR) genes that undergo V(D)J recombination to generate an extensive repertoire of B and T cell receptors. Fish B cells express membrane-bound immunoglobulins and secrete antigen-specific antibodies in response to immune challenge. As described above, three classes of antibodies are present in zebrafish: IgM, IgD, and IgZ (Fillatreau et al., 2013; Mashoof & Criscitiello, 2016). Zebrafish encode TCRα, TCRβ, TCRγ, and TCRδ which pair to form TCRα/β

and TCRγ/δ heterodimers (Haire, Rast, Litman, & Litman, 2000; Laing & Hansen, 2011; Meeker et al., 2010; Schorpp et al., 2006; Seelye et al., 2016; Yazawa et al., 2008). Zebrafish immunoglobulin and TCR diversity is influenced by V(D)J recombination and somatic mutation (Fillatreau et al., 2013; Marianes & Zimmerman, 2011; Wienholds et al., 2002). Although zebrafish encode activation-induced cytidine deaminase (AID), there is no evidence for class switch recombination (Barreto et al., 2005; Wakae et al., 2006).

Immunoglobulin Structure and Genomic Organization

An individual immunoglobulin is comprised of two identical heavy chains (IgH) and two identical light chains (IgL) that are linked by disulfide bonds. A monomeric immunoglobulin is bivalent with the antigen-binding domain comprised of the carboxy-terminal variable (V) domains from a heavy and a light chain. Antibody diversity is achieved, in part, through V-D-J recombination of IgH and V-J recombination of IgL. An IgH includes a single recombined VDJ and a variable number of constant (C) domains. An IgL includes a single recombined VJ and a single C domain. The IgH C domains define the antibody isotype. The heavy chain genes for IgM (IgHμ), IgD (IgHδ), and IgZ (IgHζ) are encoded in a single locus on chromosome 3 (Fig. 19.3A) (Danilova et al., 2005; Fillatreau et al., 2013). Four IgL loci are encoded on chromosomes 3, 19, 24, and 25 (Fig. 19.3B) (Edholm, Wilson, & Bengten, 2011a; Haire et al., 2000; Hsu & Criscitiello, 2006; Zimmerman, Romanowski, & Maddox, 2011a).

IgHμ

The IgM class of antibodies is defined by possessing a μ heavy chain, IgHμ. IgM, which is present in all vertebrates (Flajnik & Kasahara, 2010), can be expressed at the surface of B cells or secreted as a multimeric protein (Fig. 19.3C). The IgHμ locus encodes 4 Cμ domains which are encoded in the secreted form (VDJ-Cμ1-Cμ2-Cμ3-Cμ4); however, the fourth domain, Cμ4, is removed from the transmembrane form by mRNA splicing (VDJ-Cμ1-Cμ2-Cμ3-M1-M2) (Danilova et al., 2005). Transcripts of an alternative transmembrane form have been described that possesses only a single C domain (VDJ-Cμ1-M1-M2) (Hu, Zhu, Xiang, & Shao, 2011). Upon stimulation, zebrafish B cells can secrete soluble IgM as a tetramer, which is the major antibody class in serum. Although polymerization of mammalian IgM is highly dependent on a J chain to link the individual Igs (Sørensen, Rasmussen, Sundvold, Michaelsen, & Sandlie, 2000), a J chain sequence has not been identified in fish (Zhang, Salinas, & Oriol Sunyer, 2011). In general, IgM transcript levels are higher than both IgD and IgZ (Wang, Wu, Hu, & Li, 2015b; Zimmerman, Moustafa, Romanowski, & Steiner, 2011b).

IgHδ

The IgD class of antibodies is defined by possessing a δ heavy chain, IgHδ. IgD can be expressed as a membrane-bound or secretory form (Fig. 19.3C). The zebrafish IgHδ locus encodes 16 Cδ domains; however, the second domain (Cδ2) carries a frameshift mutation (Danilova et al., 2005). As a result, zebrafish IgHδ encodes a chimeric protein consisting of the Cμ1 domain followed by 14 Cδ domains (VDJ-Cμ1-Cδ3-Cδ4-Cδ5-Cδ6-Cδ7-Cδ8-Cδ9-Cδ10-Cδ11-Cδ12-Cδ13-Cδ14-Cδ15-Cδ16-M). This is feasible as fish IgM and IgD are coproduced through alternative splicing of a common premRNA containing the recombined VDJ region along with Cμ and Cδ exons (Fillatreau et al., 2013; Zimmerman et al., 2011b). Although IgD is present in most taxa of jawed vertebrates, and thus, considered an ancient antibody (Edholm, Bengten, & Wilson, 2011b; Ohta & Flajnik, 2006), a definitive functional role for IgD in fish immunity remains to be defined (Edholm et al., 2011b; Mashoof & Criscitiello, 2016).

IgHζ

The IgZ class of antibodies is defined by possessing a ζ heavy chain, IgHζ (Danilova et al., 2005). In some teleost species, IgZ is referred to as IgT with a τ heavy chain (Hansen, Landis, & Phillips, 2005; Rombout, Yang, & Kiron, 2014). In rainbow trout, IgT has been identified as membrane-bound and as two different secreted forms: secreted IgT in serum is monomeric, whereas, secreted IgT in gut mucus is primarily polymeric (likely tetrameric) (Zhang et al., 2010, 2011). It is likely that zebrafish IgZ antibodies are present in the same forms (Fig. 19.3C). Research in aquaculture species has demonstrated that IgZ/IgT is the predominant immunoglobulin in the mucosa and plays important roles in mucosal immunity (Zhang et al., 2010, 2011). It is likely that the specialization of teleost IgZ/IgT and mammalian IgA in mucosal immunity arose independently through convergent evolution (Zhang et al., 2010).

IgL

In zebrafish, immunoglobulin light chain genes (IgL) are arranged in multiple "clusters" defined as independently rearranging loci consisting of a few V segments with a single J and one or 2 C domains (Hsu & Criscitiello, 2006; Zimmerman, Yeo, Howe, Maddox, & Steiner, 2008, 2011a). Although the orientation of gene segments differs between loci requiring different modes

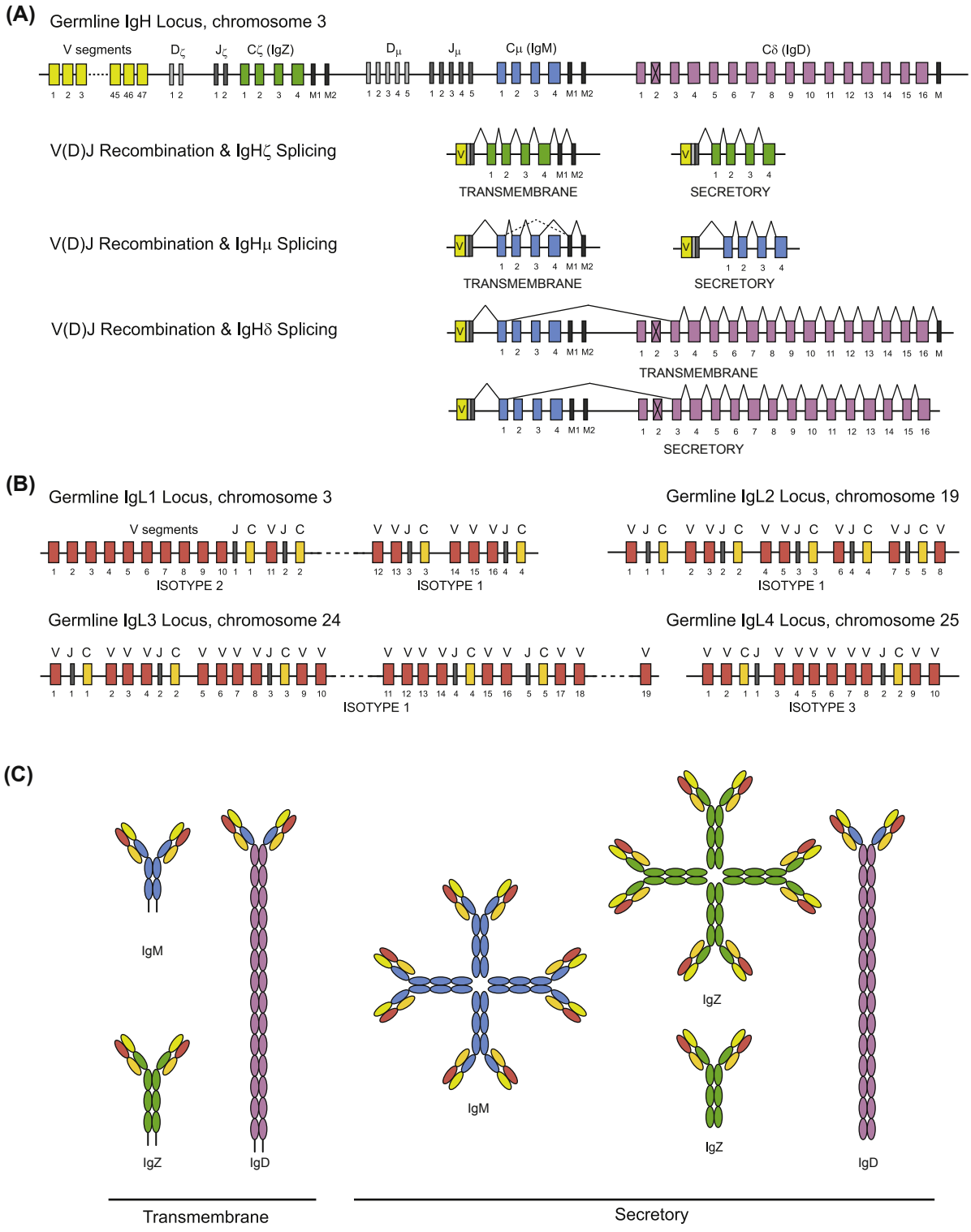

FIGURE 19.3 **Zebrafish immunoglobulins.** (A) The zebrafish immunoglobulin heavy chain locus on chromosome 3 encodes IgHζ, IgHμ, and IgHδ. Forty-seven V domains have been identified that can recombine either with D and J segments upstream of Cζ domains, and then, splice to the Cζ domains producing IgHζ or with D and J segments upstream of Cμ domains, and then, splice to the Cμ or Cδ domains to produce IgHμ and IgHδ, respectively. Note that IgHδ includes the Cμ1 domain and excludes Cδ1 and Cδ2 domains (Cδ2 possesses a frameshift) (Danilova et al., 2005). An alternatively spliced IgHμ has been reported with a single C (Cμ1) (Hu et al., 2011) and is indicated by a dashed line. (B) Zebrafish immunoglobulin light chain loci. IgL isotype 1 sequences are encoded on chromosome 3 (V_{12}–V_{16}), 19 and 24. IgL isotype 2 sequences are encoded on chromosome 3 (V_1–V_{11}). IgL isotype 3 sequences are encoded on chromosome 25 (Zimmerman et al., 2011a). DNA sequences are not to scale. (C) Predicted protein structures of predominant zebrafish antibodies.

of recombination (Hsu & Criscitiello, 2006; Zimmerman et al., 2008, 2011a), each locus is capable of producing a light chain with a V-J-C configuration. Three isotypes of immunoglobulin light chains have been defined based on C region identity (Haire et al., 2000), and are encoded in four chromosomes, which is reflected in their nomenclature (IgL1, IgL2, IgL3, and IgL4; Fig. 19.3B).

T Cell Receptor (TCR)

In teleost fish, TCR expression is limited to IgM- and IgT-negative lymphocytes in which surface expression of TCRα/β or TCRγ/δ heterodimers define T cell type (Laing & Hansen, 2011). TCR diversity is generated, in part, through V-D-J recombination of TCRβ and TCRδ and V-J recombination of TCRα and TCRγ. Zebrafish TCRα and TCRδ are encoded in a single cluster on chromosome 2 with the general organization of Dδ-Jδ-Cδ-Jα-Cα-Vα/δ in which TCRα and TCRδ share the same pool of 141 Vα/δ domains (Fig. 19.4A) (Seelye et al., 2016). Eight Dδ and 2 Jδ segments have been defined, whereas 111 Jα domains have been reported. The TCRβ locus on chromosome 17 encodes 51 Vβ segments, a single Dβ, and 28 Jβ segments along with two alternate Cβ domains (Fig. 19.4B) (Meeker et al., 2010). The genomic organization of the zebrafish TCR Cγ domain has been described (Wan et al., 2016) and Vγ and Jγ segments are identified in the reference genome (Yazawa et al., 2008). The GRCz10 version of the reference genome identifies 8 Vγ and 7 Jγ segments (Fig. 19.4C).

Major Histocompatibility Complex Genes

Zebrafish MHCI and MHCII sequences are present on different chromosomal loci which differs from most mammalian species where they are present at the same locus. As in mammals, class I genes encode alpha domain proteins, many of which heterodimerize with β2M. Class II genes encode either an alpha or beta chain which form heterodimers. Zebrafish encode three lineages of MHCI sequences termed "U" (for uno), "Z" and "L" (Dirscherl, McConnell, Yoder, & de Jong, 2014; Grimholt et al., 2015) and two lineages of MHCII sequences termed "DA" and "DB" (Dijkstra, Grimholt, Leong, Koop, & Hashimoto, 2013), although further work is needed to define the DA and DB lineages within zebrafish (Grimholt, 2016). The zebrafish MHCI gene nomenclature uses "*mhc1*" followed by three letters to designate the class I lineage (U, Z, or L), the identity of the gene, and "a" to indicate alpha chain (e.g., *mhc1uba*). The zebrafish MHCII gene nomenclature uses "*mhc2*" followed by three letters to designate the class II lineage ("d" for duo), the identity of the gene, and "a" or "b" to indicate the alpha or beta chain, respectively (e.g., *mhc2dab*). The majority of zebrafish genes have been identified based only on sequence homology to the well-described mammalian MHC genes that have been classified as "classical" (class I U and class II DA) and "nonclassical" (class I L and class II DB). Class I Z lineage sequences share features of both classical and nonclassical MHC. Classical genes tend to be highly polymorphic and possess a peptide-binding groove for antigen presentation. Nonclassical MHCI genes are minimally polymorphic and provide a range of functions (e.g., MHCI-like CD1 binds lipids, and MHCI-like MR1 presents vitamin B metabolites) (Grimholt et al., 2015). Zebrafish genes encoding other proteins required for antigen processing and presentation, including two β2M proteins (Chen et al., 2015), transporter ATP-binding cassette proteins (TAP), TAP-binding proteins (TAPBP), immunoproteosome subunits (PSMB) and two MHCII-associated invariant chains have been described (Dijkstra et al., 2013; Grimholt et al., 2015; Kuroda, Figueroa, O'hUigin, & Klein, 2002; McConnell et al., 2016; Sambrook, Figueroa, & Beck, 2005; Yoder, Haire, & Litman, 1999).

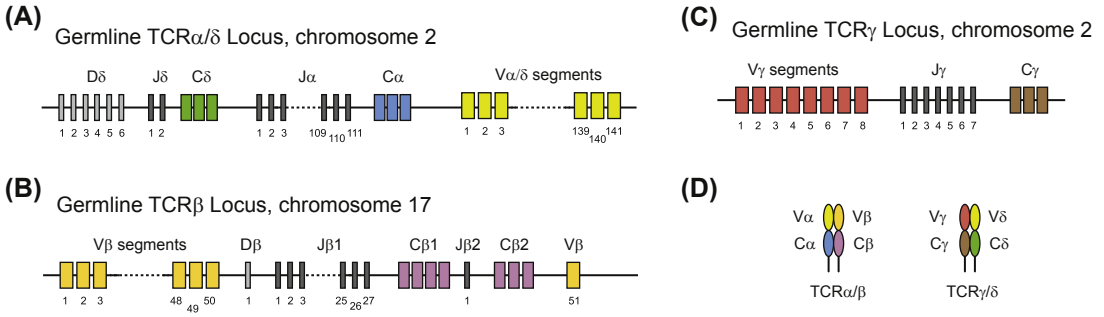

FIGURE 19.4 Zebrafish T-cell receptors. (A) TCRα and TCRδ are encoded at the same locus on chromosome 2 (Seelye et al., 2016), (B) TCRβ is encoded on chromosome 17 (Meeker et al., 2010), and (C) TCRγ is encoded on chromosome 2—4.2 Mbp from the TCRα/δ locus (Schorpp et al., 2006; Yazawa et al., 2008). (D) The predicted structures of TCRα/β and TCRγ/δ protein heterodimers are shown. DNA sequences are not to scale. Description of TCRα, TCRβ, and TCRδ gene segment orientation and recombination are described elsewhere (Meeker et al., 2010; Seelye et al., 2016). TCRγ C exons were described previously (Schorpp et al., 2006). TCRγ V, J, and C segments were identified in the GRCz10 zebrafish reference genome hosted on ENSEMBL (chromosome 2: 31,873,024—31,894,607).

MHCI "U" Lineage

U lineage MHCI sequences, which are present in all bony fish analyzed, share conserved synteny with the mammalian MHC and are encoded on zebrafish chromosome 19 (Fig. 19.5A). Additional MHCI U genes are present on zebrafish chromosome 22 (Fig. 19.5B), display characteristics of non-classical sequences but remain functionally undefined (Dirscherl & Yoder, 2015). Based on conserved residues, most U proteins are predicted to associate with β2M, bind CD8, and display peptides (Dirscherl et al., 2014; Grimholt et al., 2015). Zebrafish MHCI U sequences display high levels of polymorphic and haplotypic variation (Dirscherl & Yoder, 2015; Dirscherl et al., 2014; McConnell, Restaino, & de Jong, 2014, 2016). Gene content variation at the MHCI locus on chromosome 19 includes differing numbers of MHCI genes and differing numbers of antigen processing genes (Fig. 19.5A) suggesting that different class I haplotypes influence immune function through the presentation of alternative peptide classes (McConnell et al., 2016). In other fish models, U proteins have been shown to associate with peptide and β2M and are associated with allograft rejection and pathogen resistance (Chen et al., 2017, 2010; Grimholt et al., 2003; Sarder et al., 2003) - it is presumed that most U lineage sequences in zebrafish function in a similar manner. Transplantation studies in zebrafish confirm that grafts with matching U sequences on chromosome 19 are more successful than transplants with mismatched graft and host (de Jong et al., 2011).

MHCI "Z" Lineage

Twelve MHCI Z lineage genes have been reported in zebrafish chromosomes one and three (Fig. 19.5C,D), yet their function remains unknown (Dirscherl & Yoder, 2014). Although zebrafish Z sequences are predicted to present peptides, their limited sequence diversity indicates that they may present a restricted set of sequences (Dirscherl et al., 2014; Dirscherl & Yoder, 2014).

MHCI "L" Lineage

Fifteen MHCI L lineage genes have been reported in zebrafish chromosomes 3, 8, and 25. Although functional data is lacking, the presence and absence of specific residues and peptide motifs suggest that MHCI L sequences provide a non-classical function (Dirscherl et al., 2014), such as displaying nonpeptide antigens or binding innate immune receptors.

MHCII Sequences

Multiple MHCII genes have been described in zebrafish chromosomes 4, 8, and 18 (Fig. 19.5E–G) (Dijkstra et al., 2013). These genes are predicted to encode the alpha and beta chains of an MHCII molecule which would present peptide (Fig. 19.5H) (Dijkstra et al., 2013; Kuroda et al., 2002; Sambrook et al., 2005). The polymorphic characteristics of the zebrafish MHCII loci remain to be reported.

Toll-Like Receptors (TLRs)

TLRs bind a range of pathogen-associated molecular patterns (PAMPs) providing an early activation of the immune system in response to infection. More than 20 TLR genes have been described from zebrafish (Jault, Pichon, & Chluba, 2004; Kanwal, Wiegertjes, Veneman, Meijer, & Spaink, 2014; Meijer et al., 2004). Six subfamilies of TLRs (TLR1, TLR3, TLR4, TLR5, TLR7, and TLR11) have been described in vertebrates with each subfamily generally recognizing a different class of PAMP (Roach et al., 2005). Although the PAMPS recognized by a number of zebrafish TLRs have not been defined, representatives of each TLR subfamily are present in zebrafish (Table 19.2) (Jault et al., 2004; Kanwal et al., 2014; Meijer et al., 2004; Palti, 2011).

TLR1 Subfamily

The TLR1 subfamily often has more species-specific adaptations than the other subfamilies. The human TLR1 subfamily includes TLR1, TLR2, TLR6, and TLR10. The zebrafish TLR1 subfamily includes Tlr1, Tlr2, and Tlr18 (Jault et al., 2004; Meijer et al., 2004). Mammalian TLR2 generally forms heterodimers with TLR1 and TLR6 and is involved in the recognition of bacterial lipoproteins, lipoteichoic acid, peptidoglycan, and yeast zymosan (Kanwal et al., 2014; Palti, 2011). Zebrafish Tlr2 possesses sequence characteristics similar to human Tlr2, suggesting that it also forms heterodimers. Although ligand binding studies have not been reported for these zebrafish TLRs, the transcriptional immune response of zebrafish embryos is activated by exposure to synthetic lipopeptide (Tokarz et al., 2017; Yang, Marín-Juez, Meijer, & Spaink, 2015), and this response is dependent on Tlr2 (Yang et al., 2015).

TLR3 Subfamily

Mammalian TLR3 binds dsRNA as an indicator of viral infection (Matsumoto, Kikkawa, Kohase, Miyake, & Seya, 2002). Zebrafish encode a single member of the TLR3 subfamily, Tlr3 (Jault et al., 2004; Meijer et al., 2004). As in mammals, zebrafish Tlr3 recognizes dsRNA as an indicator of infection (Matsuo et al., 2008; Sahoo et al., 2015).

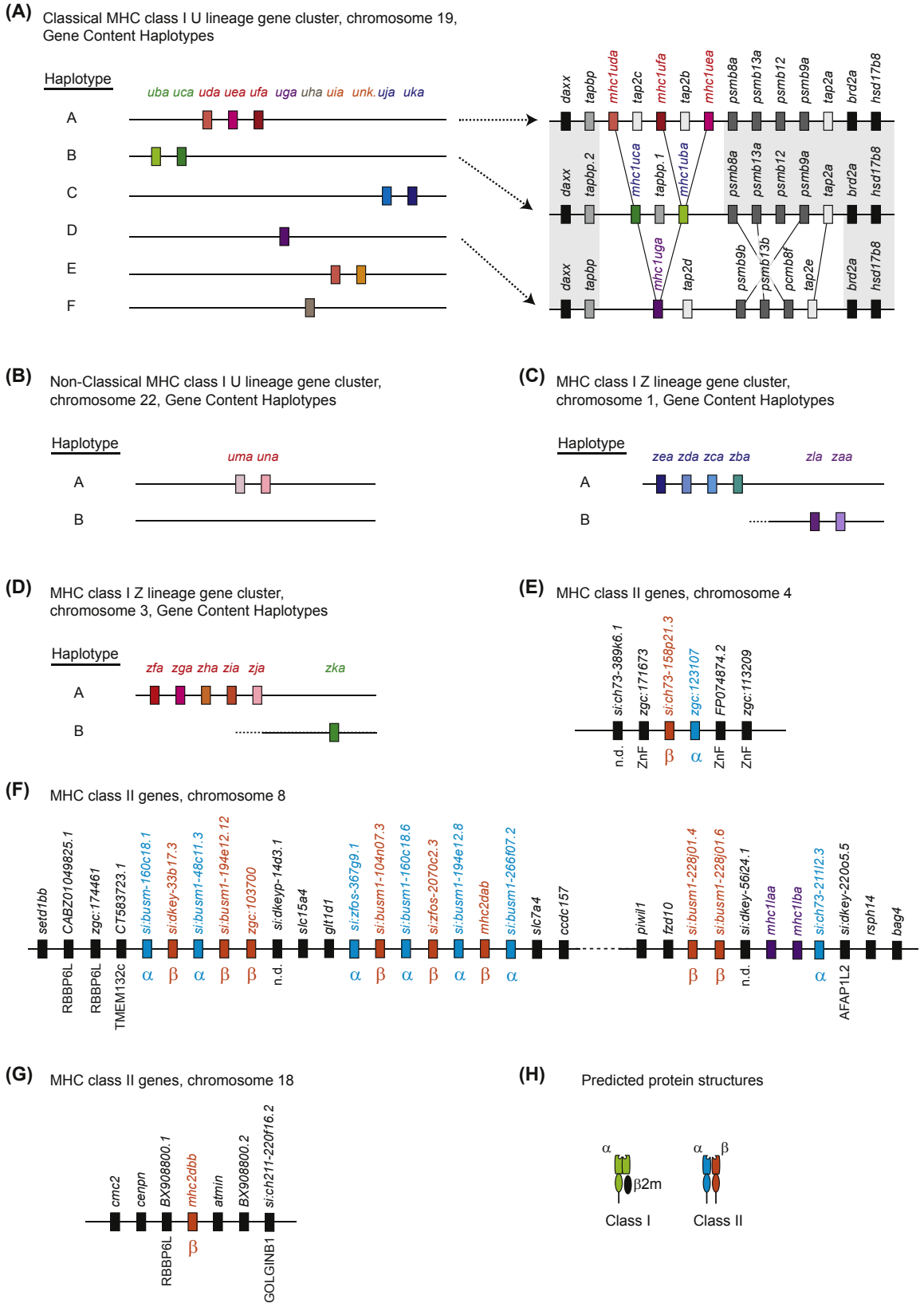

FIGURE 19.5 **Zebrafish MHC gene loci.** Genomic organization of MHCI U and Z lineage genes and MHCII genes are indicated. Sequences are not to scale. (A) Six gene content haplotypes (left) have been described for the classical MHCI U genes encoded on chromosome 19 (McConnell et al., 2014). Three of these haplotypes (right) have been resolved by demonstrating that different haplotypes can encode different TAP, TABP, and PSMB genes (McConnell et al., 2016). Shading indicates the sequence homology between haplotypes. Lines connect genes that are predicted to share a common origin. (B) Two gene content haplotypes have been described for MHCI U genes encoded on chromosome 22 (Dirscherl & Yoder, 2015). Haplotype B reflects a genomic loss of *mhc1uma* and *mhc1una*. (C) Two gene content haplotypes have been described for MHCI Z genes encoded on chromosome one and (D) two gene content haplotypes have been described for MHCI Z genes encoded on chromosome 3 (Dirscherl & Yoder, 2014). (E–G) MHCII genes have been identified on chromosomes 4, 8, and 18 (Dijkstra et al., 2013). Gene symbols from the reference genome (GRCz10) are shown above and characteristics of the encoded sequence shown below. E.g. class II sequences are identified as encoding alpha (α) or beta (β) chains; "n.d. " = not defined; Znf = zinc finger protein; RBBP6L = RB Binding Protein 6-like, Ubiquitin Ligase; AFAP1L2 = actin filament-associated protein 1-like two; and GOLGINB1 = golgin subfamily B member 1. Note that nonclassical MHCI genes (*mhc1laa* and *mhc1lba*) are linked to the MHCII genes on chromosome 8. (H) Predicted structures of class I proteins associated with β2M and class II heterodimers. Not all class I proteins associate with β2M.

TABLE 19.2 Zebrafish Toll-like receptors.

TLR subfamily	Zebrafish gene	Chromosome	Ligand	References
TLR1	*tlr1*	14		(Jault et al., 2004; Meijer et al., 2004)
	tlr2	1	Lipopeptide	(Jault et al., 2004; Meijer et al., 2004; Yang et al., 2015)
	tlr18	16		(Jault et al., 2004; Meijer et al., 2004)
TLR3	*tlr3*	1	dsRNA[a]	(Jault et al., 2004; Matsuo et al., 2008; Meijer et al., 2004)
TLR4	*tlr4al*	13		ZFIN ID: ZDB-GENE-090507-2
	tlr4ba	13		(Jault et al., 2004; Meijer et al., 2004; Sullivan et al., 2009)
	tlr4bb	13		(Jault et al., 2004; Meijer et al., 2004; Sullivan et al., 2009)
TLR5	*tlr5a*	20	Flagellin	(Jault et al., 2004; Meijer et al., 2004; Stockhammer et al., 2009)
	tlr5b	20	Flagellin	(Meijer et al., 2004; Stockhammer et al., 2009)
TLR7	*tlr7*	9		(Jault et al., 2004; Meijer et al., 2004)
	tlr8a	Unmapped		(Jault et al., 2004; Meijer et al., 2004)
	tlr8b	10		(Jault et al., 2004; Meijer et al., 2004)
	tlr9	8	CpG ODNs	(Jault et al., 2004; Meijer et al., 2004; Yeh et al., 2013)
TLR11	*tlr19*	16		(Meijer et al., 2004)
	tlr20.1	9		(Jault et al., 2004; Meijer et al., 2004; Pietretti et al., 2014)
	tlr20.2	9		(Jault et al., 2004; Meijer et al., 2004; Pietretti et al., 2014)
	tlr20.3	9		(Pietretti et al., 2014)
	tlr20.4	9		(Pietretti et al., 2014)
	tlr20f	9		(Jault et al., 2004; Meijer et al., 2004)
	tlr21	16	CpG ODNs	(Jault et al., 2004; Meijer et al., 2004; Yeh et al., 2013)
	tlr22	21	dsRNA[a]	(Jault et al., 2004; Matsuo et al., 2008; Meijer et al., 2004)

[a]*Evidence for ligand binding is from studies in pufferfish.*

TLR4 Subfamily

Although mammalian TLR4 binds lipopolysaccharides (LPS), a major component of bacterial cells walls (Hoshino et al., 1999; Poltorak et al., 1998; Qureshi et al., 1999; Takeuchi et al., 1999), neither of two tandemly encoded TLR4 proteins in zebrafish (Tlr4ba and Tlr4bb) bind LPS (Sepulcre et al., 2009; Sullivan et al., 2009). In fact, zebrafish and fish, in general, are highly resistant to LPS-induced endotoxic shock (Berczi, Bertók, & Bereznai, 1966). A third TLR4 gene (*tlr4al*) has been annotated in the reference genome (GenBank NM_001328605), but ligand binding assays have not been reported.

TLR5 Subfamily

Mammalian TLR5 binds flagellin as an indicator of bacterial infection (Hayashi et al., 2001). Zebrafish encode two TLR5 genes (*tlr5a* and *tlr5b*) and morpholino gene knock-down experiments indicate that both TLR5 proteins bind flagellin (Stockhammer et al., 2009; Yang et al., 2015). X-ray crystallography reveals the Tlr5b binding site for flagellin (Yoon et al., 2012). Although certain fish species possess an additional TLR5 gene that encodes a secreted protein (*TLR5S*), this gene has not been identified in zebrafish (Palti, 2011).

TLR7 Subfamily

The zebrafish TLR7 subfamily includes Tlr7, Tlr8a, Tlr8b, and Tlr9 (Jault et al., 2004; Meijer et al., 2004). Mammalian TLR7 and TLR8 recognize single-stranded RNA, and TLR9 recognizes CpG oligonucleotides. Ligands for zebrafish Tlr7, Tlr8a, and TLR8b remain to be defined, but Tlr9 has been shown to bind CpG oligonucleotides (Yeh et al., 2013).

TLR11 Subfamily

Zebrafish encode multiple TLR11 subfamily members, including TLR19, TLR20, TLR21, and TLR22 representatives (Jault et al., 2004; Meijer et al., 2004). Two TLR19 sequences (*tlr19a/tlr13/LOC110437753* and

tlr19b) have been reported in zebrafish, although TLR19 ligands remain undefined (Wang et al., 2015a). Zebrafish encode four highly similar, tandem copies of TLR20 (*tlr20.1*, *tlr20.2*, *tlr20.3*, and *tlr20.4*) and two likely TLR20 pseudogenes (*tlr20e* and *tlr20f*). Indirect evidence suggests that TLR20 proteins may recognize parasitic rather than bacterial or viral infections (Pietretti et al., 2014). Zebrafish Tlr21, like Tlr9, binds CpG oligonucleotides, but with a different sequence specificity (Yeh et al., 2013). Zebrafish Tlr22, like Tlr3, binds dsRNA as an indicator of viral infection, but structural modeling predicts that Tlr3 recognizes shorter dsRNA and Tlr22 recognizes longer dsRNA (Matsuo et al., 2008; Sahoo et al., 2015).

TLR Signaling

Major cytoplasmic components of the TLR signaling pathway are well conserved between fish and mammals and include zebrafish Myd88, Tirap, Trif, TRAF proteins, and IRAK proteins (Jault et al., 2004; Kanwal et al., 2014; Meijer et al., 2004; van der Sar et al., 2006). It has been suggested that sequence differences between human and zebrafish Tirap may result in weaker signaling in zebrafish (Liu et al., 2010). Notably, orthologs of *MD2* and *TRAM*, which function in ligand binding and cytoplasmic signaling of mammalian TLR4, respectively, have not been identified in zebrafish (Kanwal et al., 2014).

Fish-Specific Families of Innate Immune Receptors

The zebrafish genome encodes a number of fish-specific immunoglobulin-domain containing innate immune receptors (IIIRs) which are encoded in clusters throughout the genome (Rodríguez-Nunez, Wcisel, Litman, & Yoder, 2014; Wcisel & Yoder, 2016). The extracellular immunoglobulin domains within a family usually display sequence variation, suggesting that different family members bind different ligands. Although ligands for these families remain undefined, each family typically includes inhibitory and activating forms, as well as membrane-bound and secreted forms (Fig. 19.6). Activating forms possess an intramembrane charged residue that would permit association with signaling membrane proteins such as Dap12, Dap10, CD3ζ or FcRγ that possess cytoplasmic activation motifs such as an immunoreceptor tyrosine-based activation motif (ITAM) (Yoder, Orcutt, Traver, & Litman, 2007). Inhibitory forms possess one or more cytoplasmic immunoreceptor tyrosine-based inhibition motif (ITIM) (Wcisel & Yoder, 2016).

Novel Immune-Type Receptor (NITR) Family

Zebrafish NITRs are encoded on chromosomes 7 and 14, and individual proteins possess one or two immunoglobulin domains (Fig. 19.6A) (Rodríguez-Nunez et al., 2014; Yoder et al., 2004, 2008). The zebrafish NITR family includes 14 subfamilies based on sequence variation in the N-terminal immunoglobulin domain (e.g., Nitr1a, Nitr1b, Nitr1c comprise the NITR1 subfamily). The NITR9 subfamily includes a single activating gene, *nitr9*, that encodes three different isoforms through alternative mRNA splicing. Nitr9 associates with and signals through the Dap12 adaptor protein (Wei et al., 2007). While Nitr9 represents the only activating NITR gene in zebrafish, more than 30 different inhibitory NITR genes have been predicted along with a few ambiguous forms and secreted immunoglobulin domains (Rodríguez-Nunez et al., 2014; Yoder et al., 2004, 2008). Alternative gene content haplotypes have been described for these loci that encode different combinations of NITR genes (Wcisel & Yoder, 2016).

NITRs do not have genetic orthologs in mammalian species and are likely fish-specific. NITRs have been proposed to be functional orthologs to mammalian NK cell receptors, such as human killer cell immunoglobulin-like receptors (KIRs) (Yoder & Litman, 2011). When expressed on the surface of human NK cells, NITRs can activate/inhibit NK signaling pathways (Wei et al., 2007; Yoder et al., 2001, 2004). Although NITR ligands remain undefined, an activating NITR has been implicated in allorecognition (Cannon et al., 2008). Single-cell transcriptome analyses of zebrafish lymphocytes demonstrate that certain NITRs may be markers for NK cells (Carmona et al., 2017; Tang et al., 2017).

Diverse Immunoglobulin Domain-Containing Protein (DICP) Family

Zebrafish DICPs are encoded on chromosomes 3, 14, and 16 and individual proteins are predicted to possess one, two or four immunoglobulin domains (Fig. 19.6B) (Haire et al., 2012; Rodríguez-Nunez et al., 2014; Wcisel & Yoder, 2016). The zebrafish DICP family includes three subfamilies based on their genomic loci—the DICP1, DICP2, and DICP3 subfamilies are encoded on chromosomes 2, 14, and 16, respectively (Haire et al., 2012). A single activating DICP (Dicp2.1) and 11 inhibitory DICPs have been predicted. Ambiguous and secreted forms have also been described. Alternative gene content haplotypes have been described for these loci that encode different combinations of DICP genes (Wcisel & Yoder, 2016).

Genetic orthologs of DICPs have not been identified in mammals suggesting DICPs are fish-specific.

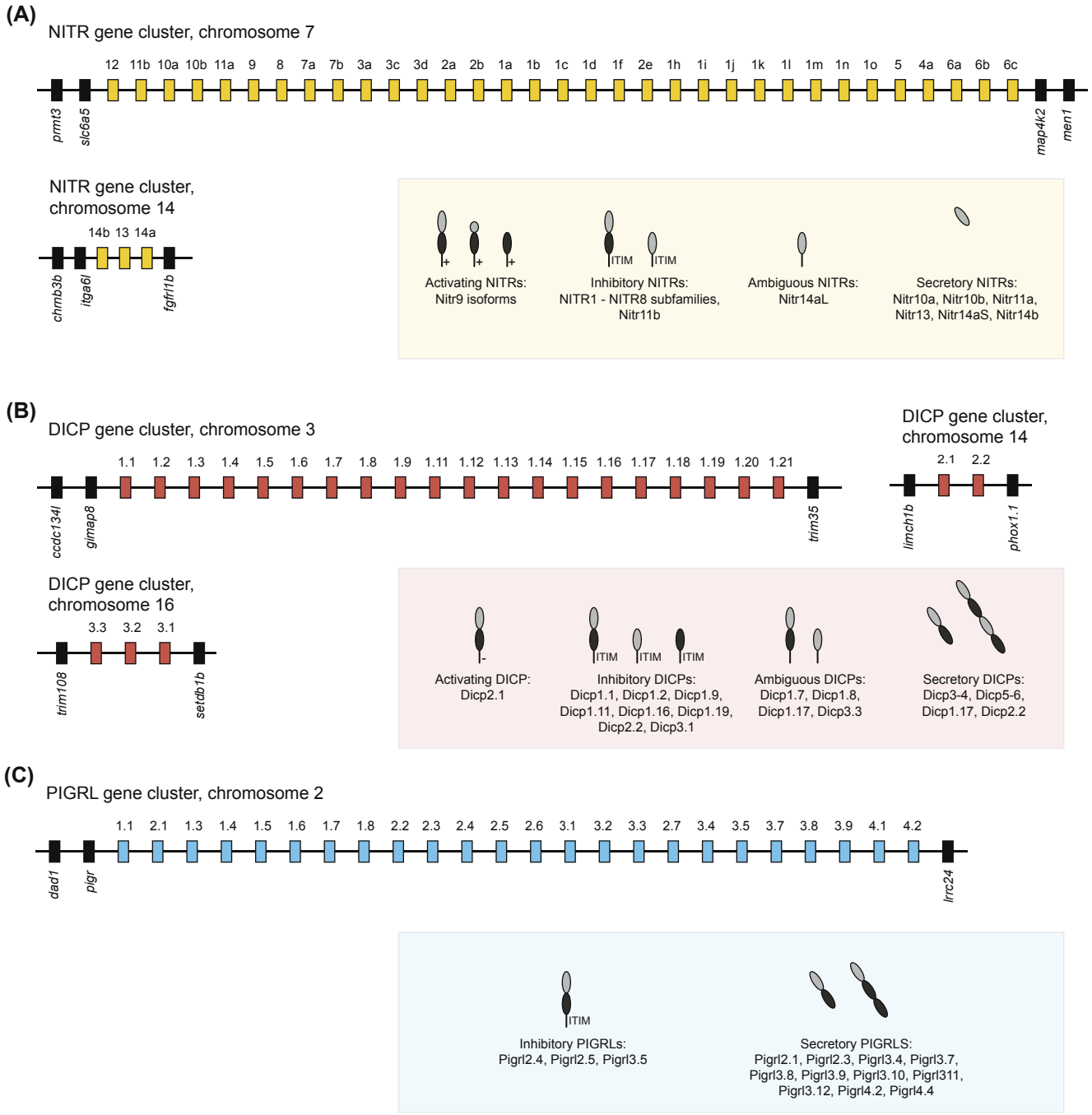

FIGURE 19.6 **Zebrafish Immunoglobulin Domain-Containing Innate Immune Receptors (IIIRs).** Genomic organization and predicted protein structures of zebrafish IIIRs. (A) NITR genes are encoded on chromosomes 7 and 14 and include activating, inhibitory, ambiguous, and secreted forms. (B) DICPs are encoded on chromosomes 3, 14, and 16 and include activating, inhibitory, ambiguous, and secreted forms. (C) PIGRL genes are encoded on chromosome two and include inhibitory and secreted forms. Each gene is represented by a color-coded rectangle. Genes flanking IIIR clusters are shaded black. Pseudogenes are excluded from the figure, and DNA sequences are not to scale. Predicted protein structures inferred from transcript sequences are shown in panel insets with each oval representing an immunoglobulin domain. Inhibitory receptors are indicated by a single "ITIM" in the figures but may possess one to five ITIM or ITIM-like sequences. *Figure adapted from Rodríguez-Nunez, I., Wcisel, D. J., Litman, G. W., and Yoder, J. A. (2014). Multigene families of immunoglobulin domain-containing innate immune receptors in zebrafish: Deciphering the differences.* Developmental and Comparative Immunology, *46 24–34; Wcisel, D. J., Yoder, J. A. (2016). The confounding complexity of innate immune receptors within and between teleost species.* Fish and Shellfish Immunology, *53 24–34.*

Although DICPs share sequence and structural homology with the mammalian CD300/TREM family of receptors, there is not enough evidence to indicate that these families share a common origin. A specific function for DICPs remains to be reported; however, certain recombinant DICPs bind microbially derived phospholipids, possibly reflecting the overall function of these receptors (Haire et al., 2012). The same single-cell transcriptome analyses that reported NITRs as possible markers of zebrafish NK cells, also demonstrated that certain DICPs may be markers for NK cells (Carmona et al., 2017; Tang et al., 2017).

Polymeric Immunoglobulin Receptor-Like (PIGRL) Family

Zebrafish PIGRLs are encoded on chromosome two, and individual proteins are predicted to possess two or three immunoglobulin domains (Fig. 19.6C) (Kortum et al., 2014; Rodríguez-Nunez et al., 2014; Wcisel & Yoder, 2016). The PIGRL family is adjacent to the single copy *pigr* gene and likely arose through tandem gene duplication events. The zebrafish PIGRL family includes four subfamilies based on sequence variation in the N-terminal immunoglobulin domain (e.g., Pigrl1.1, Pigrl1.3, Pigrl1.4 comprise the PIGRL1 subfamily). Although an activating PIGRL has yet to be identified, it is likely that additional PIGRL genes remain to be described (Kortum et al., 2014). Three inhibitory PIGRLs and 11 secreted PIGRLs have been reported (Kortum et al., 2014; Rodríguez-Nunez et al., 2014). Alternative gene content haplotypes have been described for these loci that encode different combinations of DICP genes (Wcisel & Yoder, 2016).

There is no evidence for a PIGRL gene expansion at the *PIGR* locus in mammals. In fact, PIGRL genes are not present in all teleosts studied and might be a more derived feature of certain fish species (Kortum et al., 2014). As observed with DICPs, two recombinant PIGRL proteins (Pigrl1.1 and Pigrl1.7) bind microbially derived phospholipids indicating that PIGRLs may play a role in pathogen recognition (Kortum et al., 2014).

Novel Immunoglobulin-Like Transcript (NILT) Proteins

Zebrafish NILTs have not been annotated on the genomic level. NILTs have been described in carp, trout, and salmon and NILT-like immunoglobulin domains reported from the zebrafish reference genome (Montgomery, Cortes, Mewes-Ares, Verheijen, & Stafford, 2011; Wcisel & Yoder, 2016). NILTs typically possess one, two, or six immunoglobulin domains, share sequence homology with the mammalian CD300/TREM family of immune receptors and are expressed in immune-related tissues. NILTs are considered fish-specific genes, and their function is currently undefined.

Leukocyte Immune-Type Receptors (LITRs)

Zebrafish LITRs have not been annotated on the genomic level. LITRs have been best described in channel catfish (*Ictalurus punctatus*) and LITR sequences reported in the zebrafish reference genome (Montgomery et al., 2011). Described LITRs possess one to six immunoglobulin domains, share sequence homology with mammalian FcR, LILR and KIR family members, and are expressed in immune-related tissues and a range of hematopoietic cell lineages (Montgomery et al., 2011; Stafford et al., 2006). Activating LITRs associate with the FcRγ-like adaptor protein (Montgomery et al., 2011). The observations that certain LITRs are expressed on catfish cytotoxic cells and are predicted to bind MHCI proteins led to the hypothesis that LITRs may be analogous to mammalian NK receptors (Stafford, Bengtén, Du Pasquier, Miller, & Wilson, 2007; Taylor et al., 2016).

Complement System

The complement system is an ancient mechanism of pathogen detection and has been identified in primitive protosomes and invertebrate deuterostomes (echinoderms and tunicates) (Boshra, Li, & Sunyer, 2006). The mammalian complement system employs more than 35 plasma and membrane-bound proteins for three activation pathways which recognize microbes: the classical pathway, the alternative pathway, and lectin pathway. Components of all three activation systems have been identified in zebrafish (Zhang & Cui, 2014) including orthologs of C1q, C1r, C1s (classical pathway), MBL, MASP2, and MASP3 (lectin pathway), and factor D, factor P (alternative pathway). All three pathways converge at a common amplification step involving the formation of C3-convertases (proteases), and zebrafish orthologs of genes involved in the subsequent pathway include C3, C5, C6, C7, C8, and C9. Although the genes are present in the genome, functional evidence for the lectin pathway in zebrafish is lacking. Gene duplication may have diversified the repertoire of functional complement components in zebrafish. For example, the three copies of C3 (C3-1, C3-2, and C3-3) in zebrafish may display complementary and/or redundant function (Boshra et al., 2006; Zhang & Cui, 2014).

Summary

Zebrafish possess the same major hematopoietic cell lineages as mammals and encode rearranging immunoglobulin genes to produce highly diverse antibodies and TCRs. Zebrafish also possess the major components of the major histocompatibility complex, including MHCI and MHCII sequences. Zebrafish possess a range of TLRs including some that are conserved with mammals and others that are fish-specific. A number of fish-specific putative innate immune receptors have been described including NITRs, DICPs, and PIGRLs. Although not discussed in detail here, zebrafish possess a range of cytokines reflecting all the major families (including IL-1β, TNF-α, IL-6, IFN-γ, TGF-β1) (Zou & Secombes, 2016), as well as a number of diverse antimicrobial peptides (Liu et al., 2017b; Pereiro et al., 2015). Thus, although much knowledge about zebrafish immunity can be inferred from the mammalian immune system, defining the roles of fish-specific sequences requires targeted studies in zebrafish or other fish models.

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CHAPTER

20

Physiology: Hematology and Clinical Chemistry, Gas Exchange, and Regulatory Osmolality

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Introduction

The basic physiology of vertebrate animals is highly conserved, making the zebrafish a relevant model in many fields of study. This chapter will emphasize some of these similarities and explore them in greater depth, while highlighting key differences between teleosts and warm-blooded species, especially those which have been demonstrated specifically in *Danio rerio*.

The teleost lineage branched from the vertebrate phylogenetic tree approximately 350 million years ago with a large-scale duplication of the entire genome (Amores et al. 1998). Today, the zebrafish genome contains roughly 25% of the original duplicates, termed **paralogs** (Busby, Roch, & Sherwood, 2010). Other teleost species contain varying numbers of these paralog genes and express them to varying degrees. Some paralogs have evolved unique physiologic functions while others are not expressed or appear to play a backup/redundant role that could compensate for an inactivating mutation in the first copy. The teleost duplication is an obvious disparity from our own genome and can be used to study the role of specific genes in the physiology of many systems. **Orthologs** to mammalian genes are roughly equivalent in structure or function to those found in mammals (without necessarily meeting a specific threshold for nucleic acid or amino acid identity). **Novel genes** are those found in an organism of interest, which do not have a known ortholog in other species.

The zebrafish in particular has served a critical role in advancing our understanding of gene function during vertebrate development. Following publication of the zebrafish genome, scientists have been able to map genes of interest and identify new orthologs, paralogs, and novel genes and track their expression and function using modern techniques in recombinant genetics.

Nomenclature

This chapter will adhere to the Zebrafish Information Network nomenclature for genes and gene products in zebrafish. This is summarized briefly in Table 20.1.

Chapter Contents

A fundamental understanding of the similarities and differences between teleosts and mammals or other vertebrates informs the use of zebrafish as a model organism. Basic hematology and clinical chemistry is presented here, as well as information on normal zebrafish physiology involving gas exchange and regulation of body osmolality. Details on zebrafish as a model for specific diseases and physiologic processes can be found in the final section of this book, beginning with Chapter 45. A comprehensive look at zebrafish immunology and leukocyte function is presented in Chapter 19. More details on hormonal control of diverse body systems is presented in Chapters 16 and 17. Specific information on water quality parameters and systems to maintain these is contained in Chapter 29.

Additional Resources

The current scientific knowledge of teleost physiology is better expressed in multiple volumes of textbooks than in a few pages summarized here. For

The Zebrafish in Biomedical Research
https://doi.org/10.1016/B978-0-12-812431-4.00020-8

TABLE 20.1 Zebrafish Information Network (ZFIN) Nomenclature summary.

Species	Gene	Protein
Human (*Homo sapiens*)	*GNRH*	GNRH
Mouse (*Mus musculus*) and other mammals	*Gnrh*	GNRH
Zebrafish (*Danio rerio*)	*gnrh*	Gnrh

comprehensive treatments of general fish physiology, these texts are among many valuable resources:

- Farrell, A. P., Stevens, E. D., Cech, J. J., & Richards, J. G. (2011) *Encyclopedia of fish physiology: from genome to environment*
- Perry S.F., Ekker M., Farrell A.P., Brauner C.J., editors. (2010) *Fish Physiology, Zebrafish: Volume 29*

In addition, there are many review papers that cover similar and additional material to expand one's knowledge of selected topics in teleost and zebrafish physiology. These include but are not limited to those papers listed below:

- Carradice, D. and Lieschke, G. J. (2008) *Zebrafish in hematology: sushi or science?*
- Evans, D. H., Piermarini, P.M., and Choe, K. P. (2005) *The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste*
- Hwang, P. P. and Chou, M. Y. (2013) *Zebrafish as an animal model to study ion homeostasis*
- Jagadeeswaran, P., Kulkarni, V., Carrillo, M., and Kim, S. (2007) *Zebrafish: from hematology to hydrology*
- Lin, C. H. and Hwang, P. P. (2016) *The control of calcium metabolism in zebrafish* (*Danio rerio*)
- Weyand, A. C. and Shavit, J. A. (2014*) Zebrafish as a model system for the study of hemostasis and thrombosis*

Hematology and Clinical Chemistry

Developmental Hematopoiesis

Zebrafish hematopoiesis occurs in four waves, characterized by different anatomic locations and genetic control of cell differentiation. The first two waves, together termed "primitive hematopoiesis," take place within the zebrafish embryo, unlike mammals and birds in which primitive hematopoiesis takes place within mesoderm-derived blood islands of the yolk sac. In the zebrafish, primitive myelopoiesis (formation of myeloid lineage cells) occurs before 30 hours postfertilization (hpf) in anterior lateral mesoderm (ALM) and primitive erythropoiesis (formation of erythroid lineage cells) in the posterior lateral mesoderm, which later forms the intermediate cell mass (ICM) of the embryo. Cranial to the ICM, cells of the rostral blood island give rise to primitive macrophages and granulocytes (Stachura & Traver, 2016; Weber, Choe, Dooley, Paffett-Lugassy, Zhou & Zon, 2005). Although these hematopoietic precursors are specified within the first 12 hpf, circulation of primitive blood cells does not begin until after 24 hpf, with the onset of early myocardial contractions (Chen & Zon, 2009).

Despite the differences in anatomic location, genetic control of primitive hematopoiesis is highly conserved among vertebrates. The gene *tal1* (also known as *stem cell leukemia* or *scl*), a basic helix-loop-helix transcription factor, is highly expressed in both areas described above by 10 hpf, when the embryo is at the 2-somite stage. These cells develop into either hematopoietic stem cells (HSCs) or vascular endothelial precursors (Chen & Zon, 2009). Functional expression of the *cloche* locus appears to be required to initiate this process, as zebrafish *cloche* mutants lack *tal1* expression in both ALM and ICM (Thompson et al., 1998). Also downstream of *cloche* are *lmo2* and early erythroid gene *gata1* (Stachura & Traver, 2016; Weber et al., 2005).

The second two waves of developmental hematopoiesis are termed "definitive," beginning around 24 hpf and 32–36 hpf with some overlap. The third wave creates erythromyeloid progenitors, and the fourth generates pluripotent HSCs. These cells are self-renewing and, unlike mammalian HSCs, develop in separate areas but eventually converge in what becomes the primary hematopoietic tissue of the anterior kidney (Khandekar, Kim, & Jagadeeswaran, 2012). These precursor cell types are readily distinguished microscopically (Stachura & Traver, 2016). Expression of *runx1* is conserved among zebrafish and mammals during HSC development (Chen & Zon, 2009). Zebrafish HSCs express integrin *CD41*, also called *itga2b* in zebrafish (Stachura & Traver, 2016). HSCs are formed in the posterior blood island, which later becomes the caudal hematopoietic tissue around 48 hpf.

Similar to mammals, a subset of HSCs migrate to the developing thymus to become lymphoblasts. Zebrafish T lymphocytes express conserved genes, such as *rag1* and *rag2*. Zebrafish B cell maturation occurs in renal hematopoietic tissue rather than in the bone marrow as in mammals (Chen & Zon, 2009). Invertebrate models *Drosophila* and *Caenorhabditis elegans* lack an adaptive immune system, so the zebrafish has been a valuable resource to understand the genetic control of the adaptive immune system using saturation mutagenesis and phenotype screens typically not feasible in mammalian models (Carradice & Lieschke, 2008).

Adult Hematopoiesis

Like other teleost fish, adult hematopoiesis occurs primarily in the kidney of zebrafish. The anterior portion, also called the head kidney, contains a higher ratio of hematopoietic cells to renal tubules than the

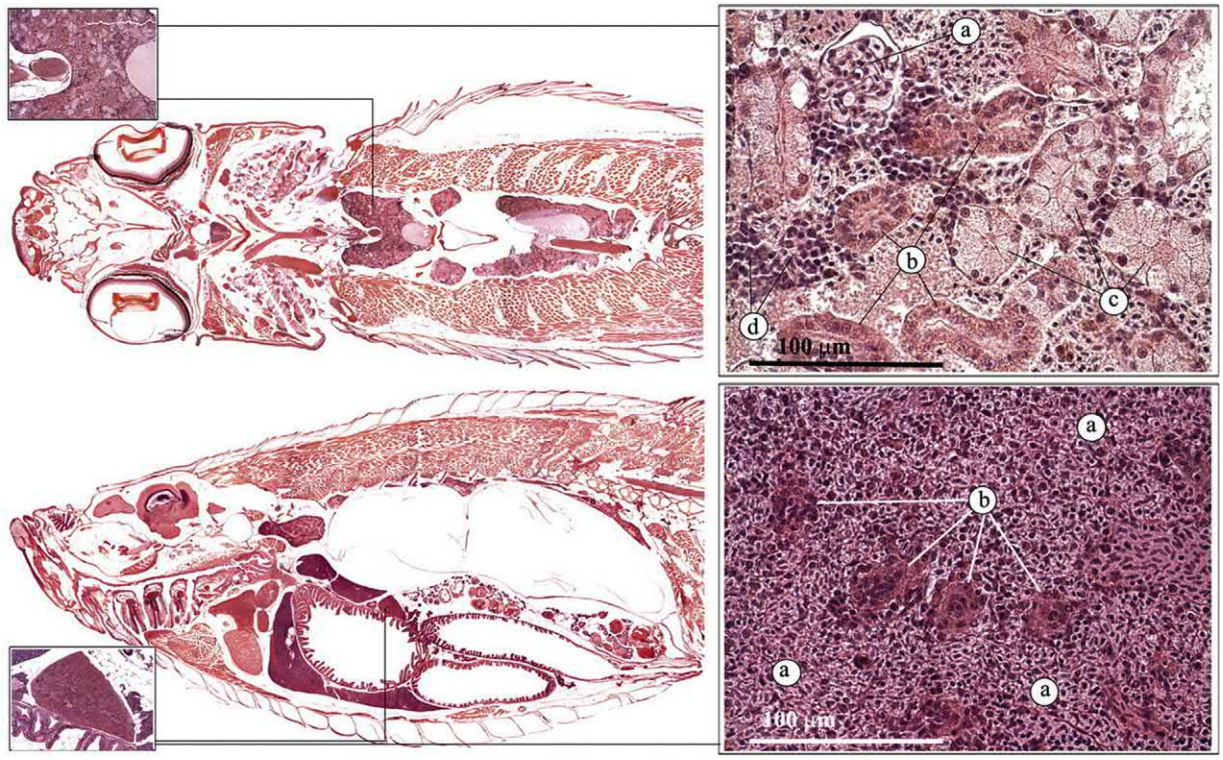

FIGURE 20.1 Histology of zebrafish hematopoiesis, the kidney, and the spleen. Top: coronal histology of adult zebrafish with insets of the head kidney (a) glomerulus, (b) proximal/distal tubule, (c) collecting duct, and (d) hematopoietic tissue. Bottom: sagittal histology of adult zebrafish with insets of the spleen (a) red pulp and (b) ellipsoid. *Adapted from Menke, A. L., Spitsbergen, J. M., Wolterbeek, P. M., & Woutersen, R. A. (2011). Normal anatomy and histology of the adult zebrafish.* Tox Path, 39, *759–775. https://ds.doi:10.1177/0192623311409597.*

posterior portion (tail or trunk kidney), though both contribute to blood cell production. While much has been learned about zebrafish HSCs through gene reporter lines, application of other techniques used to study mammalian hematopoiesis and immunology has been limited by a paucity of available antibodies to zebrafish proteins. Single-cell RNA sequencing technology, in conjunction with available genetic mutants, has been used to identify new cell types within the kidney marrow that contribute to hematopoiesis in adult zebrafish (Tang et al., 2017). Unlike mammals, the zebrafish spleen does not contain germinal centers and is characterized by a relatively simple histologic structure (Stachura & Traver, 2016). See Fig. 20.1, histology of the head kidney and spleen in adult zebrafish.

Transcriptional regulation of hematopoiesis displays a high degree of conservation between zebrafish and mammals. For example, genes *tal1 (scl)* and *lmo2* initiate hematopoiesis, *gata1* denotes an erythroid lineage, and *spi1 (pu.1)* a myeloid fate (Stachura & Traver, 2016). The staggered temporal expression of these and other orthologous genes also implies conserved regulation of hematopoietic gene expression.

Humoral regulation of hematopoiesis demonstrates a greater divergence between zebrafish and mammals. Tyrosine kinase type III receptors, which play a critical role in mammalian blood cell development, are present in zebrafish but are not essential. Nontyrosine kinases are suspected to play a more important role. These include erythropoietin, thrombopoietin, and two paralogs of granulocyte-colony stimulating factor (*gcsf*) (Paffett-Lugassy et al., 2007; Stachura et al., 2013; Svoboda et al., 2014).

Normal Values

To the author's knowledge, a 2003 study by Murtha et al. is currently the only comprehensive resource on normal blood values for zebrafish. These values are provided here for reference (Tables 20.2 and 20.3). As for other species, validation of normal values is recommended for each zebrafish colony using their own analyzers and collection methods. Data in the Murtha paper were collected from mature (approximately 1 year old) zebrafish at a single institution. These fish

TABLE 20.2 Leukocyte –zebrafish.

	Mean ± SD (%)	Range (%)
Lymphocytes	82.95 ± 5.47	71–92
Monocytes	9.68 ± 2.44	5–15
Neutrophils	7.10 ± 4.75	2–18
Eosinophils	0.15 ± 0.53	0–2
Basophils	0.13 ± 0.40	0–2

*From Murtha, J. M., Qi, W., & Keller, E. T. (2003). Hematologic and serum biochemical values for zebrafish (*Danio rerio*).* Comparative Medicine, 53*(1), 37–41.*

TABLE 20.3 Serum biochemical analytes for adult zebrafish.

	Mean ± SD	Range
Albumin	3.0 ± 0.2 g/dL	2.7–3.3 g/dL
ALP	2.0 ± 4.5 U/L[a]	0.0–10.0 U/L[a]
ALT	367.0 ± 25.3 U/L	343.0–410.0 U/L
Amylase	2331.4 ± 520.6 U/L	1898.0–3195.0 U/L
Total bilirubin	0.38 ± 0.1 mg/dL	0.2–0.6 mg/dL
BUN	3.2 ± 0.4 mg/dL	3.0–4.0 mg/dL
Calcium	14.7 ± 2.3 mg/dL	12.3–18.6 mg/dL
Phosphorus	22.3 ± 1.5 mg/dL	20.3–24.3 mg/dL
Creatinine	0.7 ± 0.2 mg/dL	0.5–0.9 mg/dL
Glucose	82.2 ± 12.0 g/dL	62.0–91.0 g/dL
Potassium	6.8 ± 1.0 mEq/L	5.2–7.7 mEq/L
Total protein	5.2 ± 0.5 g/dL	4.4–5.8 g/dL
Globulins	2.1 ± 0.6 g/dL	1.3–2.8 g/dL

[a]*ALP values were inconsistent and ranged from negative results to 10 IU/L.*
*From Murtha, J. M., Qi, W., & Keller, E. T. (2003). Hematologic and serum biochemical values for zebrafish (*Danio rerio*).* Comparative Medicine, 53*(1), 37–41.*

were acquired from a commercial supplier and were known to be clinically healthy with historically negative results on colony health screening for several common pathogens. The colony was housed in 10 gallon glass aquaria with individual filtration at the tank level, provided 25%–33% weekly water changes with conditioned tap water, at a density of 2.5 fish/gal. Blood was collected from the dorsal aorta of fish anesthetized in unbuffered MS-222 in ice water (Murtha, Qi, & Keller, 2003). These conditions may differ significantly from other institutions' housing and handling practices, and comparisons should therefore be interpreted cautiously.

Manual leukocyte counts can be made from 1 to 10 μL blood smears from individual fish, though variations in sample collection and slide preparation may affect total and relative leukocyte numbers reported. In a 2008 review, Carradice and Lieschke provide an overview of molecular diagnostic markers to identify zebrafish leukocytes using flow cytometry, whole mount in situ hybridization, histochemical stains, and in transgenic reporter fish (Carradice & Lieschke, 2008). An updated list of transgenic lines expressing genetic markers on particular leukocytes and hematopoietic cells is provided in Stachura & Traver, 2016. In the Murtha study, samples were pooled for manual hemocytometer counts, and separate samples were pooled for serum chemistry measurements (Murtha et al., 2003). The following sections describe and depict the major blood cell types in zebrafish, also depicted in Fig. 20.2.

Leukocytes

Lymphocytes are the most abundant leukocyte in zebrafish, accounting for approximately 80% of leukocytes under apparently healthy conditions (Murtha et al., 2003). Lymphocyte gene expression, function, and development appear highly conserved among vertebrates. Zebrafish lymphocytes express recombination activating genes *rag1* and *rag2* and undergo VDJ rearrangement (Willett, Cherry, & Steiner, 1997). T cells develop in the thymus and B cells in the hematopoietic tissues of the kidney, roughly equivalent to mammalian bone marrow. Lymphocytes can also be identified molecularly using riboprobe markers (Carradice & Lieschke, 2008). Ongoing research has identified αβ and γδ T-cell types with very similar functions to these subtypes in mammals (Schorpp et al., 2006; Wan, Hu, Ma, Gao, Xiang & Shao, 2016). The spleen has not been observed to contain mammalian-like germinal centers and is not a prominent feature in zebrafish lymphocyte biology (Stachura & Traver, 2016).

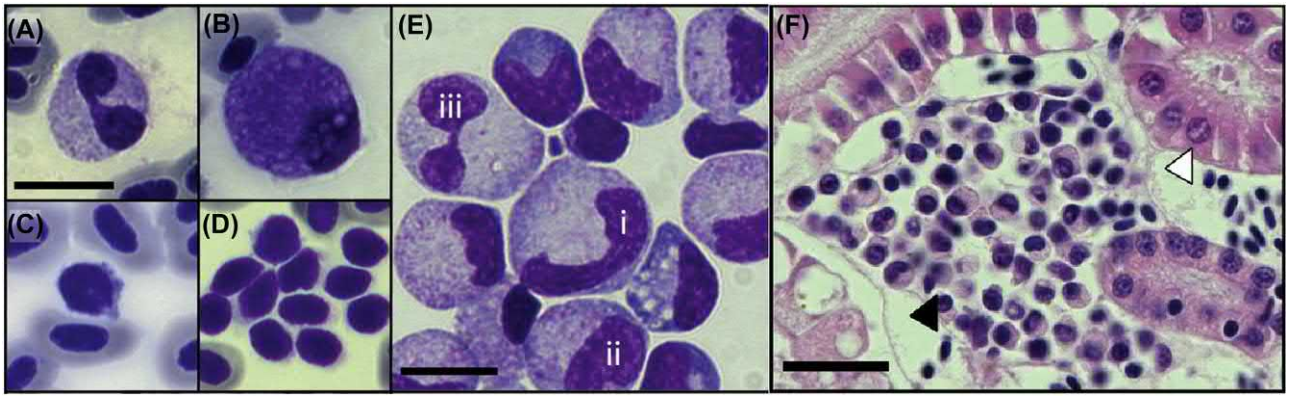

FIGURE 20.2 Zebrafish leukocytes: (A) bilobed neutrophil, (B) eosinophil, (C) lymphocyte and nucleated erythrocytes, (D) aggregate of thrombocytes, (E) progression of granulocyte maturation from immature (i, ii) to mature (iii), and (F) cluster of hematopoietic cells (closed arrowhead) nestled between renal tubules (open arrowhead). *Adapted from Carradice, D., & Lieschke, G. J. (2008). Zebrafish in hematology: Sushi or science?* Blood, 111*(7), 3331–3342. https://ds.doi:10.1182/blood-2007-10-052761.*

The most abundant fish granulocyte has been termed both neutrophil and heterophil in the literature. These cells express *myeloperoxidase* (*mpx*) beginning early in development (Lieschke, Oates, Crowhurst, Ward, & Layton, 2001). Neutrophils mature within the hematopoietic tissue of the kidney and have a two- or three- lobed nucleus with clear cytoplasmic granules at maturity (Carradice & Lieschke, 2008). During embryonic development, early neutrophils are functional, responding to acute sterile inflammation (Lieschke & et al., 2001). Zebrafish neutrophils are phagocytic cells with a preference for surface-associated microbes (vs. those suspended in a fluid within the coelom or bloodstream), as seen during *in vivo* larval imaging experiments (Colucci-Guyon, Tinevez, Renshaw, & Herbomel, 2011). Compared with a murine inflammatory response to microbial infection, zebrafish recruit relatively few neutrophils. This facilitates software-driven individual cell tracking for high resolution of cellular movement and intercellular interactions (Henry, Loynes, Whyte & Renshaw, 2013; Renshaw, Loynes, Trushell, Elworthy, Ingham & Whyte, 2006).

The function of granulocytes with eosinophilic granules on standard H&E stains has only recently been elucidated. In 2010, Balla described molecular identification of *gata2*^{hi} cells within kidney hematopoietic tissues that express genes related to mammalian eosinophil function and that degranulate when exposed to helminth antigens. Infection with *Pseudocapillaria tomentosa* also caused an increase in intestinal inflammation with the identified eosinophil cells, supporting the conserved role of this leukocyte among vertebrates (Balla et al., 2010).

A separate granulocyte cell with heterogeneous, eosinophilic granules has recently been recognized as a functional mast cell in *D. rerio* (Sfacteria, Brines, & Blank, 2015). Despite its differences in staining properties when compared with mammalian mast cells, zebrafish mast cells (also called eosinophilic granule cells) show conserved mast cell—like roles in adaptive and innate responses to inflammatory stimuli (Da'as et al., 2011).

Macrophages are the primary phagocytic leukocyte of zebrafish. These cells are the first identifiable leukocytes in the zebrafish embryo, and their functional activity can be assessed using dyes taken up by pinocytosis (Carradice & Lieschke, 2008; Herbomel, Thisse, & Thisse, 2001). These cells are very motile, migrate within tissues, and readily phagocytose bacteria, cellular detritus, or other foreign materials (Herbomel, Thisse, & Thisse, 1999). Like mammalian macrophages, zebrafish macrophages participate in a wide variety of inflammatory reactions, and tissue-resident macrophages assume specialized functions (Herbomel, Thisse, & Thisse, 2001). In addition, Nguyen-Chi et al. have recently reported the existence of M1-like and M2-like polarization of zebrafish macrophages, with *in vivo* functions and gene expression similar to these subsets of mammalian macrophages (Nguyen-Chi et al., 2015).

Erythrocytes

As in birds, reptiles, and other teleosts, zebrafish erythrocytes are elliptical and nucleated, measuring approximately 7×10 μm. Definitive erythrocytes are made in the anterior and posterior kidneys of adult fish, similar to most leukocytes. Zebrafish hemoglobin has similar oxygen-carrying functions to that in mammals but with adaptations for aquatic living discussed later in this chapter. Unlike mammals, α- and β-hemoglobin chains are not separated in the genome but are located in two intermixed groups on chromosomes 3 and 12 (Brownlie et al., 2003). The spleen is a major depot for zebrafish erythrocytes and phagocytes that manage the red cell population (Carradice & Lieschke, 2008). Despite the zebrafish erythrocyte's morphologic dissimilarity from human and other mammalian red cells, conserved developmental and functional gene and protein expression facilitate the use of zebrafish as a model for studying erythrocyte abnormalities in many warm-blooded taxa (Carradice & Lieschke, 2008).

Thrombocytes

Zebrafish thrombocytes have orthologous function and similar cytoarchitecture to mammalian platelets, despite their relatively large size and retained nucleus. Thrombocytes are difficult to distinguish from erythrocytes on basic hemocytometer counts but can be identified by surface proteins (e.g., Itga2b or *CD41*) (Lin et al., 2005), transcriptional markers (e.g., *zfpm1*) (Pham et al., 2007), and by staining characteristics on blood smear (Carradice & Lieschke, 2008; Murtha et al., 2003). Zebrafish do not have megakaryocytes; however, some of the same genes appear to regulate thrombocyte and platelet development, such as *fog1* (Carradice & Lieschke, 2008; Khandekar et al., 2012).

Again, despite morphologic differences, many platelet functions and regulatory processes are conserved in zebrafish thrombocytes, including aggregation in response to injury and platelet agonists such as collagen and arachidonic acid. Thrombocytes also express several platelet-like receptors for proteins such as von Willebrand factor and thromboxane (Jagadeeswaran, Sheehan, Craig, & Troyer, 1999; Weyand & Shavit, 2014). Studies using specific and nonspecific cyclooxygenase inhibitors have found that the cyclooxygenase pathway as it relates to coagulation is conserved, observing decreased zebrafish thrombocyte function in the presence of aspirin and indomethacin (Grosser, Yusuff, Cheskis, Pack, & FitzGerald, 2002; Jagadeeswaran et al., 1999). Additionally, proteases released into the water by zebrafish epithelium may have a role in initiating the clotting

cascade and can provide insights into the physiology of underwater wound stabilization in terrestrial vertebrates (Jagadeeswaran, Kulkarni, Carrillo, & Kim, 2007). Clotting tests analogous to activated partial prothrombin time, prothrombin time, and thrombin generation have been developed for use in zebrafish (Jagadeeswaran & Sheehan, 1999; Schurgers, Moorlag, Hemker, Lindhout, Kelchtermans & de Laat, 2016).

Serum Chemistry

Zebrafish serum chemistry analysis was performed on pooled samples from 5 groups of 50 healthy zebrafish from the population described in Murtha et al. (2003). Small blood volumes have historically limited the ability to measure multiple analytes in single fish. Human and veterinary serum chemistry analyzers typically require a volume of serum much greater than the total blood volume of the zebrafish, which necessitates pooling of samples for large chemistry panels.

A summary of the serum chemistry values in pooled samples from mature, healthy zebrafish is provided in Table 20.3. Moderate hemolysis was noted in these samples before analysis. The authors found increases in alanine aminotransferase, amylase, and phosphorus in zebrafish relative to mammalian species and some other fish species. Others have reported techniques that may be appropriate for serial blood collections and a limited panel of measurements in individual fish (Zang, Shimada, Nishimura, Tanaka, & Nishimura, 2015).

Gas Exchange

The basic principles of zebrafish gas exchange are similar among all teleosts. The water current contains gasses, which diffuse into the fish by way of the gills, driven by partial pressure gradients. The large surface area and short distance of diffusion within a healthy gill makes it a relatively efficient gas exchange system. Blood is transported from the gills to the tissues by way of the circulatory system, and diffusion mediates transport of the gasses from hemoglobin molecules into the tissues. Oxygen acts at the level of the intracellular mitochondria to power ATP generation via the Krebs cycle.

Cutaneous Gas Exchange in Zebrafish Embryos and Larvae

The primary means of gas exchange in the zebrafish embryo and early larval stages is via the integument, termed cutaneous gas exchange. This developmental mechanism is typical of other cyprinid fish. At these early stages, blood flow is not required for oxygen delivery. Rather, it reaches all areas of the developing fish via bulk diffusion until approximately 10 days postfertilization (dpf), after formation of the gills but around the time of development of secondary lamellae (Rombough, 2007).

To achieve successful diffusion of oxygen into the developing embryo and larva, oxygen must traverse three layers: the diffusive boundary layer (DBL), the chorion, and the perivitelline fluids of the embryo or surface epithelium of a hatched larva. The DBL is the limiting factor for gas exchange within the embryo and is comprised of the layer of fluid surrounding the surface of the egg capsule and later the skin and gill (Pelster & Bagatto, 2010). The thickness of the DBL determines gas exchange rates, and the DBL may accumulate metabolic by-products such as carbon dioxide (CO_2) and unionized ammonia (NH_3) making this thin layer more acidic than the surrounding environment. This serves as a proton trap for ionized ammonia (NH_4^+ ions) (Pelster & Bagatto, 2010). In static environments, where water and therefore oxygen surrounding the egg is not replenished rapidly, oxygen levels can become depleted. Within the egg, movement of the embryo mixes the perivitelline fluid and facilitates gas diffusion into the embryo.

At these early stages in development, temperature changes can have a profound impact on metabolic rate in the zebrafish embryo. Increased temperature increases molecular Brownian motion, thereby increasing the diffusion coefficient of oxygen. However, increased temperature also decreases gas solubility in aqueous solutions. The net effect on gas diffusion is about 1.1x for every 10°C change in temperature (Pelster & Bagatto, 2010).

Enzymatic reactions are more drastically altered with temperature change, approximately doubling for every 10°C in increased temperature. As temperature increases, so does the metabolic demand for oxygen. In developing zebrafish, some enzymatic activity can increase by a factor of 4 or 5 before cellular hypoxia becomes a limiting factor (Barrionuevo & Burggren, 1999). Increased metabolic oxygen demand does not have a major impact on larval heart rate, further supporting the conclusion that respiration is largely independent of the cardiovascular system at this stage (Barrionuevo & Burggren, 1999).

As the zebrafish larva grows, eventually the diffusion distances and surface area relative to metabolic demand are no longer sufficient to meet metabolic demand. The thickness of the larval DBL is related to water velocity and larval movement; increased water velocity will thin the DBL in exposed areas, and increased pectoral fin movement can have similar effects. Relative to the size of the zebrafish, the larval DBL is thicker than during embryonal stages (Rombough, 2011).

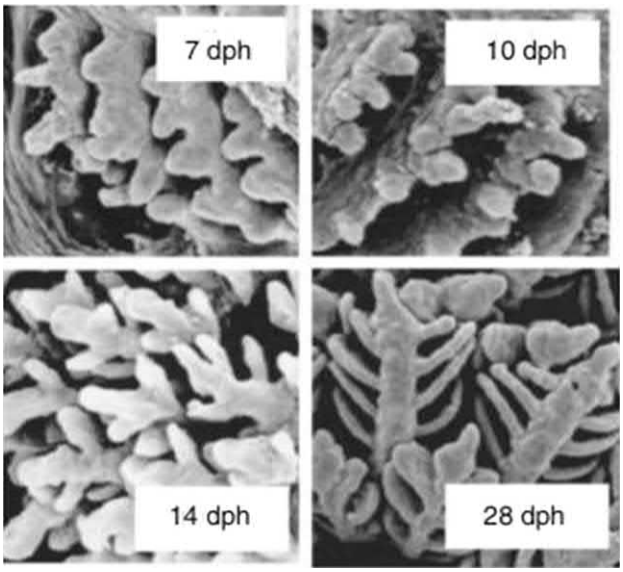

FIGURE 20.3 Development of the gills of zebrafish larvae. At 7 days posthatch (dph) (approximately 10 days postfertilization), filament buds can be seen forming on the gill arches. Lamellar buds first appear at about 10 dph. By 14 dph, one to three basal lamellae have formed closest to the gill arch. Aside from being smaller, the gill of a 28 dph larva is similar to that seen in juvenile and adult fish. *From Rombough, P.J. (2011). Respiratory gas exchange during development: Respiratory transitions. In A. P. Farrell, E. D. Stevens, J. J. Cech, & J. G. Richards, (Eds.), Encyclopedia of fish physiology: From genome to environment: Vol. 2. London; Waltham, MA: Academic Press, an imprint of Elsevier.*

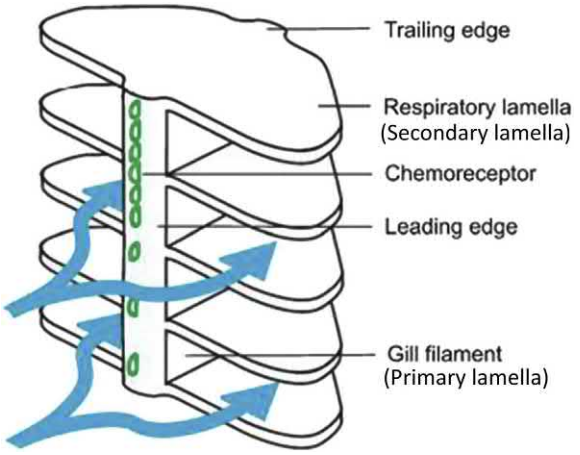

FIGURE 20.4 Schematic representation of a teleost gill filament (primary lamella) that gives rise to several respiratory lamellae (secondary lamellae), where gas exchange occurs. The leading edge of the filament faces the flow of water (shown by *blue arrows*) over the gills during ventilation, while the trailing edge represents the filament tissue furthest away. Oxygen-sensitive chemoreceoptors (i.e., neuroepithelial cells shown in green) reside within the epithelium of the gill filaments, where they may sense changes in oxygen from the water or arterial blood flow. *From Jonz, M. G. (2011). Oxygen sensing in fish. In A. P. Farrell, E. D. Stevens, J. J. Cech, & J. G. Richards, (Eds.),* Encyclopedia of fish physiology: From genome to environment, Vol. 2. *London; Waltham, MA: Academic Press, an imprint of Elsevier*bib_Jonz_2011.

As the cardiovascular system develops, the highly vascularized yolk sac is thought to take over as the primary site of gas exchange in larval teleosts. Developmental experiments performed in hypoxic environments demonstrate increased yolk sac vascularity, supporting this conclusion (Pelster & Bagatto, 2010).

Zebrafish gill arches do not play a major role in gas exchange until the development of secondary (also termed respiratory) lamellae nearly 2 weeks after fertilization (12–14 dpf) (Rombough, 2007). Instead, the rudimentary gill complements yolk sac and skin ion regulation via ionocyte activity (see more details below in Regulatory osmolality section). Until differentiation of the secondary lamellae, cutaneous respiration meets the metabolic demands of the developing embryo. Secondary lamellae vastly expand the respiratory epithelium of the maturing zebrafish and transition the fish to branchial respiration (Rombough, 2011) (Fig. 20.3).

Branchial Gas Exchange in Adult Zebrafish

Gills serve as the primary organ for gas exchange, acid/base balance, and ion regulation in the mature zebrafish. The latter two functions are detailed in later sections. Gas exchange occurs via a countercurrent mechanism of blood and water flow over the secondary, or respiratory, lamella (Fig. 20.4). Compared with earlier stages in development, the capacity for cutaneous gas exchange is not diminished but is overshadowed by the increased efficiency of branchial respiration (Rombough, 2011). For zebrafish and other teleosts, the size of respiratory epithelium can be calculated by determining the total surface area of the gill lamellae. The water–blood barrier in healthy gills is thin, on the order of 10 μm, about one to two cell layers thick (Rombough, 2011). This can change drastically in disease states, when gills are inflamed, thickened, edematous, and/or lamellae are blunted.

Ventilation of the gills is accomplished by continuous water flow. The first ventilatory movements are evident in 3 dpf zebrafish larvae but do not become a regular beat/pattern until 10 dpf (Jonz & Nurse, 2005; Turesson, Schewerte, & Sundin, 2006). As noted above, this is before efficient, functional gill respiration. These movements are controlled by highly conserved mechanisms within the central nervous system. The reticular formation within the medulla has been determined to be the primary respiratory rhythm generator in teleosts. This pattern is modulated by midbrain areas and stabilized by peripheral inputs from mechanoreceptors on the gills via the vagal sensory nucleus (Taylor, 2011). Receptors for gamma-aminobutyric acid (GABA), as well as alpha-amino-3-hydroxy-5-methyl-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors for glutamate, have been shown to modulate these

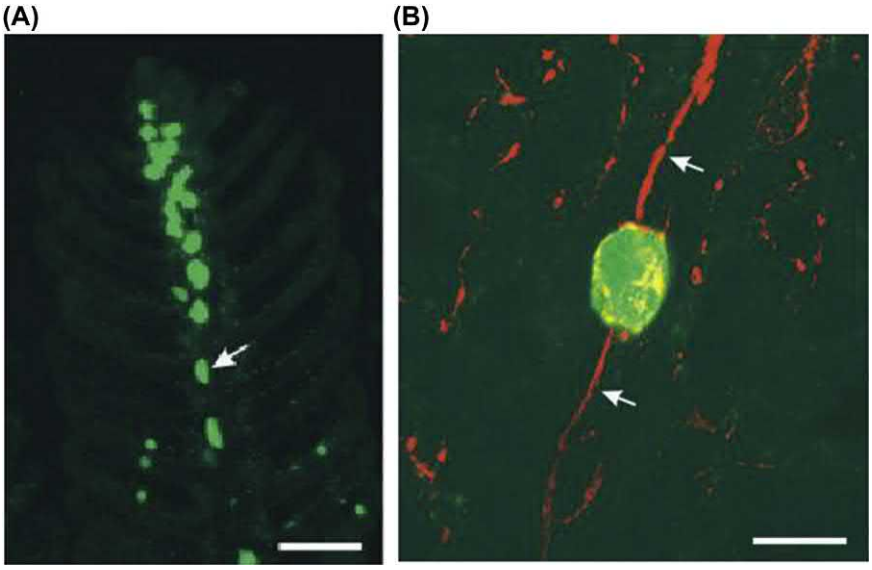

FIGURE 20.5 Neuroepithelial cells (NECs) in zebrafish gill filaments. (A) NECs (labeled in green and indicated with *white arrows*) are usually solitary and are organized along the longitudinal axis of the filament. NECs are colored green because they are labeled with antibodies that identify cells that contain the neurotransmitter, serotonin. Scale bar = 30 μm. (B) Innervation of a zebrafish NEC (green) by nerve fibers (*red and indicated with arrows*). Scale = 10 μm. *Adapted from Jonz, M. G. (2011). Oxygen sensing in fish. In A. P. Farrell, E. D. Stevens, J. J. Cech, & J. G. Richards, (Eds.),* Encyclopedia of fish physiology: From genome to environment, Vol. 2. *(2011) London; Waltham, MA: Academic Press, an imprint of Elsevier.*

signals at varying stages of growth and development (Pelster & Bagatto, 2010; Turesson et al., 2006). As in terrestrial species, increased carbon dioxide levels (hypercarbia) also stimulate increased ventilation rates.

Oxygen-dependent receptors detect the availability of oxygen in the environment and can modify the ventilation rate. For example, a reduction in environmental oxygen increases the ventilation rate and increases the heart rate. Neuroepithelial cells (NECs) in gill filaments are one source of these oxygen chemoreceptor signals. Unlike in the mammalian carotid body where these cells live in clusters, zebrafish NECs are solitary (Jonz, 2011) (Fig. 20.5). They are first identifiable in gill filament primordia of 5 dpf larvae and are fully innervated by 7 dpf. These cells store and release neurotransmitters, which can be seen microscopically as abundant intracellular vesicles (Jonz, 2011). The actual neurotransmitters and molecules involved in this process have yet to be conclusively identified, though an oxygen-sensitive potassium channel has been described (Jonz, Fearon, & Nurse, 2004). The physiologic mechanisms are thought to be largely conserved among mammals and cyprinids.

Oxygen Transport

As in other vertebrates, fish erythrocytes transport oxygen from the respiratory epithelium to sites of oxygen consumption. Water has a lower carrying capacity for oxygen than air; therefore, the respiratory epithelium must be exposed to a larger volume of water to access the same amount of oxygen molecules. As opposed to other molecules exchanged in the blood, oxygen transport is driven by diffusion only. Differences in partial pressure, rather than a metabolically active transport process, determine the uptake of oxygen and delivery to tissues.

Hemoglobin enhances the oxygen-carrying capacity of zebrafish blood, as it does in nearly all other vertebrates. Erythrocytes containing hemoglobin are present by 24 hpf in the zebrafish embryo (Brownlie et al., 2003), though under normal oxygen (normoxic) conditions, hemoglobin is not required to meet metabolic demands. Functional ablation of hemoglobin (via carbon monoxide or phenylhydrazine treatment) in zebrafish larvae up to 15 dpf does not affect heart rate, cardiac output, or even lactic acid concentration, indicating that dissolved oxygen is sufficient to meet the demands of developing zebrafish larvae without resorting to anaerobic metabolism (Jacob, Drexel, Schwerte, & Pelster, 2002). Even under severe hypoxic conditions (1% −5% oxygen), aerobic metabolism still occurs in zebrafish up to 42 dpf. After this stage in development, hemoglobin's oxygen-carrying capacity is needed for normal metabolism and development (Rombough & Drader, 2009).

Similar to other vertebrates, teleost fish including zebrafish make distinct embryonic or larval globin chains and adult globin chains. In zebrafish, scientists have found evidence of class switching similar to that documented in many mammals. Embryonic zebrafish express only low levels of alpha and beta globulin, which comprise the adult hemoglobin molecule (Chan et al., 1997). Brownlie et al. (2003) have cloned at least six embryonic hemoglobin genes in zebrafish, which

could create a diverse library of oxygen-carrying proteins. Relative to adult hemoglobin, larval hemoglobin has a very high affinity for oxygen, which may make hemoglobin-carried oxygen less available to tissues. The different physical properties of these hemoglobin classes are not fully understood, though temporal and physical waves of expression of the different globin genes during development may indicate important functional differences (Brownlie et al., 2003). The *zinfandel* (*zin*) mutant zebrafish strain develops hypochromic anemia as an embryo, which resolves in adulthood. The *zin* trait locus matches the linkage group of embryonic globin genes, but no mutations were detected in the identified cDNA, suggesting the phenotype may be due to alterations in a noncoding region with effects on the regulation of hemoglobin gene expression during development (Brownlie et al., 2003).

As adults, oxygen binding by hemoglobin is essential for normal oxygen delivery to zebrafish tissues. Similar to other vertebrates, reversible binding of oxygen to the globin molecule requires heme iron in its unstable, ferric state. The oxygen dissociation curve can be used to describe the saturation of hemoglobin with oxygen under different partial pressures. Hemoglobin-oxygen saturation is also dependent on the structure of the hemoglobin molecule, pH, temperature, and nucleoside triphosphate anions (e.g., ATP or GTP). The Bohr effect describes a pH-induced decrease of oxygen affinity without decrease in maximal oxygen saturation; as pH increases, oxygen affinity decreases. The Root effect describes a pH-induced decrease in the apparent maximal oxygen saturation; as pH decreases, so does hemoglobin's maximum carrying capacity for oxygen. Increased temperature also decreases hemoglobin-oxygen affinity. Binding of nucleoside triphosphates to the hemoglobin molecule stabilizes the nonoxygenated form, thereby also decreasing hemoglobin-oxygen affinity (summarized in Nikinmaa, 2011).

The delivery of circulating blood to tissues also regulates the delivery of oxygen. In mammals, capillary function is partially regulated by erythrocytes, including oxygen-dependent release of ATP and hemoglobin–nitric oxide interactions, and capillary hydrogen sulfide (H_2S) concentration. These mechanisms have not been definitively shown in fish but may be similar to what has been described in mammalian systems (Nikinmaa, 2011).

Excretion of Metabolites

The gills also function to eliminate waste products of metabolism, such as carbon dioxide (CO_2) and ammonia (NH_3 and NH_4^+ ions). For additional information on acid/base regulation and regulatory osmolality, see Regulatory osmolality section.

Carbon Dioxide

Compared to air, water has a similar carrying capacity for carbon dioxide, which is facilitated by the rapid transition to bicarbonate in aqueous solutions. Carbon dioxide produced by cellular metabolism readily diffuses into the blood, which has a low CO_2 partial pressure. Within erythrocytes, carbonic anhydrase rapidly hydrates CO_2 into bicarbonate and a proton. At least 16 forms of carbonic anhydrase are expressed in zebrafish, including at least four isoforms that are expressed in embryonic and larval stages, before the differentiation of erythrocytes or development of a functional circulatory system (Gilmour, Thomas, Esbaugh, & Perry, 2009). Externally oriented carbonic anhydrase on ionocytes in the gills may provide oxygen hydration during early developmental stages. Hemoglobin buffers the resulting proton by releasing oxygen. The Bohr effect is thought to have evolved independently several times throughout the kingdom Animalia. Relative to many other vertebrates, teleost hemoglobin has a relatively low buffering capacity for CO_2, and therefore, small pH changes can result in profound physiologic effects (Nikinmaa, 2011). In zebrafish and other teleosts, movement of carbon dioxide and oxygen is closely linked due to the high concentration of carbonic anhydrase within the erythrocyte and low concentration in plasma.

Ammonia

Early models of ammonia excretion in teleosts focused on passive diffusion of the molecule through the cellular lipid bilayer; however, NH_3 is poorly soluble in lipid. Instead, the family of Rhesus (Rh) glycoproteins is thought to facilitate transport of NH_3 and NH_4^+ ions, similar to the function of aquaporin proteins, in water transport across cellular membranes (Hwang & Perry, 2010). Rh proteins are expressed in zebrafish as early as 1 dpf, around the time that circulation begins. Knockdown experiments have demonstrated that Rh glycoproteins are important for NH_3 excretion in zebrafish larvae by 3 dpf (Braun, Steele, Ekker, & Perry, 2009). Rhcg1 and Rhcg2 colocalize with a proton exchanger (NHE3b) on gill ionocytes, which may coordinate sodium uptake with ammonia excretion (Hwang & Chou, 2013). See the section on regulatory osmolality for more details.

Ammonia secretion also aids in pH regulation, influenced by sodium–hydrogen exchange and proton secretion by ionocytes in the gills and carbon dioxide/bicarbonate exchange by carbonic anhydrase. See the section on acid/base regulation for additional information.

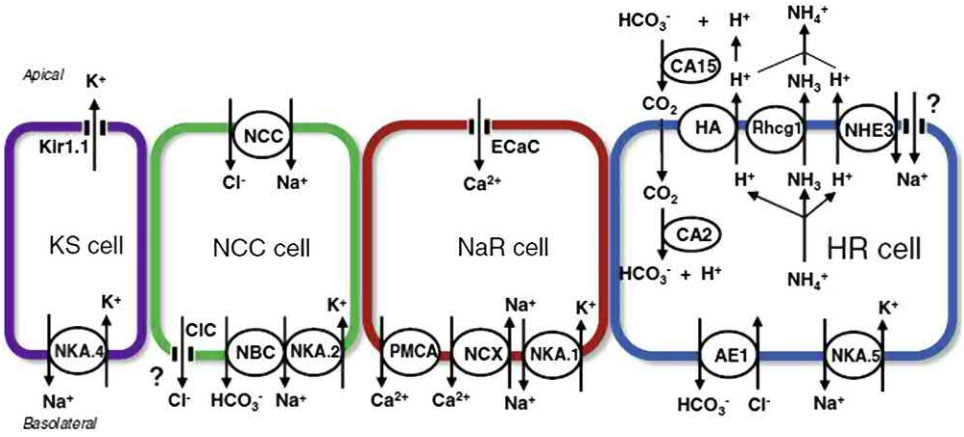

FIGURE 20.6 Ionocytes in zebrafish with proposed ion transport mechanisms. Four types of ionocyte have been identified: H^+−ATPase-rich (HR), Na^+−K^+−ATPase-rich (NaR), Na^+−Cl^- cotransporter (NCC), and K^+-secreting (KS) cells. AE1 = anion exchanger 1b (previous name of *Slc4a1b*), CA2 = carbonic anhydrase 2-like a, CA15 = carbonic anhydrase 15a, ClC = Cl^- channel, ECaC = epithelial Ca^{2+} channel, HA = H^+−ATPase, ROMK = ortholog of the mammalian renal outer medullary K^+ channel (*Kir1.1*), NBC = Na^+−HO^{3-} cotransporter 1b, NCX = Na^+/Ca^{2+} exchanger 1b, NHE = Na^+/H^+ exchanger 3b, NKA.1−5 = Na^+−K^+−ATPase α1 subunit subtypes 1−5, PMCA = plasma membrane Ca^{2+}-ATPase 2, Rhcg1 = rhesus glycoprotein. *Question mark* indicates unidentified transport pathways. *From Hwang, P. P., & Chou, M. Y. (2013). Zebrafish as an animal model to study ion homeostasis.* European Journal of Physiology, 465, *1233−1247.*

Regulatory Osmolality

The natural history of zebrafish as floodplain inhabitants provides context for an understanding of their ionic and acid−base regulation. Zebrafish can tolerate the wide range of water conditions found in slow moving or stagnant bodies of water, such as rice paddies. A field survey of zebrafish habitats in India and Thailand found that wild zebrafish commonly live in warm water (24−35°C) with moderate clarity and moderate pH (6.6−8.2) (McClure et al., 2006). However, the conditions of their natural habitat can fall far outside the average conditions with temperatures as low as 6°C and as high as 38°C (Spence et al., 2008). In the laboratory, zebrafish can survive in relatively extreme environments, including a pH range from 4 to 10, extremely soft water with hyposmotic sodium, chloride, and calcium concentrations in the range of 0.03 mM, or water with high ammonia concentrations (5 mM) (summarized in Hwang & Perry, 2010). To cope with such diverse environments, zebrafish require capable mechanisms to maintain their acid−base and ionic homeostasis.

Ionocytes and Ion Homeostasis

The gills of freshwater fishes contain ion-transporting cells with functions similar to the renal tubule cells of the mammalian kidney. To maintain physiologic concentrations and a normal pH, freshwater fish must actively absorb ions and secrete acid. The cells responsible for this function are grouped together as ionocytes, and at least four distinct types of these cells have been identified in the gills of adult zebrafish. Each cell type expresses a different group of ion transporters (Fig. 20.6). Initially, ionocytes develop within the skin of the zebrafish embryo, becoming functional around 24 hpf, long before functional gill development (Hwang & Chou, 2013). Ionocytes also differentiate from the gill arches around 5-7 dpf, prior to the development of functional gill lamellae by 14 dpf. Thus, the gills are critical for ion exchange before they are a major contributor to gas exchange physiology (Rombough, 2002). Near the ionocytes are glycogen-rich cells, thought to provide a ready energy source for neighboring ionocyte ion transporters (Hwang & Perry, 2010).

H^+−ATPase−Rich Ionocytes

Hydrogen−ATPase-rich ionocytes, or HR cells, are responsible for the majority of acid secretion from embryonic skin as well as larval and adult gill cells. They also have a role in sodium uptake and ammonium secretion. HR cells are similar to proximal tubular cells and type A intercalated cells of the mammalian kidney, both in transporter expression and function. Exchange of sodium and hydrogen ions by HR cells is dependent on carbonic anhydrase, much like mammalian proximal tubules and collecting ducts (Hwang & Perry, 2010).

Na^+−K^+−ATPase-Rich Ionocytes

Sodium−potassium−ATPase-rich ionocytes, or NaR cells, express calcium transporters and direct calcium uptake. Their physiology is conserved in mammals, which express similar transporters on renal and intestinal calcium absorption cells. The epithelial calcium channel (ECaC) in zebrafish facilitates movement of calcium from the external environment into the cell. EcaC is the single ortholog of *TRPV5* and *TRPV6*, which have

redundant function in mammals (Hwang & Chou, 2013). ECaC expression is the rate-limiting step for calcium absorption and is affected by stanniocalcin, cortisol, vitamin D, hydrogen sulfide, and calcitonin. Insulin-like growth factor 1 (*Igf1*) signaling stimulates NaR cell proliferation (Hwang & Perry, 2010).

A sodium–calcium exchanger, NCX1, moves calcium from the NaR cell into the bloodstream. Two isoforms of the sodium–calcium exchanger are expressed in zebrafish: *NCX1a* in the heart and *NCX1b* in gill/skin ionocytes (Liao, Deng, Chen, Chou, & Hwang, 2007). Differential expression of *NCX1a* facilitates specific studies of the transporter's role in ion homeostasis without impacting heart function. This is a limiting factor in mammalian models, which express only a single isoform in the heart and kidney (Hwang & Perry, 2010).

NA^+–Cl^- Cotransporter Expression

Ionocytes expressing sodium–chloride cotransporters are termed NCC cells. These function similarly to mammalian distal convoluted tubular cell cotransporters, regulating sodium and chloride absorption. Zebrafish and mammals both retain functional redundancy in sodium uptake, with multiple ionocytes regulating sodium. NCC cells function at a different osmolarity than the sodium–hydrogen exchangers (NHE) on HR cells, which makes direct comparison of ion transport kinetics and stoichiometry a challenge (Hwang & Chou, 2013). Loss of HR cells in zebrafish embryos can lead to an increased number of NCC cells, suggesting a compensatory mechanism and functional redundancy (Chang et al., 2013). This redundancy is also found in mammals, with NHE in the proximal tubules and NCC in the distal convoluted tubules both contributing to sodium uptake (Hwang & Perry, 2010).

In mammals, the distal convoluted tubules also contain chloride channels (*SLC12A3*), but equivalent channels in zebrafish are yet to be identified (Lewis & Kwong, 2018).

K^+-Secreting Ionocytes

Potassium-secreting ionocytes, KS cells, have been postulated based on knockout studies of the potassium channel *Kir1.1*, an ortholog of mammalian renal outer medullary potassium channel *ROMK*. Knockout of this receptor led to transient tachycardia followed by bradycardia, similar to the effects of hyperkalemia (Abbas et al., 2010). This suggests that *Kir1.1* has similar function to *ROMK*, which is needed for potassium secretion in the thick ascending limb of the distal mammalian nephron. While the KS cell has not been definitively identified, these functional studies support the existence of this fourth ionocyte in zebrafish (Hwang & Chou, 2013).

Ionocyte Function and Differentiation

To maintain homeostasis in a freshwater environment, ionocytes in gills and skin continuously regulate transport activities. These iterations are driven by aquatic environmental changes in temperature, pH, ion levels, and salinity and can occur over short-term (minutes to hours) or long-term (days to weeks) timescales.

For example, in response to an acute drop or rise in pH, the apical structure of ionocytes within the skin of larval zebrafish can change within minutes to adjust acid secretion (Hwang & Perry, 2010). Similarly, exposure to a hypotonic or hypertonic shock can alter chloride ion transport and change the number and size of openings in the apical surface to permit transport of chloride ions (communication cited in Hwang & Perry, 2010). A sudden drop in temperature results in impaired calcium influx through gill ionocytes (Hwang & Chou, 2013). The mechanisms for these rapid changes are not well-understood in zebrafish and other teleosts but may mirror mammalian kidney intercalated cells that traffic transporters from the apical membrane to the cytoplasm and back in response to local environmental changes (Brown, Pantescu, Breton, & Marshansky, 2009).

To acclimate to environments over longer periods of time, transporter gene expression is altered and new ionocytes develop from undifferentiated progenitor cell populations in zebrafish embryos, larvae, and adults (summarized in Hwang & Chou, 2013). Low pH, for example, will initiate increased proliferation and differentiation of HR cells from progenitor cells. Conditions of cortisol treatment or low temperatures can also stimulate ionocyte differentiation (Chou, Hsiao, Chen, Chen, Liu, Hwang, 2008; Cruz, Chao, & Hwang, 2013). In development, ionocyte progenitor cells arise from *foxi3a*-expressing cells. A cold environment has been shown to increase *foxi3a*, after which an increased number of ECaC-expressing cells were found in zebrafish gills (Chou et al., 2008).

As mentioned previously, before gills are functional, ionocytes within the skin of zebrafish embryos direct ion homeostasis. Proliferation of these cells is regulated by isotocin, an ortholog of oxytocin in mammals. Expressed in magnocellular neurons of the neurohypophysis, isotocin acts via *foxi3a* expression leading to increased ion absorption in low-ion environments (Chou et al., 2011). This finding in zebrafish implies a possible role for oxytocin in mammalian ion homeostasis (Lin & Hwang, 2016). Differentiation of ionocytes is stimulated by cortisol, which does not have a major role in ionocyte progenitor proliferation (Hwang & Chou, 2013).

The mechanisms driving specification of ionocyte subtypes (HR, NaR, NCC, KS cells) are not well-understood

but are likely similar during embryonic, larval, and adult ionocyte development. The gene groups with demonstrable roles in the process are similar to those that drive differentiation of mammalian intercalated cells (Hwang & Chou, 2013). Early in zebrafish development, coexpression and mutual inhibition of *deltaC* and *foxi3a* among epidermal cells drives differentiation of zebrafish keratinocytes and ionocytes in proportion (Hsiao, You, Guh, Ma, Jiang, & Hwang, 2007). Later, the balance between *Foxi3a* and *Foxi3b* is thought to signal NaR versus HR cell specification. Transcription factor *gcm2* transcription factor expression (within the Notch pathway) has a very similar pattern to *foxi3a* expression and can also lead to HR cell differentiation (Chang, Horng, Yan, Hsiao, & Hwang, 2009). *foxi1* mutant studies have also demonstrated a role for this gene in differentiation for HR but not NaR cells (Esaki et al. 2009).

Control of Sodium Levels

Apical and basal transport of sodium across the ionocyte is achieved through mechanisms similar to mammalian renal proximal tubule cells, with a few possible exceptions. In zebrafish, no ortholog of the mammalian *ENaC* transporter has been identified, yet some evidence supports an ATP-driven system (Hwang & Perry, 2010). An energy-neutral exchange mechanism for inward flow of sodium and outward flow of hydrogen ions may also play a role in ion balance, depending on water conditions such as high sodium concentration or pH (Evans, Piermarini, & Choe, 2005). These NHEs are present in HR cells within the skin of larval zebrafish and can also aid in acid–base balance (see Acid-base regulation section below).

On the basolateral side of the ionocyte, sodium may be pumped out of the cell via sodium–potassium transporters to provide a negative intracellular potential for a sodium–bicarbonate cotransporter ortholog of *NBC1*. A different anion exchanger, *Slc4a* (formerly *AE1*), has been found to colocalize on ionocytes of other teleost fish, suggesting that the precise mechanism of basolateral sodium transport may differ from that of renal proximal tubule cells, though the outcome is similar (Hwang, 2009).

Hormones also play a major role in controlling sodium balance within freshwater fish and zebrafish in particular. Cortisol and neurohormones such as catecholamines, prolactin, atrial natriuretic peptide (ANP), arginine vasotocin, and renin may all be involved.

Exogenous cortisol in the environment induces increased sodium uptake by zebrafish embryos acclimated to an acidic environment. This effect was blocked by antagonism of glucocorticoid receptors and not by treatment with agonists of mineralocorticoid receptors (Kumai, Nesan, Vijayan, & Perry, 2012a). In a normal, neutral pH environment, blocking glucocorticoid receptors had little or no effect on sodium balance of the zebrafish embryos (Kumai et al., 2012a), but it may suppress differentiation of ionocytes more generally, including HR cells, which could conceptually affect sodium uptake (Cruz et al., 2013).

Catecholamines exert biphasic control over ion regulation in zebrafish and other teleosts (Hwang & Chou, 2013). Epinephrine and norepinephrine acting through α-adrenergic receptors inhibit ionic transport via gill ionocytes (Evans et al., 2005). The same catecholamines stimulate ionic transport via β-adrenergic activity, especially in an acidic or ion-poor environment. In zebrafish, sodium uptake appears to be unaffected by dopamine and serotonin (Kumai, Ward, & Perry, 2012b).

Prolactin, named for its role in stimulating milk production in mammals, also has a more phylogenetically ancient role in salt balance. Increased prolactin expression occurs when the environmental salt concentration is low, which reduces water uptake and ion loss (Liu et al., 2006). Other hormones, including ANP and renin, have differential responses to environmental ionic composition (Hosijima & Hirose, 2007).

Control of Chloride Levels

Zebrafish have a remarkably high affinity for chloride uptake compared with other fish species, permitting survival in very dilute aquatic environments (Hwang & Perry, 2010). Although further research is needed to understand the full regulation of chloride in zebrafish, two major pathways have been described.

The first is roughly analogous to chloride absorption from the distal convoluted tubule in the mammalian kidney, where sodium–chloride cotransporter (NCC) expressing cells take in chloride from the lumen. On the basolateral side of the cell, chloride–potassium channels release chloride into the extracellular environment. In zebrafish embryos, ionocytes expressing *ncc2b* and *clc-2c* serve a similar role. Prolactin, isotocin, and calcitonin gene–related peptide (Cgrp) have been shown to regulate the expression of *ncc2b* and therefore play a significant role in regulating extracellular chloride levels (Shu et al., 2016; Wang, Lafont, Lee, & Hwang, 2016).

In zebrafish larvae and adults, genes in the *SLC26* ion transporter family, including *A3*, *A4*, and *A6*, may also play a significant role in chloride homeostasis (Hwang & Chou, 2013). The expression of these chloride–bicarbonate exchangers in the gill is regulated by ambient chloride levels, and knockdown of all three results in impairment in chloride uptake (Bayaa, Vulesevic, Esbaugh, Braun, Ekker, Perry, 2009).

Control of Calcium and Phosphate Levels

As in other vertebrates, calcium in zebrafish is critical for diverse physiologic processes including muscle contraction, neurotransmission, and bone remodeling. These intracellular and intercellular processes rely on close control of calcium homeostasis. While mammals primarily rely on ingestion and intestinal absorption of calcium, zebrafish acquire calcium from their aquatic environment. Like other critical ions discussed so far, the ionocytes responsible for calcium absorption are primarily in the skin of zebrafish larvae and gill epithelium of adults (Lin & Hwang, 2016).

Transepithelial transport of calcium is an active process conducted by sodium-rich ionocytes (NaRCs), which express the ECaC, sodium−calcium exchanger (NCX), and plasma membrane calcium-ATPase (PMCA) (Lin & Hwang, 2016). Multiple isoforms of these proteins have been identified and are expressed in different tissues to mediate calcium homeostasis (Liao et al., 2007). Paracellular, passive transport of calcium through tight junctions has also been described in zebrafish though control of this process is poorly understood (Lin & Hwang, 2016).

Calcium uptake and excretion is controlled by at least five interacting hormone pathways, which in turn are (at least partly) regulated by calcium sensing receptors (CaSR). These G-protein−coupled receptors are expressed in the kidney-adjacent corpuscles of Stannius and are activated by high calcium levels (Lin & Hwang, 2016). While CaSR do not seem to directly regulate ionocyte proliferation, differentiation, or expression of ECaC, their activation modulates signaling through calcium-influencing hormone pathways (Lin & Hwang, 2016) (Fig. 20.7).

i. **Calcitonin**: reduces circulating calcium, as in mammals. In zebrafish, calcitonin suppresses ECaC expression while increasing stanniocalcin expression (Lafont et al., 2011). Calcitonin is expressed in the ultimobranchial bodies of zebrafish larvae by 60 hpf (Alt, Reibe, Feitosa, Elsalini, Wendl, & Rohr, 2006).
ii. **Stanniocalcin**: analogous function to calcitonin hormone expressed in the corpuscles of Stannius and downregulates calcium levels by suppressing ECaC expression (Tseng et al., 2009). Stanniocalcin also decreases *foxi3a* expression to limit NaR cell differentiation. Other signaling pathways of stanniocalcin are still under investigation (Hwang & Chou, 2013).
iii. **Parathyroid hormone**: has the opposite effect of calcitonin and reduces phosphate absorption from the zebrafish kidney. Zebrafish have no distinct parathyroid glands but two parathyroid hormones and two receptors. Only PTH1 appears to play a role in calcium homeostasis. A low-calcium environment stimulates expression of *pth1* to increase calcium absorption and expression of ECaC (Lin & Hwang, 2016).
iv. **Vitamin D**: mammalian interactions between vitamin D and its receptor complex with ECaC are likely conserved in zebrafish. Vitamin D signaling with ECaC leads to enhanced calcium absorption, primarily through the VDRa receptor (Lin, Su, Tseng, Ding, & Hwang, 2012). Knockdown of this receptor, but not paralog VDRb, resulted in downregulation of ECaC mRNA expression (Lin et al., 2012). Feedback mechanisms controlling vitamin D synthesis are also similar between zebrafish and mammals (Hwang & Chou, 2013; Lin & Hwang, 2016).
v. **Cortisol**: in teleosts, cortisol acts as both a glucocorticoid and an aldosterone signaling molecule. It is primarily made in the interrenal tissue of the anterior kidney and acts via the glucocorticoid receptor to increase calcium absorption. When exposed to exogenous cortisol, zebrafish expression of ECaC is increased while glucocorticoid receptor expression is decreased and mineralocorticoid receptors remain unaffected (Cruz et al., 2013). Cortisol also mediates calcium absorption by increasing vitamin D receptor expression and simultaneously increasing calcitonin (Hwang & Chou, 2013; Lin & Hwang, 2016).
vi. Prolactin and growth hormone may also play a role in calcium homeostasis in teleost fishes, though more studies are needed to elucidate the pathways and demonstrate them clearly in zebrafish (Hwang & Chou, 2013).

Hydrogen sulfide is a gaseous signaling molecule with a role in calcium homeostasis in addition to regulating inflammation, cytoprotection, and the cardiovascular system (Li, Rose, & Moore, 2011). Hydrogen sulfide is produced endogenously by cytosolic enzymes, including cystathionine-beta-synthase (CBS) and cystahionine-gamma-lyase (CSE). Both are expressed in some zebrafish ionocytes, and CBSb expression is increased with zebrafish exposure to low-calcium water (Kwong & Perry, 2015). Additionally, knockdown experiments have shown that CBSb is important for maintaining calcium homeostasis (Kwong & Perry, 2015). It is thought that hydrogen sulfide stimulates ECaC activity via the PKA-cAMP pathway to upregulate calcium absorption, similar to its actions on mammalian TRPV5 (Lin & Hwang, 2016).

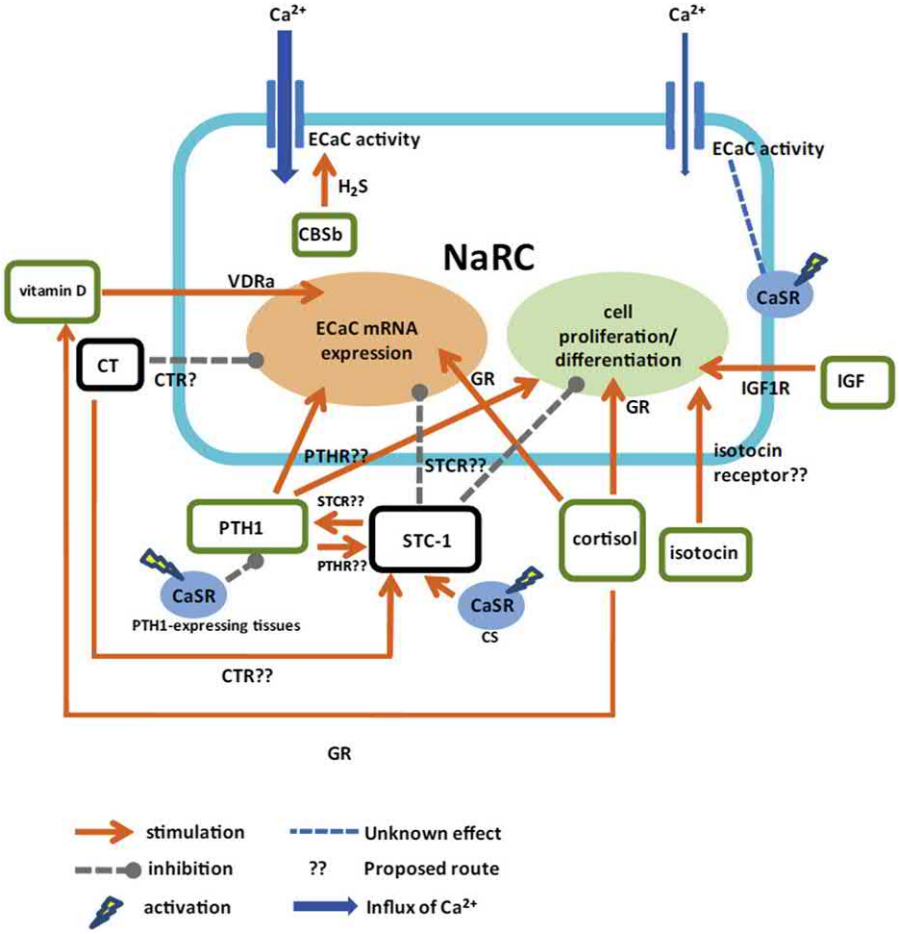

FIGURE 20.7 A proposed model for the actions of calcitropic hormones on zebrafish NaRCs. Calciotropic hormones are suggested to directly and indirectly (via the effector hormone(s)) regulate the cell proliferation/differentiation and the mRNA expression or activity of ECaC in NaRCs. *CaSR*, calcium-sensing receptor; *CBSb*,cystathionine-β-synthase b; *CS*, corpuscle of Stannius; *CT*, calcitonin; *CTR*, calcitonin receptor; *ECaC*, epithelial Ca^{2+} channel; *GR*, glucocorticoid receptor; *H_2S*, hydrogen sulfide; *IGF*, insulin-like growth factor; *IGF1R*, insulin-like growth factor 1 receptor; *NaRC*, Na^{+}–K^{+}–ATPase-rich cell; *PTH1*, parathyroid hormone 1; *PTHR*, parathyroid hormone receptor; *STC-1*, stanniocalcin 1; *STCR*, stanniocalcin receptor; *VDRa*, vitamin D receptor A. *From Lin, C. H., & Hwang, P. P. (2016). The control of calcium metabolism in zebrafish (*Danio rerio*).* International Journal of Molecular Sciences, 17, *1783. http://ds.doi:10.3390/ijms1711783.*

Acid–Base Regulation

Intracellular and extracellular pH is regulated by several body systems, including the erythrocytes, gills, and kidneys. As in mammals, carbonic anhydrase plays an important role in acid–base regulation in developing and adult zebrafish. Several isoforms with differential expression in a variety of tissues facilitate the excretion of carbon dioxide and ion exchange, especially within erythrocytes and the gill epithelium (Gilmour et al., 2009).

Ion transport across the erythrocyte membrane includes a sodium–proton exchanger and potassium–chloride cotransporter. The sodium–proton exchanger secretes protons from erythrocytes, effectively increasing intracellular pH. This is linked to acute stress via adrenergic stimulation and increases erythrocyte volume as well as hemoglobin–oxygen affinity. The potassium–chloride cotransporter has the opposite effect, decreasing erythrocyte pH, volume, and hemoglobin–oxygen affinity. This facilitates the release of oxygen from the erythrocyte into tissues (summarized in Hwang & Perry, 2010).

Aquatic environmental or internal hypercarbia and acidosis stimulate gill ventilation to control the body's pH. Chemoreceptors along the gill arches generate these signals via glossopharyngeal and vagus nerves. These have a common embryologic origin in vertebrates and are analogous to the carotid bodies in terrestrial vertebrates. Ionocytes within the gills are responsible for the vast majority of excretion of acid–base equivalents in zebrafish (Claiborne, Edwards, & Morrison-Shetlar, 2002). Ionocyte function is altered by exposure to acidic environments, by increasing the density of HR, NCC, and NaR cells, as well as increasing expression and activity of sodium–proton exchangers, sodium–chloride

cotransporters, and epithelial calcium channels, respectively. Bicarbonate levels are modified by decreasing expression and activity of anion exchangers on the apical surface of ionocytes (e.g., *SLC26A4*) and increasing expression of other anion exchangers (e.g., *SLC4A*) on the basolateral surface (summarized in Lewis & Kwong, 2018). Together, these mechanisms function to increase excretion of protons and increase absorption of bicarbonate.

As in regulation of osmolarity, discussed above, several hormones are involved in acid–base regulation. Those with important roles demonstrated in zebrafish include cortisol, endothelin, angiotensin II, catecholamines, and stanniocalcin. In response to acidosis, expression of most of these hormones results in increased sodium absorption, facilitating excretion of protons via active exchangers (summarized in Lewis & Kwong, 2018). In contrast, acid exposure decreases expression of stanniocalcin to enhance calcium uptake and regulate acid levels (Chou et al., 2015). Other hormones, including thyroid hormone, growth hormone, prolactin, and somatolactin, have been shown to play a role in acid regulation in other freshwater species, but more research is needed to demonstrate their importance in zebrafish (Lewis & Kwong, 2018).

The kidney also plays an important role in acid–base regulation, regulating the amount of bicarbonate reabsorbed from urine. Ion exchangers and cotransporters expressed in zebrafish nephrons include sodium–proton exchangers, sodium–bicarbonate cotransporters, anion exchangers, sodium–glucose cotransporters, and chloride and potassium channels (Hwang & Perry, 2010).

Summary and Conclusion

While specific anatomic features of zebrafish may diverge from terrestrial mammals, many of the same physiologic processes can be demonstrated in both taxa, making zebrafish a relevant animal model for many fields of study. The use of zebrafish to model diverse physiologic systems continues to expand, promoting a greater understanding of these processes in *D. rerio*. This creates a positive feedback loop that facilitates more detailed comparisons between zebrafish and other vertebrates and continues to expand the use of zebrafish as a model of vertebrate physiology.

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CHAPTER

21

Zebrafish in Biomedical Research: the Retina and Vision

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Introduction

The retina is a highly organized tissue with limited cell types and easy access, making it one of the most informative neuronal tissues to study. The orderly arrangement of the retina allows us to directly modulate neural transmission and measure functional output, map neuronal circuitry and synaptic connections, and test how neurons form support systems with different neuronal classes and Müller glia to maintain homeostasis and cell health.

With the aid of pharmacological agents, zebrafish can remain transparent well after larval retina function is established, making them an ideal model for visual studies. Imaging experiments using fluorescent probes (dyes, genetically encoded sensors) can be used to monitor function in a living eye in its native environment. Zebrafish genetics are sophisticated and quick to manipulate using a variety of methods, including Tol2-kit (Don et al., 2017; Kwan et al., 2007) and CRISPR/Cas9 (Ablain, Durand, Yang, Zhou, & Zon, 2015; Li, Zhao, Page-McCaw, & Chen, 2016), allowing the expression of a variety of fluorescent and mutant proteins. Zebrafish are cone-dominant similar to the human macula and mimic a variety of blindness pathologies discovered to affect humans, such as cone dystrophy (Stearns, Evangelista, Fadool, & Brockerhoff, 2007), and Leber congenital amaurosis (Iribarne et al., 2017). Disease and degeneration phenotypes can be monitored in real-time (Lewis, Williams, Lawrence, Wong, & Brockerhoff, 2010; Ma et al., 2013), and the permeability of larval fish allows for measuring effects of pharmacological treatment (Wang et al., 2015). Zebrafish retinas can regenerate their neurons, whereas human retinas do not, making them informative in gene therapy and genetic approaches to curing blindness (Gallina, Todd, & Fischer, 2014; Rao, Didiano, & Patton, 2017). There are a large number of complementary behavioral assays and techniques, discussed in this chapter, used to monitor retinal function and disease.

The Organization and Function of the Zebrafish Retina Organization

Both human and zebrafish retinas are polarized tissues that contain the same major cell classes in three nuclear layers: outer nuclear layer (ONL), which contains light-sensing photoreceptors, inner nuclear layer (INL), made up of bipolar, amacrine, and horizontal cells, and the ganglion nuclear layer (GCL) nearest to the lens, which contains ganglion cell bodies. Two plexiform layers, outer (OPL) and inner (IPL), contain synaptic connections between cells in different nuclear layers (Fig. 21.1).

Light Detection and Signal Transmission

Photoreceptors are polarized neurons with an opsin rich outer segment, an inner segment that contains the nucleus, a dense mitochondrial cluster, which regulates cell health and homeostasis, and a synapse, which transforms cell membrane potential into the controlled release of glutamate (Dowling, 1987). In the dark photoreceptors are in a depolarized state. Absorption of light causes a conformational change in opsin receptors located in the outer segment that initiate a signal transduction cascade that hyperpolarizes the neuron, thus reducing the release of the neurotransmitter glutamate at the synapse. Unlike other neurons, photoreceptors communicate via graded potentials rather than action potentials. Bipolar cells form synapses with either rods

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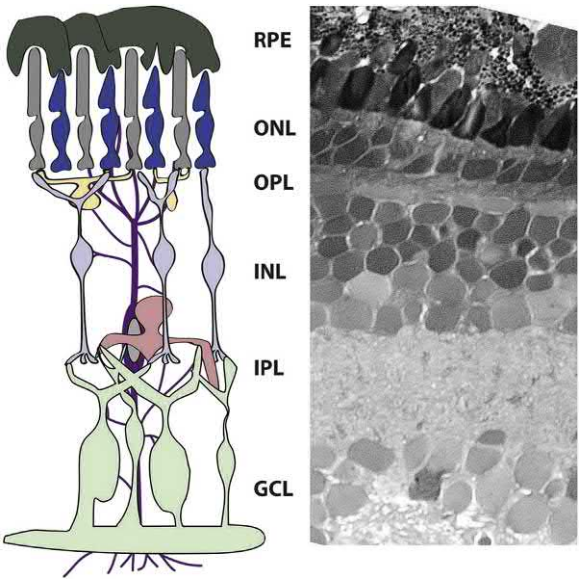

FIGURE 21.1 **The retina. (Left)** This schematic of the vertebrate retina highlights the cellular layering and different cell classes. The retinal pigment epithelium (RPE; dark green) is at the back of the eye where it extends processes that surround both rod and cone outer segments. The outer nuclear layer (ONL) contains rod (gray) and cone (blue) photoreceptors (PRs). PRs make synaptic connections to horizontal (yellow) and bipolar (light purple) cells within the outer plexiform layer (OPL). Amacrine cells (pink) reside at the vitreal side of the inner nuclear layer (INL). Cells within the INL form synapses with the ganglion cells (light green) in the ganglion cell layer (GCL) at the inner plexiform layer (IPL). Muller glia (purple) extend through much of the retina. **(Right)** This micrograph shows the larval zebrafish retina at six dpf. Already at this age, the retina is laminated with differentiated photoreceptors and robust visual behavioral responses (see text).

or cones in the outer plexiform layer, where they receive signals via glutamate released from photoreceptors (Ayoub & Copenhagen, 1991). Activated bipolar cells transmit information onto ganglion cells, the innermost layer of retinal neurons. Ganglion cells are the only cell type in the retina that project their axons directly into the brain for signal integration and interpretation.

Regulation and Maintenance of Retinal Neurons

There are two types of regulatory roles maintained by the additional retinal neurons. First, horizontal and amacrine cells directly modulate the light response by influencing photoreceptor-bipolar or bipolar-ganglion cell interactions, respectively. Horizontal cells laterally inhibit photoreceptor outputs from producing a controlled input to bipolar neurons, and the amacrine cells integrate and modulate the output message from the bipolar cells to ganglion cells. These interneurons allow for neuronal cross-talk between layers and are active within the IPL (Masland, 2012). Second, Muller glia act as support structures and span nearly the entire length of the retina, starting in the INL and reaching just within the ONL to photoreceptor nuclei. Muller glia have a range of functions that are vital to the health of all retinal neurons, including removal of toxic waste and neuronal debris created by excited or dying neurons (Bejarano-Escobar, Sanchez-Calderon, Otero-Arenas, Martin-Partido, & Francisco-Morcillo, 2017), uptake of excess glutamate from postsynaptic connections to prevent cell toxicity (Derouiche & Rauen, 1995), supply of critical nutrients to fuel neurons, and replenishment of neurotransmitters by providing glutamate precursor glutamine to neurons (Lindsay et al., 2014).

Neuronal Classification in the Zebrafish Retina

While the overall retina organization and presence of major cell types are conserved in vertebrates, there are species-specific features that reflect the unique visual requirements of different animals. For example, whereas mice contain primarily rod photoreceptors because they are nocturnal animals, zebrafish are cone dominant with a sophisticated color vision adapted for detecting a broad range of wavelengths extending from approximately 350 to 560 nm. As described in the following paragraphs, several studies report the subclasses of cells within the zebrafish retina. This information sets the stage for studies aimed at dissecting determinants of circuitry formation underlying visual behavioral specializations.

Photoreceptors

Zebrafish photoreceptors located in the ONL have four distinct cone subtypes, UV-sensitive, blue, and red-green double cones, which are spatially organized into a strictly arranged mosaic pattern (Allison et al., 2010). The photoreceptor population within the zebrafish retina contains further subdivisions of duplicated opsins, totaling 10 different opsin genes, and eight of these define eight unique cone subtypes. Zebrafish have four green-sensitive opsin genes with peak absorption maxima (λmax) each slightly shifted in wavelength sensitivity: RH2−1 (467 nm), RH2−2 (476 nm), RH2−3 (488 nm), and RH2−4 (505 nm), with RH2-2 being the most predominant form in the adult retina (Chinen, Hamaoka, Yamada, & Kawamura, 2003). Zebrafish also have two different red opsins that are spectrally distinct: LWS-1, 558 nm, the most abundant form, and LWS-2, with a peak absorbance at 548 nm. These subtype variants have unique spatial distributions in both larvae and adults: shorter wavelength sensitive opsin subtypes LWS-2, RH2-1, and RH2-2, are expressed in the central to dorsal retina, whereas longer wavelength subtypes LWS-1, RH2-3, and RH2-4 are found in the ventral retina (RH2-3 surrounds the central retina, and RH2-4

circumscribes RH2-3 in the ventral region (Takechi & Kawamura, 2005; Tsujimura, Chinen, & Kawamura, 2007). SWS1 (355 nm), or UV cones, and SWS2 (416), or blue cones, are each single-copy shorter wavelength opsins expressed throughout the retina mosaic (Chinen et al., 2003).

Rods in the zebrafish retina are arranged in regularly spaced rows. The rod cell bodies extend through the cone mosaic in a predictable pattern (Fadool, 2003). In most vertebrates, rhodopsin is a highly conserved single-copy gene. However, zebrafish express two different rod opsins with nearly identical peak sensitivity: RH1-1 (501 nm) and slightly blue-shifted RH1-2 (496 nm) (Morrow, Lazic, & Chang, 2011, 2017). While the functional characteristics of RH1-2 appear to mimic those of RH1-1, RH1-2 releases retinal at a rate similar to rhodopsin, RH1-1 is the predominant form with RH1-2 expression restricted to the ventral peripheral retina, a defined spatial distribution similar to what is seen for the other opsin duplicates. This defined distribution is not uncommon in teleosts, as specific expression patterns of precisely spectrally tuned opsin variants may be an advantage for detecting downwelling light (Morrow et al., 2011; Temple, 2011).

Diversity and Connectivity of Retinal Circuits

Downstream of photoreceptor neurons, distinctions in cell composition and circuitry also vary across species. The zebrafish retina contains 17 different types of bipolar neurons (Connaughton, Graham, & Nelson, 2004) (compared to the mouse, with 12 bipolar cells (Wassle, Puller, Muller, & Haverkamp, 2009), at least 11 ganglion neurons (Mangrum, Dowling, & Cohen, 2002; Ott, Walz, Paulsen, Mack, & Wagner, 2007) (22 ganglion cells, mouse (Volgyi, Chheda, & Bloomfield, 2009)), at least 28 types of amacrine cells (Jusuf & Harris, 2009), and four types of horizontal cells (Li, Matsui, & Dowling, 2009) (compared to the macaque, which has two (Dacey, 1999), or the mouse, with only one (Peichl & Gonzalez-Soriano, 1994)). The connectivity maps of neuronal circuits within the retina are highly complex. Each class of bipolar cell synapses with multiple photoreceptors to create and ON- OFF- center-surround array, while ganglion neurons, characterized by dense asymmetric dendritic branching patterns, make most connections with respective bipolar neurons in the IPL. However, the subclass of every neuronal type in the retina is defined by many factors: the unique number and pattern of dendrites; dendritic and synaptic projections into the retinal sublaminar; cell shape and size; and the classes of other retinal neurons with which they form synaptic connections. For example, of the four zebrafish horizontal cells, three subtypes connect with specific cones: H1 forms contacts with L-, M-, and S-cones, H2 sample M-, S-, and UV-cones, and H3 horizontal cells only receive input from S and UV cones (Li et al., 2009). The last horizontal cell subtype, H4, exclusively receives input from rods (Li et al., 2009). While circuitry and function of the retina are generally conserved between vertebrates, the zebrafish retina contains one notable difference: rod bipolar cells receive input from not only rod photoreceptors, but also from L-type cones (Li, Tsujimura, Kawamura, & Dowling, 2012). Therefore, unlike mice and monkeys, zebrafish do not have a neuronal pathway that selectively only represents the rod population.

Function

One of the properties that make zebrafish an excellent experimental system for vision studies is the rapid development of the visual function. Zebrafish develop *ex utero* and are free swimming with highly sensitive and accurate visual function already at 5 days postfertilization (dpf). The first visual responses are detected at 3dpf (Branchek, 1984; Easter & Nicola, 1996). Zebrafish larvae rely on vision, particularly cone photoreceptor function, to efficiently capture prey. By five dpf, larvae have depleted their endogenous food supply, and prey capture is required for survival. Larvae with impaired cone function have difficulty surviving without extra food provided to make foraging more successful (Brockerhoff et al., 2003). This rapid development of the visual system has been exploited by investigators interested in identifying genes critical for visual function: genetic screens analyzing visual behavior can be conducted early in development reducing the cost and labor associated with maintaining fish for long periods of time. To this end, many different behavioral screening strategies have been established for quantifying zebrafish (particularly larval zebrafish) visual function (Fleisch & Neuhauss, 2006; Neuhauss, 2003). Because many of the visually mediated behavioral responses are so robust and reproducible, they have recently been used to begin to define the circuits that are used to transform sensory input into action (Bianco & Engert, 2015; Dunn et al., 2016; Naumann et al., 2016).

Behavioral Assays

The Optokinetic Response (OKR)

The OKR is a reflex in which the eye moves in response to the movement of a visual stimulus. There are two components to the OKR, a smooth pursuit tracking movement in the same direction as the moving

stimulus and a subsequent rapid saccade back to the starting position. The zebrafish OKR is a very robust response that develops early and rapidly; 25% of larvae display an OKR at 72hpf, and 100% respond at 80hpf (Easter & Nicola, 1996). For eliciting the OKR, larvae are immobilized by being placed in a viscous aerated aqueous media. A moving grate is then provided as a stimulus, and eye movements are measured. Because this assay is robust and responses are easy to detect and measure, forward genetic screens have used this strategy to identify novel mutants with visual function defects (Brockerhoff et al., 1995; Muto et al., 2005). Many different OKR tracking systems have been reported. In the simplest versions, stripes are manually placed inside a drum whose rotational speed and direction can be mechanically controlled (Brockerhoff, 2006; Neuhauss et al., 1999). Other versions use computer-generated moving gratings that are projected onto the drum using a digital light projector that is placed either on the plane of the subject (linear projection) or below the subject (Huber-Reggi, Mueller, & Neuhauss, 2013). Eye movements are recorded and then quantified. In addition to leading to the successful identification of visually impaired zebrafish, the OKR has been used to analyze the development of visual acuity (Haug, Biehlmaier, Mueller, & Neuhauss, 2010; Rinner, Rick, & Neuhauss, 2005).

The initial analyses of zebrafish vision using the OKR were done using larvae. The OKR systems used for these studies were not designed for analyzing adults. The main difference is that adult zebrafish are strong swimmers and so they must be held tightly in place, as well as kept wet to ensure that oxygen is exchanged across the gills. Larvae can be easily restrained in viscous solutions, and oxygen readily diffuses across the skin. Adults require underwater restraint systems that keep their bodies from moving and their heads visible so that eye movements can be recorded and analyzed. Several groups have now developed OKR systems for quantifying adult vision (Cameron et al., 2013; Mueller & Neuhauss, 2010; Tappeiner et al., 2012).

Phototaxis

While the OKR is perhaps the most robust vision-dependent behavioral response, it is also relatively slow to perform since eye movements are analyzed on individual fish. The optomotor and phototactic responses, in contrast, can be measured on groups of larval zebrafish. Phototaxis describes the movement of an organism either toward (positive phototaxis) or away (negative phototaxis) from a light stimulus. Early studies reported that zebrafish larvae display both positive and negative phototactic behavior but it appeared highly variable (Brockerhoff et al., 1995; Gerlai, Lahav, Guo, & Rosenthal, 2000; Orger & Baier, 2005; Serra, Medalha, & Mattioli, 1999). Conditions establishing the relationship between the robustness and rate of phototaxis and relative light intensity were reported in 2010 (Burgess, Schoch, & Granato, 2010). Both the maximal number of responding larvae and the speed of movement toward a spot of light occur when the target illumination is 10-fold less intense than the uniform background light. Pharmacological manipulations and visually impaired mutants were used to demonstrate that the visual pathway responding to light on (the ON pathway) controls the rate of approach, while the visual pathway responding to light decrements (the OFF pathway) elicits turns that enable steering (Burgess et al., 2010).

The Optomotor Response (OMR)

The OMR is a reflexive swimming behavior that can be elicited in response to moving visual stimuli, such as rotating stripes. This behavior is robust in both larvae and adults. For larvae, the response is strong at seven dpf and can be evaluated on groups of different clutches in parallel (Neuhauss et al., 1999; Orger, Smear, Anstis, & Baier, 2000). Larvae are placed in a swimming container and moving sinusoidal gratings are presented to them either from below or from the side. Detection of the "stripes" causes a majority of larvae to swim in the same direction as the apparent motion. These properties have made this assay useful for screening for recessive mutations that cause blindness (Muto et al., 2005). This assay has also been used to measure the dominant chromatic inputs for motion detection, which varies with age and species (Orger & Baier, 2005).

In adults, the OMR is measured on individual fish since schooling can interfere with this behavioral response. Single fish are placed in a circular drum with a rod in the center to prevent swimming across the apparatus. The gratings are projected onto the drum and swimming is evoked in the apparent direction of rotation. This assay has been used to measure how spatial and temporal properties of repetitive stimuli influence the OMR and to measure the color contribution to motion detection (Krauss & Neumeyer, 2003; Maaswinkel & Li, 2003).

The Adult Escape Response

The adult escape response is initiated by the appearance of a visual stimulus that elicits a reverse in swimming direction (i.e., an escape). The experimental setup is the same as for the OMR except the stimulus is a single "threatening" stripe. The adult escape

response has been used to identify fish with visual defects (Li & Dowling, 1997; Maaswinkel, Mason, & Li, 2003a, Maaswinkel, Ren, & Li, 2003b).

Physiological Assays

Electroretinogram (ERG)

The ERG is a diagnostic test used to measure the function of the outer retina. It is a noninvasive procedure that records the light-induced changes in electrical potential across the eye measured at the corneal surface. These changes are the result of altered sodium and potassium fluxes occurring in both neuronal and nonneuronal retinal cell types. Vertebrate ERG recordings have four principal components: the a-wave, a cornea-negative potential derived from phototransduction within the photoreceptor cell; the b-wave, a cornea-positive potential derived from the ON bipolar cell activity; the c-wave, which appears more slowly and is thought to originate from the RPE and; the d-wave, which originates from OFF bipolar cell activity and can be separated from the b-wave by long-duration flashes.

Early functional studies indicated that only cones contribute to zebrafish vision until approximately 2 weeks of development (Bilotta, Saszik, & Sutherland, 2001; Branchek, 1984). The larval ERG has a photopic response dominated by the b-wave. The photoreceptor component (a-wave) can be selectively isolated using the pharmacological agent L-(+)-2-amino-4-phosphonobutyric acid (L-AP4), an agonist for the metabotropic glutamate (mGluR6) receptor expressed by the ON bipolar cells. Combinations of drugs and subtractions of waveforms have been used to isolate many ERG elements that reflect the retinal organization and summation of multiple cone signals (Nelson & Singla, 2009). This has made the ERG useful for identifying zebrafish mutants that selectively lack all cone photoreceptor function (Brockerhoff et al., 2003; Stearns et al., 2007) and also for characterizing and defining defects in mutants with subtle circuitry defects (Lewis et al., 2011).

ERGs can be recorded using an isolated eye or eyecup dissected from a larval or adult zebrafish, or they can be done on the intact eye in a living anesthetized animal. Using either strategy, an electrode, placed either on the cornea or on the vitreal side of the retina, transmits the electrical signal through an amplifier, an analog to digital converter, then to a computer for analysis and processing. The original zebrafish ERGs were done on whole animals (Brockerhoff et al., 1995). Larvae are kept moist but are not submerged in water. Adults have water continuously flushed through the gills by placing a tube with flowing water into the mouth. Adults also require treatment with a muscle stabilizer gallamine triethiodide (Li & Dowling, 1997). Recordings using whole animals are still routine (Korenbrot, Mehta, Tserentsoodol, Postlethwait, & Rebrik, 2013; Lin et al., 2016), but studies using isolated eyes or eyecups result in robust recordings for significantly longer times than with whole animals, and drugs permeate isolated eyes more readily (Wong, Gray, Hayward, Adolph, & Dowling, 2004).

Single-cell Recordings

While the ERG is a powerful strategy for analyzing retinal function, it is a summed response and does not reflect the activity of individual cells. Two general types of cell preparations are used to record activity from individual cells. Cells either are identified in the context of the retina (in an eyecup or retinal slice), or they are dissociated and isolated from neighboring cells. Several groups have reported physiological recordings of individual retinal neurons (Aquila, Benedusi, Fasoli, & Rispoli, 2015; Connaughton, Nelson, & Bender, 2008; Connaughton & Nelson, 2000; Fan & Yazulla, 1997; Klaassen et al., 2011). These strategies have been used on adults and juveniles, but not on larvae due to the small size of their neurons. The retinal slice preparation has several advantages (Connaughton, 2003). Since cells are not dissociated, the cellular arrangement and synaptic connections present *in vivo* are maintained. Further, since the slice is a tangentially cut section of the retina, all neurons in the different retinal layers are visible and accessible.

Fluorescent Strategies

Genetically Encoded Indicators

Another strategy commonly used to measure the functional activity of single cells or cellular circuits is to make transgenic zebrafish expressing genetically encoded indicators. Retinal slices from transgenic fish can be placed in a perfusion chamber, and then specific cellular responses due to changing the media can be measured in real-time at subcellular resolution (Giarmarco, Cleghorn, Sloat, Hurley, & Brockerhoff, 2017; Giarmarco, Cleghorn, Hurley, & Brockerhoff, 2018) (Fig. 21.2). A more common strategy is to image the entire eye and recently the entire fish brain; there are genetic and pharmacological ways to maintain zebrafish transparently, and thus, it is possible to image fluorescent sensors in real-time in the living animal. In the retina these strategies have been used to image events, such as dying photoreceptors (Lewis et al., 2010; Ma et al., 2013), formation of synaptic connections

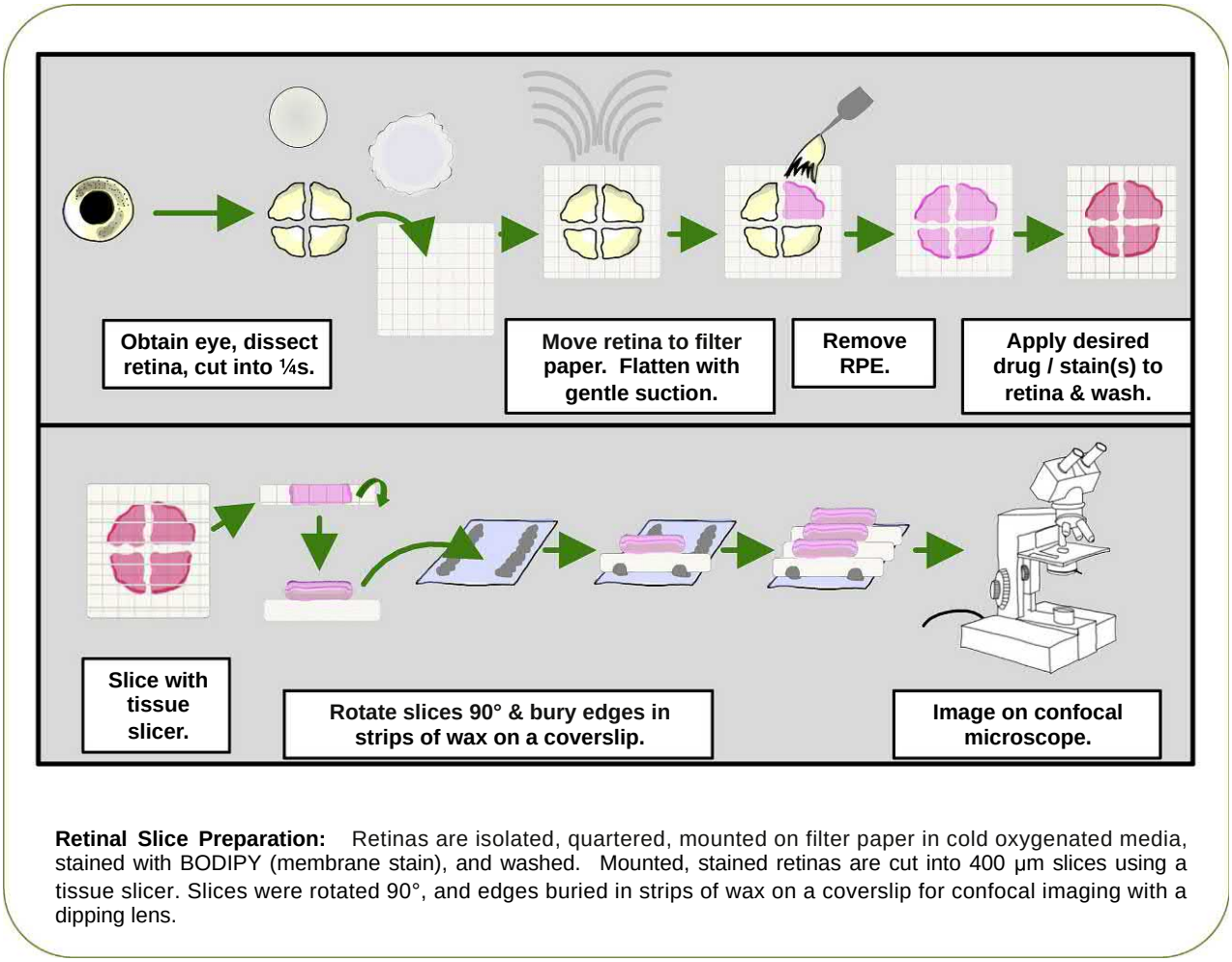

FIGURE 21.2 **The retinal slice preparation.** Retinal slices are useful for many different experiments involving adult retinas (see text).

(Williams et al., 2010), vesicular trafficking (George, Hayden, Stanton, & Brockerhoff, 2016) and dedifferentiation of Mueller glia (Bernardos, Barthel, Meyers, & Raymond, 2007). Due to the small size of the zebrafish larva, it is possible to image the vast majority of neurons in the brain at the same time (Feierstein, Portugues, & Orger, 2015) in the intact whole animal, while it is performing a stereotyped behavior in response to visual stimuli. This is an amazing achievement and a very powerful approach for dissecting the complete circuitry underlying visual behavior. Calcium sensors have often been used for these studies since Ca^{2+} dynamics reflect neuronal activity, and there are many varieties of genetically encoded Ca^{2+} indicators with different sensitivities and kinetics (Lin & Schnitzer, 2016). These types of experiments are putting zebrafish at the forefront of research aimed at defining the neuronal sequence from sensory input to behavioral output for visual, as well as other stimuli (Kawashima, Zwart, Yang, Mensh, & Ahrens, 2016; Naumann et al., 2016; Oteiza, Odstrcil, Lauder, Portugues, & Engert, 2017; Randlett et al., 2015).

Summary

This review describes the structural organization of the zebrafish retina and the many behavioral, physiological, and fluorescent approaches that can be used to measure visual function. This provides the foundation for dissecting mechanisms underlying disease and for developing therapeutic strategies to treat blindness. The sophisticated genetic, cell biological, behavioral, physiological, and biochemical approaches available combined with rapid development and high fecundity promise to ensure the continued use of the zebrafish model for important discoveries in vision research.

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CHAPTER

22

The Mechanosensory Lateral Line System

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Introduction

The lateral line is a sensory system utilized by a wide variety of aquatic vertebrates to detect changes in surrounding water flow. This sense, which utilizes mechanotransduction, mediates a wide variety of behaviors, from predator detection to schooling. Thus, the lateral line can be viewed as an extension of the vestibular system, mediating "touch at a distance"(Coombs, Bleckmann, Fay, & Popper, 2014; Dijkgraaf, 1963). In particular, the lateral line of the zebrafish has proven to be an invaluable model system for the study of sensory development, collective cell migration, and regeneration.

Anatomy of the Lateral Line

The lateral line is comprised of sensory organs called *neuromasts*. These neuromasts are arranged in a stereotyped fashion along the surface of the body (Fig. 22.1). This system is divided into two parts: the anterior lateral line (aLL), which consists of the neuromasts on the head of the fish; and the posterior lateral line (pLL), consisting of neuromasts arranged along the trunk. Neuromasts are comprised of clusters of mechanosensory hair cells surrounded by nonsensory support cells along with the afferent and efferent neural connections to the hair cells (Fig. 22.2). Each of these cell types plays a major role in the function of the neuromast.

Hair Cells

Lateral line hair cells are arranged in a rosette pattern at the center of the neuromast (Fig. 2.22, bottom). These hair cells are structurally and functionally analogous to the hair cells of the inner ear, in particular vestibular hair cells, and are polarized, containing basally localized nuclei and synapses and an apically localized mechanosensory apparatus. This apparatus consists of rows of actin-based stereocilia and a larger microtubule-based kinocilium, which project out into the environment and are surrounded by a gelatinous structure called the *cupula*. The kinocilium is located asymmetrically to the stereocilia, providing the hair cell with directional sensitivity: deflection of stereocilia toward the kinocilium results in hair cell activation (Nicolson, 2005). Each neuromast contains hair cells with apical bundles polarized along the same axis with two populations 180 degrees out of phase. Depending on the developmental origin of the neuromast, these apical bundles are aligned along either the anteroposterior or the dorsoventral axis of the body, thus establishing an axis of planar cell polarity. The orientation of hair cells is regulated by planar cell polarity genes (López-Schier & Hudspeth, 2006; López-Schier, Starr, Kappler, Kollmar, & Hudspeth, 2004).

Support Cells

Lateral line hair cells are surrounded by a seemingly homogenous group of cells called *support cells* (Fig. 22.2, top). Many of these cells are located basally to the hair cells and project interdigitating processes between them, acting to isolate hair cells from one another. In addition to serving as the source for new hair cells in the event of damage (as will be discussed in the Regeneration section), they also provide structural and trophic support. The most peripheral support cells, often referred to as mantle cells, are believed to contribute to the formation of the cupula.

Innervating Fibers

Lateral line hair cells are innervated by both afferent and efferent nerve fibers on their basal surfaces

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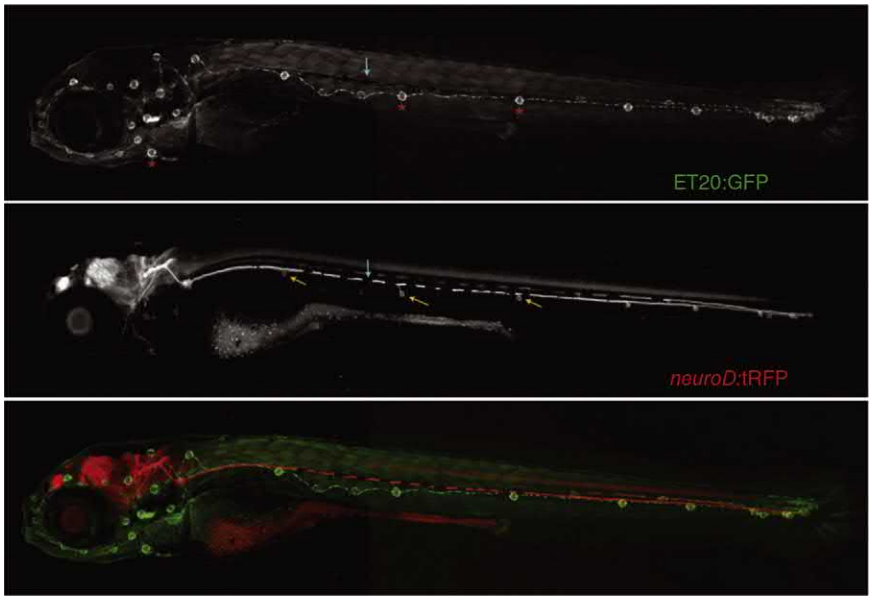

FIGURE 22.1 The larval zebrafish lateral line system. (Top) Neuromasts and interneuromast cells are labeled by the transgenic marker ET20:GFP at 5 dpf. *Red asterisks* indicate both aLL and pLL neuromasts. *Blue arrow* indicates a melanocyte located in the horizontal myoseptum. (Middle) The *lateral line* nerve is labeled by neuroD:tRFP. *Blue arrow* indicates the same melanocyte as in the top panel. *Yellow arrows* indicate afferent fibers innervating neuromasts. (Bottom) Composite image of the top and middle panels. From Thomas et al., 2015.

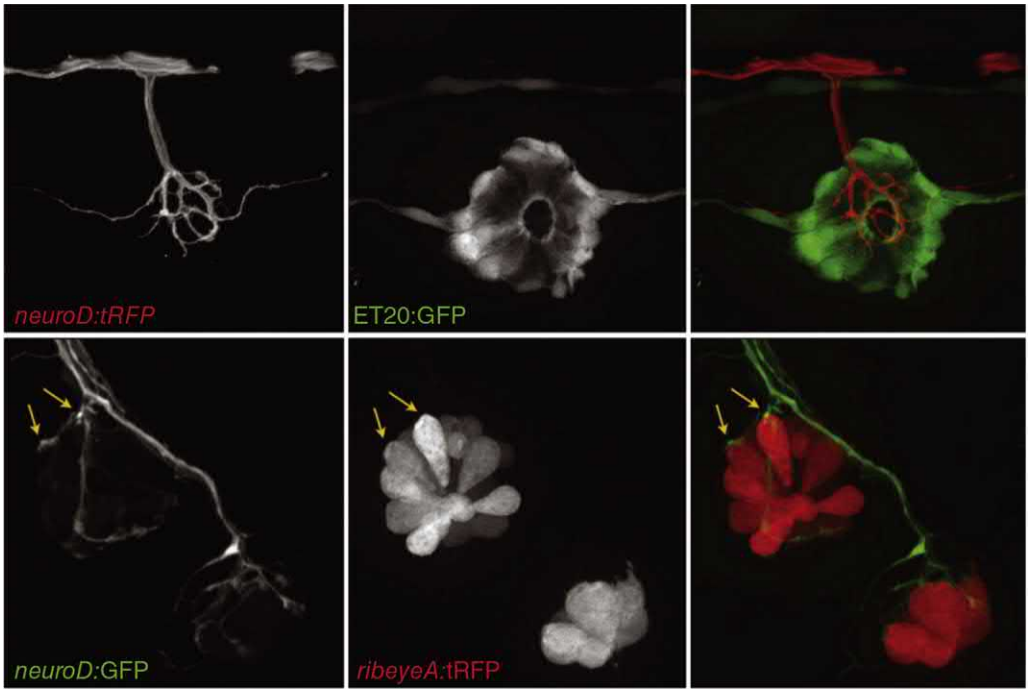

FIGURE 22.2 An individual neuromast at 5 dpf. The *lateral line* nerve and afferent terminals are labeled in red by neuroD:tRFP (top and bottom, left). Support cells are labeled in green by ET20:GFP (top middle). Hair cells are in red, marked by ribeyeA:tRFP (bottom middle). *Yellow arrows* indicate afferent terminals (left) and their synaptic partners (middle). From Thomas et al., 2015.

(Fig. 22.2). These fibers extend down the length of the trunk as part of the lateral line nerve, branching off at each neuromast (Raible & Kruse, 2000). Hair cells form specialized synapses with afferent neurons comprised of a presynaptic ribbon that serves to organize synaptic vesicles for multivesicular release. Afferent neurons can innervate multiple hair cells within a neuromast, and can also innervate multiple neuromasts. However, they only innervate hair cells of the same planar polarity (Faucherre, Pujol-Martí, Kawakami, & López-Schier, 2009;

Nagiel, Andor-Ardó, & Hudspeth, 2008). The cell bodies of afferents form the pLL ganglion (pLLG) and project to the hindbrain, exhibiting a somatotopic map of neuromast position (Alexandre & Ghysen, 1999; Liao, 2010). Efferent fibers originate from cell bodies in the hindbrain to innervate hair cells and release dopamine, modulating activity at afferent synapses (Toro et al., 2015).

Lateral Line Function

The lateral line is responsible for encoding information about fluctuations in water flow around the fish. Directional deflection of the apical hair cell bundles results in the opening of mechanotransduction channels and an influx of calcium, with corresponding depolarization and release of the neurotransmitter glutamate onto the afferent terminals, which propagate the signal to the central nervous system (Trapani & Nicolson, 2011). Proper lateral line function can be examined by measuring rheotaxis: fish are normally able to align themselves against water currents but are unable to do so when lateral line hair cell functionality is lost (Suli, Watson, Rubel, & Raible, 2012). Furthermore, loss-of-function mutations in hair cell mechanotransduction (*sputnik* and *mariner*) or hair cell development (*atoh1a*) all disrupt lateral line function (Millimaki, Sweet, Dhason, & Riley, 2007; Nicolson et al., 1998).

Development of the Lateral Line

The pLL is derived from a migratory column of cells called the *posterior lateral line primordium* (pLLP). This primordium forms adjacent to the otic placode around 20 h postfertilization and travels posteriorly along the horizontal myoseptum toward the tip of the tail, depositing clusters of cells in its wake that will form neuromasts (Sarrazin et al., 2010). The pLLP forms close contacts with the growth cones of pLLG axons, which associate with deposited cell clusters. These initial depositions form the primary lateral line. The pLLP is organized along its migratory axis: cells in the posterior third (known as the leading zone) are mesenchymal and highly proliferative, whereas cells in the anterior two-thirds (called the *trailing zone*) are organized into epithelial rosettes. The cells in these rosettes (often called *protoneuromasts*) will be specified as hair cells or support cells following their deposition. As the fish grows, the lateral line becomes far more elaborate, with more lines being added on the head and trunk.

pLLP Migration

Migration of the pLLP is regulated by the chemokine Sdf1a (also known as Cxcl12a) and its receptors Cxcr4b and Cxcr7b. Cells along the horizontal myoseptum express Sdf1a, whereas the pLLP expresses both Cxcr4b and Cxcr7b. Disrupting expression of Sdf1a or of either receptor prevents pLLP migration (David et al., 2002; Haas & Gilmour, 2006; Valentin, Haas, & Gilmour, 2007). Unlike other contexts, pLLP migration is not regulated by an expression gradient of its guidance cue, as Sdf1a is expressed uniformly along the horizontal myoseptum. Rather, it is the asymmetric expression of the chemokine receptors that allows for the directional migration of the primordium. Cxcr7b, which has a much higher affinity for Sdf1a than does Cxcr4b, is expressed in the trailing zone, whereas Ccxr4b is expressed in the leading zone (Dambly-Chaudière, Cubedo, & Ghysen, 2007). Upon binding to Cxcr7b, Sdf1a is internalized and degraded (Balabanian et al., 2005). This difference in binding affinity allows Cxcr7b to serve as a molecular sink, preventing Cxcr4b receptors expressed near the trailing zone from binding the chemokine (Donà et al., 2013; Venkiteswaran et al., 2013). Thus, a gradient of Sdf1a expression is generated solely across the primordium, with its levels highest at the leading edge, where binding to Cxcr4b can promote actin polymerization in the direction of migration (Xu et al., 2014).

The Roles of Wnt and FGF Signaling During pLLP Migration

Cellular activity within the pLLP is determined by the interplay between canonical Wnt and FGF signaling. Wnt signaling is high in the leading zone, whereas FGF signaling is high in the trailing zone. These signaling pathways also inhibit each other, thus restricting their activity to their respective regions of the primordium. Within the leading zone, Wnt signaling drives expression of Cxcr4b, as well as mediates proliferation, allowing the primordium to maintain its size throughout its migration (Aman & Piotrowski, 2008; Laguerre, Ghysen, & Dambly-Chaudière, 2009). These proliferating cells leave the leading zone and expand throughout the pLLP as it migrates, contributing to multiple protoneuromasts. Inhibiting proliferation reduces the number of neuromasts formed by decreasing the rate at which neuromasts are deposited (Aman, Nguyen, & Piotrowski, 2011; Gamba, Cubedo, Lutfalla, Ghysen, & Dambly-Chaudiere, 2010; Matsuda et al., 2013; McGraw et al., 2011; Valdivia et al., 2011). Wnt signaling also drives the expression of the ligands Fgf3 and Fgf10a (Aman & Piotrowski, 2008). Overall, inhibition of Wnt signaling results in defects in pLLP migration.

Within the trailing zone, FGF signaling regulates the morphogenesis of the epithelial rosettes that will become neuromasts. As cells enter the trailing zone, expression of *fgf10a* becomes restricted to a few

individual cells, with the surrounding cells expressing *fgfr1* (Lecaudey, Cakan-Akdogan, Norton, & Gilmour, 2008; Nechiporuk & Raible, 2008). Activation of the surrounding receptors results in the apical localization of the Rho-activated kinase Rock2a, which induces apical constriction via phosphorylation of myosin regulatory light chain (Ernst et al., 2012; Harding & Nechiporuk, 2012). Inhibition of FGF signaling prevents the formation of these rosettes, and thus the formation of neuromasts (Aman & Piotrowski, 2008; Lecaudey et al., 2008; Nechiporuk & Raible, 2008).

Neuromast Maturation

After protoneuromasts are deposited by the migrating pLLP, the cells within them are specified as hair cells or support cells via Notch-mediated lateral inhibition. Prior to deposition, Fgf10a, localized centrally within rosettes, induces expression of the bHLH transcription factor *atoh1a*, which is necessary for hair cell development (Matsuda & Chitnis, 2010). *Atoh1a* maintains the expression of *fgf10a*, as well as driving expression of the Notch ligand *deltaD*. Activation of Fgfr1 in the surrounding cells maintains the expression of the Notch receptor *notch3*. Binding of Notch3 to DeltaD results in the cleavage of the Notch intracellular domain (NICD), which translocates to the nucleus and prevents the expression of *atoh1a*. Thus, the central *atoh1a*-expressing cells will form hair cells, whereas the surrounding cells will become support cells (Matsuda & Chitnis, 2010; Millimaki et al., 2007). Inhibition or loss of Notch signaling, as occurs in the *mind bomb* mutant, results in the formation of supernumerary hair cells at the expense of support cells (Itoh & Chitnis, 2001). Loss of atoh1a function, on the other hand, prevents hair cell differentiation altogether.

The lateral line nerve migrates down the trunk of the fish along with the pLLP, branching off to innervate neuromasts as they are deposited (Metcalfe, Kimmel, & Schabtach, 1985). Inhibition of pLLP migration also prevents nerve growth (Gilmour, Knaut, Maischein, & Nüsslein-Volhard, 2004). This comigration of nerve and primordium is regulated by glial cell line-derived neurotrophic factor (GDNF) signaling. The pLLP itself produces GDNF, whereas the growth cones of the lateral line nerve express its receptor GFRα1 (Shepherd, Beattie, & Raible, 2001). Inhibition of GDNF signaling has no impact on pLLP migration, but greatly reduces the extension of the lateral line nerve (Schuster, Dambly-Chaudière, & Ghysen, 2010). The pLLG is not necessary for pLLP migration or neuromast differentiation, and even presynaptic components can develop within hair cells without innervation (Grant, Raible, & Piotrowski, 2005; Suli et al., 2016).

Growth of the Lateral Line

The developmental processes described above apply to the primary lateral line. However, as fish grow to adulthood, the lateral line grows as well. Adult zebrafish have four posterior lateral lines, which are organized into groups of neuromasts called *stitches* (Nuñez et al., 2009; Sapède, Gompel, Dambly-Chaudière, & Ghysen, 2002; Wada et al., 2010) (Fig. 23.3, bottom). The anterior lateral line also becomes more elaborate, with some neuromasts becoming incorporated into bony canals (Wada, Iwasaki, & Kawakami, 2014; Webb & Shirey, 2003) (Fig. 22.3, top). This growth of the lateral line occurs via three different mechanisms: migration of new primordia; expansion of latent neuromast precursors; and budding from existing neuromasts. The initial pLL primordium described above is known as primI. A second primordium, primII, migrates down the trunk later, depositing neuromasts in between those derived from primI. Collectively, these neuromasts form the L line (Sapède et al., 2002). A third primordium, primD, migrates along the dorsal midline and establishes the D line (Sarrazin et al., 2010). Two

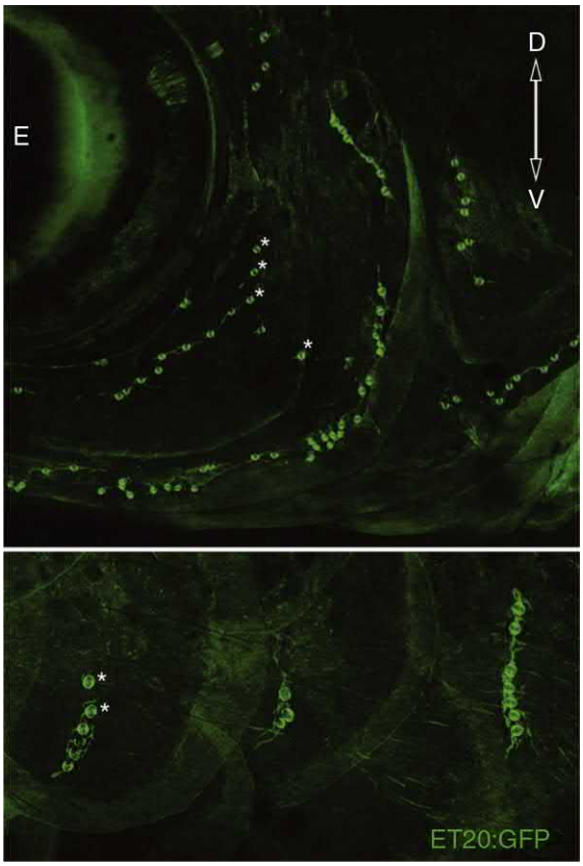

FIGURE 22.3 Lateral line organization in adult zebrafish. (Top) Anterior *lateral line* in ET20:GFP transgenic adult fish. "E," eye. *White asterisks*, neuromasts. Note the expansion of neuromast patterning compared to that of Fig. 22.1. (Bottom) Stitch patterning in the adult *posterior line*. New neuromasts bud dorsally and ventrally off of existing neuromasts. From Thomas et al., 2015.

additional lines (the L′ and D′ lines) are derived from interneuromast cells, cells derived from the primordia that are deposited in between neuromasts. These cells serve as latent neuromast precursors, as they divide and generate entire neuromasts, known as intercalary neuromasts. This process is regulated by signals from glial cells that are associated with the lateral line nerve. These intercalary neuromasts develop precociously in the absence of glia or the nerve itself, indicating that glial cells maintain interneuromast quiescence (Grant et al., 2005; Lush & Piotrowski, 2014). Finally, budding of new neuromasts from existing neuromasts is responsible for the formation of stitches as well as of the neuromasts located on the rays of the caudal fin (the caudal lateral line or cLL) (Wada, Hamaguchi, & Sakaizumi, 2008). This is regulated by Wnt signaling between the budding neuromasts and their innervating nerves. Innervating axons express the Wnt activator R-spondin (*rspo2*), which binds to LGR receptors (specifically *lgr6*) in the budding neuromasts. Ablation of the innervating neurons diminishes Wnt signaling, and inhibition of Wnt signaling prevents the formation of the cLL (Wada, Dambly-Chaudière, Kawakami, & Ghysen, 2013a; Wada et al., 2013b, pp. 1–7).

Regenerative Capacity of the Lateral Line

The neuromasts of the lateral line also have the capacity to regenerate. Exposure to a number of different toxins, such as aminoglycoside antibiotics (Harris et al., 2003; Williams & Holder, 2000), chemotherapeutics (Ou, Raible, & Rubel, 2007; Ton & Parng, 2005), and metal ions (Faucher, Floriani, Gilbin, & Adam-Guillermin, 2012; Hernández, Moreno, Olivari, & Allende, 2006; Linbo, Stehr, Incardona, & Scholz, 2006), results in the death of lateral line hair cells. However, following damage, new hair cells are gradually added, reaching their original numbers around 3 days after treatment (Hernández, Olivari, Sarrazin, Sandoval, & Allende, 2007; Ma, Rubel, & Raible, 2008; Williams & Holder, 2000) (Fig. 22.4). Hair cell polarity and innervation are also restored (López-Schier & Hudspeth, 2006; Nagiel et al., 2008), as is the fish's ability to perform rheotaxis (Suli et al., 2012), indicating the restoration of lateral line functionality. These regenerated hair cells derive from the surrounding support cells, which divide symmetrically to form two daughter hair cells (López-Schier & Hudspeth, 2006; Mirkovic, Pylawka, & Hudspeth, 2012; Wibowo, Pinto-Teixeira, Satou, Higashijima, & López-Schier, 2011). Administration of thymidine analogs, such as bromodeoxyuridine (BrdU) during the first 24 h of regeneration labels the vast majority of regenerated hair cells (Ma et al., 2008), and mitotic inhibitors block regeneration (Mackenzie & Raible, 2012; Namdaran, Reinhart, Owens, Raible, & Rubel, 2012). It is likely that the few hair cells that do not show BrdU incorporation reflect developmental hair cell addition that is ongoing in the absence of damage, although the ability of support cells to transdifferentiate into hair cells cannot be completely ruled out. This regenerative capacity is retained throughout adulthood. In fact, zebrafish as old as 3 years are still capable of regenerating their hair cells (Cruz et al., 2015).

Identity of Hair Cell Progenitors

Whether all support cells within a neuromast, or only a specialized subset, are capable of generating new hair cells in response to damage remains unknown. Time-lapse studies, as well as localization of proliferating cells, have indicated that dividing precursors are generally localized in the dorsal and ventral regions of the neuromast (Ma et al., 2008; Mirkovic et al., 2012; Wibowo et al., 2011). However, these progenitors can migrate to these regions from elsewhere in the neuromast, suggesting that these dorsal and ventral regions are not inherent stem cell niches (Wibowo et al., 2011). Since progenitors divide symmetrically to generate new hair cells, repeated damage over time could deplete the progenitor pool, and thus, diminish the regenerative capacity of the lateral line. However, regeneration occurs normally in adult zebrafish after 10 consecutive damage and regeneration cycles (Cruz et al., 2015). Thus, there must be a mechanism through which these progenitors are replenished. Future studies are needed to determine whether the cells responsible for this are distinct from the hair cell progenitors themselves.

Genetic Regulation of Regeneration

It is unclear whether regeneration in the lateral line occurs via a recapitulation of development or through its own distinct mechanisms. As in development, both Notch and Wnt signaling play important roles in hair cell regeneration. Many Notch signaling components are upregulated in neuromasts following hair cell death, and inhibition of Notch signaling increases the number of proliferating progenitors and the number of new hair cells formed. However, Notch inhibition in the absence of damage has no effect of hair cell number, indicating that Notch signaling serves to regulate the number of regenerated hair cells and restore quiescence to the neuromast (Ma et al., 2008). On the other hand, canonical Wnt signaling serves to regulate proliferation during regeneration, as during development. Pharmacological activation of Wnt signaling increases proliferation, and the overall size of the neuromast, whereas blocking Wnt signaling decreases proliferation. Wnt-induced proliferation is restricted to the dorsal and ventral poles of the

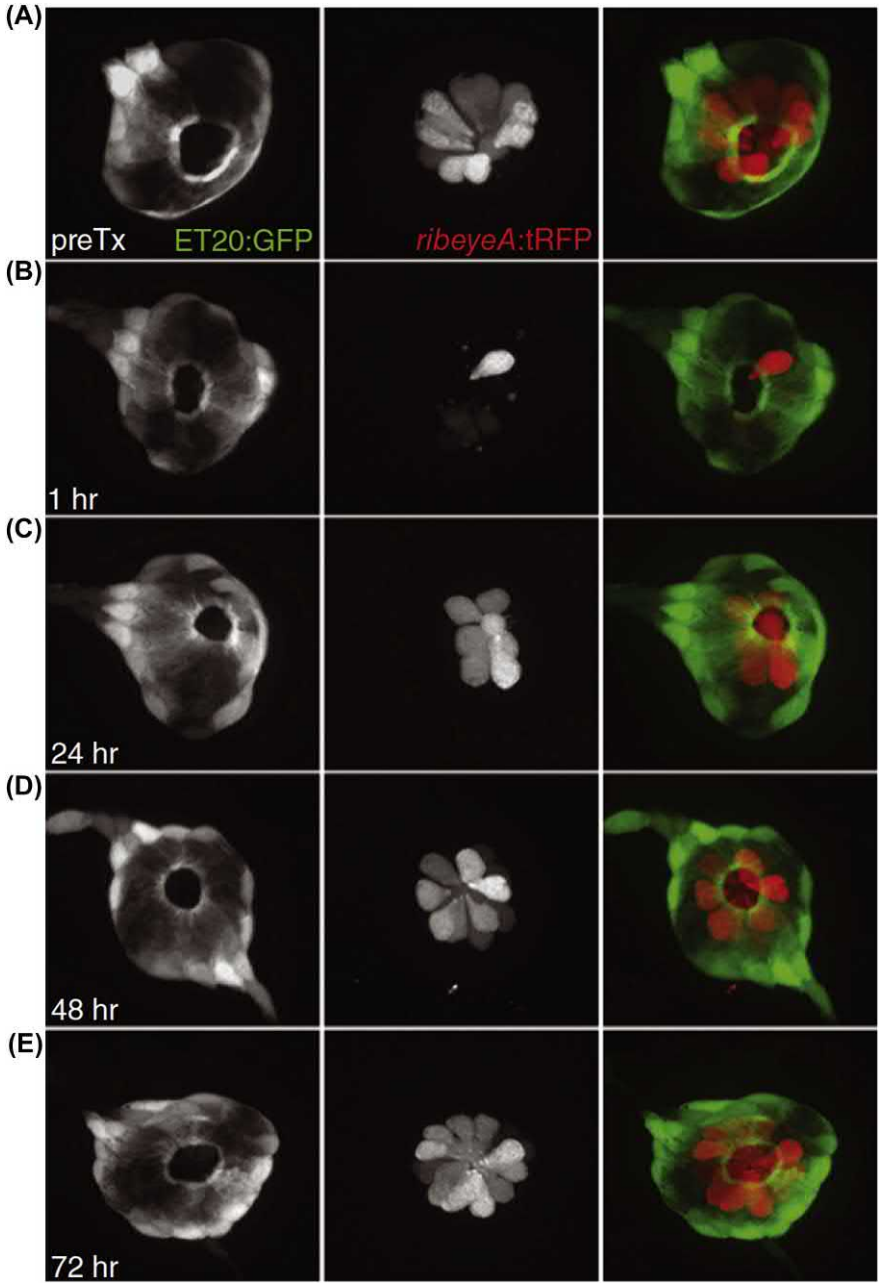

FIGURE 22.4 Hair cell regeneration. *Lateral line* neuromasts are shown support cells labeled in green (left), hair cells labeled in red (center), and overlay (right). (A) Neuromast at 5 dpf prior to neomycin treatment (B) After 1 h post neomycin treatment, almost all hair cells are killed, while the support cells are largely unaffected. By 24 h post treatment (C), some hair cells have regenerated, and these increase through 48 h (D) and 72 h (E). From Thomas et al., 2015.

neuromast by Notch signaling, which blocks Wnt signaling in the center via expression of the Wnt-inhibitor *dkk2* (Head, Gacioch, Pennisi, & Meyers, 2013). However, the mechanisms responsible for initiating Wnt signaling in these regions are unknown.

Conclusion

The zebrafish lateral line system has emerged as a model for studying the fundamental processes of development, including tissue patterning, morphogenesis, and growth. Its position on the surface of the body makes it well-suited to visualization and manipulation. The regeneration of functional mechanosensory cells after damage offers the potential to uncover processes involved in the maintenance, proliferation, and differentiation of sensory precursors. Future studies have the potential for understanding circuit development regulating fundamental behaviors and the evolution of tissue patterning. The lateral line system has bright prospects for future studies.

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CHAPTER

23

Inner Ear and Hearing

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Introduction to the Auditory System

This subchapter discusses the structure and function of the zebrafish inner ear, focused largely on development but with some attention to studies in adult animals. Zebrafish are a popular model for understanding inner ear development, owing to rapid ex utero development and the optical clarity of the embryo, facilitating real-time visualization. Elegant morphological time series have relied on techniques, such as time-lapse imaging, sequential histology, and paint-filling methods, providing a wealth of information about early ear development (e.g., Haddon & Lewis, 1996; Bever & Fekete, 2002). Coupled with analysis of mutant phenotypes from saturating ENU-mutagenesis screens (Malicki et al., 1996; Whitfield et al., 1996) and more recent electrophysiological analyses (Yao, DeSmidt, Tekin, Liu, & Lu, 2016), these studies inform our understanding of vertebrate ear development. For more information, see excellent reviews by Whitfield (2002), Riley (2003), and Nicolson (2005).

The embryonic zebrafish ear consists of two sensory maculae (Fig. 23.1). The anterior macula matures into the utricle, which is a primary balance organ in zebrafish and most other teleost fishes, while the posterior macula becomes the saccule, the primary auditory organ. The third macula, the lagena, appears at 8–9 days postfertilization (dpf) and clearly separates from the saccular pouch by 12–15 dpf (Bever & Fekete, 2002). The precise function of the lagena is unknown, although it may play both auditory and vestibular roles (Popper, Fay, Platt, & Sand, 2003). The zebrafish ear resembles the inner ear of other vertebrates, although there are differences in epithelial specificity—the amniote saccule is a vestibular organ, rather an auditory organ as in zebrafish and most fishes, and amniotic vertebrates possess a specialized auditory organ, the cochlea. Each sensory epithelium contains populations of sensory hair cells (HC) interdigitated with nonsensory supporting cells (SC), and these cells are homologous across vertebrates.

After the first few days of life, fish development depends more on size than absolute age, with factors, such as food availability, temperature, and rearing density contributing to size differences. We use absolute age for fish in the first 2 weeks of life and indicate both body length and approximate age for older animals.

Otic Induction

The zebrafish ear is derived from the otic placode, a region of ectodermal thickening adjacent to the hindbrain that becomes visible at 13.5–14 h postfertilization (hpf), comparable to many other vertebrates (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). The placode further thickens and forms a ball of cells, which then hollows, forming an internal lumen (Haddon & Lewis, 1996). All inner ear cells are derived from this otic vesicle (OV). OV development in zebrafish differs significantly from OV formation in amniotes; in the latter, the placode invaginates and pinches off, rather than hollowing out from within (Haddon & Lewis, 1996). Thickened epithelial patches appear at the anterior and posterior ends of the OV, marking the future sites of the anterior and posterior maculae.

Around 42 hpf, three protrusions develop from the nascent OV and extend into the lumen (Haddon & Lewis, 1996). These protrusions fuse in the center of the otocyst and form pillars, defining the semicircular canals that sense rotational movement. The anterior and posterior canals form by 60 hpf, while the horizontal canal forms by 72 hpf. By 5 days dpf, the larval ear has two clear maculae and three distinct semicircular canals.

Otic placode formation is induced by fibroblast growth factor (FGF) secreted from the neighboring hindbrain (Léger & Brand, 2002; Maroon et al., 2002;

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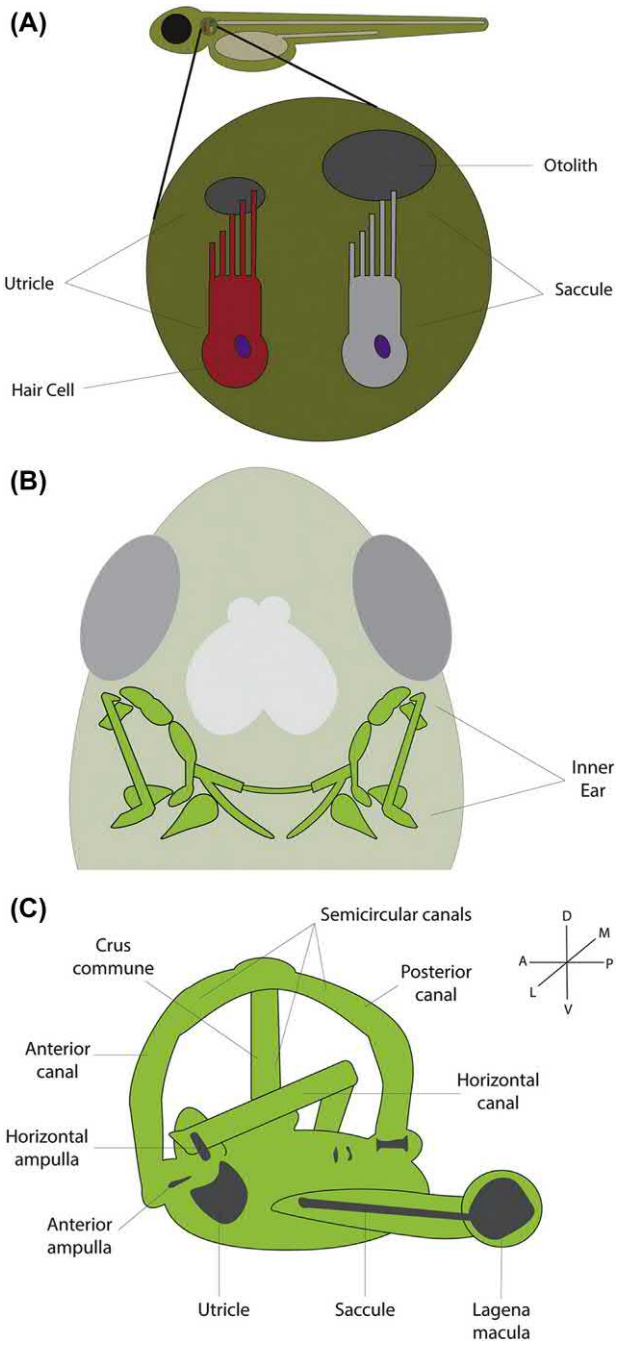

FIGURE 23.1 Anatomy of the larval and adult zebrafish inner ear. (A) Larval zebrafish hearing organs (saccule and utricle) consist of hair cells and otoliths. The otoliths deflect the hair bundles that transduce the relative motion into graded potentials within hair cells. (B) Three-dimensional transverse plane of the adult inner ears. (C) Three-dimensional view of the adult inner ear.

Phillips, Bolding, & Riley, 2001). FGF signaling induces expression of the transcription factor *pax8* (Mackereth, Kwak, Fritz, & Riley, 2005), which works with *pax2a* to maintain otic fate after induction (Hans, Liu, & Westerfield, 2004). Later in development, FGF and retinoic acid (RA) operate in a negative feedback loop to specify both anterior-posterior patterning of the OV and the ventral prosensory domains that will form the sensory maculae (Maier & Whitfield, 2014).

Macular Development

Macular patterning and cell fate decisions require members of the Notch signaling pathway, which acts via lateral inhibition to specify alternating patterns of HC and SC. Mutations in the Notch pathway result in excess HC, with a concomitant reduction in SC (Haddon, Jiang, Smithers, & Lewis, 1998; Malicki et al., 1996). The transcription factor *Atoh1* is required for HC specification; knockdown of both *atoh1a* and *atoh1b* abolishes HC (Millimaki, Sweet, Dhason, & Riley, 2007). These findings are consistent with mammalian studies, where ablation of the single *Atoh1* homolog is sufficient to prevent HC formation (Bermingham et al., 1999). In zebrafish, *atoh1b* is required for early HC development, while *atoh1a* is necessary for later-forming hair cells (Millimaki et al., 2007). *Atoh1* genes and Notch signaling show complex interactions, with *atoh1* likely acting both upstream and downstream of Notch (Millimaki et al., 2007; Sweet, Vemaraju, & Riley, 2011).

While both maculae develop from the ventral otocyst, development is asynchronous and depends on distinct transcription factors. Tether cells develop ~20–24 hpf in both maculae, becoming the first HC. Later-forming HC are present in the utricular macula by 27–30 hpf, while saccular hair cells develop ~34–36 hpf (Sapéde & Pujades, 2010). Hedgehog signaling is necessary for saccular development, while *pax5* and *six1* are required for survival and maintenance of utricular hair cells (Bricaud & Collazo, 2006; Kwak et al., 2006; Sapéde & Pujades, 2010).

Otolith Development

Otoliths precursor particles appear in the otocyst lumen ~18 hpf and otoliths are visible by 24 hpf (Haddon & Lewis, 1996; Riley, Zhu, Janetopoulos, & Aufderheide, 1997). The formation of two discrete otoliths is reliant on tether cells, which are the first HC to form in each macula. One pair of tether cells reside in the anterior otocyst and attract the precursors of the utricular otolith, while the second pair of tether cells develops in the posterior otocyst, the site of the developing saccule (Haddon & Lewis, 1996; Riley et al., 1997).

Tether cell kinocilia produce an otolith attracting factor which localizes the nascent otoliths to the appropriate regions of the otocyst (Stooke-Vaughan, Huang, Hammond, Schier, & Whitfield, 2012). In the absence

of tether cells, otolith formation is largely disrupted, often resulting in a single misshapen otolith per ear. Otogelin and α-tectorin are strong candidates for otolith tethering factors, and mutations in either gene are associated with deafness and vestibular defects in humans (Stooke-Vaughan, Obholzer, Baxendale, Megason, & Whitfield, 2015). These data reinforce the utility of zebrafish for understanding inner ear development and function, including modeling human hearing disorders.

Hair Cell Development

Hair cell development involves several morphological steps, including both apicobasal and lateral polarization. HC development in zebrafish involves many of the same molecular players as in other vertebrates; reviewed in Atkinson, Najarro, Sayyid, and Cheng (2015) and Barr-Gillespie (2015).

Functional HC maturation occurs by 40 hpf and requires both assembly of the mechanotransduction (MET) apparatus and appropriate synaptic contacts onto VIIIth nerve afferents (Fig. 23.2). While the molecular identity of the MET channel is still debated, one likely set of candidates is the transmembrane channel proteins TMC1 and TMC2 (Pan et al., 2013). Zebrafish have two *tmc2* paralogs, and *tmc1*, *tmca2a*, and *tmc2b* are all expressed in the adult inner ear (Maeda et al., 2014). Zebrafish tmc1 and tmc2 interact with the tip link protein protocadherin15, and disruption of *tmc2* reduces mechanotransduction sensitivity (Maeda et al., 2014). Another MET channel candidate, *tmie*, is also expressed in zebrafish hair cells, and mutation results in auditory and vestibular defects (Gleason et al., 2009). Zebrafish are, therefore, a viable model for identification of hair bundle proteins and delineation of their roles in mechanotransduction.

Like other vertebrate hair cells, zebrafish hair cells have specialized ribbon synapses along the basolateral membrane that tether clusters of glutamatergic vesicles near the synapse. One core ribbon component is ribeye, with both zebrafish paralogs (*ribeye a* and *ribeye b*) expressed in the ear ∼20 hpf (Sheets, Trapani, Mo, Obholzer, & Nicolson, 2011). Knockdown of either *ribeye* gene results in hearing and balance defects in 4 dpf larvae, suggesting reduced synaptic transmission.

VIIIth Nerve Development

Neurons of the statoacoustic ganglion (SAG) are derived from neuroblasts of the developing otocyst, with the axons forming the VIIIth cranial nerve. From 22 hpf, these neuroblasts delaminate from the ventral otocyst and migrate to form a rudimentary ganglion (Haddon & Lewis, 1996). Delamination is complete by 42 hpf, resulting in a few hundred cells destined for a neuronal fate. This small pool expands by proliferation, then differentiates into mature SAG neurons (Dyballa et al., 2017). The neuronal specification requires *neurog1*, while *neuroD* is required for neuronal differentiation and maintenance (Andermann, Ungos, & Raible, 2002). Hoijman, Fargas, Blader, and Alsina (2017) revealed that a subset of *neurog1*+ SAG precursors, known as "pioneer cells", arise outside the otic vesicle. These cells then migrate into the inner ear and become the first otic neuronal progenitors, activating *neurog1* in neighboring cells and committing them to a neural fate. Hair cell innervation is apparent as early as 20 hpf, before neuroblast delamination. The relative position of neuroblasts prior to delamination determines the future innervation pattern of the ear, with anterior neuroblasts innervating the utricle and posterior neuroblasts innervating the saccule (Dyballa et al., 2017; Vemaraju, Kantarci, Padanad, & Riley, 2012).

Early Development of Hearing

VIIIth nerve afferents project to hindbrain auditory centers. In fishes, these afferents also synapse on Mauthner cells, paired reticulospinal neurons in the hindbrain that mediate fast escape responses. Mauthner cell-VIIIth nerve synapses form ∼23 hpf, and electrophysiological recordings demonstrate sound-evoked Mauthner cell activity by 40 hpf (Kimmel, Hatta, & Metcalfe, 1990; Tanimoto, Ota, Horikawa, & Oda, 2009).

Microphonic recordings demonstrating physiological sensitivity to acoustic cues (up to 400 Hz) in 3 dpf larvae, and thresholds improve significantly by 7 dpf (Yao et al., 2016). By contrast, a behavioral study demonstrated acoustic sensitivity up to 1070 Hz in 5–6 dpf larvae, with maximum sensitivity between 90 and 310 Hz (Bhandiwad, Zeddies, Raible, Rubel, & Sisneros, 2013).

Growth of Sensory Maculae

The zebrafish inner ear undergoes widespread morphological changes between 5 dpf and adulthood (Fig. 23.1). As the inner ear matures, each macula has a unique HC polarity pattern, with the mature saccule containing two populations of oppositely oriented HC (Platt, 1993). Unlike mammals, HC production continues well into adulthood (Higgs, Souza, Wilkins, Presson, & Popper, 2002). The rate at which HC are added in adult zebrafish varies between maculae, with

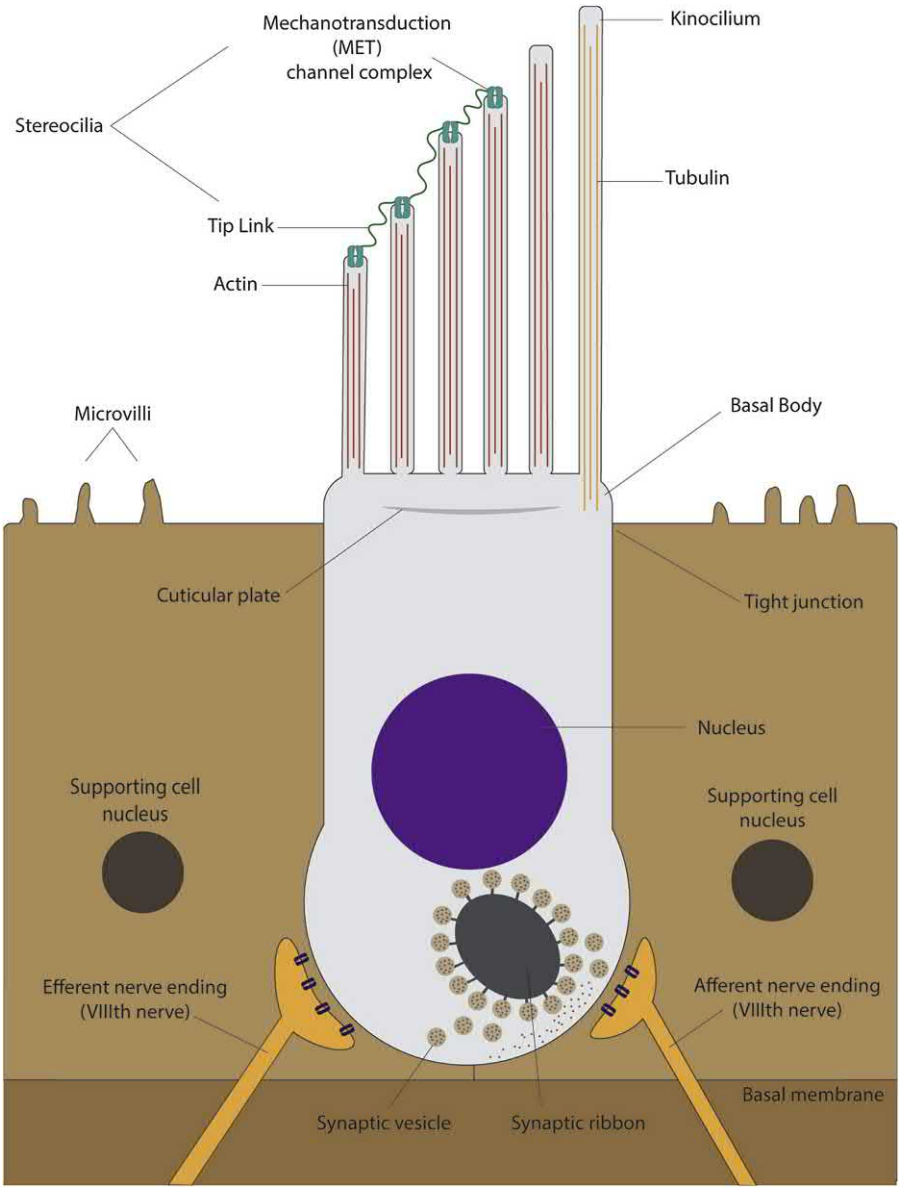

FIGURE 23.2 Anatomy of a zebrafish hair cell, showing the sensory hair bundle (kinocilium and stereocilia) and other key features, such as the ribbon synapses at the basolateral surface. Also shown are the surrounding support cells and afferent and efferent innervation.

HC addition occurring most quickly in the lagena and slowest in the saccule (Bang, Sewell, & Malicki, 2001). HC density also varies among and within maculae (Higgs et al., 2002). Just like in mammals, zebrafish HCs are damaged by a variety of stimuli. Unlike mammals, however, fish can regenerate their HCs due to supporting cell division and subsequent differentiation of daughter cells into HC or by direct transdifferentiation of supporting cells into HC, making for a powerful model to study vertebrate HC regeneration (Monroe, Rajadinakaran, & Smith, 2015).

Hearing Thresholds in Adults

Auditory Evoked Potentials (AEP) are useful to determine zebrafish auditory sensitivity. Adult zebrafish detect tones from 100 to 4000 Hz with an optimal hearing frequency ~127 Hz (Higgs et al., 2002). Higgs, Rollo, Souza, and Popper (2003) found no change in auditory sensitivity or response latency during later development, despite a gradual expansion in the frequency range. However, Wang et al. (2015) report size-dependent changes in AEP thresholds but no change

in the frequency range. Further, this group reports AEP responses up to 12 kHz, inconsistent with data from other otophysan fishes. Although there is a lack of consensus about hearing sensitivity during maturation, hearing sensitivity differs between transgenic lines that are commonly used in auditory research (Monroe et al., 2016). Transgenic lines and rearing conditions likely account for differences in hearing thresholds and should be taken into account when conducting auditory studies.

Accessory Auditory Structures

Adult otophysan fishes respond to both the particle motion and pressure components of auditory stimuli, owing to a set of modified vertebrae (Weberian ossicles) that couple the swim bladder to the saccule (Popper & Fay, 2011). Ossification of the Weberian apparatus begins around 22 dpf (4.1 mm standard length), but is not complete until ~56 dpf (36–37 mm total length) (Grande & Young, 2004). The swim bladder is inflated at 4–5 dpf, before ossicles develop, but swim bladder deflation in 5 dpf larvae does not alter behavioral thresholds (Zeddies & Fay, 2005). These data suggest that the swim bladder alone is insufficient to serve as a pressure detector, but the effect of ossicle development on auditory sensitivity is still debated (Higgs et al., 2003; Wang et al., 2015).

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SECTION III

Husbandry

CHAPTER

24

Introduction to Zebrafish Husbandry

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Husbandry is defined in the Merriam-Webster dictionary as (1) the cultivation or production of plants or animals or as (2) the scientific control and management of a branch of farming and especially of domestic animals. In the following chapters, we will describe the cultivation, control, and management of zebrafish (*Danio rerio*) for biomedical research. However, the information in this section and other sections of this book can be used as well for the organization of husbandry of zebrafish kept in other contexts.

The history of zebrafish in biomedical research is discussed in detail in an independent chapter in this text. However, we wish to remember here that George Streisinger is recognized as the founding father of zebrafish husbandry. Streisinger began his work, in 1960, at the University of Oregon where he worked with phage and made major contributions in determining the structure of the T4 phage genome, deciphering the genetic code, and increasing the understanding of frameshift mutations. Outside the laboratory, he was an experienced fish hobbyist, and this led him to propose using zebrafish to extend his studies into a vertebrate model. The use of zebrafish was revolutionary, and many of his colleagues were skeptical of his efforts. In reality, it took him about 10 years to describe the normal morphology and development of the zebrafish and to develop efficient research and husbandry approaches. In 1981, his efforts resulted in the development of a method for using zebrafish to screen for mutants among parthenogenetic offspring of mutagenized females. Colleagues soon took notice of this and also began to use zebrafish, described by Dr. Streisinger as "phage with a backbone."

Since then, the use of zebrafish has grown tremendously, with the annual number of zebrafish research publications surpassing Drosophila, the most commonly used genetic model, in 2011. In 2018, a search of the NIH Research Portfolio Online Reporting Tools (RePORT) for grants with abstracts listing the use of zebrafish identified 888 grants. Zebrafish are predicted to continue their rise in popularity for a number of reasons, including their small size, rapid development, short generation time, optical transparency during development, fecundity, the economy of maintenance, genetic similarity to humans, and suitability for genetic manipulation.

While the zebrafish is a vertebrate, there are major differences in biology and physiology between this species and many of the more commonly used vertebrate animal models, such as mice and rats, and these differences directly determine best approaches for their optimal husbandry. These differences are reviewed in detail in the chapters in the biology section of this book, but it seems appropriate to briefly summarize some of the most important differences here in the context of their relationship to husbandry.

Zebrafish differ dramatically from most vertebrates in respiratory function, as they have no lungs or diaphragm and use gills instead to absorb oxygen from their aqueous environment. Zebrafish have no hair, but instead, have scales and a mucus coat important for their health. This integument affects the balance of important substances between the tissues of their body and the water in which they live. They are also dependent upon the temperature of the water in which they live for their own body temperature. They lack a true stomach, which makes it necessary for them to eat often, as they cannot store food. They also do not perform internal fertilization and lack mammary glands. Instead, eggs and sperm are released into the water, where fertilization occurs, and there is no parental support during the embryonic or fetal periods. All of these characteristics underscore the fundamental importance of the quality of the water in which this species lives. Therefore, the most important consideration for husbandry of this species is that air is not their main environment, but water is, and the water must be of correct composition and physical character to support zebrafish optimally at all

The Zebrafish in Biomedical Research
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life stages. How that water is to be contained as its environment is also critical.

There are a variety of housing strategies for zebrafish, and these will be described in the chapters on facility design (Aquatics Facilities Design) and housing types (Aquatic Housing). The cleaning and disinfection of these housing systems and the room environment are critical and will be discussed in a separate chapter (Cleaning and Disinfection of Life Systems). Additionally, each facility should have standard operating and emergency procedures, as is discussed in the chapter on systems maintenance and troubleshooting (Zebrafish Aquatic Systems: Preventative Maintenance and Troubleshooting).

As is well described in several of the following chapters, there are many crucial water parameters that the zebrafish culturist must control in order to successfully rear them in the biomedical research setting (see the chapters titled Water Quality for Zebrafish Cultures, and Recirculating Aquaculture Systems (RAS) for Zebrafish Culture). These parameters include temperature, salinity, pH, and oxygen content. It is also important to be vigilant for the presence of other substances in the water that could harm the zebrafish, including nitrogenous and other wastes, metals, toxins, and microorganisms. If present in the tank water, many of these potentially harmful substances can lead to at least variation in research results and at most death. Since zebrafish housings are submerged in water, room humidity and air exchange are not as important as for other species. These two factors can, however, affect water quality because of their effect on the evaporation of water from tanks and sumps, and thus, both should be assessed with regard to their effects on water quality.

Another important variable in zebrafish husbandry is the provision of light. In the research setting, it is possible that these fish may be exposed to light sources that differ from what they would encounter in the wild. While this may cause some research variations, it can be minimized if the type of light provided in different facilities is comparable. However, the cycle of the light provided is critical for reproduction, as zebrafish tend to breed most often at dawn. Most facilities use a 14/10 (on/off) light cycle in zebrafish facilities, and it has been shown that interruption of or lack of a dark cycle can completely block reproduction in this species. Factors to be considered include the presence of unusual sources of light, such as exit signs in the animal areas. Lighting is discussed in some chapters (Aquatics Facilities Design and Zebrafish Breeding and Colony Management).

Since most biomedical research requires sufficient sampling, reproduction is a critical subject. Best practices are described in two chapters (Zebrafish Breeding and Colony Management and Larviculture).

An area that needs more work is in the determination of zebrafish nutritional needs and the best methods for meeting those needs. It is important to optimize the nutritional content of feeds used, as well as, how and when it is provided. This is discussed in the chapter titled Zebrafish Nutrition.

In recent years, the importance of alleviation of pain and distress in this species has been recognized, and there has been a progression in the methodology. Please see the chapter titled Analgesia, Anesthesia, and Euthanasia for a thorough review of this field.

Several chapters in this husbandry section, focus on the overlapping areas of health surveillance, transportation, and biosecurity. These chapters discuss practices that are critical for the establishment and maintenance of healthy colonies, the basis of good research. Please see the chapters titled Health Surveillance Programs, Importation and Quarantine, and Export and Transportation of Zebrafish for information on these important topics.

Finally, regulation of husbandry for this species has grown with its increased use, and this is addressed in the chapter titled Regulations, Policies, and Guidelines Pertaining to the Use of Zebrafish in Biomedical Research.

In closing, this editor is very grateful to the authors who have so diligently contributed to the chapters in the husbandry section of this book. Each author was selected based on his or her expertise. These chapters could not have been completed without the support and resources of these authors and their institutions that allowed them time to contribute to this reference. This editor also is very appreciative of the work of the many reviewers, who also had contributed to this text. We hope that this section will be a valuable guide to those culturing zebrafish and will serve as a foundation for work leading to further refinements and improvements in husbandry for this species.

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CHAPTER

25

Aquatics Facility Design Considerations: Incorporating Aquatics into an Animal Facility

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Introduction

As with any emerging research subject, the first few steps are purely experimental. At first, aquatic research facilities shared many commonalities with aquaria. Educated guesses and experimentation with techniques and applications, such as different housing systems and water quality devices, established a baseline understanding of successful and unsuccessful approaches to aquatic facility design. Initially, the predominant facility designs for rodents were adapted to accommodate the specific needs of aquatic research. As greater numbers of research fields discovered the broad applicability of the zebrafish, *Danio rerio*, as well as that of other aquatic species, the demand for facilities to support research with these species steadily grew. Collectively, there is information available now from several decades of research and experience for the programming, planning, design, and construction of zebrafish aquatics facilities.

While there are commonalities in facility design features between those for aquatic species and those for rodents and other "traditional" research species, there are also differences, from nuances to highly specific requirements. In this chapter, we will provide the reader with an understanding of aquatic research processes that delineate the design and construction of a successful aquatic research facility.

Species Dependence

As with any animal research facility design, the first step in the process is to establish the species used in the research program. The needs of saltwater mollusks as compared to those of the common zebrafish are significantly different, resulting in vastly different facility requirements. While the focus of this chapter will be predominantly on zebrafish facilities, one should discuss the range of research species to be housed early in the programming and planning process to avoid inconveniences and perhaps incompatibilities with the research program. Most importantly, the determination of fresh versus saltwater species will have the greatest impact on facility design, from material and finish selection to HVAC (heating, ventilation, and air conditioning) design.

Similarities to Rodent Facilities

The baselines for most vivarium standards were established to support research utilizing the predominant model for animal research for the past 70 years, the mouse. The ILAR (Institute for Laboratory Animal Research) Guide for the Care and Use of Laboratory Animals (The Guide) describes generally accepted facility standards for mice and other common research species. Zebrafish are relatively new to the research arena, and as such, descriptions of requirements for the design of facilities for their care in "The Guide" is less prescriptive and somewhat ambiguous. Nevertheless, the fundamental principles of zebrafish facility design follow those for facilities housing other species. Specifically, the facility should be designed to support animal health, welfare, and husbandry in a safe and robust environment. Provision of basic life-support is paramount to any animal research facility; therefore, the armature around which any aquatics facility is developed, the foremost ishealth, safety, and welfare centric.

Unique Features

In contrast to mammalian centered facilities, in which provision of properly conditioned airflow is critical to

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life-support, aquatic facilities require provision and flow of properly conditioned water to keep fish safe and healthy. While this seems basic, it is the fundamental design driver in fish facilities. In terms of space allocation and facility layout, aquatics and in particular zebrafish facilities, require a proportionately greater amount of support space than do rodent facilities. This is especially true in "centralized" system facilities, as will be discussed later in this Chapter. A general requirement for smooth, cleanable, and sanitizable surfaces is similar; however, special attention to corrosion resistance and vapor barrier design is necessary because of elevated humidity levels in aquatics facilities.

Program Components

The beginning of a design for an animal facility starts with the definition of the space program, or in other words, determination of spatial needs for the intended use of the facility. The "building blocks" that address function are established and conceptually designed to meet the needs of research and operations.

Housing

The largest space allocation in a zebrafish facility is associated with housing. As we will discuss later in this chapter, temperature and humidity control are critically important to animal health and welfare, and the temperature is more successfully maintained in larger rooms that serve as slow-changing thermal masses than in smaller segregated rooms. Clearly, room size should be discussed within the context of research support, study size, and biosafety and security; however, early consideration of temperature and humidity maintenance is crucial.

A primary determinant for the size of housing rooms for zebrafish is the type of housing to be used. There are three commonly used types of housing for zebrafish in biomedical facilities, known as centralized, distributed, or stand-alone (sometimes alternatively described as a packaged or pocket system). As described in more detail in other chapters, centralized systems have a central water reservoir or resource that supplies to multiple connected racks that are usually located in the same room. This centralized water reservoir and its support equipment is usually located in a separate room from that holding the racks, and so a separate space must be allocated for this purpose. Distributed housing is characterized by the allowance of one or more water sources that each supply a number of racks in separate locations. Stand-alone racks each have their own built-in water processing equipment. As shown in Fig. 25.1, the arrangement of racks in the room will vary based upon housing type. In the left panel, a centralized system is illustrated, with all water processing and storage equipment located elsewhere (not shown), so the room space is all dedicated to racks for holding housing tanks, while in the right panel, the lower portion of each rack contains an independent sump and water processing equipment. Two other important factors determining the size of housing rooms are the consideration of tank change processes to be used (where and how this occurs), and the number of animals and potentially concurrent disparate studies to be located within a single housing room (i.e., whether incompatibilities require separation of studies).

Procedural Space

Procedural spaces for fish are somewhat different from those for rodents or other species. Common fish procedures include breeding, microinjection, fin clipping, tagging, and microscopy. Since zebrafish require

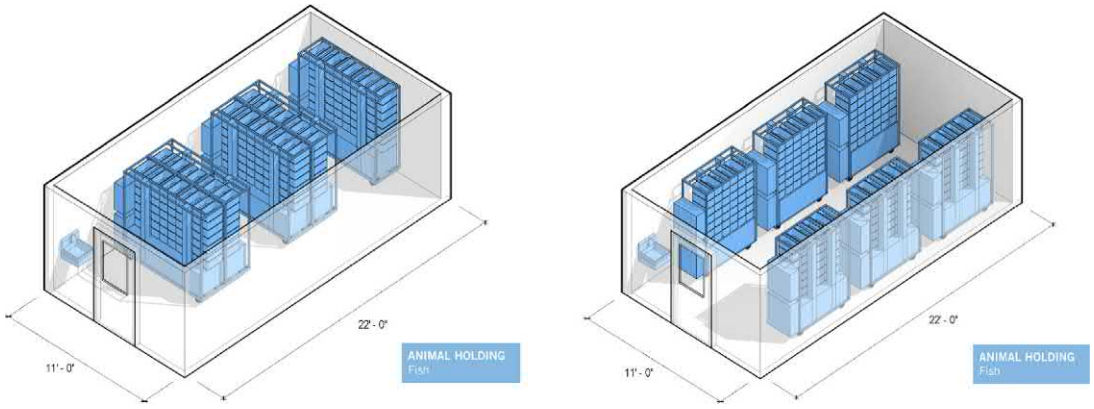

FIGURE 25.1 The diagram on the left illustrates a centralized arrangement of housing racks serviced by a water source in an adjacent room (not shown), while that on the right illustrates a set of stand-alone racks, each with its own water processing equipment.

water for life support, provision of this support during extended procedures should be carefully considered. Space assignment should take into account the space needed for instruments used during procedures, as well as that needed for staging and movement, often involving transfer carts. Airflow devices, such as biological safety cabinets, may be included but are less likely to be used as often as in rodent facilities. As procedural spaces are likely to be designed to meet highly specific needs, discussions about the types and ranges of procedures to be done will be critical in designing spaces to meet programmatic needs. From a utility perspective, in addition to electrical, vacuum, and medical gas support present in many procedural spaces, aquatics facilities may have other specialized needs. If microinjection is a required procedure, available fine-tunable compressed air will be required. Often aquatic procedural spaces also require the provision of "system water" (described later in this chapter) to maintain consistent habitat requirements before, during, and after procedures (Fig. 25.2).

Breeding

In aquatic programs, breeding space is often separate from other housing space. To ensure that proper breeding conditions are met (as discussed in the chapter on Breeding and Colony Management), dedicated housing units for breeding are recommended. These spaces typically resemble the housing rooms; however, they may also include compartmentalization for provision of alternative day-night lighting cycles and sometimes segregated water systems. The provision of a standardized program of light (on/off cycle) in all rooms housing fish is very important as alterations will negatively affect the breeding activity and normal growth and development (Adatto; Villamizar). Breeding spaces are commonly acoustically improved from surrounding spaces to ensure that acoustic disturbances will not negatively influence breeding.

FIGURE 25.2 Example of procedural space for microscopy work in a zebrafish facility.

Behavioral Testing

Depending on the research program needs, some spaces may be dedicated for behavioral testing. Functional considerations for these spaces are directly related to the types and modalities of testing to be done; therefore, timely discussion of types, ranges, and possible future testing methods are recommended since many features of testing spaces would be difficult and potentially prohibitively costly to retrofit. As with breeding rooms, behavioral testing spaces often have acoustically improved partitions to ensure outside influences do not confound the research. Spaces for behavioral testing often need to accommodate large pieces of equipment such as complicated mazing. Consideration for the accommodation of large-scale apparatus may include oversized doors and overhead uni-strut racks used for placement of monitoring equipment, sensors or cameras. Access into and out of these spaces is an important aspect of design, since some behavioral testing vessels are single-piece assemblies holding 400 gallons or more. Considerations should be made for acoustic noise attenuation, dimmable or otherwise adjustable lighting, and robust data capture. With increasing frequency, we also see need for RF/EMI (radio frequency or electromagnetic interference) filtered testing rooms, something that the previous generations of behavioral testing apparatus did not require.

Support Spaces

Even as zebrafish research, husbandry, and care continue to evolve, we anticipate seeing a greater need for specialized spaces to adequately support program needs not currently understood or routinely demanded. An example of this type of space is a specialized space for producing or preparing feeds. Diet preparation will be discussed in detail in the chapter on Nutrition, but related space needs will be discussed in this section. Preparation of live feeds (i.e., Artemia, rotifers) requires significant space for oxygenated columns supporting their growth and maturation. Provision of compressed air or oxygen in volumes sufficient to support these columns may come from "house" systems capable of producing 25 to 50 scfm (square cubic feet per minute) of flow (Fig. 25.3).

FIGURE 25.3 Picture of area for diet preparation in a zebrafish facility.

Marshalling/Storage

An important, but often overlooked, need for space in aquatics facilities is for storage and preparation of spare tanks. It is recommended that space for accommodating 10% of the overall tank census be dedicated for this purpose. Often, this marshaling of space also allows for preparation of tanks with enrichment or similar apparatus before they are used for fish.

Cagewash

The cage-washing portion of a vivarium is often referred to as the "heart" of the facility. Whereas caging for other animal species can often be washed using shared equipment, this may not be possible for zebrafish tanks due to concerns about possible deleterious effects of residual detergent on the fish. For this reason, separate wash equipment may be used. Typically, cages are washed in a "cage and rack" washer or cabinet washer. Recently equipment manufacturers have begun manufacturing "aquatics specific" wash machinery. This equipment can be colocated with other wash equipment or in a dedicated wash facility proximately located to the aquatics housing areas. Details of the wash facility components will be discussed later in this chapter.

Planning Approaches

The difference between an efficient zebrafish facility and a mediocre one is often determined early in the design process, when an overall planning model is selected. Animal facilities often have a process and flow-based design, in which the desired operational methodologies heavily influence the facility plan. A valuable technique to use as a guide in planning a zebrafish facility is that of a process and flow diagram in which the design team illiustrates each of the critical process required. This technique allows the processes and flows required in a facility to guide the facility layout. Often, plan layouts selected directly correspond to the diagrams produced in this phase.

Organization

Often a zebrafish facility is a subset of a larger vivarium that may house multiple species. As the environmental parameters and associated support systems required to provide the proper environment are typically different from those of rodent and large animal counterparts, consolidation of aquatics spaces makes practical and economic sense in planning a facility. While zebrafish spaces can technically be interchangeable with rodent spaces, consolidation of species not only helps organize the utilities and systems, but also assists in operational parameters of these unique facilities. The overall organization of zebrafish facilities tends to group like-functioned spaces together in order to optimize their individual and collective spatial efficiency. However, each facility will likely have unique programmatic drivers that determine the ultimate use distribution. Evaluation of the pros and cons of a consolidated versus distributed versus stand-alone model should be undertaken during the initial programming of a new facility or renovation. Critical factors to these decisions are the amount of physical space allocated versus census needs, the associated budget, and the number of full time employees available for husbandry and technical staffing.

Structural Support

When considering the location of a zebrafish facility, the primary consideration must be the size of the facility and the associated weight of the water it holds. Since water weighs approximately 8.3 pounds per gallon, the weight of a rack of zebrafish is approximately 60% more than that of a comparably sized rack of rodent caging. This has traditionally biased zebrafish spaces to slab-on-grade conditions to avoid elevated structural costs. However, there are many examples of zebrafish facilities on elevated structural slabs to be close to associated, nonvivarium research spaces. Careful evaluation of the location of a new facility, or structural assessment of an existing building, should be undertaken at the onset of the design process to allow estimation of associated costs.

Adjacencies

For ensuring repeatability in research outcomes, the variables affecting zebrafish research must be understood and documented. The organization and distribution, that is, the relationship between program elements, such as housing or holding spaces to areas in which procedures are performed, is important to reinforcing repeatability. In general, procedure spaces should be located as close to housing areas as is possible so that bio-stressors associated with the transport of fish do not become a research confounding issue.

Separations

Some of the functions within an aquatics facility are incompatible with potentially adjacent program elements. For example, dry feed and bedding storage for rodents is likely incompatible with the humidity present in a live-diet preparation area for zebrafish; so while functionally there are similarities, separation is desirable. These types of program separations should be carefully discussed with the design team during the programming and planning of a facility. For example, as noise is transmitted approximately 4.4 times faster through water than through air, adjacencies to mechanical spaces must be carefully considered. Ideally, building systems serving aquatic facilities are remotely located. However, in centralized system facilities there are distinct cost and maintenance issues when water filtration and treatment facilities are placed far from housing areas. In this situation, attention to sound mitigation/migration by use of partitions should be considered.

Housing System Types

Selection of housing type will influence almost every aspect of facility design. As discussed in detail in several other chapters in this book, there are three predominant housing types, distinguished by the way in which they provide life support (water) to the housing tank units, the centralized system, the distributed system, and the stand-alone system.

Centralized System

A centralized system facility is one in which there is one central water production and filtration plant space that serves one or more separate housing units and/or procedural spaces. This approach is common to larger facilities, usually accommodating more than 2000 tanks. The relatively large volumes of water used in centralized facilities are produced through treatment and or conditioning of water from various water sources to become "fish ready." Fish wastewater exiting the tanks is filtered and processed for reuse. In a centralized facility, it is possible to effectively consolidate service and maintenance needs while providing process separation for control of acoustic and vibration variations (Fig. 25.4).

Distributed System

If there are anticipated differing water needs or significant distances between the housing units in a facility, a distributed system approach is optimal. This consists of multiple, smaller water production and filtration centers that are located closest to each point-of-use. A distributed or multizone system can also be useful if research needs call for multiple water temperatures or differing water quality parameters or treatments. Each zone of housing is served by an independent water treatment plant space connected to a housing unit or units.

Stand-Alone System

In these systems, sometimes referred to as pocket or packaged systems, the housing and the water treatment/filtration is provided by a manufacturer-packaged product that is located in a housing unit (rack). These units often have a filtration unit located at the base of a rack holding the housing tanks. These types of units frequently require little more than a cold-water supply and a drain, making them extremely popular for small-scale aquatic operations (Fig. 25.5).

FIGURE 25.4 A large centralized facility with many racks sharing one water source.

FIGURE 25.5 Photograph of a stand-alone rack.

Environmental Parameters

As in any animal research laboratory, the facility itself plays a critical role with respect to life-support of the research population, including both human and animal occupants. It is important to ensure adequate temperature, humidity and lighting to support the health and wellbeing of the zebrafish colony and to create a safe and healthy workplace for personnel working in the facility. Close coordination between environmental parameters and those facility features that support them is crucial.

Temperature

One of the key aspects contributing to the health of fish and to the reproducibility of research results is the maintenance of consistent temperature with little short-term fluctuation. Zebrafish in biomedical research are most often housed at 28.5°C (83.3°F) which has been previously recommended as the optimal temperature for their housing (Reed). Most water systems for zebrafish contain indwelling water heaters that maintain the temperature of the water provided to tanks, but room temperature is also critical for fish kept "off system" during experiments or in breeding tanks overnight. While that may seem a simple and straightforward task to accomplish, careful consideration of the coordination of HVAC with room construction methodology is required to successfully maintain this target. The vast majority of spaces in an animal care facility or research building will be about 10°F lower to accommodate the comfort of personnel. The design parameters for partitions and high-performance coatings that will be applied to them to accommodate these temperature differences between spaces in a facility requires attention. Insulation to reduce heat migration is required in most circumstances. Insulating the interior partitions will assist in temperature maintenance as well as reduce overall energy cost over time by encouraging a greater thermal mass to exist in the desired space and by reducing the need for additional heating or cooling to maintain the temperature within the required parameters. In the following section, we will discuss the means to achieve this in further detail.

Humidity

The humidity must be maintained at a relatively high level to reduce thermal reduction due to evaporative cooling. The recommended level of relative humidity (RH) for zebrafish facilities is 70%. The mechanical means to provide these high levels of humidity will be discussed in more detail later in this chapter; however, at this point, it should be noted that attaining and maintaining high levels of humidity will require supplemental equipment in most locations. Unlike in rodent facilities, where humidity is important for the health and welfare of the animals, in aquatics facilities the high level of humidity is predominantly to prevent rapid evaporation from the tanks and sumps. Rapid evaporation due to low room humidity can impact temperature maintenance within the tank (due to evaporative cooling) and require constant addition of make-up water to maintain tank water levels.

While 70% RH is a normal occurrence in some natural environments every day, within a building it can cause significant problems and even safety issues. Improper detailing and or vapor transmission mitigation can lead to mold growth and other facility damage that could also confound research outcomes. The key feature of maintaining humidity levels while minimizing damaging humidity transmission is referred to as a "vapor barrier." This is an impermeable membrane that prevents the migration of humidity from its intended areas into others. In practical terms, this often takes the form of high-performance coatings applied as finishes to the surfaces and partitions. Proper detailing is required to ensure that all vulnerable areas are sealed, especially if these areas are positively pressurized. A vestibule is recommended to maintain elevated levels of humidity in a space where personnel often enter and exit. This is most often created by using a set of two

doors in series for access, sometimes (erroneously) referred to as an airlock.

Lighting

Lighting within an aquatics facility serves two roles: to ensure the health and welfare of the animals and to allow for the safety of personnel in the space. Sometimes, these goals are in conflict with one another, so a clear direction on how both can be simultaneously achieved should be discussed early in the programming process.

Light Intensity

Provision of light in excess of 300 lux for extended periods can have deleterious effects on zebrafish. It recommended that light levels be targeted at 300 lux at a 1 m height from the room floor and at the center of an aisle within housing spaces. Too much light will also lead to excessive growth of algae in tanks, which can interfere with the visualization of fish.

Light Source

As energy efficiency and maintenance are of increasing importance in animal facility design, lighting is currently most commonly provided via a light-emitting diode (LED) source. An excellent means of providing significant illumination at a low-wattage cost and long (sometime 30,000 h) lifespan, LED fixtures have revolutionized research facility lighting. It is important to note that not all LED lighting is created equally. LED light blends different color spectra in order to provide the appearance of white light; red, blue, yellow and green wavelengths appear in varying concentrations depending on the desired light intensity and color rendering. From a human occupant perspective, the ability to tune light is of great benefit; however, research has shown that certain color wavelengths can have negative effects on aquatic species—notably zebrafish. Impacts on growth, development and behavior due to red-light inclusion have been published (Villamizar, Vera, Foulkes, and Sanchez-Vazquez, 2014). In many existing facilities in which fluorescent light sources are still in use, careful attention to the ballasts and "flicker" rates is an important consideration.

Light Cycle

As alluded to above, the light cycle in a zebrafish room is critical. Most zebrafish rooms follow a 14:10 lights on:off cycle. This has been shown by many to support optimal fish health and breeding. This cycle is generally controlled by automation of room lighting. Some facilities incorporate dimmers in order to provide a slow on and a slow off of lights that more closely models dawn and dusk. Many facilities also provide a similar light cycle in incubators housing embryos and larvae.

Housing Room Planning and Design

The design of the housing spaces in a zebrafish facility will be directly based on the housing system selection made in combination with the scientific techniques to be supported. Additionally, the biological safety aspects of design are accommodated more easily by distinct approaches. For example, large scale behavioral studies may be best performed in large housing rooms to ensure that different study groups receive equivalent environmental conditions that otherwise may affect the study. Conversely, infectious disease studies may be better accommodated by the use of stand-alone systems in smaller compartmental housing rooms. In every applicable approach, it is recommended that an 11′-0″ (3300 mm) wide planning module be applied to allow for sufficient space for movement of tanks and carts between the racks, as well as for adequate lighting at all levels in the zebrafish rack. This 11′-0 module accommodates two opposing 3′-deep pieces of equipment (two racks) separated by an aisle. The 5′ aisle width that results is a minimum because of requirements by the Americans with Disabilities Act; this also allows one researcher to pass another without interference. Lighting in the aisle should be placed such that the fixture is parallel to the long side of the rack. The following are general features to be considered when designing and constructing these spaces.

Sizes

Centralized system approaches tend to favor larger rooms of tanks as the nature of these systems is to produce mass quantities of the same water type and quality. It is recommended that centralized systems are designed using modular components. This approach allows for augmentation of individual components over time. Since many centralized systems will not serve their full load at initial start-up, consideration of the degree of expansion or future growth possible should be considered during the design of associated centralized water treatment equipment.

Features

Housing rooms tend to be basic spaces built for the specific purpose of housing tanks of zebrafish. Tank racks are most commonly arranged in rows, oriented perpendicular to the room partitions, allowing for

back-to-back placement of racks into a double-sided orientation with an efficient distribution of supply and return water. In spaces where light control is critically important to research outcomes, a center-aisle configuration (center placement of lighting over aisles separating flanking single-sided tank racks) may be preferred. As previously mentioned, the general layout recommended is to follow an 11′-0″ (3300 mm) planning module when possible; that is, racks should be placed to allow enough aisle space to accommodate easy access to the tanks. Special consideration to rack spacing may be required if robotic or automated feeding systems are utilized. Generally, this spacing allows for a change and crossing cart to be located in the aisle between racks and allows for sufficient personnel movement during tank changing. Since water-filled tanks provide a very different lift-load for a husbandry worker, the ergonomics of tanks changing should be considered in the design. This often translates to one or perhaps two rows of fewer tanks on a rack than as compared to rodent-based counterparts.

Finishes

Finish selection in zebrafish facilities is important not only to the health and welfare of the animals but also the safety of personnel and the ultimate longevity of the facility. In general, finishes should be hydrophobic, seamless, and impervious to heat, chemicals, and solvents. It is important to avoid finishes that continue to off-gas volatile organic chemicals (VOCs) as this can have deleterious effects on the animals housed in these facilities. Selected finishes should be:

Durable

Finishes must be able to withstand abrasions, compression, and the normal use "wear and tear" from moving carts, equipment, and personnel on a frequent basis. In an environment of high humidity and water exposure, finishes should be at least rust resistant and preferable rust proof.

Cleanable

The finishes selected must be able to be cleaned with the chemicals or agents identified for the sanitization of the facility. These range greatly, from simple bleach solutions to harsh chemicals. Discussion of the types of cleaning agents and methods appropriate in zebrafish facilities can be found in the chapter on Cleaning and Disinfection in this book. Types of agents and methods to use should be considered in the design phase of a project to allow for the selection of compatible finishes.

Safe and Maintainable

In the typically wet areas of aquatic facilities, particular attention to slip resistance and traction are important. As slip-resistant treatment (aggregates or textures) tend to degrade after significant use, considerations for renewal of these surfaces is of particular importance in their selection.

Repairable

As most animal facilities are prone to damage due to the movement of carts, racks, and materials, finishes must be able to be repaired in the field without impacting the health of the animals or human personnel.

Flooring

When selecting floor finishes, one must take into account the ability of the flooring to support point loads from racks and equipment (compressive strength qualities). On elevated slabs, it is also important to consider the slab's ability to bridge substrate cracking or movement (elasticity). Given the amounts of water used in aquatics facilities, resinous monolithic floor systems with integrated seamless cove-bases are most commonly used. Some of the most common resinous systems are novolac-based epoxy systems, urethane systems, and methyl methacrylate (MMA) systems (Fig. 25.6).

Partitions and Walls

There are two aspects of partition and wall systems to consider. These are the substrate and the finish. Substrate choices vary and include concrete masonry units and engineered panel systems. Most commonly, zebrafish facilities utilize a modified gypsum wallboard

FIGURE 25.6 Photograph of flooring.

(GWB) product with moisture and/or mold-resistant properties. This is a critical consideration in environments with high humidity and high water use. There are a number of options for finishes. It is recommended that a multilayer epoxy or urethane-based system be applied to meet a dry-film thickness (DFT) of at least 15 mils. In order to achieve this, three or more layers (base, intermediate, and top) are required. Consumer available single or two coat epoxy paint products are generally not robust enough to withstand wash-down and disinfection regimens in most facilities.

Ceilings

As a general principle in animal facilities, monolithic ceilings are preferred. This can typically be achieved with a mold/moisture resistant GWB product. In situations in which a significant amount of access is required, gasketed resin panels or sealed fiberglass ceiling systems may be most appropriate. In all cases, the use of nonferrous materials to prevent rusting in high humidity is recommended.

Procedure Room Planning and Design

Procedure rooms in zebrafish facilities are usually separated from housing rooms for practical and operational reasons. Undesirable variables of movement, noise, and light intrusion in housing spaces can be prevented by conducting procedures in separate spaces.

Sizing

In general, most procedures can be accommodated within a typical 11′-0″ planning module based room. Common sizes are 11′-0″ × 22′-0″ or 11′-0″ × 24′-0″. Sizing will be dependent on the number of simultaneous procedures, and workstations to accomplish the workload. For behavioral studies, larger spaces may be required to accommodate mazing and other special tanks.

Special Features

Special building features in procedure rooms may include:

Procedure Lighting

High-intensity lighting that can be directed toward a work surface may be required for micromanipulation or other fine procedures.

Snorkel Exhausts

When working with some chemicals or noxoids that do not require a primary containment device (biological safety cabinet or fume hood), a properly utilized snorkel will remove fumes or vapors on an open surface.

Compressed Air

This may be necessary in procedural rooms to drive microinjection equipment and float vibration isolation tables.

Dimmable Lighting

This may be used to minimize eyestrain or for some microscopy (fluorescence work).

Cage/Tank Wash Planning and Design

Equipment types

A wash facility for aquatic species has features and equipment that are similar to wash facilities for rodents, but there are also some distinct differences. While the ultimate goal of both types of facilities is to both clean and sanitize housing units via (generally) a mechanical wash method, the equipment used to do so can often vary significantly. For large rodent facilities that require the throughput of 8000–10,000 cages per day, tunnel washers are common. Given the issues of biofilm buildup and surface scaling common in zebrafish tanks, the short duration of this wash exposure is typically inadequate to thoroughly clean the tanks. Given longer potential exposure/wash times, cage-and-rack washers and cabinet washers are much more appropriate for the wash and sanitization of zebrafish tanks. As is discussed in the chapter on cleaning and disinfection, the use of surfactants must be carefully managed—so the selection of wash equipment that can work within parameters acceptable for aquatic use is of importance. From this perspective, while equipment could technically be shared between species, in practice, it is rarely done because of vastly different cycle times and detergent parameters (Fig. 25.7).

Flow Considerations

Most washing facilities share a typical tripartite configuration of soiled side, wash equipment, and clean side, including aquatic washing facilities; however, the space required for each side may be different depending on the washing procedures and equipment used. Because it can be difficult to remove biofilms that build-up on zebrafish tank surfaces, pretreatment of dirty tanks and accessories with chemicals (bleach) is often done. Space must be allowed for this, and is often

FIGURE 25.7 Fish tank washer, manufactured by Tecniplast.

needed to accommodate "bleach baths," large storage vessels containing bleach solution into which dirty tank components are placed to be soaked before further cleaning is done. As the time required to release some of the biofilm material might be considerable (depending on the level of deposits), adequate space to allow for this "soaking" must be provided.

Throughput Considerations

As is discussed in-depth in the chapter on cleaning and disinfection, this process is nuanced and requires specialized techniques and processes. With this in mind, adequate space for these activities should be carefully considered. However, when we look at typical throughputs, there are significant differences in the expectations and or ability to process fish tanks. As a general rule of thumb, our practice has seen that the throughput time is approximately double that of an efficient rodent cage operation. For example, a theoretical facility of 500 cages can be used to evaluate the differences in expected throughput. If the wash-load was typical mouse cages, washed in an efficient cage washer (cabinet) configuration, we might expect to this process to take about two and a half hours to complete. However, due to longer cycle times to remove tank deposits without the aid of surfactants, the same load of 500 tanks (equivalent to a cage) would likely take about 5.1 h—double the time to process rodent caging. It is important to consider in planning the degree to which these throughput numbers will likely require additional queuing space and potentially additional wash equipment to accommodate the required workload in adequate time.

Features

Casework

Given the issues of high-humidity and wet environment that have been previously raised, careful consideration of the casework systems is required in aquatics facilities. Most commonly, either phenolic resin or plastic casework is used in housing areas—where humidity is of significant threat to the degradation of casework. Metal casework has been used in many facilities—however, regardless of the finish, it tends to rust and degrade rapidly. Epoxy benchtops with marine edges to contain and direct spilled water to drains are recommended. In locations adjacent to sinks, benchtops with integral drain-boards (grooved drainage ways) help with water and spill mitigation.

Doors

Doors take a great amount of abuse within an aquatics facility. To resist the warm, humid environmental issues present, the preferred type of door for zebrafish facilities is an FRP (Fiber-Resin Polyester) door. These fully sealed, hydrophobic assemblies will resist the humid environment and provide a long-lasting function (Fig. 25.8).

FIGURE 25.8 Photograph of animal facility doors and hallway.

Mechanical, Electrical and Plumbing System Design Considerations

As in all animal research facilities, supporting mechanical, electrical and plumbing systems are critical to the operations and life-support of the animal population housed within. While there are many commonalities between HVAC design for a prototypical rodent facility and that for an aquatics facility, there are also notable differences. In this section, common differentiators will be identified.

HVAC Design

Fro achieving the temperature and humidity requirements outlined earlier in this chapter, it is recommended that dedicated systems be provided for the aquatics portion of an animal program and facility. As animal research facilities are most commonly 100% outdoor air HVAC systems, the energy penalty to condition air serving aquatics programs is more significant than that for rodent facilities in many climatic conditions. As approximately 65% of the energy cost of a research facility is in the conditioning and movement of air, it is prudent to "right-size" the air handling capacity of a system serving an aquatics facility. Increasingly popular is the utilization of "fan-wall" systems that allow air-handling units to operate efficiently while also allowing additional capacity for future expansion by sizing the overall air-handling unit to allow for this expansion. Material selection for HVAC systems and associated components must be selected to support a warm and humid environment. The specification of aluminum or stainless steel ductwork is recommended for the distribution system and diffuser materials given the potential for corrosion in humid environments. While stamped steel diffusers with a high-performance paint or coating are common in animal facilities, investment in an aluminum diffuser will eliminate the issue of metal rust and associated sanitation issues and provide a long-term solution in aquatics programs. The placement of diffusers to ensure proper room mixing and avoid stratification of conditioned air, without "hot-spotting" (or cold-spotting) tank locations is also a consideration for HVAC design. The relative uniformity of the macro environment will encourage uniformity the environment in the housing units (tanks), therefore, leading to a more stable comparative baseline for research. Another area requiring attention is the HVAC control devices, in particular, the temperature sensors. Since zebrafish facilities are warm, humid spaces, it is recommended to avoid the use of electronic devices that could be susceptible to rapid degradation, decay, and malfunction.

Electrical

By their nature, aquatics facilities are wet spaces. As such, proper provision of safe and secure electrical devices is critical to the operation of the facility. Given the wet nature of the spaces, it is highly recommended that GFCI circuiting be utilized throughout any aquatics facility, with particular attention to wet workspace areas. Discussing with the electrical engineer during facility design the advantages and disadvantages of receptacle based GFCI versus Whole Circuit GFCI is recommended. The second critical decision related to electrical systems is the selection of receptacle protection devices. Again, since aquatics spaces are wet by nature, protection against the introduction of water and shock exposure is paramount. There are two primary choices for electrical receptacle protection: "weatherproof" and "weatherproof while in use." Each of these may be appropriate depending on the intended use; however, the implications on space usage are quite different. Weatherproof receptacles when closed are watertight. These are primarily used in spaces where ancillary equipment is unplugged for washdown or where water exposure is somewhat predictable. In contrast, weatherproof while in use receptacles commonly have a plastic dome that allows for water exposure while devices are plugged into the power source. The latter require three to five inches additional space to accommodate the domed cover.

Lighting

In addition to power provision, another critical aspect of electrical design in aquatics facilities pertains to lighting. Recent research has shown that color rendering has a significant impact on aquatic species, zebrafish in particular. As LED illumination sources become more common in animal research facilities, due to their energy efficiency and nearly maintenance free assemblies, careful attention to their color light output is required. As identified in "The Red Light District and Its Effects on Zebrafish Reproduction (Adatto, Krug and Zon; 2016), red colored light can be detrimental to the zebrafish, impacting reproduction rates and significantly effecting behavior. Even a common feature such as the red illumination of an emergency egress "EXIT" sign can produce enough illumination to impact the behavior of the fish within the vicinity of the lights (Fig. 25.9).

Plumbing

It may seem obvious for an aquatics research facility, but special attention to the provision and removal of water is required. On the supply side, not all water is created equally. The definition of the type of water

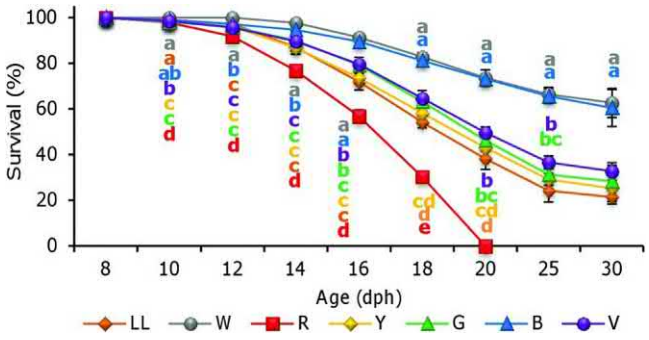

FIGURE 25.9 Survival (%) of zebrafish larvae reared under different light spectra between 8 and 30 dph (days posthatching). Values are mean ± SEM. Survival in constant white light (LL) or light–dark cycles of white (W), red (R), yellow (Y), green (G), blue (B), and violet (V) light are shown. Different lower case letters indicate statistically significant differences between each treatment with each colored letter representing the corresponding wavelength (blue letters for blue light treatment, etc.). For example, at 16 dph there was not a statistically significant difference in survival between larvae reared under light–dark cycles of either white or blue light (represented by the letter a); or between survival of larvae reared under light–dark cycles of violet or green light (indicated by the letter b); or of survival of larvae reared under light–dark cycles of green or yellow light, or of constant white light (indicated by the letter c). However, the survival of larvae reared under light–dark cycles of red light (indicated by the letter d) was significantly different from all other groups. Thus, the survival of larvae at this age was significantly different between groups a, b, c, and d. (ANOVA, $P < 0.05$, n = 2). Published with permission from Liebert Publishing.

required to feed the housing system is a critical first step in the design process. Common options for water quality are defined by ASTM standards, and are commonly referred to as water "type." The most prevalent type of water in use is reverse osmosis filtered water. The filtration system that produces this type of water is usually within the scope of the plumbing engineer, whereas further filtration and treatment to create "system water" is the responsibility of the housing system vendor. It should be noted that delineation of scope in plumbing should be clearly defined at the outset of the project. In other terms, whether specific water supply and waste water plumbing is the responsibility of the plumbing engineer or the aquatic housing vendor must be clearly understood. The drainage or effluent side of the design problem is the next issue to define. Location type and features of floor drains are determined by the process and methodology for washdown as well as the layout of the racks and preferred supply water approach. Differing vendors of aquatic housing and life-support systems have widely varied approaches to accommodation, so early inclusion and coordination are critical to the success of the design project. In the design of the plumbing systems, it is also critically important that the selection of the materials takes into account the possibility of chemical, mineral, and metal leaching

FIGURE 25.10 Photograph of plumbing and other equipment in a zebrafish pump room.

into the water carried by the system. In general, copper and metallic piping and/or fittings used for domestic water distribution are prohibited in aquatic facilities due to their potential water quality impact and possible negative effect on aquatic animal health. While an experienced vendor generally designs the life-support systems, the feed-water supplies are often designed by plumbing engineers who may or may not have experience in the design of aquatic facilities. It is important to ensure that posttreatment water systems are designed to be copper free. It is also critically important to determine that the amount of water that can be produced is sufficient for all the needs of the system, including not only system and tank water but also extra water for setting up breeding tanks and other experimental resources (Fig. 25.10).

Special Considerations

Facility Sustainability

As fresh water is an increasingly scarce resource globally, the design of zebrafish facilities must be considered from a sustainability perspective. As a large consumer of potable water, zebrafish facilities can serve as exemplars for reuse strategies. Instead of flowing the entire daily turnover load to the sanitary waste system, with minimal filtering, the turnover water can be used for other purposes. Some opportunities for water reuse could include toilet flushing, site irrigation, and cooling system recharge. The research community should responsibly explore ways to minimize water turnover rates while maintaining water quality. Perhaps this will be one outcome of increased efficiency of filtration media as those technologies continue to evolve.

Redundancy

Provision of basic life support is paramount in both rodent and aquatic facilities. For aquatic life support, a slightly different perspective may need to be considered. It is standard to plan for redundancy in electricity (optional standby power or emergency power), HVAC function, and other primary utility systems in animal facilities. However, with aquatic programs, the timeframe of loss of life associated with loss of life support to tanks is different when compared to that of rodent programs. Since oxygen and temperature levels in the water will dissipate at a relatively slow rate, the rate at which systems need to respond to maintain life support is different. This is not to suggest that standby power is not important; however, the provision of standby to the air-side systems could be costly and unnecessary. However, providing optional standby for direct water heating and filtration systems in the case of an extended power loss may be an important strategy. For example, biofilters can lose their effectiveness for ammonia conversion due to loss of their microbial population within an hour of power loss, leading to health and welfare concerns for the fish (personal communication, Susan Farmer, UAB).

Lessons Learned

Over the past 2 decades of development in the design of zebrafish facilities, there have been many lessons learned. It would be an impossible task to catalog and qualify them all. With that said, there are two important lessons learned by the design community from which everyone involved in zebrafish facility development can learn:

Early Vendor Selection – The art and science of zebrafish housing are greatly varied in the present industry. While all vendors strive to achieve the same goals, they do so via very different systemic approaches. This leads to differences in layout, utilities, and ultimately operations. Understanding these differences early in the design or modification process will allow for targeted and cost-effective design responses. Issues such as aisle width, lighting layout, supply water distribution, and drainage are all significantly dependent on the type of zebrafish housing selected and the unique layout associated with a vendor's preferred approach. Early onboarding and coordination save time, money, and frustration.

Non-Transference – Amongst many, all animal facility issues require the same approaches. Understanding that there are many similarities in design features, finishes, and layouts; however, there are also very specific environmental, operational and functional differences in aquatics and in particular, zebrafish facilities will lead to a zebrafish research-ready space; rather than a rodent facility adapted to aquatic use. Even something as small as the red light from an exit sign can have deleterious effects on zebrafish and requires thought during the design process (Adatto, Krug, and Zon).

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CHAPTER

26

Aquatic Housing

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Introduction to Aquatic Housing

When zebrafish first emerged as a novel genetics model system, the availability of strategic housing options was extremely limited. Despite this, the Streisinger Laboratory at the University of Oregon was able to devise a life-support system that enabled the fish to flourish under their care. The early genetics revolutions that led to zebrafish becoming today's second most used animal model after mice began in common glass aquaria, purchased from a local pet shop provider and covered with custom-cut acrylic lids. The fish were housed in a semiflow-through environment where water exchange occurred during the day by filtration of influent mains water through a household carbon filter and a thermostatic mixing valve; water exchange was then turned off in the evenings (Lawrence & Mason, 2012).

Today aquatic housing solutions share common threads with this precursor system, but technology has made possible many advances, from significant enhancements in tank design and construction to the development of smartphone-interfaced, fully automated electronic water quality monitoring and control instrumentation. These advancements allow the luxury of multiple strategic choices when considering how best to house this durable model organism.

This chapter will explore available choices in detail, but before decisions can be made with regards to tank design, materials selection, system design (single-pass or recirculating water), or scientific consequences (such as effects on gut flora), one must first understand the impact that scale and application have on these choices, as this is the key driver for determining which aquatic solution will best enable one's research goals.

As the zebrafish model continues to break new ground beyond its traditional biomedical discipline, many research institutions are making a strategic commitment to its application by building core aquatic animal facilities designed to facilitate the complexity of cross-disciplinary research programs. This commitment often requires the use of specialized housing equipment, especially when project designs evaluate the fish over many weeks or months (Traver et al., 2004). While the zebrafish has proven itself as an adaptable research model and a critical core model organism, the institutional commitment worldwide to its use is still very cautious compared to century-old rodent models. One would be hard pressed to find an institution with a biomedical research discipline in its faculties lacking a rodent vivarium supported by dedicated laboratory animal technicians, yet the commitment to centrally staffed aquatic facilities is still in an emergent phase. As a result, it is commonplace for significantly lower scale zebrafish aquariums to be utilized as the housing solution for the model. Regardless of the commitment level toward the zebrafish model system, all facilities strive to provide the fish with the best care possible within the restrictions of the aquarium's design. Thus, housing systems need to provide the fish with a stable and favorable culture condition while also supporting the specific research goals. In addition, as the application of fish in biomedical research increases, oversight bodies are requiring aquatic research facilities to meet and adhere to more stringent regulatory requirements worldwide (Lawrence et al., 2009).

When it comes to deciding on the appropriate scale for a zebrafish commitment, the most influential drivers are (1) whether multiple research groups are interested in applying the model, and (2) whether the investigative questions can be addressed with simple short-term developmental experiments, or whether longer term, multigenerational approaches are required. If the desire for zebrafish application is being driven by only a small number of investigators who are asking questions that can be assessed without the need to rear multiple strains, then the most appropriate housing solution may be as simple as a glass aquarium tank on a bench.

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If, however, the questions require multiple generations of controlled pedigree crosses, then the need to provide husbandry for multiple life stages will require a more involved commitment to the model, and an expanded commitment toward the housing technology applied. This commitment may take the shape of a custom solution designed and developed specifically for the application. However, the most common answer is likely to come in the shape of a commercially available housing solution that elegantly combines principles of commercial aquaculture, laboratory animal housing, and research genetics, and that can be tailored and scaled to meet the needs of multiple research programs in one facility.

Beyond defining the application scale, culture condition is the next most critical element to be addressed in order to ensure that the fish become a productive research model. While the major elements of culture condition will be discussed in other chapters of this text, there is one element of culture conditions that requires attention within the discussion of housing solutions. This is stocking density and its impact on stress.

The density at which fish are kept in captivity exerts profound effects on their health, productivity, and welfare. Holding densities also ultimately have significant implications on the operating costs of an aquarium, the space required for the housing solution, and the labor required for maintaining the aquatic facility. In general, higher stocking densities will ensure that costs, space, and labor are kept to a minimum, but it may not be the best approach for achieving a healthy, productive zebrafish colony.

It is widely accepted that crowding fish at high stocking densities can cause chronic stress, resulting in changes in growth rate, feeding, behavior, immune function, and health (Ellis et al., 2002; Procarione, Barry, & Malison, 1999; Suomalainen, Tiirola, & Valtonen, 2005; Wedemeyer, 1996). It is also recognized that stocking density effects on fish welfare are complex and species-specific; these effects can comprise numerous interacting factors including water quality, social interaction, the carrying capacity of the holding environment, as well as the special and behavioral needs of the particular species (Pavlidis et al., 2013).

As a gregarious species, zebrafish benefit from the ability to form loose shoals with their tank mates, where they can establish and express social hierarchies. Zebrafish are also known to display aggressive behavior associated with the establishment of territories and dominance hierarchies (Larson, O'Malley, & Melloni, 2006); thus, both crowding and low-density housing are less than ideal for zebrafish. This highlights the importance of matching holding conditions to their behavioral tendencies.

The currently accepted guideline for establishing a stocking density policy that is conducive to productive and healthy zebrafish is based on the approach outlined by Harper and Lawrence (2011), who suggest that densities of 40–50 fish per liter are appropriate for early larval culture, but that density should be gradually reduced to five fish per liter for adults. This commonly applied strategy was supported by Pavlidis et al. (2013) who showed that zebrafish held at a density of 10 fish per 2 L of available water volume adequately expressed normal behaviors while avoiding elevated cortisol stress reactions; this suggests that even when water quality is maintained, a minimum water volume is required for zebrafish welfare.

With this knowledge, it is possible to determine the necessary scale of the intended application, based on the target number of animals required to meet the research program's needs. With appropriate scale commitment established, the desired application will be the key driver for determining the type of enclosures needed for housing the fish.

Primary Enclosures (Tanks)

In an aquaculture facility, the primary housing enclosures are the tanks. Depending on the scale and application of the projects employing the zebrafish model system, the tanks used will vary in shape, size, and materials from which they are constructed. They will also vary in how they handle water exchange and how they contain the specimens. These factors all affect the welfare of the fish, the functionality of the life-support system, and the pace and efficiency of research being conducted. Thus, aquatic housing choice for any application needs to include careful consideration of how a particular system, and its associated tank types, help investigators achieve their research and husbandry objectives.

Materials Selection

Material toxicity is a concern when introducing any new equipment that will contact the fish. For this reason, the most common materials that zebrafish holding tanks are made from are glass, acrylic, fiberglass, polyethylene, polycarbonate, or polysulfone. These materials have an established record for use as aquatic housing solutions, each with its benefits and limitations, and thus, the appropriate material selection will depend on the application.

Thermoplastic Polymers

In modern high-density installations, tanks are most likely to be made from polycarbonate. This plastic is durable and relatively inexpensive, and it can be clear or pigmented. The pigments chosen for zebrafish enclosures are typically shades of blue or green, focused on filtering out the light spectrum utilized by chloroplasts, thus reducing the development of algae on tank surfaces as the aquarium's water begins to accumulate phosphates and nitrates. Polycarbonate tank construction is made via pressure injection molding, and it is, therefore, amenable to the production of tanks in a wide range of shapes and sizes. Most commercial zebrafish aquarium manufacturers offer a range of polycarbonate tanks, allowing a significant level of customization to any aquarium installation. One of the most significant benefits with the use of polycarbonate tanks is that they are readily movable within the facility. One can thus temporarily relocate fish to a workbench for investigation, directly exchange tanks for cage cleaning, or transfer fish from a fouled tank into a fresh new tank. This accommodates significant flexibility in cleaning and disinfection protocols. Although polycarbonate tanks are lightweight and can withstand mechanical washing processes, thereby reducing manual labor and zoonosis risks present with some other tanks, polycarbonate degrades at high temperatures, and thus, cannot be repeatedly sterilized. For countering this, several manufacturers of polycarbonate tanks also offer a range of tanks made from polysulfone.

Polysulfone is slightly more costly, with a greyish hue, and is marketed as having a higher temperature and chemical tolerance than polycarbonate. Tanks made from polysulfone can be a suitable substitute for applications, where regular tank sterilization is required.

Both polycarbonate and polysulfone tanks are produced using bisphenol-A (BPA). This is the main drawback to the application of these thermoplastic polymers in aquarium holding tanks, as it has been established that both types can leach BPA into the water. Bisphenol-A is an estrogen mimic that can cause serious reproductive problems in vertebrates, including fish. Howdeshell et al. (2003) demonstrated that new polycarbonate and polysulfone tanks both leach BPA into the water. However, leaching is significantly higher in aged polycarbonate tanks, thus elevating this as a potential concern for established zebrafish facilities. Howdeshell et al. (2003) also discussed the leaching levels being similar to those previously reported to skew sex ratios toward females in the South African clawed frog, *Xenopus laevis* (Kloas, Lutz, & Einspanier, 1999), and that resulted in the presence of ovo-testes and testicular abnormalities, including a decrease in the number of spermatozoa, in the medaka *Oryzias latipes* (Metcalfe et al., 2001). While definitive effects of BPA leaching in a zebrafish colony remains to be tested, it is reasonable to conclude that BPA leaching may have similar effects on the diminutive minnow. The benefits gained from the use of thermoplastic polymer tanks are nevertheless significant, suggesting that regular tank replacement may be the most reasonable strategy for managing this side effect until a satisfactory BPA-free substitute material can be found.

Glass

If BPA effects are likely to cause issues for the research program, the inert properties of silica-based glass may make it the material of choice. Glass tanks are the most common housing solution implemented in small-scale operations, as basic rectangular glass aquaria are readily available at most pet shops. Glass can also be applied in large-scale operations, and it is the next most common material used in primary enclosures after thermoplastic polymer tanks. The principle benefits of glass are that it is inert, and thus, poses no chemical leaching risk to its inhabitants; it does not scratch easily, which, in turn, means it resists the establishment of biofilms and other tank fouling, and it is relatively inexpensive. Glass, however, lacks the flexibility necessary for design variability, making it difficult to apply to as wide a range of housing solutions as other materials. Glass also has the significant limitations of being fragile and heavy, posing major health and safety challenges to personnel during routine cleaning.

There are health and safety challenges that glass aquaria present to the personnel who maintain them. There are manual handling risks associated with moving, servicing, and replacing the tanks. Large glass aquaria are heavy, often requiring multiple people to move and position them. This means they do not readily lend themselves toward automated washing. As a result, most glass aquaria will need to be cleaned in place, often resulting in a laboratory animal technician arm deep in fish water. While this in itself is not a dangerous activity, there is a significantly elevated risk of skin abrasions from this method of tank maintenance, a risk that is compounded if there are minor chips to the rims of the tanks. This can prove to be a major obstacle for facilities that opt to utilize glass for their primary enclosures, as there is a significant zoonotic disease common to zebrafish colonies; the atypical tuberculosis, *Mycobacterium marinum* (Matthews, 2004). To overcome this risk, facilities using glass tanks need to be mindful of their biosecurity efforts, to ensure they do not inadvertently contract a mycobacterium infection. Strategies for maintaining a mycobacterium free facility are discussed later in this book.

Acrylic

As the zebrafish model expanded beyond traditional developmental genetics studies, there grew a need for specially designed primary enclosures to enable specific experimental work. As a result, poly(methyl methacrylate), commonly referred to as acrylic, has become an important material of choice. Although technically also a thermoplastic polymer, acrylic has significant differences in how it can be applied as a primary enclosure, and thus, is being discussed separately. Acrylic does not contain the potentially harmful bisphenol-A subunits found in polycarbonate and polysulfone plastics, and it is an economical alternative to polycarbonate when extreme strength is not necessary. This transparent thermoplastic is often used in sheet form as a lightweight, shatter-resistant alternative to glass. It is approximately half the weight of glass, and while it is significantly stronger and more flexible than glass, it is also significantly more prone to scratching. Scratches may be easily removed by polishing the damaged surface of the material with appropriate solvents available from hobby retailers. Acrylic can be damaged by organic solvents such as ethanol and by other chemicals. It also has low heat tolerance, which can limit sanitation options. Even high-quality acrylic will begin to disfigure and melt at temperatures above 80°C. Acrylic is very resistant to the effects of strong alkali solutions, which are extremely effective germicides. Sodium hypochlorite (bleach) can be applied safely to acrylic tanks at concentrations up to 5%, and any biofilms or algae can then be subsequently wiped off with a soft cotton cloth. When using this method of sanitation, it is necessary to detoxify any residual free chlorine before repopulating the tank.

Polyethylene and Fiberglass

Although less commonly applied, polyethylene and fiberglass-reinforced polyester (FRP) also play an important role in zebrafish housing. These materials share similar beneficial traits and are most commonly applied when the application requires either holding a large volume of water or controlling illumination to the culture, thus eliminating the option of using the optically transparent acrylic.

Emerging Materials

In recent years, 3D printing has significantly enhanced the ability to construct tools and in this case, housing solutions. A prime example, the Screen Cube, is a 3D-printed housing solution that is used for the transfer of fish between primary holding tanks and treatment baths, allowing for rapid and cost-effective chemical screening of adult zebrafish (Monstad-Rios, Watson, & Kwon, 2017). While 3D printing offers immensely powerful flexibility for creating novel primary enclosures, it is important to note that not all 3D printing polymers are considered safe. Macdonald et al. (2016) assessed the biocompatibility of four commercially available 3D printing polymers, and via the observation of key markers in developing zebrafish embryos, showed all of the photopolymers in an untreated state to be lethally toxic to zebrafish embryos. When parts fabricated from one of these photopolymers, Fototec SLA 7150 Clear, were washed with an organic solvent (99% ethanol), biocompatibility significantly improved, making it suitable for use in zebrafish culture. This is an important message with regards to employing new materials in a zebrafish aquarium. It is critically important to run a basic Fish Embryo Toxicity (FET) test, such as that described by Schulte and Nagel (1994), to determine the biocompatibility and safety of employing the new material.

Tank Design

Armed with the knowledge of application and appropriate material selection, it is possible to consider tank design for research program outcomes. Zebrafish tanks come in a variety of shapes and sizes, defined by their intended function. Although tank design varies between vendor and application, all tanks are generally designed to serve the common purpose of containing the fish within a specified space and volume of water, while also maintaining suitable life-supporting culture conditions for its inhabitants.

Box Tanks

Although becoming less common in the modern era, the traditional box tank still serves an important role for those who are keen to pilot small experiments but are unable to access a large-scale production facility. These tanks are typically 20−50 L in volume, allowing an all in one approach for the keeping of the fish. The large water volume allows the fish the space to exhibit their natural shoaling behavior, while also providing sufficient volume to buffer against shifts in water quality. The box tank can be used as a freestanding static aquarium, or with some modification, as a flow-through aquarium, in a semirecirculating life support system, or in a Recirculating Aquaculture System.

Serial Tanks

Serial tanks are most commonly made from glass and are applied in large-scale aquariums. Serial tanks are essentially an advanced modification of a box tank, resulting in long aquaria that are divided transversely by partitions to provide multiple compartments for keeping adult fish (Fig. 26.1). A typical serial tank is

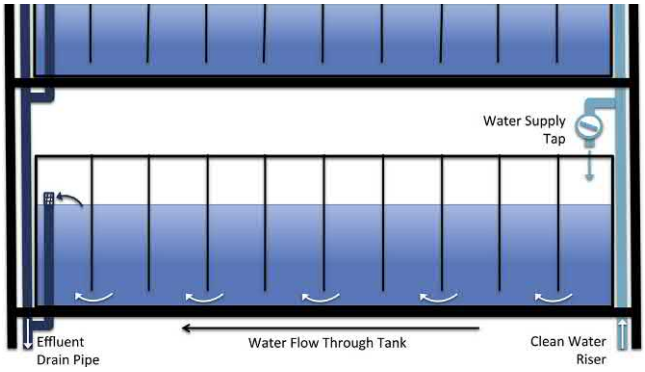

FIGURE 26.1 Serial Tanks. Serial tanks typically comprise a long glass box tank, which is divided into housing compartments by partitions (*black vertical lines*). These partitions leave a small gap at the base of the tank, resulting in a series of interconnected compartments. Influent water enters the series of tanks in the compartment at one end and flows through each tank compartment via the gaps at the base of the partitions (*white arrows*). Effluent water exits the tank in the last compartment via a standpipe (passive overflow), setting the water volume throughout the series. A grill fitted to the head of the standpipe prevents fish from escaping containment.

120 cm wide, 60 cm deep, and 22 cm high (Brand, Granato, & Nüsslein-Volhard, 2002). The long box is typically subdivided by glass plates that leave a small 1.5 mm slit at the bottom, or via perforated plastic plates, resulting in a series of 8–10 interconnected compartments of about 12 L each. Each compartment has a separate lid, allowing the holding of up to 60 adult fish per compartment at a stocking density of five fish per liter. The defining feature of serial tanks is that the water in a row of tanks flows into the compartment at one end, and then passes through the slits from one compartment to the next, carrying waste particles with it. The water is drained from the last compartment at the far end of the row by overflowing into a standpipe, thus determining the water level across the row. In order to prevent loss of fish, the standpipe is typically fitted with a coarse mesh or grill.

There is potential for capital savings to be made with the implementation of serial tanks, as there is only one influent and one effluent point requiring attention for supporting 10 tanks; however, the fact that serial tanks are too large to be removed from their support racks means cleaning must be done in place, making long-term maintenance of the system inefficient. Thus, the benefits gained from serial tanks are often at best neutralized by longer-term inefficiencies.

There is also one significant limitation to consider before committing to the use of serial tanks. Due to their interconnectivity, they are not able to control the spread of horizontally transmitted diseases, as all tanks share a common water source. Thus, if a population of fish in chamber one were infected with a disease like microsporidia (*Pseudoloma neurophilia*), then fish in the other nine compartments would be exposed to spores released into the water as it moves through the serial chambers toward the outflow pipe. To avoid this problem, the use of individual, self-contained primary enclosures may be necessary instead; this is the main reason why this tank design has lost favor in recent years.

Passive Overflow Tanks

There are many ways a passive overflow tank can be applied in a large-scale facility, and these tanks offer the most flexibility in design, shape, and customization. The simplest implementation of the passive overflow tank is described by Brand et al. (2002), in which individual containers (plastic or glass) are placed in a row on to a bunded shelf. Water enters into each tank through an influent hose or pipe and exits through an overflow point on to the shelf, from which it is drained into a common water treatment unit. However, this implementation of the passive overflow tank design often results in fish waste accumulating around the base of the tanks, and thus, more frequent cleaning is needed. An alternative implementation of the passive overflow tank utilizes a standpipe or tank flange, which allows the overflow point of the tank to couple to a plumbed waste line, keeping all effluent water contained within the wastewater drainage pipes. Both strategies typically rely on fine mesh grills, or filter media, at the overflow point to avoid fish escaping containment, and this can be tailored to suit adult or juvenile fish. Examples of both strategies are illustrated in Fig. 26.2.

Passive overflow is the most common strategy applied to specialty application tanks when they are incorporated into system solutions rather than existing as stand-alone housing chambers. Some of these solutions to unique applications will be discussed subsequently.

Active Overflow Tanks

Despite the significant flexibility advantage passive overflow tanks offer, the nature of the hydrodynamics within the tanks means these solutions offer limited ability to maintain tank hygiene, and frequent cleaning intervention is needed to keep these tanks functioning as intended. To overcome this challenge, it is commonplace for large-scale aquariums to implement the use of solids-lift (active) overflow tanks as their principle housing device. In these solutions, the tanks employ the use of either siphons and/or specially designed partitioning baffles to alter the hydrodynamics within the tank, such that an undertow current is established, creating an active draw across the base of the tank (Fig. 26.3). Providing there is an adequate velocity of water entering the tank via the influent pipe, the undertow

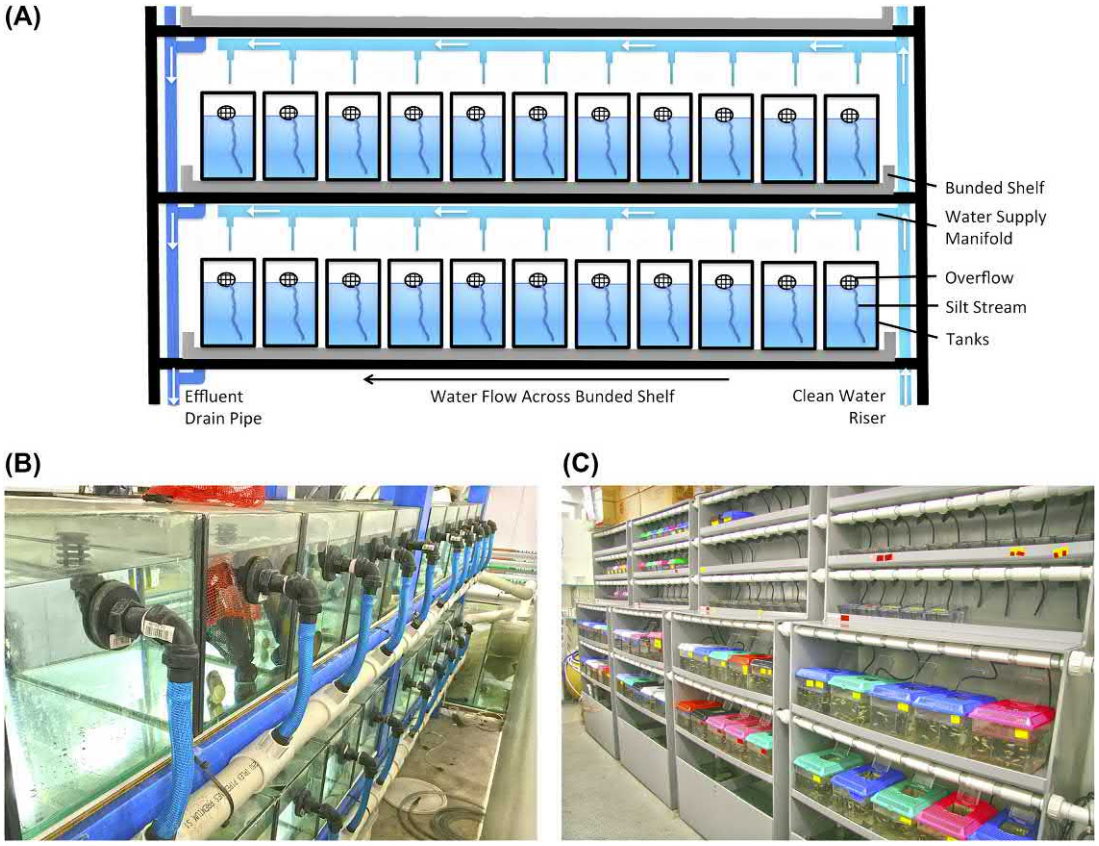

FIGURE 26.2 Passive Overflow Tank Application. (A) In this schema, individual holding tanks are placed onto a bunded shelf. Water enters into each tank via a small outlet from a header pipe manifold that runs along the length of the shelf. Water exits the tank through a grill, or series of small holes, at the top of the tank. Suspended particulate waste (fines) trickles down the tank onto the shelf (silt stream). Wastewater pools on the bunded shelf, and is collected into a common drainpipe that sequesters the wastewater to the filtration assembly. (B) Passive overflow, glass box tanks coupled to a wastewater drainpipe via tank flanges (C) Passive overflow, polycarbonate tanks on a bunded shelf.

current will have sufficient strength to carry waste particles out of the tank.

Specialty Application Tanks

Beyond the range of designs that make up the housing tanks, there are a number of unique primary enclosures designed to facilitate specific application needs, the most common enabling controlled breeding. Mating tanks are a critical element of an aquarium supporting research projects that focus on developing zebrafish embryos.

Mating Tanks

Historically, adult zebrafish were encouraged to spawn their eggs via a process known as "marbling," where an investigator would cover the entire bottom of the housing tank with a layer of marbles, effectively creating a series of nooks and crannies that allow fertilized eggs to drop beyond the reach of hungry tank mates (Westerfield, 2000). The fertilized embryos would then be siphoned from the tank every morning. While effective, marbling lacks the finesse that is required to meet the productivity demands of many research aquariums today; hence the development of purpose-designed breeding tanks.

Mating tanks come in various shapes and sizes, ranging from just over half a liter up to 2L volumes (Fig. 26.4). These devices can be applied directly in the main housing tanks; however, are most commonly applied as short-term, static enclosures, housing a pair of fish, or in the larger cases, small groups of fish, based on a technique that was first described by Mullins, Hammerschmidt, Haffter, and Nüsslein-Volhard (1994). They all share the same fundamental design characteristics: a tank to hold the water, a trap to separate the adult fish from their spawn, a divider to segregate the adults by sex and so enable timed mating, and a lid to prevent the fish from jumping out of the tank. While there is some handling stress associated with the use of these tanks, their independence from the main housing enclosures gives them significant advantages for both the harvesting of the embryos and for sanitation after use.

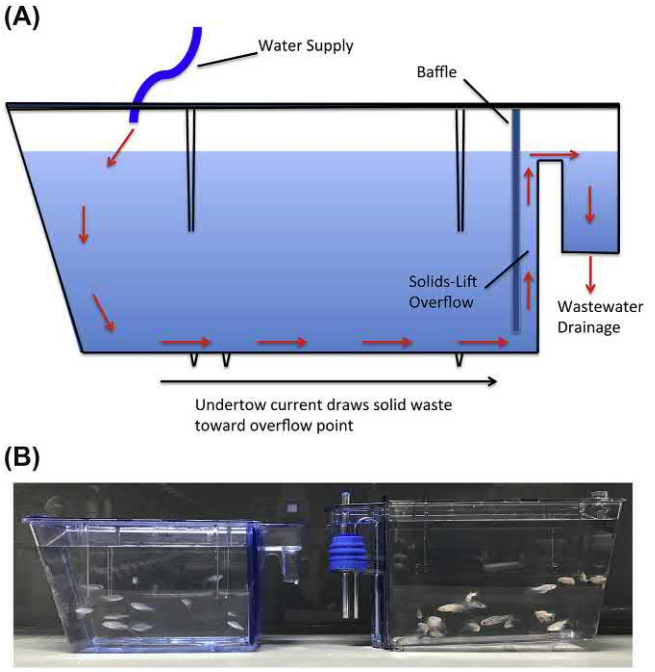

FIGURE 26.3 Active Overflow Tanks. (A) This shows the basic hydrodynamic design of a solids-lift tank. Water is delivered (*blue line*) into the tank toward the front of the tank. The unique design of the partitioning baffle (or siphon) at the back of the tank results in a pressure differential between the holding space and the overflow. A strong undertow current (*red arrows*) draws particulate waste toward the back of the tank and lifts it up to the tank overflow. (B) Examples of typical active overflow tanks.

FIGURE 26.4 Mating Tanks. Examples of various polycarbonate mating tank designs.

For many years, the vast majority of mating tank designs followed a simple, flat-bottom approach to the trap, which although satisfactory to yield sufficient quantities of embryos from the fish, did not play to the fish's natural spawning site preferences. Following research conducted by Sessa et al. (2008) that illustrated a preference to spawn in the shallows, as evidenced by the observation that the zebrafish actively chose to spawn at the shallow end of a sloped trap, many aquariums began to establish gradients in their mating tanks by placing them on a sloped platform or by wedging the trap with the divider, such that one end sat higher in the tank. The widespread adoption of this behavior led to one commercial manufacturer refining its mating tank design, resulting in a beach style trap, with a sloping layered surface, that allows the fish to select their preferred depth upon it for spawning.

Mass Embryo Production Systems

While the above mating tank designs are effective, there are scenarios where their application cannot easily meet the production demand for fertilized embryos. When obtaining large quantities of developmentally synchronized embryos is more important than tracking pedigree of individual contributors, Mass Embryo Production Systems (MEPS) offer a unique solution.

The main impetus for shifting breeding tank design to capitalize upon the group spawning dynamics of the zebrafish came as a result of the model expanding into the realms of toxicological studies, chemical screens, and other applications that demand increasingly large embryo numbers. Adatto, Lawrence, Thompson, and Zon (2011) describe placing up to 300 fish inside a custom-designed, 100 L static group spawning chamber capable of yielding 10,000 fertilized embryos within 10-minute harvesting windows.

There are several different models of MEPS currently available commercially (Fig. 26.5), each capable of use as a static breeding tank, or of being coupled to an aquarium system as a passive overflow device. One of the most significant differences between the MEPS solutions from various commercial suppliers is the base material with which these tanks are constructed. One design is completely transparent, constructed from acrylic, allowing the tank to leverage the existing facility

FIGURE 26.5 Mass Embryo Production Systems (MEPS). Large numbers of broodstock fish can be housed and spawned directly inside these devices, facilitating on-demand collection of synchronized embryos. From left to right, an example of a polyethylene MEPS, large (60 L, 200 fish) and small (13 L, 40 fish) acrylic MEPS, and a fiberglass-reinforced polyester MEPS. *Courtesy of Aquaneering Inc., Courtesy of Tecniplast Aquatic Solutions and Courtesy of Pentair Aquatic Eco-systems.*

photoperiods; another has opted to construct their MEPS solution from polyethylene; while a third supplier chose to fabricate their tank using FRP. The latter designs both allow photoperiods to be established independent of the main facility light cycle. Other differences between the various MEPS include size, holding capacity, spawning platforms, mode of egg collection, and patterns of water circulation; but regardless of design, the scale and reliability of function of these tools are tightly coupled to scientifically grounded management of the broodstock fish placed inside (Lawrence, 2011).

Respirometers and Mazes

While traditionally the model's strength lay with the developing embryo in biomedical discoveries, and so too with the majority of specialty application tank solutions, the adult zebrafish has started to become a more significant model to other disciplines; and with this, new applications for the model have emerged. Swim-tunnel respirometers, puzzle mazes, and operant conditioning shuttle boxes have all emerged as significant tools in the zebrafish toolkit in recent years.

One such tool, the respirometer, has gained particular focus for investigators interested in the physiology of the zebrafish, examining traits such as swimming speed, stamina, oxygen demand, and body shape drag. The most commonly applied, Brett-style Swim-tunnel Respirometers (Fig. 26.6) have a rounded-rectangular shape and contain a propeller that drives water circulation in one direction, while the fish are restricted to a long compartment known as the "working section," where the water passes in a laminar flow profile (Brett, 1964). A variable-voltage motor typically drives the propeller in the swim-tunnels, such that a range of water speeds can be obtained. These devices are typically constructed from acrylic, allowing optical transparency for simplifying data collection, and can be applied statically, fitted with a hang-on filter or connected to an aquarium system as a passive overflow tank. Respirometers can be applied as open swim-tunnels for projects such as examining gross morphology impact on stamina (Conradsen, Walker, Perna, & McGuigan, 2016); but when they are used for determining physiological processes, such as oxygen demand in exercising fish, it is necessary to seal the experimental chamber from the surrounding water bath to prevent water mixing, so that precise measurements can be achieved. In this situation, a flush pump should be used to refresh the water within the treatment chamber at regular intervals, to ensure the dissolved oxygen levels within the respirometer remain above 80% saturation at all times to minimize the chance of measurements being influenced by hypoxia-induced metabolic adjustments (Clark, Sandblom, & Jutfelt, 2013).

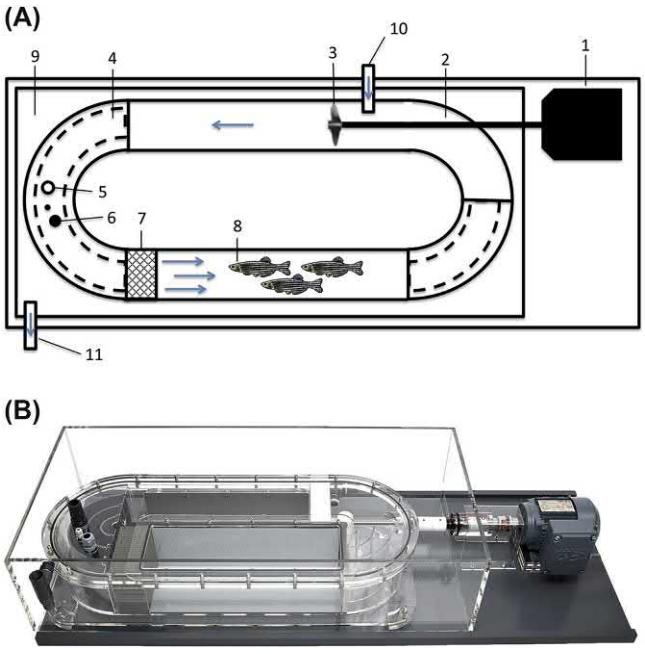

FIGURE 26.6 A Brett-style Swim-tunnel Respirometer can be used for measuring the physiology, energetics, behavior, biomechanics, and kinematics of swimming fish. (A) Overhead schema: (1) variable speed motor, (2) propeller shaft, (3) propeller, (4) baffles to assist with achieving laminar flow, (5) overflow pipe, which extends above water surface, (6) sealable port for oxygen sensor, (7) honeycomb grid to assist with establishing laminar flow, (8) working section where fish are housed, (9) water bath, (10) water inlet, one-way flow (11) water bath passive overflow (B) Swim-tunnel respirometer. *Courtesy of Loligo Systems.*

Mazes and shuttle boxes have also made inroads as specialty application tanks for zebrafish in recent years. Employing the use of a shuttle box, Sneddon (2011) demonstrated that when housed in an environment with the choice of a barren or structurally enriched condition, zebrafish exposed to a painful acid stimulus will override their preference for the enrichment and self-administer analgesics offered only on the barren side.

Environmental Enrichment

One common trait across most of the tank designs discussed thus far is that they are designed to operate as a barren enclosure. This has been a strong barrier against the use of structural enrichment in zebrafish production facilities. In addition, it remains unclear whether structural enrichment, designed to mimic the habitat complexity that wild zebrafish can experience, also benefits laboratory strains, particularly when such interventions are balanced against costs, such as the increased difficulty of observing fish and the increased accumulation of mulm (detritus), which in itself can lead to morbidity and mortality (Parichy, 2015).

While there is mounting evidence suggesting that zebrafish may indeed benefit psychologically from structurally enriched environments (Collymore, Tolwani, & Rasmussen, 2015; Manuel et al., 2015; Maximino, De Brito, de Mattos Dias, Gouveia, & Morato, 2010; Spence, Magurran, & Smith, 2011; Weber & Ghorai, 2013), little work has been done to address the concerns relating to colony-wide health impacts that may emerge as a result of changes in tank hydrodynamics, and the consequential diminished tank hygiene due to the presence of alien structures within the water body. Also, the material selection needs to be carefully considered when opting to implement the use of a structural enrichment device to ensure there is no leaching of unwanted chemicals into the water column. Fig. 26.7 illustrates how some facilities are implementing the use of structural enrichment.

While it is still relatively uncommon to utilize structural enrichment in large-scale production facilities, it is commonly accepted that it can be significantly detrimental to house individuals in isolation (Fox, White, Kao, & Fernald, 1997; Harris & Bird, 2000; Larson et al., 2006; McCarthy, Carter, & Houlihan, 1992). In low-density situations, such as keeping a carrier pair segregated, fish can exhibit enhanced aggression and will often fight until one fish either escapes the conflict by jumping out of the tank or is harassed to the point of death. This is a major concern for ensuring the longevity of critically important animals.

In a situation where structural enrichment may not be readily available for providing refuge for the subordinate, or a territory for the dominant to defend, the simplest way to manage the aggressive behavior of the fish is through social enrichment. As discussed previously, zebrafish have a preference to form small shoals. This behavior allows a more balanced social hierarchy to form, and this can be easily established by combining phenotypically different strains of fish. For example, if a carrier pair has been identified on an AB background, adding a small number of TL or TLN strain fish can preserve the social welfare of all fish in the tank. This allows the fish to maintain a natural social hierarchy, while also being easily identified as the specimens of interest to the investigator.

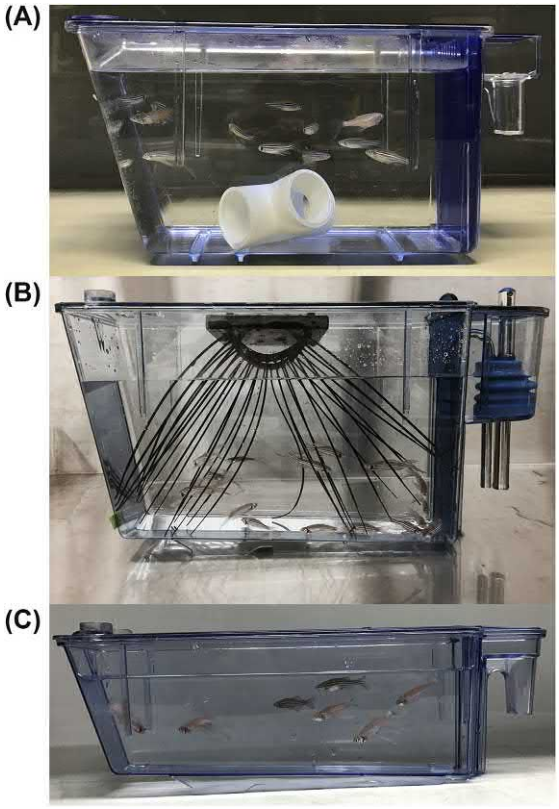

FIGURE 26.7 Structural and Social Enrichment. (A) A small PVC T-joint provides a tunnel refuge for subordinate fish. (B) The Zeb device, a structural enrichment solution offered by Otto Environmental, provides a floating canopy designed to mimic the dense vegetation of the native habitat of the zebrafish. (C) For avoiding the detrimental effects of housing zebrafish in low densities, important carrier pairs can be housed with phenotypically distinct zebrafish, allowing them to form social shoals and mediate their aggression by establishing dominance hierarchies with the other fish. *(B) Photo courtesy of Lucie Nedved.*

Secondary Enclosure Systems

While in a small-scale operation, the tank may serve as both the primary and secondary enclosure, but in large-scale production facilities, the zebrafish tend to be housed in relatively small primary enclosures, to maximize the number of specimens contained within a workspace. If the water within these tanks is not constantly cycled and cleaned, the fish will quickly suffer from the toxicity of their metabolic wastes accumulating in the water. For overcoming this, the tanks are integrated into much larger secondary enclosures, making up dedicated life-support systems designed to allow influent fresh water to replace the waste accumulating effluent water.

Static Enclosures

The challenge with static enclosures is that from the moment fish are added into the tank, the life-support condition, specifically the water quality, begins to deteriorate. As the fish respire, they consume dissolved oxygen and release carbon dioxide and ammonia into the water. As these metabolic wastes accumulate in the water, the environment becomes less favorable for its inhabitants. For this reason, static tanks need to be kept at significantly lower densities than enclosures in

which constant water exchange is possible, and thus, the situations where static enclosures are appropriate should be carefully considered.

A significant advantage of static enclosures is that they avoid all costs associated with establishing and servicing filtration assemblies, and are, therefore, very cheap to implement. However, because these devices lack the ability to clean the water, in comparison to other life-support solutions, they require significantly more space per capita. An additional consequence of avoiding the complexity of filtration assemblies is that static enclosures require high labor commitment in order to keep the life-support condition within suitable parameters for the fish.

As a general guide, if the fish are to be maintained long term in a static enclosure, there are a few basic tips that will help improve the success of the colony. To keep water degradation within manageable tolerances, Brand et al. (2002) advise densities in static tanks should not exceed two fish per liter. Only feed the fish to satiation, so that uneaten food does not amass in the tank, releasing ammonia and rapidly degrading the water quality. Allow the fish opportunity to digest and defecate before performing a tank clean and water change. Keep a small sponge, or other porous material, in the tank to act as a basic biofilter. Even without aeration, these measures will assist with the natural biological process associated with converting toxic ammonia into the significantly less toxic, oxidized state (nitrate). Also, where possible, aerate the water to increase gas exchange, removing dissolved carbon dioxide and regenerating dissolved oxygen.

On a more temporary timeframe, adult fish can be comfortably maintained in static tanks for up to 2 weeks at low densities without water exchange if feeding is withheld and the fish are prevented from stressful interactions, such as breeding or fighting. This is an ideal strategy to exploit when genotyping individuals and can be easily achieved by placing two fish in a 1-L mating tank with a divider separating them.

As zebrafish alevin (yolk sac larvae) transition into fry (exogenous feeders), they are also often kept in static enclosures for the first few days of their foraging existence. As described by Best, Adatto, Cockington, James, and Lawrence (2010), first-feeding zebrafish fry can thrive at a density of 50 fish in 200 mL (250 fish per liter), highlighting that the challenges associated with static enclosures are also life stage-dependent. By far the greatest application for static enclosures, however, is their use in spawning, as was discussed earlier in this chapter.

Before moving onto the more sophisticated secondary enclosure solutions, it is worth noting that the addition of a basic hang-on filter can significantly enhance the life-support condition of a static tank. By adding one of these devices to the side of a tank, a semirecirculating system is achieved, in which the main body of water within the aquarium is pulled out of the tank by a small powerhead pump, and then passes through a basic mechanical filter (usually a pad akin to the scotch scourer) and a small chamber containing extruded activated carbon. The water may also pass through a more porous sponge-like material that acts as a biological filter before the water flows back into the tank, encouraging agitation and enhancing gaseous exchange at the water surface (Fig. 26.8). The principal benefit achieved by the use of a hang-on filter, is the minimization of mulm, the sludge that collects at the bottom of an aquarium, consisting of fish fecal pellets, decaying food matter, and other assorted detritus. By keeping a steady water turnover, the hang-on filter is able to second waste particles into the filter before they have a chance to settle in the base of the tank, significantly extending the useful life of the water within the tank.

FIGURE 26.8 Hang-on Filter. With the application of a hang-on filter to an otherwise static holding tank, the useable lifespan of the culture water can be significantly extended. A small powerhead pump draws water up and out of the tank. The water then passes through a basic mechanical filter pad, activated carbon filter, and biological filter sponge before freefalling back into the holding tank, offering an opportunity for gas exchange.

Recirculating Aquaculture Systems

Recirculating Aquaculture Systems (RAS) are the predominant secondary enclosure solution applied in zebrafish facilities of all sizes. The distinguishing feature defining RAS aquaria is that the water used to house the fish is cleaned and reused, minimizing water consumption and waste. This allows the total water volume of a RAS aquarium to be shared by a number of primary enclosures, thereby, greatly reducing the space required for housing the research colony. Mastering the process of cleaning the recirculating water is the greatest challenge for applying a RAS solution, and its implementation can vary significantly from one aquarium design to another, but at its core are five key elements: mechanical filtration, biological filtration, gas exchange, chemical filtration, and disinfection.

Mechanical filtration is typically the first step of a RAS filtration assembly and targets the isolation and removal of suspended solids from the effluent water. Solid waste enters the system as a result of uneaten food or fecal pellets and must be quickly removed from the circulating water because as the waste decomposes, becoming a food source for heterotrophic bacteria, dissolved oxygen is significantly reduced and ammonia is released into the water column. Particle filters used for mechanical filtration may be either depth or surface filters that trap solids as the water flows through it.

With the solid carbonaceous waste eliminated, the next phase of water treatment typically focuses on biofiltration. Biological filtration describes a process harnessing the power of chemolithotrophic bacteria that oxidize ammonia into nitrite, and then nitrate, to detoxify the nitrogenous waste. Biofiltration is a highly aerobic process, requiring double the ammonia concentration in dissolved oxygen within the reaction chamber (Chen, Ling, & Blancheton, 2006). For this reason, the biofilter is often coupled with gas exchange (oxygen in, carbon dioxide out).

Oxygen is a staple for aquatic life, just as it is for terrestrial life; therefore, as water circulates through the holding tanks, oxygen will be consumed, and carbon dioxide added into the water column in its place. If the active replacement of the water in the holding tanks is insufficient, dissolved oxygen will be depleted, and carbon dioxide concentrations will rise to stressful levels, diminishing the life-support characteristics of the holding tank. A minimum of three to four tank changes per hour is generally recommended (Baensch & Riehl, 1997), with most aquarium operators aiming for at least five changes per hour through each tank.

Some aquariums employ the use of macroalgae or additional filtration elements to further digest the nitrogenous waste, filtering nitrate out of the water also. However, this is not common practice within zebrafish facilities at this time, with most opting to perform regular water exchange to keep the nitrates from rising to dangerous levels. While the precise measure of water exchange will depend on the rate nitrates accumulate in the water column, most facilities apply an exchange rate of 10% over 24 h.

Following the biofilter, the water passes through chemical filtration, focused on removing colloidal solids and dissolved organic compounds (DOCs). While not essential for life support, this additional filtration step significantly enhances the clarity of the water, which is essential for achieving ultimate efficiency in water disinfection if an ultraviolet (UV) filter is employed.

There are many different technologies available to achieve these five key elements; thus, a significant factor driving the underlying decisions that shape the filtration assembly of a RAS aquarium is the application scale and system volume.

Small to Medium Scale RAS Aquaria

When a research program expands beyond one or two production tanks, independently maintaining the individual tanks becomes very labor intensive and impractical as a viable option for maintaining the colony. The hang-on semicirculating filtration solution is inadequate as this can only service the tank it is directly coupled to, and thus, a true RAS solution is necessary to provide optimal life support for the fish in a cost-effective manner. Most manufacturers of research aquaria enter into the market at this level, designing small RAS solutions affectionately referred to as "Bench-Top" or "Stand-Alone" systems depending on size.

Stand-alone systems typically take the form of a rack, resembling a library bookcase, filled with tanks on the top section, and with a compact filtration assembly condensed into the space immediately below the tank holding space (Fig. 26.9). The rack provides the structural support for the tanks and the necessary plumbing to deliver and remove water. The rack is typically constructed from marine grade stainless steel, and it is divided into several presentation rungs. The exact size and shape of the rack will vary between manufacturers, with the smallest "bench-top" solution comprising two rungs of 10 tanks, and the larger stand-alone racks comprising up to six rungs of 10–20 tanks. A typical space footprint for a stand-alone system would be 1620 mm × 565 mm × 2360 mm but will vary with manufacturer.

The dominant feature of a stand-alone aquarium is the water treatment unit (WTU) residing directly

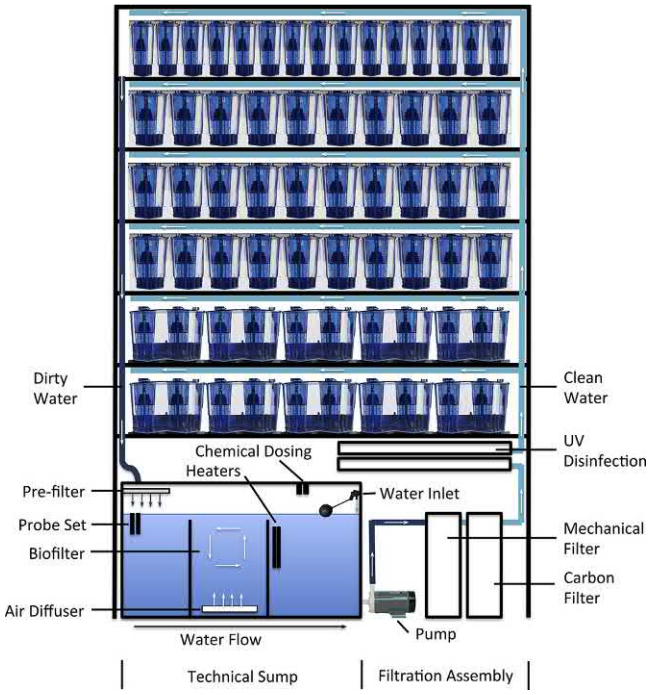

FIGURE 26.9 Stand-Alone RAS Aquarium. A single rack of tanks, equipped with an integrated water treatment unit, enables a space-efficient housing solution for a small colony of zebrafish. Effluent wastewater flows through a prefiltration device as it enters the technical sump, where the buffering water volume resides, and the culture condition is monitored and manipulated. The pump draws the water from the technical sump, driving it through the mechanical and chemical (carbon) filters, and through the disinfection chamber before cycling the water up the vertical riser into the header pipes and then to the fish tanks.

under the bottommost rung of the system. Wastewater enters the WTU by gravity via a downpipe, typically passing through a coarse mechanical filter, often referred to as the prefilter. The water then accumulates in a technical sump, where essential culture conditions such as temperature, pH, and conductivity are manipulated. A pump then drives the water through the filtration assembly, up a vertical riser, and to the tanks via header pipes and taps. The water then passes through the tanks, exiting via passive or active overflow into gutters that connect into the downpipe, thus closing the circle.

Large Scale RAS Aquaria

When the aquarium application demands a larger scale operation than can be achieved with a stand-alone rack or two, the most common solution is to combine several racks together with a central filtration assembly. These multilinked systems offer the advantage of high holding capacity with enhanced space efficiency at a relatively low operating cost. While the basic elements defining RAS aquaria remain in large-scale production facilities, there are additional elements to consider that impact the function of large multilink, central life-support (CLS) aquariums.

The greatest benefit that comes with a multilinked CLS aquarium is that hundreds to thousands of relatively small tanks can reside on a single secondary enclosure; thus, maintenance only needs to be performed on one life-support system making the labor contribution per tank significantly lower than other aquarium designs. There is, however, a significant trade-off that comes with this gain in efficiency, and it must be weighed against the risks to the research application before its suitability can be accepted. With one life-support system supporting so many tanks, biosecurity in the aquarium is a significantly enhanced challenge, as a disease outbreak can rapidly circulate to the other tanks and animals residing in the common recirculating water. Therefore, as more racks are committed to a multilinked CLS aquarium during the design phase of the facility, it is essential to ensure that adequate capital is allocated to the filtration assembly in order to ensure maximum biosecurity for the system. As a guide, the "Plus 10" approach is an excellent means of ensuring sufficient CLS capability. This approach suggests that if the final RAS aquarium will consist of 50 racks, and 18,000 L of water, then the CLS should be designed to support the biological load of a 55 rack, 20,000 L system, or 10% more than the maximum carrying capacity of the aquarium. This will ensure that even if the aquarium is pushed to capacity for an extended period of time, water quality and life-support condition will not be compromised.

As the scale of the aquarium is considered, it is critical to ensure sufficient water volume is available in the CLS to buffer against water chemistry changes in the system. For example, as the fish respire, and as the biofilter converts ammonia waste, the water in the aquarium is acidified as a result of an introduction of carbon dioxide and hydronium (H^+) into the water column respectively. If there is not sufficient hydro volume in the aquarium, this change will progress quickly, making it difficult to manage appropriate chemical additions into the water that would be necessary to compensate the culture condition change.

Currently, there are two common strategies for ensuring a sufficient volume of buffering water in a zebrafish RAS aquarium: a single large technical sump or a series of multilinked sumps that reside under the main holding racks. While both methods are effective, each has its limitations.

The under-rack sump option is an ideal solution when there is little flexibility with regards to useable space for the aquarium (Fig. 26.10). By storing the buffering water in the space below the tank holding space of a rack, it is possible to store the majority of the aquarium's volume within the tank room, requiring only a

FIGURE 26.10 Under-rack Sumps. A bank of four holding racks, fitted with under-rack sumps.

small additional space for the filtration assembly and technical sump. As a result, it is possible to house significantly larger systems in relatively small facilities.

The most significant drawback of the under-rack sump solution is the fact that the main buffering volume of the aquarium is stored as "dirty" water. As wastewater leaves the tanks and enters the rack's drainage system, the water flows into the downpipe and typically passes over a coarse prefilter pad before it accumulates in the under-rack sump. The water does eventually pass into the filtration assembly and is cleaned before being reused in the housing tanks, but the wastewater that accumulates in the under-rack sumps moves significantly slower than the rest of the aquarium's water flow, and as a result suspended solids (fines) settle out and begin to accumulate in the base of the sumps. While this accumulated mulm itself is not necessarily harmful to the aquarium system, it can become a food source for a plethora of micro-fauna, including potentially pathogenic bacteria. The accumulation and digestion of mulm also result in significant inefficiency within the biological system of the aquarium, as heterotrophic bacteria compete with the nitrifying bacteria for resources, such as alkalinity and dissolved oxygen within the water as they feed on the mulm. This side effect of leveraging an under-rack sump solution is readily managed by regularly servicing the sumps. If the aquarium is fitted with a disposable depth filter (sock), this service may be as simple as agitating the sumps to re-suspend the accumulated mulm so that the mechanical filter can remove the fines. This should obviously be performed prior to a scheduled filter service. If the aquarium is instead fitted with a surface filter, such as a drum filter, then some of the solid waste particles will be finer than the filter screen and will simply pass through the filter. In this instance, siphoning the mulm from the bottom of the sumps would be an appropriate means of managing the situation.

If there is more space available for the CLS when the aquarium is being designed, it is possible to avoid the complications of under-rack sumps by increasing the size of the technical sump to accommodate the buffering water volume. Allowing opportunity for increasing the depth of the sump this large central sump can either be sunk into the floor of the facility, or it can simply reside as a large above-ground holding tank (Fig. 26.11).

The main benefit that comes from a central sump design is that it allows the buffer water to be stored in a somewhat cleaner state. As wastewater leaves the rack, the downpipe directs it into another wastewater pipe that channels the water to the filtration assembly, then typically, directly into a first stage mechanical filter

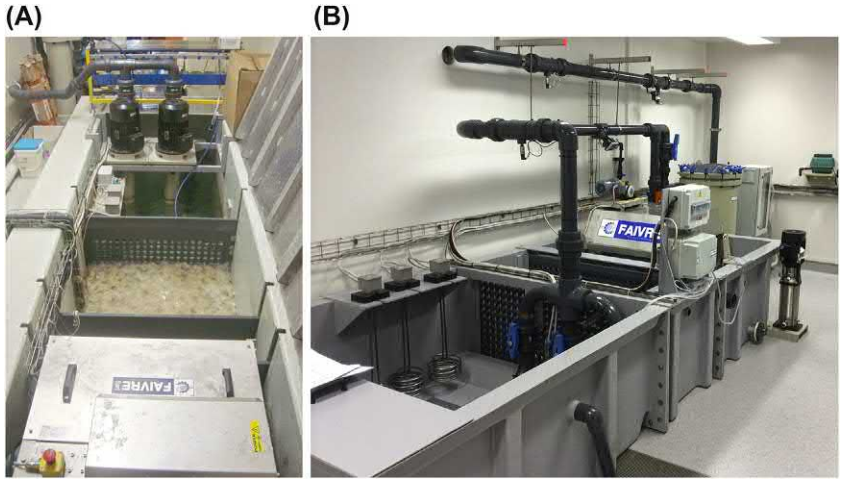

FIGURE 26.11 CLS Sumps. Large central life-support sumps can be sunk into the floor of the facility (A), increasing the maximum depth of the sump, effectively holding the system's buffering water volume vertically. When this option is not available, the CLS sump can reside as a large above-ground holding tank but will take up significantly more floor space as the water volume is held horizontally (B), unless the system racks are at a significantly higher elevation. *Photo B courtesy of Glen Turner.*

or dirty sump. Thus, the water can be processed by the filtration assembly prior to being "stored," limiting the volume of water capable of supporting unwanted microbiota. While this solution allows the first stages of the RAS process to complete (removing solid wastes via mechanical filtration, converting nitrogen wastes via biofiltration, and possibly even stripping the water of DOCs and other colloidal solids in the chemical filter), it is prudent to ensure that the disinfection step always occurs immediately prior to the water being returned to the holding tanks, as effective disinfection is transitory in a recirculating system.

Water Loop

With buffer volume addressed, the next major decisions will center on the recirculation of the water. A major challenge in designing a large-scale zebrafish aquarium is being able to manage the dynamic pressure changes that occur within the aquarium as depth filters foul and as more weep points emerge in response to the opening of taps as tanks are added to the system. These challenges are addressed in the water loop strategy of the aquarium.

A dual-loop system, similar to that operating at ZIRC, the Zebrafish International Resource Center (Varga, 2011), requires the use of multiple pumps to circulate the water through the aquarium and is typically employed when the filtration assembly is not able to rely on gravity pressure to drive the mechanical filtration step. In this scenario, the aquarium will typically drain into a wastewater collection sump from which the filtration pump will draw water. The dirty water is driven through the filtration assembly and returns into a clean sump. The clean sump is typically fitted with an overflow that returns water to the dirty sump if unused. This is the first loop. The second loop is driven by the circulation pump, which drives water from the clean sump via a disinfection chamber, through the header pipes to the tanks. The water then drains from the holding tanks into the dirty sump. The circulation loop is also typically fitted with a rack bypass, allowing water to be diverted directly into the dirty sump when the holding racks are sparsely populated. An example of a dual-loop system is illustrated in Fig. 26.12.

The benefit of a dual-loop system is that it allows greater flexibility in filtration assembly design, while also ensuring that tank circulation is not impacted by changes in the hydrodynamics of a fouling filter. Dual-loop systems also have a significant disadvantage, however, which is the need to balance the pumps. If the circulation pump has a greater draw from the clean sump than the filtration pump is delivering, water level

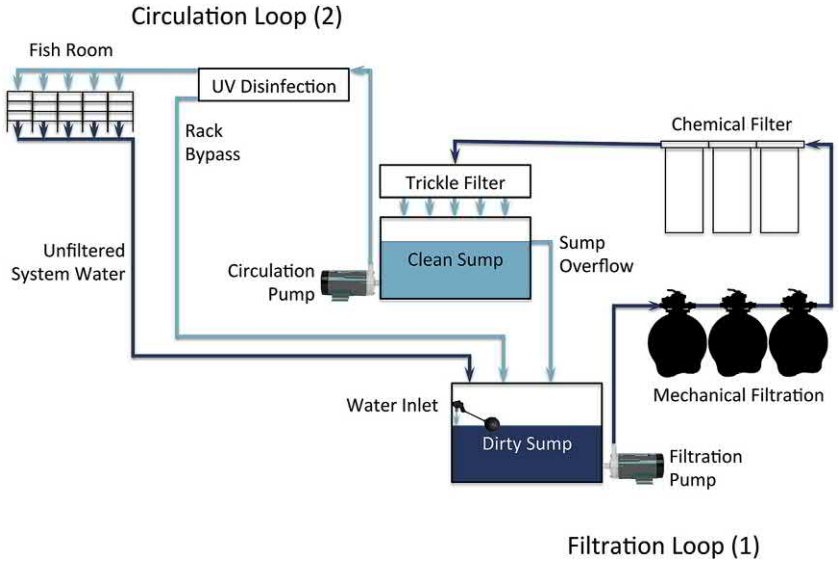

FIGURE 26.12 Dual-loop RAS aquarium. The Filtration Loop (1) processes the effluent (used) water, making it suitable for reuse. Used water drains from the holding tanks and is collected in the Dirty Sump. The used water is pumped through the mechanical filtration assembly, eliminating the large particulate wastes. The water is then cycled through an activated carbon chemical filter, removing the fines and colloidal solids, including dissolved organic compounds, the carbonaceous wastes that yellow the water and act as a food source for heterotrophic bacteria. Finally, the water passes over a trickle filter before entering the Clean Sump. The trickle filter serves as both a biofilter and gas exchange column. Denitrifying bacteria populate the oxygen-rich filter, converting ammonia waste into less toxic nitrite and nitrate. Water from the Clean Sump can reenter loop one by overflowing into the Dirty Sump, or it is pumped into loop 2 as required. The Circulation Loop (2) pumps water from the Clean Sump through the UV disinfection chamber to the header pipes servicing the tanks in the fish room. In the fish room, water passes through the holding tanks, emerging as used water entering a central wastewater drain and returns to the Dirty Sump (Loop 1). Loop two also contains a rack bypass, allowing water to be cycled directly from the Clean Sump to the Dirty Sump, reducing rack pressure of a lightly populated fish room, and simplifying the pump balancing process.

will begin to drop, and could lead to the circulation pump pulling air into the water as a result of a vortex forming near the pump pickup, which in turn, could result in a Gas Bubble Disease (GBD) event.

For any given temperature, salinity, and barometric pressure, there is a maximum stable level of dissolved gases within the water. When this is exceeded, the total gas pressure (TGP) is in an unstable, super-saturated state. In a recirculating aquarium system, the TGP will remain supersaturated while in the header pipes, because the water is under pressure; however, the dissolved gases begin to release from the water as soon as it is freed from the pressurized condition. In a RAS aquarium, this occurs in the holding tanks, exposing the fish to a potentially lethal situation. In extreme cases, it is possible to observe tiny bubbles effervescing out of solution; however, GBD is usually far subtler, manifesting as microscopic bubbles in the soft tissues of the fish, leading to death from asphyxiation following ischemic necrosis of gill lamellae, as the air bubbles disrupt the blood flow (Pauley & Nakatani, 1967; Weitkamp & Katz, 1980).

There are several options available to control the risks associated with GBD and pump imbalance. A TGP sentinel probe offers a safeguard against GBD by allowing the aquarium system to have an automatic cutoff trigger. In a dual-loop aquarium, the TGP sentinel probe is coupled to the circulation pump and monitors the TGP in the header pipes. If the TGP rises above 100%, a relay is triggered that cuts power to the circulation pump. The filtration pump can continue to circulate without risk to the fish, depending on system design. Although the fish are now at risk from complications due to the lack of circulation, the lethal effects are significantly slower than GBD, giving the operator an opportunity to address the fault. While effective, TGP sentinel probes require regular maintenance and calibration to ensure they are functioning correctly.

To effectively protect the aquarium from GBD, it is important to measure TGP and not rely solely on shifts in dissolved oxygen as a guide for GBD risk, as nitrogen is a significantly greater percentage of the atmosphere. It is thus, possible to experience a GBD event when dissolved oxygen is below saturation.

While there is no better substitute available for safeguarding against GBD, in a dual-loop aquarium it is prudent to also install level sensors in the sumps that allow an operator to shut down pumps to ensure there is no risk of drawing air into the system if the pumps get out of balance. In some cases, an aquarium life-support computer can also manage balancing, if the system is fitted with Variable Frequency Drive (VFD) pumps.

A VFD is used for adjusting the flow of a pump to the system's actual demand by controlling the frequency of electrical energy running the pump. It is most typically applied on aquariums that employ larger three-phase pumps. However, recent advancements in this technology have allowed the development of VFDs also to control smaller single-phase pumps. Aside from allowing enhanced control over a pump's performance, VFDs also allow the pump to constantly run at peak efficiency, by matching the pump curve to the system curve.

In a single loop, or dead-leg system, the challenge of pump balancing does not exist, but there are other challenges coupled with relying on a single drive point to maintain both filtration and circulation. As with the dual-loop aquarium, a dead-leg system will experience significant shifts in the hydrodynamic resistance as filters foul, and tanks require more available water flow. As a result, VFD pumps are a staple element of a single loop aquarium.

In order to achieve sufficient flow through every tank at maximum holding capacity, the pump needs to be capable of delivering sufficient flow velocity to overcome the resistance that mounts as more taps are opened and as filtration media fouls. In addition to these obvious sources of resistance, it is important to remember that zebrafish aquarium system curves have a significantly greater resistance than other aquaculture facilities of comparable size, because of the number of header pipes carrying water to the tanks, and the narrow diameter of the final outlet from which the water must pass into the tank. Without the sophistication of VFD pumps, a large dead-leg zebrafish aquarium would need to either run the system at capacity all the time, creating a significant burden from the need to clean unpopulated tanks in order to maintain system hygiene, or it would need a large bypass valve that could be gradually throttled as the system population grew.

Flow-Through Systems

While the vast majority of commercially available zebrafish housing solutions employ recirculating aquaculture technology, there are applications when recycling the culture water is not desirable. The application of flow-through technology is the solution to this need. In recirculating aquaculture systems, the culture water used to house and grow the aquatic organisms is captured, treated, and reused, thereby, greatly reducing the total water volume consumed in the rearing process. In flow-through or single-pass systems, new clean water is delivered to the tanks, and the wastewater flowing out of the tanks is rejected from the aquarium, typically to the municipal sewerage system. For this reason, flow-through aquarium systems are normally used only for applications that require the complete isolation of one tank from another.

Toxicology and environmental manipulation experiments are the archetypal applications that implement the single-pass system. With flow-through technology, each tank or series of tanks can be exposed to different chemical agents without contamination of others. As the water exits the tank, it is routed to the sewer, and thus, poses no cross-contamination risk to other tanks on the system. Flow-through technology is also appropriate to apply to high-risk quarantine scenarios in which imported fish may be suspected of carrying a disease that can have high pathogenicity and be difficult to contain. Such a disease is likely to have a significant detrimental impact on other fish residing under the heightened biosecurity control of a quarantine aquarium, and use of a flow-through system can greatly reduce the dangers of cross-contamination that would be present in a RAS.

Aside from the increased operational cost associated with replacing 100% of the system's water volume every pass, there is also a significant challenge associated with controlling the culture conditions of the incoming new water. In a RAS aquarium, it is logical to choose demineralized water as the source water for the aquarium, giving the operator complete control over the fish ready water that circulates through the aquarium. The operator simply changes the chemical mix added into the makeup water to alter the culture condition. In a flow-through system, manipulating the water chemistry to this extreme would be prohibitively expensive. As a result, most flow-through aquaria need to accept that the source water will largely determine the culture condition. For most operators, this means being limited to using the municipal water supply, and applying basic treatment and disinfection to the incoming water on its way to the header pipes. As zebrafish are tolerant of a wide range of culture conditions, having the basic water quality dictated by the municipal authorities is not a major issue provided the operator is aware of the potential for fluctuation in the water chemistry as the seasons change. In some cases, a facility may be able to utilize a natural water source, such as a local stream or bore; however, the basic water chemistry of the source water will still be the defining culture condition of the system. As a minimum, the incoming water should pass through an activated carbon filter, to strip heavy metals and chloramines from the water, and then pass through a UV disinfection chamber to knock down potential pathogens residing in the water source.

Advanced Applications

Beyond the major decisions surrounding the core housing solutions for a zebrafish facility, consideration also needs to be applied as to whether there is need for additional special housing elements.

Genotyping Racks

One of the most common activities that occurs in a large-scale zebrafish production facility is the need to isolate individual fish while they are genotyped. Traditionally this meant that fish would need to be maintained in small static tanks for several days while their genotype results were obtained. During this time the fish cannot be fed, as uneaten food, and wastes released from the fish after eating, rapidly degrades the water quality, even to levels that could result in death.

Recognizing this technology limitation, some commercial manufacturers now offer specialized genotyping racks in the approximate footprint of a typical housing rack and designed to hold up to 288 fish in isolation while maintaining water circulation (Fig. 26.13). This means the fish can continue to be fed while in isolation, addressing the first of the five freedoms. This is a significant enhancement in the animal welfare associated with the challenge of genotyping.

Automated Heat Shock Systems

Heat shock is a powerful technique for inducible gene expression systems, and the zebrafish is well suited to exploit the system, with the existence of over 1300 transgenic fish lines carrying a construct under the control of the heat shock promoter 70 (*hsp70*; Zfin transgenic database; http://zfin.org/). Heat shock treatment of developing zebrafish embryos is relatively simple to perform because the fish are largely stationary, but more importantly, they do not require water circulation.

Heat shock treatment of adult fish is more involved as there is a need to maintain circulation as their culture medium is raised to the necessary shock temperature.

FIGURE 26.13 Genotyping Rack with under-rack sump. *Photo courtesy of Tecniplast Aquatic Solutions.*

While this challenge can be addressed by simply transferring the fish from housing tanks to treatment tanks, this approach requires significant handling, which is labor-intensive and increases stress levels in the fish. In larger recirculating systems, when only a few tanks of adult zebrafish require regular heat shocking, it is possible to place in-tank heaters with activation timers into each of the required tanks to avoid the need for excessive handling (Duszynski, Topczewski, & LeClair, 2011). This technique, however, also requires regular calibration of equipment, and strict monitoring of treatment conditions to ensure that tanks are exposed to statistically equivalent conditions. While this works well in treating a handful of tanks for short-term experiments, if a more pronounced commitment to heat shock techniques is desired, the major limitation of the RAS system is that it is not amenable to scalability.

While, currently, there are no off-the-shelf, scalable solutions to heat shocking adult zebrafish, it is possible to modify existing aquarium housing designs to enable heat shock treatment in conditions of continuous water flow, without impacting the standard culture condition of the main circulation loop. By adding an additional manifold to an aquarium holding rack, and by modifying the wastewater return path to circulate water from a treatment water bath, it is possible to deliver heat shock conditions to an entire row of tanks with precision and minimal variability between treatment tanks (Saera-Vila, Kish, & Kahana, 2015). This system can be easily scaled to suit demand and offers greater assurance of maintaining optimal culture condition throughout the RAS aquarium. In addition, this design modification, illustrated in Fig. 26.14, can be further enhanced through the use of solenoid valves to control manifold change over, offering a fully automated heat shock solution that avoids unnecessary handling stress, and ensures best water quality and fish welfare at all times.

Introduction to Defined Flora

Zebrafish are also breaking new ground in the realm of gnotobiology. The term "gnotobiology" (*gnos*, known; *bios*, life) is used to describe the study of animals raised

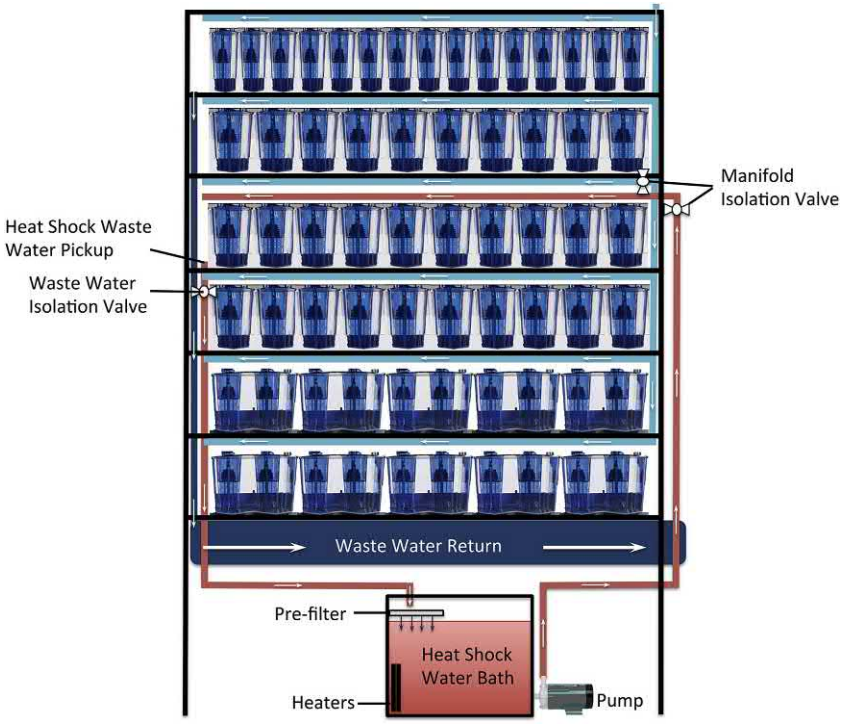

FIGURE 26.14 Heat Shock modification of a multilink zebrafish housing rack. The recirculating heat shock (35°C) system (shown in *red*) resides under the housing rack. A small pump circulates hot water to an additional water distribution manifold, and a secondary wastewater drain returns the effluent water to the heat shock water bath, closing the loop. The manifolds and drain of the regular (28°C) system are shown in *blue*. For exposing the fish to the treatment, the manifold isolation valves are switched (closing the regular manifold valve, and opening the heat shock manifold valve) and the heat shock circulation pump is turned on. The main system drain of the gutter of the heat shock tanks' shelf is closed, diverting the heated effluent water into the extra heat shock drain, returning the water to the heat shock water bath, avoiding heat contamination to the rest of the system. On conclusion of the treatment, the heat shock circulation pump is turned off, the main system drain is opened, and the manifold isolation valves are returned to their normal state, allowing normal system water to mix with the heated water in the tanks, gradually returning holding conditions to normal.

in the absence of microorganisms or in the presence of known microbial strains or communities (Reyniers et al., 1949). Just as a geneticist performs genetic manipulations to study loss and gain of function, gnotobiologists test the necessity of microbiota by rearing animals in the absence of microorganisms and then adding one or more defined microbial strains to test sufficiency (Melancon et al., 2017). The first concept of studying germ-free and defined flora animals began back in 1885 when Louis Pasteur hypothesized the impossibility of animal life surviving in the absence of microorganisms. Since then, mammalian models have dominated gnotobiology, with various fish models playing only a minor role. The plethora of attributes that make the zebrafish model system popular with geneticists, including rapid external development, optical transparency, large brood size and ease of housing, are also the key attributes propelling zebrafish as a gnotobiotic model. In fact, the use of zebrafish is enabling vertebrate gnotobiotic experimentation on a scale not possible with the traditional mammalian models (Melancon et al., 2017).

The vast majority of gnotobiotic studies employing zebrafish to date have focused on the use of larvae, in part due to the challenges of long-term germ-free zebrafish husbandry methods. For successfully administering long-term gnotobiotic zebrafish husbandry requires consideration of animal housing, water quality, and exchange, waste removal, nutritional value of food, live food culturing, work flows and space requirements, record keeping, supplies, expenses, and labor. Procedures for long-term gnotobiotic husbandry are evolving. Melancon et al. (2017) have reported success in raising germ-free zebrafish for up to 1 month; however, the process was extremely labor-intensive. For more information on techniques, please also refer to the chapter in this book on gnotobiotics.

Presently, two general housing systems can be employed to raise gnotobiotic zebrafish. Germ-free zebrafish can be maintained outside of a gnotobiotic isolator by rearing the fish in sterile tissue culture flasks or multiwell plates within a biosafety cabinet, using standard sterile techniques (Melancon et al., 2017). Each flask or well can represent a different microbial condition, allowing multiple microbiotic conditions to be studied in a single experiment (Pham, Kanther, Semova, & Rawls, 2008). This brings gnotobiotic experimentation within the grasp of most investigators working with the zebrafish model. However, scalability of growing gnotobiotic zebrafish in flasks has significant limitations. The daily requirement to service these experimental housing tanks increases husbandry labor, which, in turn, increases the opportunity for contamination; this can become financially limiting as throughput and longevity increase. Culture flasks and plates are, therefore, ideal for experiments that require analysis of multiple different microbial conditions with relatively few subject animals per condition and/or can be completed within a short time course with minimal manipulations to reduce the risk of contamination (Pham et al., 2008).

The second and more rigorous method of rearing germ-free zebrafish is to rear them within a purpose-built gnotobiotic isolator as described by Pham et al.

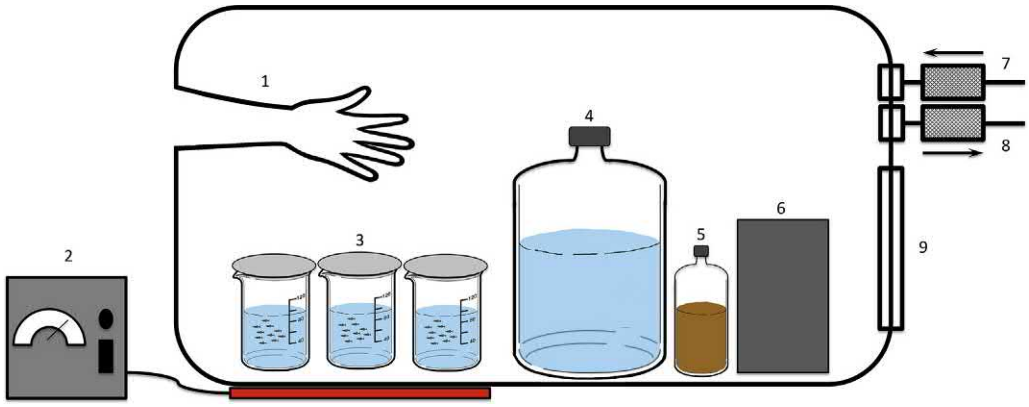

FIGURE 26.15 Schematic diagram of isolator equipped for gnotobiotic zebrafish husbandry. The gnotobiotic isolator allows zebrafish to be reared for extended periods in a defined flora environment. The gnotobiotic isolator includes attached gloves, allowing sterile interaction with the inhabitants (1). A heating pad or heated water pump located directly below the specimen holding tanks allows control of isolator operating temperature (2). Foil-covered beakers containing zebrafish in gnotobiotic zebrafish medium (GZM) act as static specimen holding tanks (3). Carboy of sterile GZM allows routine water changes in holding tanks (4), and a second carboy collects the wastewater. Bottles of sterile zebrafish food (5), and other supplies (6) are also stored within the isolator. For maintaining a sterile environment, the isolator is kept under positive pressure, with HEPA-filtered air supply (7). Air exhaust is likewise typically HEPA-filtered (8). The gnotobiotic isolator also has a sealed port to enable the transfer of materials to and from the sterile enclosure (9). *Figure adapted from Pham, L. N., Kanther, M., Semova, I., Rawls, J. F. (2008). Methods for generating and colonizing gnotobiotic zebrafish.* Nature Protocols, *3(12), 1862.*

(2008). In this work, fish were kept in a flexible film isolator maintained under positive pressure and supplied with high-efficiency particulate air (HEPA) filtered air (Fig. 26.15). This device allows sterilized food, water, and supplies to be introduced into the isolator via a sealed port, and manipulations are performed using attached gloves. Providing the gnotobiotic isolator is of sufficient size, large quantities of sterile food, water and supplies can be maintained within the gnotobiotic environment, reducing the need to open the isolator port to the external environment, and thereby, reducing the risk of isolator contamination. A single isolator can house a relatively large number of zebrafish, but the limitation of this device is that all animals within the isolator are subject to the same microbial condition. In a typical experiment utilizing a gnotobiotic isolator, it is possible to house 360 germ-free zebrafish embryos for a single experiment by distributing them into 12 400 mL, foil-covered glass beakers, each containing 30 fish in 100 mL of gnotobiotic zebrafish medium (water) (Pham et al., 2008).

Acquisition of gnotobiotic isolators requires a significant initial financial investment, and their routine maintenance is relatively laborious; thus, gnotobiotic isolators are best suited to experiments that require gnotobiotic animals be reared for extended periods of time or for experiments that require large numbers of animals to be exposed to the same microbial condition (Pham et al., 2008).

As zebrafish gnotobiology expands, it is likely that specialized aquarium systems will be developed to allow the maintenance of gnotobiotic zebrafish throughout their life cycle. Melancon et al. (2017) postulate that a combination of bioreactor-like stand-alone aquarium systems, similar to vessels that have already been developed for other fish (Forberg, Arukwe, & Vadstein, 2011), could be modified as follows for this purpose. A system combining equipment that would allow automated media changes, control of feeding and colonization, and prevention of accumulation of toxic byproducts together with interconnected minibioreactor technology previously developed for culturing microorganisms (Auchtung, Robinson, & Britton, 2015), might allow large-scale screening of gnotobiotic zebrafish. This could allow zebrafish to become the only gnotobiotic vertebrate model for which large-scale, long-term husbandry is possible.

Summary

It is an exciting time to be involved in the husbandry of zebrafish. A steadfast and deliberate commitment for the strategic development of housing solutions is needed to support its continued development as a preeminent model. The extent of research program support will determine the scale and sophistication of the available solutions to be developed. While a simple box tank on the bench may be sufficient to meet the needs of an independent investigator seeking to address straightforward questions, a complex central life-support RAS aquarium will allow hundreds to thousands of genetically unique lines to be maintained in a cost-effective manner, supporting a number of investigators focused on answering more complex problems. The experimental application of the model will dictate the materials required to construct optimal primary and secondary enclosures and the types of manufacturer options needed for appropriate housing flexibility. Specific tank design will depend on the goals of the intended research programs utilizing the aquarium. Housing and breeding for maintenance may be all that is required for some research programs, but others may require the production of vast quantities of synchronized embryos on demand, an ability to monitor physiological response to oxygen limitation, or the effects of defined gut flora.

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CHAPTER

27

Cleaning and Disinfection of Life Systems

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Abstract

A. Understanding the differences between Cleaning, Sanitizing, Disinfection, and Sterilization

a. Cleaning

Cleaning refers to the removal of both organic and inorganic debris or soils by means of physical impingement, such as scrubbing, scraping, wiping, or brushing. Some common examples of soils in aquatic animal housing include fecal casts, uneaten food, and biological fouling agents, such as bacterial biofilm, algae, diatoms, and bryozoans. The object of cleaning is simply to remove this debris. Scraping tools and abrasive pads are commonly used to remove debris, but the powerful spray of water from commercial cage wash machines, especially when combined with suitable chemical solvents, may be capable of removing some or all of these soils.

b. Sanitization

Sanitization refers to the process of reducing the levels of pathogens on an object to safe levels and consequently reducing the likelihood of perpetuating a contaminant.

c. Disinfection

Disinfection refers to the process of destroying or preventing the growth of pathogenic organisms. Disinfection has the specific aim of a four or five log reduction in the number of microorganisms initially present on a surface. Disinfection of aquatic animal housing and equipment should follow the cleaning process and may be achieved using specialized processes including moderate to high heat, high-pressure sprays, and chemical or ultraviolet light exposure.

d. Sterilization

Sterilization refers to the process through which, statistically, all microorganisms and their spore cells and pathogenic or toxic products are destroyed, sometimes referred to as a log six kill, where we expect a 99.9999% reduction of microorganisms initially present on a surface. Sterilization may be achieved through autoclaving, gas plasma, irradiation, or chemical exposure to compounds, such as sodium hydroxide, chlorine dioxide, hydrogen peroxide, peracetic acid, ozone, or glutaraldehyde.

B. Sources and Kinds of Pathogens Relevant to Aquatics Facilities

a. Pathogens that may have an untoward effect on the overall health status of an aquatics facility may be introduced from either an aquatic animal or human vector, or from fomites such as a dip net, aquaria components, or apparel worn by husbandry technicians and researchers.

b. Some feeds may also be vectors of pathogens. Examples include tubifex worms, Artemia cysts, rotifers, and virtually any of a wide array of wild-caught products commonly used to feed fish cultures. Adequate and regular testing of such feed items is warranted to ensure that the risks of importing a pathogen are minimized.

C. Hygiene as a Preventative Medicine Program and Biosecurity Measure

a. A sound Quarantine procedure must be in place and strictly adhered to in order to minimize the risks of introducing pathogens from other facilities. This involves taking procedural steps that minimize the risks that fish imported from other sources will infect your colony with a pathogen, parasite, or biofouling agents such as bryozoans, mollusks, or algae.

b. Environmental hygiene practices must be designed to effectively target the specific pathogens common to aquatics facilities. Not all pathogens require the same agents or level of

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exposure to disinfecting agents; thus, special care must be taken when developing cleaning practices to ensure that the measures taken are effective.

c. While it is uncommon to find a dirty-side/clean-side approach in aquatics facilities, and perhaps even more rare to find barrier facilities, where aquatics species are concerned, implementing proper workflow can have a positive impact toward minimizing cross-contamination from within the facility.

d. The use of disposable surgical gloves is common in the laboratory animal field, and aquatics facilities are no exception. Pathogens, including zoonotic species, such as *Mycobacterium marinum*, may be spread through contact with one's hands, and often disposable gloves can mitigate these risks. However, gloves that contain powder should be avoided as they may contribute to health risks to your fish. Moreover, the use of disposable gloves should always be accompanied by a rigorous hand-washing policy, which is likely to be more effective at preventing outbreaks in the aquatic facility.

e. Chemical footbaths are often found in aquatics facilities. If used, they must be appropriately designed, positioned, and maintained to target the specific pathogens of concern.

Recirculating Aquaculture Systems (RAS) Components Overview

a. Fixed Components

i. Fish Holding and Spawning Racks are those units, where the fish housing units are placed in order to receive flowing water from the RAS. These are often made from stainless steel and are designed to withstand the substantial weight of water held in the numerous tanks.

Once fish holding system racks are set into place, we may anticipate that they will not be moved. In fact, it is not uncommon to find that the racks have been affixed to the floors, walls and/or, ceilings due to building codes relating to seismic zone and occupational health and safety guidelines. Special care will need to be taken to ensure that cleaning around this equipment is both effective and safe for the fish and people. Much of this equipment is either washed or wiped down by hand, and chemicals that are volatile or toxic to fish should be avoided.

ii. Supply and Return Plumbing

The piping that carries water to, and away from, the fish holding tanks can be differentiated as supply and return plumbing. Cleaning and sanitizing of this plumbing should only be undertaken when no fish are living on the system. The choice of chemical and method(s) of application chosen will depend on the goals and aims of the task and the identification of any known pathogens and biofouling agents. Developing an action plan should include consulting a qualified fish pathologist.

iii. Life Support Systems

1. The pumps used to distribute fish system water may vary in design and location depending on the design of the RAS, but virtually all can be removed for maintenance, including sanitizing the components that come in contact with the RAS water. The design of some of these components can be quite convoluted and may be of specific concern when attempting to eliminate all possible biofilms. It is recommended that you confirm the compatibility of the components with any cleaning and disinfecting agents you may choose to employ.
2. All RAS employ filters to perform various tasks.
   (a) Mechanical filters remove suspended and settleable solids and may be disposable, re-useable, or permanent components within the RAS design. Regardless of the nature of the mechanical filter, all of them must be adequately sanitized to prevent reinfecting your RAS.
   (b) Chemical filtration in RAS is often performed using activated carbon/charcoal media. Activated carbon is most commonly used to remove undesirable odors and colors or stains (e.g., tannins) from the RAS water. This media should be removed during any sanitization procedures as it may reduce the efficacy of some chemical agents.
   (c) Ultraviolet (UV) filters are often used to minimize the growth of algae, and pathogens, by direct exposure to UV-C radiation. It is critical that these UV reactors and the quartz sleeves that contain the UV-C bulbs are properly sanitized, and that the UV-C is powered off to prevent interfering with any chemical treatment used for sanitizing your RAS. These reactors and bulbs should be maintained on a strict schedule to ensure their efficacy in your regular RAS operations.
   (d) Biofiltration is a component of all RAS. This is the process whereby bacteria perform the process of nitrification and in

some cases, denitrification. Most attempts at sanitization of a RAS will kill all of the beneficial bacteria living in the biofilter. Biofilters, by their very design, must have tremendous amounts of surface area in which the bacteria colonize. Please ensure that any sanitization procedure adequately addresses this important concern. And be prepared to properly cycle, or inoculate, the biofilter when you decide to return the RAS to service in the future.

3. Heaters/chillers
   (a) Heaters are often submerged in sumps and will require special attention when employing any sanitization efforts.
   (b) Chillers are often stand-alone items through which the RAS water is passed into a circuitous route to cool the water to the desired temperature before it exits the equipment. Consequently, chillers may pose a difficulty when attempting to sanitize any RAS.
4. Aeration
   (a) RAS often require supplemental aeration, and zebrafish RAS often employ air-stone diffusers for this purpose. These diffusers are very porous, and may be disposable, or may have their own instructions for proper cleaning and disinfection. It is best to consult the manufacturer.

b. Modular Components

i. Fish Holding Tanks and Parts

Fish holding tanks may be constructed of a variety of plastics and glass. While most are truly modular and are routinely moved around for purposes of access to fish and clean tank exchange, some are essentially stationary and will require manual cleaning and sanitization. Modular zebrafish aquaria and their associated components, such as lids, siphons, and baffles, are typically constructed from polycarbonate plastic, silicone rubber, and stainless steel. These items lend themselves to being cleaned and sanitized in industrial cage wash machines that differ very little from those used in a rodent or rabbit facility. Some are constructed from materials that may be autoclaved, but it is advisable to follow the manufacturer's guidelines concerning the temperature and duration of autoclave cycles.

ii. Spawning Chambers and Traps

1. Spawning chambers or cages are typically modular items that can be used as stand-alone units or placed into the top of a fish housing tank. These items are typically constructed from the same materials as the fish housing tanks and may be cleaned and sanitized in the same way.
2. Spawning traps are items that are typically placed into a larger fish housing tank with the intent to collect fertilized eggs as the fish spawn over them. These traps may include artificial plants, mesh-screen panels, or a substrate such as marbles. Due to the substantial surface area of these items, special attention will be required to ensure adequate cleaning and sanitization.

iii. Culturing Equipment for Live Feeds

Equipment used for the production and feed out of living feeds, such as paramecia, Artemia, rotifers, and other planktons represent potential sinks for unwanted microorganisms and pathogens. A well-written and effective SOP for the disinfection and cleaning of this equipment should be followed to ensure that your live feeds do not become the vector for disease in your facility.

iv. Mobile Carts and Racks

Mobile carts and racks are commonly used as work surfaces and transport vehicles within and without the zebrafish facility. Regular cleaning and sanitizing of this equipment are necessary to prevent the transmission of any possible pathogens and microorganisms from one use to the next. Facility policies should be established to prevent these carts and racks from traveling between facilities and laboratories.

c. Room Floors, Walls, Ceilings

The floors, walls, and ceilings of your zebrafish holding facility should be designed and built to a wash-down specification. This means that you can apply surface disinfectants and rinse them off as needed without destroying the construction materials. Although you may have a wash-down spec facility, it is still best to test an area with the sanitizing agents you are interested in using before committing to a specific action plan. Further, it is always prudent to consider the volatile nature of some cleaning agents and to err on the side of caution when using them in areas where RAS water may be exposed since this may pose a threat to the health of your fish colony.

Types of Soils and Dirt Found in and on RAS Equipment

a. A variety of algae and diatoms may be found in a healthy zebrafish RAS, but efforts should be made to minimize its presence since it may have negative outcomes on animal welfare and system operations.

While some algae are single cells that remain in the water column, others form colonies that may interfere with the ability to perform proper daily health checks of the fish. These algal colonies are often tenacious and will require mechanical removal or physical impingement with high-pressure sprays. Some algae are toxic or produce toxic substances, so it is a best practice to prevent or minimize the occurrence of algae in a zebrafish RAS. Figs. 27.1–27.3 show tanks with accumulations of algae/diatoms.

b. Bryozoans are invertebrate animals known to colonize and encrust surfaces in zebrafish (and other) RAS. In addition to creating a barrier to performing proper health checks of the fish in the tanks, bryozoans also act as biofouling agents that may impede the proper flow of water in and out of the tanks and throughout the entirety of the RAS. Importantly, bryozoans are also a secondary host for one or more myxozoans known to cause Proliferative Kidney Disease (PKD) in aquaculture fish species. Finally, bryozoans are notoriously difficult to remove from tank surfaces and will require prolonged exposure to harsh chemicals such as bleach, and high temperatures to control or eliminate from the RAS. Figs. 27.4 and 27.5 show bryozoans in fish tanks.

FIGURE 27.1 Examples of algae and diatoms on tank surfaces. Close up view from each tank is on the right in the inset view. *Photos courtesy of IWT/Tecniplast.*

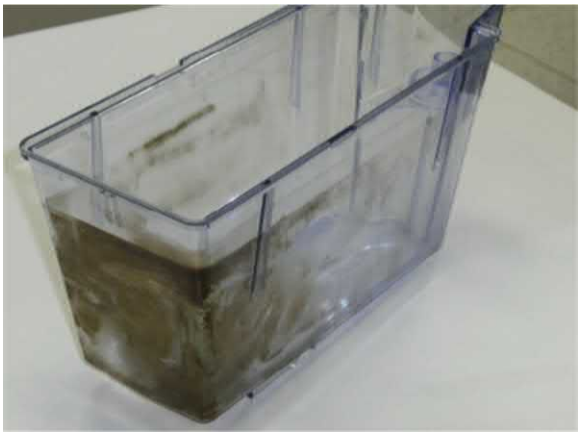

FIGURE 27.2 Example of algae on tank surfaces. *Photo courtesy of IWT/Tecniplast.*

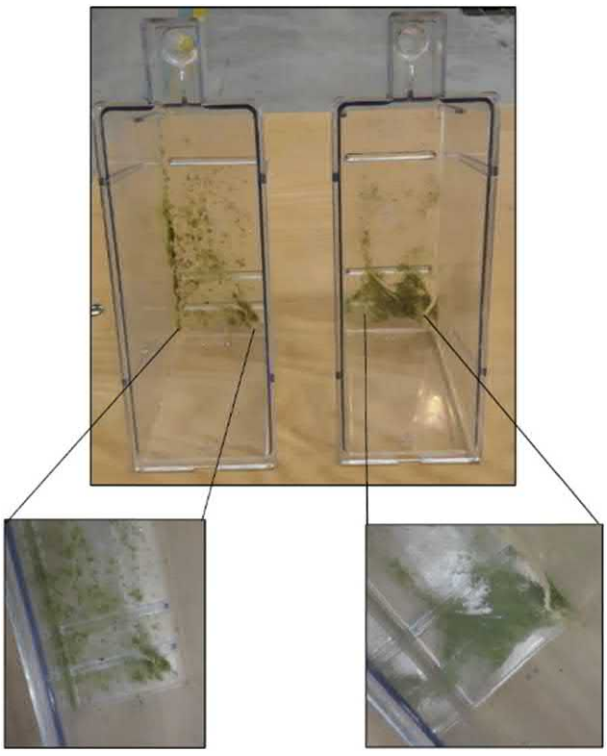

FIGURE 27.3 Examples of algae on tank surfaces. Close up view from each tank is underneath in inset view. *Photos courtesy of IWT/Tecniplast.*

c. Biofilms form when bacteria adhere to wet surfaces and excrete molecular strands and consequently form three-dimensional communities. Biofilms accumulate over time and are an integral part of all RAS. In zebrafish RAS, the biofilms that pose the most direct obstacle to proper daily operations occur at the tank-level. Those biofilms impede the proper flow of water in and out of the tanks and create a visual barrier to those who must perform the daily observations of fish in their home tank. A number of factors will influence

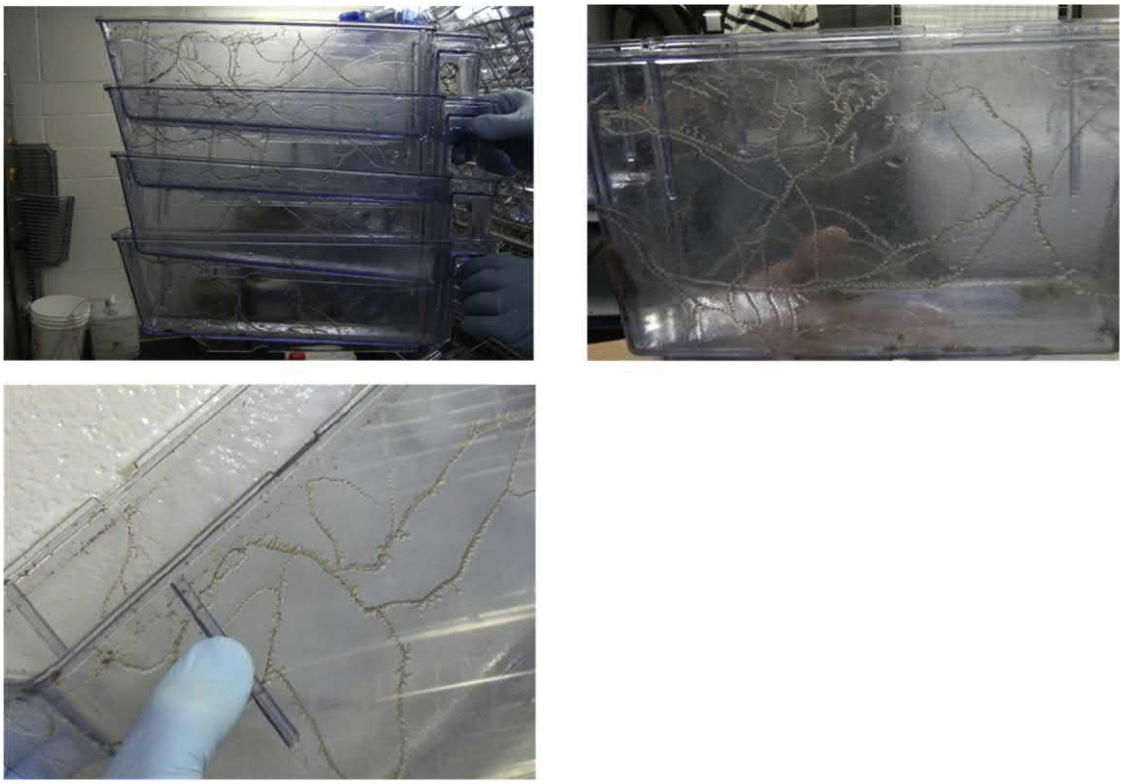

FIGURE 27.4 Images of bryozoans on tanks. *Photographs courtesy of IWT Tecniplast.*

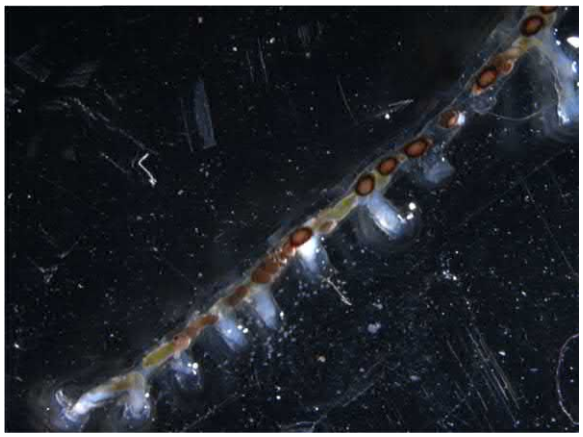

FIGURE 27.5 Image of bryozoa (Plumatella rugose) on a tank surface with visible statoblasts. *Photograph courtesy of Erik Sanders.*

FIGURE 27.6 Image of zebrafish tank with heavy biofilm coating as evidenced by the hazy appearance. *Photograph courtesy of Erik Sanders.*

the growth of biofilms in your fish tanks, including light intensity, nutrient load, and many consecutive days of operation. Fig. 27.6 shows biofilm accumulated on a tank.

d. Fish feces or fecal casts or pellets are a natural consequence of providing your fish with their daily dietary needs. The size, consistency, and amount of this waste is a direct corollary of the amount, type, and frequency of the feeds you provide to your fish. In most cases, the properly functioning tank should be able to remove this waste as it is produced. However, when a tank's self-cleaning function is impaired, this waste may accumulate rapidly, and a mitigating response from the husbandry provider is required.

e. Detritus commonly found in zebrafish RAS will commonly consist of fish waste, uneaten food, and other decomposing organic matter. This detritus must not be allowed to accumulate in excess as it offers a

FIGURE 27.7 Image of soiled zebrafish tank with accumulated biofilm (hazy appearance), bryozoa, and food water, and fecal detritus. *Photograph courtesy of Erik Sanders.*

lattice for pathogens and intermediate hosts of pathogens. A properly functioning tank should have no trouble removing this waste, but if not, it may need to be removed manually, and a tank exchange is warranted. Fig. 27.7 illustrates accumulated biofilm, bryozoans, and food/fecal detritus in a tank.

f. Suspended solids, in zebrafish RAS, should be well below the size permitted to pass through the mechanical filter in service. If abundant, they may be an indication that other problems need addressing, such as replacing or repairing the mechanical filter. These solids alone should not pose a significant contribution to the needs for cage wash or tank exchange.

Settleable solids are those solids that will settle to the bottom of the tank when the velocity of the water is inadequate to keep them in suspension. In zebrafish RAS, these should consist predominately of uneaten food and fish feces. These are often captured in mechanical filters.

Cleaning Agents and Techniques used in Aquatics Facilities

A. Cleaning Agents

a. In the case of cleaning zebrafish equipment, acids are typically used to neutralize alkalis rather than as a primary cleaning agent. Examples include citric and acetic acid.

b. Alkalis are often the primary cleaning agent used to clean and sanitize zebrafish tanks and components. Alkalis hydrolyze organic matter, making it more susceptible to removal and destruction by other chemicals or high temperature. Because of their high pH, alkalis should be neutralized and rinsed away fully before returning an item to service in the RAS. Despite being volatile and highly toxic to fish, household bleach (sodium hypochlorite) is still commonly used in zebrafish facilities as a cleaning and disinfecting agent. Other examples of alkalis used for cleaning and disinfection in zebrafish facilities include sodium hydroxide and Tosylchloramide sodium (chloramine-T).

c. Oxidizers can be powerful cleaning and disinfecting agents in the aquaculture setting. Because the oxidation of a substrate often contributes to the degradation of that substrate, oxidizers are very effective at removing many soils common to aquaculture and zebrafish RAS. Hydrogen peroxide is a readily available, relatively inexpensive, and when used properly, fish-safe chemical oxidizer. This is because, when added to water, hydrogen peroxide breaks down into oxygen and water. Other commonly used oxidizers used in aquaculture cleaning and disinfection include ozone and peracetic acid.

d. Soaps and Detergents are both surfactants, which are chemicals that lower surface tension, and may act as foaming agents and emulsifiers. For this reason, they are useful in removing soils from hard surfaces but pose a real danger to both fish and beneficial bacteria cultured in RAS.

e. Solvents are a substance, usually a liquid, which dissolves a solute. While not a particularly effective cleaning agent for aquaculture related soils, ethanol (70%) is commonly found in laboratories and zebrafish facilities, where it is often used as a disinfectant and drying agent for common-use equipment, such as microscopes and hand-tools.

B. Cleaning Techniques

a. Manual washing of RAS equipment and components is perhaps the most commonly employed cleaning technique. When manually washing zebrafish tanks, accessories, and related equipment, there is often a need for pretreatment in baths of water in order to keep the soils wet since, once dried, many of the soils common to zebrafish RAS become tenacious and require additional force to remove. It is common to use household bleach to assist in this process, but this approach will require adequate posttreatment to neutralize the chlorine, which may pose a direct threat if left on the tanks. Sodium thiosulfate is commonly used for this purpose. The manual scrubbing of tanks and accessories is often aided by the use of scrapers, sponges, and so-called *nonabrasive pads* or electric-powered tools borrowed from the restaurant and bar industry. The use of these tools will ultimately abrade surfaces making tanks that were once optically clear, become foggy and scratched. Fig. 27.8

FIGURE 27.8 Image of abraded surfaces from cleaning a zebrafish tank. *Photograph courtesy of Erik Sanders.*

illustrates a tank with abraded inner surfaces. Fig. 27.9 illustrates properly cleaned tanks. This abrasion represents a manifold increase in surface area and will create a surface that will continue to be more and more rapidly colonized by algae and biofilms, effectively decreasing the useful service life of the equipment. From a staffing and labor cost standpoint, manual washing is quite expensive when analyzed on a per-unit basis. Areas of undesirable variability in manual washing include the level of productivity, quality control, and the potential for mistakes with chemicals leading to a risk of human and fish colony health.

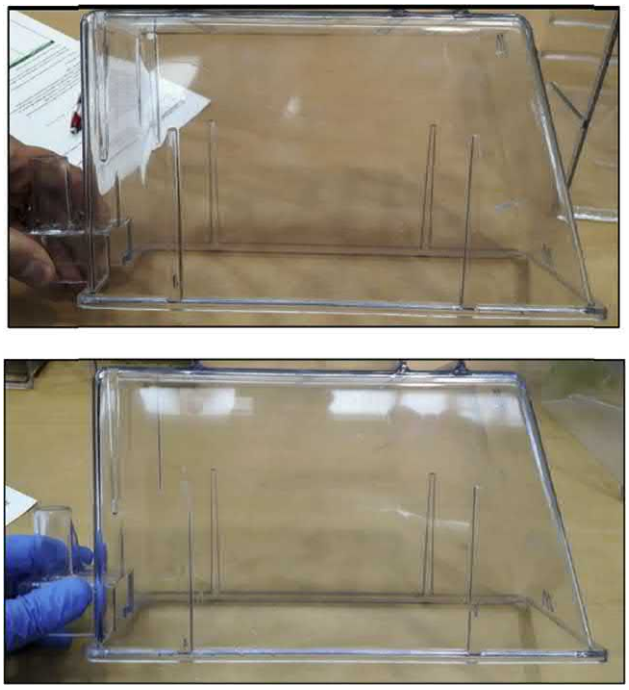

FIGURE 27.9 Images of properly cleaned tanks. *Photographs courtesy of Erik Sanders.*

b. Automated washing, while common in rodent facilities, where it is commonly referred to as cage wash, is relatively new to the field of zebrafish, only becoming benchmarked in 2012. The machines common to this kind of washing are cabinet washers, cage, and rack washers, and tunnel washers. This kind of washing should require a minimum of pretreatment of the tanks and accessories (lids, baffles, etc.) and should render the items washed completely free of any and all soils, and preferably, at a high level of disinfection. All chemicals employed should be free of detergents, soaps, and surfactants since these can cause serious problems by creating foaming in RAS and by damaging the slime-coat of fish and biofilter bacteria. Wash chemicals typically consist of alkalis and oxidizers but may also include acids. While municipal water may be acceptable for the wash phases of the process, the final rinse should be performed only with reverse-osmosis or suitably purified water. Importantly, the presence of any chemical residue on the tanks at the completion of the wash cycle is unacceptable considering the highly sensitive nature of aquatic species and biofilter bacteria and the types of research being performed with zebrafish (embryology, toxicology, etc.). In contrast to rodent cage wash standards, the drying phase of the process is not as important for aquatics cage wash standards. Although automated cage wash requires a capital investment, the cost of washing on a per-unit basis is often at least 50% less, and the level of cleaning and disinfection is substantially higher than that which can be expected using a manual approach. Fig. 27.10 illustrates the Calypso Aquatic Cabinet Washer from Tecniplast. Fig. 27.11 illustrates a Tecniplast Presentation Rack for tanks prepared to be rolled into a Tecniplast Cage and Rack Washer.

c. Autoclaving, considered to be complete sterilization of an object, is performed using high temperature at elevated atmospheric pressure for an extended period of time. All items to be autoclaved must be fully cleaned before undergoing the process. While many zebrafish housing components are capable of withstanding these conditions, the recommendations of the manufacturer should be carefully followed to avoid damage to the items being autoclaved.

C. Cage wash Area Planning

a. Design Challenges

i. Work Flow

While rodent facilities predominantly employ the clean-dirty corridor concept, it is far less common in aquatics facilities. Much of

FIGURE 27.10 Images of the Tecniplast Calypso Aquatic Cabinet Washer, closed and open, showing crates for accessories, and in service. *Photographs courtesy of IWT/Tecniplast.*

FIGURE 27.11 Image of Tecniplast cage wash presentation rack loaded with fish tanks and components, prepared for entry to Tecniplast Cage and Rack Washer. *Photograph courtesy of IWT/Tecniplast.*

this difference can be attributed to the historical design and cost expectations of zebrafish facilities, which often, and mistakenly, are thought to be much less complex and costly than rodent facilities. Dirty aquatics equipment is prone to drip and fall to the floor; thus, it is essential that this be considered when selecting cage wash operations locations. A failure to do so may result in a situation, where researchers are constantly crossing paths with staff carrying out cage wash operations.

ii. Space Requirements

Because the allotment of space for cage wash in aquatics facilities is not standard practice as it is in rodent facilities, we must borrow from the rodent facilities in order to arrive at a best-practice approach. There exist relatively simple formulae for planning throughput and equipment requirements for rodent facilities, which may readily be adjusted for aquatics facilities. Factors for establishing a baseline in a full-capacity facility are the total quantity of cages in use, the percentage of these cages changed daily, the number of days/week cage wash is performed, the number of shifts in a day, and an efficiency factor of a full workday. From these factors, it is possible to define the daily demand. Daily demand will describe how many cages must be processed each day at peak capacity. With this figure, it is possible to specify the type and number of machines needed, and consequently, the space they will require. Additionally, you will be able to determine the total inventory of cages and related components required to maintain the daily operations at full capacity. Considering that colony size may vary wildly from week to week within zebrafish facilities, you should expect to have space to store at least 25% of your total cages and components as clean supply.

iii. Finishes

Since cage wash operations involve high temperatures, steam, corrosive chemicals, water, and high humidity, all materials in the

cage wash areas must be constructed from durable materials, such as stainless steel, epoxy resins, fiberglass, etc. Given the high traffic volume, the floors and doors should be specified to handle both a high degree of wear and a wet environment.

iv. Waste Handling

Most solid waste in a zebrafish facility is disposed of by the RAS mechanical filtration, but some will make its way into the cage wash area. It is important to have the ability to spray down equipment to remove or loosen solid wastes, and that the areas have adequate floor drains to handle its removal.

v. Safety

Safety of personnel and animal colony are of critical importance. This is especially true where caustic or corrosive chemicals are concerned and where volatile chemicals are used. Always ensure your operational guidelines are aligned with the Environmental Health and Safety (EH&S) coordinators for your institution. The need for eyewash and chemical showers will be dictated by EH&S and will need to be incorporated into your space planning and budget. There will likely be additional requirements from EH&S for personal protective equipment to be worn and used by personnel.

b. Operational Philosophy

Many variables will impact the time that a zebrafish cage may stay on a rack until it becomes necessary to exchange it for a clean one. It is critical that daily health checks be performed, and if our view of the fish is impeded by the growth of algae, then the tank must be exchanged. If the accumulation of solid waste in the tank has begun to impede the self-cleaning action of the tank or has caused the water level in the tank to rise above its normal operating level, then the tank must be changed. Your daily operations will guide you in this regard, but as a general rule, tanks should be changed at least every 90 days.

Cleaning Validation Techniques

A. Microbial/Biological Techniques:

These are used to establish the efficacy of the cleaning process as it pertains to removing the biological load. There are several readily available and reliable test methods to consider.

a. Replicate Organism Detection and Counting (RODAC) plates are inexpensive and very useful for detecting viable organisms that may be left behind after cleaning. These plates should be formulated and incubated in a manner that would allow detection of possible organisms of concern in a zebrafish facility. Fig. 27.12 illustrates a set of RODAC plates.

FIGURE 27.12 A set of RODAC plates. The top two plates have no growth indicating a complete cleaning and sanitation of the tested surfaces; the bottom two plates show the growth of microorganisms from pre-washed surfaces. *Photograph courtesy of Erik Sanders.*

b. ATP Detection Swabs are available from several manufacturers. Most utilize a combination of enzymes and buffer which, when combined with ATP, emit light that is detected and enumerated by a handheld device. ATP detection is fast (10-second or less), produces recordable and numeric data, is reliable, and is very sensitive, detecting contamination well below the threshold for RODAC plates.

Conclusions

Using proper cleaning and disinfection techniques are crucial for the culture of zebrafish for many reasons. Incorrect procedures can result in catastrophic failures, including loss of animals and data. Those working in the field must be well trained, familiar with their systems, and observant to ensure that the systems are kept in a manner that supports optimal animal health and valid research results. For further reading on this topic, please refer to the references listed below.

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CHAPTER

26

Aquatic Housing

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Introduction to Aquatic Housing

When zebrafish first emerged as a novel genetics model system, the availability of strategic housing options was extremely limited. Despite this, the Streisinger Laboratory at the University of Oregon was able to devise a life-support system that enabled the fish to flourish under their care. The early genetics revolutions that led to zebrafish becoming today's second most used animal model after mice began in common glass aquaria, purchased from a local pet shop provider and covered with custom-cut acrylic lids. The fish were housed in a semiflow-through environment where water exchange occurred during the day by filtration of influent mains water through a household carbon filter and a thermostatic mixing valve; water exchange was then turned off in the evenings (Lawrence & Mason, 2012).

Today aquatic housing solutions share common threads with this precursor system, but technology has made possible many advances, from significant enhancements in tank design and construction to the development of smartphone-interfaced, fully automated electronic water quality monitoring and control instrumentation. These advancements allow the luxury of multiple strategic choices when considering how best to house this durable model organism.

This chapter will explore available choices in detail, but before decisions can be made with regards to tank design, materials selection, system design (single-pass or recirculating water), or scientific consequences (such as effects on gut flora), one must first understand the impact that scale and application have on these choices, as this is the key driver for determining which aquatic solution will best enable one's research goals.

As the zebrafish model continues to break new ground beyond its traditional biomedical discipline, many research institutions are making a strategic commitment to its application by building core aquatic animal facilities designed to facilitate the complexity of cross-disciplinary research programs. This commitment often requires the use of specialized housing equipment, especially when project designs evaluate the fish over many weeks or months (Traver et al., 2004). While the zebrafish has proven itself as an adaptable research model and a critical core model organism, the institutional commitment worldwide to its use is still very cautious compared to century-old rodent models. One would be hard pressed to find an institution with a biomedical research discipline in its faculties lacking a rodent vivarium supported by dedicated laboratory animal technicians, yet the commitment to centrally staffed aquatic facilities is still in an emergent phase. As a result, it is commonplace for significantly lower scale zebrafish aquariums to be utilized as the housing solution for the model. Regardless of the commitment level toward the zebrafish model system, all facilities strive to provide the fish with the best care possible within the restrictions of the aquarium's design. Thus, housing systems need to provide the fish with a stable and favorable culture condition while also supporting the specific research goals. In addition, as the application of fish in biomedical research increases, oversight bodies are requiring aquatic research facilities to meet and adhere to more stringent regulatory requirements worldwide (Lawrence et al., 2009).

When it comes to deciding on the appropriate scale for a zebrafish commitment, the most influential drivers are (1) whether multiple research groups are interested in applying the model, and (2) whether the investigative questions can be addressed with simple short-term developmental experiments, or whether longer term, multigenerational approaches are required. If the desire for zebrafish application is being driven by only a small number of investigators who are asking questions that can be assessed without the need to rear multiple strains, then the most appropriate housing solution may be as simple as a glass aquarium tank on a bench.

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If, however, the questions require multiple generations of controlled pedigree crosses, then the need to provide husbandry for multiple life stages will require a more involved commitment to the model, and an expanded commitment toward the housing technology applied. This commitment may take the shape of a custom solution designed and developed specifically for the application. However, the most common answer is likely to come in the shape of a commercially available housing solution that elegantly combines principles of commercial aquaculture, laboratory animal housing, and research genetics, and that can be tailored and scaled to meet the needs of multiple research programs in one facility.

Beyond defining the application scale, culture condition is the next most critical element to be addressed in order to ensure that the fish become a productive research model. While the major elements of culture condition will be discussed in other chapters of this text, there is one element of culture conditions that requires attention within the discussion of housing solutions. This is stocking density and its impact on stress.

The density at which fish are kept in captivity exerts profound effects on their health, productivity, and welfare. Holding densities also ultimately have significant implications on the operating costs of an aquarium, the space required for the housing solution, and the labor required for maintaining the aquatic facility. In general, higher stocking densities will ensure that costs, space, and labor are kept to a minimum, but it may not be the best approach for achieving a healthy, productive zebrafish colony.

It is widely accepted that crowding fish at high stocking densities can cause chronic stress, resulting in changes in growth rate, feeding, behavior, immune function, and health (Ellis et al., 2002; Procarione, Barry, & Malison, 1999; Suomalainen, Tiirola, & Valtonen, 2005; Wedemeyer, 1996). It is also recognized that stocking density effects on fish welfare are complex and species-specific; these effects can comprise numerous interacting factors including water quality, social interaction, the carrying capacity of the holding environment, as well as the special and behavioral needs of the particular species (Pavlidis et al., 2013).

As a gregarious species, zebrafish benefit from the ability to form loose shoals with their tank mates, where they can establish and express social hierarchies. Zebrafish are also known to display aggressive behavior associated with the establishment of territories and dominance hierarchies (Larson, O'Malley, & Melloni, 2006); thus, both crowding and low-density housing are less than ideal for zebrafish. This highlights the importance of matching holding conditions to their behavioral tendencies.

The currently accepted guideline for establishing a stocking density policy that is conducive to productive and healthy zebrafish is based on the approach outlined by Harper and Lawrence (2011), who suggest that densities of 40–50 fish per liter are appropriate for early larval culture, but that density should be gradually reduced to five fish per liter for adults. This commonly applied strategy was supported by Pavlidis et al. (2013) who showed that zebrafish held at a density of 10 fish per 2 L of available water volume adequately expressed normal behaviors while avoiding elevated cortisol stress reactions; this suggests that even when water quality is maintained, a minimum water volume is required for zebrafish welfare.

With this knowledge, it is possible to determine the necessary scale of the intended application, based on the target number of animals required to meet the research program's needs. With appropriate scale commitment established, the desired application will be the key driver for determining the type of enclosures needed for housing the fish.

Primary Enclosures (Tanks)

In an aquaculture facility, the primary housing enclosures are the tanks. Depending on the scale and application of the projects employing the zebrafish model system, the tanks used will vary in shape, size, and materials from which they are constructed. They will also vary in how they handle water exchange and how they contain the specimens. These factors all affect the welfare of the fish, the functionality of the life-support system, and the pace and efficiency of research being conducted. Thus, aquatic housing choice for any application needs to include careful consideration of how a particular system, and its associated tank types, help investigators achieve their research and husbandry objectives.

Materials Selection

Material toxicity is a concern when introducing any new equipment that will contact the fish. For this reason, the most common materials that zebrafish holding tanks are made from are glass, acrylic, fiberglass, polyethylene, polycarbonate, or polysulfone. These materials have an established record for use as aquatic housing solutions, each with its benefits and limitations, and thus, the appropriate material selection will depend on the application.

Thermoplastic Polymers

In modern high-density installations, tanks are most likely to be made from polycarbonate. This plastic is durable and relatively inexpensive, and it can be clear or pigmented. The pigments chosen for zebrafish enclosures are typically shades of blue or green, focused on filtering out the light spectrum utilized by chloroplasts, thus reducing the development of algae on tank surfaces as the aquarium's water begins to accumulate phosphates and nitrates. Polycarbonate tank construction is made via pressure injection molding, and it is, therefore, amenable to the production of tanks in a wide range of shapes and sizes. Most commercial zebrafish aquarium manufacturers offer a range of polycarbonate tanks, allowing a significant level of customization to any aquarium installation. One of the most significant benefits with the use of polycarbonate tanks is that they are readily movable within the facility. One can thus temporarily relocate fish to a workbench for investigation, directly exchange tanks for cage cleaning, or transfer fish from a fouled tank into a fresh new tank. This accommodates significant flexibility in cleaning and disinfection protocols. Although polycarbonate tanks are lightweight and can withstand mechanical washing processes, thereby reducing manual labor and zoonosis risks present with some other tanks, polycarbonate degrades at high temperatures, and thus, cannot be repeatedly sterilized. For countering this, several manufacturers of polycarbonate tanks also offer a range of tanks made from polysulfone.

Polysulfone is slightly more costly, with a greyish hue, and is marketed as having a higher temperature and chemical tolerance than polycarbonate. Tanks made from polysulfone can be a suitable substitute for applications, where regular tank sterilization is required.

Both polycarbonate and polysulfone tanks are produced using bisphenol-A (BPA). This is the main drawback to the application of these thermoplastic polymers in aquarium holding tanks, as it has been established that both types can leach BPA into the water. Bisphenol-A is an estrogen mimic that can cause serious reproductive problems in vertebrates, including fish. Howdeshell et al. (2003) demonstrated that new polycarbonate and polysulfone tanks both leach BPA into the water. However, leaching is significantly higher in aged polycarbonate tanks, thus elevating this as a potential concern for established zebrafish facilities. Howdeshell et al. (2003) also discussed the leaching levels being similar to those previously reported to skew sex ratios toward females in the South African clawed frog, *Xenopus laevis* (Kloas, Lutz, & Einspanier, 1999), and that resulted in the presence of ovo-testes and testicular abnormalities, including a decrease in the number of spermatozoa, in the medaka *Oryzias latipes* (Metcalfe et al., 2001). While definitive effects of BPA leaching in a zebrafish colony remains to be tested, it is reasonable to conclude that BPA leaching may have similar effects on the diminutive minnow. The benefits gained from the use of thermoplastic polymer tanks are nevertheless significant, suggesting that regular tank replacement may be the most reasonable strategy for managing this side effect until a satisfactory BPA-free substitute material can be found.

Glass

If BPA effects are likely to cause issues for the research program, the inert properties of silica-based glass may make it the material of choice. Glass tanks are the most common housing solution implemented in small-scale operations, as basic rectangular glass aquaria are readily available at most pet shops. Glass can also be applied in large-scale operations, and it is the next most common material used in primary enclosures after thermoplastic polymer tanks. The principle benefits of glass are that it is inert, and thus, poses no chemical leaching risk to its inhabitants; it does not scratch easily, which, in turn, means it resists the establishment of biofilms and other tank fouling, and it is relatively inexpensive. Glass, however, lacks the flexibility necessary for design variability, making it difficult to apply to as wide a range of housing solutions as other materials. Glass also has the significant limitations of being fragile and heavy, posing major health and safety challenges to personnel during routine cleaning.

There are health and safety challenges that glass aquaria present to the personnel who maintain them. There are manual handling risks associated with moving, servicing, and replacing the tanks. Large glass aquaria are heavy, often requiring multiple people to move and position them. This means they do not readily lend themselves toward automated washing. As a result, most glass aquaria will need to be cleaned in place, often resulting in a laboratory animal technician arm deep in fish water. While this in itself is not a dangerous activity, there is a significantly elevated risk of skin abrasions from this method of tank maintenance, a risk that is compounded if there are minor chips to the rims of the tanks. This can prove to be a major obstacle for facilities that opt to utilize glass for their primary enclosures, as there is a significant zoonotic disease common to zebrafish colonies; the atypical tuberculosis, *Mycobacterium marinum* (Matthews, 2004). To overcome this risk, facilities using glass tanks need to be mindful of their biosecurity efforts, to ensure they do not inadvertently contract a mycobacterium infection. Strategies for maintaining a mycobacterium free facility are discussed later in this book.

Acrylic

As the zebrafish model expanded beyond traditional developmental genetics studies, there grew a need for specially designed primary enclosures to enable specific experimental work. As a result, poly(methyl methacrylate), commonly referred to as acrylic, has become an important material of choice. Although technically also a thermoplastic polymer, acrylic has significant differences in how it can be applied as a primary enclosure, and thus, is being discussed separately. Acrylic does not contain the potentially harmful bisphenol-A subunits found in polycarbonate and polysulfone plastics, and it is an economical alternative to polycarbonate when extreme strength is not necessary. This transparent thermoplastic is often used in sheet form as a lightweight, shatter-resistant alternative to glass. It is approximately half the weight of glass, and while it is significantly stronger and more flexible than glass, it is also significantly more prone to scratching. Scratches may be easily removed by polishing the damaged surface of the material with appropriate solvents available from hobby retailers. Acrylic can be damaged by organic solvents such as ethanol and by other chemicals. It also has low heat tolerance, which can limit sanitation options. Even high-quality acrylic will begin to disfigure and melt at temperatures above 80°C. Acrylic is very resistant to the effects of strong alkali solutions, which are extremely effective germicides. Sodium hypochlorite (bleach) can be applied safely to acrylic tanks at concentrations up to 5%, and any biofilms or algae can then be subsequently wiped off with a soft cotton cloth. When using this method of sanitation, it is necessary to detoxify any residual free chlorine before repopulating the tank.

Polyethylene and Fiberglass

Although less commonly applied, polyethylene and fiberglass-reinforced polyester (FRP) also play an important role in zebrafish housing. These materials share similar beneficial traits and are most commonly applied when the application requires either holding a large volume of water or controlling illumination to the culture, thus eliminating the option of using the optically transparent acrylic.

Emerging Materials

In recent years, 3D printing has significantly enhanced the ability to construct tools and in this case, housing solutions. A prime example, the Screen Cube, is a 3D-printed housing solution that is used for the transfer of fish between primary holding tanks and treatment baths, allowing for rapid and cost-effective chemical screening of adult zebrafish (Monstad-Rios, Watson, & Kwon, 2017). While 3D printing offers immensely powerful flexibility for creating novel primary enclosures, it is important to note that not all 3D printing polymers are considered safe. Macdonald et al. (2016) assessed the biocompatibility of four commercially available 3D printing polymers, and via the observation of key markers in developing zebrafish embryos, showed all of the photopolymers in an untreated state to be lethally toxic to zebrafish embryos. When parts fabricated from one of these photopolymers, Fototec SLA 7150 Clear, were washed with an organic solvent (99% ethanol), biocompatibility significantly improved, making it suitable for use in zebrafish culture. This is an important message with regards to employing new materials in a zebrafish aquarium. It is critically important to run a basic Fish Embryo Toxicity (FET) test, such as that described by Schulte and Nagel (1994), to determine the biocompatibility and safety of employing the new material.

Tank Design

Armed with the knowledge of application and appropriate material selection, it is possible to consider tank design for research program outcomes. Zebrafish tanks come in a variety of shapes and sizes, defined by their intended function. Although tank design varies between vendor and application, all tanks are generally designed to serve the common purpose of containing the fish within a specified space and volume of water, while also maintaining suitable life-supporting culture conditions for its inhabitants.

Box Tanks

Although becoming less common in the modern era, the traditional box tank still serves an important role for those who are keen to pilot small experiments but are unable to access a large-scale production facility. These tanks are typically 20−50 L in volume, allowing an all in one approach for the keeping of the fish. The large water volume allows the fish the space to exhibit their natural shoaling behavior, while also providing sufficient volume to buffer against shifts in water quality. The box tank can be used as a freestanding static aquarium, or with some modification, as a flow-through aquarium, in a semirecirculating life support system, or in a Recirculating Aquaculture System.

Serial Tanks

Serial tanks are most commonly made from glass and are applied in large-scale aquariums. Serial tanks are essentially an advanced modification of a box tank, resulting in long aquaria that are divided transversely by partitions to provide multiple compartments for keeping adult fish (Fig. 26.1). A typical serial tank is

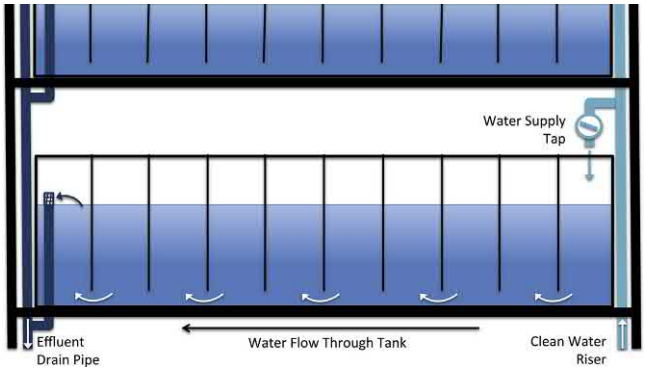

FIGURE 26.1 Serial Tanks. Serial tanks typically comprise a long glass box tank, which is divided into housing compartments by partitions (*black vertical lines*). These partitions leave a small gap at the base of the tank, resulting in a series of interconnected compartments. Influent water enters the series of tanks in the compartment at one end and flows through each tank compartment via the gaps at the base of the partitions (*white arrows*). Effluent water exits the tank in the last compartment via a standpipe (passive overflow), setting the water volume throughout the series. A grill fitted to the head of the standpipe prevents fish from escaping containment.

120 cm wide, 60 cm deep, and 22 cm high (Brand, Granato, & Nüsslein-Volhard, 2002). The long box is typically subdivided by glass plates that leave a small 1.5 mm slit at the bottom, or via perforated plastic plates, resulting in a series of 8–10 interconnected compartments of about 12 L each. Each compartment has a separate lid, allowing the holding of up to 60 adult fish per compartment at a stocking density of five fish per liter. The defining feature of serial tanks is that the water in a row of tanks flows into the compartment at one end, and then passes through the slits from one compartment to the next, carrying waste particles with it. The water is drained from the last compartment at the far end of the row by overflowing into a standpipe, thus determining the water level across the row. In order to prevent loss of fish, the standpipe is typically fitted with a coarse mesh or grill.

There is potential for capital savings to be made with the implementation of serial tanks, as there is only one influent and one effluent point requiring attention for supporting 10 tanks; however, the fact that serial tanks are too large to be removed from their support racks means cleaning must be done in place, making long-term maintenance of the system inefficient. Thus, the benefits gained from serial tanks are often at best neutralized by longer-term inefficiencies.

There is also one significant limitation to consider before committing to the use of serial tanks. Due to their interconnectivity, they are not able to control the spread of horizontally transmitted diseases, as all tanks share a common water source. Thus, if a population of fish in chamber one were infected with a disease like microsporidia (*Pseudoloma neurophilia*), then fish in the other nine compartments would be exposed to spores released into the water as it moves through the serial chambers toward the outflow pipe. To avoid this problem, the use of individual, self-contained primary enclosures may be necessary instead; this is the main reason why this tank design has lost favor in recent years.

Passive Overflow Tanks

There are many ways a passive overflow tank can be applied in a large-scale facility, and these tanks offer the most flexibility in design, shape, and customization. The simplest implementation of the passive overflow tank is described by Brand et al. (2002), in which individual containers (plastic or glass) are placed in a row on to a bunded shelf. Water enters into each tank through an influent hose or pipe and exits through an overflow point on to the shelf, from which it is drained into a common water treatment unit. However, this implementation of the passive overflow tank design often results in fish waste accumulating around the base of the tanks, and thus, more frequent cleaning is needed. An alternative implementation of the passive overflow tank utilizes a standpipe or tank flange, which allows the overflow point of the tank to couple to a plumbed waste line, keeping all effluent water contained within the wastewater drainage pipes. Both strategies typically rely on fine mesh grills, or filter media, at the overflow point to avoid fish escaping containment, and this can be tailored to suit adult or juvenile fish. Examples of both strategies are illustrated in Fig. 26.2.

Passive overflow is the most common strategy applied to specialty application tanks when they are incorporated into system solutions rather than existing as stand-alone housing chambers. Some of these solutions to unique applications will be discussed subsequently.

Active Overflow Tanks

Despite the significant flexibility advantage passive overflow tanks offer, the nature of the hydrodynamics within the tanks means these solutions offer limited ability to maintain tank hygiene, and frequent cleaning intervention is needed to keep these tanks functioning as intended. To overcome this challenge, it is commonplace for large-scale aquariums to implement the use of solids-lift (active) overflow tanks as their principle housing device. In these solutions, the tanks employ the use of either siphons and/or specially designed partitioning baffles to alter the hydrodynamics within the tank, such that an undertow current is established, creating an active draw across the base of the tank (Fig. 26.3). Providing there is an adequate velocity of water entering the tank via the influent pipe, the undertow

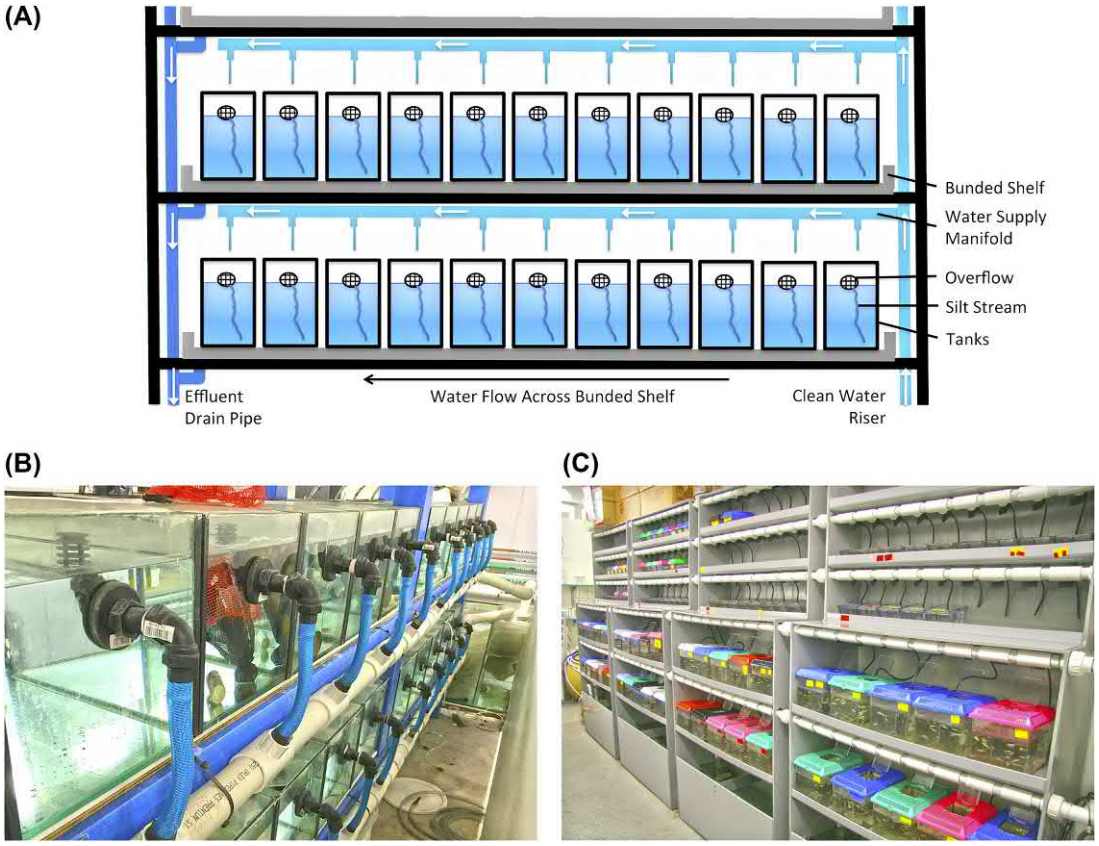

FIGURE 26.2 Passive Overflow Tank Application. (A) In this schema, individual holding tanks are placed onto a bunded shelf. Water enters into each tank via a small outlet from a header pipe manifold that runs along the length of the shelf. Water exits the tank through a grill, or series of small holes, at the top of the tank. Suspended particulate waste (fines) trickles down the tank onto the shelf (silt stream). Wastewater pools on the bunded shelf, and is collected into a common drainpipe that sequesters the wastewater to the filtration assembly. (B) Passive overflow, glass box tanks coupled to a wastewater drainpipe via tank flanges (C) Passive overflow, polycarbonate tanks on a bunded shelf.

current will have sufficient strength to carry waste particles out of the tank.

Specialty Application Tanks

Beyond the range of designs that make up the housing tanks, there are a number of unique primary enclosures designed to facilitate specific application needs, the most common enabling controlled breeding. Mating tanks are a critical element of an aquarium supporting research projects that focus on developing zebrafish embryos.

Mating Tanks

Historically, adult zebrafish were encouraged to spawn their eggs via a process known as "marbling," where an investigator would cover the entire bottom of the housing tank with a layer of marbles, effectively creating a series of nooks and crannies that allow fertilized eggs to drop beyond the reach of hungry tank mates (Westerfield, 2000). The fertilized embryos would then be siphoned from the tank every morning. While effective, marbling lacks the finesse that is required to meet the productivity demands of many research aquariums today; hence the development of purpose-designed breeding tanks.

Mating tanks come in various shapes and sizes, ranging from just over half a liter up to 2L volumes (Fig. 26.4). These devices can be applied directly in the main housing tanks; however, are most commonly applied as short-term, static enclosures, housing a pair of fish, or in the larger cases, small groups of fish, based on a technique that was first described by Mullins, Hammerschmidt, Haffter, and Nüsslein-Volhard (1994). They all share the same fundamental design characteristics: a tank to hold the water, a trap to separate the adult fish from their spawn, a divider to segregate the adults by sex and so enable timed mating, and a lid to prevent the fish from jumping out of the tank. While there is some handling stress associated with the use of these tanks, their independence from the main housing enclosures gives them significant advantages for both the harvesting of the embryos and for sanitation after use.

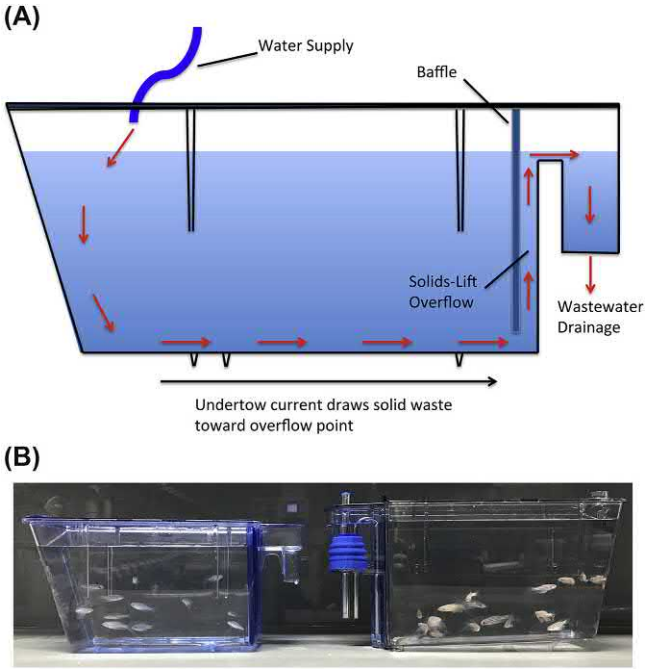

FIGURE 26.3 Active Overflow Tanks. (A) This shows the basic hydrodynamic design of a solids-lift tank. Water is delivered (*blue line*) into the tank toward the front of the tank. The unique design of the partitioning baffle (or siphon) at the back of the tank results in a pressure differential between the holding space and the overflow. A strong undertow current (*red arrows*) draws particulate waste toward the back of the tank and lifts it up to the tank overflow. (B) Examples of typical active overflow tanks.

FIGURE 26.4 Mating Tanks. Examples of various polycarbonate mating tank designs.

For many years, the vast majority of mating tank designs followed a simple, flat-bottom approach to the trap, which although satisfactory to yield sufficient quantities of embryos from the fish, did not play to the fish's natural spawning site preferences. Following research conducted by Sessa et al. (2008) that illustrated a preference to spawn in the shallows, as evidenced by the observation that the zebrafish actively chose to spawn at the shallow end of a sloped trap, many aquariums began to establish gradients in their mating tanks by placing them on a sloped platform or by wedging the trap with the divider, such that one end sat higher in the tank. The widespread adoption of this behavior led to one commercial manufacturer refining its mating tank design, resulting in a beach style trap, with a sloping layered surface, that allows the fish to select their preferred depth upon it for spawning.

Mass Embryo Production Systems

While the above mating tank designs are effective, there are scenarios where their application cannot easily meet the production demand for fertilized embryos. When obtaining large quantities of developmentally synchronized embryos is more important than tracking pedigree of individual contributors, Mass Embryo Production Systems (MEPS) offer a unique solution.

The main impetus for shifting breeding tank design to capitalize upon the group spawning dynamics of the zebrafish came as a result of the model expanding into the realms of toxicological studies, chemical screens, and other applications that demand increasingly large embryo numbers. Adatto, Lawrence, Thompson, and Zon (2011) describe placing up to 300 fish inside a custom-designed, 100 L static group spawning chamber capable of yielding 10,000 fertilized embryos within 10-minute harvesting windows.

There are several different models of MEPS currently available commercially (Fig. 26.5), each capable of use as a static breeding tank, or of being coupled to an aquarium system as a passive overflow device. One of the most significant differences between the MEPS solutions from various commercial suppliers is the base material with which these tanks are constructed. One design is completely transparent, constructed from acrylic, allowing the tank to leverage the existing facility

FIGURE 26.5 Mass Embryo Production Systems (MEPS). Large numbers of broodstock fish can be housed and spawned directly inside these devices, facilitating on-demand collection of synchronized embryos. From left to right, an example of a polyethylene MEPS, large (60 L, 200 fish) and small (13 L, 40 fish) acrylic MEPS, and a fiberglass-reinforced polyester MEPS. *Courtesy of Aquaneering Inc., Courtesy of Tecniplast Aquatic Solutions and Courtesy of Pentair Aquatic Eco-systems.*

photoperiods; another has opted to construct their MEPS solution from polyethylene; while a third supplier chose to fabricate their tank using FRP. The latter designs both allow photoperiods to be established independent of the main facility light cycle. Other differences between the various MEPS include size, holding capacity, spawning platforms, mode of egg collection, and patterns of water circulation; but regardless of design, the scale and reliability of function of these tools are tightly coupled to scientifically grounded management of the broodstock fish placed inside (Lawrence, 2011).

Respirometers and Mazes

While traditionally the model's strength lay with the developing embryo in biomedical discoveries, and so too with the majority of specialty application tank solutions, the adult zebrafish has started to become a more significant model to other disciplines; and with this, new applications for the model have emerged. Swim-tunnel respirometers, puzzle mazes, and operant conditioning shuttle boxes have all emerged as significant tools in the zebrafish toolkit in recent years.

One such tool, the respirometer, has gained particular focus for investigators interested in the physiology of the zebrafish, examining traits such as swimming speed, stamina, oxygen demand, and body shape drag. The most commonly applied, Brett-style Swim-tunnel Respirometers (Fig. 26.6) have a rounded-rectangular shape and contain a propeller that drives water circulation in one direction, while the fish are restricted to a long compartment known as the "working section," where the water passes in a laminar flow profile (Brett, 1964). A variable-voltage motor typically drives the propeller in the swim-tunnels, such that a range of water speeds can be obtained. These devices are typically constructed from acrylic, allowing optical transparency for simplifying data collection, and can be applied statically, fitted with a hang-on filter or connected to an aquarium system as a passive overflow tank. Respirometers can be applied as open swim-tunnels for projects such as examining gross morphology impact on stamina (Conradsen, Walker, Perna, & McGuigan, 2016); but when they are used for determining physiological processes, such as oxygen demand in exercising fish, it is necessary to seal the experimental chamber from the surrounding water bath to prevent water mixing, so that precise measurements can be achieved. In this situation, a flush pump should be used to refresh the water within the treatment chamber at regular intervals, to ensure the dissolved oxygen levels within the respirometer remain above 80% saturation at all times to minimize the chance of measurements being influenced by hypoxia-induced metabolic adjustments (Clark, Sandblom, & Jutfelt, 2013).

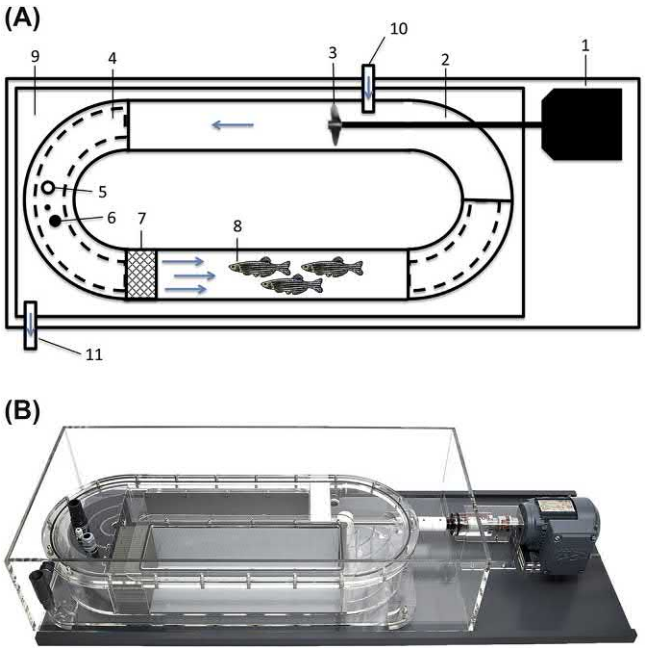

FIGURE 26.6 A Brett-style Swim-tunnel Respirometer can be used for measuring the physiology, energetics, behavior, biomechanics, and kinematics of swimming fish. (A) Overhead schema: (1) variable speed motor, (2) propeller shaft, (3) propeller, (4) baffles to assist with achieving laminar flow, (5) overflow pipe, which extends above water surface, (6) sealable port for oxygen sensor, (7) honeycomb grid to assist with establishing laminar flow, (8) working section where fish are housed, (9) water bath, (10) water inlet, one-way flow (11) water bath passive overflow (B) Swim-tunnel respirometer. *Courtesy of Loligo Systems.*

Mazes and shuttle boxes have also made inroads as specialty application tanks for zebrafish in recent years. Employing the use of a shuttle box, Sneddon (2011) demonstrated that when housed in an environment with the choice of a barren or structurally enriched condition, zebrafish exposed to a painful acid stimulus will override their preference for the enrichment and self-administer analgesics offered only on the barren side.

Environmental Enrichment

One common trait across most of the tank designs discussed thus far is that they are designed to operate as a barren enclosure. This has been a strong barrier against the use of structural enrichment in zebrafish production facilities. In addition, it remains unclear whether structural enrichment, designed to mimic the habitat complexity that wild zebrafish can experience, also benefits laboratory strains, particularly when such interventions are balanced against costs, such as the increased difficulty of observing fish and the increased accumulation of mulm (detritus), which in itself can lead to morbidity and mortality (Parichy, 2015).

While there is mounting evidence suggesting that zebrafish may indeed benefit psychologically from structurally enriched environments (Collymore, Tolwani, & Rasmussen, 2015; Manuel et al., 2015; Maximino, De Brito, de Mattos Dias, Gouveia, & Morato, 2010; Spence, Magurran, & Smith, 2011; Weber & Ghorai, 2013), little work has been done to address the concerns relating to colony-wide health impacts that may emerge as a result of changes in tank hydrodynamics, and the consequential diminished tank hygiene due to the presence of alien structures within the water body. Also, the material selection needs to be carefully considered when opting to implement the use of a structural enrichment device to ensure there is no leaching of unwanted chemicals into the water column. Fig. 26.7 illustrates how some facilities are implementing the use of structural enrichment.

While it is still relatively uncommon to utilize structural enrichment in large-scale production facilities, it is commonly accepted that it can be significantly detrimental to house individuals in isolation (Fox, White, Kao, & Fernald, 1997; Harris & Bird, 2000; Larson et al., 2006; McCarthy, Carter, & Houlihan, 1992). In low-density situations, such as keeping a carrier pair segregated, fish can exhibit enhanced aggression and will often fight until one fish either escapes the conflict by jumping out of the tank or is harassed to the point of death. This is a major concern for ensuring the longevity of critically important animals.

In a situation where structural enrichment may not be readily available for providing refuge for the subordinate, or a territory for the dominant to defend, the simplest way to manage the aggressive behavior of the fish is through social enrichment. As discussed previously, zebrafish have a preference to form small shoals. This behavior allows a more balanced social hierarchy to form, and this can be easily established by combining phenotypically different strains of fish. For example, if a carrier pair has been identified on an AB background, adding a small number of TL or TLN strain fish can preserve the social welfare of all fish in the tank. This allows the fish to maintain a natural social hierarchy, while also being easily identified as the specimens of interest to the investigator.

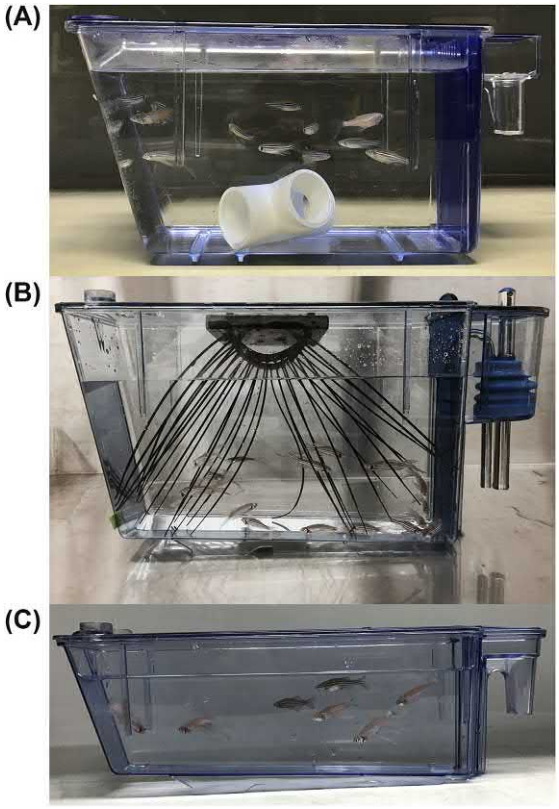

FIGURE 26.7 Structural and Social Enrichment. (A) A small PVC T-joint provides a tunnel refuge for subordinate fish. (B) The Zeb device, a structural enrichment solution offered by Otto Environmental, provides a floating canopy designed to mimic the dense vegetation of the native habitat of the zebrafish. (C) For avoiding the detrimental effects of housing zebrafish in low densities, important carrier pairs can be housed with phenotypically distinct zebrafish, allowing them to form social shoals and mediate their aggression by establishing dominance hierarchies with the other fish. *(B) Photo courtesy of Lucie Nedved.*

Secondary Enclosure Systems

While in a small-scale operation, the tank may serve as both the primary and secondary enclosure, but in large-scale production facilities, the zebrafish tend to be housed in relatively small primary enclosures, to maximize the number of specimens contained within a workspace. If the water within these tanks is not constantly cycled and cleaned, the fish will quickly suffer from the toxicity of their metabolic wastes accumulating in the water. For overcoming this, the tanks are integrated into much larger secondary enclosures, making up dedicated life-support systems designed to allow influent fresh water to replace the waste accumulating effluent water.

Static Enclosures

The challenge with static enclosures is that from the moment fish are added into the tank, the life-support condition, specifically the water quality, begins to deteriorate. As the fish respire, they consume dissolved oxygen and release carbon dioxide and ammonia into the water. As these metabolic wastes accumulate in the water, the environment becomes less favorable for its inhabitants. For this reason, static tanks need to be kept at significantly lower densities than enclosures in

which constant water exchange is possible, and thus, the situations where static enclosures are appropriate should be carefully considered.

A significant advantage of static enclosures is that they avoid all costs associated with establishing and servicing filtration assemblies, and are, therefore, very cheap to implement. However, because these devices lack the ability to clean the water, in comparison to other life-support solutions, they require significantly more space per capita. An additional consequence of avoiding the complexity of filtration assemblies is that static enclosures require high labor commitment in order to keep the life-support condition within suitable parameters for the fish.

As a general guide, if the fish are to be maintained long term in a static enclosure, there are a few basic tips that will help improve the success of the colony. To keep water degradation within manageable tolerances, Brand et al. (2002) advise densities in static tanks should not exceed two fish per liter. Only feed the fish to satiation, so that uneaten food does not amass in the tank, releasing ammonia and rapidly degrading the water quality. Allow the fish opportunity to digest and defecate before performing a tank clean and water change. Keep a small sponge, or other porous material, in the tank to act as a basic biofilter. Even without aeration, these measures will assist with the natural biological process associated with converting toxic ammonia into the significantly less toxic, oxidized state (nitrate). Also, where possible, aerate the water to increase gas exchange, removing dissolved carbon dioxide and regenerating dissolved oxygen.

On a more temporary timeframe, adult fish can be comfortably maintained in static tanks for up to 2 weeks at low densities without water exchange if feeding is withheld and the fish are prevented from stressful interactions, such as breeding or fighting. This is an ideal strategy to exploit when genotyping individuals and can be easily achieved by placing two fish in a 1-L mating tank with a divider separating them.

As zebrafish alevin (yolk sac larvae) transition into fry (exogenous feeders), they are also often kept in static enclosures for the first few days of their foraging existence. As described by Best, Adatto, Cockington, James, and Lawrence (2010), first-feeding zebrafish fry can thrive at a density of 50 fish in 200 mL (250 fish per liter), highlighting that the challenges associated with static enclosures are also life stage-dependent. By far the greatest application for static enclosures, however, is their use in spawning, as was discussed earlier in this chapter.

Before moving onto the more sophisticated secondary enclosure solutions, it is worth noting that the addition of a basic hang-on filter can significantly enhance the life-support condition of a static tank. By adding one of these devices to the side of a tank, a semirecirculating system is achieved, in which the main body of water within the aquarium is pulled out of the tank by a small powerhead pump, and then passes through a basic mechanical filter (usually a pad akin to the scotch scourer) and a small chamber containing extruded activated carbon. The water may also pass through a more porous sponge-like material that acts as a biological filter before the water flows back into the tank, encouraging agitation and enhancing gaseous exchange at the water surface (Fig. 26.8). The principal benefit achieved by the use of a hang-on filter, is the minimization of mulm, the sludge that collects at the bottom of an aquarium, consisting of fish fecal pellets, decaying food matter, and other assorted detritus. By keeping a steady water turnover, the hang-on filter is able to second waste particles into the filter before they have a chance to settle in the base of the tank, significantly extending the useful life of the water within the tank.

FIGURE 26.8 Hang-on Filter. With the application of a hang-on filter to an otherwise static holding tank, the useable lifespan of the culture water can be significantly extended. A small powerhead pump draws water up and out of the tank. The water then passes through a basic mechanical filter pad, activated carbon filter, and biological filter sponge before freefalling back into the holding tank, offering an opportunity for gas exchange.

Recirculating Aquaculture Systems

Recirculating Aquaculture Systems (RAS) are the predominant secondary enclosure solution applied in zebrafish facilities of all sizes. The distinguishing feature defining RAS aquaria is that the water used to house the fish is cleaned and reused, minimizing water consumption and waste. This allows the total water volume of a RAS aquarium to be shared by a number of primary enclosures, thereby, greatly reducing the space required for housing the research colony. Mastering the process of cleaning the recirculating water is the greatest challenge for applying a RAS solution, and its implementation can vary significantly from one aquarium design to another, but at its core are five key elements: mechanical filtration, biological filtration, gas exchange, chemical filtration, and disinfection.

Mechanical filtration is typically the first step of a RAS filtration assembly and targets the isolation and removal of suspended solids from the effluent water. Solid waste enters the system as a result of uneaten food or fecal pellets and must be quickly removed from the circulating water because as the waste decomposes, becoming a food source for heterotrophic bacteria, dissolved oxygen is significantly reduced and ammonia is released into the water column. Particle filters used for mechanical filtration may be either depth or surface filters that trap solids as the water flows through it.

With the solid carbonaceous waste eliminated, the next phase of water treatment typically focuses on biofiltration. Biological filtration describes a process harnessing the power of chemolithotrophic bacteria that oxidize ammonia into nitrite, and then nitrate, to detoxify the nitrogenous waste. Biofiltration is a highly aerobic process, requiring double the ammonia concentration in dissolved oxygen within the reaction chamber (Chen, Ling, & Blancheton, 2006). For this reason, the biofilter is often coupled with gas exchange (oxygen in, carbon dioxide out).

Oxygen is a staple for aquatic life, just as it is for terrestrial life; therefore, as water circulates through the holding tanks, oxygen will be consumed, and carbon dioxide added into the water column in its place. If the active replacement of the water in the holding tanks is insufficient, dissolved oxygen will be depleted, and carbon dioxide concentrations will rise to stressful levels, diminishing the life-support characteristics of the holding tank. A minimum of three to four tank changes per hour is generally recommended (Baensch & Riehl, 1997), with most aquarium operators aiming for at least five changes per hour through each tank.

Some aquariums employ the use of macroalgae or additional filtration elements to further digest the nitrogenous waste, filtering nitrate out of the water also. However, this is not common practice within zebrafish facilities at this time, with most opting to perform regular water exchange to keep the nitrates from rising to dangerous levels. While the precise measure of water exchange will depend on the rate nitrates accumulate in the water column, most facilities apply an exchange rate of 10% over 24 h.

Following the biofilter, the water passes through chemical filtration, focused on removing colloidal solids and dissolved organic compounds (DOCs). While not essential for life support, this additional filtration step significantly enhances the clarity of the water, which is essential for achieving ultimate efficiency in water disinfection if an ultraviolet (UV) filter is employed.

There are many different technologies available to achieve these five key elements; thus, a significant factor driving the underlying decisions that shape the filtration assembly of a RAS aquarium is the application scale and system volume.

Small to Medium Scale RAS Aquaria

When a research program expands beyond one or two production tanks, independently maintaining the individual tanks becomes very labor intensive and impractical as a viable option for maintaining the colony. The hang-on semicirculating filtration solution is inadequate as this can only service the tank it is directly coupled to, and thus, a true RAS solution is necessary to provide optimal life support for the fish in a cost-effective manner. Most manufacturers of research aquaria enter into the market at this level, designing small RAS solutions affectionately referred to as "Bench-Top" or "Stand-Alone" systems depending on size.

Stand-alone systems typically take the form of a rack, resembling a library bookcase, filled with tanks on the top section, and with a compact filtration assembly condensed into the space immediately below the tank holding space (Fig. 26.9). The rack provides the structural support for the tanks and the necessary plumbing to deliver and remove water. The rack is typically constructed from marine grade stainless steel, and it is divided into several presentation rungs. The exact size and shape of the rack will vary between manufacturers, with the smallest "bench-top" solution comprising two rungs of 10 tanks, and the larger stand-alone racks comprising up to six rungs of 10–20 tanks. A typical space footprint for a stand-alone system would be 1620 mm × 565 mm × 2360 mm but will vary with manufacturer.

The dominant feature of a stand-alone aquarium is the water treatment unit (WTU) residing directly

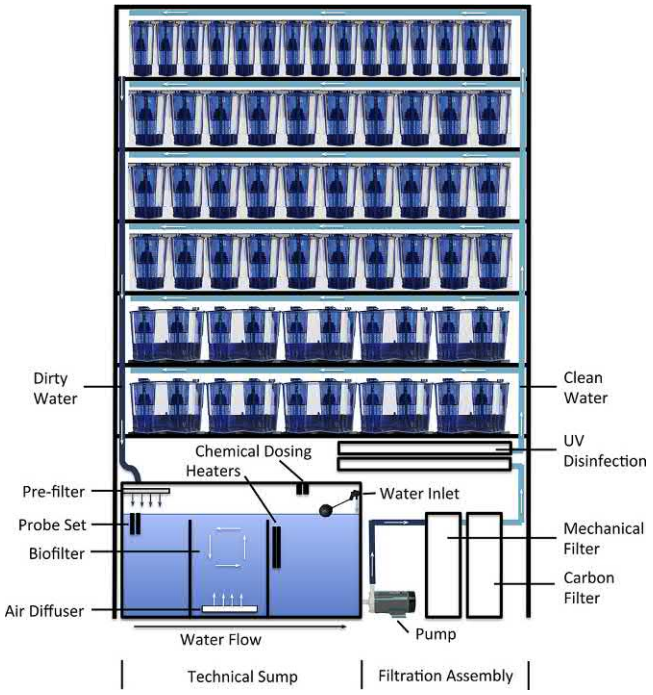

FIGURE 26.9 Stand-Alone RAS Aquarium. A single rack of tanks, equipped with an integrated water treatment unit, enables a space-efficient housing solution for a small colony of zebrafish. Effluent wastewater flows through a prefiltration device as it enters the technical sump, where the buffering water volume resides, and the culture condition is monitored and manipulated. The pump draws the water from the technical sump, driving it through the mechanical and chemical (carbon) filters, and through the disinfection chamber before cycling the water up the vertical riser into the header pipes and then to the fish tanks.

under the bottommost rung of the system. Wastewater enters the WTU by gravity via a downpipe, typically passing through a coarse mechanical filter, often referred to as the prefilter. The water then accumulates in a technical sump, where essential culture conditions such as temperature, pH, and conductivity are manipulated. A pump then drives the water through the filtration assembly, up a vertical riser, and to the tanks via header pipes and taps. The water then passes through the tanks, exiting via passive or active overflow into gutters that connect into the downpipe, thus closing the circle.

Large Scale RAS Aquaria

When the aquarium application demands a larger scale operation than can be achieved with a stand-alone rack or two, the most common solution is to combine several racks together with a central filtration assembly. These multilinked systems offer the advantage of high holding capacity with enhanced space efficiency at a relatively low operating cost. While the basic elements defining RAS aquaria remain in large-scale production facilities, there are additional elements to consider that impact the function of large multilink, central life-support (CLS) aquariums.

The greatest benefit that comes with a multilinked CLS aquarium is that hundreds to thousands of relatively small tanks can reside on a single secondary enclosure; thus, maintenance only needs to be performed on one life-support system making the labor contribution per tank significantly lower than other aquarium designs. There is, however, a significant trade-off that comes with this gain in efficiency, and it must be weighed against the risks to the research application before its suitability can be accepted. With one life-support system supporting so many tanks, biosecurity in the aquarium is a significantly enhanced challenge, as a disease outbreak can rapidly circulate to the other tanks and animals residing in the common recirculating water. Therefore, as more racks are committed to a multilinked CLS aquarium during the design phase of the facility, it is essential to ensure that adequate capital is allocated to the filtration assembly in order to ensure maximum biosecurity for the system. As a guide, the "Plus 10" approach is an excellent means of ensuring sufficient CLS capability. This approach suggests that if the final RAS aquarium will consist of 50 racks, and 18,000 L of water, then the CLS should be designed to support the biological load of a 55 rack, 20,000 L system, or 10% more than the maximum carrying capacity of the aquarium. This will ensure that even if the aquarium is pushed to capacity for an extended period of time, water quality and life-support condition will not be compromised.

As the scale of the aquarium is considered, it is critical to ensure sufficient water volume is available in the CLS to buffer against water chemistry changes in the system. For example, as the fish respire, and as the biofilter converts ammonia waste, the water in the aquarium is acidified as a result of an introduction of carbon dioxide and hydronium (H^+) into the water column respectively. If there is not sufficient hydro volume in the aquarium, this change will progress quickly, making it difficult to manage appropriate chemical additions into the water that would be necessary to compensate the culture condition change.

Currently, there are two common strategies for ensuring a sufficient volume of buffering water in a zebrafish RAS aquarium: a single large technical sump or a series of multilinked sumps that reside under the main holding racks. While both methods are effective, each has its limitations.

The under-rack sump option is an ideal solution when there is little flexibility with regards to useable space for the aquarium (Fig. 26.10). By storing the buffering water in the space below the tank holding space of a rack, it is possible to store the majority of the aquarium's volume within the tank room, requiring only a

FIGURE 26.10 Under-rack Sumps. A bank of four holding racks, fitted with under-rack sumps.

small additional space for the filtration assembly and technical sump. As a result, it is possible to house significantly larger systems in relatively small facilities.

The most significant drawback of the under-rack sump solution is the fact that the main buffering volume of the aquarium is stored as "dirty" water. As wastewater leaves the tanks and enters the rack's drainage system, the water flows into the downpipe and typically passes over a coarse prefilter pad before it accumulates in the under-rack sump. The water does eventually pass into the filtration assembly and is cleaned before being reused in the housing tanks, but the wastewater that accumulates in the under-rack sumps moves significantly slower than the rest of the aquarium's water flow, and as a result suspended solids (fines) settle out and begin to accumulate in the base of the sumps. While this accumulated mulm itself is not necessarily harmful to the aquarium system, it can become a food source for a plethora of micro-fauna, including potentially pathogenic bacteria. The accumulation and digestion of mulm also result in significant inefficiency within the biological system of the aquarium, as heterotrophic bacteria compete with the nitrifying bacteria for resources, such as alkalinity and dissolved oxygen within the water as they feed on the mulm. This side effect of leveraging an under-rack sump solution is readily managed by regularly servicing the sumps. If the aquarium is fitted with a disposable depth filter (sock), this service may be as simple as agitating the sumps to re-suspend the accumulated mulm so that the mechanical filter can remove the fines. This should obviously be performed prior to a scheduled filter service. If the aquarium is instead fitted with a surface filter, such as a drum filter, then some of the solid waste particles will be finer than the filter screen and will simply pass through the filter. In this instance, siphoning the mulm from the bottom of the sumps would be an appropriate means of managing the situation.

If there is more space available for the CLS when the aquarium is being designed, it is possible to avoid the complications of under-rack sumps by increasing the size of the technical sump to accommodate the buffering water volume. Allowing opportunity for increasing the depth of the sump this large central sump can either be sunk into the floor of the facility, or it can simply reside as a large above-ground holding tank (Fig. 26.11).

The main benefit that comes from a central sump design is that it allows the buffer water to be stored in a somewhat cleaner state. As wastewater leaves the rack, the downpipe directs it into another wastewater pipe that channels the water to the filtration assembly, then typically, directly into a first stage mechanical filter

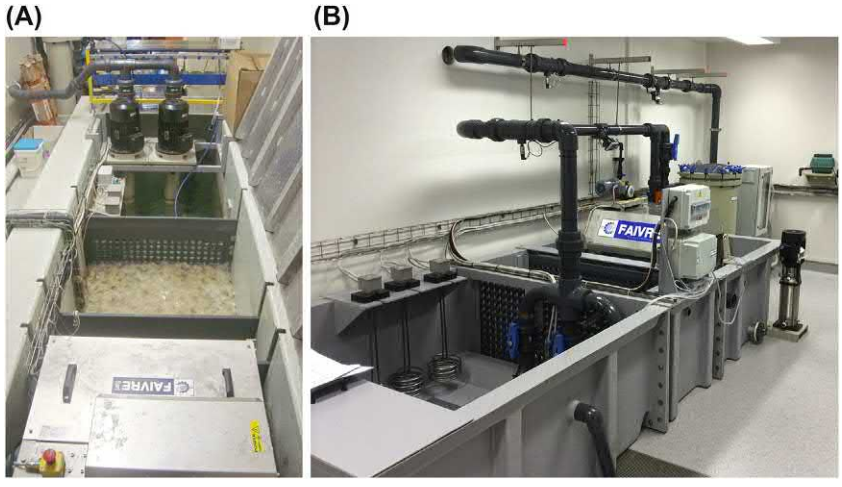

FIGURE 26.11 CLS Sumps. Large central life-support sumps can be sunk into the floor of the facility (A), increasing the maximum depth of the sump, effectively holding the system's buffering water volume vertically. When this option is not available, the CLS sump can reside as a large above-ground holding tank but will take up significantly more floor space as the water volume is held horizontally (B), unless the system racks are at a significantly higher elevation. *Photo B courtesy of Glen Turner.*

or dirty sump. Thus, the water can be processed by the filtration assembly prior to being "stored," limiting the volume of water capable of supporting unwanted microbiota. While this solution allows the first stages of the RAS process to complete (removing solid wastes via mechanical filtration, converting nitrogen wastes via biofiltration, and possibly even stripping the water of DOCs and other colloidal solids in the chemical filter), it is prudent to ensure that the disinfection step always occurs immediately prior to the water being returned to the holding tanks, as effective disinfection is transitory in a recirculating system.

Water Loop

With buffer volume addressed, the next major decisions will center on the recirculation of the water. A major challenge in designing a large-scale zebrafish aquarium is being able to manage the dynamic pressure changes that occur within the aquarium as depth filters foul and as more weep points emerge in response to the opening of taps as tanks are added to the system. These challenges are addressed in the water loop strategy of the aquarium.

A dual-loop system, similar to that operating at ZIRC, the Zebrafish International Resource Center (Varga, 2011), requires the use of multiple pumps to circulate the water through the aquarium and is typically employed when the filtration assembly is not able to rely on gravity pressure to drive the mechanical filtration step. In this scenario, the aquarium will typically drain into a wastewater collection sump from which the filtration pump will draw water. The dirty water is driven through the filtration assembly and returns into a clean sump. The clean sump is typically fitted with an overflow that returns water to the dirty sump if unused. This is the first loop. The second loop is driven by the circulation pump, which drives water from the clean sump via a disinfection chamber, through the header pipes to the tanks. The water then drains from the holding tanks into the dirty sump. The circulation loop is also typically fitted with a rack bypass, allowing water to be diverted directly into the dirty sump when the holding racks are sparsely populated. An example of a dual-loop system is illustrated in Fig. 26.12.

The benefit of a dual-loop system is that it allows greater flexibility in filtration assembly design, while also ensuring that tank circulation is not impacted by changes in the hydrodynamics of a fouling filter. Dual-loop systems also have a significant disadvantage, however, which is the need to balance the pumps. If the circulation pump has a greater draw from the clean sump than the filtration pump is delivering, water level

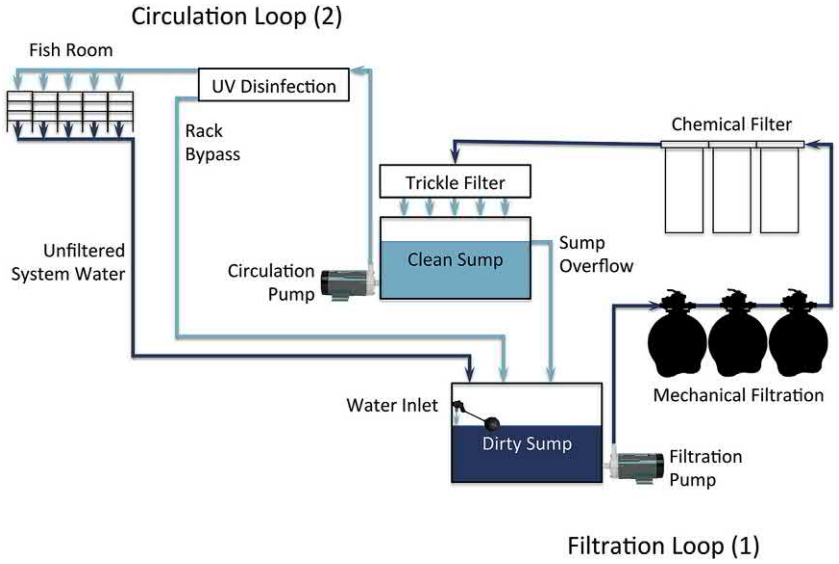

FIGURE 26.12 Dual-loop RAS aquarium. The Filtration Loop (1) processes the effluent (used) water, making it suitable for reuse. Used water drains from the holding tanks and is collected in the Dirty Sump. The used water is pumped through the mechanical filtration assembly, eliminating the large particulate wastes. The water is then cycled through an activated carbon chemical filter, removing the fines and colloidal solids, including dissolved organic compounds, the carbonaceous wastes that yellow the water and act as a food source for heterotrophic bacteria. Finally, the water passes over a trickle filter before entering the Clean Sump. The trickle filter serves as both a biofilter and gas exchange column. Denitrifying bacteria populate the oxygen-rich filter, converting ammonia waste into less toxic nitrite and nitrate. Water from the Clean Sump can reenter loop one by overflowing into the Dirty Sump, or it is pumped into loop 2 as required. The Circulation Loop (2) pumps water from the Clean Sump through the UV disinfection chamber to the header pipes servicing the tanks in the fish room. In the fish room, water passes through the holding tanks, emerging as used water entering a central wastewater drain and returns to the Dirty Sump (Loop 1). Loop two also contains a rack bypass, allowing water to be cycled directly from the Clean Sump to the Dirty Sump, reducing rack pressure of a lightly populated fish room, and simplifying the pump balancing process.

will begin to drop, and could lead to the circulation pump pulling air into the water as a result of a vortex forming near the pump pickup, which in turn, could result in a Gas Bubble Disease (GBD) event.

For any given temperature, salinity, and barometric pressure, there is a maximum stable level of dissolved gases within the water. When this is exceeded, the total gas pressure (TGP) is in an unstable, super-saturated state. In a recirculating aquarium system, the TGP will remain supersaturated while in the header pipes, because the water is under pressure; however, the dissolved gases begin to release from the water as soon as it is freed from the pressurized condition. In a RAS aquarium, this occurs in the holding tanks, exposing the fish to a potentially lethal situation. In extreme cases, it is possible to observe tiny bubbles effervescing out of solution; however, GBD is usually far subtler, manifesting as microscopic bubbles in the soft tissues of the fish, leading to death from asphyxiation following ischemic necrosis of gill lamellae, as the air bubbles disrupt the blood flow (Pauley & Nakatani, 1967; Weitkamp & Katz, 1980).

There are several options available to control the risks associated with GBD and pump imbalance. A TGP sentinel probe offers a safeguard against GBD by allowing the aquarium system to have an automatic cutoff trigger. In a dual-loop aquarium, the TGP sentinel probe is coupled to the circulation pump and monitors the TGP in the header pipes. If the TGP rises above 100%, a relay is triggered that cuts power to the circulation pump. The filtration pump can continue to circulate without risk to the fish, depending on system design. Although the fish are now at risk from complications due to the lack of circulation, the lethal effects are significantly slower than GBD, giving the operator an opportunity to address the fault. While effective, TGP sentinel probes require regular maintenance and calibration to ensure they are functioning correctly.

To effectively protect the aquarium from GBD, it is important to measure TGP and not rely solely on shifts in dissolved oxygen as a guide for GBD risk, as nitrogen is a significantly greater percentage of the atmosphere. It is thus, possible to experience a GBD event when dissolved oxygen is below saturation.

While there is no better substitute available for safeguarding against GBD, in a dual-loop aquarium it is prudent to also install level sensors in the sumps that allow an operator to shut down pumps to ensure there is no risk of drawing air into the system if the pumps get out of balance. In some cases, an aquarium life-support computer can also manage balancing, if the system is fitted with Variable Frequency Drive (VFD) pumps.

A VFD is used for adjusting the flow of a pump to the system's actual demand by controlling the frequency of electrical energy running the pump. It is most typically applied on aquariums that employ larger three-phase pumps. However, recent advancements in this technology have allowed the development of VFDs also to control smaller single-phase pumps. Aside from allowing enhanced control over a pump's performance, VFDs also allow the pump to constantly run at peak efficiency, by matching the pump curve to the system curve.

In a single loop, or dead-leg system, the challenge of pump balancing does not exist, but there are other challenges coupled with relying on a single drive point to maintain both filtration and circulation. As with the dual-loop aquarium, a dead-leg system will experience significant shifts in the hydrodynamic resistance as filters foul, and tanks require more available water flow. As a result, VFD pumps are a staple element of a single loop aquarium.

In order to achieve sufficient flow through every tank at maximum holding capacity, the pump needs to be capable of delivering sufficient flow velocity to overcome the resistance that mounts as more taps are opened and as filtration media fouls. In addition to these obvious sources of resistance, it is important to remember that zebrafish aquarium system curves have a significantly greater resistance than other aquaculture facilities of comparable size, because of the number of header pipes carrying water to the tanks, and the narrow diameter of the final outlet from which the water must pass into the tank. Without the sophistication of VFD pumps, a large dead-leg zebrafish aquarium would need to either run the system at capacity all the time, creating a significant burden from the need to clean unpopulated tanks in order to maintain system hygiene, or it would need a large bypass valve that could be gradually throttled as the system population grew.

Flow-Through Systems

While the vast majority of commercially available zebrafish housing solutions employ recirculating aquaculture technology, there are applications when recycling the culture water is not desirable. The application of flow-through technology is the solution to this need. In recirculating aquaculture systems, the culture water used to house and grow the aquatic organisms is captured, treated, and reused, thereby, greatly reducing the total water volume consumed in the rearing process. In flow-through or single-pass systems, new clean water is delivered to the tanks, and the wastewater flowing out of the tanks is rejected from the aquarium, typically to the municipal sewerage system. For this reason, flow-through aquarium systems are normally used only for applications that require the complete isolation of one tank from another.

Toxicology and environmental manipulation experiments are the archetypal applications that implement the single-pass system. With flow-through technology, each tank or series of tanks can be exposed to different chemical agents without contamination of others. As the water exits the tank, it is routed to the sewer, and thus, poses no cross-contamination risk to other tanks on the system. Flow-through technology is also appropriate to apply to high-risk quarantine scenarios in which imported fish may be suspected of carrying a disease that can have high pathogenicity and be difficult to contain. Such a disease is likely to have a significant detrimental impact on other fish residing under the heightened biosecurity control of a quarantine aquarium, and use of a flow-through system can greatly reduce the dangers of cross-contamination that would be present in a RAS.

Aside from the increased operational cost associated with replacing 100% of the system's water volume every pass, there is also a significant challenge associated with controlling the culture conditions of the incoming new water. In a RAS aquarium, it is logical to choose demineralized water as the source water for the aquarium, giving the operator complete control over the fish ready water that circulates through the aquarium. The operator simply changes the chemical mix added into the makeup water to alter the culture condition. In a flow-through system, manipulating the water chemistry to this extreme would be prohibitively expensive. As a result, most flow-through aquaria need to accept that the source water will largely determine the culture condition. For most operators, this means being limited to using the municipal water supply, and applying basic treatment and disinfection to the incoming water on its way to the header pipes. As zebrafish are tolerant of a wide range of culture conditions, having the basic water quality dictated by the municipal authorities is not a major issue provided the operator is aware of the potential for fluctuation in the water chemistry as the seasons change. In some cases, a facility may be able to utilize a natural water source, such as a local stream or bore; however, the basic water chemistry of the source water will still be the defining culture condition of the system. As a minimum, the incoming water should pass through an activated carbon filter, to strip heavy metals and chloramines from the water, and then pass through a UV disinfection chamber to knock down potential pathogens residing in the water source.

Advanced Applications

Beyond the major decisions surrounding the core housing solutions for a zebrafish facility, consideration also needs to be applied as to whether there is need for additional special housing elements.

Genotyping Racks

One of the most common activities that occurs in a large-scale zebrafish production facility is the need to isolate individual fish while they are genotyped. Traditionally this meant that fish would need to be maintained in small static tanks for several days while their genotype results were obtained. During this time the fish cannot be fed, as uneaten food, and wastes released from the fish after eating, rapidly degrades the water quality, even to levels that could result in death.

Recognizing this technology limitation, some commercial manufacturers now offer specialized genotyping racks in the approximate footprint of a typical housing rack and designed to hold up to 288 fish in isolation while maintaining water circulation (Fig. 26.13). This means the fish can continue to be fed while in isolation, addressing the first of the five freedoms. This is a significant enhancement in the animal welfare associated with the challenge of genotyping.

Automated Heat Shock Systems

Heat shock is a powerful technique for inducible gene expression systems, and the zebrafish is well suited to exploit the system, with the existence of over 1300 transgenic fish lines carrying a construct under the control of the heat shock promoter 70 (*hsp70*; Zfin transgenic database; http://zfin.org/). Heat shock treatment of developing zebrafish embryos is relatively simple to perform because the fish are largely stationary, but more importantly, they do not require water circulation.

Heat shock treatment of adult fish is more involved as there is a need to maintain circulation as their culture medium is raised to the necessary shock temperature.

FIGURE 26.13 Genotyping Rack with under-rack sump. *Photo courtesy of Tecniplast Aquatic Solutions.*

While this challenge can be addressed by simply transferring the fish from housing tanks to treatment tanks, this approach requires significant handling, which is labor-intensive and increases stress levels in the fish. In larger recirculating systems, when only a few tanks of adult zebrafish require regular heat shocking, it is possible to place in-tank heaters with activation timers into each of the required tanks to avoid the need for excessive handling (Duszynski, Topczewski, & LeClair, 2011). This technique, however, also requires regular calibration of equipment, and strict monitoring of treatment conditions to ensure that tanks are exposed to statistically equivalent conditions. While this works well in treating a handful of tanks for short-term experiments, if a more pronounced commitment to heat shock techniques is desired, the major limitation of the RAS system is that it is not amenable to scalability.

While, currently, there are no off-the-shelf, scalable solutions to heat shocking adult zebrafish, it is possible to modify existing aquarium housing designs to enable heat shock treatment in conditions of continuous water flow, without impacting the standard culture condition of the main circulation loop. By adding an additional manifold to an aquarium holding rack, and by modifying the wastewater return path to circulate water from a treatment water bath, it is possible to deliver heat shock conditions to an entire row of tanks with precision and minimal variability between treatment tanks (Saera-Vila, Kish, & Kahana, 2015). This system can be easily scaled to suit demand and offers greater assurance of maintaining optimal culture condition throughout the RAS aquarium. In addition, this design modification, illustrated in Fig. 26.14, can be further enhanced through the use of solenoid valves to control manifold change over, offering a fully automated heat shock solution that avoids unnecessary handling stress, and ensures best water quality and fish welfare at all times.

Introduction to Defined Flora

Zebrafish are also breaking new ground in the realm of gnotobiology. The term "gnotobiology" (*gnos*, known; *bios*, life) is used to describe the study of animals raised

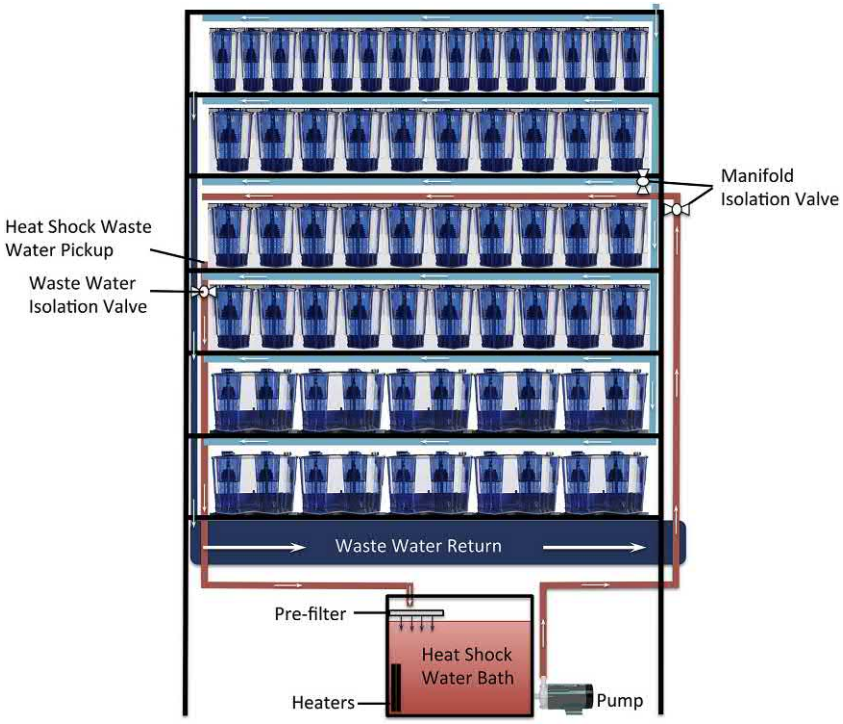

FIGURE 26.14 Heat Shock modification of a multilink zebrafish housing rack. The recirculating heat shock (35°C) system (shown in *red*) resides under the housing rack. A small pump circulates hot water to an additional water distribution manifold, and a secondary wastewater drain returns the effluent water to the heat shock water bath, closing the loop. The manifolds and drain of the regular (28°C) system are shown in *blue*. For exposing the fish to the treatment, the manifold isolation valves are switched (closing the regular manifold valve, and opening the heat shock manifold valve) and the heat shock circulation pump is turned on. The main system drain of the gutter of the heat shock tanks' shelf is closed, diverting the heated effluent water into the extra heat shock drain, returning the water to the heat shock water bath, avoiding heat contamination to the rest of the system. On conclusion of the treatment, the heat shock circulation pump is turned off, the main system drain is opened, and the manifold isolation valves are returned to their normal state, allowing normal system water to mix with the heated water in the tanks, gradually returning holding conditions to normal.

in the absence of microorganisms or in the presence of known microbial strains or communities (Reyniers et al., 1949). Just as a geneticist performs genetic manipulations to study loss and gain of function, gnotobiologists test the necessity of microbiota by rearing animals in the absence of microorganisms and then adding one or more defined microbial strains to test sufficiency (Melancon et al., 2017). The first concept of studying germ-free and defined flora animals began back in 1885 when Louis Pasteur hypothesized the impossibility of animal life surviving in the absence of microorganisms. Since then, mammalian models have dominated gnotobiology, with various fish models playing only a minor role. The plethora of attributes that make the zebrafish model system popular with geneticists, including rapid external development, optical transparency, large brood size and ease of housing, are also the key attributes propelling zebrafish as a gnotobiotic model. In fact, the use of zebrafish is enabling vertebrate gnotobiotic experimentation on a scale not possible with the traditional mammalian models (Melancon et al., 2017).

The vast majority of gnotobiotic studies employing zebrafish to date have focused on the use of larvae, in part due to the challenges of long-term germ-free zebrafish husbandry methods. For successfully administering long-term gnotobiotic zebrafish husbandry requires consideration of animal housing, water quality, and exchange, waste removal, nutritional value of food, live food culturing, work flows and space requirements, record keeping, supplies, expenses, and labor. Procedures for long-term gnotobiotic husbandry are evolving. Melancon et al. (2017) have reported success in raising germ-free zebrafish for up to 1 month; however, the process was extremely labor-intensive. For more information on techniques, please also refer to the chapter in this book on gnotobiotics.

Presently, two general housing systems can be employed to raise gnotobiotic zebrafish. Germ-free zebrafish can be maintained outside of a gnotobiotic isolator by rearing the fish in sterile tissue culture flasks or multiwell plates within a biosafety cabinet, using standard sterile techniques (Melancon et al., 2017). Each flask or well can represent a different microbial condition, allowing multiple microbiotic conditions to be studied in a single experiment (Pham, Kanther, Semova, & Rawls, 2008). This brings gnotobiotic experimentation within the grasp of most investigators working with the zebrafish model. However, scalability of growing gnotobiotic zebrafish in flasks has significant limitations. The daily requirement to service these experimental housing tanks increases husbandry labor, which, in turn, increases the opportunity for contamination; this can become financially limiting as throughput and longevity increase. Culture flasks and plates are, therefore, ideal for experiments that require analysis of multiple different microbial conditions with relatively few subject animals per condition and/or can be completed within a short time course with minimal manipulations to reduce the risk of contamination (Pham et al., 2008).

The second and more rigorous method of rearing germ-free zebrafish is to rear them within a purpose-built gnotobiotic isolator as described by Pham et al.

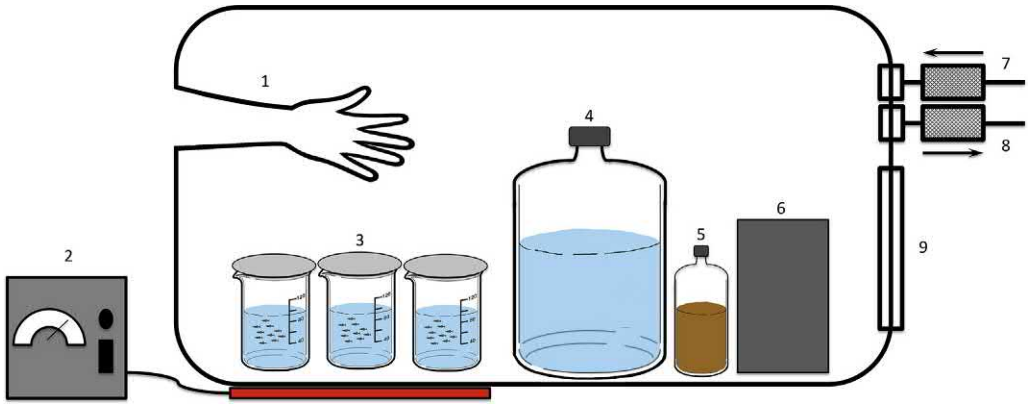

FIGURE 26.15 Schematic diagram of isolator equipped for gnotobiotic zebrafish husbandry. The gnotobiotic isolator allows zebrafish to be reared for extended periods in a defined flora environment. The gnotobiotic isolator includes attached gloves, allowing sterile interaction with the inhabitants (1). A heating pad or heated water pump located directly below the specimen holding tanks allows control of isolator operating temperature (2). Foil-covered beakers containing zebrafish in gnotobiotic zebrafish medium (GZM) act as static specimen holding tanks (3). Carboy of sterile GZM allows routine water changes in holding tanks (4), and a second carboy collects the wastewater. Bottles of sterile zebrafish food (5), and other supplies (6) are also stored within the isolator. For maintaining a sterile environment, the isolator is kept under positive pressure, with HEPA-filtered air supply (7). Air exhaust is likewise typically HEPA-filtered (8). The gnotobiotic isolator also has a sealed port to enable the transfer of materials to and from the sterile enclosure (9). *Figure adapted from Pham, L. N., Kanther, M., Semova, I., Rawls, J. F. (2008). Methods for generating and colonizing gnotobiotic zebrafish.* Nature Protocols, *3(12), 1862.*

(2008). In this work, fish were kept in a flexible film isolator maintained under positive pressure and supplied with high-efficiency particulate air (HEPA) filtered air (Fig. 26.15). This device allows sterilized food, water, and supplies to be introduced into the isolator via a sealed port, and manipulations are performed using attached gloves. Providing the gnotobiotic isolator is of sufficient size, large quantities of sterile food, water and supplies can be maintained within the gnotobiotic environment, reducing the need to open the isolator port to the external environment, and thereby, reducing the risk of isolator contamination. A single isolator can house a relatively large number of zebrafish, but the limitation of this device is that all animals within the isolator are subject to the same microbial condition. In a typical experiment utilizing a gnotobiotic isolator, it is possible to house 360 germ-free zebrafish embryos for a single experiment by distributing them into 12 400 mL, foil-covered glass beakers, each containing 30 fish in 100 mL of gnotobiotic zebrafish medium (water) (Pham et al., 2008).

Acquisition of gnotobiotic isolators requires a significant initial financial investment, and their routine maintenance is relatively laborious; thus, gnotobiotic isolators are best suited to experiments that require gnotobiotic animals be reared for extended periods of time or for experiments that require large numbers of animals to be exposed to the same microbial condition (Pham et al., 2008).

As zebrafish gnotobiology expands, it is likely that specialized aquarium systems will be developed to allow the maintenance of gnotobiotic zebrafish throughout their life cycle. Melancon et al. (2017) postulate that a combination of bioreactor-like stand-alone aquarium systems, similar to vessels that have already been developed for other fish (Forberg, Arukwe, & Vadstein, 2011), could be modified as follows for this purpose. A system combining equipment that would allow automated media changes, control of feeding and colonization, and prevention of accumulation of toxic byproducts together with interconnected minibioreactor technology previously developed for culturing microorganisms (Auchtung, Robinson, & Britton, 2015), might allow large-scale screening of gnotobiotic zebrafish. This could allow zebrafish to become the only gnotobiotic vertebrate model for which large-scale, long-term husbandry is possible.

Summary

It is an exciting time to be involved in the husbandry of zebrafish. A steadfast and deliberate commitment for the strategic development of housing solutions is needed to support its continued development as a preeminent model. The extent of research program support will determine the scale and sophistication of the available solutions to be developed. While a simple box tank on the bench may be sufficient to meet the needs of an independent investigator seeking to address straightforward questions, a complex central life-support RAS aquarium will allow hundreds to thousands of genetically unique lines to be maintained in a cost-effective manner, supporting a number of investigators focused on answering more complex problems. The experimental application of the model will dictate the materials required to construct optimal primary and secondary enclosures and the types of manufacturer options needed for appropriate housing flexibility. Specific tank design will depend on the goals of the intended research programs utilizing the aquarium. Housing and breeding for maintenance may be all that is required for some research programs, but others may require the production of vast quantities of synchronized embryos on demand, an ability to monitor physiological response to oxygen limitation, or the effects of defined gut flora.

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CHAPTER

28

Zebrafish Aquatic Systems: Preventative Maintenance and Troubleshooting

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Preventative Maintenance

Preventative maintenance is an important safeguard against systems failure. A preventative maintenance program can protect against the loss of valuable research animals and equipment while avoiding costs for their replacement. "Where possible the carrying capacity of a system must be increased with-out increasing cost or sacrificing system re-liability" (Losordo & Westerman, 1994). The size, scale, and design of the maintenance program needed in a facility is determined by carefully weighing the cost for its implementation in time, labor, and financial and intellectual resources against the costs incurred, as a consequence of failures. Defined tasks and timelines for their performance must be organized into a maintenance schedule, which should then be followed to assure that the system components are routinely and systematically evaluated and preserved. These tasks, including but not limited to cleaning, replacement of worn parts, observation of performance, and adjustments made based on observations, will increase the longevity of the equipment and the system. These tasks should be performed while the equipment is still functioning normally to prevent the risk of breakdown or failure. Preventative maintenance is also an essential component of disaster planning for aquatics facilities and can prevent catastrophic failures of a system.

The first source for critical maintenance information is any materials provided by a manufacturer with the equipment or the manufacturer's customer service website or call center. These resources often include troubleshooting guides that can be beneficial for identifying components requiring maintenance or servicing. Facilities must identify key equipment that requires servicing, the recommended frequency of servicing, equipment needed to perform the service, and risks encountered during the service to the life support system and the employees.

There is a finite shelf life for most components of an aquaculture system, and it is important to understand the life expectancy of equipment and materials so that a plan for appropriate replacement can be made. All equipment will deteriorate over time, and that timeline can be accelerated with heavy usage. Regular monitoring and testing of equipment is a good way to identify components that need replacement prior to failure. Replacing components before failure is much less stressful to the life support system and the animals.

"The quality of a reliability analysis strongly depends on the analyst's ability to identify all the required functions–and hence all the failures–of the item that is subject to analysis. Classification of all required functions including essential, auxiliary, protective, information, interface, and superfluous function of equipment must be made and then from this the failure points and expectations can be assessed" (Rausand & Øien, 1996). Understanding the rates of expected failures can assist with keeping a stock of backup or replacement parts that meets the needs of the system while avoiding waste from the expiration of these parts before their use. For example, rubber O-rings are frequently used at machinery junctions in aquaculture systems and often wear out from use and/or age. Spares kept on a shelf too long or in an area that is dry can also age before use and undergo dry rotting and crumbling. PVC and other piping materials can also become brittle and rotten from age or exposure to heat, UV, or harsh weather. So, while it is important to keep spare parts, these must also be assessed periodically for functionality so that useful spares are available when needed.

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Preventative maintenance should be performed on any piece of equipment critical to systems operation and to any equipment with a high likelihood of failure over time. Maintenance tasks may require engineering, plumbing, electrical, and digital monitoring expertise. Monitoring alarms and digitally controlled components may have specialized modes, in addition to failure modes, that will need to be identified and understood.

Many manufacturers and technical service companies offer preventative maintenance or service agreement contracts. For those facilities that do not have employees with the right technical expertise or skills to perform routine maintenance, such contracts can be a useful means for obtaining service of equipment required for maintaining proper function.

This chapter will discuss aquatics facility life support components and directions for establishing and carrying out a preventative maintenance program, including monitoring of facility life support systems; documenting of maintenance performed; providing for redundancy; troubleshooting; and verifying the correct function of facility equipment.

Record Keeping

It is essential to systems maintenance to have clear and reliable records of all tasks performed to maintain the facility, including the dates of their performance. These records should document tasks performed for operation and maintenance of both the Physical Plant and the equipment in that plant. Records should be inspected frequently to ensure that critical maintenance work is being performed on schedule. Some parameters should be monitored daily, while others may be monitored less often.

Records should be maintained for:

1. Daily individual animal room environmental conditions: light cycle, temperature, and ventilation. This data may be gathered by computer monitoring equipment or read directly by handheld instrumentation, such as a thermometer.
2. Daily life support maintenance
3. Other maintenance work to be done
4. Completed work
5. Emergency maintenance calls
6. Loss of electrical power
7. Monthly life support maintenance
8. Semiannual life support maintenance
9. Annual life support maintenance

With good, clear record keeping and regular review, facility functionality can be monitored, and trends noted. Observation of trends in the data can allow prediction of facility abnormalities before they become failures. Record keeping allows a facility to learn from component failures by examining previous trending data. Regular record review is valuable for prevention of missed critical maintenance. It also allows for a seamless exchange of critical information should multiple, or new staff members need to coordinate review and performance of maintenance.

Redundancy

Aquaculture life support equipment must remain in operation continuously in order to maintain an aquatic environment that is ideal for the species of interest. This need is shared among all operations relying on mechanical systems, which are critical to functionality. As the NASA Preferred Reliability Practices state, "The probability of 2 redundant components failing during a critical time period is much less likely than 1 component failing during the same period" and "All other factors being equal, the elimination of redundancy in the system described in this practice would result in a considerably higher probability of failure, the identification of additional critical items and increased probability of loss of life" (Practices, 1995). The constant usage of equipment and the need to minimize life support downtime for a repair or maintenance event makes component redundancy a wise thing to consider. Having redundant equipment minimizes the impact on water quality and safety for the animals in the event of a repair and can allow facilities to avoid complete shutdowns of life support. This reduces potential irregularities in water quality, temperature, and other critical environmental parameters.

There are pros and cons to be considered when designing for functional redundancy. Redundancy of critical components increases the reliability and efficiency of the system. It can also reduce critical time loss in an emergency or repair situation. However, redundancy can create a more complicated operating system, which may be more prone to error or accident. Redundancy can also lead to increased human neglect because of personnel reliance on redundancy in place of day-to-day care. In a limitless environment where space and cost are not a factor, having redundant critical components in place allows for a seamless transition when needed. However, often, the ability to include redundancy must be tempered by space and financial resources available in a facility. If having integral redundancy is not an option, facilities should consider keeping an inventory of parts that are critical, hard to find, or take a long time to acquire or fabricate. Examples include water pumps and motors, water monitoring probes, and spare valves.

At a bare minimum, facilities should be able to provide redundancy to components critical for the provision of power and control of temperature as part of disaster planning. As systems increase in size and complexity, there is a higher likelihood of failure and a higher need for redundancy. For systems that rely on computer automation for their life support controls, manual override features should be required to ensure that the system can still be manipulated should the control system fail.

Redundancy should also be considered for hardware, software, and record storage. For areas that do not utilize physical redundancy, designing methods to allow for addressing failures even in a "fail-safe" condition can also protect against mechanical damage and animal loss. Creating a plan with a defined flow for how redundancy operates will help ensure that these redundancies are in place and working as needed. "Redundancy is indispensable to a world where technological risks must be closely regulated and where 'reliability' is construed as a variable that can be defined, calculated and designed" (Downer, Risk, Regulation, Economics, & Science, 2009).

Monitoring

A life support system (LSS) is a system that has been designed to sustain normal biological functions of a target living being or species. By replicating an organism's native environment through careful observation and available technology, this sustaining, native environment can be viably created where normally it would not be found. The target species is defined as the intended occupant of the LSS. In many cases, there can be other biological entities occupying the same space; however, the environment is not tailored to their optimum parameters for life, and so, these additional entities may not thrive. In some cases, this is an advantage as the other species could be pathogens that could harm the target species when present. By its nature, an LLS is often a struggle against entropy. The creation and maintenance of these artificially induced environments require energy proportional to the scale of the particular LSS to exist. Once that energy in the form of staff effort, power (electricity, consumable fossil fuels, etc.) and human prioritization and interest fades, so does the LSS. In short, if the LSS fails, the target species dies. For zebrafish used in biomedical research, the LSS required is an aquatic environment with the optimal characteristics but on land, and generally in a building, where there is increased control of temperature, light, and presence of pathogens.

Many zebrafish LSSs are designed as a closed-loop or Recirculating Aquaculture System (RAS). In a RAS, the water is recirculated so that a large portion of this water is reused over and over. "The RAS is a complex system requiring a high level of monitoring and maintenance, and solids management and biofilter operation and management are often the most difficult tasks in an RAS, constituting the main reasons for system failures" (Badiola, Mendiola, & Bostock, 2012). It is important to remember that this increased complexity results in a higher probability of system failure. Most large aquaculture facilities employ some level of automated monitoring. This can alleviate the necessity to have staff constantly monitor the system, make adjustments, and react to abnormal conditions. Operators must still, however, use the available resources wisely and, at times, sparingly.

Prioritization of Maintenance

All LSS need regular monitoring and maintenance. However, most facilities have finite resources to devote to these needs, and managers may often be faced with the dilemma of prioritizing certain or imminent failures over others in order to form a priority task list for operators to use when monitoring system functionality. "This list usually encompasses corrective maintenance for things that have failed, and preventative maintenance and can be broken down into Deferred Action, Immediate Action, Scheduled, and Scheduled as needed" (Niu, Yang, & Pecht, 2010). In an even more advanced breakdown, maintenance needs can be categorized as critical failures, medium priority failures, and low priority failures. Critical failures include losses in the function of system pumps/motors, large-scale plumbing failures, and failures of automated or alarm devices, which could result in imminent animal loss. Medium priority failures include subsystem failures like those of a sodium bicarbonate dosing system, of water quality monitoring systems, or of regenerative blowers for an auxiliary air input source, which could result in harm or death to the target species within 3 days. Low priority failures include small isolated plumbing problems or low-grade inconsistent system performance over time, which could lead to harm to the target species in a week's time. An operator must then be able to respond to critical failures immediately even if working on low priority failures simultaneously.

Fault Tolerances

Within every fabricated device, or even within parts of a device, lies an Achilles' heel particularly vulnerable to failure or to abnormal operation from excess pressure,

speed, temperature, or wear and tear. Many engineered devices have a built intolerance. In other words, a motor that is designed to last 100,000 h of operation might fail just before this or just after this. For accommodating this, a very rough rule of thumb is to plan to service that device at +/− 5%−15% of the specified predicted failure rate. This way, an operator can be prepared to apply appropriate service or replacement of that device when the actual need arrives.

Monitoring of Specific Devices and Subsystems

Electronic water quality devices are in continual use and are critical for the maintenance of the life support environment. These probes not only monitor and alert users to water quality concerns but often also drive the use and function of water chemistry control events, such as water changes or additions of chemicals to adjust pH and conductivity. It is important to verify probe readings with other secondary devices to ensure that they are taking accurate and correct water quality readings; otherwise, the aquaculture system will not provide an environment within the preferred water quality ranges for your animal species. The relays and electrical communications connections must also be regularly tested to ensure the monitor and control probes are able to properly alert and command as needed. In an aquatic environment, water is an added complication to monitoring probe equipment. Probes must be inspected regularly for water intrusion into the probes that will lead to failure of the device. The manuals provided by the manufacturer will contain the recommended inspection and servicing needs of the device. They will also contain recommendations on life expectations for parts and for expected expiration rates of calibration solutions. Replacing parts and calibration or cleaning solutions prior to expiration is important to avoid failure or inaccuracy of the device readings. Equipment manuals provide guidance, but depending on the device use and its location on the system, it may be necessary to check or service an item more or less frequently than recommended. It is important to remember that these manuals are a guideline, but realistic needs may differ.

Certain equipment used within aquatic animal facilities must be certified or calibrated on a regular basis and should be tested and serviced on a set schedule. Equipment may need annual, semi-annual, monthly, weekly, or daily testing, depending on the type of equipment used and the reliability of the equipment. Most of the general room macro-environment equipment should be calibrated and certified annually. This equipment includes instruments used to verify room temperature, humidity, light cycle and intensity, HEPA filtration, and autoclave and fume hood functionality. All annually certified/calibrated equipment should be tracked using an equipment log, often maintained by the buildings facility support staff. This document should note the equipment type, location, serial/property/ID number, and expiration if applicable of the items. This document should be reviewed monthly to ensure that all items are within their specific date ranges.

Facility aquaculture system monitoring probes should be calibrated in accordance with their accompanying manufacturer's manual. This is usually monthly or biweekly for most probes and involves reconditioning the probe by cleaning the electrode contacts, rinsing out the well encircling the electrodes with proper cleaning solution, and inspecting and replacing the membranes.

Pump Speeds, Water Flow, Water Change Cycles

For every aquaculture system, the requirements for pump sizing, water flow, and water change cycles should be calculated and incorporated into the system design by engineers. These specifications will be documented and set upon commissioning. Once the system is up and running, these values may need to be adjusted. Keeping clear records on the performance of these parameters is essential to ensuring that overall flow dynamics, energy use, and resource use fall within target ranges.

Biological Filtration Maintenance

Most modern aquatic life support systems have some form of biofiltration. This can range from something as simple as a mesh bag of biomedia that rests in a sump tank to a fully functioning biofiltration component in the life support loop. Biofilters rely on aerobic and anaerobic bacteria to break down chemical waste (ammonia) to less harmful compounds, and although the bacteria are not mechanical, the environment they need to thrive must be cared for and maintained.

"There are many factors and degrees of functionality that are needed to allow the biomedia filter to function, and proper water flow through the biomedia must be verified daily. This makes the function of a biofiltration unit a fuzzy logic problem that requires the understanding of degrees of functionality for multiple aspects" (Lee, Lea, Dohmann, Prebilsky, Turk, Ying, et al., 2000). Therefore, it is also recommended to check certain water quality parameters daily (ammonia, nitrite, nitrate) to verify biomedia functionality. If ammonia levels appear higher than normal, it may be important to check dissolved oxygen levels in the biofilter unit since low

oxygen can inhibit the function of the bacteria present. Establishing a record of the expected normal values for these readings will help to identify when the system is not performing to the best of its ability.

The goal is to ensure that the system will perform with maximum efficiency and longevity. Inspection of fluidized bed media is important to ensure that it is properly suspended. If it is not properly suspended, fluidized beds may channel or collapse. Channeling will prevent the bed from performing chemical filtration, and collapse will suffocate and kill the biomedia. It is also important to measure the level of biomedia present in the biofilter. Calculations can be done to determine the required amount of media needed on a system, and if media is lost, it must be replaced to maintain proper filtration capability. As the biofilter is an active live culture filter, detritus may accumulate on top of the biomedia. If an excessive layer of detritus has built up, it must be removed to ensure it is not suffocating or fouling the bacterial environment.

Degassing Device

The various dissolved gases (nitrogen, oxygen, argon, etc.) that many aquatic animals such as zebrafish rely on to respire and regulate pressure in the water column can also be the source of illness if not controlled. Supersaturation of the system with these dissolved gases can lead to Gas Bubble Disease, which occurs when the concentration of Total Dissolved Gases (TDG) of the water column is higher than the atmospheric pressure of those gases. When this happens, the gases will migrate into lower pressure areas, such as back into the atmosphere or into the tissues of aquatic animals on the system. This can damage the animal's soft tissues and can even lead to death. Supersaturated water can be prevented from reaching culture tanks and aquatic animals by placing a Degassing Device on the system. These range in design but are universally one of the most simple and least maintenance-intensive components of an aquaculture facility. Some rely on gravity water flow over very coarse media to break up the water column for a certain duration. Others use a countercurrent airflow over the water column area. The working principle of all Degassing Devices is to equalize the TDG pressure inside the water column with that of the gas pressure of the atmosphere, usually by increasing the surface area and contact time of the water column and allowing simple pressure equalization to occur. Because of their usually simple design and placement in line with plumbing, very little maintenance is needed; however, they still should be checked routinely. Verifying correct functionality can be achieved by installing TDG monitoring devices that measure the gas pressure in the water column and compare it with atmospheric pressure. These can be placed just after the Degassing Device and can alert system operators of a potential dissolved gas supersaturation event.

Mechanical Filtration Maintenance

Mechanical filtration on aquatic systems can be accomplished by a wide variety of equipment and servicing. The maintenance required for this filtration will vary, depending on the equipment used. Having a clear understanding of the end goals for the mechanical filtration on a particular system is the foundation for selection and care of its mechanical filtration components. The filters must be chosen to remove the appropriate size and volume of physical waste debris, and periodic evaluation of waste effluent released from the filter should be measured to verify its proper function. Things to be considered are types of solids, the number of solids, mesh or filtration size, flow capacity, and head pressure loss. If the filters are too small to capture the bioload or the filtration screening is too large to capture smaller particle sizes, mechanical filtration cannot perform efficiently to meet the needs of the aquaculture system. Some common mechanical filtration equipment examples and basic care are listed in Table 28.1.

If mechanical filtration is not properly maintained, various functionality problems can arise. If the media is not correctly sized, the filters are not cleaned or changed frequently enough, or the pumps, valves, or plumbing are not functioning appropriately, mechanical filtration can fail. Solids buildup can stress the filtration seals, valves, and pumps due to excessive pressure buildup. Regular waste removal by element changing or backwash must be performed based on manufacturer recommendations for pressure limitations and burden maximums. Flow dynamics are an important part of mechanical filtration. Mechanical filters must be able to capture the waste material, allow the filtered water to continue to pass through the filter, and properly backwash themselves if they operate through a backwashing mechanism. Inadequate or improper flow to the filtration devices can prevent filters from achieving these critical functions. Pressure before and after the filter units must be maintained within range to ensure this functionality. As a filter clogs, the pressure to the unit will increase, and pressure leaving the unit will decrease. Pump and pipe size, filtration mesh sizing, and frequency of backwash or cartridge change out will affect this.

TABLE 28.1 Various mechanical filtration methods.

Filtration type	Considerations	General maintenance
Fluidized bed filters ex- sand or bead filters	- Sufficient media - Seals and O ring condition - Backwash schedule - Pump, sensor, and valve function	Daily: Functionality and leak inspection Periodic: Mechanical servicing to inspect media, pumps, seals
Gravity filters ex- drum or screen filters	- Condition of screen - Backwash sensor switches - Backwash schedule - Pump, sensor, and valve function	Daily: Screen condition, clogs, functionality, and leak inspection Periodic: Mechanical servicing to inspect pumps, sensors, and functionality
Pressure filters ex- cartridge or sock filters	- Pressure differential limitations - Filter cartridge change frequency - Seals and O ring condition - Pump, sensor, and valve function	Daily: Functionality, pressure, and leak inspection Scheduled: Filtration element changes Periodic: Mechanical servicing to inspect pumps, sensors, and seals

Biological Filtration

Biological Filtration is utilized in the LSS as a mechanism to break down chemical waste created from animal feces, urine, and feed and to process harmful components, such as ammonia and nitrites into less harmful components. For this filtration, the facility typically employs culture tanks that host particular aerobic and anaerobic bacteria. The microenvironment in these tanks must be examined and managed. Biological filtration relies on proper flow dynamics in order to create an environment where the bacteria can move about within the media to encounter food resources. These resources are both the waste from the animals and a steady streaming flow of water to flush out processed materials and bring in new nutrient sources. If the flow dynamics are not balanced, the bacterial colony can starve, suffocate under its own weight, or encounter an environment that is toxic.

For preventing waste buildup from interfering with the flow, the biofloc or bacterial colony must be kept in balance. The physical detritus from feces and feed waste must be properly aerated and flowing through the media suspension, and fouling must be prevented. If there is too much detritus, the bacterial colony will die from excessive waste chemicals. If there is not enough, it will starve. When maintaining a good biological filtration system, technicians must:

1. Inspect the water quality pre and postbiological filtration to ensure that the bacteria are breaking down the waste chemicals.
2. Periodically inspect and clean the biological media to remove excessive physical detritus.
3. Examine and ensure that there is appropriate movement of the biological media. All media should be rotating and turning over within the biological filter. If the balance of the water flow is incorrect, there will be channeling. Channeling means that the water is passing through a limited area of least resistance rather than all media. This allows the majority of the chemical waste to bypass the biological filtration, elevating harmful chemicals in the environment, and starving the biological filter bacteria colony.

Ultraviolet Lamps

Ultraviolet (UV) lamps are used in aquaculture for reducing bacterial growth and pathogen contaminants within a recirculating water system. They are only effective for these purposes if the UV bulbs are operating at appropriate strength and the UV light can penetrate the water to properly expose microbes to the UV. The bulbs have a very specific shelf life for effective strength and must be replaced before their light intensity decreases below this threshold. The quartz sleeves that encase them must also be regularly cleaned to ensure their light is able to pass through effectively to treat the water. Electrical wiring and control connections must also be carefully monitored and inspected to ensure there are no hazards and that there is proper control of function. As with any other filtration

equipment, flow rates, seals, and connections must be routinely inspected to ensure proper UV lamp function.

System Water Generation

Water used to make the system water for an aquatics facility can come from a variety of sources, including municipal, unprotected, or artificial; and maintenance of the equipment used will vary depending on the source. Municipal sources or tap water are in reality treated sewage, and these sources may contain chlorine or pathogens. Unprotected sources, including springs, wells, lakes, or reservoirs, can contain contaminants from agricultural runoff or wild flora and fauna. Artificial sources of water include water produced by reverse osmosis, distillation, and desalination. Typically for zebrafish systems, since chemicals and pathogens are a concern, no matter what the source water, an artificial method of cleaning the water is used before the water is made available to the animals. Artificial methods help to remove chemicals or pathogens from the original water source but also strip the water of many key elements needed to maintain the environment.

For proper maintenance of aquatic water generation, the source, equipment types used, and output result must be understood. Speaking with personnel from facilities support and local municipalities can help identify key information needed about water sources. For artificial water sources, often a preventative maintenance contract with the equipment provider is required if special servicing of the equipment is needed. Membranes, filter elements, UV bulbs, and other components will need changing and cleaning on a set schedule. It is important to understand this schedule and ensure that these tasks are being performed properly and on time. Emergency repair service options should also be considered.

Maintenance and monitoring that can be performed by facility staff includes monitoring of the pressure of the water coming in and out of the mechanical units, the temperature of the water, and provision of any chemical supplies, such as water softener, to ensure operation within the manufacturer's recommended range. Facility staff could also inspect electrical components, valve configuration and functionality, and alarm equipment. Artificial water generators produce wastewater as a byproduct of the purification process. This wastewater output should be monitored to ensure that the correct ratio of product versus. wastewater is being produced. If the machine is unable to process the expected amount of product water and is diverting a larger portion to wastewater, it needs servicing.

An example of the maintenance care for a large Reverse Osmosis water generator would be as follows:

(1) Daily
- **(a)** General System Check, to include:
  - (i) Unit Power (on and not in alarm)
  - (ii) No obvious leaks,
  - (iii) Appropriate incoming pressure (30–60 psi)
  - (iv) Appropriate incoming water temp (77° F, ±10°)
- **(b)** Identification of booster pump running lead, 1 or 2
- **(c)** Unit discharge temp (82–88°F)
- **(d)** Permeate Flow rate (between 3.0 – 3.5 gpm)
- **(e)** Unit Conductivity (0–15 micro-Siemens/cm)
- **(f)** RO Tank Conductivity (0–50 micro-Siemens/cm)

(2) Weekly
- **(a)** Confirm booster pump lead/lag switch
- **(b)** Check for salt in water softener; if depleted, add solar salt

(3) Monthly
- **(a)** Cleaning of RO storage tank
- **(b)** Testing of high and low-level sensors in the RO storage tank.
- **(c)** Record visits by the vendor

Makeup water often needs to have the salts, and other essential environmental components that have been removed in purification added back in. This is often done by hand or by chemical dosing equipment. This equipment operates based upon probe readings, and as with any other probe-based system, upkeep, calibration, and testing must be performed regularly to ensure proper function of these probes. Doser pumps must also be examined, and solution dosing measurement must be controlled and measured. There are very specific salt and elemental requirements for aquaculture systems. For saltwater species, this can be very complex, but for zebrafish the general use or custom premixed salts commercially available are acceptable. It is important to be aware of the levels of elements, including magnesium and calcium in the salt mixes. Refer to the systems design and water quality chapters for more information on requirements for water quality.

Pump/Motor Monitoring

Modern Closed-loop aquaculture systems rely on the constant recirculation of the aquatic environment through various waste removing and filtration devices before its return to the culture tanks. Water must be constantly moved through the system by submersible,

horizontal, or vertical water pumps, or by air pumps utilizing air-lift. When failures of the pumps/motors occur, target species can face imminent harm and perhaps death. Pumps are thus critical components and must be put very high on the list of items to monitor. Most frequently, motors and pumps need to be checked and possibly corrected for vibration, and internal bearings should be greased (if unsealed) on a routine basis. The recommended time interval is generally quarterly. If excessive vibrations occur, bearings might need replacement. This would require a shutdown unless another pump (redundant) is present.

Automation

"Automated monitoring and control systems can be found in many modern facilities. Automation can come in the form of Data Acquisition and Control systems (DAC) or Supervisory Control and Data Acquisition (SCADA) " (Lee, 2000). Although they may differ in scale and customization, they accomplish the same goal; both will monitor already designed system parameters, such as water quality (pH, temperature, oxidation-reduction potential, conductivity, dissolved oxygen, total dissolved gas pressure, etc.), tank water levels, pump speed status, and more. These may be programmed to have predesigned instructions through either an individual component Programmable Logical Controller (PLC) or a more centralized, all component encompassing SCADA like Siemens Power Process Control Language (PPCL). These must be observed for correct function.

In general, a facility incorporates a DAC or SCADA to reduce necessary overall human labor and frequency of intervention during normal system operation. For example, a SCADA can perform what would otherwise take a small army of individuals on constantly rotating shifts to perform. However, as with all mechanisms, maintenance is required. The more automation (relays, junction boxes, solenoids, etc.) present, the more labor required for maintenance. If the amount of labor/time needed to service the SCADA is greater than the amount of labor/time to service the LSS directly, the automation may not be worthwhile. Software is mostly conditional based, so that if a predefined condition is detected, then a control command is sent to a device or devices to correct it. However, automated monitoring and control can never fully replace a skilled and well-experienced LSS operator. It should also be considered that automation is not a predictive technology. It is reactive with software controls based on If/Then statements, and its reactions are programmed and hardwired by an operator. More on the advantages and systems for setting up a Process Control System for Aquaculture can be found in Lee's article for Aquaculture Engineering (Lee, 2000).

Troubleshooting or Diagnosing Nonnormal Events

Human Factor

Human intuition is one of the most valuable qualities that an operator can possess and apply during troubleshooting. Failures will arise in systems, and without previous experience of that particular failure, an operator must rely on intuition, deduction, and inference.

Failure is possible for all devices, systems, processes, etc. Eventually, everything can break down. Problem-solving is an essential method for operators and others attempting to maintain these devices and systems and fix them when failures occur.

Pattern Recognition

The goal for dealing with a failure is to identify the root cause or causes leading to the nonnormal state present. Several approaches exist to accomplish this; however, Fig. 28.1 illustrates the most straightforward approach in establishing a problem and diagnosing it appropriately.

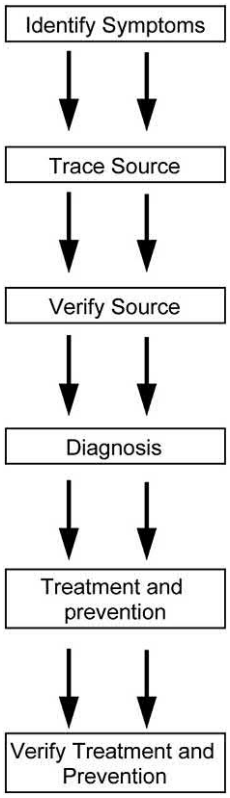

FIGURE 28.1 Generic diagnosing flow chart.

Flowchart Approach

In this approach, we identify symptoms, trace the source of the symptoms, diagnose the cause of the symptoms, formulate a treatment and prevention plan, and verify that the treatment and prevention methods worked.

Symptoms are any nonnormal operational states. This could be something as nebulous as an operator noting an unusual sound in the Mechanical Room of the facility or as specific as a puddle of a liquid on the floor.

Diagnosis is establishing the specific cause and effect that generated the identified symptoms. Usually, this involves further investigation of the symptoms. "Sufficient and efficient decision support would be crucial to maintenance personnel's decisions on taking maintenance actions. Techniques for maintenance decision support in a (*condition-based maintenance*) program can be divided into two main categories: diagnostics and prognostics … fault diagnostics focuses on detection, isolation and identification of faults when they occur. Prognostics, however, attempts to predict faults or failures before they occur" (Jardine, Lin, & Banjevic, 2006). For instance, if the first symptom is a puddle of liquid on the floor, the liquid substance may be determined to be water. Observation of surrounding areas may identify a problem in the aquatic system water pipes above the puddle. An operator examines the pipework and identifies the normal water path. By following this path up to an area where pipes are dry, the operator can identify the location of the problem area from which gravity is pulling water into the puddle. The second symptom is the leak. A break in the fitting of the piping is observed. The break is determined to be caused by a substandard fitting. The Diagnosis is a substandard fitting caused a leak and puddle. Treatment is a course of action that will return the affected parts of the system to a state of normality. Forms of treatment can include replacement, adjustment, or repair. This could be a multistep process of addressing the diagnosed area and fixing it. For the puddle example, replacement of the leaking plumbing area would be sufficient.

Case Studies: NIH Examples

The National Institute of Health is home to one of the world's largest and most heavily automated centralized zebrafish facilities and so possesses a unique opportunity for the study of the many troubleshooting events that arise out of its sheer complexity. This facility has a high degree of automation in which thousands of physically integrated and virtual points work together through PPCL (see Fig. 28.2) communications, so that defined conditions are met or not, which then may trigger other points. Along with feedback, these algorithms control and regulate operator-defined parameters. For instance, pH is constantly monitored with an in-line sensor, and when the pH is reading lower than a predetermined value (condition), an algorithm turns on a sodium bicarbonate dosing pump (trigger) to add sodium bicarbonate into the system, thus increasing pH value. This pH dosing will continue to add sodium bicarbonate until the desired pH level is reached (feedback). In another example, if the conductivity is higher than a predetermined value (condition), a different algorithm opens a valve (trigger) that allows reverse osmosis water (water devoid of most impurities, in particular, salt) to enter into the system, and thus, reducing conductivity. Again, this conductivity subroutine will continue to run until conductivity is at the desired level (feedback) or if the tank has reached a high-water level state, will close the Reverse Osmosis valve, this being a separate condition, trigger, and feedback system.

One can quickly see how these controlling subroutines and routines can be layered on top of one another to create more complex control of the system by corresponding physical devices linked to each condition and trigger. And, this only illustrates in-system troubleshooting. Most zebrafish facilities will be located within a building, which will more than likely have its own controls and automation that eventually will function abnormally. All this amounts to a fascinating level of involved and multilayered troubleshooting.

The following is just a few of those Pattern Recognized events.

During two feeding times per day, pH is recorded and observed to noticeably drop. For maintaining a relatively stable pH throughout the day, a sodium bicarbonate dosing pump is automatically controlled through the pH sensor, activating the pump to dose at a speed and frequency proportional to the pH drop. This demonstrates the normal fall of pH concentration from the increased digestion in the system, and the automated dosing system put in place to compensate without an operator's interaction (Fig. 28.3).

In this example, observe the pH dropping during the morning feeding session as is normal around 9:30 a.m. However, the doser malfunctioned, causing pH to drop lower than normal, and an alarm occurred. Note that the doser was engaged manually by an operator, at up to 100% speed, twice while it was being repaired. This demonstrates that at times, automation can fail and an operator, once notified by an alarm, can remedy the situation (Fig. 28.4).

Low water level will activate a Low-Level Alarm, triggering a system shut down. We can verify that the water level was indeed low by examining the recorded

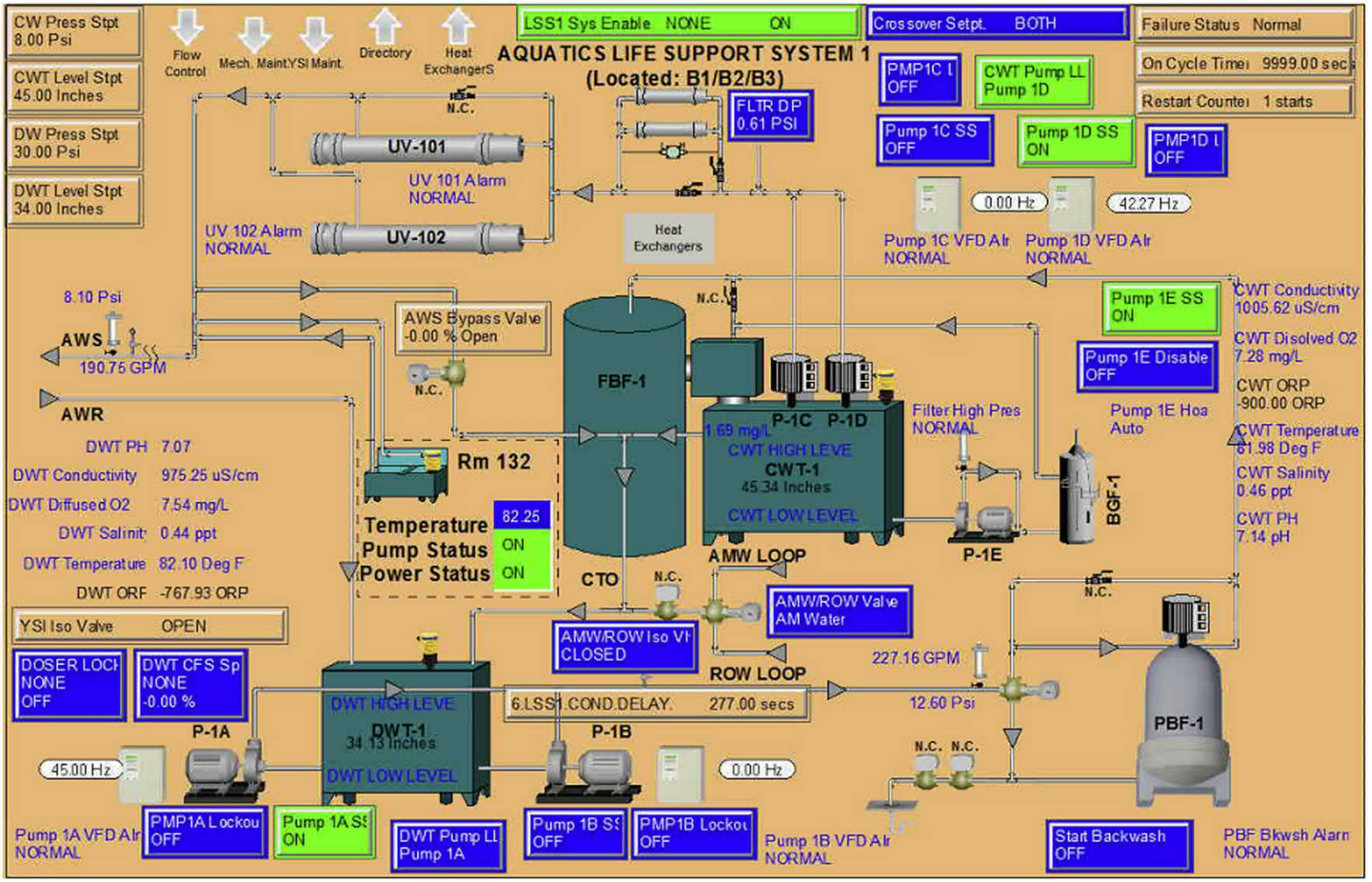

FIGURE 28.2 A screen capture of a small portion of the PPCL (controlling code) of Insight, a Siemens produced environmental monitor and control software used as a SCADA.

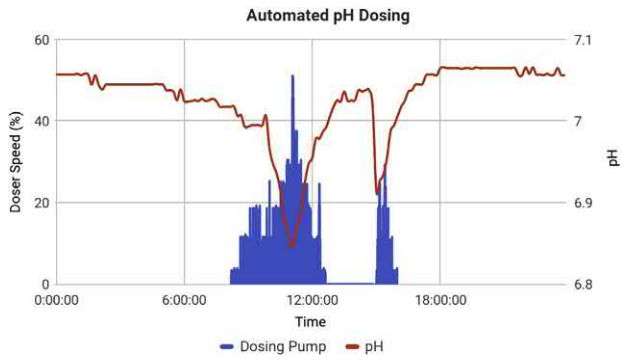

FIGURE 28.3 Nominal pH concentration with a low pH alarm and Sodium Bicarbonate Doser pump speed during a 24-h period.

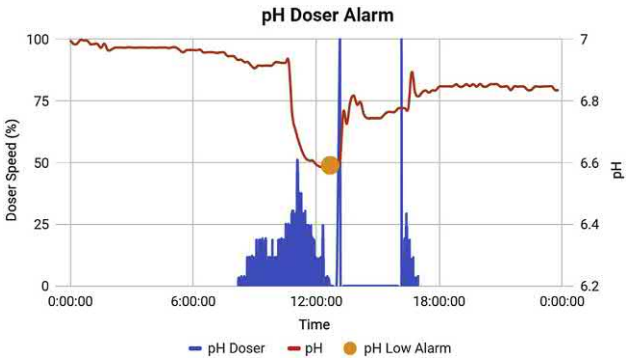

FIGURE 28.4 pH concentration with a low pH alarm and Sodium Bicarbonate Doser pump speed during a 24-h period.

dissolved oxygen around the time of the alarm. If the water level is low, a venturi is created, drawing air into the pump, thereby creating a momentary elevated spike in dissolved oxygen. This demonstrates the normal interaction/observable pattern of a spike in dissolved oxygen with a low tank water level event (Fig. 28.5).

Normally, low levels will be accompanied by a momentary high spike in dissolved oxygen; however,

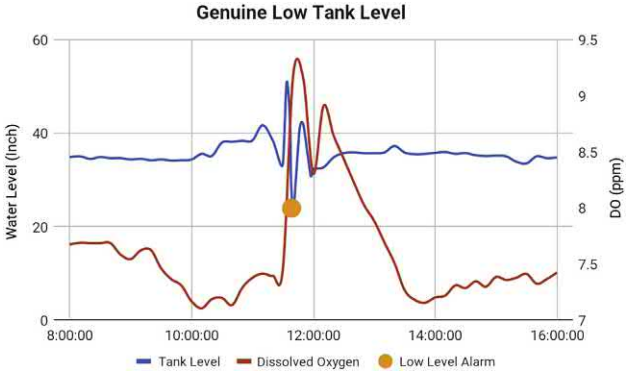

FIGURE 28.5 Water level and Dissolved Oxygen concentration with a genuine low water level event.

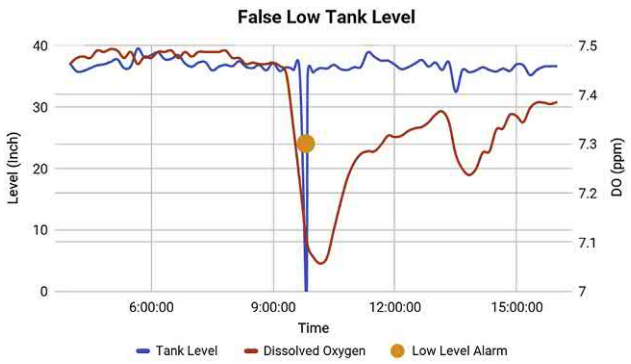

FIGURE 28.6 Water level and Dissolved Oxygen Concentration with a False Low water level.

in this case, it dropped slightly, as expected, during a feeding session but did not seem to be impacted by an apparent low-level alarm. Dissolved oxygen, in this case, acted as a secondary indicator, responding normally, and thereby verifying that the low-level alarm was malfunctioning. This demonstrates that the various sensors meant to protect critical devices from damage, in this case, air damaging pumps due to low tank water level, can themselves malfunction and need either repair or replacement (Fig. 28.6).

Observe that as the Differential Pressure (Prefilter Pressure/Postfilter Pressure) increases, meaning that the filter is becoming increasingly clogged with waste and debris, and the pump speed increases to compensate so that a constant 8 psi pressure is supplied to the aquaria water (filtered and cleaned water that supplies each culture tank). This demonstrates that changing filters proactively, prior to filters clogging completely and not allowing sufficient flow, allows operators to maintain a stable flow and, therefore, a stable environment for the zebrafish. **** This is an important example of the **paramount goal** of systems maintenance: to achieve and maintain system performance considering dynamic system function (Fig. 28.7).

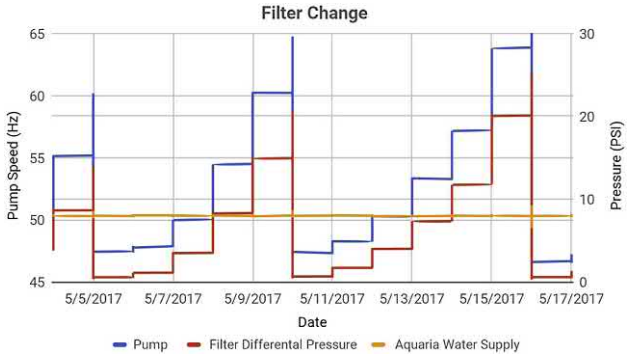

FIGURE 28.7 Pump speed, differential pressure, and water supply pressure (postfilters) over about 2 weeks.

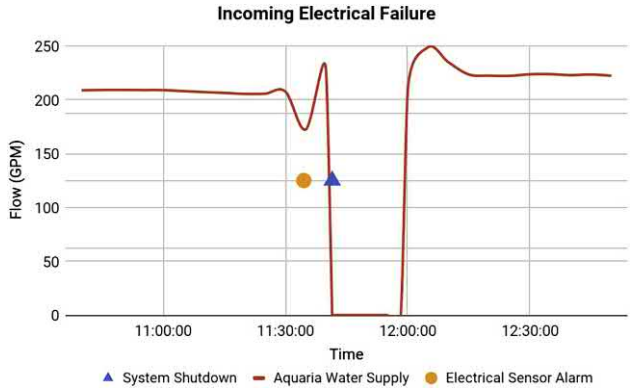

FIGURE 28.8 Electrical sensor reading fluctuation in grid initiating a system shutdown with water flow dropping off.

Observe here an Electrical Sensor, registering an electrical fault, triggers an automated system shutdown including pumps supplying water. Electrical faults, voltage or amperage lows and/or highs, could damage critical system assets such as, but not limited to, pumps, UV sterilizing lamps, actuated valves, dosing pumps, etc. Note that after the sensed electrical fault and shutdown, operators restored normal function. This demonstrates that utilizing automatic sensors and controls can avoid possible equipment damage and/or loss of zebrafish (Fig. 28.8).

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CHAPTER

29

Water Quality For Zebrafish Culture

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Introduction

Fish live in water, and water quality serves as the most fundamental and critical environmental control in zebrafish culture. Poor water quality can have direct effects on growth, metabolism, development, immune function, physiology, behavior, stress, reproduction, and many other parameters. In addition to the effects on fish, water quality will critically affect the biological filter performance, as this process requires relatively narrow conditions to function optimally. Husbandry professionals (culturists) must be uniquely knowledgeable and skilled to monitor, maintain, and manipulate the living environment of the fish to reduce opportunities for nonprotocol-based variation in research studies. Water quality is perhaps the most underrated and overlooked control variable in aquatic animal research.

Fish are uniquely adapted to water that contacts them both externally and internally through their skin, gills, digestive tract, and other surfaces. The hypotonic environment of all freshwater fish, including zebrafish, creates a concentration gradient that allows the passive penetration of water into their internal environment (tissues) and the passive loss of ions from their tissues to the external environment in a continuous manner. Water carries dissolved gases, salts, chemicals, and other organisms that may be able to enter or exit the fish at various exposed surfaces (Evans, Piermarini, & Choe, 2005).

The Aquatic Environment

Water, as we currently understand it, is the compound that makes life possible. Water has several properties that result from the polar covalent bonds between the hydrogen atoms and the strongly electronegative oxygen atom; these displaced electron densities in the bonds create regions of partial positive charge near each hydrogen atom and partial negative charge near each oxygen atom. Attractive forces among positive and negative charges in adjacent molecules give rise to hydrogen bonds that create the foundation for water's unique properties.

One important property of water is its high specific heat relative to many other molecules. It takes one calorie, a relatively large amount of energy, to raise 1 gram of water by one degree Celsius. As a result, aquatic environments are much more thermally stable than terrestrial environments, and aquatic organisms are typically more sensitive to rapid changes in temperature. Culture systems of a larger volume are more thermally stable, fluctuate less rapidly, and are preferred by culturists.

Water is a nearly universal solvent, meaning that more types of molecules are soluble in water than any other solvent. The chemistry of living organisms occurs under aqueous conditions, and fish, being immersed in water, are constantly interacting with the molecules dissolved in the water. Gas exchange, osmoregulation, and waste removal are a few examples of physiological processes that rely on the relative concentrations of dissolved molecules in the living environment (Evans et al., 2005). Stability in the concentration of molecules in the culture environment is critical to the health of fish, and therefore, to the validity of the research.

The unique properties of water create not only physiological challenges for the culturist but also infrastructural challenges. At a weight about 1 metric ton per m^3, structures housing large aquatic systems will typically have to be structurally reinforced, and considerable energy must be employed in the form of water pumps to circulate the water through filters to maintain optimal water quality for the fish in culture.

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Testing, Units, and Measurement

Water quality is measured using a variety of different methods, including test strips, test kits, and electronic probes. Fish culturists should have two different testing methods available for each critical water quality variable so that irregular readings can be checked with a second method prior to action being taken. Many a fish culturist has mistakenly created water quality issues based upon action taken from irregular readings later discovered to be false. Culturists should use only high-quality testing equipment from trusted sources, and water quality results should be frequently checked using a second method to confirm accuracy. Many of the specially made rack systems used for zebrafish culture have electronic probes for pH, conductivity, temperature, and sometimes dissolved gases. Many of these systems will automatically adjust water quality, especially pH and conductivity, using dosing pumps and culturist-prepared stock solutions. All of these probes need to be routinely maintained and calibrated to ensure proper function and accurate readings. Additionally, delivery tubes for dosing pumps need to be cleaned periodically to prevent blockage. Please consult the manuals, companies, and/or rack system providers for instructions to maintain the water quality probes and automated adjustment systems. Culturists should familiarize themselves with automated systems to prevent equipment failures that could potentially be stressful or lethal to cultured fish.

There is some debate and a great deal of variation among fish culture facilities as to which water quality parameters should be tested and how often. This author agrees with the guidelines for the testing frequency of water quality parameters for zebrafish as proposed by Harper & Lawrence (2011). Similar testing guidelines were also adopted by a recently completed online zebrafish husbandry course created by a team of more than 30 experts in zebrafish culture, aquaculture, veterinary science, and other related fields. The consensus of these parties is that temperature, pH, and conductivity should be monitored daily in each system. Alkalinity, hardness, carbon dioxide, and dissolved oxygen should be tested weekly but more often if changes to the system occur or if fish behavior appears irregular. Ammonia, nitrite, and nitrate should be monitored at least weekly and more often in situations where biological filters likely experience disturbances, such as during cleaning, new system start-up, power outages, or with changes in biological loading.

Each of the water quality parameters has particular units used to quantify that parameter. In general, most of the units used are typically within the international system (SI) of measurement as is standard for the sciences. Many water quality parameters used in practical fish culture utilize specialized measurements of concentration not commonly used amongst chemists. Two such units describe parts of solute per liter of solvent using terms, such as parts per thousand (ppt) or parts per million (ppm). Parts per thousand (ppt) is a frequently used unit to describe salinity and is understood to be grams of solute per liter of solvent (g/L). Parts per million (ppm) is a frequently used unit to describe dissolved minerals (alkalinity, hardness) dissolved gases (oxygen, nitrogen, carbon dioxide), and dissolved nitrogenous wastes (ammonia, nitrite, nitrate); parts per million is understood to be milligrams of solute per liter of solvent (mg/L). The electrical conductance of water, herein called *conductivity*, is described using the SI unit called *Siemens* and is abbreviated as "S." More commonly for zebrafish culture, the unit micro-Siemens abbreviated as "μS" is used. Highly purified water has a conductivity approaching zero μS, while full strength seawater has a conductivity approaching 54,000 μS.

Making Water For Controlled Aquatic Environments

Several sources of water may be utilized for aquatic research facilities, including groundwater, surface water, and municipal water. Each of these has advantages and disadvantages that the culturist should be aware of so that appropriate steps are taken to ensure the health of aquatic laboratory animals. Any of these sources, if managed and treated correctly, can be used successfully.

Groundwater and surface water are among the most plentiful, inexpensive, and most variable sources of water utilized in traditional aquaculture. The quality, quantity, and composition of the water vary greatly with location, season, and local rainfall totals. Additionally, the source water can change in composition as it is moved through pipes and fittings to the laboratory. It is imperative that any water source used for laboratory animal research be thoroughly tested for chemical and microbial impurities on a regular schedule so that appropriate treatments are used to alleviate any issues regarding composition. Such a comprehensive analysis can be acquired through private water testing laboratories or municipal water treatment facilities for a fee.

Most zebrafish research facilities located in or near urban areas will likely use a municipal water source. This has the advantages of consistent availability, defined quality and composition, and regular, thorough monitoring with historical records. The primary disadvantages of municipal water use are the presence of disinfectant chemicals and the cost associated with service.

Two different disinfectants commonly used by municipalities to reduce pathogenic bacterial loads in drinking water are chlorine and chloramine. Chlorine is more common, less stable, and easier to remove from water supplies. Options for chlorine removal include off-gassing for 24 h with vigorous aeration, treatment with sodium thiosulfate at 7 ppm (mg/L) for every ppm of chlorine detected (Noga, 2010) or removal of chlorine by the passing of source water through granular activated carbon (GAC). Chloramine is more stable and more difficult to remove from source water. Options for the removal of chloramine include treatment with sodium thiosulfate at 7 ppm (mg/L) for every ppm of chloramine detected followed by ammonia removal by treatment with zeolite (Noga, 2010) or removal of chloramine by the passing of source water through surface activated "catalytic" granular activated carbon (GAC_c). Note that off-gassing is NOT an effective method for the removal of chloramine from source water. Commercially available water conditioners useful to fish culturists for chlorine/chloramine removal from municipal water include Ultimate Water Conditioner and Cloram-X (Aquascience Technologies, LLC), Prime and Proline Aqua-coat (Pentair Aquatic Ecosystems Inc.), Amquel and Novaqua (Kordon LLC), and Ammo-Lock (Aquarium Pharmaceuticals). Each manufacturer provides directions for their product's use. It is recommended that culturists contact their source water municipality to determine which disinfectant is present and to obtain copies of recent analyses for chemical and microbial impurities. Keep in mind that water composition (particularly for heavy metals such as lead, zinc, and copper) can change as water travels through pipes to the laboratory. It is always a good idea to have water periodically tested at the final destination to rule out additional contamination from pipes and fittings.

Source water quality can present a serious risk of non-protocol-based variation in research studies. Even municipal water quality will vary greatly with significant rainfall or other natural events. To maintain a high level of water quality and consistency, industry leaders have generally agreed upon a standard course of preferred water treatment (Harper & Lawrence, 2011). The concept is to highly filter the source water removing all possible impurities to achieve a near-zero total dissolved solids (TDS) reading for the purified base water. High-quality synthetic sea salts are added back to the purified base water to reach a standard conductivity that is fish-ready water.

The recommended water treatment is a four-step process using (1) a mechanical cartridge prefilter followed by (2) a granular activated carbon (GAC) prefilter followed by (3) a reverse osmosis (RO) filter followed by (4) a deionization (DI) filter (Fig. 29.1). The mechanical prefilter cartridge will remove all suspended particles larger than 5 to 25 microns (depending

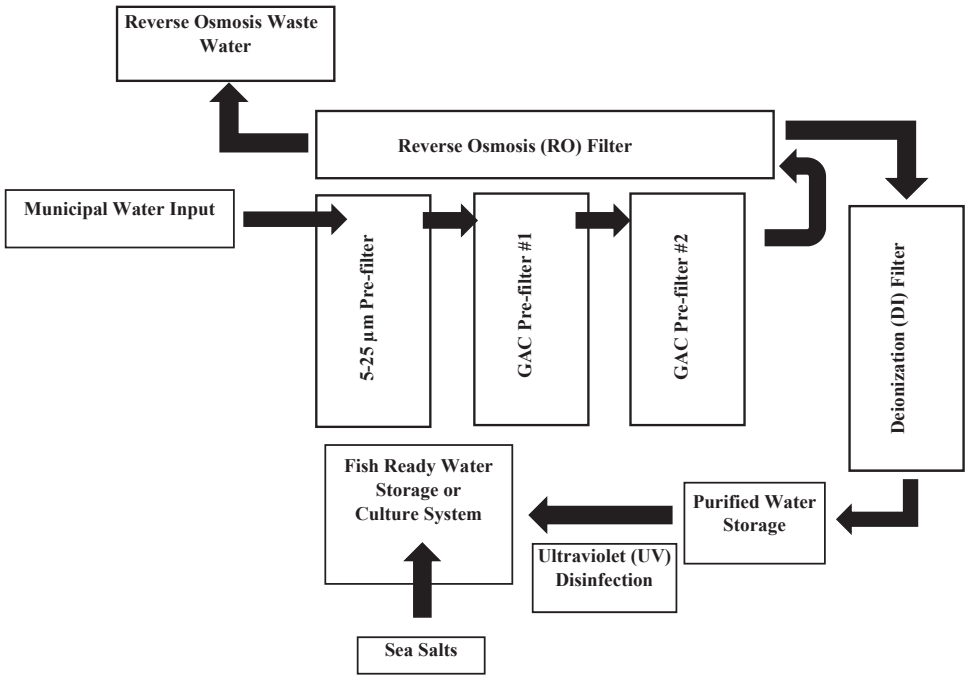

FIGURE 29.1 A schema showing the recommended filtration process for municipal water filtration to purified water and the addition of salts to create fish-ready water. Municipal water first passes through a mechanical prefilter, a series of two redundant granular activated carbon filters (GAC), a reverse osmosis filter, and a deionization filter. Purified water in storage has a small amount of sea salt added to create fish-ready water of appropriate conductivity.

on the prefilter type). These filters will greatly increase the working life of the carbon prefilter and the reverse osmosis filter. Water from the mechanical prefilter cartridge will go to the granular activated carbon (GAC) prefilter. This prefilter must be specific for the type of disinfectant used for the source of municipal water. Standard GAC is used to treat water disinfected with chlorine, and surface activated "catalytic" (GAC_c) is used for chloramine-treated water. GAC will also remove heavy metals, toxins, pesticides, and many dissolved organic molecules. The reverse osmosis (RO) unit uses water and/or pump pressure to force water through a very small micron semipermeable membrane against the concentration gradient for water. RO filters will remove most molecules larger than water molecules. RO units produce high volumes of wastewater compared to purified water. The RO purified water will lastly enter the deionization (DI) filter. This filter is composed of a synthetic resin with positively and negatively charged particles attached to the resin. These charged resins will bind any remaining ions that are dissolved in the RO filtered water. Water exiting the DI filter will have total dissolved solids (TDS) reading near zero and go to a storage container. It is notable that some small pathogens can pass through the RO membrane and find their way to the storage container. For this reason, base water is typically UV disinfected before it is considered fish-ready (Harper & Lawrence, 2011).

Water Quality For Zebrafish Culture

Temperature

Temperature is defined as the average intensity of heat and is measured in the laboratory using a thermometer in units of degrees Celsius (°C). Most fish are poikilotherms in which the body temperature conforms to the temperature of the ambient environment (Prosser & Heath, 1991). As a result, the temperature has the greatest impact of all environmental parameters on the culture of fish. Temperature fluctuations in fish culture affect growth, development, metabolism, appetite, physical activity, waste production, reproduction, immunity, and other parameters (Noga, 2010; Timmons & Ebeling, 2013). For example, a small fish (1g total weight) can cool at a rate of 1.8°C/minute x °C gradient resulting in rapid changes in metabolic rate (Prosser & Heath, 1991). Fluctuations in culture temperatures need to be immediately addressed, as they can modify physiology or cause mortality in extreme circumstances. Fish transported into the laboratory should be carefully acclimated by floating the closed transport bags in receiving water to equilibrate the temperature prior to introducing fish to the receiving system.

Fish and other poikilothermic organisms indicate varied tolerance to environmental temperatures based on the temperatures encountered in nature (Prosser & Heath, 1991). Zebrafish are a eurythermal species that exist in nature at temperatures between 8 and 35°C (McClure, McIntyre, & McCune, 2006; Spence, Fatema, Reichard, Huq, Wahab, Ahmed, et al., 2006; Zahangir, Haque, & Mostakim, 2015). Furthermore, laboratory studies have indicated that zebrafish can survive acclimated temperatures that range from 7 to 42°C (Cortemeglia & Beitinger, 2011). Zebrafish indicate an optimal range of 24–28°C with the highest growth rates occurring at 28°C (Schaefer & Ryan, 2006). High water temperature leads to increased oxygen consumption and heart rate in zebrafish (Barrionuevo & Burggren, 1999). Thermal history during early development in zebrafish causes irreversible influence on thermal tolerance (Schaefer & Ryan, 2006) and swimming performance (Sfakianakis, Leris, & Kentouri, 2011). Zebrafish colonies indicate genetic sex determination that is sensitive to environmental temperature influence and masculinization at high culture temperatures (Uchida, Yamashita, Kitano, & Iguchi, 2004; Ospina-Alvarez & Piferrer, 2008; Luzio, Santos, Fontainhas-Fernandes, Monteiro, & Coimbra, 2016; Ribas, Chang Liew, Diaz, Sreenivasan, Orbán, & Piferrer, 2017). Most zebrafish studies are conducted at 28°C to coincide with optimal growth (Schaefer & Ryan, 2006) and described developmental sequence (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995).

Temperature not only has effects on the fish but also on other water quality parameters. The equilibrium of total ammonia nitrogen (TAN) depends on both temperature and pH (Timmons & Ebeling, 2013). Increasing water temperature shifts the TAN equilibrium toward the prevalence of nonionized and more toxic form of ammonia (NH_3). In addition, increasing water temperature significantly reduces the saturation of dissolved oxygen (DO) (Noga, 2010; Timmons & Ebeling, 2013). The effects of temperature on DO can become limiting in some circumstances, as fish and aerobic bacteria in biological filters compete for DO.

The temperature in zebrafish culture systems should be monitored and recorded daily. This author recommends that temperature be maintained at a stable level between 26 and 29°C. This should be accomplished through a combination of adjusting the temperature of the culture room and adjusting the temperature of the water supply for fish using specialized heaters and chillers that utilize thermostats. Heaters and chillers regulate water temperature via thermostats that measure water temperature and turn them on or off as required to maintain water within the limits defined by the culturist. Heater elements will require routine cleaning, as they will accumulate a layer of solids over

time. Elements should be cleaned by (1) unplugging the element and allowing it to completely cool, (2) immersing the element in concentrated muriatic acid or other cleaning acid to loosen and dissolve solids (personal protective gear is required) (3) thoroughly rinsing the element with clean tap water, and (4) wiping the element with a clean cloth. Heaters, chillers, and thermostats frequently blow electrical fuses. It is recommended that husbandry professionals familiarize themselves with equipment fuses and keep extra fuses on hand in case of equipment failures. It is also suggested that facilities keep extra heaters and chillers available to replace failed equipment.

pH

The pH is defined as the log of the hydrogen ion (H^+) concentration and describes the relative concentration of acids and bases in the culture water (Timmons & Ebeling, 2013). The pH scale is logarithmic going from zero (strongest acid) to 14 (strongest base) with pH 7 representing a neutral solution that is neither acidic nor basic. Changes in one unit of pH represent a change in hydrogen ion concentration by 10-fold thus even changes of 1 pH unit are highly significant. The water in recirculating aquaculture systems is continuously becoming more acidic over time due to acids being generated and buffers being consumed as nitrogenous wastes, such as total ammonia nitrogen (TAN) are being converted to less toxic nitrate by nitrifying bacteria (Timmons & Ebeling, 2013). Sodium bicarbonate is added to culture water to counteract the production of acids and increase buffering capacity to keep pH at near optimal levels. Buffers are chemical species that can resist changes in pH by donating or accepting hydrogen ions from solutions. Freshwater fish culture systems typically use sodium bicarbonate as the primary buffer, although some laboratories will use reactors that contain sources of calcium carbonate, such as aragonite, dolomite, crushed coral, or limestone.

Most freshwater fish tolerate pH levels between 6.5 and 9 (Harper & Lawrence, 2011; Noga, 2010; Timmons & Ebeling, 2013). Zebrafish found in nature exist in slightly basic water with pH ranging from 6.6 to 8.2, and that fluctuates slightly with the season (McClure et al., 2006; Spence et al., 2006). The optimal pH for *Danio rerio* has not been determined (Reviewed by Lawrence, 2007). The median LC_{50} for acute exposure of zebrafish to pH for 2 h was 3.9 and 10.8 (Zahangir et al., 2015). No behavioral changes were observed for acute pH exposures between pH 5.5 and 9.0 (Zahangir et al., 2015). Similarly, De Paiva Magalhães, Forsin Buss, Da Cunha, Linde-Arias, and Fernandes Baptista (2012) indicated a 24 h LC_{50} at pH 3.0–3.2 and 10.5–11 for acute exposure of zebrafish. Zebrafish culturists strive to maintain pH levels between 7 and 8 as these pH values agree with the natural pH range for zebrafish and are also near optimal microbial conditions for the nitrifying bacteria in biological filters (Chen, Ling, & Blacheton, 2006; Harper & Lawrence, 2011; Timmons & Ebeling, 2013).

The physiological effects of pH occur primarily at the gill epithelium. Acute or chronically low, pH exposure, results in several different physiological stressors, such as excessive mucus production that interferes with ion and gas exchange, the passive loss of sodium and chloride ions, an increase in cortisol levels, the modulation of gene expression, and the loss of acid-base balance (Kwong, Kumai, & Perry, 2014; Noga, 2010; Zahangir et al., 2015). Acidic pH at the gills lowers the binding affinity of hemoglobin and increases the amount of oxygen required for hemoglobin to reach oxygen saturation, also called the *Bohr Effect* (Burggren, McMahon, & Powers, 1991). In some instances of low pH, hemoglobin may lose the ability to become oxygen saturated regardless of oxygen concentration, also called the *Root Effect* (Burggren et al., 1991). The lower the pH, the more difficult it is for the fish to bind and transport oxygen. Acute exposure of zebrafish to pH near the lower (pH 4) and upper (pH 11) limits resulted in excessive mucus production covering the skin and gills, the destruction of the respiratory epithelium, the loss of swimming balance, convulsive behavior, and death (Zahangir et al., 2015). Zebrafish acclimated to pH of 5.0, 7.2, and 10.0 for 30 days experienced initial respiratory distress and increased blood glucose levels at the pH extremes but recovered to normal levels after 12–24 h (Zahangir et al., 2015). Long-term effects of pH exposure included a decrease in red blood cell (RBC) numbers and an increase in white blood cell (WBC) numbers (Zahangir et al., 2015). De Paiva Magalhães et al. (2012) indicated increased levels of hypoactivity with acute exposure to acidic and basic sublethal pH values when swimming velocity was measured.

As with all water quality parameters, pH stability is important to fish health. This author recommends maintaining the pH between 7 and 8 through the slow addition of sodium bicarbonate (for low pH) and the slow addition of dilute hydrochloric acid or acetic acid (for high pH). Many centralized filtration systems and stand-alone racks have pH probes and automatic dosing systems to maintain pH within narrowly defined limits. Cleaning and maintenance of pH probes and equipment are necessary to prevent water quality problems. It is recommended that pH probes in continuous use be calibrated monthly and that hand-held units be calibrated weekly using fresh standard buffers and a two- or three-point calibration. Consult the probe manufacturer directions for proper calibration. Probes

used to monitor pH require proper storage when not in use and periodic cleaning when in use. Consult the probe manufacturer for directions for proper storage and cleaning of pH probes. Generally, pH probes can be cleaned by (1) soaking the probe for 10–15 min in a mild solution of dishwashing detergent, (2) thoroughly rinsing the probe with clean water, and (3) calibrating and testing the probe for performance. If the probe fails to achieve a stable reading within 2 min: (1) soak the probe for 30 s in 1 M HCl solution (proper personal protective gear is required), (2) rinse the probe thoroughly with clean water, and (3) calibrate and test the probe for performance. If the probe still fails to achieve a stable reading within 2 min, call the probe manufacturer for further assistance. Never attempt to clean a probe with any type of brush or cloth as this can scratch the surface and destroy the probe. Always store probes not in use in a brand name pH probe storage solution or clean pH 4 buffer solution. Note that the storage of probes in purified water can destroy them.

Conductivity/Salinity

Conductivity describes the concentration of dissolved ions or salts in freshwater and is typically expressed in units called *micro-Seimens* (μS). The conductivity of freshwater solutions is low and typically ranges from 100 to 1000 μS, while the conductivity of seawater solutions can exceed 54,000 μS. Conductivity examines the sum of dissolved ions and will include such ions as sodium (Na^+), potassium (K^+), calcium (Ca^{+2}), magnesium (Mg^{+2}), chloride (Cl^-), bicarbonate (HCO_3^-), and others. At high ion concentrations, the term salinity is frequently used and is generally measured in units of parts per thousand (ppt). Conductivity and salinity encompass both alkalinity and hardness values, and thus, it is recommended to test alkalinity and hardness separately to get a more accurate picture of the water quality in a given system.

Zebrafish are freshwater fish adapted to live in hypotonic environments where water is passively entering the fish by diffusion at the gills, skin, and other surfaces, and ions (primarily Na^+ and Cl^-) are being passively lost to the external environment on a continuous basis (Evans et al., 2005). To maintain salt and water balance, freshwater fish avoid consuming water, secrete copious amounts of dilute urine, reabsorb useful salts in the kidneys, absorb useful ions from food intake, and actively transport Na^+, Cl^-, and Ca^{+2} ions into the body fluid (Randall, Burggren, & French, 2002; Chen, Lu, & Hwang, 2003; Evans et al., 2005). Maintaining osmoregulatory balance comes at an energetic cost that increases, as the environment (system water) deviates from isosmotic concentrations (Randall et al., 2002). In nature, zebrafish are found in conductivities ranging from 10 to 2000 μS (0.006–1.3 ppt); and in laboratory culture, systems are frequently maintained at 200 to 3000 μS (0.5–2 ppt) (Harper & Lawrence, 2011). Embryos tolerate 0.3–2 ppt, and tolerance increases with development (Sawant, Zhang, & Li, 2001). Boisen, Amstrup, Novak, & Grosell, 2003 suggested that zebrafish were able to tolerate environments with extremely low ionic concentrations for long periods and that they are adept at maintaining osmotic balance by the rapid upregulation of Na^+ and Cl^- uptake. Long-term exposure to low ionic or high ionic concentrations is likely to have negative effects on energy expenditure as the fish struggle to balance salt and water intake with losses (Boisen et al., 2003).

Alkalinity

Alkalinity is defined as the concentration of titratable bases that exist in a solution (Boyd, Tucker, & Somridhivej, 2016). In freshwater fish culture, those bases are most often forms of carbonates ($CO3^{-2}$), bicarbonates (HCO_3^-), and hydroxides (OH^-) (Boyd et al., 2016). In addition to these weak bases, alkalinity is also strongly linked to pH and the production of CO_2 through the acid-base reactions involved in nitrification (Summerfelt, Vinci, & Piedrahita, 2000). From a practical standpoint, alkalinity refers to the buffering capacity or the ability of water to resist changes in pH. Alkalinity is most frequently measured in parts per million (ppm or mg/L) of calcium carbonate ($CaCO_3$). Alkalinity is a component of conductivity that is important for keeping the pH optimal in the recirculating system and supplying bicarbonate ions to the reactions of nitrification (biological filtration) (Timmons & Ebeling, 2013). The process of nitrification produces $4\,H^+$ ions and consumes seven molecules of HCO_3^- for every molecule of TAN converted to nitrate. Nitrification, in healthy recirculating systems, is a continuous process, and thus, the pH in these systems is continuously dropping (becoming more acidic). As the pH drops, CO_2 becomes more available, through a shift in the bicarbonate/carbonate equilibrium, to serve as the primary carbon source for beneficial nitrifying bacteria in biological filters (biofilters) (Summerfelt & Sharrer, 2004). Often more CO_2 is released than bacteria can utilize, and the systems produce a net increase in CO_2 concentrations (Summerfelt & Sharrer, 2004). Increases in biological load or feeding rate only increase these processes and hasten the drop in system pH. In addition to the direct effects of nitrification, other natural reactions lower system pH, such as the CO_2 production from the respiration of fish and aerobic microbial colonies. The culturist is constantly battling with system alkalinity and

pH. The more the culturist understands the process, the better equipped they are to correctly manage and stabilize the water quality of the system.

The most frequent source of alkalinity used in recirculating system management is sodium bicarbonate ($NaHCO_3$) although forms of calcium carbonate ($CaCO_3$) such as aragonite and crushed coral are also used. Alkalinity levels are typically adjusted to meet the pH requirements of biological filters since optimal alkalinity values have not been determined for many fish species. It is recommended that systems maintain alkalinity levels of 50–75 ppm although higher concentrations may not be problematic if the pH is stable. Alkalinity levels below 20 ppm will allow for large fluctuations in pH and can damage the nitrifying bacterial community of biological filters, leading to spikes in total ammonia nitrogen (TAN) (Wurts, 2002). Many centralized filtration systems and stand-alone rack systems have automatic dosing systems linked to pH probes. These systems will activate peristaltic dosing pumps to add concentrated sodium bicarbonate solutions in a slow and methodical manner until pH values normalize then shut off when the target pH is reached. The concentrated solution is powdered sodium bicarbonate dissolved in RO/DI water at 16 g/L. Automated dosing systems should be tested and cleaned periodically to prevent the formation of sodium bicarbonate plugs in the delivery line. The sudden release of a plug in the delivery line followed by the sudden release of concentrated sodium bicarbonate solution can cause a rapid change in system pH that can stress or damage zebrafish.

Hardness

Hardness is the measurement of divalent ions, such as calcium (Ca^{+2}), magnesium (Mg^{+2}), ferrous iron (Fe^{+2}), strontium (Sr^{+2}), and Manganous manganese (Mn^{+2}) (Boyd et al., 2016; Wurts, 2002). These ions are crucial for many physiological processes, including brain function, bone formation, blood clotting, muscular function, neurological function, osmoregulation, and many others (Noga, 2010; Wurts, 2002). Hardness in natural waters depends largely on the regional soils and the presence of these divalent ions in the soils. As a result, the hardness of municipal water varies tremendously by location. If the culturist is using the recommended four-step filtration process for source water (mechanical, GAC, RO, DI), then hardness will be added back to purified base water using high-quality sea salts. Fish will also get many of the divalent ions they need through the consumption of high-quality diets, as mineral premixes are a common component among diets formulated for fish. Note that fish-ready water made from high-quality sea salts, as described in this chapter, will have significantly more sodium (Na^{+}) and chloride (Cl^{-}) than would be expected in most natural freshwater sources, and how this may affect zebrafish is not known. Optimal water hardness, including the energetic costs of osmoregulation, has not been examined in zebrafish, so it is suggested for levels to be kept stable between 75 and 200 ppm representing a range that is similar to the natural range of freshwater environments for *Danio rerio* (Chen et al., 2003; Lawrence, 2007). It is recommended that husbandry professionals maintain levels greater than 75 ppm (mg/L) hardness and that levels be tested weekly. Chen et al. 2003 determined that the calcium regulatory capacity of zebrafish acclimated to low calcium environments was lower than that for goldfish, meaning that zebrafish were less well equipped to deal with changes in calcium-depleted environments than goldfish. Boisen et al., 2003 indicated that whole-body content of Na^{+}, K^{+}, Cl^{-}, and Ca^{+2} ions did not differ significantly among zebrafish acclimated to soft water and hard water after acclimation for 40 days although breeding success and larval survival were low in the soft water acclimated fish. Craig, Wood, & McClelland, 2007 indicated a high degree of phenotypic plasticity to maintain homeostasis in zebrafish that were acclimated to soft water 51 μM Ca^{+2} and 26 μM Mg^{+2}.

Some laboratories are currently using calcium reactors to add both alkalinity and hardness to the system water. Calcium reactors are typically cylindrical vessels filled with pellets of calcium carbonate ($CaCO_3$) that are highly fluidized so that the collision of $CaCO_3$ particles solubilizes small amounts of Ca^{+2} and CO_3^{-2} on a continuous basis over many months. The calcium carbonate can take the form of agricultural limestone, dolomite, aragonite, or crushed coral. Note that calcium carbonate is sparingly soluble in water, so immediate changes are not frequently observed with this method; however, stable levels of both Ca^{+2} and CO_3^{-2} (hardness and alkalinity) can be achieved over time. The calcium carbonate source will need to be replaced when levels begin to decrease, and hardness should be tested on a weekly basis so that adjustments can be made if necessary.

Dissolved Gases (O_2, CO_2, N_2)

Although many gases are highly soluble in water, dissolved oxygen (DO), carbon dioxide (CO_2), and nitrogen (N_2) are the most important from a fish culture perspective. The atmospheric concentration of these gases is about 21% O_2, 0.03% CO_2, and 78% N_2 yet the solubility of these gases in water under standard conditions is 10.08 ppm (mg/L) O_2, 0.69 ppm (mg/L) CO_2, and 16.36 ppm (mg/L) for N_2 (Timmons & Ebeling, 2013).

Of these gases, the most noteworthy is that of dissolved oxygen (DO). Oxygen is only sparingly soluble in water under standard conditions, and its solubility greatly decreases with increasing water temperature, altitude, and salinity. In addition to water's low solubility under these conditions, fish and aerobic bacteria have increased needs for DO with increasing water temperature, high feeding rates, and high fish density. In zebrafish aquaculture systems, oxygen can become limiting when tanks are removed from racks prior to a procedure. Under these circumstances, the DO can drop to dangerously low levels in a short time. Uneaten food and decaying solids in recirculating systems can also create DO-limiting conditions as aerobic bacteria proliferate and consume DO from the system water. For this reason, culture tanks, sumps, and filters should be thoroughly cleaned of waste solids on a routine time schedule. The aerobic microbial communities found in biological filters can greatly affect the overall DO of the system water. Summerfelt and Sharrer (2004) directly examined the DO consumed by fluidized sand biofilters attached to a salmonid production system and reported a DO loss of nearly 4 ppm from the biofilter alone. This accounted for 35% of the total DO removed by the aquaculture system. They suggested that water be aerated after leaving the biofilter and prior to re-entering the culture system for this reason. The DO should be monitored weekly in zebrafish systems under normal conditions and more frequently in systems with heavy biological loads (high fish densities) or when there is a significant increase in biological load. It is recommended that DO be maintained at or near saturation (6–8 ppm) at all times although traditional food aquaculture systems set a lower standard of 5–6 ppm (Timmons & Ebeling, 2013).

Carbon dioxide (CO_2) is not only the by-product of respiration from zebrafish and aerobic microbial communities, but it is also created by the acid-base reactions (bicarbonate/carbonate equilibrium) that occur within recirculating aquaculture systems (Summerfelt et al., 2000). Because of its relatively high solubility in water, CO_2 can sometimes become problematic in recirculating systems (Timmons & Ebeling, 2013). Heavily loaded systems, systems located in areas with reduced airflow, or periods of reduced water circulation can cause net accumulation of CO_2 in system water (Timmons & Ebeling, 2013). In addition, high nitrification rates in biofilters can also increase system CO_2 concentrations as nitrifying bacteria create a shift in the bicarbonate/carbonate equilibrium to produce a net increase in CO_2. Summerfelt and Sharrer (2004) reported that a fluidized sand biofilter receiving water from a salmonid production system increased the CO_2 concentration of the biofilter effluent by more than 4 ppm (mg/L) and accounted for 37% of the total CO_2 production of the system. This study suggested that water effluent from a biofilter should have the CO_2 removed prior to its re-entering the culture tanks. In stand-alone zebrafish culture systems, carbon dioxide is typically off-gassed to the atmosphere in aerated sumps or as it falls down through the open gutter systems of zebrafish culture racks. Centralized filtration systems should provide a greater emphasis on aeration and carbon dioxide removal for biofilter effluent prior to water re-entering culture tanks.

Carbon dioxide concentrations affect system pH and can have several negative physiological effects in fish. Increased CO_2 concentrations in RAS can cause a decrease in the ability of hemoglobin to transport oxygen (Bohr effect), reduce the binding affinity of hemoglobin (Root effect), and lower blood pH (Summerfelt et al., 2000). Safe limits for CO_2 and the effects of elevated CO_2 concentrations on zebrafish have not been determined. This author recommends that carbon dioxide levels be kept as low as possible. Carbon dioxide concentrations approaching 15–20 ppm require immediate action as such levels can have anesthetic effects on fish (Timmons & Ebeling, 2013). Carbon dioxide levels should be tested weekly. If high levels are detected, culturists should increase aeration, remove waste solids from the system, increase water exchange, reduce fish density, increase water circulation through the system if possible, and/or increase air circulation in the room using floor fans or HVAC.

Nitrogen (N_2) is the most prevalent gas in the atmosphere and has a high solubility in water. Nitrogen gas is seldom problematic unless it is compressed and super-saturated into the water of the system. Compressed, super-saturated nitrogen is the leading cause of gas-bubble disease in zebrafish systems. Signs of gas super-saturation include small bubbles on the walls and other tank surfaces, sporadic water flows from system pumps with small bubbles exiting the supply lines feeding the system tanks, fish uncharacteristically sitting on the tank bottom, and fish showing indications of gas-bubble disease. Gas super-saturation is caused by (1) a low water level in the sump, which allows the pump to pull in both water and air or (2) by a small leak on the intake (impeller) side of a water pump. Gas bubble disease is highly lethal, so any signs of gas super-saturation should be addressed immediately. All pumps should be examined daily for proper function, and sumps should be maintained nearly full of water to prevent gas bubble disease.

Nitrogenous Wastes (NH_3, NO_2^-, NO_3^-)

Vertebrate animals consume protein to provide amino acid building blocks for growth, protein turnover

(maintenance), or energy production. Dietary proteins are digested to amino acids that can be absorbed through the gut or excreted with the feces. Dietary amino acids that are absorbed and in excess of those required for growth or protein turnover are utilized in the liver for energy production by amino acid gluconeogenesis or by oxidation to CO_2 in the Citric Acid Cycle (Campbell, 1991). As a precursor to energy production, the α-amino group of excess dietary amino acids is removed and excreted primarily as ammonia (NH_3) so that the carbon skeletons can be utilized for energy production (Campbell, 1991). Animals excrete nitrogenous wastes as urea (ureotelic), uric acid (uricotelic), or ammonia (ammonotelic). Most fishes and aquatic/marine invertebrates are considered to be primarily ammonotelic and excrete ammonia directly to the water by passive diffusion and monovalent cation (Na^+) exchange from the gills (60%–90%) and, to a lesser extent, through urinary discharge (Campbell, 1991; Hagopian & Riley, 1998; Randall et al., 2002). In addition to ammonia, fish also excrete small amounts of other nitrogenous wastes, including urea, uric acid, trimethylamine oxide (TMAO), creatine, creatinine, and nucleic acids (Poxton, 2003). Culture variables, such as temperature, feeding rate, feed protein concentration, and stocking density will affect metabolic activity, and therefore, ammonia excretion (Poxton, 2003). These variables need to be carefully considered as fish culturists attempt to keep nitrogenous waste concentrations at or near optimum concentrations.

Nitrogenous wastes not only arise from metabolic processes in fishes but can also come from environmental sources through the decomposition of organic materials by a wide variety of heterotrophic bacteria in aquatic systems (Blancheton, Attramadal, Michaud, Roque d'Orbcastel, & Vadstein, 2013; Rurangwa & Verdegem, 2015; Schreier, Mirzoyan, & Keiko, 2010). This is particularly noteworthy in recirculating aquaculture systems (RAS) where the heterotrophic bacterial decomposition of waste solids, such as uneaten foods, feces, biofilms, detritus, or dead organisms can greatly increase ammonia levels (Blancheton et al., 2013; Timmons & Ebeling, 2013). Since fish excrete metabolic ammonia mainly by passive diffusion, it is possible for high environmental ammonia concentrations to result in a net influx of ammonia into the blood of fish (Poxton, 2003).

Metabolic production from fish and environmental production from bacterial decomposition are the sources of ammonia in fish culture systems. In these systems, ammonia is sequentially oxidized in the biological filter (biofilter) by ammonia-oxidizing bacteria (AOB) to nitrite (NO_2^-), and then again by nitrite-oxidizing bacteria (NOB) to nitrate (NO_3^-). This process is the central dogma of biological filtration in recirculating aquaculture systems (RAS). The overall general equation for this process is provided below (reviewed in Schreier et al., 2010; Timmons & Ebeling, 2013).

$$NH_4^+ + 1.5O_2 \overset{AOB}{\rightarrow} NO_2^- + 2H^+ + H_2O \overset{NOB}{\rightarrow} NO_3^- + 2H^+ + 2e^- \quad (6.1)$$

The overall equation for microbial nitrification in biological filters. Ammonium ion (NH_4^+) is converted to nitrite (NO_2^-) and two hydrogen ions (H^+) by ammonia-oxidizing bacteria (AOB) and eventually to nitrate (NO_3^-) by nitrite-oxidizing bacteria (NOB).

Ammonia

Ammonia is present in two forms that exist in equilibrium: unionized ammonia (NH_3) and ammonium ion (NH_4^+). Together the sum of these chemical species is total ammonia nitrogen or TAN. Ammonium ion (NH_4^+) is much less toxic than unionized ammonia (NH_3). The prevalence of the unionized ammonia (NH_3) largely depends on the pH and, to a lesser extent, the temperature of the culture system (Timmons & Ebeling, 2013). The unionized (toxic) level of ammonia is calculated from Table 29.1 using the temperature and pH of the system being examined. For most fish species, levels of unionized ammonia (NH_3) should not exceed 0.05 ppm, and total ammonia nitrogen (TAN) should not exceed 1 ppm for long-term exposure (Timmons & Ebeling, 2013). Culturists should strive to keep TAN levels as close to 0 ppm as possible.

It is clear that ammonia is highly toxic to living organisms, although the exact mechanisms of this toxicity remain unclear (Campbell, 1991; Noga, 2010). Several

TABLE 29.1 The percentage of toxic unionized ammonia (NH_3) present in the water of fish culture systems with respect to water temperature and pH. TAN levels (ppm) X percentage of unionized ammonia (from the table) = ppm unionized ammonia. Example: A total ammonia nitrogen (TAN) value of 3 ppm at 28°C and a pH of 7.5 has a percentage of unionized (toxic) ammonia of 2.17%. The unionized ammonia concentration is 0.065 ppm (3 ppm X 0.0217 = 0.065 ppm).

	Water temperature					
pH ⬇	**20°C**	**22°C**	**24°C**	**26°C**	**28°C**	**30°C**
6.0	0.04	0.05	0.05	0.06	0.07	0.08
6.5	0.13	0.15	0.17	0.19	0.22	0.25
7.0	0.4	0.46	0.53	0.61	0.70	0.80
7.5	1.24	1.43	1.65	1.89	2.17	2.48
8.0	3.82	4.39	5.03	5.75	6.56	7.46
8.5	11.2	12.7	14.4	16.2	18.2	20.3
9.0	28.4	31.5	34.6	37.9	41.2	44.6

negative effects at the subcellular, cellular, and tissue level have been described, but from a practical standpoint, the lethality of ammonia is probably related to the combined physiological effects (Campbell, 1991; Noga, 2010; Randall et al., 2002).

Nitrite

Ammonia is oxidized in nitrification to nitrite (NO_2^-) another form of nitrogenous waste that is toxic at low concentrations. Toxicity occurs when NO_2^- molecules in culture water are actively transported across the gill epithelium and oxidize hemoglobin molecules resulting in conversion to met-hemoglobin (Noga, 2010; Poxton, 2003; Timmons & Ebeling, 2013). The prevalence of met-hemoglobin reduces oxygen transfer efficiency and in high concentrations results in potential asphyxiation and death. The pathology for this condition is frequently called *brown blood disease* and refers to the change in color of hemoglobin when it is converted to met-hemoglobin (Noga, 2010). Nitrite influx from the environment occurs through channel proteins for monovalent ions, such as Cl^-, Br^-, and HCO_3^-; thus, the increased presence of these ions will function as a competitive inhibitor for nitrite and reduce toxicity (Noga, 2010; Poxton, 2003). Nitrite toxicity is reduced, but not eliminated, in water with chloride: nitrite ratios of 20:1 or higher (Timmons & Ebeling, 2013). Although seawater has very high chloride concentrations, some marine fish species may still indicate nitrite poisoning at very high nitrite concentrations (Noga, 2010). Nitrite toxicity varies greatly among species. Median LC50 values can range from 0.6 to 200 ppm, depending on the species and culture conditions (Noga, 2010). Noga (2010) recommended that nitrite concentrations remain below 0.1 ppm to avoid possible long-term exposure toxicity. Voslářová, Pištěková, Svobodová, and Bedáňová (2008) detected growth suppression in adult zebrafish at 28 days during chronic nitrite exposure at a concentration of 73 ppm that was statistically significant for 28 days at 130 ppm (mg/L). The same study reported a 96 h LC50 for adult zebrafish at 386 ppm (Voslářová et al., 2008). We recommend that culturists strive to keep nitrite levels as close to 0 ppm as possible and to take action if concentrations approach 0.5 ppm.

Nitrate

Nitrite is oxidized to nitrate (NO_3^-), the end product of nitrification, and is much less toxic than either ammonia or nitrite. In recirculating aquaculture systems (RAS), nitrate concentrations will increase over time unless regular water exchanges are performed or additional remediation by denitrification or aquatic plant uptake occurs. The rate at which nitrate levels rise in the system is tightly linked to the amount of ammonia that enters the system and the rate of nitrification. Culture variables that affect ammonia excretion, such as temperature, feeding rate, feed protein concentration, and stocking density will also affect nitrate synthesis rates (Poxton, 2003). The mechanism of toxicity for nitrate is similar to that of nitrite in that it converts hemoglobin to met-hemoglobin, and thus, reduces the efficiency of oxygen transport (Camargo, Alonso, & Salamanca, 2005; Noga, 2010). Nitrate is less able to permeate gill membranes than nitrite, and this accounts for the relatively high levels of nitrate required to cause toxicity (Noga, 2010). Nitrate toxicity increases with nitrate concentration and time of exposure and is suggested to decrease with increasing body size, water salinity, and environmental adaptation (Camargo et al., 2005). In addition, freshwater animals appear to be more sensitive to nitrate than marine animals.

The toxic effects of nitrate vary greatly among fish species with salmonids, appearing to be the most sensitive, indicating physiological problems at concentrations of nitrate less than 10 ppm (mg/L) (Camargo et al., 2005). Some fish species indicate the ability to handle nitrate concentrations ranging from 200 to 2000 ppm for 24–96 h exposures (Noga, 2010). Nitrate is, however, more of a chronic issue, so studies of acute toxicity have limited value (Noga, 2010). Camargo et al. (2005) suggest a safe level of nitrate below 10 ppm (mg/L) for most freshwater species and less than 2 ppm (mg/L) for sensitive freshwater species. A nitrate level at or below 50 ppm (mg/L) is considered a safe level for most species (Noga, 2010; Poxton, 2003). Learmonth and Paulo Carvalho (2015) studied the effects of acute and chronic nitrate exposure on zebrafish and found no negative effects on embryos, newly hatched larvae, or swim up larvae at nitrate concentrations less than 1000 ppm. The same study found no negative effects on zebrafish over the first 23 days postfertilization (dpf) at nitrite concentrations up to 200 ppm. At nitrate concentrations of 400 ppm zebrafish indicated greatly reduced survival, growth, and development (Learmonth & Paulo Carvalho, 2015). This study suggested a safety level of 200 ppm (mg/L) for nitrate in zebrafish. More recently, Pereira, Paulo Carvalho, Cruz, and Saraiva (2017) examined the chronic effects of nitrate exposure on juvenile zebrafish and reported that exposure to nitrate levels above 100 ppm for 28 days indicated significant histopathology differences in the skin, gills, kidney, liver, and intestine. Although growth suppression was not significantly different among the groups, the study indicated a strong negative correlation in growth with increasing nitrate concentrations above 100 ppm (the lowest concentration examined). No mortality was detected up to 200 ppm, but mortality was reported at 47% for zebrafish exposed to 400 ppm for 28 days (the highest concentration tested) (Pereira et al., 2017). Pereira et al. (2017) recommend a safe

nitrate level of less than 100 ppm (the lowest level examined). This author recommends a nitrate safety level of less than 50 ppm for long-term exposure.

Biological Filtration

A recirculating aquaculture system (RAS) is a highly complex microenvironment with a diversity of microorganisms occupying microniches within the system (reviewed in Schreier et al., 2010; reviewed in Blancheton et al., 2013; reviewed in Rurangwa & Verdegem, 2015). The biological filter (biofilter) is the apparatus in the RAS that houses the surface area (biomedia) for colonization of nitrifying bacteria that perform the reactions of nitrification for fish survival. The microbiology of an RAS is affected by: feed type and regimen, system management, animal exposure, microflora associated with fish species, water source and quality, cleanliness of equipment used, cleanliness of staff and visitors, and the selective pressures that exist within the culture system (Blancheton et al., 2013; Rurangwa & Verdegem, 2015). One such microbial niche is nitrification. The process of nitrification, also referred to as biological filtration, describes the sequential oxidation of ammonia to nitrate by aerobic nitrifying bacteria (Eq. 6.1). Nitrifying bacteria are gram-negative chemoautotrophs classified into two broad groups based upon the substrate that they oxidize; ammonia-oxidizing bacteria (AOB) that oxidize ammonia (TAN) to nitrite (NO_2^-) and nitrite-oxidizing bacteria (NOB) that oxidize nitrite (NO_2^-) to nitrate (NO_3^-) (Chen et al., 2006; Hagopian & Riley, 1998; Koops & Pommerening-Röser, 2001; Schreier et al., 2010). The taxonomy of AOB and NOB is complicated, as a diversity of species catalyze these reactions in different natural and artificial environments (Burrell, Phalen, & Hovanec, 2001; Chen et al., 2006; Hagopian & Riley, 1998; Koops & Pommerening-Röser, 2001; Rurangwa & Verdegem, 2015; Schreier et al., 2010). Ammonia-oxidizing bacteria (AOB) in freshwater belong to strains of the genera *Nitrosomonas* sp., *Nitrosococcus* sp., and *Nitrosospira* sp. and nitrite-oxidizing bacteria (NOB) belong to strains of the genera *Nitrospira* sp. and *Nitrobacter sp* (Hagopian & Riley, 1998; Koops & Pommerening-Röser, 2001; Burrell et al., 2001; Chen et al., 2006; Schreier et al., 2010; Rurangwa & Verdegem, 2015). The strains of nitrifying bacteria present in any biofilter are known to differ among freshwater and marine systems and with the specific conditions of a given system (Blancheton et al., 2013). In addition to strains of AOB and NOB, a group of ammonia-oxidizing archaea (AOA) have also been detected in the biological filters of marine aquaculture systems (Urakawa et al., 2008) and are likely to be found in freshwater systems as well (Rurangwa & Verdegem, 2015; Schreier et al., 2010).

The optimal culture conditions for nitrifying bacteria in RAS biofilters have been described in numerous publications (Alleman & Preston, 1991; Hagopian & Riley, 1998; Chen et al., 2006, and DeLong & Losordo, 2012). Heterotrophic bacteria decompose solid wastes to generate ammonia and other nitrogenous wastes. Both nitrifying bacteria and heterotrophic bacteria compete for resources in the microbial communities of biofilters. Heterotrophic bacteria grow at a relatively rapid rate with an average generation time of 2.7 h, while nitrifying bacteria grow slowly with an average generation time of 18–70 h (Chen et al., 2006; Hagopian & Riley, 1998; Rurangwa & Verdegem, 2015). In addition, it appears that AOB has a faster generation time (26 h) than that for NOB (60 h) (Hagopian & Riley, 1998). Good mechanical filtration and the prompt removal of solid organic material from systems should be a priority in RAS systems to reduce the competition by heterotrophic bacteria in biofilters. In addition to the organic load in the biofilter, the success of nitrification is also influenced by the concentrations of ammonia and nitrite, the concentration of DO and mixing (turbulence), the pH and alkalinity of the system water, the salinity, and the temperature (Chen et al., 2006).

The biofilters frequently used for the culture of zebrafish in RAS are called *fixed-film biofilters* because the nitrifying bacteria reside in layers of biofilm fixed to a media substrate. Several studies have indicated that the preferred species of AOB and NOB in biofilters only function optimally at relatively low substrate concentrations of TAN and nitrite and that these processes are inhibited by high concentrations of substrates. Nitrification is inhibited by ammonia concentrations greater than 10 ppm and nitrite concentrations greater than 1 ppm and is also limited by very low concentrations of nitrogenous wastes at TAN concentrations less than 0.07 ppm (Hagopian & Riley, 1998; Chen et al., 2006). Fish culturists need to keep some ammonia in the system to keep nitrifying bacteria alive and functioning but not so much that fish are stressed and nitrification is inhibited.

Nitrifying bacteria are aerobic chemoautrophs and need high dissolved oxygen (DO) levels and turbulent mixing to function at high rates of nitrification. Every 1 g of ammonia (NH_3) converted to nitrate (NO_3^-) requires 4.57 ppm DO (Chen et al., 2006; Timmons & Ebeling, 2013). Nitrifying bacteria will function at DO levels from 0.6 to 60 ppm; however, the rate of nitrification is often DO limited due to a lack of water circulation past fixed biofilms (turbulence), limited diffusion rates within biofilms, or by the reduced oxygen saturations at high water temperatures (Chen et al., 2006). Nitrifying bacteria have demonstrated survival under prolonged anaerobic conditions in natural environments and have quickly rebounded when conditions became better

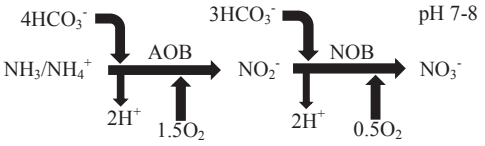

FIGURE 29.2 Important inputs and outputs for nitrification of TAN by ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). Nitrification produces water acidification in the form of H^+ and consumes approximately 7 molecules of alkalinity in the form of bicarbonate (HCO_3^-) and 2 molecules of oxygen for every gram of ammonia converted to nitrate.

(Hagopian & Riley, 1998). These findings did not examine the particular nitrifying bacterial species present in freshwater aquarium biofilters. It is, therefore, recommended that DO levels in biofilters be kept well mixed at near DO saturation and that the aeration of biofilters is among the highest priorities for back-up power should a power outage occur (Fig. 29.2).

Nitrifying bacteria have the highest levels of nitrification at basic pH with high concentrations of alkalinity. The optimal pH for nitrification in RAS is reported to be between pH 7.0 and 9.0 with the optimal range for AOB and NOB being 7.2 to 8.8 and 7.2 to 9.0 respectively (reviewed in Chen et al., 2006; Timmons & Ebeling, 2013). DeLong and Losordo (2012) cite a pH of 6.8–7.2 to be best for biofilter start-up. Nitrification rates drop off quickly in acidic pH (Chen et al., 2006). Alkalinity in the form of carbonate (CO_3^{-2}) or bicarbonate (HCO_3^-) serves as a carbon source for nitrifying bacteria (Chen et al., 2006; DeLong & Losordo, 2012) and buffers the water of RAS from the acidification that occurs during nitrification. Nitrification consumes alkalinity at a rate of 7.05 g for every 1 g of ammonia converted to nitrate (Chen et al., 2006; Timmons & Ebeling, 2013). It is, therefore, important that plenty of alkalinity or (HCO_3^- (bicarbonate)) be available for nitrifying bacteria and that fish culturists closely monitor systems for optimal pH and alkalinity levels (Fig. 29.2).

One of the circumstances that often eludes novice and experienced fish culturists alike is how to best establish the process of nitrification in a new system and biofilter. The literature clearly indicates that without assistance it takes considerable time, often up to 50 days, to get a biofilter to a fully functional status (DeLong & Losordo, 2012; Manthe & Malone, 1987; Timmons & Ebeling, 2013). There are several ways to seed new biofilters that have been used successfully either alone or in combination. Procedures used to start up new biofilters include the application of commercial bacterial preparations, the introduction of seasoned media from established filters, fishless chemical startups using ammonium chloride and sodium nitrite, and low fish biomass start-ups with expendable organisms. Regardless of the procedure used to start up the biofilter, there are common conditions that will help to speed up the acclimation process. Most culturists prefer to do a fishless start up as this provides the culturist more freedom to manipulate the microbial environment. The culturist should first optimize water quality for nitrification, including pH 7-8, higher alkalinity than normal system function (100–150 ppm), DO near saturation, warm water temperatures, and low levels (3–5 ppm) of both TAN and nitrite to "feed" AOB and NOB (DeLong & Losordo, 2012). The biofilter should have an appropriate biomedia with high surface area that is well mixed and aerated. If commercial nitrifying bacterial preparations are used, it is very important to follow the provider guidelines closely for optimal results. Finally, it is important to monitor water quality conditions for pH, alkalinity, TAN, and nitrite during the start-up for best results. DeLong and Losordo (2012) provide an excellent guide on starting a biofilter that can be consulted for further details. A typical nitrogenous waste profile for the establishment of a new biofilter is provided in Fig. 29.3.

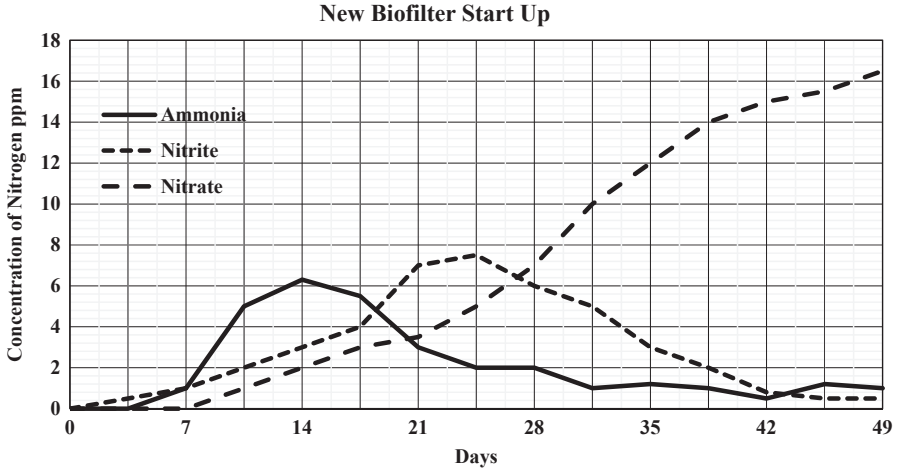

FIGURE 29.3 A typical profile of nitrogenous waste concentrations during the establishment of a new biofilter. Ammonia peaks first at approximately 14 days, but fast-growing ammonia-oxidizing bacteria (AOB) quickly decrease levels. Nitrite levels then peak at approximately 24 days, but slower growing nitrite-oxidizing bacteria (NOB) take longer to decrease nitrite levels. Nitrate levels increase starting at about day 7 and continue to increase until water is exchanged (not shown).

TABLE 29.2 Summary of water quality parameters, testing frequency, and suggested adjustments to water quality for zebrafish culture systems. All values reflect the current normal values based on the literature as of the writing of this text. Testing frequency and adjustments should depend on potential disturbances to the system, such as filter cleaning, new system start-up, power outages, changes in feeding, and changes in fish density, or other potential issues.

Water quality parameter	Testing frequency	Recommended range	Adjustment if Low	Adjustment if High
Temperature	Daily	26–29°C	Increase heater thermostat	Decrease heater thermostat
pH	Daily	7–8	Slow addition of sodium bicarbonate solution	Slow addition of dilute hydrochloric or acetic acid solution
Conductivity/Salinity	Daily	200–3000 µS, 0.5–2 ppt	Slow addition of sea salt solution	Slow addition of RO/DI purified water
Alkalinity	Weekly	50–75 ppm	Slow addition of sodium bicarbonate solution	Slow addition of RO/DI purified water
Hardness	Weekly	100–200 ppm	Slow addition of sea salt solution	Slow addition of RO/DI purified water
Dissolved oxygen (DO)	Weekly	6–8 ppm near saturation	Increase aeration, remove waste solids, increase water exchange, decrease stocking density	Reduce aeration, investigate for possible gas bubble disease
Carbon dioxide (CO_2)	Weekly	Close to 0 ppm as possible, prompt action at 15 ppm	Not applicable	Increase aeration, remove waste solids, increase water exchange, ventilate area
Total ammonia nitrogen (TAN)	Weekly	Close to 0 ppm as possible, less than 1 ppm TAN	Not applicable	Remove waste solids, increase water exchange, check pH and alkalinity
Nitrite (NO_2^-)	Weekly	Close to 0 ppm as possible, less than 0.5 ppm	Not applicable	Remove waste solids, increase water exchange, check pH and alkalinity, slow addition of sea salt solution
Nitrate (NO_3^-)	Weekly	Less than 50 ppm	Not applicable	Increase water exchange

Conclusion

Water quality is one of the most important but still overlooked variables in zebrafish research. Water quality parameters affect almost every aspect of fish physiology, and are, therefore, an important source of nonprotocol-based variation in studies. It is important that fish culturists understand each critical water quality parameter, its potential impact on fish physiology, and how to properly adjust each parameter in aquatic systems. Important water quality parameters include temperature, pH, conductivity/salinity, alkalinity, hardness, dissolved oxygen, carbon dioxide, ammonia, nitrite, and nitrate. It is important that fish culturists monitor and record the values of water quality parameters on a frequent and regular basis as this information verifies the conditions of the research. Additionally, this information may be useful to troubleshoot possible problems in experimental procedures, results, and outcomes. A brief summary of critical water quality parameters, recommended ranges, and adjustments, is provided in Table 29.2.

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CHAPTER

30

Recirculating Aquaculture Systems (RAS) for Zebrafish Culture

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Introduction

Recirculating aquaculture systems or recirculating systems are most simply defined as aquaculture production systems in which water is moved from a culture tank, through a treatment process, and then returned to the culture tank (Timmons & Ebeling, 2013). The greatest advantage of recirculating systems is that the culturist has nearly complete control over the culture environment and the organisms in culture. This is also its greatest weakness as recirculating systems have the greatest opportunity for catastrophic failure due to this high degree of control. Nevertheless, the high degree of control is desirable from the scientific research perspective of reducing potential experimental variation, and is, therefore, the preferred method of culture for zebrafish used in biomedical research.

Recirculating aquaculture systems used for research are complex and require the oversight of a knowledgeable and committed culturist to be consistently successful. The challenges of managing a recirculating system include (1) that they contain many different types of apparatus required to optimize culture conditions. These apparatus will be in continuous or near continuous use and must be understood, maintained, and manipulated correctly. (2) Recirculating system components, due to continuous use, are particularly prone to equipment failures, power outages, and technician errors. (3) Recirculating systems are particularly unforgiving of mistakes as they have relatively small water volumes, complex microbial communities, and high animal densities. As a result, the culturists manipulating the recirculating system must be well trained, experienced, detail-oriented, and careful. Experienced culturists understand a facility's normal sights, sounds, and smells; they habitually verify everything at least twice and never leave a facility without checking the function of critical valves and basic life support equipment. (4) Recirculating systems are expensive due to costs for infrastructure and apparatus, electricity, water, testing of water quality, replacement of consumables, labor, and necessary redundancy. Many aquaculture businesses have failed due to the expense of growing organisms in recirculating systems (Timmons & Ebeling, 2013). Zebrafish, aquacultured for research, are housed in specialized and sophisticated recirculating systems with daily water exchange rates of 5%–20% of system volume. Flow-through systems, where water is used once in a single pass through the system before being discarded, are sometimes used in specialized applications, such as toxicology studies. For more information on flow-through systems, consult chapter 29 Aquatic Housing of this text. This section will focus on the housing systems and water treatment of zebrafish recirculating systems used for research.

Required and Optional Components

In captivity, fish have four basic requirements, which are water, food, oxygen, and waste disposal. The required components of any recirculating system include a tank to hold the fish, a pump for circulation of water, equipment to control oxygen and carbon dioxide in the water, a mechanical filter to remove waste solids (clarification), and a biological filter to remediate toxic dissolved nitrogenous wastes (biofiltration) (Malone, 2013). In addition to these basic components, zebrafish culture systems often employ a wide variety of additional apparatus to optimize aquatic environments. Optional apparatus include heaters to maintain

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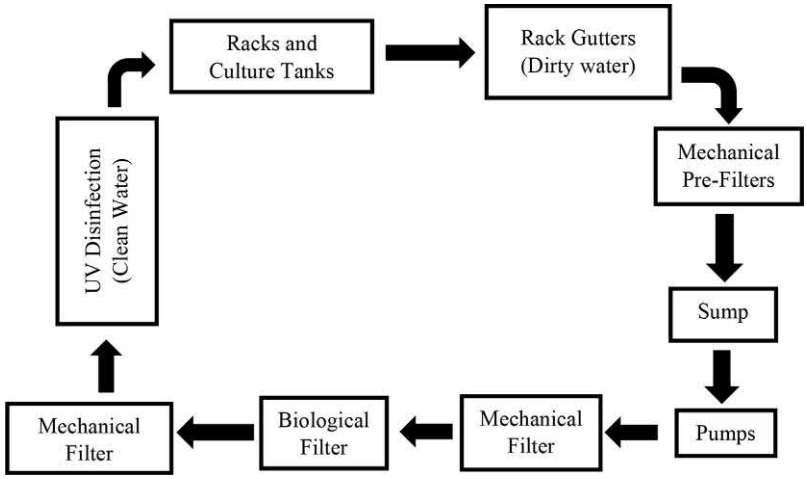

FIGURE 30.1 A typical order of water treatment processes for the culture of zebrafish. Dirty water exits the tank into the rack gutter. The rack gutter delivers water through a mechanical pre-filter, such as a polyester filter pad, filter sock, or stainless steel screen to the sump. Water pumps move water from the sump to the mechanical filter for further solids removal before entering the biological filter for the bacterial oxidation of nitrogenous wastes. Water exiting the biological filter is mechanically filtered to increase the efficiency of the ultraviolet (UV) disinfection unit that eliminates pathogens before being returned as clean water to the culture tank.

warm water temperatures, chemical filters to add or remove chemicals from the water, ultraviolet (UV) and/or ozone disinfection units to reduce circulating pathogens, chemical probes connected to dosing pumps to automatically monitor and adjust water quality, and computer interfaces to monitor and regulate the above processes and alert culturists to problems. A generalized water treatment process is provided in Fig. 30.1.

The rapid increase in popularity of zebrafish used for research has provided a unique niche for the mass production of specialized tanks, racks, and integrated recirculating system components. Lawrence and Mason (2012) stated that fish housing systems should (1) provide a stable and favorable growing environment for fish health and productivity, (2) support the research goals of the investigators, and (3) be designed to adhere to the regulatory requirements for fishes used in biomedical research. Several companies have successfully combined lessons learned from experimental rodent culture, aquaculture, and various engineering fields to provide unique multi-shelf zebrafish rack systems that support a large number of replicate tanks of various sizes and shapes (Lawrence & Mason, 2012). These companies have helped research institutions construct zebrafish facilities of all sizes. At the time of this writing, within major research institutions globally, there are a small but growing number of zebrafish culture facilities, where each facility contains more than 100 racks.

Racks and Tanks

The racks used to hold zebrafish culture tanks are typically constructed from high-grade stainless steel, aluminum, or powder-coated metal. They are usually designed to have four to six evenly spaced shelves that maximize personnel access to the tanks. It is paramount that racks are constructed of corrosion-resistant materials, as zebrafish culture is conducted in warm, humid, salty environments that lead to eventual corrosion of exposed metal surfaces (Lawrence & Mason, 2012). The racks are integrated with water delivery lines (one or more per tank) from above the tanks, and tanks typically drain toward the rear of the rack into open gutter systems that return water to under-rack collection sumps. Water is delivered to tanks on a rack through a hole in the tank lid. This is done either from specially designed spigots that route the water directly into each tank (example: Zebtec by Tecniplast or the Aquarius System by Aquatic Enterprises) or through a tube that routes water from a spigot into the tank (example: Aquaneering Inc. and Pentair Aquatic Eco-Systems). Racks can be constructed with light-cycle cabinets and control interfaces on one or more shelves to provide flexibility in photoperiod (Lawrence & Mason, 2012). Racks can be single-sided or double-sided and can be stand-alone with under-rack collection sumps and water treatment components or modular with centralized water treatment components. Individual commercially available single-sided racks (stand-alone or modular) appear to fall into a typical footprint of approximately 229 cm high by 154 cm wide and 36 cm deep (Lawrence & Mason, 2012). In the modular approach, water is collected from each of the racks into a common water return line, delivered to a separate filtration area for treatment, and then circulated back to the racks. The primary advantage of this approach is that a physically separated

water treatment area greatly reduces noise and vibration in the fish culture area. The noise and vibration associated with water pumps and treatment apparatus have been implicated as a potential stressor to fish in culture (Smith, Kane, & Popper, 2004; Vandenberg, Stevenson, & Levin, 2012; Wei, Lin, Chen, Tseng, & Shao, 2017). The modular approach also allows for relatively simple expansion of current facilities by addition of racks to the system. In addition to full-size racks, several companies have also made smaller tabletop or benchtop racks for researchers or educators with limited space. Many commercially available racks can accommodate tanks of different volumes on the same shelf, which adds flexibility to a system (Lawrence & Mason, 2012). For additional information and perspective, please consult chapter 26 Aquatic Housing within this text.

Zebrafish tanks are typically small and hold volumes ranging from 0.3 to 11 L (Lawrence & Mason, 2012). Tanks can be made of acrylic, fiberglass, glass (toxicology applications), polysulfone, or polycarbonate. Many facilities use polycarbonate, as this material is transparent, sturdy, inexpensive, and can be autoclaved without damage (Lawrence & Mason, 2012). One drawback of polycarbonate and polysulfone is its ability to leach bisphenol-A (BPA), a known estrogen mimic (Howdeshell et al. 2003), especially true for older tanks. Despite this issue, many facilities will continue to use polycarbonate, due to its other advantages, until a desirable replacement material becomes available (Lawrence & Mason, 2012). Tanks are designed with angled bottoms and specialized rear baffles or siphons to aid in the removal of solids. The removal of waste solids by this "self-cleaning" feature of tanks is a paramount consideration in tank selection (Timmons & Ebeling, 2013). Each of the commercial tank manufacturers has a unique design to promote "self-cleaning" while preventing fish from escaping from the tanks into the system. Escaping fish can create issues with the genetic integrity of fish colonies and can cause the spread of communicable diseases (Lawrence & Mason, 2012). The rear baffles of tanks frequently come in a variety of different pore sizes to maximize water circulation while preventing the escape of fry, juvenile, or adult zebrafish. Fry baffles have very small pores to minimize escape of very small zebrafish (2−3 mm) in tanks with continuous water circulation on a rack. In addition to interchangeable baffles, many of the commercial tank manufacturers have specialized tank inserts for spawning zebrafish on or off the rack system. The inserts have a slotted bottom to allow zebrafish eggs to fall through the floor of the insert onto the tank bottom (away from cannibalistic parents) and removable dividers to separate males and females prior to spawning. Commercial companies have also constructed spawning tanks with inserts, removable dividers, and in some cases a shallow end to promote shoaling behavior in zebrafish spawning; these are often designed to be used off the system.

The lids of zebrafish tanks are designed to contain fish, accommodate the entry of water, and the delivery of food without lid removal. The holes in tank lids must be large enough to allow the delivery of water and food into the tank but small enough to prevent fish from jumping out. It has been anecdotally reported that fish have been observed to jump through a hole in a lid onto the lid and then into an adjacent tank, creating a potential contamination problem for researchers (Farmer personal communication). Some tank lids come in colors that reduce light penetration and the growth of undesirable algae. Regardless of the lid color, tanks on the upper shelves (closer to ceiling lights) grow algae at a faster rate. Illumination intensity in culture rooms should be reduced to limit algae growth in tanks but still allow technicians to work efficiently. For additional information and perspective on tanks and their components, please consult chapter 26 Aquatic Housing within this text.

Pumps and Circulation

Water pumps for zebrafish research facilities are usually sized, engineered, and supplied by the commercial vendor for the system. Nevertheless, competent aquatic technicians should have a basic understanding of water pumps. All recirculating systems require a way to move water vertically to higher elevations to supply culture tanks or to increase system pressure and move water for filtration, disinfection, or other necessary treatment (Timmons & Ebeling, 2013). In zebrafish culture facilities, the water pumps are required to supply clean oxygenated water to fish, remove solid and toxic nitrogenous wastes from fish tanks, and supply oxygenated water and nitrogenous wastes to aerobic nitrifying bacteria in the biological filter. The loss of water circulation to aquatic systems from equipment failure or power outage can be problematic or catastrophic. Consequently, systems should be designed with redundant pumps, a reliable source of backup power, and an alarm system to notify technicians when problems occur. Priority for backup power should be provided first to the pump supporting the biological filter, as the high aerobic bacterial loads in this apparatus will quickly go anoxic without water circulation, and secondly to system circulation. In addition, facilities should keep extra water pumps in storage, and technicians should be familiar with pump maintenance and installation.

Centrifugal pumps are the most common type of water pump used in zebrafish culture systems. Centrifugal pumps work by supplying water to the center of the pump head, where it meets a rapidly spinning impeller blade that thrusts the water out at a right angle to the incoming water (Malone, 2013; Timmons & Ebeling, 2013). The impeller provides water pressure (lift) and flow (discharge) to the water exiting the pump head. Centrifugal pumps are capable of producing moderate to high water pressures and moderate to high flow rates depending on the type of pump utilized (Malone, 2013; Timmons & Ebeling, 2013). Wide varieties of pumps are commercially available to meet most desired combinations of pressure and flow. With all pump designs, water pressure (lift) is inversely related to water flow (discharge), meaning that pumps working at higher pressures will provide less flow (liters per minute) than pumps working at lower water pressures (Timmons & Ebeling, 2013). Commercial companies that supply water pumps for aquacultural use provide pump performance curves to describe the relationship between pressure and flow for various pumps. Zebrafish rack systems, by design, have large numbers of small diameter valves and fittings that supply water to a large number of tanks, and therefore, require powerful centrifugal pumps rated for high water pressures. Centrifugal pumps on larger centralized zebrafish systems and some stand-alone racks are typically located outside the tanks, while those for some stand-alone racks may have submersible pumps located underwater in the sump. The motor for submersible pumps is a strong electromagnet located in a plastic waterproof housing that spins a magnetized impeller blade in the pump head (Malone, 2013). Most centrifugal pumps, including submersible pumps, require positive water pressure at the inlet and are not capable of "self-priming," or drawing necessary water significant distances toward the impeller. To avoid issues with priming, most centrifugal pumps should be placed where they can draw water from the bottom of the sump where there is significant positive water pressure to feed the impeller (Timmons & Ebeling, 2013). The positive water pressure going to the impeller can be important because insufficient water depth in the sump can lead to Gas Bubble Disease (GBD). This disease of aquatic organisms occurs when supersaturated dissolved gases (typically nitrogen) enter fish via respiration and then come out of solution, due to insufficient counteracting hydrostatic pressure inherent to the shallow housing tanks, creating bubbles/emboli in fish tissues. GBD is associated with very high mortality rates and is most often caused by water pumps with small air leaks on the pump head near the impeller housing. It can also occur when there is insufficient water in the sump near the pump intake, causing the pump to take in a combination of air and water and thus compressing the gases. Symptoms of GBD include the presence of small bubbles exiting the water spigots into the tanks, small bubbles on the tank walls, and fish remaining near the bottom of the tank. GBD can be prevented by routinely inspecting pumps, placing a float switch in the sump to automatically turn pumps off when water levels are too low, and by using total gas pressure probes linked to an alarm system that will notify technicians when gas pressures become abnormal (Lawrence & Mason, 2012).

Many of the larger centrifugal water pumps have small, attached filter baskets, or pump traps, in front of the pump head or impeller housing. The purpose of these baskets is to capture any large solid items that might interfere or damage the impeller blade, such as plastic filter media, tank labels, plastic bags, or escaped fish. Baskets clogged with debris will decrease the positive water pressure of the pump and greatly decrease pump efficiency. These baskets should be routinely checked and cleaned to prevent unnecessary stress on the pump and decreased pump efficiency.

Centrifugal pumps are designed to move water at a continuous water pressure and flow rate through an aquaculture system. In reality, zebrafish culture systems, in particular, have highly varied needs for water pressure and flow. The reality of zebrafish culture facilities is that tanks are frequently added or removed from systems, valves are frequently turned on or off, and at times entire racks may be temporarily added or removed from the system circulation. These activities constantly change the pressure and flow of the water in a system and add stress to water pumps, resulting in reduced pumping efficiency, reduced energy efficiency, and ultimately increased operating costs. Traditionally, aquaculture systems were designed with oversized centrifugal water pumps that could handle the system's maximum capacity even though this resulted in a loss of pump energy and cost efficiency when the system was being run at less than maximum capacity. More recently, the arrival of variable frequency drive (VFD) or variable speed drive (VSD) pumps have greatly increased efficiency (Kalaiselvan, Subramaniam, Shanmugam, & Hanigovszki, 2016). In a sense, VFD pumping systems adjust energy use and system demand to always run the pumps for optimal performance and efficiency. VFD pumps and the configuration of water circulation in zebrafish facilities are discussed further in chapter 26 Aquatic Housing within this text.

Aeration

Dissolved oxygen (DO) is critical to the survival of fish and the beneficial aerobic bacteria that reside within

biological filters. The saturation of DO in water is affected by water temperature, altitude, salinity, and several other water quality parameters. Zebrafish are small tropical fish with high metabolic rates that are cultured at warm water temperatures (26–29°C). The DO of zebrafish culture systems should remain nearly saturated at 6–9 parts per million (ppm or mg/L) and the carbon dioxide (CO_2) concentration should remain at less than 20 ppm. DO and CO_2 concentrations should be tested weekly and recorded accurately on water quality data sheets to assist in trouble-shooting system trends (see chapter 29 entitled Water Quality for Zebrafish Culture in this text). Stable DO and CO_2 levels in zebrafish culture systems are supported by the small size and low weight of these fish, following a relatively low stocking density of between 5 and 15 fish per liter, offering a low total feed weight, and providing relatively high water turnover rates. The turbulence of water exiting the back of the tanks into the gutters and falling into the collection sumps assists in both oxygenation and carbon dioxide removal, also called *degassing*. It is also helpful to aerate/degas sumps and biological filters with pressurized air stones/air diffusers; these areas contain high aerobic bacterial concentrations that can greatly reduce DO and increase CO_2 concentrations in system water prior to returning to culture tanks. In addition, when a system includes a moving bed bioreactor (MBBR) for biological filtration, the "bed"/media is often fluidized by an aeration system. Air pumps and blowers are typically employed to drive air to diffusers. Operators should routinely observe and inspect for proper airflow through the diffusers. Inadequate airflow may result in reduced oxygen and increased carbon dioxide throughout the system and damage to air pumps and blowers from overheating. Routine maintenance of an aeration system should include periodic cleaning of air diffusers and inspection and cleaning of the air pump and blower filters. Preventive maintenance may include replacement of air filters, air pump piston seals, and air blower bearings. Aeration is considered a critical unit process and redundancy or the stocking of critical spare units and replacement parts is highly recommended. In large centralized facilities with higher biological loads, it may also be advantageous to utilize trickle filters or aeration/degassing columns for water treatment. Trickle filters or aeration/degassing columns are simple plastic columns filled with high void volume plastic media. Water is pumped to a pore plate or showerhead at the top of the filter and then allowed to trickle down through this media to exit at the bottom of the filter as highly oxygenated and carbon dioxide-free water (Eding, Kamstra, Verreth, Huisman, & Klapwijk, 2006). In addition to performing aeration and degassing, trickle filters can serve as relatively good biological filters that remove toxic nitrogenous wastes (Eding et al. 2006).

Mechanical Filtration (Solids Removal)

The solid wastes in a recirculating system arise from a variety of different sources, including uneaten food, feces, dead organisms, microbial biofilms, and other organic solids (Blancheton, Attramadal, Michaud, Roque d'Orbcastel, & Vadstein, 2013; Timmons & Ebeling, 2013). Most of these sources are linked directly or indirectly to the feed (Blancheton et al. 2013; Timmons & Ebeling, 2013) and nearly half of the feed consumed by fish is excreted as solids (Malone, 2013). As a result, solids management in a recirculating system really begins with the avoidance of overfeeding by the staff in a zebrafish culture facility. The accumulation of organic solids in a recirculating system has detrimental effects on nearly every aspect of fish culture, and solids removal is the most important process in the successful management of a recirculating system (Blancheton et al. 2013; Timmons & Ebeling, 2013). The accumulation of waste solids can increase the concentrations of nitrogenous wastes in water, increase the oxygen consumption and CO_2 production by aerobic bacteria, damage the gills in fish, harbor fish pathogens, reduce the visibility of fish in tanks, and obstruct the flow of water through the system (Lawrence & Mason, 2012; Malone, 2013; Timmons & Ebeling, 2013).

The total solids (TS) in any recirculating system can be divided into four size classes based on the ability of these solids to be removed from the system. These size classes are settleable solids, suspended or "fine" solids, colloids, and dissolved solids (Lawrence & Mason, 2012; Malone, 2013; Timmons & Ebeling, 2013). The settleable solids are those large solids, typically greater than 100 microns that readily settle out of still water, and are, therefore, the easiest to remove. Suspended or "fine" solids are those solids that will not readily settle out of still water and are typically less than 100 microns and usually between 1 and 30 microns (Timmons & Ebeling, 2013; Malone, 2013). These solids are the hardest to remove but account for the majority of solids by weight in intensive fish culture systems (Timmons & Ebeling, 2013). Colloids are solids that are indefinitely suspended in water and typically measure 0.1 to 1 micron, and dissolved solids are those that are dissolved at a molecular level (Malone, 2013; Timmons & Ebeling, 2013). Both colloids and dissolved solids are not removed from the recirculating system by filtration. Ideally, all of the settleable solids and as many of the suspended "fine" solids as possible should be removed from wastewater prior to biological filtration

(Lawrence & Mason, 2012; Timmons & Ebeling, 2013). Effective removal of waste solids will increase the efficiency of biological filtration by limiting the competition of heterotrophic bacteria on biological filter media (Lawrence & Mason, 2012). For further details on the microbial communities in biological filters, please refer to chapter 29 water quality for zebrafish culture in this text.

Solids removal in zebrafish culture facilities occurs at multiple levels, including the tank, the sump, the primary mechanical filter, and often a secondary mechanical filter prior to (UV) disinfection (Lawrence & Mason, 2012). Zebrafish tanks are designed to be "self-cleaning" with sloped tank bottoms and baffles in the rear of the tanks that promote the siphoning of solids into the rear gutters (see the more detailed description of tanks above and in the chapter 26 Aquatic Housing within this text). Once the solids enter the gutter system, the wastewater typically travels through mechanical pre-filters to the collection sumps. The mechanical pre-filters associated with the collection sumps are typically polyester filter pads, filter socks, or stainless steel screens (Lawrence & Mason, 2012). Polyester filter pads or filter socks are simple, inexpensive, and effective at removing large settleable solids. The disadvantages of filter pads and socks are that they are labor-intensive to clean, can clog quickly, and require periodic replacement, adding to the cost of labor and consumables. Stainless steel screens have recently started to replace polyester pads or socks, as they are easier to clean and seldom require replacement, reducing the cost of labor and consumables. Mechanical filters above the collection sumps are relatively effective at removing large settleable solids but will not capture smaller solids; these may settle in the bottom of collection sumps, especially those linked together to move wastewater toward a centralized filtration area. Solids settling in these areas will have to be periodically removed by siphoning (Lawrence & Mason, 2012).

Wastewater in the collection sump of a stand-alone rack system is typically moved to a biological filter for the remediation of nitrogenous wastes, and then it is pumped through a second mechanical cartridge filter, a granular activated carbon filter, and ultraviolet (UV) disinfection unit before being returned to the culture tanks. The mechanical cartridge filters can be made of polyester wound string, small pore polyester tubes, or a series of small micron stainless steel screens to polish water prior to entering UV disinfection units.

Wastewater that returns to the collection sumps of centralized water treatment facilities may receive further mechanical filtration prior to biological filtration; additional mechanical filtration often occurs prior to UV disinfection. The devices most frequently used for primary mechanical filtration in these larger centralized facilities include rotating drum microscreen filters, pressurized bag or cartridge filters, and granular, rapid sand or bead filters. Each of these filters has advantages and disadvantages summarized (Table 30.1).

Rotating Drum Microscreen Filters

Rotating drum microscreen filters (Fig. 30.2) are among the most effective and cost-efficient mechanical filters for commercial aquaculture loads (Malone, 2013). Rotating drum microscreen filters are cylindrical drums made from stainless steel or other noncorrosive metals. These drums are covered on the outside by small micron stainless steel or nylon mesh. The pore size of the mesh can vary from 20 to 200 microns depending on the size and amount of anticipated solids moving through the filter (Malone, 2013; Timmons & Ebeling, 2013). The drum is placed long side down and is approximately half-submerged in water inside a large tank or sump (Fig. 30.2). Wastewater enters the end of the drum near its middle axis and passes through the microscreen mesh that captures solids as the water moves through the mesh into the sump. If the mesh becomes congested with solids, the water level inside the drum will rise. Rising water triggers rotation of the drum and initiates automated high-pressure spray nozzles to wash solids into a gutter, cleaning the mesh and removing the waste solids from the filter to the sewage stream. Rotating drum microscreen filters remove solid wastes from water circulation before significant decay or leaching can occur, unlike the rapid sand or bead filters described below. These filters, thus, have high solids removal rates that actually increase as the solids concentration increases (Timmons & Ebeling, 2013). It is very important to have rotating drum microscreen filters, correctly sized, for the facility to reduce backwash frequency and consequent water losses. Zebrafish culture facilities have much lower feed rates, and therefore, solids loads than commercial aquaculture facilities. It may be possible to use smaller mesh sizes (closer to 30 μm) to remove more suspended "fine" solids. Rotating drum microscreen filters are expensive compared to other mechanical filtration options, and due to the mechanical nature of the filters, redundancy should be strongly considered. Many large zebrafish facilities with centralized filtration are using rotating drum filters as the primary mechanical filtration. Tecniplast has incorporated a small rotating drum filter into the filtration of the Active Blue Stand-Alone rack for zebrafish culture (Tecniplast). Routine drum filter maintenance includes periodic inspection of the drum seal, observation of backwash spray patterns, and logging of backwash pressures and the number of cycles per hour. Preventive maintenance may include the greasing

TABLE 30.1 Comparison of mechanical filter types used for the aquaculture of zebrafish for research with the advantages and disadvantages of each filter type.

Mechanical filter	Advantages	Disadvantages
Rotating drum microscreen filters	Large biological loads	Expensive equipment
	Filtration of particles 20–100 μm	Redundancy necessary
	Solids removed from the culture water before decay occurs	Potential for equipment failures
	Low operating cost	Greater water loss than other methods
	Low water pressure	
	Small footprint	
	Automation saves labor	
	No consumables	
Rapid sand filters	Filtration of particles 20–100 μm	Small biological loads
	Inexpensive filter and media	High water pressure
	Small footprint	Water loss with frequent backwash
	Automation saves labor	Heavy media
	No consumables	Potential for anoxia with power outages
	Some biological filtration	Potential for channeling
		Solids remain in the filter until backwash
		Variable/reduced flow over time until next backwash cycle
Floating bead filters	Filtration of particles 30–100 μm	Solids remain in the filter until backwash
	Handles large biological loads	Optimal performance requires management of backwash intensity and frequency
	Light-weight media easier to clean	Solids residing in the filter decay until backwash
	Low pumping costs	Variable/reduced flow over time until next backwash cycle
	Small footprint	
	Automation saves labor	
	No consumables	
	Biological filtration	
	Low volume of water loss during backwash	
Pressurized bag filters and cartridge filters	Filtration of particles 10–100 μm	Small biological loads
	Flexibility in particle sizes	Consumables used
	High flow rates	Labor to clean and replace filter bags

Continued

TABLE 30.1 Comparison of mechanical filter types used for the aquaculture of zebrafish for research with the advantages and disadvantages of each filter type.—cont'd

Mechanical filter	Advantages	Disadvantages
	Small footprint	High water pressure
		High pumping cost
		Variable/reduced flow over time until next filter change
Foam fractionators (protein skimmers)	Removal of the smallest suspended "fine" solids	Marginal beneficial impacts in freshwater systems
	Removal of some other organic solids	Inconsistent efficiency in freshwater applications
	Works great in marine applications	Requires frequent adjustment
	Can be used with ozone to increase efficiency	

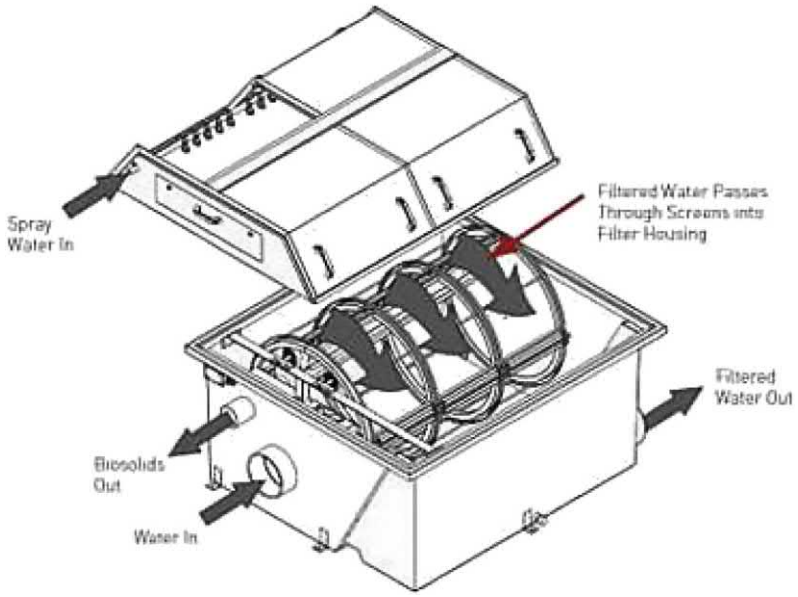

FIGURE 30.2 A schema of an example rotating drum microscreen filter. Dirty water enters the filter from the pipe. Water moves from inside the filter through the microscreen mesh that captures the solids and moves into the filter casing, where it exits the filter through the filtered water outlet pipe, as in the schema. PR Aqua™ Rotofilter Drum Filter. *Schematic of PR Aqua Rotofilter Drum Filter courtesy of Pentair Aquatic Eco-Systems.*

of drive chains and bearings, cleaning and or replacement of the spray nozzles, replacement of drum seals and drive belts, cleaning or replacement of filter screens, and the replacement of backwash pump bearings and shaft seals. The advantages and disadvantages of rotating drum microscreen filters are summarized in Table 30.1.

Rapid Sand Filters

Rapid sand filters are among the most recognizable filters as they are widely used as mechanical filters in backyard swimming pools and public aquariums (Fig. 30.3). This type of filter is sufficient for zebrafish culture facilities, especially as a secondary solids

FIGURE 30.3 A rapid sand filter used as a mechanical filter. *Picture courtesy of McWane Science Center Birmingham AL.*

removal process, but is not appropriate for the moderate to high biological loads of commercial aquaculture facilities (Timmons & Ebeling, 2013). Rapid sand filters work by passing water through a bed of sand under moderate to high water pressure. Solids are captured as water passes through the spaces between the sand grains and exits the filter clean. With use, the sand will become congested with solids, and the pressure will rise inside the filter, signaling the need for a backwash. Backwashes can be manual or automated. During a backwash, high-pressure water is pushed into the filter in the direction opposite that of filtration to free up debris and remove it from the filter. Rapid sand filters will provide some biological filtration as nitrifying bacteria will colonize the sand. Rapid sand filters with high bacterial titers can go anoxic quickly when circulation is interrupted, and sand compresses. Anoxic sand may produce toxic hydrogen sulfide capable of killing fish at very low concentrations. Rapid sand filters should be immediately backwashed after any significant interruption in circulation to remove toxic hydrogen sulfide prior to reestablishing water circulation with fish culture systems. With extended use or failure to consistently backwash the filter, the sand will accumulate organic material that congeals into solid masses, causing the water to bypass filtration in a process called "channeling." When channeling occurs, the filter must be disassembled and the sand replaced. This can be a cumbersome, messy, and a labor-intensive process. The advantages and disadvantages of rapid sand filters are summarized in Table 30.1.

Floating Bead Filters

Floating bead filters were developed as a commercial aquaculture alternative to rapid sand filters (Fig. 30.4). Floating bead filters provide most of the benefits of rapid sand filters without many of the disadvantages (Timmons & Ebeling, 2013). These filters utilize a 30–90 cm layer of small diameter (3–5 mm) floating plastic bead media to capture solids as water passes between the spaces in the beads (Malone, 2013). Plastic beads differ from sand in that the spaces between beads are larger and the beads are lighter, both of which make it easier to clean the beads of solids. These differences provide some important advantages over rapid sand filters. Bead filters have reduced backwash frequency, less water loss, lower operational costs, more biological filtration, a lower risk of anoxia, and a lower risk of channeling. Floating bead filters were designed to handle the high biological loads associated with commercial aquaculture (Timmons & Ebeling, 2013; Malone, 2013). Like rapid sand filters, the beads of floating bead filters must be backwashed when internal water pressures rise. The "backwash" typically involves using motorized propellers (large units) or air bubbles (small units) to mix the beads, free up the solids, and release the waste solids from the filter as discharge (Timmons

FIGURE 30.4 A floating bead filter used as a biological filter. *Picture courtesy of McWane Science Center Birmingham, AL.*

& Ebeling, 2013). Both incoming and outgoing water lines in bead filters have bead-retaining screens to prevent beads from exiting the filters during filtration and backwash (Timmons & Ebeling, 2013). A distinct disadvantage of bead filters and rapid sand filters is the retention of waste solids inside the filter until the backwash. This delay promotes heterotrophic bacterial growth and solids decay to ammonia (Timmons & Ebeling, 2013). For managing this issue, the frequency and intensity of the backwash need to be closely managed (Timmons & Ebeling, 2013). More recent bead filter designs (Polygeyser made by Aquaculture Systems Technologies AST) include a pneumatic backwash; backwashed water is then recycled internally to concentrate solids and greatly reduce water losses (Malone, 2013). Zebrafish culture facilities have begun to recognize the utility of floating bead filters as they provide a number of advantages when compared to other mechanical filters (Table 30.1). Well-managed floating bead filters in zebrafish culture facilities can serve as both mechanical and biological filters in the same unit process (Malone, 2013).

Pressurized Bag Filters and Cartridge Filters

Pressurized bag filters use polyester felt filter bags to strain the solids from pressurized water at high flow rates (Fig. 30.5). The bags are interchangeable for a wide variety of particles between one and 100 μm. Pressurized filter bags can remove nearly all sizes of suspended "fine" solids at very high flow rates (>300 L per minute). Bag vessels are equipped with pressure gauges to notify technicians that bags need to be cleaned or replaced. Pressurized bag filters located just prior to UV disinfection equipment will greatly increase the efficiency of UV disinfection as water clarity (absence of suspended "fine" solids) is one of the most important factors in efficient UV disinfection (Malone, 2013). Pressurized bag filters have small footprints and high solids removal rates but can be labor-intensive, require consumables, and have a high water pressure requirement (Table 30.1). For smaller applications with lower flow rates, cartridge filters can be used. Many of the standalone zebrafish racks have integrated cartridge filters prior to chemical filters, and UV disinfection equipment for reasons stated above. Cartridge filters are available in a wide variety of particle removal sizes from 20 to 50 μm and can be comprised of stainless steel mesh to reduce consumable costs. Cartridge filter canisters are also equipped with pressure gauges to notify technicians that cartridges need to be cleaned or changed. Cartridge filters are also very good at removing the smallest suspended "fine" solids from system water (Table 30.1) (Figs. 30.5 and 30.6).

FIGURE 30.5 Pressurized bag filters to provide mechanical filtration prior to ultraviolet (UV) disinfection. Note the pressure gauges on top of the canisters to notify technicians that the bags need to be cleaned or changed. Note the UV disinfection units behind the pressurized bag filters. *Picture courtesy of The University of Alabama at Birmingham.*

Foam Fractionation (Protein Skimming)

Foam fractionators, also called protein skimmers, use air bubbles and organic surfactants to create stable foams that remove suspended "fine" solids from culture water (Malone, 2013; Timmons & Ebeling, 2013). Surfactants are chemicals that have a hydrophobic "water-hating" portion and a hydrophilic "water-loving" portion (Timmons & Ebeling, 2013). Surfactants form when proteins are degraded in the system water. Protein surfactants that come into contact with air bubbles orient the charged hydrophilic portion of the molecules outward where they attract oppositely charged molecules in the water (suspended "fine" solids in particular) and trap them, creating a stable foam that overflows into a collection cup for disposal. The amount of skimming accomplished depends on the amount of surfactant and the amount of surface tension in the water. While foam fractionation is very effective in marine systems due to the high surface tension of the water, it is more erratic in freshwater systems and will probably have only marginal significance for freshwater zebrafish culture systems (Malone, 2013). This author is not aware of any zebrafish facilities that currently use foam fractionation as part of the filtration process though it has been discussed.

Biological Filtration

Biological filtration is defined as the process by which dissolved wastes are removed from aquaculture water by bacterial action (Malone, 2013). The term biological

filtration, in the literature, most frequently refers to the process of converting dissolved nitrogenous wastes, such as toxic ammonia (NH_3) and nitrite (NO_2^-) to less toxic nitrate (NO_3^-). Often overlooked are the important processes of mineralization by the heterotrophic microbial communities also found in these filters (Malone, 2013; Blacheton et al. 2013). Heterotrophic microbial communities within biofilters rapidly and efficiently mineralize a wide variety of dissolved organic materials, including carbohydrates, fats, and proteins (Malone, 2013; Schreier, Mirzoyan, & Keiko, 2010; Blacheton et al. 2013). Biological filters house the greatest variety and number of microbial organisms in recirculating systems (Rurangwa & Verdegem, 2015). Schreier et al. (2010) provides a list of the microbial diversity found in the biofilters of recirculating systems. The management of these microbial communities is reviewed in Rurangwa and Verdegem (2015).

The process of nitrification is most important to the discussion of biological filtration for zebrafish husbandry. Two different groups of aerobic nitrifying bacteria drive this process: (1) the ammonia-oxidizing bacteria (AOB) that convert ammonia (NH_3) to nitrite (NO_2^-) and (2) the nitrite-oxidizing bacteria (NOB) that convert nitrite to nitrate (NO_3^-). Ammonia production comes ultimately from the feed either as the nitrogenous waste from protein metabolism or from the mineralization of solid organic wastes, such as feces or uneaten feed, by heterotrophic microbial communities (Blancheton et al. 2013; Malone, 2013; Rurangwa & Verdegem, 2015).

FIGURE 30.6 Two fluidized sand filters. *Picture courtesy of McWane Science Center.*

Ammonia comes in two forms that exist in equilibrium and that are collectively referred to as total ammonia nitrogen (TAN). The optimal water quality conditions for the growth of AOB and NOB are generally a pH between seven to eight, alkalinity of 50–100 ppm (mg/L), dissolved oxygen (DO) levels near saturation (6–9 ppm), and low concentrations of ammonia and nitrite. For detailed information regarding TAN, nitrite, nitrate, and nitrification, please refer to chapter 29 entitled Water Quality for Zebrafish Culture in this book.

There are two basic types of biological filters found in aquaculture. Suspended growth biofilters grow suspended bacteria in the same tank as the culture organisms, and fixed film biofilters circulate treatment water in close proximity to bacteria attached to media in a location separate from the culture tank (Malone, 2013). Both biofilter types rely on the diffusion of dissolved organic materials for either mineralization or nitrification to occur. For this reason, both aeration and water circulation within biofilters is of paramount importance to optimize processes. Fixed film biofilters are the overwhelming favorite for most aquaculture applications and are the only method of biological filtration currently used for the culture of zebrafish in the research environment.

Characteristics that influence the effectiveness of biological filtration include: the size and volume of the biofilter, the circulation of the water through the media, the retention time of the water in the biofilter, the water quality conditions within the biofilter, and the type of media used (Malone, 2013; Rurangwa & Verdegem, 2015; Timmons & Ebeling, 2013). It is currently presumed that the ammonia movement into biofilms is the single most important factor affecting the rate of biofiltration (Malone, 2013). The aquaculture industry is placing a strong emphasis on filter types that maximize the surface area of biofilms available to oxidize ammonia (Malone, 2013). Fixed film biofilters use a wide variety of media types with high surface area to volume ratios (Table 30.2). Several characteristics are considered when media are selected for biofilters (Timmons & Ebeling, 2013):

(1) The void volume of the media. Media with larger open spaces among the surface area for bacterial attachment encourage water circulation, oxygen transfer, and reduce potential clogging.
(2) The specific surface area of the media per unit of volume (ft^2 per ft^3 or m^2 per m^3). The greater surface area provides greater space for bacterial growth and more TAN removal per unit of media volume.
(3) The TAN conversion rate of the media type (area and volume) as grams of TAN converted per ft^2 or m^2 of media per day or grams of TAN converted per ft^3 or m^3 of media per day.

(4) The cross-sectional area and hydraulic loading rate. These are the area of media directed at the water flow (ft^2 or m^2) and the volume of water moved through the biofilter per unit of cross-sectional area per unit of time (gallons per ft^2 per day, or m^3 per m^2 per day) respectively.

Different media types and the characteristics of each are shown in Table 30.2.

A large number of different biofilter types are commercially available for use with centralized filter systems in zebrafish culture facilities. There is less variety found in the stand-alone rack systems provided by the commercial suppliers. The ideal biofilter would have a maximal surface area, remove 100% of toxic ammonia and nitrite, maximize DO transfer, have a small footprint, use inexpensive media, have low pumping requirements, require little maintenance, and not capture solids (Timmons & Ebeling, 2013). Such a filter does not exist. There are many types of filters that can be used, and this chapter will focus on those types best suited for the culture of zebrafish used in research.

Moving Bed Bioreactors

The moving bed bioreactors (MBBR) use 6–13 mm plastic media with moderate void volumes and protected internal surface areas (Kaldnes K1 media) for the attachment of bacteria (Malone, 2013; Timmons & Ebeling, 2013). Media typically occupies 50%–70% of the reactor volume and is contained within the reactor by the screened pipe at the water outlet (Timmons & Ebeling, 2013). The MBBR is continuously and vigorously aerated, creating maximal water circulation for maximal diffusion of dissolved oxygen (DO) and nitrogenous wastes (Malone, 2013). These filters have only about ¼ to ½ the nitrification capacity of fluidized sand biofilters but feature high levels of aeration and CO_2 removal (Malone, 2013). Guerdat et al. (2010) researched an MBBR from an intensive warm-water tilapia aquaculture system and observed a TAN removal rate of 267 g TAN/m^3/day and a modest increase in DO as water exited the MBBR. MBBRs have a small footprint, low maintenance requirements, no requirement for backwash, a low construction cost, and are conceptually simple (Malone, 2013; Timmons & Ebeling, 2013). These filters cause moderate shearing of biofilms as media collide with each other inside the reactor (Timmons & Ebeling, 2013). MBBR-like biofilters are found in the sumps of several commercially available zebrafish racks, such as the Aquarius System stand-alone rack by Aquatic Enterprises and the Z-Hab System by Pentair Aquatic Eco-Systems. The advantages and disadvantages of MBBR are summarized in Table 30.3.

TABLE 30.2 Biological filter media examples provided with a brief description, the approximate size of the media, specific surface areas m^2/m^3, relative void volume, and approximate TAN conversion rates for warm-water aquaculture systems.

Media	Description	Size	Surface area m^2 per m^3	Void volume	TAN conversion rate
Bio-Balls[a]	Plastic spheres	3.8 cm	322[a]	High	1–2 g/m^2/day[d]
Bio-Balls[a]	Plastic spheres	2.5 cm	526[a]	High	1–2 g/m^2/day[d]
Bio-Fill[a]	PVC ribbon	Ribbon	822[a]	Medium	No reference
Bio-Strata[a]	Plastic blocks	Block	223–361[a]	Medium	1–2 g/m^2/day[d]
Bio-Barrels[a]	Plastic cylinders	2.5 cm	210[a]	High	1–2 g/m^2/day[d]
Kaldnes K1[d]	Plastic cylinders with protected surface area	1 × 0.7 cm	500[d]	High	267 g/m^3/day[e]
Standard bead[b]	Spherical plastic beads	3–5 mm	1150–1475[b]	Low	380–518 g/m^3/day[f]
Enhanced nitrification[b]	Pressed plastic beads	8–10 mm	1150–1475[b]	Low	586 g/m^3/day[e]
Sand[c]	Sand or silica glass of various sizes	0.1 – 0.3 mm[c]	4000-20,000 m^2/m^3[c]	Low	667 g/m^3/day[e]

[a] *Values derived from the Pentair Aquatic Eco-Systems 2016 Master Catalog.*
[b] *Values from Malone, Chitta, and Drennan (1993).*
[c] *Values from Summerfelt 2006.*
[d] *Values from Timmons and Ebeling (2013) for Kaldnes K1 media surface area and TAN removal rates for trickle filter media with specific surface areas from 100 to 300 m^2/m^3*
[e] *Observed values from Guerdat, Losordo, Classen, Osborne, and Delong (2010) for tilapia grown under intensive conditions at average temperature of 29 C.*
[f] *Observed values for TAN removal rates from Sastry, DeLosReyes, Rusch, and Malone (1999) for tilapia and bubble-wash bead filters that were performing both mechanical and biological filtration.*

TABLE 30.3 Comparison of biological filter types (biofilters) used for the aquaculture of zebrafish for research with the advantages and disadvantages of each filter type.

Biofilter	Advantages	Disadvantages
Moving bed bioreactors (MBBR) Media: Bioballs, biobarrels, kaldnes K1	Aeration increases DO Good CO_2 removal Small footprint Low maintenance No backwash needed Low-cost construction	Nitrification rates are ¼ to ½ of fluidized sand filters Surface area of media is about ½ that of fluidized sand filters Not good removal of excess biofilms Requires good water circulation
Fluidized sand filters (FS) Media: Sand of various sizes Silica glass beads	Highest surface area Highest TAN removal rate Small footprint Highest biological loads Excellent transfer of DO and TAN Inexpensive media No backwash or water loss	High DO consumption High CO_2 production Biofouling of sand media and bed growth High pumping costs Backup pumps and power necessity Heavy media Sand beds go anoxic quickly with loss of water flow
Floating bead filters (FB) Media: Floating plastic beads Standard beads or enhanced nitrification "EN" beads	High TAN removal rates High surface area media Light-weight media Easy to install and operate Small footprint Backwash removes excessive biofouling Mechanical and biological filtration Low pumping requirement Low water loss on the backwash	DO is consumed CO_2 produced Relatively high head loss that can be variable Require frequent backwashing to work optimally If used for both mechanical and biological filtration nitrification rates are lower
Trickling media filters (TM) Media: Bioballs, biobarrels, biofill, kaldness K1	Simple to construct and operate Excellent aeration Excellent CO_2 removal No mechanical or electrical requirement	Low surface area media Lower nitrification and TAN removal rates Biofouling of media no biofilm removal Requires maintenance

Fluidized Sand Filters

Fluidized sand (FS) filters are tubular filter casings containing a bed of sand that is expanded by the up-flow of water from the bottom of the filter casing (Malone, 2013) (Fig. 30.6). The up-flow of water greatly expands the distance between individual sand grains and fluidizes the sand, thus expanding the entire sand bed within the filter casing. Well-engineered FS filters have excellent transport of DO, ammonia, and nitrite to nitrifying bacteria (Timmons & Ebeling, 2013). FS filters feature the greatest specific surface areas and the highest TAN removal rates (Malone, 2013). FS filters can remediate TAN at 0.6–1.0 kg/m^3/day and typically maintain very low ammonia and nitrite levels; it has been reported that as much as 50%–90% of the TAN is removed each time the water passes through the filter (Malone, 2013; Timmons & Ebeling, 2013). The continuous collisions of adjacent sand particles help to remove excessive biofilm accumulations, and this increases nitrification efficiency (Malone, 2013). FS filters produce large amounts of carbon dioxide (CO_2) and consume large amounts of oxygen (DO). Summerfelt and Sharrer (2004) reported that an FS filter attached to a salmonid production system produced an average of 4 ppm (mg/L) CO_2 and consumed an average of 3.8 ppm (mg/L) of DO. These values represented 37% of the total CO_2 produced and 35% of the total DO consumed by the entire system. These values are likely to be higher in warm water culture systems. The authors recommend that aeration/degassing would be most effective if located immediately after the biofilter (Summerfelt & Sharrer, 2004). Additional problems with FS filters include maintenance problems associated with biofouling (fine solids capture) and the high pumping costs associated with fluidizing sand (Timmons & Ebeling, 2013). Davidson, Helwig, and Summerfelt (2008)

described an effective method for managing sand bed growth due to biofouling around sand grains. Sand with excessive biofouling has a low density and will float to the top of the FS bed. Authors used a submersible pump to move this low-density sand to the base of the FS filter. At the base of the filter, increased shearing removed the biofilm that then simply floated out of the filter. Managing FS filters in this way increased TAN and nitrite removal rates, decreased management labor, reduced bed growth, and reduced sand loss (Davidson et al. 2008). One pitfall of this FS filter management strategy is that it increases the amounts of fine solids that could irritate the gills of sensitive species (Summerfelt, 2006). FS filters can become compact and go anoxic quickly during a loss of pumping pressure or loss of power. For this reason, reliable back up pumps and power supplies are a necessity for FS filters. Different sizes and types of sand media can be used in FS filters. Davidson et al. (2008) studied FS filters using two different sizes of sand at 0.11 and 0.19 mm. They reported increased TAN and nitrite removal and increased DO consumption with the smaller sand size. Aquaneering Inc., for example, features an FS filter with a small spherical glass media (<1 mm). These filters are adapted for the small stand-alone zebrafish rack systems and large FS filters found in the centralized filtration systems of large zebrafish culture facilities (Aquaneering Inc.). The advantages and disadvantages of FS filters are summarized in (Table 30.3).

Floating Bead Filters

Floating Bead filters (FB) use floating plastic bead media (3–10 mm) with high specific surface area (>1000 m^2/m^3) and low void volume to support bacterial growth (Fig. 30.4). Water to be treated typically upflows through a static bed of floating beads that function as either a mechanical filter, a biological filter, or both simultaneously (Malone, 2013; Timmons & Ebeling, 2013). Aquaculture Systems Technologies (AST) has developed three different types of FB that suit a wide variety of culture applications. The bubble-wash bead filter, propeller-wash bead filter, and polygeyser bead filter, all have advantages and disadvantages that will not be discussed here. All FB filters have the advantage of high surface area and light-weight floating bead media that helps to facilitate good bacterial growth with effective wash capability to remove trapped solids. Beads are routinely washed by hydraulic, pneumatic, or mechanical means to remove accumulated solids and excessive biofilms (Malone, 2013; Timmons & Ebeling, 2013). FB filters have low pump pressure requirements, compact design, conserve water better than many other filters, and are easy to install, operate, and backwash. The nitrification rates of FB filters approximate those of FS filters when used only for biological filtration (Malone, 2013). Nitrification rates for FB filters performing as both mechanical and biological filters are much lower at 380–518 g TAN/m^3/day (Sastry et al. 1999). FB filters are not commonly used in zebrafish culture facilities at this time, although these filters have several desirable characteristics that could no doubt benefit such facilities. The advantages and disadvantages of FB filters are summarized in Table 30.3.

Trickling Media (TM) Filters

Trickle filters (TM) use an open-top filter column packed with high void volume media to combine nitrification with aeration and degassing capability. The specific surface area and TAN conversion rates of the media used for TM filters is lower than that of other types of biofilters, but these issues are offset by the aeration and CO_2 removal that these filters provide. Authors have reported a range of TAN removal rates from 0.1 to 0.9 g of TAN/m^2/day for various TM filters operated at temperatures from 15 to 25 C (Eding et al. 2006; Timmons & Ebeling, 2013). The efficiency of these filters greatly depends on the water distribution at the top of the column and the efficiency of solids removal prior to water entering the TM filter (Eding et al. 2006; Timmons & Ebeling, 2013). Designs use flattened pore plates, spray nozzles, or other mechanisms to evenly distribute water to the top of the column. Water exiting TM filters is typically DO saturated and has very low CO_2 concentrations. Solids not removed by efficient mechanical filtration upstream of TM filters will clog filter media, increase oxygen demand, and reduce TAN removal rates (Eding et al. 2006). TM filters are also easy to construct, relatively inexpensive, and have no mechanical or electrical requirements (Lawrence & Mason, 2012; Malone, 2013; Timmons & Ebeling, 2013). The advantages and disadvantages of TM filters are summarized in Table 30.3.

Chemical Filtration and Modification of Water Quality

Chemical filtration is broadly interpreted herein to include any water treatment process where the water chemistry is altered by the addition or removal of chemicals from the water. This may occur by the processes of physical adsorption, water conditioning, or ion exchange.

Granular Activated Carbon

Granular activated carbon (GAC) can be made from wood, charcoal, nutshells, fruit pits, bituminous coals,

lignite, peat, bone, and paper mill waste (Tchobanoglous, Burton, & Stensel, 2003; Yu, Yong, Han, & Ma, 2016). Material is heated at high temperature (700°C) in the absence of the oxygen necessary to sustain combustion. The material is then activated by oxidizing gases, such as steam and CO_2, create a porous structure. The internal surface area created in the activated carbon has macropores >25 nm, mesopores from 1 to 25 nm, and micropores <1 nm. The internal structure of GAC will vary based on the raw material and the treatment process (Tchobanoglous et al. 2003). GAC works through the process of adsorption, which is the process of transferring and accumulating substances from solution onto a solid phase (Tchobanoglous et al. 2003). Adsorption onto the solid phase GAC occurs via hydrogen bonding, covalent bonding, van der Waals forces, dipole-dipole interactions, and other chemical interactions (Tchobanoglous et al. 2003).

GAC has been used for decades by the wastewater treatment and water treatment industries to remove a wide variety of different dissolved chemicals, including pesticides, herbicides, aromatic solvents, high molecular weight hydrocarbons, and PCBs. GAC will not remove ammonia, nitrite, nitrate, alkalinity or hardness to any appreciable extent. In aquaculture, GAC is frequently used to remove organic dyes, colors, odors, dissolved organic carbon (DOC), residual ozone where ozonation is used, and for the final polishing of water. Spotte and Adams (1984) observed the removal of DOC in aquaria using GAC made from coconut shell, hardwood, anthracite, and bone. They found that hardwood was superior for DOC removal. Aitcheson, Arnett, Murray, and Zhang (2001) suggested that coal-based GAC was effective for the removal of the aquaculture therapeutics Malachite Green, Oxytetracycline, Chloramine-T, and Formalin, alone or in mixtures. GAC is also useful for the removal of chlorine, chloramine, and other chemicals from municipal water. For further information on this process, please refer to chapter 29 entitled "Water Quality for Zebrafish Culture" in this book. GAC is consumed over time and must be replaced. GAC replacement will vary based on the system load, feeding rate, quality of mechanical filtration, and other factors. The use of new GAC may contribute to spikes in the pH of zebrafish culture systems, and it is recommended that only acid-washed GAC be used (Lawrence & Mason, 2012). GAC needs to be thoroughly rinsed with reverse osmosis water prior to use as the material powders during transport and small particles can accumulate in tanks, tubes and other areas of the recirculating system. Like all reactors of this type, the efficiency of removal processes can be increased with increased surface area and contact time (Lawrence & Mason, 2012). GAC cartridges are used in several of the stand-alone zebrafish rack systems, including those by Tecniplast, Aquaneering, and Pentair. In large centralized filtration systems, GAC can be placed in pressurized canister filters located after the mechanical and biological filters and before UV disinfection equipment. GAC functional life can be extended if fine solids capture via small micron cartridge or canister filters occurs prior to GAC canisters.

Calcium Limestone Reactors

A calcium limestone reactor (CLR) is a simple enclosed tubular casing containing a small particulate form of calcium carbonate ($CaCO_3$) media, such as crushed coral, oyster shell, or aragonite. Water up-flows through the reactor casing, fluidizing the media and causing abrasions among the media particles, which solubilizes small amounts of $CaCO_3$ on a continuous basis. Once a CLR comes online and reaches equilibrium, hardness and alkalinity values should remain relatively constant for long periods. Such reactors are used for the aquaculture of hard corals in recirculating systems since corals continuously deplete the calcium carbonate from the system water to build skeletons for growth. A CLR was described in Harper and Lawrence (2011) for use with a zebrafish culture system to maintain alkalinity and hardness levels. It is recommended to thoroughly rinse the media with reverse osmosis water prior to use to remove small particles that could potentially cause problems.

Zeolite Reactors (Ammonia Towers)

A zeolite reactor (ZR) is a simple large diameter tube (column) of varying length sealed at the bottom and having a bulk-head fitting on its side near its bottom. Water to be treated enters at the top of the tube and flows downward through ammonia absorbing media, such as zeolite contained in the tube. Ammonia is removed from the treatment water that falls by gravity into a sump. Alternatively, zeolite material can be added to pressurized cartridge vessels as part of a filtration loop. ZR reactors are not cost-effective or desirable as a replacement for good biological filters and are most useful when added as a separate temporary loop for systems in ammonia crisis. Zeolite material, like any other reactor material, will be consumed over time and will require replacement. Like all reactors of this type, the efficiency of ammonia removal can be increased with an increased surface area of zeolite and increased contact time.

Chemical Probes

Small stand-alone racks and large centralized zebrafish facilities frequently utilize electronic probes to

monitor water quality. Systems may utilize probes for determining temperature, pH, conductivity, dissolved oxygen (DO), total gas pressure, and oxidation/reduction potential (ORP). Readings from all electronic probes should be recorded daily. In addition, probes for critical water quality parameters, such as temperature, pH, and conductivity should be routinely validated using a second testing method (hand-held probes or water quality test kit) to be certain that results are accurate. Each probe has unique characteristics that require regular maintenance and calibration by personnel that are familiar with the equipment and procedures. Consult the appropriate manuals or manufacturer representatives for specific instructions.

Dosing Systems

Many centralized filtration systems and stand-alone rack systems have automatic dosing systems linked to pH and conductivity probes. These systems will activate peristaltic dosing pumps to add concentrated sodium bicarbonate (pH and alkalinity) or sea salt (conductivity, alkalinity, hardness) solutions in a slow and methodical manner until pH or conductivity values normalize to user-set parameters respectively and shut off when the target level is reached. The concentrated solution for pH is made from powdered sodium bicarbonate dissolved in RO/DI water at 16 ppt (g/L), and the concentrated conductivity solution is made from high-quality sea salts dissolved in RO/DI water at 25 ppt (g/L). Solution levels should be monitored regularly and replenished on a periodic schedule before they become depleted. Automated dosing systems should be tested and cleaned periodically to prevent the formation of solid plugs in the delivery lines. The sudden release of a plugin a delivery line followed by the sudden release of a concentrated solution in a system sump can cause a rapid change in system pH or conductivity that can stress or damage zebrafish. Peristaltic tubing should be lubricated (if applicable) and or replaced per the manufacturer's recommended preventive maintenance schedule.

Disinfection

Ultraviolet (UV) Disinfection Units

There are a wide variety of microorganisms that reside within the culture tanks and biological filters of recirculating aquaculture systems (Rurangwa & Verdegem, 2015; Schreier et al. 2010). Some of these microorganisms are potential pathogens of cultured fish and could pose a threat to experimental results. Commercial culture systems for zebrafish, including small stand-alone racks and centralized filtration rooms of large facilities, utilize a disinfection step immediately after mechanical, biological, and chemical filtration (Lawrence & Mason, 2012). Ultraviolet (UV) disinfection systems are commonly used for this purpose (Harper and Lawrence 2011).

UV disinfection systems can be nonpressurized open-cell units or pressurized tube and shell units (Timmons & Ebeling, 2013). The latter is most common among zebrafish facilities as they are the easiest to install and operate. The UV bulbs in these systems are contained within quartz sleeves that run down the center of a plastic or stainless steel tube and extend through the long axis of the tube (Fig. 30.7). The quartz sleeve maximizes transmittance while sealing the bulb away from the water that enters the tubular shell and surrounds the bulb. Water travels uni-directionally through the long-axis of the tube, and rubberized seals keep the electrical connections, wires, and the UV bulb dry. The UV bulbs used in disinfection units are typically low-pressure mercury vapor bulbs that are monochromatic, emitting single wave-length UV-C light at 254 nm. This type of bulb and wavelength of UV-C light has been shown to be most effective in the elimination of fish pathogens (Rurangwa & Verdegem, 2015; Summerfelt, 2003; Timmons & Ebeling, 2013). UV light penetrates and alters the DNA of microorganisms, creating pyrimidine dimers that either block DNA replication or mutate the organism enough to prevent successful reproduction (Timmons & Ebeling, 2013). UV irradiation intensity is described as microWatts per square centimeter ($\mu W/cm^2$) and the dosage is described as microWatt seconds per square centimeter ($\mu Ws/cm^2$) or milli-joules per square centimeter (mJ/cm^2) where $1000\ \mu Ws = 1\ mJ$ (Summerfelt, 2003). Important factors in the effectiveness of UV disinfection include the irradiation intensity (bulb wattage), dosage ($\mu Ws/cm^2$ or mJ/cm^2), contact time (10–30 s), particle size of materials still in the water after filtration, particle density, and transmittance (Lawrence & Mason, 2012; Rurangwa & Verdegem, 2015; Summerfelt, 2003). Transmittance is the ability of the UV light to reach the target organism through the water. Transmittance is related to the turbidity (clarity) of the water to be treated. Excellent mechanical filtration immediately prior to UV exposure will enhance transmittance and lead to better overall results (Malone, 2013; Rurangwa & Verdegem, 2015; Summerfelt, 2003; Timmons & Ebeling, 2013).

UV bulb intensity begins to decline immediately after installation. Timmons and Ebeling (2013) stated that users should expect the bulb intensity to decrease by 3% per month, and by 40% over 1 year of service. UV disinfection should be designed based on the predicted bulb intensity at the end of the bulb life and at the lowest expected transmittance for the system in order to be

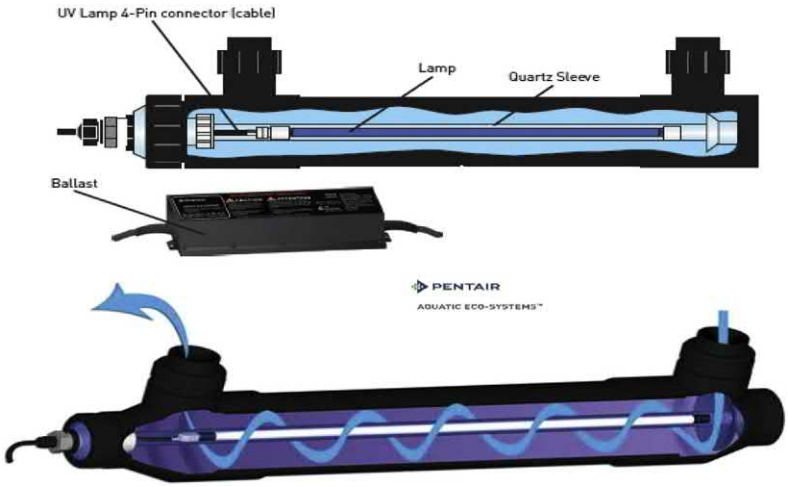

FIGURE 30.7 Schema of an ultraviolet (UV) disinfection unit. The UV bulbs in these systems are contained within quartz sleeves that run down the center of a plastic or stainless steel tube and extend through the long axis of the tube. The quartz sleeve maximizes transmittance while sealing the bulb away from the water that enters the tubular shell and surrounds the bulb. Water travels uni-directionally through the long-axis of the tube, and rubberized seals keep the electrical connections, wires, and the UV bulb dry. *Schematic courtesy of Pentair Aquatic Eco-Systems.*

certain that target UV intensity is always maintained (Summerfelt, 2003; Timmons & Ebeling, 2013). Transmittance at 254 nm for system water can be tested using a UV transmittance analyzer or UV spectrophotometer to be certain that UV disinfection is optimal. Maintenance for UV disinfection units should include annual replacement of bulbs and seals and cleaning the quartz sleeves.

The pathogen eliminating the ability of UV lamps varies widely by target organism and throughout the literature (Annett, 2016; Rurangwa & Verdegem, 2015; Summerfelt, 2003; Timmons & Ebeling, 2013). The recommended UV dose ranges from 100,000 μWs/cm^2 (100 mJ/cm^2) for most bacteria to 250,000 μWs/cm^2 (250 mJ/cm^2) for most fungi (Rurangwa & Verdegem, 2015). In general, viruses appear to require much higher UV doses, ranging from 122,000 μWs/cm^2 (122 mJ/cm^2) to 900,000 μWs/cm^2 (900 mJ/cm^2) for infectious pancreatic necrosis (IPN) and white spot syndrome baculovirus of shrimp (WSSB) respectively (Rurangwa & Verdegem, 2015). Pentair Aquatic Eco-Systems, a supplier of aquaculture supplies, published a list of target organisms with effective UV dosages (Table 30.4). This list ranged from a low of 4000 μWs/cm^2 (4 mJ/cm^2) for Koi herpesvirus (KHV) to a high of 315,000 μWs/cm^2 (315 mJ/cm^2) for the parasitic protozoan *Costia necatrix*. Many of the pathogens of interest to zebrafish culturists, including *Mycobacteriium* sp. and *Pseudoloma nuerophilia*, have no reported effective UV dosages. It is best to take a conservative approach to UV dosage for pathogen reduction in zebrafish. Most commercial zebrafish culture systems offer UV disinfection at 110,000 μWs/cm^2 (110 mJ/cm^2) for the end of UV bulb life (Lawrence & Mason, 2012).

Ozone (O_3)

Another powerful disinfection tool used for aquaculture is ozone. Ozone is a free-radical form of oxygen that is a very strong oxidant. Ozone treatment of water can destroy fish pathogens, oxidize organic wastes, such as color and nitrite, increase nitrification rates, and improve water quality for culture animals (Malone, 2013; Summerfelt, 2003). Electric corona discharge units take dry oxygen-enriched air and create ozone (Malone, 2013). Ozone application to RAS water requires (1) ozone generation, (2) ozone transfer to the water, (3) ozone contact time with the water, and (4) the destruction of residual ozone (Summerfelt, 2003). Ozone must be tightly controlled, monitored using oxidation/reduction potential (ORP) probes, and managed to reduce residual ozone. High concentrations of residual ozone entering culture systems can kill fish. Ozone is often dosed into the water using a venturri, but ozone can also be applied in the same manner as aeration (Malone, 2013). Ozone entering RAS water reacts with dissolved organic material first, then with bacteria, viruses, and protozoans, respectively (Malone, 2013). For maximum efficiency, ozone must have adequate contact time (several seconds) with the water. Contact time can be increased using a designated ozone chamber; subsequent treatment with either granular activated carbon (GAC) or UV disinfection to destroy residual ozone. One limitation of UV as the solitary method of disinfection is that UV light penetration is often hindered by particle density as microorganisms hide in biofilms, fine solids, or larger aggregates of bacteria that UV cannot penetrate. Ozone at 0.1−0.2 min mg/L used in combination with UV disinfection at 50,000 μWs/cm^2 was highly effective

TABLE 30.4 A table demonstrating the required UV dosage (mj/cm^2) to reduce various groups of fish pathogens.

	UV DOSE
ALGAE	
Chlorella Vulgaris	22 mJ/cm2
BACTERIA	
Aeromonas salmonicida	3.6 mJ/cm2 (log-3)
Pseudomonas fluorescens (fin rot)	11 mJ/cm2 (log-3)
Listeria monocytogenes	16 mJ/cm2 (log-5)
Streptococcus sp. (seawater)	20 mJ/cm2
Bacillus subtilis (spores)	22 mJ/cm2 (log-3)
Vibrio anguillarum	30 mJ/cm2
Yersinia ruckeri	30 mJ/cm2
BKD (Bacterial Kidney Disease)	60 mJ/cm2 (estimate)
Flavobacterium psychrophilum (Salmonid Bacterial Coldwater Disease)	126 mJ/cm2
Vibrio sp. (oyster)	155 mJ/cm2
FUNGI	
Saprolegnia diclina (zoospores)	40 mJ/cm2 - 170 mJ/cm2
PROTOZOA	
Sarcina lutea (Micrococcus luteus)	26 mJ/cm2 (log-3)
Ceratomyxa shasta	30 mJ/cm2 (log-3)
Perkinsus marinus (Dermo disease)	30 mJ/cm2
Trichodina sp.	35 mJ/cm2 (log-3)
Myxobolus cerebralis (TAMs, Whirling Disease)	40 mJ/cm2
Ichthyophthirius multifiliis (freshwater white spot)	100 mJ/cm2
Amyloodinium ocellatum	105 mJ/cm2
Trichodina nigra	159 mJ/cm2
Cryptocaryon irritans (marine white spot)	280 mJ/cm2
Costia necatrix	318 mJ/cm2 (log-3)
VIRUS	
KHV (Koi herpesvirus)	4 mJ/cm2
ISA (Infectious Salmon Anemia)	8 mJ/cm2
CCV (Channel Catfish Virus)	20 mJ/cm2
IHNV (Infectious Hematopoietic Necrosis Virus/CHAB)	20 mJ/cm2
OMV (Oncorhynchus Masou Virus Disease)	20 mJ/cm2
IHNV (Infectious Hematopoietic Necrosis/RTTO)	30 mJ/cm2
VHS (Viral Hemorrhagic Septicemia)	32 mJ/cm2
CSV (Chum Salmon Virus)	100 mJ/cm2
AHNV (Atlantic Halibut Nodavirus)	105 mJ/cm2
IPNV (Infectious Pancreatic Necrosis Virus)	246 mJ/cm2

Log-1 = 90% • Log-2 = 99% • Log-3 = 99.9% • Log-4 = 99.99% • Log-5 = 99.999%

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Table provided courtesy of Pentair Aquatic Eco-Systems.

and often brings heterotrophic bacteria and total coliform bacteria counts to near zero in outflow water (Sharrer & Summerfelt, 2007). High ozone concentrations will oxidize plastics, making them brittle, so parts of the RAS in contact with high concentrations of ozone need to be made of ozone resistant materials. ORP probes and appropriate solenoid switches can automatically adjust ozone in a reliable fashion. Appropriate alarm systems should be used to alert culturists when residual ozone levels exceed optimal levels. This author does not know of any zebrafish culture facilities using ozone at the time of this writing. Occupational health and safety concerns have previously discouraged ozone use in zebrafish facilities. Two decades ago, ozonation was difficult to control, and culturists were concerned that residual ozone would damage zebrafish and could potentially harm human culturists. More recently, the technology has developed to make ozone use very safe and reliable for both the aquaculture organisms and the culturists. The advantages of ozone use greatly outweigh the cost and other disadvantages of this tool. Of interest to the community would be studies on the effect of ozone in combination with UV on *Mycobacterium* sp. and *Pseudoloma neurophilia* control, as both of these pathogens are of great concern to this audience.

Additional Apparatus

Temperature Control

The zebrafish is a subtropical species with an apparent optimal range of 24–28°C, with the highest growth rates reported at 28°C (Schaefer & Ryan, 2006). The temperature in zebrafish culture systems should be monitored and recorded daily. This author recommends that water temperature be maintained at a stable level between 26 and 29°C. This can be accomplished through a combination of adjusting the air temperature of the culture room and adjusting system water temperature using specialized heaters and chillers having thermostats. Heaters and chillers regulate water temperature via thermostats that measure water temperature and switch apparatus on or off as required to maintain water within the limits defined by the culturist. Heater elements will require routine cleaning, as they will accumulate a layer of solids over time. Elements should be cleaned by (1) unplugging the element and allowing it to cool completely, (2) immersing the element in concentrated muriatic acid or other cleaning acids to loosen and dissolve solids (personal protective gear is required), (3) rinsing the element thoroughly with clean tap water, and (4) wiping the element with a clean cloth. Heaters, chillers, and thermostats frequently blow electrical fuses. It is recommended that husbandry professionals familiarize themselves with equipment fuses and keep extra fuses on hand in case of equipment failures. It is also suggested that facilities keep extra heaters and chillers available to replace failed equipment (Table 30.4).

Automation Monitoring, Control, and Alarm Systems

Lawrence and Mason (2012) stated that a stable and favorable growing environment for research fish is the central operating objective. As the scale and intensity of recirculating aquaculture systems for zebrafish housing have increased, so has the need and desire for the automation of routine monitoring, adjustment, and system functions (Lawrence & Mason, 2012). Many of the commercial zebrafish housing units are capable of automating routine tasks, such as water quality testing and adjustment (temperature, pH, conductivity), mechanical and biological filter backwashes, daily water exchanges, and in some instances, feeding. The rise of sophisticated software packages, real-time sensors, and remote monitoring through mobile phones, tablets, and computers has created a reliable reality for many of these processes (Lawrence & Mason, 2012; Timmons & Ebeling, 2013). Automation provides the culturist with continuous real-time monitoring, a higher degree of control over the husbandry environment and system parameters, alarms to notify staff of any abnormalities, and the ability to shut down components automatically or remotely to prevent catastrophic losses of fish or equipment (Lawrence & Mason, 2012). Overly sophisticated and complex alarm systems can be problematic if alarms are so frequent or unreliable that they annoy staff and create a desire to turn them off (Timmons & Ebeling, 2013). It is wise to compile a list of the critical life support priorities and the necessary response times to personnel in the event of a failure (Timmons & Ebeling, 2013). Items, such as electrical power continuity, circulation pump operation, and sump water level maintenance may have the highest priority and shortest necessary response times, while alarms for fluctuations in temperature, conductivity, and other slowly changing parameters may not cause an immediate problem (Timmons & Ebeling, 2013). Regardless of automation, monitoring, and alarms, there is no substitute for competent, properly trained, knowledgeable, and experienced human labor (Timmons & Ebeling, 2013).

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CHAPTER

31

Zebrafish Breeding and Colony Management

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Introduction

The zebrafish (*Danio rerio*) has adapted to tolerate, thrive, and spawn in a wide variety of environmental conditions in South Asia. These adaptations are one of the reasons that zebrafish have become an excellent laboratory animal model. Even under conditions that would be considered suboptimal for many other fish species, zebrafish are able to survive, thrive, and produce large numbers of viable offspring.

In the laboratory, a single pair of zebrafish can produce one to two hundred eggs on average, with clutches of over 1000 eggs possible (Castranova et al., 2011). Zebrafish are routinely spawned every few days, weekly, or every other week; however, they can also go much longer without spawning. Zebrafish eggs are externally fertilized, surrounded by a strong nonsticky chorion, and negatively buoyant, making them easy to manipulate and culture. Zebrafish can begin spawning as early as 60 days postfertilization (dpf) and can remain reproductively viable up to five and a half years, although a decline in reproductive performance is often seen after 2 years. Flexibility in spawning frequency, the qualities of the embryos, and the reproductive lifespan of zebrafish are all attributes that make zebrafish a good fit for the laboratory animal setting.

Spawning and the Sexes

Wild zebrafish prefer stagnant bodies of water, such as rice paddies, ponds, and slow-flowing streams and rivers (Spence, Gerlach, Lawrence, & Smith, 2008). Spawning occurs in the shallows with negatively buoyant eggs being scattered and falling into the substrate. In the wild, zebrafish appear to be seasonal spawners with spawning being linked to the rainy season (Engeszer, Patterson, Rao, & Parichy, 2007). The seasonality of spawning is believed to be associated with the increased food availability of the monsoon season rather than other seasonal cues because gravid females have been found in the wild outside of the rainy season (Spence et al., 2006).

In the wild, genetic factors have a large influence on sex differentiation (described elsewhere in this book), while in domesticated zebrafish strains, it appears that the sex-determining locus has been lost (Wilson et al., 2014), making the prediction of sex ratio difficult. There are a few environmental factors identified as influences of sex determination in laboratory-reared zebrafish, and having a good understanding of these factors is important to aid in colony management. Lack of sex-determining loci can lead to extremely skewed sex ratios, including entirely male or entirely female clutches. As inbreeding increases, the prevalence of highly skewed sex ratios also increases (Brown et al., 2012). Hybridization (crossing different strains of zebrafish) has also been shown to cause skewed sex ratios in favor of females (Brown et al., 2012; Lawrence, Ebersole, & Kesseli, 2008). When zebrafish larvae are reared in water with low dissolved oxygen, the chances that the fish develop into males increases (Shang, Yu, & Wu, 2006); while larvae housed at low densities and provided with an abundant food source are more likely to develop as females (Lawrence et al., 2008).

All zebrafish begin their sexual development with primitive ovaries. In fish that grow into females, their ovaries mature; while in the fish that become males, ovaries regress, and testis develop (Liew & Orban, 2014). The mechanisms behind these changes have not been fully elucidated. High levels of cortisol, a stress hormone, during sexual differentiation is believed to drive zebrafish to become males (Ribas, Valdivieso, Diaz, & Piferrer, 2017).

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It takes from two to 4 months for laboratory zebrafish to become sexually mature in most facilities (Lawrence, Adatto, Best, James, & Maloney, 2012). Variables that affect age to the first spawn include husbandry factors, such as water quality, food type, and availability, as well as genetic background. With sexual maturity comes sexual dimorphism, subtle differences between male and female fish. Gravid female zebrafish have a rounded whitish abdomen, and a yellow tint to their dorsal fin, pelvic fins, and the tips of their tail (Fig. 31.1A). Male zebrafish have torpedo-shaped bodies, a pinkish hue, and an orange coloration in their anal fin (Fig. 31.1B). Male fish also have breeding tubercles attached to their pectoral fins that are believed to be used during physical contact with females during spawning (McMillan et al., 2013). Tubercles are visible under a dissecting microscope and can be used to positively identify male fish (Fig. 31.1C) (McMillan et al., 2013; McMillan, Geraudie, & Akimenko, 2015). Because female fish that are carrying smaller numbers of eggs can have less pronounced bellies and male fish can have bellies filled with food or testicular tumors that can cause a swollen abdomen; it is strongly recommended that users differentiate males and females based on coloration and not solely on body shape.

There are several cues believed to stimulate spawning in laboratory zebrafish, the most well-known being light, specifically, the turning on of the lights in the morning simulating artificial dawn. The environmental change also helps stimulate spawning with the most commonly used change being removing fish from their recirculating housing tanks and placing them in specially designed static breeding tanks (Fig. 31.2A–D). Some researchers add plastic plants to spawning tanks to provide additional substrate, cover, and environmental enrichment (Wafer et al., 2016). Although setting up fish in breeding tanks the night before an experiment is standard, fish can spawn when they are set up in breeding tanks in the morning. Further research is needed to determine how much an evening set up improves reproductive performance. The pheromonal exchanges between male and female fish are believed to drive spawning behavior, including that of prostaglandin F2 alpha (Yabuki et al., 2016). When female zebrafish are exposed to pheromones produced by other females, reproduction is suppressed; but when females are exposed to pheromones from male zebrafish, reproduction is stimulated (Gerlach, 2006). A male fish will chase a female fish and rub his body against hers to stimulate the female to release her eggs. Eggs and sperm are released very closely together in the water column, where the change in osmotic pressure causes sperm activation and subsequent egg fertilization. Unlike mammalian sperm, fish sperm is active for less than 2 min (Wilson-Leedy, Kanuga, & Ingermann, 2009), so if fertilization does not happen at the time of gamete release, it is unlikely to happen at all. Most off-system breeding tanks have a removable insert with holes or a screen on their bottom wall that allows eggs to fall out of the insert onto the bottom surface of the tank, separating them from the spawning adult zebrafish (Fig. 31.2A–D). Without this separation, zebrafish adults will eat the eggs on the bottom of the tank.

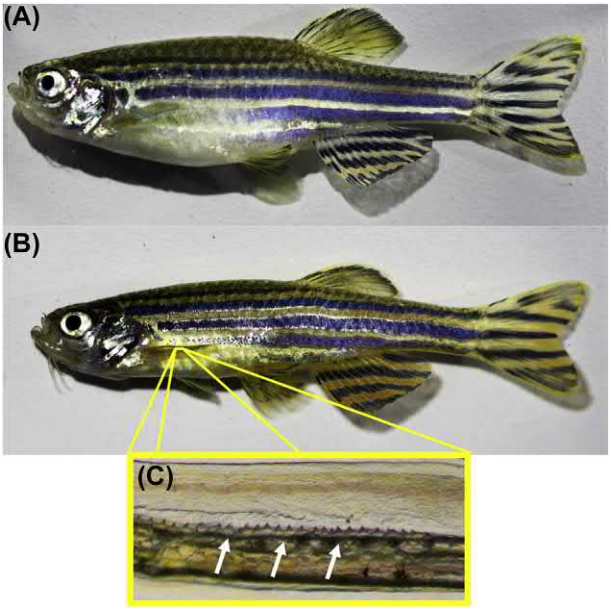

FIGURE 31.1 Adult female zebrafish (A) with a rounded belly, and an adult male zebrafish (B) showing breeding tubercles (C) on the pectoral fin.

Zebrafish adults are often housed in groups of varying size and in tanks of different sizes depending on the facility design. Many zebrafish researchers choose to cohouse males and females together in the same tanks. Fish housed in such mixed-sex arrangements will periodically spawn in their housing tanks, and the eggs will be eaten, either by the adult fish or will be captured by the system's mechanical filtration. Adequate mechanical filtration is essential in zebrafish facilities to prevent tank-spawned eggs and larvae from ending up in fry tanks and causing genetic contamination. In-tank spawning allows for continued production and replacement of gametes and is believed to extend the reproductive lifespan. Male and female zebrafish that are spawned regularly are sometimes housed separately by sex to prevent in-tank spawning events and increase reproductive performance. If female fish are separated for too long from males, and are thus, not stimulated to spawn, the female's eggs will undergo follicular atresia, in which the oocytes are reabsorbed (Connolly, Dutkosky, Heah, Sayler, & Henry, 2014). Sometimes, the reabsorption of eggs by the female is incomplete, and the oviduct can become clogged with

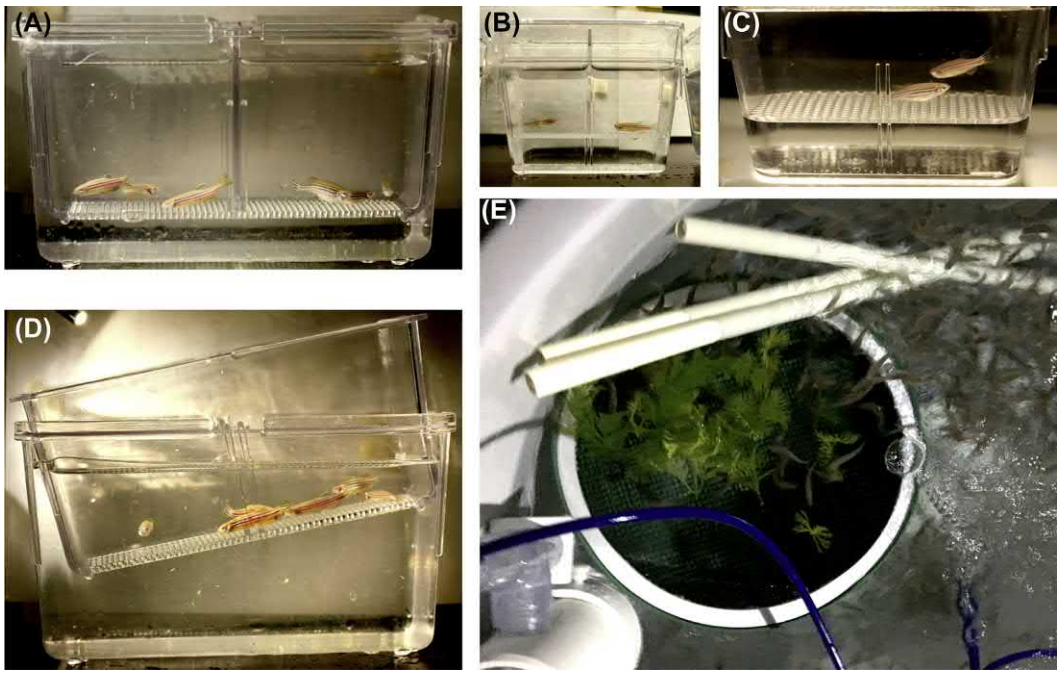

FIGURE 31.2 An array of breeding tanks showing a large static breeder with males and females separated (A), a small static breeder with the male and female separated (B), a small static breeder with fish spawning and eggs at the bottom of the tank (C), a large static breeder with the insert tilted and fish spawning (D), and a mass spawning system with zebrafish spawning over an egg collector (E).

the resulting nonviable egg material. Females with this condition are often referred to as "egg bound" (Connolly et al., 2014). Clogged oviducts can sometimes be cleared by gentle abdominal squeezing, and the female may then generate new viable eggs in a few weeks.

Laboratory Spawning

Zebrafish breeding or spawning tanks are available in many different shapes, sizes, and designs, but all can be divided into two main categories, namely, on-system and off-system. Most commonly, zebrafish are removed from their housing tanks and placed into off-system spawning tanks that often consist of two tanks nested together with the inside tank having holes or a screen to allow eggs to fall through to the bottom of the outer tank (Fig. 31.2A–D). These types of tanks are available in different sizes depending on the number of fish to be grouped for a spawn. It is important to use adequately sized breeding tanks that are appropriately filled because when using spawning chambers containing 200 mL or less of water, a decrease in spawning performance has been seen (Goolish, Evans, Okutake, & Max, 1998). Many of these tanks have an option of a removable barrier that can be placed in the tank to separate males and females for a predetermined time period before spawning (Fig. 31.2A–B). When the researcher is ready to harvest eggs, the barrier or divider is removed from the tank, allowing males and females to comingle, and spawning usually commences within a few minutes.

Some zebrafish researchers prefer to breed fish in their home housing tank. This can be done by inserting an egg collection device therein. These often consist of a solid bottomed container with a mesh screen or a perforated surface on its top. These are often placed toward the top of the water column, to take advantage of the zebrafish's natural preference to spawn in shallow water (Sessa et al., 2008). Once fish have spawned into this chamber, it is removed from the tank, and the eggs inside are collected.

Regardless of spawning technique, the most common method of egg collection from a breeding container is to pour the water containing the eggs from the spawning tank through a tea strainer. The strainer will collect the eggs while the water exits it. The eggs are then rinsed out of the tea strainer and into a Petri dish using an embryo medium.

For laboratories that need to collect many thousands of eggs at one time, large scale breeding systems are used (Adatto, Lawrence, Thompson, & Zon, 2011). Like smaller scale breeding, large scale breeding can take place off the main aquaculture system, or in a large housing tank connected to the main system. Large-scale spawning systems are available from several zebrafish aquaculture systems manufacturers. These systems often consist of a large housing tank (10–100 gallons)

that can support hundreds to thousands of zebrafish. Egg collection devices are placed inside these tanks, and fish swim over them and spawn (Fig. 31.2E). The eggs are then collected from the devices.

The frequency at which laboratory zebrafish are placed in breeding tanks to be spawned is somewhat variable. Some laboratories give fish a 2-week break between spawns while other laboratories spawn fish every few days. There appears to be a correlation between frequency of spawning and the longevity of the reproductive lifespan, with fish being spawned more frequently having shorter reproductive lifespans. When male and female fish are housed together, they can go many months without being set up to spawn and still spawn well when needed. Reproductive lifespan varies depending on many conditions, including husbandry, nutrition, and the genetic hardiness of the stock. Under standard laboratory conditions, fish spawn well until between 2 and 3 years of age. As the fish get older, the reliability of successful spawning decreases, making stock regeneration the best option to continue experiments and maintain valuable lines. Viable sperm has been harvested from male zebrafish even at five and a half years of age.

Although zebrafish are known for their reproductive vigor, sometimes laboratory stocks do not spawn when placed in breeding tanks. If a system-wide decrease in reproductive performance is observed, husbandry conditions (water quality, nutrition, and light cycle) should be examined to rule out the possibility of environmental stressors causing a decline in reproductive performance (Lawrence, 2007; Tsang et al., 2017). Keeping and reviewing records of system-wide reproductive performance is a great way for quickly detecting changes in the reproductive performance caused by environmental changes. Reproductive performance can be viewed as the "canary in the coal mine" of fish facilities, often being the first noticeable sign that there is a systemic problem. If the problems are not addressed, disease outbreaks and mortalities often follow.

In Vitro Fertilization

If individual or groups of fish fail to spawn when placed in breeding tanks, and environmental changes are ruled out because other fish on the system are performing well, there are several factors that can contribute to spawning decline including advanced age, inbreeding, disease, or experimentally induced health issues like ENU-induced or targeted mutations. If spawning these fish is critical to the success of the research program, In Vitro fertilization (IVF) is often performed. Variability in IVF techniques are common, including the order of gamete extraction, and the selection of buffers for gamete preservation and extension, but the basic principles are the same (Hagedorn & Carter, 2011; Lieschke, Oakes, & Kawakami, 2009; Westerfield, 1993). Most protocols start by setting up fish in an off-system breeding tank with a divider separating males and females.

Sperm can be extracted from an anesthetized male zebrafish by drying its urogenital pore before covering it with the open end of a capillary tube or pipette tip (Fig. 31.3A). Gentle abdominal massage will cause the sperm to be released, and it is then collected into the tube or tip. Sperm volumes of 0.5–3.0 ul are common, with milky colored sperm being preferred; clear sperm will also often have enough sperm cells to fertilize hundreds of eggs (Fig. 31.3B). Sperm can be placed into a small volume of Hank's balanced salt solution to prevent sperm activation (Westerfield, 1993) (Fig. 31.3C) and kept on ice for at least an hour. This method is preferred if sperm from multiple males needs to be pooled. Alternatively, the sperm sample can be placed directly onto high-quality eggs, or in a drop of system water next to the eggs, followed by the addition of more system water and immediate mixing (Fig. 31.3F).

Eggs are collected by drying the urogenital pore of an anesthetized female zebrafish and applying gentle pressure to the abdomen (Fig. 31.3D). If viable eggs are present, they will be expelled quickly, and little pressure is needed. Eggs can be pooled if the experiment or management strategy requires it. When manipulating eggs, it is important to use a Teflon coated spatula because the use of metal spatulas has been shown to reduce the fertilization rate (Hagedorn & Carter, 2011). It is important that, like for sperm, the eggs are not exposed to water. The chorion or outer membrane of the egg has a small hole in it called the micropyle. The sperm cell enters the egg through the micropyle (Sharma & Kinsey, 2008); and shortly after eggs are exposed to water, the micropyle closes, making fertilization impossible. High-quality eggs have a uniform yellow color and a round shape and will remain viable, if kept dry and covered, for 30 min to an hour. Eggs that are white and lumpy are in some stage of degradation and will not be fertilizable. Occasionally eggs of high quality and low quality are expressed at the same time, but it is difficult to tell if any eggs are viable until they are fertilized and begin to develop. If excessive force is needed to express eggs from the female, even if the eggs appear to be high quality, they are often not fertilizable because they were not fully ovulated. Sometimes when harder squeezing is necessary, a plug of degraded eggs can be expressed, and high-quality eggs follow. If eggs of poor quality follow the plug, the female can be returned to the system, and she will often begin to spawn normally again in a few weeks.

As with any procedure that involves fish anesthesia and handling, there are risks of injury or death

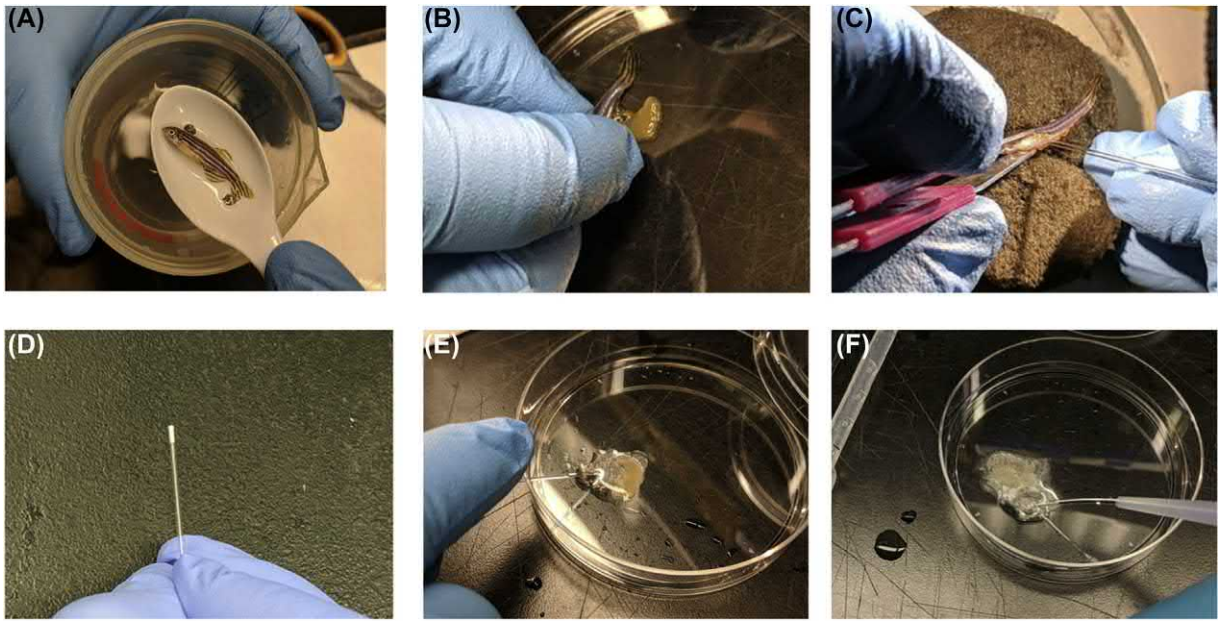

FIGURE 31.3 Some of the steps of In Vitro fertilization include anesthetizing zebrafish (A), gently squeezing the female's abdomen to express eggs into a Petri dish (B), collecting sperm from a male fish into a capillary tube (C), evaluate sperm quality and volume (D), placing sperm on eggs with a small amount of system water (E), and waiting 2 min before adding embryo media (F).

associated with IVF. These risks can be minimized by handling fish gently, not leaving them in the anesthesia solution too long, and making sure they "wake up" after the procedure by gently mixing the water that they are in to ensure that water flows over their gills, maximizing the rate so that the anesthetic leaves their system.

In Vitro fertilization is an important tool for zebrafish researchers, allowing them to generate large numbers of developmentally synchronized embryos, and to generate embryos from fish that will not spawn naturally. IVF is also valuable when fish from different facilities need to be crossed. For example, if fish are brought into a quarantine facility and need to be crossed with fish in the main facility but there are no extra fish to bring to the quarantine room, gametes can be harvested from the fish in the main facility and brought to the quarantine facility for fertilization.

Cryopreservation

Unlike for many mammalian laboratory animal models, the freezing of zebrafish embryos has not been perfected for standard use, although there have been some promising advances in this area recently with the first successful freezing and thawing of zebrafish embryos (Khosla, Wang, Hagedorn, Qin, & Bischof, 2017). Although this is a limitation for the model, many effective protocols to freeze zebrafish sperm have been developed. The Zebrafish International Resource Center (ZIRC) has cryopreserved tens of thousands of lines and will thaw sperm samples, fertilize wild type eggs, and send them to researchers for a fee. ZIRC will accept male fish for sperm cryopreservation from fish lines that have been used and published in peer-reviewed journal articles. This is an excellent way to back up precious stocks that have been published and relieves the laboratory of the responsibility of distributing published lines.

Many larger zebrafish laboratories cryopreserve sperm to back up important fish lines and transgenic lines that have to be still published or to preserve mutant alleles from forward and reverse genetic screens. Many zebrafish sperm cryopreservation protocols are available (Draper & Moens, 2009; Morris et al., 2003; Yang, Carmichael, Varga, & Tiersch, 2007; Yang et al., 2016), and laboratories interested in starting an in-house cryopreservation protocol will need to find the protocol that works best for them. Although the actual protocols vary, the principle remains the same. Sperm is extracted from the male fish and into a pipette tip or capillary tube as is done for In Vitro fertilization (described above), or the male fish are euthanized, and the testes are dissected and homogenized (Yang et al., 2007). The advantage to testis removal protocols is that all the viable sperm cells are harvested, allowing the sample to be split, and subsamples are frozen separately; while the disadvantage is that the procedure is terminal. Regardless of how the sperm samples are harvested, they are placed into a cryoprotectant to prevent cell damage from ice crystal formation and then frozen at a controlled rate before they are placed in liquid nitrogen (Yang & Tiersch, 2009). The rate of freezing is important and is controlled by placing tubes in dry ice for a certain amount of time before they are submerged in liquid nitrogen (Bai et al., 2013; Berghmans, Morris, Kanki, &

Look, 2004). The rate of freezing is important for successful cryopreservation because the sperm will become activated by contact with water and the cryoprotectants are toxic to the cells; thus, practice is needed to master any of the published protocols.

Colony Management

Over the past decade, techniques to more easily create transgenic and mutant zebrafish have been developed. These techniques have been a boon for research but create challenges for managing zebrafish colonies. It is not uncommon for laboratories to maintain hundreds of wild type, mutant, and transgenic lines, and keeping them all healthy and spawning can be challenging. Most laboratories use a database to keep track of their fish lines. Custom databases written in FilemakerPro are common, with commercial and open-source options (Yakulov & Walz, 2015) available as well. Unlike other lab animal models, most individual zebrafish are not tracked; rather fish from the same spawning events (either group or individual) are tracked with genotype information and date of birth being the minimum information needed. Many groups go further and include parental stock information, whether the fish have been identified as carrying the transgene or mutation of interest, the number of fish in the cohort, and information about where in the facility the fish can be found.

Some databases go further and help keep track of large scale experiments like ENU mutagenesis screens and CRISPR projects. In addition to including lineage and experimental treatment information, these databases can include picture fields to more easily describe, record, and store phenotype information.

Recording husbandry-related information in a database is also useful. Information about spawning success and mortality rate is not only important if they are relevant to specific fish or experiments, but they can also act as an early indicator for the facility and can alert the researchers and staff to problems that have not yet become obvious.

An important advantage to keeping track of fish lines with a database is that it allows researchers to go through the genealogical history of fish lines. Family lineage is important in case an unexpected transgene or phenotype is seen. Lineage information is also important to help researchers avoid inbreeding of their zebrafish lines. The easiest way for researchers to maintain transgenes and mutations in homozygous or heterozygous states is to incross them. Although this is a reasonable approach for a few generations, as fish become more inbred, their reproductive performance declines, and overall hardiness suffers (Monson & Sadler, 2010). It is important to outcross transgenic and mutant lines to a robust wild type stock to maintain overall hardiness and reproductive vigor. An in-depth review of wild type zebrafish strains and crossing strategies was written by Trevarrow and Robison (Trevarrow & Robison, 2004). Sequenced lines have been generated to help reduce experimental variability caused by high rates of polymorphisms in outbred wild type lines. Two of these lines are available through the Zebrafish International Resource Center (ZIRC), the SAT line (Nasiadka & Clark, 2012) and the NHGRI1 line (LaFave, Varshney, Vemulapalli, Mullikin, & Burgess, 2014).

Maintaining a hardy, genetically diverse wild type stock is important because many experiments begin with eggs from wild type strains, and because the health of all the lines in the laboratory relies on having a hardy wild type strain for outcrossing. Informal line maintenance strategies include setting up as many pairs of fish as possible to produce the next generation. More formal strategies involve setting up individual pairwise crosses and redistributing embryos so that tanks that do not contain any siblings are created (Fig. 31.4) (Harper &

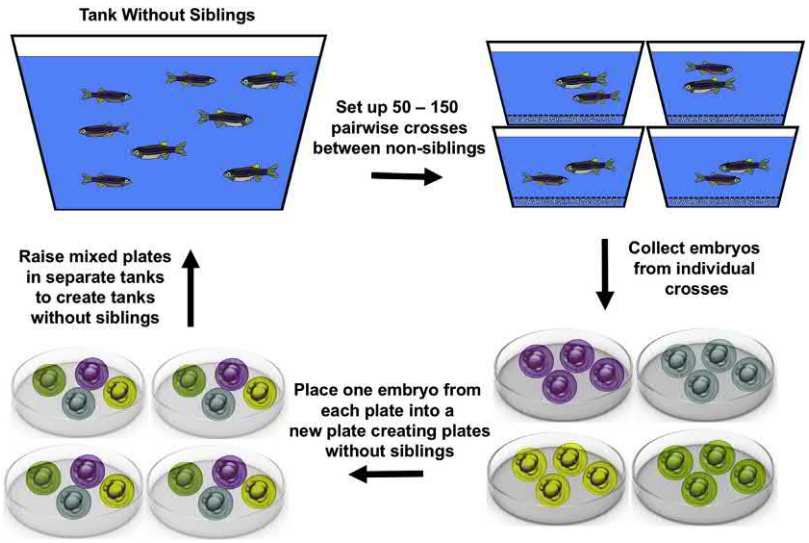

FIGURE 31.4 A spawning schematic used to help maintain genetic diversity in zebrafish lines by avoiding mating of siblings.

Lawrence, 2011). These tanks are used to generate the next generation of wild types, while extra fish from these crosses can be used for generating embryos for experiments. Additional hardiness can be infused into wild type stocks by outcrossing them to the same strain maintained at a different site (Harper & Lawrence, 2011). Keep in mind that there is a tradeoff between isogeny and reproductive hardiness, with fish being more isogenic also being more difficult to work with. Researchers need to weigh the costs of reproductive performance versus genetic homogeneity when deciding how to maintain their stocks.

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CHAPTER

32

Zebrafish Larviculture

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Introduction

The practice of larviculture is a critical part of any research program that utilizes the zebrafish model system, and it remains one of the great operational challenges in fish husbandry. It is also one of the first barriers to entry into zebrafish research, as an inability to grow animals will either severely limit the type and scale of science possible, or preclude it altogether.

Relative success in larval rearing is defined by growth and survival rates, with the goal being the maximization of both values, not only for the sake of operational efficiency but also for animal welfare. Compared to many fish species commonly cultured in captivity, zebrafish are not particularly difficult to rear, but their small size and limited swimming ability at the onset of first feeding are among the challenges that must be addressed.

Protocols for zebrafish larviculture must incorporate an understanding of (1) the natural history, biology, behavior, and nutritional requirements of the developing fish, and (2) the relationship between these traits and the physical and chemical properties of water. The best and most effective larval rearing techniques strike the proper balance between the two and require vigilant, attentive caretakers with keen observational skills to administer them.

Natural History/Biology of Larvae

Natural Distribution

Zebrafish are native to south Asia and are widely distributed across much of India, Bangladesh, and lowland Nepal (Arunachalam, Raja, Vijayakumar, Malaiammal, & Mayden, 2013; Engeszer, Patterson, Rao, & Parichy, 2007; Spence, Gerlach, Lawrence, & Smith, 2008; Talwar & Jingran, 1991). While these fish may be found in virtually any freshwater habitat type within this vast region (streams, rivers, rice paddies, ponds, lakes, etc.), they most commonly tend to occur in shallow, still, or slow-moving water in and around the margins of water bodies (Roy, Shukla, & Bhat, 2017). This level of habitat diversity, coupled with the monsoonal climate regime that defines this region, necessitates that, like many animals that inhabit this part of the world, the zebrafish are able to tolerate wide and sometimes rapid alterations in environmental conditions. Indeed, zebrafish can be considered exemplary in this regard, having been documented in habitats that experience wide temporal fluctuations in salinity, temperature, pH, and other chemical and physical parameters (Arunachalam et al., 2013; Engeszer et al., 2007; McClure, McIntyre, & McCune, 2006; Spence et al., 2006).

Natural History and Reproductive Biology

In the wild, zebrafish display a range of social behaviors and tend to associate in groups that vary in size and activity in accordance with the habitat type (Roy et al., 2017; Suriyampola et al., 2015, p. 1159). They occupy the entirety of the water column but more commonly inhabit shallower waters along banks and margins, often in association with overhanging vegetation (Spence et al., 2006). They are omnivorous, opportunistic feeders, consuming small zooplankton, terrestrial, and aquatic insects, and even plant material (Arunachalam et al., 2013; McClure et al., 2006; Spence, Fatema, Ellis, Ahmed, & Smith, 2007a). Gut content analyses reveal that terrestrial insects, presumably falling into the water from overhanging vegetation, are the predominant prey type (Arunachalam et al., 2013), but this may simply reflect relative abundance in the environment at a given time point.

Spawning commences prior to the onset of the rainy season, with the fish breeding in pairs or small groups in shallow water in and around the margins of water bodies they inhabit (Hutter, Penn, Magee, & Zala, 2010; Spence, Gerlach, Lawrence, & Smith, 2008).

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Fertilization is external; and during spawning, the nonadhesive, demersal eggs are scattered and fall to the substrate with no parental care. In the wild, female zebrafish have been shown to prefer to oviposit in shallow water among submerged vegetation and gravel (Spence, Ashton, & Smith, 2007c), presumably because these materials provide protective cover for developing embryos (Engeszer et al., 2007). The embryos hatch within a few days, and spend the first few weeks of life in shallow, nutrient-rich habitats, feeding on small zooplankton and other organisms within the water column.

Biological Characteristics and Staging of Zebrafish Larvae

There has been considerable discussion in the literature regarding the appropriateness of defining age classes in developing zebrafish based on the time since their fertilization versus distinct morphological criteria/size (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995; Parichy, Elizondo, Mills, Gordon, & Engeszer, 2009). From the moment of fertilization, a whole host of environmental conditions influence the rate of development, so the further one goes out from time zero, the less relevant that time point becomes in the defining stages.

The exception to this is during the first 24–72 h of life, if temperature, medium, density, and photoperiod are standardized (Kimmel et al., 1995). Once the fish inflates its swim bladder and begin to swim and feed, it is more appropriate to use standard length (SL). The size ranges given below are based on those given by Parichy et al. in their classic paper defining postembryological staging in zebrafish (Parichy et al., 2009).

The larval stage begins at 48–72 h postfertilization (hpf), when the embryos hatch from the chorion, which is progressively weakened by release of a hatching enzyme and spontaneous flexing movements of the embryo (Laale, 1977). Over the next several days, the developing larvae show low levels of spontaneous activity but slowly begin to make their way closer to the surface, usually by "creeping" their way up submerged structures by means of specialized adhesive cells on the epidermis of the head (Laale, 1977).

Once they reach an SL of 3.4–3.7 mm (mm), which usually occurs within 48 h after hatching, the larvae will swim up to the air-water interface and inflate their gas bladders by swallowing air and forcing it into their guts (Goolish & Okutake, 1999). This developmental event, which allows them to regulate their position within the water column, is coincident with the onset of both exogenous feeding and active avoidance behaviors (Kimmel et al., 1995; McHenry Feitl, Strother, & Van Trump, 2009; Strähle et al., 2012).

Development rapidly proceeds over the next several weeks of life. Fish are classified as larvae up until the larval fin fold disappears, and they attain a complete scaling pattern (Parichy et al., 2009). This corresponds to an SL of ~11.0 mm and may take several weeks to several months to attain (Augustine, Gagnaire, Floriani, Adam-Guillermin, & Kooijman, 2011).

Feeding

Meeting the nutritional demands of larval fish is an age-old challenge in aquaculture (Cahu & Infante, 2001; Hamre et al., 2013; Onal & Langdon, 2005), and the selection and administration of the diet to animals during this time period will define the success or failure of any rearing program. There are a number of different factors that must be considered, the most important of which will be treated below, with a specific relationship to zebrafish.

Nutrient Requirements

After hatching, larval zebrafish subsist entirely off the nutritional reserves provided within the yolk (Hanisch, Küster, Altenburger, & Gündel, 2010). Larvae will not feed until uptake and process of external nutrients become possible, a stage which is coincident with sufficient development of a functional digestive system (Ng de Jong Curtain, Mawdsley, White, Shin, Appel, Dong, et al., 2005) and the ability to regulate their position within the water column (Strähle et al., 2012). The confluence of these developmental events is the swim-up period when larvae inflate their gas bladders and begin feeding on exogenous prey (Strähle et al., 2012).

Zebrafish larvae should be presented with food immediately after this point because any energy remaining in their yolks is rapidly depleted once the animals start actively swimming. Still, it is important to consider that there is some overlap (~24–48 h depending on temperature and activity rates (Jardine & Litvak, 2003; Semova et al., 2012)) between total yolk absorption and the commencement of independent feeding. This provides the fish with a nutritional head-start during a critical period when their locomotor capacity is at its lowest and/or if suitable prey is scarce (Lawrence, et al., 2012a). If fish are not fed at all, they will starve to death within a few days after swim-up, the timing of which depends upon temperature and other environmental factors. For example, zebrafish that are never provided with feed will starve by 10 days postfertilization (dpf) when maintained at 28°Centigrade (Jardine & Litvak, 2003).

During the first few weeks of life, larval zebrafish need to feed continuously to fuel rapid growth (Hamre et al., 2013), especially the structural remodeling required for the transformation from the larval to the juvenile stage (Parichy et al., 2009; Rombough, 2002). Prey items need to be highly digestible, as the larval alimentary tract is functional, but structurally simple with a limited capacity for storage and assimilation of complex molecules (Field, Roeser, Stainier, Ober, 2003; Govoni, Boehlert, & Watanabe, 1986; Ng et al., 2005; Zambonino-Infante et al., 2008). The ability of the fish to break down and assimilate more complex nutrients increases as they mature, but in general, dietary items should be as digestible as possible to maximize efficiency, and therefore, growth rates.

As is the case with many other fish species, the precise nutrient requirements of zebrafish larvae have not been formally delineated (Watts, Powell, & D'Abramo, 2012). However, limited inferences about this can be made upon the basis of growth and survival performance of the fish when reared on different diets. Indeed, zebrafish appear to perform best when first presented with live zooplankton that mimics their diet in natural settings (Lawrence, 2007; Spence et al., 2008).

While there are a number of factors that contribute to this success, it is probably due at least in part to the basic nutritional characteristics of these feeds. Commonly cultured zooplankton species like brine shrimp (*Artemia* sp.) or rotifers (*Brachionus* sp.) generally possess basic nutritional characteristics that support larval fish development, like high protein content and higher digestibility; but it is important to consider that they will vary in nutritional composition depending on culturing conditions and feed inputs (Lavens & Sorgeloos, 1996a). Rotifers, *Artemia*, and other zooplankton species may be "bioencapsulated" to boost their profiles of essential fatty acids, key amino acids, vitamins, and minerals known to be important for fish growth and survival (Hamre, Mollan, Sæle, & Erstad, 2008; Figueiredo, van Woesik, Lin, & Narciso, 2009; Ritar et al., 2004; Thépot et al., 2016). This approach has been successfully applied to zebrafish larviculture (Best, Adatto, Cockington, James, & Lawrence, 2010; Lawrence et al., 2016).

Feeding Behavior/Anatomy

As adults, zebrafish feed effectively at the surface, benthos, and within the water column (Lawrence, 2007). As first-feeding larvae, however, they are essentially restricted to items within the water column, due mostly to their limited swimming ability at this stage of development (Harper & Lawrence, 2010). Larval zebrafish are suction feeders and capture prey by swimming up to it and opening their mouths while expanding their mouth cavity. This action creates a strong flow of water that draws the item into the mouth to be consumed (Bianco, Kampff, & Engert, 2011; China & Holzman, 2014; McElligott & O'Malley, 2005).

Given how this mechanism works, the size of item relative to the size of the fish is important, and the size of the particle that fish are able to consume is defined by the width of their mouths. This metric increases rapidly as the fish grows. For example, in one study, 3.8 mm fish had a mean mouth width of 180–200 μm (micrometers), while larvae that were 5.2 mm had a mean mouth width of 290–320 μm (Önal & Langdon, 2016). It is important to understand that these values do not necessarily correspond to the actual size limit of what a fish of a particular age and/or length can consume. This is more accurately delineated by the ratio of particle size to mouth width (Önal & Langdon, 2016). The upper limit of this value in developing zebrafish larvae has been shown to be in the range of 40%–55%, but the fish actually tend to consume much smaller items, more in the range of <20% (Önal & Langdon, 2016). Probably, this is because they have higher success rates in feeding on smaller items than items that are at the upper limit of their abilities. As they continue to grow, fish will selectively feed on increasingly larger particles, and once they transition to the juvenile stage, they become more effective at feeding first at the surface and then finally in the benthos (Harper & Lawrence, 2010).

Feed Characteristics

Prey items must be both available and attractive to larval fish. Availability is a key parameter; larvae must be able to locate and subsequently consume items while expending minimal energy to do so. For many species that are obligate water column feeders early in development, including zebrafish, this is one reason why live zooplankton is so effective; it is biologically available to larvae because when alive, the organisms swim actively throughout the water column and therefore are available for the fish to encounter (Conceição, Yúfera, Makridis, Morais, & Dinis, 2010). For the same reason, processed feeds, like microdiets, are designed to maximize their residence time in the water so that larva may encounter them. Considerable attention is devoted to sinking rates, retention of water-soluble nutrients, as well as physical characteristics and mechanisms to keep them in suspension (Bonaldo, Parma, Badiani, Serratore, & Gatta, 2011; Onal & Langdon, 2005; Person, 1989). Sinking rates also can apply to zooplankton in some situations; for example, some species only persist for finite periods once introduced to culturing tanks (i.e., *Artemia* in freshwater).

Another aspect of feed availability is the rate at which the particle moves in the water. This applies especially to live feeds, as zooplankton swim in different ways and at different speeds (Lavens & Sorgeloos, 1996a). This must be matched with the swimming ability and age of the target fish. For example, zebrafish larvae are too slow to efficiently capture *Artemia* nauplii during the first few days of exogenous feeding and are much more adept at feeding on slower moving Paramecium (Westerfield, 2007) or rotifers during that period (Best et al., 2010; Lawrence, James, & Mobley, 2015; Lawrence et al., 2016). As they grow, they more effectively feed on the larger, faster Artemia nauplii (Harper & Lawrence, 2010). Processed feeds will also move throughout the water column at different rates depending on their size, density, and composition (Girin, Halver, & Tiews, 1979; Kolkovski, 2013). As the fish grow and develop their ability to maneuver directionally, this becomes of less concern.

The attractiveness of a given feed item is also critical, as larval fish are not indiscriminate. In fact, larvae selectively track and consume prey based on a wide variety of characteristics. For example, fish display color preferences from a young age, and this will often extend to prey selectivity (Browman & Marcotte, 1987; El-Sayed, El-Ghobashy, & El-Mezayen, 2013). Zebrafish show an innate preference for red food as adults (Spence & Smith, 2008). This has not been tested in fish during the larval stage, but it is thought that the preference observed in adults reflects that the coloring of zooplankton in wild habitats is often red or orange (Spence et al., 2007a; Spence & Smith, 2008).

The smell and taste of a feed are also components of its attractiveness. The olfactory system in zebrafish is functional from swim-up (Hansen & Zeiske, 1993; Miyasaka et al., 2013), indicating the important role that it plays in helping fish to find and detect prey. Larvae can detect amino acid mixtures in the water as early as four dpf (Lindsay, 2004), and they can utilize this sensitivity, in combination with the directional sensing of currents by the lateral line (Rønnestad et al., 2013), to help locate food items in the water column. Once prey is located, fish use their taste buds to determine its palatability (HARA, 1994; Rønnestad et al., 2013). Functional taste bud cells are also present in zebrafish at the onset of independent feeding (Hansen, Reutter, & Zeiske, 2002), and so it is reasonable to presume that taste plays a role in prey selectivity in this species, even at a young age.

Another part of the attractiveness of a feed particle is the manner, as opposed to the rate, in which it moves through the water. Prey items that have active, irregular swimming motions are thought to be more conspicuous to fish larvae (Buskey, Coulter, & Strom, 1993). For example, copepods (e.g., *Arcatia* sp.) are particularly well known in this regard (Conceição et al., 2010). In this way, zooplankton are generally more attractive to fish larvae than are inanimate processed particles. Among the species of zooplankton most commonly fed to laboratory zebrafish, *Artemia* sp. probably elicit the greatest response based on their swimming behavior, which is more erratic and faster than rotifers or *Paramecium*, both of which move more slowly through the water column (Best et al., 2010; Harper & Lawrence, 2010). Processed feeds having superior buoyancy when combined with favorable upwelling flow regimes would mimic the random movement that allows fish to locate *Artemia* while feeding.

Feed Types

Both live and processed feeds can be utilized as prey items for first feeding zebrafish. Among live feeds, three species predominate in their application across the zebrafish research community. *Paramecium* sp. were among the first live feeds that pioneering zebrafish laboratories used to rear larval fish (Nüsslein-Volhard Dahm, 2002; Westerfield, 2007) and are still commonly used in the field (Varga, 2011). *Paramecium* are ciliated, unicellular, multi-nucleated organisms that reproduce both asexually and sexually, depending upon environmental conditions. Under ideal conditions, they can reproduce asexually 2–3 times daily, and they feed primarily on bacteria, as well as microalgae, yeast, and small protozoa (ALLEN & NERAD, 1978; Beale, Preer, & Harumoto, 2008; Sonneborn, 1970). Depending on the species, Paramecium may range in size but on average measure about 50 × 180 μm (width by length), which is suitable for first feeding zebrafish (Varga, 2011). They can be cultured on various diets, but protocols utilizing yeast or LB media are most common (Nüsslein-Volhard Dahm, 2002; Varga, 2011; Westerfield, 2007). *Paramecium* swim slowly throughout the water column and may be easily captured by larvae that encounter them. In addition to that fact that they may be readily acquired and cultured, these traits have made them a favorite diet for ornamental fish growers (Mitchell, 1991) and often the primary component of "infusoria" used for rearing fish in home aquaria (Mukai, Sani, & Kadowaki, 2016). Numerous reports demonstrate that they are effective prey items for first feeding larval zebrafish, especially for the first several days postswim-up (Biga & Goetz, 2006; Goolish, Okutake, & Lesure, 1999; Hensley & Leung, 2010; Mullins, Hammerschmidt, Haffter, & Nüsslein-Volhard, 1994; Nüsslein-Volhard Dahm, 2002; Wilson, 2012), although there are some indications that they may not adequately meet nutritional demands of the fish much beyond this stage (Lawrence, 2007; Wilson, 2012).

Rotifers, most especially the euryhaline *Brachionus plicatilis* or *B. rotundiformis*, are the most important live

prey for many cultured fish species during the first feeding phase, due to their small size, slow swimming behavior, and their amenability to intensive culture and bioencapsulation with nutrients essential for growth and development (Cahu & Infante, 2001; Lavens & Sorgeloos, 1996b; Lawrence, Sanders, & Henry, 2012b). The freshwater rotifer *B. calyciflorus* is also utilized because of its similar attributes but is less common due to the fact that it can be more challenging to culture than its euryhaline relatives (Aoyama et al., 2015; Arimoro, 2006). Like *Paramecium*, brachionid rotifers asexually reproduce when conditions are favorable, and switch to sexual reproduction when resources become limited (Lavens & Sorgeloos, 1996a; Lawrence et al., 2016). The goals of typical batch or continuous culturing methods for rotifers center around promoting favorable environmental conditions to promote maximal population growth through asexual reproduction (Lawrence et al., 2012b, 2016). "Type L" *B. plicatilis* range in size from ~130 to 340 μm, while "type S" *B. rotundiformis* are ~100–210 μm; both feed primarily on small phytoplankton (Lavens & Sorgeloos, 1996b). The freshwater *B. calyciflorus* are similar in size, ~260 μm (Aoyama et al., 2015). The success of brachionid rotifers as a first prey item for larval zebrafish has been well documented, with numerous publications reporting high rates of growth and survival in zebrafish fed on both euryhaline and freshwater species (Aoyama et al., 2015; Best et al., 2010; Farias & Certal, 2016; Lawrence et al., 2016; Siccardi et al., 2009).

The brine shrimp, *Artemia salina* (and other species, including *A. franciscana*) is the most important and prevalent live prey for commercially produced species of fish and shellfish (Lavens & Sorgeloos, 1996b). This small branchiopod crustacean, which is found in hypersaline lakes and lagoons in several locations globally, produces dormant embryos or "cysts" that can withstand desiccation in dry lake beds. These cysts can be harvested, processed, and stored such that they can be later "reanimated" on demand by hydration in seawater, to hatch into free-swimming nauplii that can be used as a food source for larval fish and shellfish. *Artemia* are easily cultured in batches, and a typical production cycle (hatching from cysts into stage 1 nauplii) takes ~24 h (Treece, 2000). The first instar nauplii at ~400–500 μm in length (Harper & Lawrence, 2010; Lavens & Sorgeloos, 1996b) are a suitable live prey source for a variety of first feeding fish. After hatching, the nauplii will eventually molt into the second stage larval metanauplii, which is at that point capable of exogenous feeding. From then on, the *Artemia* must be fed, or "bioencapsulated," with microalgae or fatty acid emulsifications to maintain their nutritional value for the fish (Figueiredo et al., 2009; Sorgeloos, Dhert, & Candreva, 2001). *Artemia* are perhaps the most widely utilized feed item—live or processed—in zebrafish culture, for the entire life cycle (Carvalho, Araujo, & Santos, 2006; Gonzales & Law, 2013; Lawrence, Best, James, & Maloney, 2012c, 2016; Markovich, Rizzuto, & Brown, 2007). They may be used as a prey item for first feeding zebrafish (Carvalho et al., 2006; Conceic, 2010), although this method can be challenging to reproduce because the larvae do not efficiently capture the larger and fast swimming nauplii (Lawrence et al., 2015). This difficulty is further compounded by the fact that *Artemia* may only survive in freshwater habitats for a short period of time, reducing their bioavailability to larval fish.

A number of other zooplankton species may be used as a prey item for larval zebrafish. These include ciliates (*Tetrahymena* sp.) (Kopp, Legler, & Legradi, 2016; Rendueles et al., 2012) and microworms (e.g., *Panagrellus redivivus*) (Amaral & Johnston, 2012; Westerfield, 2007). Other zooplankton, like cladocerans (water fleas), copepods, and krill, are too large for larval zebrafish, and are only suitable for fish from the subadult stage or beyond.

Larval fish may also be reared on processed diets, either exclusively, or in tandem with live feeds. The challenges associated with feeding small, first feeding fish larvae with processed diets are well-documented (Cahu & Infante, 2001; Girin et al., 1979; Hamre et al., 2013; Kolkovski, 2013; Onal & Langdon, 2005; Person, 1989), primarily because many species of fish are designed by nature to feed on zooplankton, and all of the inherent features of these organisms are difficult to incorporate into an inanimate particle. Among the most notable problems associated with processed "micro" diets is leaching of water-soluble nutrients upon hydration (Yúfera, Kolkovski, Fernández-Dıaz, & Dabrowski, 2002; Kvåle et al., 2007), a situation made more difficult by the very high surface area to volume ratios of small particles, especially those under 100 μm. Diets may be microencapsulated in such a way to minimize these losses (Yúfera, Pascual, & Fernández-Díaz, 1999), but this remains a problem, especially since residence time in the water must be maximized in order for weakly swimming fish larvae to be able to encounter them. The other issues associated with these diets include precise nutrient formulation, palatability, color, and movement in the water. Live feeds are superior in most if not all of these areas.

Despite these shortcomings, it is possible to rear fish exclusively on these diets, and this extends to zebrafish (Harper & Lawrence, 2010). First feeding zebrafish will readily consume inanimate particles (Onal & Langdon, 2000; Önal & Langdon, 2016), and there are numerous examples in the literature of them being reared on processed diets alone (Carvalho et al., 2006; Farias & Certal, 2016; Goolish et al., 1999; Hensley & Leung, 2010). It should be noted that survival and or growth is typically reduced when compared to performance on live

diets, however (Carvalho et al., 2006; Farias & Certal, 2016; Goolish et al., 1999).

Water Quality

The physical and chemical characteristics of water exert a broad range of effects on larval survival. For any species, there is a given set of parameters that must be maintained within a certain range that will promote optimal performance.

Physical

A number of physical factors impact the behavior, biology, growth, and survival of larval fish as they develop. Of all of these factors, the temperature is perhaps the most significant and pervasive as it affects both the physiology of the fish and the physics of the water (von Herbing, 2002). As poikilotherms, fish are profoundly sensitive to temperature. While zebrafish are among the most eurythermal fish species on record, with an acclimated thermal tolerance of 6.7–41.7°C under controlled conditions (Cortemeglia & Beitinger, 2005; Schaefer & Ryan, 2006), temperature exerts myriad changes on gene expression and physiology, especially during early life (von Herbing, 2002; Long et al., 2013; Villamizar, Ribas, Piferrer, Vera, & Sánchez-Vázquez, 2012; Scott & Johnston, 2012).

In general, the range within which embryonic development proceeds normally in zebrafish is between 24 and 32°C (Schirone & Gross, 1968). The thermal optima for zebrafish in controlled conditions have not been formally defined, but recommendations can be found in the literature (e.g., the 24–30°C range promoted by Matthews et al. (Matthews, Trevarrow, & Matthews, 2002)) and generally conform to the biological data. The widely cited maintenance temperature of 28.5°C (Westerfield, 2007) is derived from the classic embryological staging experiments of Kimmel et al (Kimmel et al., 1995), and this does not necessarily represent an optimum with respect to performance. However, some existing data supports the idea that zebrafish growth is maximized around this value; in one study, fish grown at 28°C were larger than cohorts reared under 22, 25, or 31°C regimes (Sfakianakis, Leris, Laggis, & Kentouri, 2011). Villamizar et al. observed a similar trend in their study on daily thermocycles in zebrafish (Villamizar et al., 2012).

Light is another major physical factor that impacts larval fish on many levels. As a physical factor, light encompasses color spectrum, photoperiod, and intensity; and its effects on larval growth, development and other performance metrics have been extensively evaluated in farmed fish species (Almazán-Rueda, Van Helmond, Verreth, & Schrama, 2005; Boeuf & Falcón, 2001; Boeuf & Le Bail, 1999; El-Sayed & Kawanna, 2004; Mclean, Cotter, Thain, & King, 2008; Ullmann et al., 2011).

There are a growing number of studies on how various aspects of light impact zebrafish embryos and larva. For example, the ambient color spectrum was found to exert profound effects on embryo hatching rates, larval growth and survival in zebrafish, with fish exposed to blue and white light showing highest rates of growth and survival along with the lowest rates of malformation (Villamizar, Vera, Foulkes, & Sánchez-Vázquez, 2013). In the same study, the authors found that exposure to constant light depressed growth and reduced survival, while fish that were reared in constant darkness showed 100% mortality by 18 days posthatch. However, it has also been shown that larval zebrafish are able to forage in the dark using the lateral line system (Carrillo & McHenry, 2016), suggesting that the negative results in some studies might be related to feed type or availability.

Interestingly, manipulation of photoperiod (i.e., subjecting fish to extended or continuous light conditions) has been utilized in a number of aquaculture species with some success (Boeuf & Falcón, 2001; Partridge et al., 2011; Valenzuela et al., 2012), presumably due to increased feed intake and feed conversion. Certainly, this could be the case for zebrafish, but none of the studies published so far in the literature of fish reared under extended daylight have evaluated performance in the presence of unlimited prey. At least in one study, a higher proportion of malformations was observed when larvae were exposed to constant light (Villamizar et al., 2013). It is critical to consider that any artificial manipulation of photoperiod and lighting should be done in the context of the visual physiology, biology, and ecology of the target species.

The color of housing enclosures has also been shown to influence growth performance in a number of aquaculture species (Imanpoor & Abdollahi, 2011; Mclean et al., 2008; Ullmann et al., 2011; Yasir & Qin, 2009). A primary causative factor for this phenomenon is thought to be contrast created between differential background colors and prey types; background colors that enhance this contrast are thought to improve capture/ingestion rates. However, it is critical to note that this relationship is entirely dependent upon species. For example, spotted grouper reared in white tanks ingested more rotifers and showed better growth than those grown in dark enclosures (Zhang et al., 2015). Striped trumpeter larvae showed enhanced growth and food consumption when grown with live feeds in dark backgrounds (Cobcroft, Shu-Chien, Kuah, Jaya-Ram, & Battaglene, 2012). The variability shown in this response across species and situation illustrates the complex relationship that

exists between color spectrum, light intensity, and prey type in larval fish.

The effect of tank color has not been well explored relative to zebrafish growth performance. However, tank background colors have been shown to impact zebrafish behavior (Blaser & Rosemberg, 2012; Vignet et al., 2013). In one study, tank wall color significantly affected depth preference in adult fish (Blaser & Goldsteinholm, 2012; Blaser & Rosemberg, 2012); illustrating the complexity of interactions between lighting, background, and behavior in this species. All of these data highlight the sensitivity of the fish to lighting conditions and support the idea that this parameter is deserving of further examination.

Water flow rates will also impact larval fish performance. At their earliest stages, most larval fish are faced with the fundamental challenge of effectively maintaining their position within the water column, especially as it relates to prey capture and ingestion (China & Holzman, 2014). Effectively, larval fish need to consume enough nutrients to satisfy their demands for both energy (required to combat drag) and growth. At the same time, the flow rate will also affect prey availability, and this can be more or less pronounced depending upon the size and density of the particle. Finally, flow rates will also impact water quality; in general, higher rates of flow will result in lower accumulations of nitrogenous wastes and higher dissolved oxygen. Larval rearing protocols must strike a balance between all of these forces while taking into account the biology of the target species.

Zebrafish larvae are not adapted to high flow conditions. Elevated rates of flow into tanks has been shown to reduce survival and growth (Bagatto, Pelster, & Burggren, 2001). This is, presumably, because the increased energy demands associated with maintaining a position in the water under elevated flow come at the expense of prey capture and growth, an idea supported by the fact that performance is poorest in the youngest fish exposed to the highest rates of flow (Bagatto et al., 2001). This is an important point to consider in the development of rearing protocols for this species, especially those that rely on higher rates of water exchange to control chemical waste.

Chemical

The chemical composition of the water profoundly impacts the physiology, health, and behavior of the fish that live in it. So it should come as no surprise that water quality is a major determinant in the success or failure of any given fish larviculture program. Various chemical parameters influence larval performance, but the most proximate of these are nitrogenous waste products, particularly unionized ammonia (NH_3) and nitrite (NO_2^-). There are numerous examples of the toxicity of these compounds to developing fish (Adelman, Kusilek, Koehle, & Hess, 2009; Barbieri & Doi, 2012; Bardon-Albaret & Saillant, 2016; Daoust & Ferguson, 1984; Jensen, 2003; Palachek & Tomasso, 1984; Rodrigues, Schwarz, Delbos, & Sampaio, 2007; Wang, Wang, Yu, & Jiang, 2015). In closed system aquaculture, nitrogenous wastes are a particular challenge because the goal of providing constant feed to larval fish tends to promote overfeeding and excessive ammonia production in rearing tanks.

Zebrafish adults and larvae appear to be well adapted to deal with relatively high levels of environmental ammonia (Braun, Steele, Ekker, & Perry, 2009; Kumai, Harris, Al-Rewashdy, Kwong Raymond, & Perry, 2015; Shih Horng, Hwang, & Lin, 2008). Larval zebrafish have been shown to survive and grow well in static culture tanks where unionized ammonia levels are as high as 0.16–0.18 parts per million (ppm) (Best et al., 2010). This elevated tolerance might be related to the salinity levels of the water in this particular study (5 parts per 1000 or ppt); in a number of other fish species, ammonia toxicity is reduced when animals are held at elevated salinities (Da Silva, Coimbra, & Wilson, 2009; Sampaio, 2006). Still, zebrafish larvae display remarkable flexibility in the way they are able to transport ammonia; in one study where they were challenged with high environmental ammonia along with conditions of high alkalinity (pH of 10.0) that inhibit ammonia excretion, they were able to either to elevate their excretion of ammonia (if not acclimated to the high alkalinity) or convert ammonia to urea (when acclimated) (Kumai et al., 2015). This is an excellent demonstration of the adaptability of the zebrafish to varying conditions, a character trait that reflects their evolutionary history in habitats subjected to wide ranges in seasonality and environmental change (Arunachalam et al., 2013; Engeszer et al., 2007).

Zebrafish larvae also display some level of tolerance to environmental nitrite. In one study, juvenile fish from 20 dpf showed significant depression in growth rates when exposed to 107 and 130 mg/L NO_2^- for 28 days (Voslářová, Pištěková, Svobodová, & Bedáňová, 2008). Earlier exposures (96 h postfertilization) cause developmental abnormalities and functional defects at concentrations as low at 100 mg/L (Simmons, Karimi, Talwar, & Simmons, 2012). Trace amounts of up to 0.15 mg/L do not inhibit growth or survival during the larval stage (Best et al., 2010).

Nitrate (NO_3^-), the tertiary product in the nitrogen cycle that is found in recirculating aquaculture systems (Swann, 1997), is generally far less toxic to larval fish than its precursors (Camargo, Alonso, & Salamanca, 2005), and this is certainly the case for zebrafish

(Lawrence, 2007). One comprehensive study recommends minimal safety values of 1450, 1855, and 1075 mg/L for embryonic, newly hatched, and swim-up larvae, respectively (Learmonth & Carvalho, 2015).

While zebrafish larvae display tolerance to higher levels of nitrogenous wastes than many other aquaculture species, the management goal should be to maintain total ammonia and nitrite values at or near zero and nitrate values well below the minimal safety values referenced above (Harper & Lawrence, 2010). This highlights the importance of making a distinction between tolerance and optima. The fish may be adapted to deal with elevated waste levels, but there is always an energy tradeoff involved; any energy expended in maintaining homeostasis under varying conditions is energy lost from growth and overall performance. Certainly, managers should look to avoid "challenging" fish with sub-optimal conditions; doing so will always result in depressed performance; in some cases, mortality. This applies not only to nitrogenous wastes, but also to any other environmental parameter that the developing fish may experience.

Given the prevalence of the zebrafish as a model organism, it is perhaps surprising that there are relatively few published studies in the literature on the impacts of water chemistry on growth and survival, other than nitrogenous wastes. The one other environmental parameter that has been delineated for zebrafish larvae is salinity. A common rearing practice involves cogrowing zebrafish with rotifers in brackish water at salinity values of 5 mg/L (Best et al., 2010; Lawrence et al., 2016) Additional studies on the salinity tolerance of zebrafish larvae show that their tolerance increases with age; 2–4 h old embryos exposed to salinities above 6 mg/L for longer than 1 h displayed significantly elevated mortality versus control, and none survived at 8 mg/L when exposed for 2 h (Sawant, Zhang, & Li, 2001). However, once embryos were beyond 10 h, they tolerated 1–2 h pulses of salinities of up to 10 mg/L before showing any appreciable signs of stress (Sawant et al., 2001). In at least one other study, larval fish (yolk-sac and five to seven dpf stages) showed elevated mortality at salinity values as low as 2 mg/L (Rothen, Curtis, & Yanong, 2002), so caution should be used when adjusting salinities; in general the fish seem to be more tolerant to salt once they inflate their gas bladders (Best et al., 2010; Lawrence et al., 2016).

Epigenetic Factors

Parental Condition

The concept that the environment experienced by the parent, especially the mother, has an influence on the phenotype of their offspring is well established in many organisms, including fishes (Donelson, Munday, & McCormick, 2009; Einum & Fleming, 1999; Platenkamp & Shaw, 1993; Vallin & Nissling, 2000; Vijendravarma, Narasimha, & Kawecki, 2010). There is evidence that maternal condition will influence egg size in a number of fish species, including brown trout (Einum & Fleming, 1999), Pacific cod (Vallin & Nissling, 2000) and mosquitofish (O'Dea et al., 2015). This phenomenon is often related to the nutritional state of the parents; offspring derived from well-fed parents survived at higher rates than offspring from feed-challenged individuals in the marine fish *Acanthochromis polyacanthus*[150]. It may also be related to other factors, including the size and age of the mother (Kindsvater, Rosenthal, & Alonzo, 2012). Stress has also been shown to influence egg quality and survival in several species (Campbell et al., 1992, 1994; Mccormick, 1998).

This relationship has been explored to some extent in zebrafish. However, several studies have highlighted the association between maternal diet and embryo survival and quality. Miller and coauthors showed that zebrafish fed a vitamin E deficient diet produced significantly more embryos with developmental abnormalities and a reduced rate of survival (Miller et al., 2012). In another study, zebrafish with access to more food produced offspring that were more likely to survive to adulthood and showed higher levels of physical activity than fish that were produced by adults subjected to feed restriction (Newman, Jhinku, Meier, & Horsfield, 2016). These data demonstrate how critical environmental conditions experienced by broodstock are for larval performance.

Methodology

Given the plethora of factors that contribute to success in larval rearing protocols, there is no standard approach to zebrafish larviculture. The fish can be reared on a variety of diets, under a wide range of conditions. Methodology can be broken down into two different basic approaches: static and recirculating. There are also approaches that utilize a blend of both.

Basic Approaches

Larval zebrafish are typically grown in either static or recirculating tanks. In static rearing approaches, the fish are cultured in containers that receive only periodic water exchanges, either automatically or manually. In general, static approaches should incorporate the use of live feeds that persist, if not thrive, in these conditions. The best-known examples of these are ciliates, brachionid rotifers, and cladocerans (e.g., *Daphnia*). The use of these types of feeds will minimize waste production and decrease the required frequency of water exchange. Processed feeds and live feeds that do not persist in freshwater (i.e., *Artemia* nauplii) tend to break down and

produce more ammonia, thus necessitating a greater frequency and volume of water exchanges to minimize pollution that will limit fish growth. This breakdown of organic waste will also consume and therefore limit dissolved oxygen (Swann, 1997).

Many culturing methods for zebrafish employ a blend of the two approaches, usually starting with live diets in static conditions before progressing to processed diets or some admixture of live and processed feeds in recirculating or flow-through tanks. The higher protein content and mass of the processed diet or admixture of live/processed feeds will necessitate a rate of water exchange that can be most practically achieved through a constant exchange to control ammonia and maintain dissolved oxygen at adequate levels.

Published Methods

There are a number of published methods for rearing zebrafish larvae in the literature from the past several decades. The first and most notable of these appeared in the *Zebrafish Book*[68], which described a basic protocol for rearing "baby" zebrafish centered on the use of first *Paramecium* sp. and then *Artemia* nauplii in static culturing containers before introduction of the larvae at 21 dpf into recirculating tanks where they continued to be fed *Artemi*a until they were eventually transitioned to a flake diet (Westerfield, 2007,). Another variation of this method was published in another popular book, *Zebrafish: A Practical Approach*, although this protocol incorporated the use of processed, powdered diet, rather than live feeds, in static conditions with zero water exchange for the first few weeks of life (Nusslein-Volhard & Dahm, 2002). These two basic approaches, or various derivations of them, have been utilized by laboratories all around the world to grow zebrafish for several decades.

Indeed, the efficacy of these methods for rearing larval zebrafish has never been in doubt; the model system experienced dramatic growth in popularity during this period. This "success" can probably be attributed to the considerable tolerance of the fish—even during the larval stages—to varying environmental conditions. However, it is important to note that these methods were never optimized or subjected to rigorous peer review. Consequently, there is no published performance on survival or data associated with these methods, and it is difficult to tell how effective they really are.

In the past five to 10 years, there has been an increased focus on optimizing larval rearing protocols beyond these early approaches. These data highlight a number of basic generalities about zebrafish larviculture. One is that *Paramecium*, while it supports good survival of first feeding zebrafish, does not promote robust growth. A number of studies demonstrate this, most notably one published by Biga and Goetz that showed that zebrafish fed on *Parmecium* only for the first 4 weeks of life attained a total mean length of only ~4.7 mm at 28 days postfertilization (Biga & Goetz, 2006). This compares very unfavorably with a number of other papers that show that larval zebrafish fed other diets (*Artemia* (Conceic, 2010), rotifers (Aoyama et al., 2015; Best et al., 2010; Farias & Certal, 2016), or processed feeds (Carvalho et al., 2006; Hensley & Leung, 2010)) all attain a significantly larger size at a similar stage of development.

In principle, it is possible to grow zebrafish exclusively on a processed diet from first feeding onward. Historically, to achieve this necessitated a sacrifice of either growth or survival (Goolish & Okutake, 1999), but recent advances in diet formulation and feeding practices allow for relatively high values of both metrics (Farias & Certal, 2016; Hensley & Leung, 2010; Onal & Langdon, 2005; Önal & Langdon, 2016). Interestingly, the addition of live diets to protocols that utilize processed feeds tends to improve growth and survival (Farias & Certal, 2016).

This supports the generally held perception that larval zebrafish, as is the case for many other aquaculture species, perform best on live diets. Zebrafish larvae fed on *Artemia*, either exclusively (Carvalho et al., 2006; Conceic, 2010), or in progression after rotifers (Lawrence et al., 2015), perform very well. The "polyculture method," where larval zebrafish and rotifers are grown together in static, brackish water for the first week of development, supports some of the highest performance metrics published in the literature (Best et al., 2010; Lawrence, Adatto, Best, James, & Maloney, 2012d, 2016). Critically, this particular approach is likely so successful because it meets many of the criteria (discussed above) that are critical to developing, first feeding larvae: a simple to digest, nutritious, and attractive prey item that can be saturated in the water without polluting it, maximizing encounter rates for the fish. The rotifer, when presented in this fashion satisfies many of these requirements, but other diets and approaches can be adapted to achieve similar results.

Future Directions

The state of zebrafish larviculture has advanced considerably in the past 5 years. Still, there are many areas in need of improvement. The first is simple: the newest methodologies need to be more widely applied than they are. A very large proportion of labs still practice very basic larval rearing approaches that have remained largely unchanged from the primitive methods published in the Nusslein-Volhard and Westerfield books (Lidster, Readman, Prescott, & Owen, 2017). It is very likely that

their efficiency and performance would dramatically improve if they applied modern techniques.

While techniques have improved, there are still gaps in the scientific understanding of larval nutrition (Watts et al., 2012, 2016). Detailed information on specific requirements for macro and micronutrients could be used to improve formulated diets and/or live feed enrichment products. Data on feed frequency and volume for early-stage zebrafish would also be beneficial. For example, it has been established that zebrafish adults perform well when fed between 3% and 5% body weight per day (Lawrence et al., 2012c, 2012d; Siccardi et al., 2009). However, the amount of feed required to support optimal growth and survival in larval and juvenile stages of fish is still unknown. These data would help define the nutritional landscape and improve and standardize rearing programs.

Another area of future growth in zebrafish larviculture is the advent of automation in facility and nursery design. For example, the automated robotic feeders that have been developed for zebrafish facilities in the last few years (Lawrence, 2011, 2016; Lawrence & Mason, 2012) can be leveraged to help solve traditional problems in larviculture. The most notable of these is the requirement for larval fish to be presented with small amounts of biologically available feed on a nearly constant basis. Robotic feeders can be programmed to meet this demand in a standardized and defined manner. Other advances in automation that could help drive the field, in the near future, include fish counting and grading tools, and behavioral monitoring software.

Conclusion

The field of zebrafish husbandry and management, after a long period of relative stagnation, is finally beginning to show signs of innovation and advance that match the pace of technology development seen in the model system overall. This change is being driven by the increasing use of the fish and expansion in scale and type of experiment that zebrafish are used in, as well as regulatory requirements. The practice of zebrafish larviculture is poised for rapid advance, as a result of the growing body of published work in the literature, and advancing technology in housing systems and fish diets. Success in larviculture—maximizing survival and growth rates—will accelerate the pace of scientific discovery and help the zebrafish realize its potential in a number of far-reaching disciplines.

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CHAPTER

33

Zebrafish Nutrition—Moving Forward

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Introduction

In research, nutrition is an essential factor in any animal study, as it can affect an organism's growth, reproduction, health, and response to stimuli. A lack of knowledge about an animal's nutritional requirements provides a challenge when that animal is used as an important biomedical model, as many diseases have nutritional confounders. Furthermore, a lack of nutritional control prevents the standardization of nutrition-based protocols for reproducible research. Several decades ago, investigators utilizing rodent models addressed the lack of knowledge in animal model nutrition and developed dietary guidelines using standardized and open formulation diets. Despite the widespread use of the zebrafish as a model in multiple areas of research, nutritional requirements and standardized feeding protocols have yet to be established. Consequently, over the past decade, the zebrafish community has begun to emphasize nutrition as an important component of animal husbandry and biomedical research. The goal of this chapter is to provide a basic understanding of nutrition, energy allocation, and feed management in fish, while specifically presenting published information related to zebrafish, or in comparison to other fish species. The criteria and methods identified below aim to provide the current understanding of nutrition in zebrafish, ultimately leading to the standardization of feeding practices and development of chemically defined, reference diets for use in zebrafish laboratories.

Nutrients

Protein and Amino Acids

Protein content and amino acid composition of the diet affects animal growth by regulating body protein synthesis and lean body mass that primarily composes organs, red muscle, and white muscle. For most fish species, as in higher vertebrates, the rate of synthesis is prioritized differentially among tissues with liver having the highest synthesis rate and white muscle having the lowest (Guillaume, 2001). Protein retention is also exhibited differentially among tissues with white muscle retaining over 50% of its synthesized protein. For *Danio rerio* (*D. rerio*), the addition of lean mass in muscle is initiated by hypertrophic growth similar to that observed in mammals (Biga & Goetz, 2006).

Zebrafish are believed to require the same ten essential amino acids (EAAs) as higher vertebrates and humans based on the results of comparative studies (Akiyama, Oohara, & Yamamoto, 1997). If a daily dietary requirement for a single EAA cannot be met, the synthesis of protein decreases and body protein is hydrolyzed, leading to reduced muscle gain or, in an extreme case, loss of lean body mass. Nonessential amino acids (NEAAs) can be synthesized by transamination of dietary carbon skeletons independent of dietary protein intake and body protein hydrolysis. Inclusion of some NEAAs in the diet is recommended to avoid the utilization of EAAs in de novo synthesis of NEAAs. In addition to amino acids being utilized for lean tissue growth or energy, several small molecules are produced from individual EAAs and NEAAs. Many nitrogenous compounds, pigments, neurotransmitters, hormones, vitamins, and other compounds that are needed to regulate metabolic functions require a specific amino acid precursor for synthesis.

Zebrafish have been observed anecdotally to reach satiation and cease consumption of available feed. It is, however, unknown if satiation is the result of reaching a particular intake level of protein intake, energy intake, volume satiation, or some other dietary component (Watts, Powell, & D'Abramo, 2012). In a diet with low protein content, the dietary ratio of protein to energy decreases as lipid and carbohydrate correspondingly

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account for a larger relative percentage of the dietary macronutrient composition and provide nonprotein energy. *Ad libitum* feeding of a single diet that is either low in total protein or deficient in one or more EAAs to zebrafish may not meet their daily protein or amino acid requirements, resulting in long-term consequences for growth, body composition, reproduction, and/or longevity, particularly during critical periods of development. Alternatively, to meet a daily protein or amino acid requirement when fed a protein-deficient diet, some animals will overconsume energy (lipid and carbohydrates) by essentially targeting an optimal daily protein or amino acid intake to reach satiation. This response is described by the well-supported protein leverage theory that describes negative metabolic consequences arising from overconsumption of energy to reach a daily protein or amino acid optimum for lean tissue growth (Simpson & Raubenheimer, 2012). Another potential response may arise from the use of high protein diets with a high protein to energy ratio. If energy from dietary carbohydrate and lipid is too low, amino acids may be utilized as an energy source rather than for synthesis of body protein. This metabolic activity is undesirable due to the protein being a costly feed ingredient, whose metabolism can lead to the presence of more nitrogenous waste that must be managed.

Deficiencies of specific amino acids have species-specific effects that are independent of body protein synthesis and lean mass accumulation. The impact of specific amino acid deficiency seen in fish species closely related to *D. rerio* should be considered, and additional studies in zebrafish are warranted. For example, a diet deficient in tryptophan results in abnormal calcium deposition in trout and salmon but did not impact *D. rerio* skeletal formation (Newsome & Piron, 1982).

Protein or specific amino acid requirements can be assessed in two ways, either as an absolute (mg/g average body weight/day) or relative requirement (percentage protein in the diet). When measured as an absolute requirement, most fish species do not have a requirement that is particularly different from that observed in higher vertebrates. This shared characteristic should be considered before providing a commercial diet that may contain upwards of 40%–60% protein, a level commonly seen in fish diets. The most recent and comprehensive study done on *D. rerio* protein requirement recommended a diet with a protein content of no greater than 44.8% and an absolute intake of approximately 14 mg/g average body weight/day for optimal growth (Fernandes, Peres, & Carvalho, 2016); however, the results of this study are only representative of a single dietary protein source (fish meal). Source of protein used among commercial diets can vary according to brand and/or batch, and many sources are not disclosed. Protein sources will vary in amino acid content and have intrinsic differences in nonprotein components. Studies demonstrate that different dietary protein sources will influence growth and body composition, independent of the amount of total protein in the diet (Smith et al., 2013; Ulloa et al., 2013). Studies utilizing whole-body proteome of zebrafish show that lysine deficient diets altered expression of proteins important for growth and metabolism, with downregulation of muscle proteins and upregulation of protein related to fasting, energy deficiency, and arrested growth (Gomez-Requeni, Conceicao, Olderbakk Jordal, & Ronnestad, 2010). Additional investigations on the impact of dietary protein source and an individual amino acid requirement for *D. rerio* are needed.

Lipids

Lipids are well known as dietary compounds that supply energy; however, they can also act as pheromones, hormones, and components of membranes, as well as aiding in the absorption of lipo-soluble vitamins and carotenoid pigments. (Meinelt, Schulz, Wirth, Kürzinger, & Steinberg, 2000; Guillaume, 2001). In many fish species, they are the predominant source of dietary energy for metabolism, development, growth, and reproduction, and also serve as transporters for the allocation of energy derived from lipids (Leaver et al., 2008). The amount and types of lipid included in a diet can influence multiple outcomes, including but not limited to weight, body composition, reproduction, behavior, vision, osmoregulation, gut microbiota populations, and immune responses (Arts & Kohler, 2009; Watts et al., 2012; Falcinelli et al., 2017). Despite the significant impact of dietary lipid on these outcomes, lipid requirements have still been only minimally evaluated in zebrafish.

Defining dietary lipid requirements is complicated by the varied chemical nature and functional role of lipids (NRC, 2011). Although optimum levels of dietary lipid quantity and quality cannot be truly defined for any species, a perceived optimum range can be estimated (NRC, 2011). The lower limit of this range should be defined as the minimum amount of lipid meeting requirements for essential fatty acids (EFAs), while the upper limit should be established as the maximum level of dietary lipid that does not elicit an adverse response (NRC, 2011). Currently, no optimum range for total dietary lipid intake has been established in zebrafish (Watts et al., 2012). However, zebrafish are classified as a tropical omnivorous freshwater fish, and other species of this type, including carp and tilapia, have demonstrated a reduced ability to tolerate high levels of dietary lipid (Lawrence, 2007; NRC, 2011; Watts et al., 2012). This intolerance translates into negative impacts on

growth performance and nutrient utilization, in addition to ectopic fat deposition within organs and visceral tissues (NRC, 2011). Studies using other tropical omnivorous freshwater species have suggested ranges of from less than 10% to as high as 18% of total dietary lipid; nonetheless, studies are needed to determine an optimal range that is specific to zebrafish (NRC, 2011). Notably, studies utilizing the zebrafish as a model for diet-induced obesity proffered diets containing total dietary lipid levels of 22%–26% dry weight of feed (Meguro, Hasumura, & Hase, 2015; Stoletov et al., 2009; Vargas & Vásquez, 2017). At these levels, zebrafish appeared to share similar phenotypes, as well as pathophysiological pathways observed in humans in the development of diet-induced obesity.

The limited studies that have been conducted on the dietary lipid "requirements" of zebrafish have focused primarily on their requirements for essential fatty acids (EFAs), specifically for polyunsaturated fatty acids (PUFAs) and long-chain polyunsaturated fatty acids (LC-PUFAs). EFAs are fatty acids that are not synthesized by most organisms but are required for cellular metabolism, maintenance of membrane structural integrity, and synthesis of prostaglandins, and similar compounds (Guillaume, 2001). For fish, diets that are EFA deficient result in reduced growth rates and feeding efficiency. Over time, EFA deficiencies are manifested by the development of fatty liver, fin erosion, and gill lesions (Guillaume, 2001).

EFA deficiencies have also been found to have significant negative impacts on the quality of reproduction in spawning fish (Guillaume, 2001). It has been well established that mechanisms affecting egg release are influenced by the n-6 and n-3 fatty acid content of the diet (Abayasekara & Wathes, 1999; Izquierdo, Fernández-Palacios, & Tacon, 2001; Mazorra et al., 2003). Polyunsaturated fatty acids regulate and are precursors to eicosanoid production; arachidonic acid (ARA; 20:4n-6) serves as the primary substrate for the proinflammatory series-2 prostaglandins (PGE_2 and PGF_{2a}), while eicosapentaeoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) give rise to the less inflammatory series-3 prostaglandins (PGE_3) (Broughton, Bayes, & Culver, 2010; Izquierdo et al., 2001). Ovaries have a high capacity to generate these prostaglandins, which have been found to significantly influence male spawning behavior, gonadal steroidogenesis, and ovulation in both mammals and fish (Murdoch, Hansen, & McPherson, 1993; Izquierdo et al., 2001; Knight & Van Der Kraak, 2015).

Specific FA requirements depend on their different functional roles and whether they can be synthesized endogenously (NRC, 2011). Fish oils, fish meal, and certain plant oils, such as flaxseed and rapeseed oil are rich in n-3 PUFAs and LC-PUFAs, while other plant oils, such as corn and safflower oil can contain 50% or more of n-6 PUFAs and LC-PUFAs. For formulating a diet that meets the EFA requirements of a certain fish species, it is important to know the dominant EFA type(s) (n-3 or n-6 PUFA/LC-PUFA) for the species and its capacity for bioconversion (elongation and desaturation) from shorter chain PUFAs (Guillaume, 2001). Zebrafish, like many other freshwater fishes, have the ability to elongate and convert the essential PUFAs linoleic acid (18:2n-6) and alpha-linolenic acid (18:3n-3) into the physiologically more important LC-PUFAs eicosapentanenoic acid (20:5n-3; EPA), docsahexaenoic (22:6n-3; DHA) and arachidonic acid (20:4n-6; ARA) (Lawrence, 2007; Bell & Tocher, 2009; NRC, 2011). Also similar to what has been determined for other freshwater fish species, zebrafish require a higher dietary proportion of n-6 to n-3 PUFA. Meinelt and colleagues conducted a series of studies that found growth and fertilization rates were positively correlated with increased dietary levels of n-6 PUFAs (Meinelt, Schulz, Wirth, Kürzinger, & Steinberg, 1999; Meinelt et al., 2000). Other studies that specifically focused on zebrafish reported that the fatty acid profiles of many organs, reproductive success, and embryonic development of offspring changed in response to the sources of dietary lipid and balance of dietary PUFAs and LC-PUFAs (Araújo et al., 2016; Jaya-Ram, Kuah, Lim, Kolkovski, & Shu-Chien, 2008). Finally, results from two other published studies suggested that dietary provision of PUFAs and LC-PUFAs also influences body composition and inflammatory responses of juvenile zebrafish (Adam, Lie, Moren, & Skjærven, 2017; Powell et al., 2015). However, additional experiments are warranted to further define EFA requirements in zebrafish, particularly for different life stages and genetic lines.

Although a requirement for dietary cholesterol in zebrafish or any other teleost has not been reported, this requires more attention in the future (NRC, 2011). Research evaluating the effects of dietary cholesterol in zebrafish has been limited to studies evaluating the effects of excessive cholesterol intake on the development of atherosclerosis. Zebrafish consuming a high cholesterol diet (4% w/w) exhibited hypercholesterolemia and elevated levels of oxidized cholesteryl esters and lipoproteins (Fang et al., 2010; Fang et al., 2011; Stoletov et al., 2009). Transgenic *fli1:EGFP* zebrafish larvae fed artificial *Artemia* enriched with 2%–10% cholesterol displayed fatty streak formation and thickening of endothelial cells in the artery walls, both of which are evidence of early atherogenesis (Stoletov et al., 2009). Results from these studies suggest that the health of zebrafish may be significantly impacted by dietary cholesterol intake, highlighting the need for future studies to determine a safe intake level.

The phospholipid is a general term comprising all lipids that contain phosphorous, including sphingomyelin, although the term is commonly used to describe phosphoglycerides, which are the predominant polar lipids (NRC, 2011). The inclusion of intact phospholipids in diet formulations can potentially improve the culture performance of various freshwater species (Tocher, Bendiksen, Campbell, & Bell, 2008). Previous studies have observed multiple beneficial effects, including improved growth in both larvae and early juveniles, higher survival, and reduced incidence of malformation in larvae, and potentially even higher resistance to stress (Tocher et al., 2008). However, the determination of an absolute dietary requirement for phospholipid in finfish has been confounded by the use of a wide range of combinations of phospholipid sources and levels of purity in nutritional studies. The majority of these studies have utilized relatively unrefined mixed phospholipid preparations, including soy and other plant lecithins, and egg yolk lecithins. These ingredients contain several phospholipid classes, making it difficult to discriminate benefits from a specific phospholipid class (Tocher et al., 2008). The number of nutrition studies in teleosts, in which one single phospholipid class has been utilized, is limited. In both zebrafish and related teleost species, dietary supplementation of phosphatidylcholine (PC) appears to promote higher growth rates compared to other phospholipids, but it is also associated with a high rate of skeletal malformations and low survivorship in progeny (Diogo et al., 2015; NRC, 2011; Tocher et al., 2008). In contrast, supplementation of phosphatidylinositol (PI) and phosphatidylethanolamine (PE) is associated with improved sperm quality and higher fecundity and survivorship of progeny when compared to broodstock fed diets supplemented with PE. The effects of other phospholipid classes or sphingolipids on fish health have not yet been identified (NRC, 2011). Another inherent limitation common to previous studies is that they have been primarily restricted to young fish. While it appears that the quantitative requirement for phospholipid generally decreases with age, dietary phospholipid requirements have yet to be studied in any adult species of fish.

Soy lecithin is used as an ingredient in aquaculture feeds and is believed to serve both nutritional and functional roles. While its nutrient content can be highly variable and poorly defined, its inclusion can improve digestion through emulsification, protect against lipid peroxidation, and improve feed palatability and manufacture by extrusion (Sealey, Craig, & Gatlin, 2001). In zebrafish, both soy lecithin and choline chloride (as a source of choline in the PC molecule) have been included in the diets as a source of phospholipids, at levels ranging from 1.9% to 4% and 0.3%–0.5% of diet dry matter, respectively (Robison et al., 2008; Siccardi, III. et al., 2010; Smith et al., 2013; Powell et al., 2015; Fernandes et al., 2016). Future studies will be needed to compare the efficacy of both sources, as well as determine dietary phospholipid requirements for all life stage of zebrafish.

Carbohydrates

Carbohydrates, which include low-molecular-weight sugars, starches, and various nonstarch polysaccharides (NSP), are stored in liver and muscle tissues and provide the major and least expensive source of energy in diets for humans and most farmed animals (NRC, 2011). Among the different forms of carbohydrate that are abundant in plant sources, only sugars and digestible starches provide nutritive value for fish; therefore, the term "carbohydrates" in this section exclusively refers to them (Kamalam, Medale, & Panserat, 2017; NRC, 2011).

In contrast to terrestrial vertebrates, fish preferentially use lipids rather than carbohydrates as an energy source (Babin & Vernier, 1989). The ability of a fish species to utilize energy from carbohydrates differs depending on their biology (feeding habits, anatomical-physiological features of the gastrointestinal tract, genotype) and diet (carbohydrate source, inclusion level, and nutrient interactions) (Kamalam et al., 2017). Overall, however, fish do not possess efficient carbohydrate utilization systems, and it is generally accepted that they do not have a specific dietary requirement for carbohydrates (Leaver et al., 2008; NRC, 2011). Rather, fish appear to satisfy structural carbohydrate and carbohydrate storage (glycogen) requirements primarily by synthesizing glucose from nonglucose precursors, such as amino acids, which are the major substrates for gluconeogenesis (Cowey & Walton, 1989; Leaver et al., 2008; NRC, 2011). However, the inclusion of appropriate levels of carbohydrates in diet formulations of some fish species can minimize the catabolism of other dietary macronutrients, such as protein and lipid for energy and glucose synthesis (NRC, 2011; Fang et al., 2013). In rainbow trout, the inclusion of digestible carbohydrate at 20% of the dry weight of the diet improved protein retention and reduced metabolic fecal nitrogen loss; these animals also had the best performance for weight gain, feed conversion, and daily growth index (Kim & Kaushik, 1992).

Wild zebrafish consume a wide variety of animal and plant matter and, similar to other omnivorous fish species, are able to utilize larger amounts of carbohydrates and demonstrate a higher degree of protein sparing as compared to carnivorous fish (Watts et al., 2012; Fang et al., 2013). Robison et al. (2008) reported that a lack of dietary carbohydrate (<5% of the dietary

dry matter) for adult zebrafish reduced growth rates and significantly affected condition factor, body composition, and hepatic gene expression differentially in male and female zebrafish, while simultaneously reducing retention efficiencies of protein and energy. Another study of sexually immature zebrafish reported that similar to the study of Robison et al. (2008), hepatic gene expression was altered in response to dietary carbohydrate intake (Seiliez et al., 2013). The magnitude of expression of glucokinase, an enzyme associated with glycolysis, differed according to dietary ratios of protein to carbohydrate. Cumulative results from these studies suggest that in zebrafish, genes involved in glucose homeostasis and carbohydrate metabolism respond rapidly to changes in dietary carbohydrate intake (Robison et al., 2008). Furthermore, the inclusion of dietary levels of specific carbohydrates may have protein and lipid sparing effects, and in turn, promote higher growth rates. Establishment of an appropriate balance of dietary carbohydrate in diet formulations is an issue that continues to require attention.

As previously stated, the extent to which zebrafish and other fish species can utilize dietary carbohydrates for energy depends on not only the amount but also on the quality of carbohydrates. The molecular structure of carbohydrates determines their receptiveness to enzymatic hydrolysis and their corresponding influence on animal physiology (NRC, 2011). Most fish have the enzymes (α-amylase) to hydrolyze glucose polymers with α-glycosidic linkages (amylose and amylopectin). While zebrafish and other omnivorous fish species can utilize multiple dietary carbohydrate sources to obtain glucose and energy sources, the two primary sources commonly used in diet formulations to conduct nutritional research with zebrafish are starch and dextrin (Kamalam et al., 2017) (Table 33.1).

Starch is a polysaccharide stored as an energy reserve in many plants (NRC, 2011). Its digestibility is highly variable according to its primary structure, granule type, size, distribution, type (simple or compound), and amylose to amylopectin ratio (Svihus, Uhlen, & Harstad, 2005). Generally, starches with smaller granule sizes, such as rice and wheat starches, have a higher digestibility than those with larger granule sizes, such as maize starch (Kamalam et al., 2017; NRC, 2011). Additionally, starches with high amylose content, such as maize starch, are less digestible than amylopectin, which is highly branched and more receptive to enzymatic breakdown (Svihus et al., 2005). Gelatinized starches produced via extrusion or cooking have increased solubility, water absorption capacity, and digestibility compared to their raw counterparts (Honorato et al., 2016; NRC, 2011). Dextrin is hydrolyzed from starch, and comparatively has higher digestibility and intestinal uptake that confers better feed utilization and protein sparing in other fish species (Kamalam et al., 2017; NRC, 2011). Previous studies have demonstrated that the digestibility and utilization of different carbohydrate sources are species-specific, and for this reason, an ideal source, as well as amount, of dietary carbohydrate still needs to be established in zebrafish.

Regardless of feeding habit, the ideal amount of dietary carbohydrates to provide to a fish species may vary with life stage (Lee & Kim, 2009). The incorporation

TABLE 33.1 Inclusion levels and sources of carbohydrates used in formulated zebrafish diets.

CHO source	Min-max level	Life stage	Reference
Wheat	8%–42%	Adult	Seiliez et al. 2013
Gelatinized starch	15%		
Maltodextrin	35%–60%	Larval-juvenile	Fang et al. 2013
Wheat starch	0%–35%	Adult	Robison et al. 2008
Raw pea starch	0%–10%	Juvenile	Rocha et al. 2014
Gelatinized pea starch	0%–10%		
Starch	0%–20%	Adult	Meguro et al. 2015
Dextrin	46.17%	Juvenile	Adam et al. 2017
Pregelled starch	10%	Adult	Jaya-Ram et al. 2008
Dextrin	12.85%–21.85%	Juvenile	Smith et al. 2013
Pre-gelatinized maize starch	11.4%–60.9%	Juvenile	Fernandes et al. 2016
Dextrin	9.77%	Juvenile-adult	Powell et al. 2015

of carbohydrates into the diets of larval zebrafish needs careful consideration because early nutritional programming effects on outcomes in growth and metabolism have been observed in adult zebrafish. Fang et al. (2013) found that including a comparatively high level of dietary carbohydrate in diets fed to larval zebrafish significantly modified their carbohydrate digestion, transport, and metabolism as adults. In contrast, Rocha et al. (2014) reported that early exposure to high levels of dietary carbohydrate showed only marginal effects on the programming of carbohydrate metabolism in zebrafish. They injected glucose into the egg yolk of developing embryos followed by a challenge using a high-carbohydrate diet when the juvenile stage was reached. While overall effects were minimal, glucose storage capacity in muscle, as well as glucose production and transport in viscera, may have been affected (Rocha et al., 2014).

One potential explanation for these confounding results between studies could be the variation in amount and source of carbohydrate used in the high carbohydrate diets. Fang et al. (2013) added maltodextrin in the diets at levels of 35% and 60% of dry matter (DM), whereas Rocha et al. (2014) used gelatinized pea starch and glucose, both at 10% DM. For researchers utilizing the zebrafish as a model for nutrition, obesity, and metabolic health, these results are a strong indicator of the importance of standardizing nutrition in the larval feeding stage of zebrafish. This developmental interval is critical to the potential modification of long-term physiological functions (Fang et al., 2013). As with other macronutrients, standardization through the provision of a single appropriate source and amount of carbohydrate in formulated diets can reduce variability in results between labs, and improve the level of confidence in the sharing and application of research findings.

Fiber

Fiber is considered any plant-based feed component that cannot be digested by endogenous enzymes. In fish feeds, ingredients that are fiber compounds can be used as bulking agents and/or binders. The primary functions of binding agents are the improvement of the stability of feed pellets in the water, increasing pellet firmness, and reduction of fines produced during processing and handling of feed (NRC, 2011). In zebrafish, binders are often added in small amounts, and examples include guar gum, alginate, hemicellulose, and gelatinized starches. Studies using terrestrial animal models and humans have demonstrated the substantial impacts that dietary fiber confers on health (Anderson et al., 2009). Currently, no study has shown a qualitative or quantitative requirement for fiber in zebrafish or that dietary fiber plays a role in zebrafish health.

Dietary fiber can impact the transit rate of food, uptake of nutrients, and fecal composition and/or volume in the gastrointestinal tract (Dias, Huelvan, Dinis, & Métailler, 1998). The impact on specific nutrient uptake is dependent on fish species and fiber type. In sea bass, an inclusion of 10%–20% dietary fiber from cellulose, silica, or zeolite caused a reduction in lipid uptake but exerted no influence on protein uptake. Feed conversion efficiency was reduced, but this response was attributed to nutrient dilution rather than a direct effect on metabolism. Fecal volume was also increased. In rainbow trout, an inclusion of 2.5%–10% guar gum or alginate reduced both lipid and protein digestibility and feed intake (Storebakken, 1985). The highest level of guar gum reduced growth and body lipid and increased the passage rate of digesta. Rainbow trout fed diets containing levels of cellulose up to 30% increased feed intake, a compensatory response that resulted in no significant differences in growth or nutrient uptake (Bromley & Adkins, 1984). At higher levels of 40 or 50% cellulose, growth and nutrient uptake were reduced by half. The only study looking at the impact of dietary fiber content in zebrafish showed that increased cellulose content from 1.5%–60% resulted in increased gut length, gut mass, surface area, and activity level of digestive enzymes, but decreased body mass (Leigh, Nguyen-Phuc, & German, 2018). However, in this study, the higher fiber diets also had decreased dietary protein, which may have impacted gut function and structure independent of fiber.

Minerals

In zebrafish, calcium, phosphorus, and magnesium are all important macro-minerals required for growth and development of bone, fins, and scales. The majority of the calcium requirement for fish can be supplied by uptake of calcium through the gills from the water of sufficient hardness (Wood & Shuttleworth, 1995). Zebrafish can mobilize calcium from scales and fins in response to calcium deficiency (Metz, Leeuwis, Zethof, & Flik, 2014). If any animal-sourced ingredients, such as animal and fish meals, egg yolk, etc., are used in the diet, calcium supplementation is generally not needed. The inclusion of egg yolk in a zebrafish diet provided adequate calcium for bone development with no additional supplementation, even in calcium-free water (Padgett-Vasquez, Siccardi, D'Abramo, & Watts, 2008). The estimated dietary level of calcium was 0.08% in the diet treatment with no calcium supplementation, suggesting that zebrafish are highly efficient in utilizing available calcium. Phosphorus is essential for bone

formation, both as an inorganic buffer of cellular fluids, and a nucleic acid constituent. In fish species, phosphorus requirements are unlikely to be met by the gills and must be supplied via the diet (Lall, 1991, p. 1991). Phosphorus can also be mobilized from scales as needed. An optimal calcium/phosphorus ratio has been determined for some species of fish, but a ratio of less than 2:1 commonly yields adequate growth and survival across many aquatic species (NRC, 2011). Magnesium is also important for bone formation and is involved as a cofactor for enzymatic reactions. In both terrestrial animals and fish, the requirement for magnesium increases as the level of dietary protein increases. Magnesium requirements cannot be completely satisfied by uptake by the gills from freshwater and must be supplied in the diet like phosphorus (Bijvelds, Velden, Kolar, & Flik, 1998). Dietary strontium, which is not considered as essential in humans and other vertebrate diets, notably increases mineral density in bone and scales (Siccardi, III. et al., 2010).

Many other minerals are hypothesized to be required in trace amounts in the diet and are used in biological reactions as enzymatic cofactors rather than for mineralization. Copper is needed in trace amounts for enzyme activity and can be supplied via the gill or in the diet. Previously, dietary copper was considered to be a much safer alternative to dissolved levels. Toxicity to both dietary and waterborne levels in water can occur and varies by species (Clearwater, Farag, & Meyer, 2002). Copper deficiency leads to decreased superoxide dismutase (SOD) and oxidase activity and a clinical symptom of cataracts (Gatlin & Wilson, 1986). Iron is needed in trace amounts for oxygen transport and as a cofactor in enzyme-mediated oxidation and reduction. Most animal-sourced dietary ingredients contain comparatively high iron content. In zebrafish, high dietary iron can decrease the uptake of waterborne cadmium and thereby mitigate waterborne cadmium toxicity. This ability is apparently due to a divalent metal transporter shared by both metals (Cooper, Handy, & Bury, 2006). Excessive dietary iron can lead to lipid peroxidation and adversely affects vitamin C availability. If not included in a diet, iodine can be absorbed through the gills. Iodine is required for thyroid hormone production, and consequentially a deficiency can influence growth, metabolic rate, reproduction, and development. When zebrafish were provided a diet of iodine enriched *Artemia*, survival was observed to exceed that achieved when a diet of nonenriched *Artemia* was proffered (Hawkyard, Sæle, Nordgreen, Langdon, & Hamre, 2011). Manganese, zinc, and selenium are needed for SOD activity and as cofactors in numerous enzymatic reactions and are available through animal-sourced ingredients (especially fishmeal for selenium). Uptake through the gills is not a major source of manganese, zinc, or selenium uptake and therefore, trace amounts (μg/kg) are required for normal physiological function. Zinc deficient zebrafish produce progeny that exhibit increased mortality and altered expression of mineral homeostasis genes (Beaver et al., 2017). When zebrafish are proffered a diet supplemented with sodium selenite, increased whole-body selenium, and altered expression of selenocysteine containing proteins were observed (Benner, Settles, Murdoch, Hardy, & Robison, 2013).

The exchange of sodium and chloride across the gill surface is sufficient to meet the requirements of these minerals. Fish require a dietary source of potassium, and a deficiency in potassium will lead to anorexia and convulsions. High dietary levels of sodium and chloride can help animals adapt to changes in water salinity (Al-Amoudi, 1987).

Vitamins

As an important and diverse nutrient class, vitamins are most commonly classified by solubility, lipid or water-soluble, and function. Water-soluble vitamins are rapidly excreted, thereby being quickly prone to the onset of a deficiency. In contrast, lipid-soluble vitamins are stored, such that any dietary deficiency would not be observed for a much longer period of time. Vitamin C and B complex vitamins are water-soluble. Zebrafish, like other species of fish, require a dietary source of Vitamin C (ascorbic acid or dehydroascorbic acid) due to the lack of an enzyme necessary to synthesize it from glucose. This requirement is the foundation for the use of zebrafish as a model for vitamin C deficiency, a benefit that the rat or the mouse cannot confer. Recently zebrafish were used to improve the understanding of the role vitamin C plays in cellular energy in addition to its characterized roles as an antioxidant and part of procollagen maturation (Kirkwood et al., 2012). Due to the comparatively high solubility of vitamin C in water, fish and crustacean feeds require an insoluble form, or alternatively, a vitamin C enriched live diet (Merchie, Lavens, & Sorgeloos, 1997). The vitamin B complex of vitamins consists of eight individual compounds that function as coenzymes in metabolism and oxidation-reduction reactions. Requirements for B vitamins increase with standard growth rate and metabolic rate. Dietary biotin (vitamin B7) provided to zebrafish in a formulated diet, increased survival, weight gain, protein conversion ratio, and specific growth rate when compared to the responses to a biotin free diet (Yossa et al., 2011, 2015). These effects are reduced according to a dose-dependent relationship when dietary avidin, a potent biotin binder, is supplied in the diet. Biotin is suggested to act also as a gene

regulator, known to have altered liver carboxylase expression, and positively affects reproduction in zebrafish. Zebrafish provided dietary cyanocobalamin (vitamin B12) at 5 ug per kg of feed did not show clinical signs of deficiency or compromised growth and did not maintain whole-body stores of vitamin B12 (Hansen, Olsvik, & Hemre, 2013).

Fat-soluble vitamins include vitamins A, D, E, and K. Fat-soluble vitamins are stored in the body so any symptoms of a deficiency will be protracted if requirements increase (due to life stage, stress, or reproductive stage) or reduced amounts in feeds are proffered. Vitamin A is an inclusive name for retinal, retinol, and retinoic acid, supplied as a dietary ingredient as either one of these forms of vitamin A or as provitamin A carotenoids. In fish diets, vitamin A is often sourced from cod liver oils or beta-carotene. In zebrafish, vitamin A has been shown to be required for proper development of vision, and a dietary deficiency causes reductions of both eye size and response to light (Le, Dowling, & Cameron, 2012). Vitamin A also serves a role in fin regeneration in zebrafish and as a hormone related to metabolic regulation and cell differentiation (White, Boffa, Jones, & Petkovich, 1994). Early hindbrain formation in zebrafish is also compromised if progeny come from parents fed a vitamin A deficient diet (Cao, Jia, & Zhao, 2012). Receptors for retinoic acid are expressed in zebrafish testes and ovaries. Inhibition of retinoic acids synthesis or a vitamin A deficient diet reduces egg production (Alsop, Matsumoto, Brown, & Van Der Kraak, 2008).

Vitamin D is important for hormonal control of calcium homeostasis and consequentially bone growth and maintenance (Lin, Su, Tseng, Ding, & Hwang, 2012). Unlike terrestrial animals that utilize UV light to synthesize vitamin D from cholesterol, de novo synthesis does not occur in fish, and all required vitamin D must be derived from the diet. Fish consuming a diet consisting of a group of live plankton species obtain an adequate level of vitamin D (Rao & Raghuramulu, 1996). Independent of calcium regulation, vitamin D regulates the production of both hematopoietic stem cells and progenitor cells, as well as fatty acid oxidation in zebrafish (Cortes et al., 2016; Peng et al., 2017). Vitamin E acts as an important reducing agent through the donation of electrons to prevent the chain reaction of peroxidation caused by the presence of radical oxygen species (ROS). Toxicity of polychlorinated biphenyls (PCB) to zebrafish, caused by oxidative damage to cells, is mitigated by the presence of vitamin E (Na et al., 2009). Vitamin E also protects vitamin A, carotenoids, PUFAs, and LC-PUFAs from oxidations. One study found that the use of α-tocopherol as a feed additive in zebrafish diets increased body weight, standard growth rate, and fecundity in a dose-dependent manner, indicating that its supplementation would benefit zebrafish health (Mehrad, Jafaryan, & Taati, 2012). Results from another study suggest that ascorbic acid and beta carotene have a role in protecting against damage caused by oxidative stress in larval zebrafish (Wu et al., 2015). However, an optimal dietary level of inclusion for zebrafish has yet to be determined for any of these additives, and their actual role as an antioxidant still requires evaluation.

Vitamin E impacts vitamin C status and diets lacking vitamin E can result in skeletal muscle degeneration leading to adverse effects on mobility via increased vitamin C depletion rates (Lebold et al., 2013). Excess dietary vitamin E is principally stored in lipid bilayers, and this abundant storage area is believed to be the reason that upper physiological limits for most species are extremely high. Improved survival and fecundity and a higher specific growth rate and body weight of zebrafish were observed when provided a 1000 mg per kg vitamin E supplement; however, there was no indication of improved egg quality (Mehrad et al., 2012). Parental vitamin E diet content influences whole-body transcriptome of progeny during the embryonic stage, with numerous transcripts affected being involved in metabolic regulation (Miller et al., 2014). Vitamin K is important for blood coagulation and bone mineralization, and vitamin K in zebrafish is required to prevent unhealthy mineralization of tissues (Mackay, Apschner, & Schulte-Merker, 2015).

Some plants and microbes can naturally synthesize two different forms of vitamin K. A popular synthetic form called menadione is used for animal feeds. Menadione can be subject to degradation and loss of activity in feed if not properly stored, resulting in feed becoming deficient in vitamin K content (Krossøy, Waagbø, & Ørnsrud, 2011). Zebrafish have shown to be a useful model to investigate vitamin K function as related to the development and drug-vitamin interactions (Hanumanthaiah, Thankavel, Day, Gregory, & Jagadeeswaran, 2001; Granadeiro, 2015).

Nonnutritive Components

Energy

Dietary Energy Utilization

Fish require energy for major cellular functions involved in the maintenance of life processes, growth, reproduction, swimming, respiration, and osmoregulation. Energy is not a nutrient; rather, it is produced from the digestion of absorbed nutrients that have been oxidized and metabolized, each having a specific role and metabolic outcome (Cho & Bureau, 1995; van Milgen, 2002). Multiple nutrients or metabolic intermediates derived from nutrients may also be used

simultaneously as part of the same process, and interactions among nutrients often occur (NRC, 2011). A diet that contains sufficient amounts of energy and the proper balance of nutrients is essential for the support of optimal growth and health in the zebrafish.

Nutritional bioenergetics examines the flow of dietary energy relative to supply, loss, expenditure, and net gain. This approach requires an examination of the physiological processes by which energy is converted in all organisms (Cho & Bureau, 1995). These physiological processes may vary among and within species due to influences that are both intrinsic (sex, species, life stage, physiological state) and extrinsic (temperature, salinity, stress) (Rosenfeld, Van Leeuwen, Richards, & Allen, 2015). During the last few decades, studies have utilized the principles of bioenergetics to measure energy utilization and expenditure in multiple fish species (NRC, 2011). In 1981, the National Research Council (NRC) developed a systematic terminology to describe the partitioning of energy intake, and this practice has been adopted by numerous researchers in fish nutrition (Fig. 33.1) (NRC, 2011). The NRC also expresses dietary energy in terms of the calorie, which is the amount of energy required to raise the temperature of 1 g of water from 14.5 to 15.5°C. A kilocalorie (kcal) is equivalent to 1000 calories and serves as the unit of reference in nutritional energetics (NRC, 2011). Studies devoted to the examination of energy requirements and nutritional bioenergetics in zebrafish have been sparse; however, the following overview of energy partitioning can be applied to all fish species.

Intake of energy, i.e., gross energy (GE) is defined as the gross amount of energy contained within the gross amount of food consumed (NRC, 2011). The GE content of a food item is determined by its chemical composition and is typically measured by combustion in a bomb calorimeter. It should be noted that feed specific nutrients contained within a wet or dry diet are not necessarily available biologically to the consumer (Watts et al., 2012). Nondigestible components egested as feces are categorized as fecal energy (FE) and therefore are unavailable. The remaining energy not lost as fecal energy and available is defined as digestible energy (DE).

DE can be defined as the component of feed that is available to the animal for energy. Some of the DE is lost to the excretion of ammonia and other waste products through the gills and urine. This metabolic waste originates from the catabolism of proteins, lipids, and carbohydrates, as well as excesses of other dietary nutrients (NRC, 2011; Lall & Dumas, 2015). After DE losses from excretion, the remaining amount of energy available from DE is defined as metabolizable energy (ME). Theoretically, ME is recognized as the appropriate amount of energy available in evaluating the potential energy actually utilized from the consumption of formulated diets (Cho & Bureau, 1995). However, the direct determination of ME requires quantitative measurements of both gill and urinary losses, and therefore,

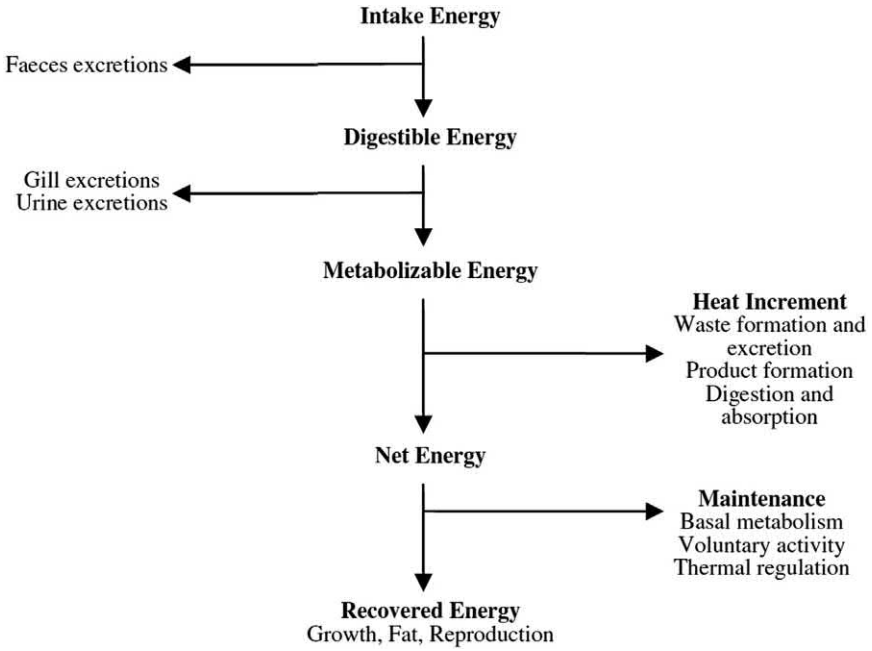

FIGURE 33.1 Factorial framework of energy partitioning in typical bioenergetic models intended to evaluate feed requirements (Dumas et al., 2010).

ME values for aquaculture diets are extremely difficult to determine. The ME values of carbohydrates, lipids, and proteins have been estimated to be 4 kcal/g, 9 kcal/g, and 4 kcal/g, respectively (Sanchez-Pena et al., 2017). Thus, overall DE is considered to be an appropriate measure of energy availability derived from feed ingredients (Lall & Dumas, 2015).

A portion of ME available to the animal becomes unavailable due to heat loss, or metabolic inefficiency. A major portion of this heat loss is from a percentage of energy that is needed to maintain basal metabolism, or metabolic rate (respiration and circulation). Basal metabolism is influenced by factors, such as body weight and water temperature in fish and may ultimately affect dietary energy requirements. Ingestion of feed by some species of fish that have been fasting also increases heat loss and is referred to as specific dynamic action (SDA). SDA is not needed in fish fed throughout the day or via demand feeders. While SDA does not have to occur in the animals after fasting, laboratory measurement of SDA does require a fasting period to determine basal metabolism. Contributing factors to SDA include digestion and absorption processes, formation and excretion of metabolic wastes, and transformation and interconversion of substrates and their retention in tissues (NRC, 2011). Physical activity is an additional contributor to heat loss (NRC, 2011).

The remaining ME after the sum of energy from heat losses has been removed is referred to as recovered energy (RE), and is retained within the body for deposition of new tissue (Cho & Bureau, 1995). In juvenile animals, RE is primarily devoted to somatic growth, and any energy retained is stored as both protein and lipid (NRC, 2011). As the animal reaches adulthood, a larger proportion of the recovered energy is allocated toward gonadal development and reproduction, and any excess energy is more likely to be stored as lipid (Lall & Dumas, 2015). In adult animals, the partitioning of lipid and protein stored becomes dependent on the availability of amino acids from sources of dietary protein, and the feed's proportional content of protein relative to energy (Cho & Bureau, 1995).

Energy Requirements

The term "energy requirement" is the amount of energy expended by fish to maintain vital body functions combined with the amount of energy spent for growth and reproduction (Lall & Dumas, 2015). Evidence found in previously published literature suggests that fish seem to adjust feed intake in response to diets with varying levels of DE from macronutrients, perhaps to ensure that adequate levels of specific nutrients are consumed to meet targets for growth and body composition (Lupatsch, Deshev, & Magen, 2010; NRC, 2011; Yamamoto et al., 2000). Fernandes et al. (2016) stated that feed intake increased linearly as the levels of protein decreased below the estimated requirement, suggesting that zebrafish regulate food intake to meet protein needs. Another study reported that when zebrafish were fed a high lipid diet, feed intakes were higher in adult females as compared to males (Meguro et al., 2015). Nonetheless, studies that have measured feed intake of zebrafish remain extremely limited due to the procedural challenge founded in their small size (Watts et al., 2012). The methods employed by Meguro et al. (2015) and Fernandes et al. (2016) to measure feed intake involved either feeding fish until apparent satiation or feeding each tank a measured amount and then scoring the amount remaining. While the results obtained from these studies were valuable, the techniques used for measuring feed intake were time-consuming and highly subjective, indicating that the development of time-saving, but still accurate methods of measurement need to be introduced in future studies.

Digestion and absorption of nutrients may vary among fish species due to comparative differences in the morphologies of the digestive tracts, enzymatic digestion, gut pH, and other factors (Lall & Dumas, 2015). These comparative differences in the digestive tract can affect multiple factors, including the rate and extent of digestion, the chemical nature of the absorbed product that is subject to metabolism, and deposition within body stores of protein, fat, and glycogen. Ultimately, these factors influence the efficiency of dietary energy utilization. To our knowledge, no studies designed to assess DE availability of feed ingredients specific to zebrafish have been conducted. Direct determination (gravimetric collection of all feces for analysis) is an effective determinant of apparent dry matter digestibility for many fish species; however, for zebrafish, collection of small feces would be a time-consuming and laborious procedure confounded by the loss of water-soluble nutrients (Watts et al., 2012). Development and testing of new technologies and procedures to directly measure digestibility of feed ingredients in zebrafish are needed (Watts et al., 2012).

We hypothesize that an optimal balance of energy and protein components of the diet is also important because an excess or deficiency of nonprotein energy (lipid and carbohydrate) may reduce growth rates and impair health status. A diet deficient in nonprotein energy sources will lead to protein being used as an energy source for energetic purposes (basal metabolism and voluntary metabolism) rather than for growth, resulting in reduced growth rates (Cho & Bureau, 1995; Lall & Dumas, 2015). Gonzales and Law (2013) reported that the body weights of adult zebrafish fed *Artemia* exclusively for a 5-week period were significantly reduced compared to those fed formulated diets. They suggest that the adverse response to *Artemia* was

due to an imbalance in the protein to energy ratio. Compared to the formulated diets, the *Artemia*-only diet contained lower levels of nonprotein energy, which may have led to the utilization of protein to meet energy needs, rather than sparing it for growth and gonadal development (Gonzales & Law, 2013). Fernandes et al. (2016) found that a crude protein to energy ratio of 22.5 g/MJ promoted maximum weight gain and protein retention in juvenile zebrafish; this ratio may not be optimal for other life stages. However, an excess of dietary energy can lead to reduced levels of food consumption such that an adequate amount of protein is not provided to meet targets for protein synthesis and growth (Cho & Bureau, 1995; Lall & Dumas, 2015). An excess of energy may also lead to excessive lipid deposition, which would be an undesirable consequence in studies utilizing zebrafish as a model to gain knowledge about obesity and metabolic health (NRC, 2011). In these areas of study, diet is a variable that must be controlled to yield reliable results that can be exchanged among laboratories. To effectively utilize the zebrafish as a model for diet-induced obesity, a standardized reference diet must be developed containing an ideal amount of dietary energy content.

Nutrient allocation and energy expenditure in fish most likely vary among life stages (Dumas, France, & Bureau, 2010; NRC, 2011; Rosenfeld et al., 2015). Young fish require more energy per unit of body weight than the adult stage (Lall & Dumas, 2015). Small zebrafish from 20 to 50 days postfertilization (dpf) are undergoing a period of rapid growth, and most recovered energy from the diet is allocated for this purpose (Gomez-Requeni et al., 2010). After 50 dpf, more dietary energy was allocated toward maturation and gonad development, and growth rates correspondingly began to decrease (Gomez-Requeni et al., 2010). These results collectively indicate that juvenile zebrafish may require a higher feed intake and/or diet with increased energy content. Thus, the ratio of digestible protein to digestible energy may need to be modified to ensure that optimal growth and health is achieved for this life stage. For determining whether this approach will be effective, further definition of energy requirements for each life stage in zebrafish is needed.

Feed Additives

In addition to essential nutrients, feeds may contain organic and/or inorganic materials that can have beneficial, detrimental, or negligible effects on animal health. Feed or diet additives are defined as nonnutritive ingredients included in formulations to either alter chemical or physical properties of the diet or to impact fish performance and health (Barrows, 2000). Their availability and utilization vary according to diet. Descriptions of some feed additives utilized in zebrafish diets follow.

Antioxidants

Lipid sources included in zebrafish diets often contain large amounts of PUFAs, which are highly vulnerable to oxidation. The presence of oxidized lipid products in the diet may directly affect fish and/or exacerbate deficiencies of antioxidant vitamins, resulting in pathological conditions that include liver degeneration, spleen abnormalities, and anemia (NRC, 2011). Tocopherol (Vitamin E), ascorbic acid/ascorbyl palmitate (Vitamin C), and carotenoids, three additives commonly included in aquaculture feeds, are known to effectively control the production of reactive oxygen species associated with lipid oxidation (Di Mascio, Murphy, & Sies, 1991; Wu et al., 2015). Their specific antioxidant effects are covered in more detail in the Vitamins and Minerals section.

Immunostimulants

Immunostimulants are naturally occurring compounds that modulate the immune system by increasing the host's resistance against diseases that are principally caused by pathogens (Bricknell & Dalmo, 2005). These immune-potentiating substances are generally included as dietary supplements during stress-induced aquaculture conditions or during specific life stages to help minimize susceptibility to pathogens and maintain good health (Kiron, 2012). β-glucans, which are extracted from microbial products, such as yeast and barley, have been extensively recognized as immunostimulants in various aquatic animals (NRC, 2011). Both individual and trans-generational effects on zebrafish immunity have been demonstrated. Oyarbide, Rainieri, and Pardo (2012) found that provision of β-glucans increases innate immunity of larval zebrafish, while a recent study by Jiang, Wang, Li, Liu, and Zhang (2016) reported that the embryos of female zebrafish fed dietary β-glucans were more resistant than control embryos to bacterial challenges (Jiang et al., 2016; Oyarbide et al., 2012). Another immunostimulant, lactoferrin, is an iron-containing glycoprotein derived from milk or other exocrine secretions of mammals (NRC, 2011). Lactoferrin, when included in a soybean-based diet fed to larval zebrafish, acted as an intestinal anti-inflammatory agent, with a level of 1.5 g/kg found to be most effective (Ulloa et al., 2016).

Antimicrobial Agents

As an additional defense against pathogenic infection, antimicrobials are another additive often included in aquaculture feeds to control disease outbreaks. Antimicrobial peptides (AMPs) are evolutionarily conserved, natural defensive weapons secreted by

prokaryotes, plants, and invertebrates. They play important roles in conferring innate immunity and in serving as antimicrobial agents (Huang & Chen, 2013). The AMP epinecidin-1 has been demonstrated to exhibit antibacterial effects against the pathogen *Vibrio vulnificus* in zebrafish, while the phosvitin-derived peptide Pt5 significantly increased survivorship of adult zebrafish infected with *Aeromonas hydrophila* (Ding, Liu, Bu, Li, & Zhang, 2012; Huang & Chen, 2013). While these results demonstrate promise for the use of AMPs as antimicrobial agents in zebrafish feeds, additional studies are required to establish optimal dietary levels.

Preventative health care through nutrition that uses feed additives is a practice that is expanding because it continues to be recognized as essential in aquaculture, particularly for intensive culture systems. While the information provided from these studies provides us with some guidelines, the use of additives and corresponding levels in zebrafish diets needs to be investigated to gain knowledge about effects specific to zebrafish.

Natural and Anthropogenic Contaminants

Many organisms have evolved several chemical defenses to predation ranging from noxious taste and smell to hormonal mimics (Levin, 1976). Improper selection or preparation of feeds and feed ingredients can result in the exposure of zebrafish to these compounds. Trypsin inhibitors occur in soybean and greatly impact growth and protein utilization in channel catfish and rainbow trout (Rumsey, Siwicki, Anderson, & Bowser, 1994; Wilson & Poe, 1985). If soybean is not heated beyond a minimum level, then trypsin inhibitors will remain active. Soy-derived ingredients can also contain isoflavones, such as genistein, a phytoestrogen that causes sex reversal effects in Nile tilapia and interferes with zebrafish development (El-Sayed, Abdel-Aziz, & Abdel-Ghani, 2012; Sassi-Messai et al., 2009). Phytoestrogen isoflavones remain present in soy-based feed ingredients in detectable amounts even in more purified soy protein isolates (Bhagwat, Haytowitz, & Holden, 2008). Other components of soybean meal (saponins and soy protein) added to zebrafish diets induced expression of genes related to innate immunity and inflammation in the gut (Fuentes-Appelgren et al., 2014). Inflammatory and immunity gene expression, in addition to nutritional programming, was also affected when zebrafish were proffered diets with soybean meal and soy protein concentrate (Perera & Yúfera, 2016). Zebrafish provided with a soybean meal diet at 5–7 dpf were less affected by reexposure to soybean meal diet at 31–50 dpf. Fish fed soy protein concentrate had a contrasting result, where later reexposure to a soy protein diet elicited a larger effect. Soybean meal, cottonseed, and sunflower oil contain phytic acid that binds (chelates) phosphorous, manganese, and zinc, limiting the availability of these important micronutrients. Dietary phytic acid was found to reduce growth and feed efficiency in rainbow trout (Spinelli, Houle, & Wekell, 1983). Low levels of gossypol, a compound found in cottonseed, was also reported to reduce the growth of rainbow trout. At higher levels, gossypol completely suppressed feed intake and/or caused high mortality (Herman, 1970; Roehm, Lee, & Sinnhuber, 1967).

Toxins present in feeds may also be the products of a chemical defense or metabolic byproduct originating from microbes that attempt to derive nutrients from feeds. Aflatoxins are produced by fungal molds (naturally occurring Aspergillus flavus and Aspergillus parasiticus) that contaminate and digest feed ingredients under favorable conditions of temperatures and humidity (Russo & Yanong, 2010). Aflatoxin effects are dose-dependent and clinical external signs of toxicity include blindness, cataracts, skin lesions, fin and tail rot, reduced appetite, yellowing of the body, and increased fingerling mortality in Nile tilapia (Ashley, 1970; Cagauan, Tayaban, Somga, & Bartolome, 2004). Internally, these toxins adversely affect the liver resulting in enlargement, yellowing, deterioration, and tumorigenesis. These consequences of toxicity are also observed in rainbow trout and walleye, but not in channel catfish, which are believed to be highly resistant to aflatoxin (Halver, 1969; Hussain, Gabal, Wilson, & Summerfelt, 1993; Manning, Li, & Robinson, 2005). Deoxynivalenol is another toxin produced by Fusarium fungus. Zebrafish provided a deoxynivalenol contaminated diet exhibit increased transcription of genes related to detoxification and growth regulation in the liver (Sanden, Jørgensen, Hemre, Ørnsrud, & Sissener, 2012). Bacterial microbiota also can be dangerous contaminates. Nesse et al. (2003) found that a distinct serotype of *Salmonella* persisted in a Norwegian fish feed manufacturing facility for more than 3 years due to poor decontamination procedures. Analysis of *Salmonella serovars* in delivered fishmeal suggests that this contamination is a part of the factory environment or machinery and not from deliveries of contaminated ingredients (Nesse et al., 2003).

Anthropogenic contamination of feeds can occur via multiple routes of the transfer. A human source of contamination can enter into an environment that is occupied by animal or plant ingredients and proliferate. Alternatively, contaminates are often unintentionally introduced during the feed production process. Various heavy metals can accumulate via diet at a much higher rate than by uptake via the gills (Dallinger & Kautzky, 1985). Mercury levels determined in commercially available pelleted fish feeds were higher than in most commercial foodstuffs, and the concentration found in

feed proffered to Sacramento blackfish correlated directly with what was measured in blood samples (Choi & Cech, 1998). High concentrations of polychlorinated biphenyls and arsenic have also been detected in fish feeds and can bioaccumulate in fish tissue (Berntssen, Lundebye, & Torstensen, 2005; Sloth, Julshamn, & Lundebye, 2005). Melamine is also a feed contaminate that recently caused the deaths of domestically owned American dogs and cats in 2004 and 2007, due to the formation of kidney stones and renal failure (Brown et al., 2007). In these cases, melamine was not included as feed ingredient to improve nutritional or binding characteristics, but rather to deceptively create the perception of higher levels of measured protein, thereby allowing an increase in sale price while maintaining the same production cost. This type of activity emphasizes the need for policies designed to ensure international food security relative to the safety of laboratory animals and commercial animal agriculture production systems, both terrestrial and aquatic. This is of particular importance in laboratory animals used in the study of human disease, which includes zebrafish.

Live Diets

Live diets have been shown to be extremely important for early husbandry of *D. rerio* due to the small size of zebrafish when first exogenous food is initially required (Westerfield, 1995). The current research community consensus is to raise zebrafish larvae on live animal diets, although alternatives would be highly desirable.

Research efforts have been devoted to the formulation of defined nonlive diets and proffering them to zebrafish larvae. One study found that larval zebrafish fed Gemma Micro, a micro-particulate dry feed, demonstrated reasonable growth (23 mm at 9 weeks of age) and survivorship (89%) (Kaushik, Georga, & Koumoundouros, 2011). However, when compared to live diets, reduced survival and early growth (up to 21 days) were observed when zebrafish were fed formulated diets, even under an automated continuous feeding regime (Carvalho, Araújo, & Santos, 2006; Goolish, Okutake, & Lesure, 1999). The existence of successful, whole-lifespan formulated diets would introduce greater reproducibility and reduction in the probability of system contamination, both benefits being very valuable to the scientific zebrafish community. However, typical successful zebrafish culture is not at the point where live diets can be excluded in early life stages.

The first live diet to be widely adopted for feeding by the zebrafish research community was paramecium. Although many zebrafish culture laboratories still utilize paramecium for early larva feeding, support of rapid growth and development in early life falls short of what is achieved now with other species, including rotifers and *Artemia* nauplii (Biga & Goetz, 2006; Carvalho et al., 2006). In addition, maintenance of paramecium populations is laborious, and paramecium can act as a vector promoting mycobacterial infections of zebrafish (Peterson et al., 2013). Also, at this point, paramecium has not been enriched with nutritional additives, further limiting their usefulness.

During early development, larval fish have a high energy demand due to rapid growth and development. To provide consistent access to live feeds, the zebrafish research community has adopted polyculture of zebrafish larva with rotifer species. This management practice offers several advantages and is possible due to the high tolerance of zebrafish larva to salt and ammonia (higher than previously considered) (Best, Adatto, Cockington, James, & Lawrence, 2010). Conditions of tank salinity and rotifer feeding rate promote rotifer reproduction and the survival of both rotifers and zebrafish larva in the same tank. Several commercially available products simplify rotifer husbandry and ensure supplementation of essential fatty acids in higher quantities needed by zebrafish larva for early development. Rotifer husbandry practices specific for zebrafish facilities are available and can be adapted to meet colony size (Lawrence, Sanders, & Henry, 2012).

Artemia nauplii are also an easily utilized live feed used in laboratories that culture zebrafish. *Artemia* are typically not used in early feedings due to their larger size and faster speed of movement compared to rotifers, both of which increase the difficulty of effective capture and consumption by young larva (Lawrence, 2007; Lawrence, James, & Mobley, 2015). Unlike rotifers and paramecium, *Artemia* are commercially available in the form of dry cysts that can be hatched and available for feeding in less than 24 h. Alternatively, zebrafish can be provided unhatched de-encapsulated *Artemia*, which reduces cost and preparation time, with no significant reduction to growth, survival, or reproduction (Tye et al., 2015). *Artemia* can also be fed diets that enrich them with essential fatty acids and other nutrients, not only to promote early development of zebrafish larvae but also to aid in the understanding of the role of supplemented dietary components in early development (Furuita, Takeuchi, Toyota, & Watanabe, 1996; Hawkyard et al., 2011). Results from previous studies indicate that zebrafish fed live diets exclusively from first feeding until the adult stage demonstrated increased growth and higher fecundity rates compared to those fed formulated diets (Karga & Mandal, 2017; Markovich, Rizzuto, & Brown, 2007).

TABLE 33.2 Classification of feeds (Watts et al., 2012).

Type	Application	Content	Relative cost
Practical	Commercial production feeds, produced in mass quantities for aquaculture operations, cannot be used to evaluate nutrient requirements	Practical ingredients; not chemically defined	Low
Semi-purified	Experimental feeds, used to evaluate macro- and some micronutrient requirements	Both purified and practical ingredients, not completely defined	Medium
Purified	Experimental feeds, used to evaluate macro- and micronutrient requirements	Purified, chemically defined ingredients, usually with defined lot numbers and specifications	High

Formulated Diets: Classification and Use

Feed formulation is the process of combining quantitative amounts of feed ingredients to meet the nutritional needs of fish to produce a mixture that can be pelleted and stored. The feed should also be relatively water-stable; must support acceptable growth, health, and wellness; and be economical to use (NRC, 2011). Formulation of feeds implies two prerequisites: (1) that the essential nutrients along with their requirements for the targeted animals are known (at least to a certain extent), and (2) that the candidate sources of these nutrients are outlined and suitable for feeding animals. By building on these two principles, feed formulas can be further refined by considering the effect of processing on the quality of raw materials, functionality of ingredients, nutrient requirements at different life stages, and the dynamics of production systems (Lall & Dumas, 2015).

Quality formulated diets used in maintenance/breeding and experimental zebrafish colonies will be essential for maintaining good health and obtaining consistent experimental results. As previously stated, the nutritional requirements of zebrafish have still not been defined. Empirical studies are needed using diets composed of chemically defined ingredients to gain a full understanding of these requirements. For development of formulated diets for zebrafish, Watts et al. (2012) recommend a step-wise evaluation of daily dietary requirements that will include an examination of nutritional values derived from specific feed ingredients. The step-wise evaluation will require a continuous reevaluation of specific nutrients as requirements for other nutrients are determined. This investigative approach will allow an evaluation of specific effects of each nutrient on growth, reproduction, and other physiological/molecular processes, particularly those relating to disease onset and progression (Watts et al., 2012).

Diets are classified based on ingredient and nutrient composition (Table 33.2) (Watts et al., 2012). Development and selection of formulated diets for zebrafish cultures should depend on desired production or experimental objectives, rather than cost. Some diets (purified research diets) are exclusively used to determine specific nutrient requirements under defined experimental conditions, while others (practical diets) are designed for application within large-scale commercial production (Watts et al., 2012). Additional characteristics of a formulated diet, such as feed particle size and density, and palatability must also be carefully considered (NRC, 2011; Lall & Dumas, 2015).

Practical diets consist of natural ingredients that have been subjected to limited refinement (e.g., raw forms of starch or unprocessed protein sources), dried, and ground. Practical ingredients are used to provide essential levels/proportions of macronutrients in feeds, as well as trace amounts of some micronutrients. While they are the most economical to manufacture, their limited refinement and often inconsistent composition precludes the ability to control the concentrations of specific nutrients and the corresponding nutritional effect. Consequently, practical ingredients are highly variable in composition based on source, vendor, and season of the collection (Watts et al., 2012). Although *Artemia*-fed juvenile zebrafish had the best survival rates and growth performance, those fed practical diets demonstrated comparable survival and growth performance, indicating that practical diets may be useful for maintenance of zebrafish populations (Carvalho et al., 2006). However, due to their inconsistent composition, they are inappropriate for use in studies evaluating the effect of a specific nutrient.

In contrast, purified diets consist of chemically defined ingredients, each identified by a specific ingredient and its lot number. Single-ingredient nutrient sources (e.g., casein as a sole protein source) are often used in purified diets to establish conditions, whereby

the accurate determination of the nutritional requirement for a specific nutrient or nutrient source can be achieved (Watts et al., 2012). Due to the high level of refinement required for single-ingredient nutrient sources, their chemical composition is typically comparable among similar products offered by different vendors (Watts et al., 2012). Compared to practical ingredients, the cost for purified ingredients used in the manufacture of a purified diet is considerably higher. However, their use is essential in experiments evaluating specific nutrient requirements in zebrafish. The semi-purified diets contain a mixture of both practical and purified ingredients, and can be used to evaluate macro- and some micronutrient requirements.

Currently, no standardized formulated diet is utilized among zebrafish research laboratories. Commercial diets are a popular choice for zebrafish culture facilities, particularly those with a primary focus on large-scale production. These diets are used because they provide reasonable growth and fecundity desired for acceptable zebrafish culture. However, as many of these commercially available diets do not report ingredient composition and content, the composition of specific nutrients (or antinutritional factors) is unknown (Watts et al., 2012). For some applications, the use of undefined diets may be of limited consequence. However, when zebrafish are used as a medical model to investigate human disease, knowledge of both quantity and quality of specific nutrients is essential (Siccardi et al., 2009). Zebrafish researchers must acknowledge the potential variability and unintended outcomes that characterize the use of undefined diets. Siccardi et al. (2009) reported that growth demographics varied significantly among zebrafish fed different formulated and commercial diets. Two additional studies found that reproductive performance and survivorship also varied significantly in zebrafish fed different commercial diets (Gonzales, 2012; Farias & Certal, 2015). Another study found that zebrafish provided flake diets as their primary source of nutrition produced significantly fewer eggs than those fed either a control diet (flake diet supplemented with *Artemia*), exclusively *Artemia*, or a commercially available trout diet (Markovich et al., 2007). Development of a defined diet consisting of chemically defined ingredients for zebrafish will permit comparisons among laboratories, strains, and experiments. In addition, specific outcomes can be evaluated in terms of not only experimental design but also nutrition.

Feed Management

Storage

Proper storage of zebrafish feeds in powder, or small pelleted forms (commercial or lab-formulated) is essential to prevent oxidation, photooxidation, and degradation by bacteria and fungus. Most zebrafish feeds contain saturated and unsaturated lipids (Addis, 1986). Therefore, oxidation is a fundamental concern because some of the amount of energy per gram of feed proffered would no longer be available, and harmful oxidized products may be formed. For controlling the oxidation of nutrients in feed ingredients, long-term storage under conditions of darkness and low levels of oxygen is strongly recommended. Removal of oxygen can be accomplished by vacuum sealing or addition of nitrogen gas prior to sealing. Minimally, all packaging should be airtight to prevent excess oxygen from interacting with feed. If feeds are stored in a lighted area, an opaque packaging or a secondary packaging surrounding the initial airtight/oxygen-depleted packaging should be used to minimize photoreactions with the feed. The packed feeds should also be stored in an environment held at less than 4°C to limit any microbial growth. If feeds are stored as whole extruded pellets, or are subject to being ground into a powder prior to feeding, grinding should be limited to only a small portion needed for either daily or weekly use.

Ration

Daily feeds are proffered to *D. rerio* using several methods. Commonly used practices are ad libitum feeding, the "x minutes" rule, or a ration based on body weight. For ad libitum feeding, the amount provided to each unit holding fish should not limit the ability to consume to satiety; thus, excess feed is always present in each holding unit. There are pros and cons associated with this type of feeding method. *Ad libitum* feeding can adversely affect water quality by increasing both ammonia waste and breakdown by oxygen-consuming microorganisms. This method also increases the cost of feeding because the excess, uneaten feed is wasted. *Ad libitum* feeding of some diets will result in a comparatively high content of body lipid, a condition that has poorly defined impacts on animal health and may compromise study outcomes (Landgraf et al., 2017). The most notable advantage of this type of feeding is the assurance that fish always consume enough food to reach satiety, and only then will consumption cease. The "x-mins rule," in which, there is a provision of an amount of visible feed that can be completely consumed in a 5–15 min period, is the easiest but most subjective feeding method. The amount proffered to fish in each holding unit is an amount that is typically consumed in x-minutes of time (Lawrence, Best et al., 2012). The subjective nature of this method introduces a level of uncertainty and consequently would be a poor choice when conducting any nutritional study or an investigation where small differences in nutritional status impact measured outcomes. The last method, of providing a ration based on body weight,

is the most labor-intensive. In this method, the body weight of experimental fish needs to be monitored regularly, and their daily ration is then a set percentage of the total body weight of the fish occupying that culture unit or a set percentage of the average body weight of fish that occupy multiple units that are replicates of a specific treatment group. The substantial amount of labor required for using this method has shortcomings because it can limit the number of treatments and/or replicate tanks for laboratories that do not have a sufficiently large labor force. Another source of confusion is that the ration size (expressed as a percent of animal body weight) for weight maintenance or growth varies according to the composition of the feed proffered because of differences in feed composition and energy content per gram of feed. Moreover, these latter differences become even more significant because the required energy per gram of body weight per day for any life stage of zebrafish has yet to be established. The principal benefit of the ration feeding method is the ability to describe the exact amount of each feed component proffered to each treatment over the whole study period (assuming a nonproprietary feed is used).

Feed Frequency and Timing

Timing and frequency of when rations are proffered to fish can play a role in outcomes of nutritional experiments, and the effects of these variables have not been fully investigated. When zebrafish were proffered 5% body weight/day with feeding frequencies ranging from every other day to 5 times daily, differences in growth and successful reproductive events were observed (Lawrence, Best et al., 2012, Lawrence, Sanders et al.,2012). A higher feeding frequency (3 or 5 times a day) was best for growth, whereas less frequent feedings (every other day or once a day) were best for reproductive success. The recommended protocol for feeding frequency may vary according to the populations of interest, i.e., populations used for broodstock versus those in growth trials or other studies. Another important consideration for feeding frequency is its impact on the physical properties of the feed. If the entire ration is fed all at once, then feed not immediately consumed by the fish will be prone to nutrient leaching of water-soluble components, lipid oxidation, and microbial degradation; this condition can be circumvented if several small rations are proffered during selected times throughout the day. Independent of feeding frequency, feed timing has been shown to affect the expression of genes related to metabolism. Many metabolic genes manifest a cyclic expression regulated by clock control network genes (Paredes, Lopez-Olmeda, Martinez, & Sanchez-Vazquez, 2015). Providing a single daily ration at a different time of day can shift the peak time of expression of metabolically important genes. This should be an active area of research in future studies.

Diet-microbiome Interactions

The relationship between gut microbiota and nutrient metabolism has emerged as a key feature of host-microbe relationships in the gut (Sekirov, Russell, Antunes, & Finlay, 2010). Microbes can affect food processing and make otherwise indigestible products available to the host (Brugman, 2016). If appropriate microflora are not present in the intestine, specific aspects of cell differentiation may be arrested, and the uptake of protein macromolecules and possibly other nutrients may be adversely affected (Bates et al., 2006).

In zebrafish, intestinal development and microbe colonization of the digestive tract occurs simultaneously during the first few weeks of life, with each developmental life stage having its own signature composition of gut microbiota (Brugman, 2016; Stephens et al., 2016). A study utilizing gnotobiotic zebrafish demonstrated that microbe colonization of the zebrafish gut influences the expression of 212 genes, of which, 59 responses are conserved between zebrafish and mice (Rawls, Mahowald, Ley, & Gordon, 2006). These responses include those associated with epithelial proliferation and nutrient metabolism.

As in mammals, the gut microbiota composition of zebrafish can be significantly affected by acute dietary alterations, and changes in microbiota composition can correspondingly affect the processing and digestion of nutrients (Wong et al., 2015). One study reported apparent microbial community shifts during periods of both constant diet and environmental conditions and in response to dietary and environmental change (Stephens et al., 2016). Microbiota in the zebrafish gut were also found to stimulate fatty acid uptake and lipid droplet formation in the intestinal epithelium and liver, and an increase in epithelial lipid droplets is diet-dependent (Semova et al., 2012). Falcinelli et al. (2017) found that the zebrafish gut microbiome structure is influenced by dietary lipid content. Additionally, the amount of dietary lipid consumed results in distinctive age-specific effects on gut community assembly (Wong et al., 2015). The microbial communities of gluten-fed zebrafish displayed increased abundances of Legionellales, Rhizobiaceae, and *Rhodobacter*, compared to their control-fed counterparts (Koo et al., 2017). Outcomes from these studies reveal that in zebrafish, host—microbe relationships are strongly influenced by diet and in turn, can confer significant effects on outcomes on digestive and metabolic outcomes.

In fish, gut microbiota manipulation can potentially promote the growth of beneficial bacterial communities. This response may translate into improvements in growth, digestion, immunity, and pathogen resistance within the host organism (Llewellyn, Boutin, Hoseinifar, & Derome, 2014; Tellez, Higgins, Donoghue, & Hargis, 2006). In zebrafish, gut microbiota manipulation can be achieved with the inclusion of probiotics in feeds (Llewellyn et al., 2014). Observations derived from two previous studies suggest that administration of probiotics increases development rate and confers a protective role against *Edwardsiella ictaluri* infection in larval zebrafish (Avella et al., 2012; Rendueles et al., 2012). Inclusion of the probiotic *Lactobacillus rhamnosus (L. rhamnosus)* as a feed additive (10^6 CFU/gram) also increased fecundity, modulated the innate immune response, and improved hepatic stress tolerance in female zebrafish (Gioacchini et al., 2010; Gioacchini et al., 2014). Male zebrafish fed another probiotic supplement, Bactocell, significantly overexpressed three fertility markers (*lepa, dmrt1*, and *bdnf*) associated with sperm quality when compared to a control group, suggesting that probiotic supplementation could also affect male reproductive performance (Valcarce, Pardo, Riesco, Cruz, & Robles, 2015).

Additional research suggests that provision of L. *rhamnosus* may also significantly alter lipid metabolism. The addition of *L. rhamnosus* to culture water of juvenile zebrafish altered the gut microbiome, which, in turn, modulated host lipid processing and metabolism (Falcinelli et al., 2015). Another study found that supplementation of *L. rhamnosus* in a high-lipid diet significantly attenuated weight gain, upregulated anorexigenic genes, and decreased expression of genes involved in cholesterol and triglyceride metabolism in adult zebrafish, as compared to zebrafish fed a high lipid diet without *L. rhamnosus* supplementation (Falcinelli et al., 2017). Taken together, these results demonstrate that probiotics in zebrafish feed formulations can significantly affect multiple outcomes, and their inclusion should be carefully considered.

Prebiotics are also often included to resolve any potential limitations associated with the administration of probiotics (Llewellyn et al., 2014). Prebiotics are defined as nondigestible dietary ingredients that beneficially affect the host by selectively stimulating the growth of and/or activating the metabolism of health-promoting bacteria in the gastrointestinal tract (Gibson & Roberfroid, 1995). Adding the prebiotic *Ecklonia cava (E. cava)* to feeds promoted the growth of lactic acid bacteria in the guts of zebrafish, and the authors from this study suggested that *E. cava* may also help to protect against pathogenic infection (Lee et al., 2016). Another study investigating the potential prebiotic effects of mannan oligosaccharides (MOS) on growth and anxiety behaviors in juvenile zebrafish reported that for fish fed diets containing MOS, growth increased and anxiety was significantly reduced as compared to the controls (Forsatkar, Nematollahi, Rafiee, Farahmand, & Martínez-Rodríguez, 2017). In the same study, the ideal MOS inclusion level was calculated to be 4 g MOS kg^{-1} of feed. Two additional studies have also investigated the usefulness of dietary sodium propionate, a short-chain fatty acid (SCFA), as a prebiotic in zebrafish diets (Hoseinifar, Safari, & Dadar, 2017; Safari, Hoseinifar, & Kavandi, 2016).

A recent concept in pursuit of the manipulation of gut microbiota in zebrafish is the use of synbiotics, which are designed to combine the effects of probiotics and prebiotics to elicit a response in the form of synergism (Nekoubin, Javaheri, & Imanpour 2012; Llewellyn et al., 2014). Growth, survival rate, and reproductive success were significantly higher in zebrafish fed the synbiotic Biomar compared to responses of control treatment fish (Nekoubin, Gharedaashi, Imanpour, Nowferesti, & Asgharimoghadam, 2012; Nekoubin et al., 2012). However, studies examining the efficacy of synbiotics on zebrafish health are scarce, and an enhanced research effort is required in this area to affirm the conditions that consistently produce desired effects.

Conclusions

Experimental variability must be minimized within and among research studies when using lab animal models for the study of human health. Effective management of lab animals is essential when controlling for experimental variability. However, lab animals cannot be effectively managed unless their basic biology, including nutrition, is understood. Over the last decade, the number of studies utilizing the zebrafish as a model system for human disease has significantly increased, and the zebrafish is currently ranked by the National Institute of Health as the second most popular model in biomedical research (Gut, Reischauer, Stainier, & Arnaout, 2017). The growing popularity of the zebrafish model in this field and in other areas of research, coupled with the desire to increase reproducibility in research, has led the zebrafish community to seek to understand nutrition in the zebrafish model. This information will allow researchers to develop and utilize high standards of nutrition for this model. In comparison, researchers using rodent models have decreased the experimental variability associated with nutrition in many studies by developing standardized reference and open formulation diets (Watts et al., 2012). While the zebrafish community is still in the early stages of defining zebrafish nutrition and diet development, significant advances in understanding nutrition have been made over the past decade and should continue.

A 2012 review article (Watts et al. 2012) provided the first review on the status of zebrafish nutrition. At the 2014 International Conference on Zebrafish Development and Genetics, it was established that the development of standardized diets is vital for reproducible research in studies utilizing the zebrafish as a model. The findings outlined in the current chapter have hopefully provided an up-to-date review of published information related to nutrition, energy allocation, and feed management in zebrafish, and wherever lacking, in other related species. We have provided multiple factors for consideration in zebrafish nutrition. Current knowledge indicates that in zebrafish and other similar cyprinid species, the availability and utilization of a nutrient is influenced by the amount (quantity) and source (quality) of that nutrient provided. As a result, the quantity and quality of a nutrient in a diet have the potential to influence multiple outcomes in studies related to growth, reproduction, and disease onset and progression. As zebrafish are hypothesized to regulate nutrient intake to meet specific macronutrient needs, the proportion of specific nutrients to total energy content in the diet should also be carefully considered. However, nonnutritive components of the zebrafish diet, while not essential for normal physiological function, must also be carefully managed as they have the potential to affect experimental results. The studies reviewed herein provide key evidence regarding the importance of developing strict nutritional regimes for the zebrafish model. While studies over the past decade have provided considerable insight into nutrition requirements for zebrafish, we still have not identified daily nutritional requirements, and this is critical for the establishment of standardized nutrition protocols for research laboratories. We hope this chapter has provided a historical perspective and future guide for zebrafish nutrition studies.

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CHAPTER

34

Anesthesia, Analgesia, and Euthanasia of the Laboratory Zebrafish

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Anesthesia

Introduction

Anesthesia results in a reversible generalized loss of sensation accompanied by a sleeplike state through the depression of the central nervous system (Ackerman, Morgan, & Iwama, 2005; Ross & Ross, 2008). The appropriate use of anesthetics is essential for minimizing pain and distress that may result from a variety of research procedures performed on zebrafish. Anesthesia of zebrafish requires proper training of personnel, appropriate materials, and equipment, and an anesthetic agent compatible with research objectives and life stage of the fish. This chapter describes commonly utilized anesthetic agents and methods, as well as the technical approaches and considerations for the appropriate handling of zebrafish during anesthesia.

Preanesthetic Assessment and Fish Handling

All fish should be acclimated to their life support system for at least 2 weeks prior to an anesthetic event. Acclimation lowers stress and prevents high morbidity and mortality following potentially stressful events. This time also allows staff the opportunity to identify any clinical signs of disease prior to anesthetizing the fish.

Health surveillance data may inform users of any potential underlying agents that may affect fish physiology, and thus, response to anesthetics. This data may also alert handlers to the presence of zoonotic agents, such as *Mycobacteria* spp., for which they may need to take additional precautions. For more detail, please see the chapters on health surveillance and diseases in this book.

Zebrafish with physical signs of illness, including, but not limited to, poor body condition, granulomas, egg-associated inflammation, and abnormal behavior should not be anesthetized for research projects. These animals should be euthanized to prevent dissemination of causative agents, minimize the risk of zoonotic disease spread to personnel, and prevent sudden death due to excessive stress. It is, therefore, important that personnel are trained to identify clinical signs of disease in zebrafish.

Fasting prior to anesthesia is commonly recommended in other species of fish and might be prudent for zebrafish (Ross & Ross, 2008). Withholding food for 12–24 h also decreases nitrogenous waste production, which helps maintain the water quality of the anesthetic immersion solution.

Soft nets and nonpowdered gloves should be used to handle zebrafish. This prevents disruption of the protective mucus layer over the scales, skin abrasions, and development of secondary bacterial infections. Fish should be transferred using quick and gentle handling as prolonged chasing increases stress.

All items necessary for the procedure should be prepared prior to anesthetizing the fish. If the fish are manipulated out of the water, moist cloths or positioning aids are recommended. These materials should be chemical-free and regularly cleaned and disinfected.

Anesthetic Support and Monitoring

The most common route of administration is immersion in an anesthetic solution. The immersion solution should be prepared using water from the life support system on which the fish are housed. This prevents stress due to physiological changes induced by

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differences in various water quality parameters. The life support system water quality should be maintained within steady ranges and monitored for fluctuations. For more information on appropriate water quality parameters, please see the chapter on water quality in this book.

Working solutions of the anesthetics should be prepared just before use. Water quality must be carefully controlled during immersion anesthesia as hardness, temperature, salinity, pH, and dissolved oxygen concentration may impact anesthetic effectiveness. If large numbers of fish are to be anesthetized at one time, ammonia levels and fecal matter may build up, altering the water quality. If waste buildup is observed in the anesthetic solution, it should be changed.

Baths should be equipped with air stones or air diffusers in order to ensure adequate oxygenation of the solution. Diffusers are preferred as the gas exchange efficiency is greater (Ross & Ross, 2008). The temperature should be close to the normal range to which the fish is acclimated unless hypothermia is to be used. Small heaters or coolers can be used to manage the temperature of the solution.

Five stages of anesthesia (Table 34.1), similar to those described in mammals, are also described for zebrafish (Chen et al., 2014; Collymore, Tolwani, Lieggi, & Rasmussen, 2014; Ross & Ross, 2008). Many animal-related factors can also influence anesthetic efficacy, including body size, stage of development, the density of fish in the bath, sex, lipid content, body condition, disease status, and stress (Ackerman et al., 2005; Martins, Valentim, Pereira, & Antunes, 2016; Readman, Owen, Murrell, & Knowles, 2013; Ross & Ross, 2008). A number of monitoring parameters may be used to judge anesthetic depth.

Monitoring Parameters

Opercular Beat Rate

Opercular beat rate decreases as zebrafish progress through each stage of anesthesia. The beat rate should be continuously monitored during anesthesia. If the opercula stop beating, fish should be immediately removed from the anesthetic solution and placed into a recovery tank. Also, opercula may "flare," or open more widely, which should prompt removal of the fish from the anesthetic solution. Time to cessation of opercular movement varies with each anesthetic agent.

Immobility

Zebrafish will lose the ability to swim upright when placed under anesthesia. In the initial stages of anesthesia, zebrafish may exhibit erratic swimming that will eventually diminish as they become anesthetized. Typically, their swim rate decreases and they will become unable to right themselves. After the loss of righting ability, they also generally stop forward progress and sink to the bottom of the tank.

Response to Tail Fin Pinch

If performing a painful procedure, the tail fin pinch reflex should be evaluated prior to applying the painful stimulus. In this case, fingers or forceps may be used to gently pinch the tail fin once the fish is immobilized. If not deeply anesthetized, the fish will dart off or wiggle in response to the pinch.

Response to Sharp Knock/Deep Vibration

Zebrafish not sufficiently anesthetized may also respond to a knock on a table or tap on the anesthetic tank. This reflex also disappears with deeper anesthesia.

Other

Coughing

During induction, stressed zebrafish might cough, which can expel food material into their gills. The food may prevent oxygen exchange from occurring and make anesthesia more dangerous. Care should be taken to fast fish prior to anesthesia or to use solutions that are less irritating to the fish.

Color

Stressed fish may darken in color during anesthesia. This is transient, and the fish will return to their normal color when they are no longer stressed.

Aversive Behaviors

Behaviors, such as erratic swimming, piping (or gulping air from the water surface), and body twitching may be observed in zebrafish exposed to irritating or aversive anesthetics. In many cases, these behaviors stop as the animal becomes progressively anesthetized.

Recovery

Once procedures are complete, fish should be moved into an anesthetic-free recovery tank. As with the anesthetic solution, the water quality should match that of the life support system on which the fish are housed. In addition, proper aeration and heating are also recommended. If resuscitation is required, such as in the event of loss of opercular movement, using a pipette to pass freshwater through the mouth and over the gills is preferred. It is not recommended to pull fish backward through the water to stimulate recovery as this can cause inefficient oxygen exchange and may damage the gills (Neiffer & Stamper, 2009).

Fish recover by first regaining increasing opercular movement followed by increasing muscle tone and fin

TABLE 34.1 Stages of anesthesia.

Stage	Plane	Level of anesthesia	General behavior	Voluntary locomotor activity	Equilibrium	Opercular movement rate	Reflex response	Heart rate	Muscle tone	Examples of procedures
0		None	Normal	Normal	Normal	Normal	Normal	Normal	Normal	
I		Light sedation	Disorientation	Decreased	Difficult to maintain	Normal	Reduced	Normal	Normal	ENU mutagenesis, imaging
II		Excitation	Agitation	Increased	Lost	Increased	Increased	Increased	Normal	
III	1	Light anesthesia	Anesthetized	None	Lost	Decreased	Reduced	Regular	Decreased	Weighing, gill scrape, skin scrape
	2	Surgical anesthesia	Anesthetized	None	Lost	Shallow	None and no response to deep vibration	Reduced	Decreased	Gill biopsy, tail fin clipping, recovery surgery
	3	Deep	Anesthetized	None	Lost	Rare movements	None	Reduced	Relaxed	Non-recovery surgery
IV		Overdose	Apparently dead	None	Lost	None	None	Cardiac failure	None	Euthanasia

Modified from Collymore et al. (2014).

movements and then less ataxia until it returns to full equilibrium and a normal swimming pattern.

Personnel Safety

Anesthetic agents may be hazardous to personnel (Neiffer & Stamper, 2009; Ross & Ross, 2008). Therefore, personnel must be familiar with the agents they are using and have access to Safety Data Sheets detailing any precautions that should be taken when working with these agents. Personal protective equipment such as gloves, waterproof coverings, and protective eyewear (if necessary) should be available to personnel. Respiratory masks or use of fume hoods may be required when measuring out powder anesthetics. Furthermore, some agents may cause skin or eye irritation requiring the use of gloves when manipulating the anesthetic. Lastly, the presence of any zoonotic agents on the life support system or in the fish should also be indicated, and appropriate precautions must be taken.

Anesthetic Agents

Characteristics of An Ideal Anesthetic Agent

Many characteristics have been described for the ideal anesthetic agent for fish (Ackerman et al., 2005; Grush, Noakes, & Moccia, 2004; Martins et al., 2016; Matthews & Varga, 2012; Neiffer & Stamper, 2009; NRC, 2011; Readman et al., 2013; Ross & Ross, 2008; Stoskopf, 2002). The agent should be water-soluble; easy to prepare and administer; chemically stable over a reasonable period of time; and biodegradable with no persisting physiological, immunological, or behavioral effects. Moreover, it should be potent, leading to induction of anesthesia in less than 3 min and complete recovery within 5 min at low doses. Ideally, it has a wide margin of safety and provides predictable levels of anesthesia that are easy to titrate to the animal and procedure. The ideal agent should also provide proper immobility and effective analgesia. Finally, availability, cost, and potentially toxic side effects to the fish and the handler should be considered. While no single agent fulfills all these criteria, ones that fulfill most of them are preferable. Selection of anesthetic agents should be made with professional judgment as to which agent best meets clinical and humane requirements for the research being performed.

Tricaine Methanesulfonate (Also Known As MS222, TMS)

Tricaine is the most widely used fish anesthetic in the research community and is very effective for rapid induction of deep anesthesia. Importantly, unlike some other compounds, it is approved for veterinary use worldwide. Tricaine induces muscle relaxation and anesthesia by blocking sodium, and to a lesser degree, potassium currents in nerve membranes. It is absorbed across the gills and skin, biotransformed in the liver and possibly kidney, and then excreted primarily through the gills (but also in urine and bile). Recovery with tricaine is usually rapid, and it has a wide margin of safety. It remains the best option for invasive procedures in zebrafish (Collymore et al., 2014). Although there were questions regarding whether tricaine leads to immobilization as opposed to unconsciousness; Attili et al. (2014) demonstrated that it leads to unconsciousness in larval zebrafish. When combined with isoflurane, it maintains the heart rate, extends the anesthetic duration, and shortens the recovery time (Huang et al., 2010; Lockwood, Parker, Wilson, & Frankel, 2017; Matthews & Varga, 2012). The effective dose of tricaine depends on fish age, size, and metabolic rate. Generally, higher concentrations are required for younger animals (Rombough, 2007). Table 34.2 provides dose ranges described for zebrafish.

Fish should be exposed to tricaine in a well-aerated tank. When they are initially exposed fish may display excitatory behavior and hypertaxia (e.g., twitching, erratic swimming, and piping) along with increased opercular movement and gill bleeding (Ackerman et al., 2005; Matthews & Varga, 2012). This behavior subsides as the fish lose equilibrium. Tricaine causes many physiological changes, including respiratory acidosis, cardiac depression, cardiac failure, death, and increasing blood glucose, plasma cortisol, lactate, and blood chemistry values (Carter, Woodley, & Brown, 2011). Recent work also suggests it is moderately aversive to zebrafish (Readman et al., 2013; Wong, von Keyserlingk, Richards, & Weary, 2014). Prolonged (>4–5 min) or repeated exposure (over consecutive days) frequently results in death due to hypoxemia in a high percentage of fish (Chen et al., 2014; Huang et al., 2010). In surviving fish, repeated anesthesia causes prolonged induction and recovery times (Matthews & Varga, 2012).

As tricaine can be highly acidic, buffering with sodium bicarbonate, or another buffer is recommended (Ackerman et al., 2005; Readman et al., 2013). Stock solutions must be stored in a dark container in a cool environment (Neiffer & Stamper, 2009). Properly stored, the stock solution may remain stable for up to 3 months (Ross & Ross, 2008). The solution should be replaced if an oily residue develops or a dark color appears as these generally indicate a decrease in potency (Neiffer & Stamper, 2009; Ross & Ross, 2008). Distilled or deionized water containing little buffering capacity should not be used to create tricaine solutions (Ross & Ross, 2008). Tricaine is generally safe to handle for personnel, but contact with eyes and mucous membranes can cause

TABLE 34.2 Anesthetic agents used for zebrafish.

Drug	Dosage	Anesthetic stage	Observations & comments
Tricaine methansulfonate	50 mg/L 50–100 mg/L 100–200 mg/L	Sedation Light anesthesia Surgical anesthesia	Also used to anesthetize larval zebrafish
Benzocaine	25–100 mg/L 35 mg/L	Light anesthesia Light anesthesia	After induction with MS222, intermittent dosing with benzocaine maintains anesthesia for median time of 7.5h
2-Phenoxyethanol	200–300 μL/L	Light anesthesia	
Clove oil/eugenol/isoeugenol	2–5 mg/L 60–100 mg/L	Sedation Surgical anesthesia	
Metomidate hydrochloride	2–4 mg/L 6–10 mg/L	Sedation Light anesthesia	
Lidocaine hydrochloride	300 mg/L 325 mg/L	Light anesthesia Surgical anesthesia	
Tricaine methanesulfonate and isoflurane	65 ppm + 65 ppm 175 ppm + 175 ppm	Light anesthesia Deep anesthesia	Maintains opercular movement for 20–60 min in adults For imaging for approximately 10 min duration
Hypothermia	12°C 10°C 0–4°C	Sedation Light anesthesia Anesthesia	Larval zebrafish can be exposed for up to 10 min

From Ackerman et al. (2005), Chen et al. (2014), Collymore et al. (2014), Grush (2004), Huang et al. (2010), Lockwood et al. (2017), Matthews and Varga (2012).

irritation. Gloves and goggles are recommended when working with the powder form (Ross & Ross, 2008). It should be immediately washed off if it falls on the skin. Some jurisdictions do not allow tricaine to be poured down drains into waterways; always verify disposal requirements in your region.

Eugenol (Clove Oil, Isoeugenol)

Clove oil is derived from the leaves, bud, and stem of the clove tree (*Eugenia* spp. or *Syzygium aromaticum*) (Ackerman et al., 2005; Grush et al., 2004; Martins et al., 2016). Its active ingredients are eugenol and isoeugenol, which constitute 70%–95% base weight of clove oil.

Eugenol induces anesthesia more rapidly and with lower doses than tricaine but may result in longer recovery times (Grush et al., 2004). It is highly lipophilic and is rapidly absorbed across the gills and skin (Davis et al., 2015). It has a wide margin of safety at a range of temperatures and produces less excitatory behavior than tricaine. Isoeugenol was reported to be highly aversive to zebrafish (Readman et al., 2013); however, clove oil is reported to be less aversive than tricaine (Wong et al., 2014).

Some components of clove oil (e.g., methyleugenol) are suspected carcinogens, and thus, it is not approved for veterinary use in all jurisdictions (Matthews & Varga, 2012). Eugenol, however, is widely available and Aqui-S (50% isoeugenol) is available for use in some countries. Eugenol is water-soluble at high temperatures with vigorous shaking; however, at lower temperatures, it must be prepared as a 10% solution in ethanol (Grush et al., 2004). Stock solutions may be effective for up to 3 months if stored at room temperature in a dark container. This compound is safe for people to handle, but contact with the eyes and mucous membranes should be avoided.

2-Phenoxyethanol (2-PE)

2-PE is a moderately water-soluble topical anesthetic whose efficacy varies with fish size and water temperature. There is little published work on the anesthetic properties of 2-PE in zebrafish. In other fish, it is reported to have a number of disadvantages. For example, it does not block the stress response or involuntary muscle reflexes, making it inadequate for surgical anesthesia. Furthermore, it significantly reduces cardiovascular activity, provides poor analgesia, and may result in hypoventilation. Habituation may occur in fish that are repeatedly exposed to 2-PE. It is also reported to be moderately aversive in zebrafish (Readman et al., 2013). Overall, 2-PE has no great advantages over other drugs except that it is relatively inexpensive (Ross & Ross, 2008). It is routinely used for anesthesia in Europe and the United Kingdom (Readman et al., 2013). Working solutions may last for up to 3 days. Finally, 2-PE has been described as a mild toxin that may cause some irritation to the skin, and any contact with the eyes should be avoided.

Benzocaine

Benzocaine is the parent compound of tricaine and has similar pharmacology, but is less acidic and less water-soluble. Induction is rapid, and recovery occurs within 10 min although usually longer than with tricaine. Drug efficacy is affected by the fish size and water temperature, but not water hardness or pH (Ross & Ross, 2008). The margin of safety is also reduced at high temperatures. Many of the side effects observed with tricaine are present with benzocaine. It is not adequate for surgical anesthesia as fish maintain some locomotor functions in all stages of anesthesia; furthermore, it is reported to be highly aversive to zebrafish (Readman et al., 2013). A dynamic perfusion system for long-term intermittent administration of benzocaine has been described for zebrafish (Wynd, Watson, Patil, Sanders, & Kwon, 2017).

Benzocaine stock solutions are prepared in ethanol, acetone, or propylene glycol prior to dissolution in water and must be stored in a dark bottle at room temperature. Stock solutions, if well maintained, are stable for up to 1 year. To overcome this insolubility, one can use benzocaine hydrochloride, a water-soluble compound that, much like tricaine, forms an acidic solution that needs to be buffered. The powder is a respiratory irritant to people; however, it is not generally toxic to at the concentrations used for zebrafish.

Metomidate and Etomidate

Metomidate is an imidazole-based nonbarbiturate hypnotic used to reduce stress during handling and transportation. Metomidate hydrochloride is an effective sedative for zebrafish (Collymore et al., 2014). It has a wide margin of safety and a rapid induction time; however, recovery is lengthy (up to 40 min) (Collymore et al., 2014). Metomidate is ineffective for use with larval fish, and muscle twitching is a common side effect. This makes it unsuitable for use as a surgical anesthetic. Metomidate also suppresses the cortisol response in fish. In addition, fish may darken in color transiently when exposed to the solution. Metomidate is less aversive than tricaine (Wong et al., 2014). In Canada, it is sold as Marini, and in the United States, Aquacalm is commercially available. Metomidate is indexed for sedation and anesthesia in the United States but cannot be used off label as it is not currently FDA-approved. It is water-soluble, requires storage in a tight light-protected container, and working solutions last up to 3 days.

There have been no published studies on the effectiveness of etomidate for the anesthesia of zebrafish, although it can be used as a sedative. Etomidate is more effective in alkaline waters, and higher temperatures render the drug less toxic. As with metomidate, it induces animals rapidly and produces a relatively long recovery. Etomidate is not aversive to zebrafish (Readman et al., 2013), but neither metomidate nor etomidate provide analgesia.

Lidocaine

Lidocaine hydrochloride is a water-soluble local anesthetic agent. It has only recently been used in zebrafish, but it leads to surgical anesthesia in this species. Lidocaine leads to rapid induction and recovery (Collymore et al., 2014; Martins et al., 2016). However, its primary disadvantage is its small margin of safety (Collymore et al., 2014). It is also reported to be moderately aversive to zebrafish (Readman et al., 2013). The addition of sodium bicarbonate may enhance its anesthetic effects.

Combinations with propofol may potentiate its effect and reduce dosages of both drugs required to anesthetize fish (Martins et al., 2016; Valentim, Felix, Carvalho, Diniz, & Antunes, 2016). Lidocaine may also provide perioperative analgesia (Martins et al., 2016).

Propofol

Propofol is a short-acting sedative-hypnotic that binds to GABA A receptors used for induction and maintenance of general anesthesia. Propofol is lipophilic and rapidly metabolized, leading to rapid induction. Other advantages include a lack of cumulative effects and rapid recovery from anesthesia. However, it is not fully soluble, and solutions should be used within 30 min of preparation (Martins et al., 2016; Valentim et al., 2016). Propofol alone does not provide sufficient analgesia for painful procedures (Valentim et al., 2016).

Lidocaine and propofol combinations result in faster induction than tricaine (Valentim et al., 2016). Furthermore, these combinations also result in general anesthesia and analgesia (based on lack of response to tail fin pinch) (Valentim et al., 2016). Still, recovery is faster in fish exposed to tricaine compared with lidocaine and propofol (Valentim et al., 2016).

Gradual Cooling (Hypothermia)

Cooling involves lowering the ambient body temperature with cold water to tranquilize or immobilize fish. Gradual cooling should be employed for recovery procedures. Adult zebrafish are anesthetized at approximately 10°C (Collymore et al., 2014). Fish recover quickly when in contact with instruments at room temperature or placed back into regular life support system water (Collymore et al., 2014). Cooling is, therefore, useful for transportation, short-term handling, and minor procedures, such as IP injections, but not surgical procedures.

Rapid chilling is an effective anesthetic method for larval zebrafish (Chen et al., 2014). Zebrafish from 1 to 14 days postfertilization (dpf) reach deep anesthesia

(loss of equilibrium, loss of all reflex activity, and no movement of opercula for more than 5s) within 10s after exposure to water at a temperature of zero. Anesthesia for up to 10 min is achieved even after 3 days of repeated exposure from 1 to 60s (Chen et al., 2014). The fish recover within 2 min when placed in a 28°C bath. However, mortality occurs when fish of all ages are exposed to rapid chilling for over 10 min.

Generally, cooling is most effective for small fish acclimated to water temperatures above 10°C. Cooling slows movement and reduces the general metabolic activities of fish. It is important to note that nerve conduction is reduced but not completely blocked using cooling. The rate of cooling must be carefully controlled to avoid mortality, and zebrafish should be physically separated from the ice. Dry ice should never be used to create hypothermia.

Others: Ketamine, Propoxate, Quinaldine, Isoflurane, Electroanesthesia

Ketamine is a dissociative anesthetic, whose use in adult zebrafish has not been described. It is reported to be neurotoxic to zebrafish larvae and interferes with complete development of embryos (Kanungo, Cuevas, Ali, & Paule, 2013).

Propoxate is a compound similar to metomidate and etomidate that is moderately aversive but has not been described for anesthesia of zebrafish (Readman et al., 2013).

Quinaldine is widely used by marine biologists, but use in zebrafish has not been described. Disadvantages of its use include continued involuntary muscular movement, lack of analgesia, and high aversiveness to zebrafish (Readman et al., 2013). It can also be a skin and eye irritant to people.

Isoflurane is a hydrocarbon volatile hypnotic anesthetic that can be injected underwater; however, alone, it causes variable anesthesia (Collymore et al., 2014; Huang et al., 2010). It also causes distressful behaviors, no loss of equilibrium at doses tested in zebrafish, and 30% mortality (Collymore et al., 2014). In order to minimize operator exposure to isoflurane, anesthetic preparation and anesthesia should be conducted in a chemical fume hood.

Electroanesthesia is used for larger species of fish; however, no device or method has been accepted for use with zebrafish.

Special Anesthetic Considerations

Anesthesia of Embryos

Embryos (1–3 dpf) require significantly higher concentrations of anesthetic than adults in order to assure immobility (Rombough, 2007). Rapid cooling is an alternative to tricaine for adequate anesthesia of embryonic zebrafish (Chen et al., 2014). Generally, embryos are considered not to have fully developed pain receptors, and thus, do not require the use of agents that provide analgesia (NIH, 2013).

Anesthesia of Larvae

Gills are required in adult zebrafish for respiratory gas exchange, ion and water balance, excretion of nitrogenous wastes, and maintenance of acid-base balance. These processes take place in embryonic and larval skin until the gills develop around 12–14 dpf (Rombough, 2002, 2007). This difference in metabolism is responsible for the resistance of larval fish to chemical agents. Rapid cooling, as well as high doses of tricaine or eugenol, may provide adequate anesthesia for embryonic and larval zebrafish (Chen et al., 2014; Strykowski & Schech, 2015). High doses of lidocaine hydrochloride were not sufficient to induce anesthesia in larvae around 14 dpf (Collymore, Banks, & Turner, 2016).

Long-Term Anesthesia

In some projects, prolonged anesthesia of adult zebrafish is required. Recently, a number of protocols have been developed using multiple agents (i.e., tricaine and isoflurane), or intermittent dosing of single agents (i.e., benzocaine) to prolong anesthesia (Huang et al., 2010 Wynd et al., 2017; Lockwood et al., 2017). In these cases, anesthesia can be achieved for durations of up to 8 h.

Repeated Anesthesia

Repeated anesthesia involves exposing zebrafish to an anesthetic agent daily for three or more consecutive days. Tricaine can lead to a high level of mortality when zebrafish (larval and adult) are exposed over multiple days (Chen et al., 2014). Rapid cooling has been suggested as an alternative to tricaine for repeated anesthesia of larval zebrafish up to 14 dpf (Chen et al., 2014). However, further work is required to determine whether other agents can be used for repeated anesthetic events without causing high morbidity and mortality.

Analgesia

Introduction

Analgesic protocols for zebrafish are relatively new, despite the well-established use of zebrafish for modeling nociceptive behavior (Correia, Cunha, Scholze, & Stevens, 2011; Curtright et al., 2015; Maximino, 2011). Anesthetics may provide analgesia while the animal is unconscious; however, they do not provide sustained analgesia after potentially painful interventions. In larger fish, analgesics may be provided through the intramuscular, subcutaneous, or intracoelomic

routes; these techniques are challenging in zebrafish. Recently, bath immersions of analgesic agents have been proposed to address some of these problems (Lopez-Luna, Al-Jubouri, Al-Nuaimy, Sneddon, 2017a, 2017b; Schroeder & Sneddon, 2017). Bath immersion allows shoals of fish to be maintained together after an intervention. The choice of agents is, however, restricted to those that are water-soluble (Schroeder & Sneddon, 2017). Further investigation into the appropriate use of analgesics for zebrafish is required. Here, what is currently reported regarding analgesic bath immersion for zebrafish is summarized.

Assessment of Pain and Discomfort

Zebrafish exposed to noxious stimuli demonstrate increased ventilation (or rapid opercular movement), increased use of cover (if provided), increased bottom-dwelling, a decreased swim rate and higher cortisol levels (Curtright et al., 2015; Davis et al., 2015; Maximino, 2011). Further, altered swimming mechanics may be observed in zebrafish injected with acetic acid near the caudal tail fin or subjected to tail fin clipping (Schroeder & Sneddon, 2017).

Analgesic Agents

Lidocaine

Exposure to lidocaine at a dose of 5 mg/L for 30 min increases activity levels and more rapidly returns opercular beat rate to baseline levels for up 90 min in adult zebrafish after fin clipping than those not receiving analgesia (Schroeder & Sneddon, 2017). It also improves activity level in zebrafish larvae exposed to heat and reduces behavioral changes in larvae exposed to noxious chemicals (Lopez-Luna 2017a, 2017b).

Aspirin

Aspirin is a nonselective COX inhibitor used to treat mild to moderate pain. When provided in immersion at a dose of 2.5 mg/L for 30 min to fish after fin clipping, it results in higher activity levels and lower opercular beat rates than fish not receiving an analgesic for up to 90 min following the procedure (Schroeder & Sneddon, 2017). Aspirin also reduces behavioral changes in larvae exposed to noxious chemicals (Lopez-Luna, 2017b).

Morphine

Morphine is an opioid analgesic used to treat mild to severe pain. Morphine at an immersion dose of 48 mg/L for 30 min prevents changes in behavior in zebrafish larvae exposed to noxious chemicals and high temperature (Lopez-Luna 2017a, 2017b).

Euthanasia

Introduction

Euthanasia is the act of providing a "good death" to animals by methods that induce rapid unconsciousness and death without pain or distress (AVMA, 2013; CCAC, 2010). There are a number of approved methods for the euthanasia of laboratory zebrafish. National guidelines on approved euthanasia methods vary by jurisdiction (i.e., US vs. Canada vs. UK) and each facility should adhere to methods approved by the regulatory agencies in their area. In all cases, staff performing euthanasia should be experienced and appropriately trained in the procedure. Any chemical agents used should be within their expiry dates, stored appropriately, and disposed of according to local regulations.

Acceptable methods of euthanasia must induce loss of consciousness and death with only momentary or no pain, distress or anxiety, be reliable and irreversible, and require a short time to induce unconsciousness. Furthermore, the method must be appropriate for the species and age of the animal, be compatible with research objectives, and be safe for personnel, as well as have a minimal emotional effect on personnel (AVMA, 2013; CCAC, 2010). Death must be confirmed by personnel trained to recognize the cessation of vital signs in zebrafish. Recent publications have also called into question the aversiveness of various chemicals used for the anesthesia and euthanasia of adult zebrafish (Readman et al., 2013; Wong et al., 2014). While best practices for zebrafish euthanasia have still not been fully elucidated, personnel should keep abreast of new findings on this subject.

Euthanasia methods should be standardized so that the procedure is predictable and controlled. The methods should be reviewed and approved by the Institutional Animal Care and Use Committee or similar body (AVMA, 2013; CCAC, 2010).

Methods of Euthanasia

Overdose With Anesthetic Agents

Adult Euthanasia

Overdose of chemical anesthetic agents is an acceptable method of euthanasia (Table 34.3) (AVMA, 2013; CCAC, 2010). Prolonged exposure to chemical anesthetics often leads to death by hypoxia; for this purpose, adults must generally remain in euthanasia solutions for at least 10 min after the opercular movement has ceased. Immersion in liquid nitrogen following anesthesia with tricaine may be required for RNA isolation and preparation or to prevent rapid degradation of biological

TABLE 34.3 Euthanasia doses for zebrafish.

Drug	Dosage	Observation & comments
Adult zebrafish		
Tricaine methanesulfonate	>200 mg/L	For 10 min after cessation of opercula movement
Eugenol/Clove oil	>100 mg/L	For 10 min after cessation of opercular movement
Lidocaine hydrochloride	>400 mg/L	For 10 min after cessation of opercular movement
2-Phenoxyethanol	≥800 mg/L	For 5 min
Hypothermia	0–4°C	For 5 min
Larval fish		
Tricaine methanesulfonate	>1800 mg/L	For larvae 3–8 dpf with exposure for over 1 h
	>1800 mg/L	For larvae 3–8 dpf followed by an adjunctive method of euthanasia
Isoeugenol	>1500 μL/L	For larvae 14 dpf with exposure for at least 20 min after cessation of heartbeat
Hypothermia	0–4°C	Followed by an adjunctive method (bleach/decapitation/maceration) after exposure for at least 20 min after cessation of heart beat for larvae <14 dpf Maintained in cold solution for at least 12h for larvae ≤14 dpf if no secondary adjunctive method used

From Collymore et al. (2014), Davis et al. (2015), Matthews and Varga (2012), NIH Guidelines (2013), Rombough (2007), Schroeder & Sneddon (2017), Strykowski and Schech (2015), Wallace et al. (2018).

material. Euthanasia with an overdose of clove oil increases trunk blood collection yield and has a smaller effect on detectable cortisol levels than tricaine (Davis et al., 2015). Maceration or another adjunctive method should follow chemical methods of euthanasia to ensure complete brain destruction (Matthews & Varga, 2012). Injectable pentobarbital has not been described in zebrafish; however, it is an AVMA-approved method of euthanasia (AVMA, 2013).

Larval Euthanasia

Recent publications demonstrate that zebrafish embryos and larvae are more resistant to chemical euthanasia agents than adults due to specific aspects of embryonic and larval metabolism (Collymore et al., 2016; Rombough, 2007; Strykowski & Schech, 2015). Death in young larvae may be due to heart failure rather than hypoxia as observed in adults. Loss of heartbeat and no loss of opercular movement may thus, be a more appropriate method for determining death in larval zebrafish (Collymore et al., 2016; Rombough, 2007; Strykowski & Schech, 2015). Exposure to very high concentrations of tricaine and eugenol leads to deep anesthesia in larvae aged 4–14 dpf. Larval fish should remain in chemical euthanasia solutions for at least 20 min after cessation of all movement and/or heartbeat. These methods should be followed by adjunctive methods to ensure brain destruction and euthanasia. Dilute sodium hypochlorite can be used for this purpose, as these fish are too small for maceration (Matthews & Varga, 2012).

Physical Methods of Euthanasia

Rapid cooling of zebrafish leads to hypothermal shock and subsequently, death. This method does not produce ice crystals in the body (Wilson, Bunte, & Carty, 2009). Death is induced quickly (less than 10 s) and with minimal pain and distress (Matthews & Varga, 2012; Wilson et al., 2009). Larval zebrafish up to 14 dpf should remain in cold solutions for at least 12 h to ensure death via rapid cooling (Wallace et al., 2018). Zebrafish aged 16 dpf and above should be exposed to rapid cooling for at least 5 min to ensure death (Wallace et al., 2018). Maceration or another adjunctive physical method should

follow rapid cooling to ensure complete brain destruction (Matthews & Varga, 2012). Embryos 3 dpf and under may be euthanized by exposure to bleach solution alone or after extended exposure to ice water (Matthews & Varga, 2012; NIH, 2013).

Euthanasia Guidelines

AVMA Guidelines on The Euthanasia of Animals The most recent version of the AVMA Guidelines provide a number of "acceptable" and "acceptable with conditions" methods of euthanasia. All methods of anesthetic overdose are approved (AVMA, 2013). In addition, euthanasia of small tropical fish via rapid cooling is also listed as acceptable. Physical and adjunctive methods, such as the use of sodium hypochlorite, decapitation followed by pithing and maceration, are also listed to confirm the death.

National Institutes of Health (NIH) Euthanasia Guidelines NIH guidelines indicate that fish older than eight dpf may be euthanized via rapid cooling or tricaine (NIH, 2013). Fish 4–7 dpf are euthanized by prolonged immersion in a cold solution followed by the addition of sodium hypochlorite to ensure death. For embryos, sodium hypochlorite alone may be used. Additional methods are described if required and with conditions.

CCAC Guidelines for The Euthanasia of Animals Current CCAC guidelines on euthanasia consider overdose of anesthetics as the only acceptable method of euthanasia for fish (CCAC, 2010). Maceration for fish less than 2 cm in length is also approved. CCAC guidelines for the use of fish recommend a two-step method of euthanasia to ensure brain death with the first step, preferably overdose with an anesthetic agent. In regard to euthanasia, they do not recommend the use of hypothermia by placing fish on ice, but they make no reference to rapid cooling in solution.

European Union Directive 2010/63/EU The 2010 Directive limits approved methods of euthanasia for fish to tricaine, electroanesthesia, and cranial concussion (European Parliament, 2010). Other methods of euthanasia are not approved and depending on the country, may not be accepted for use by local authorities (Kohler et al., 2017).

Conclusion

As the use of zebrafish as a model continues to increase in biomedical research, further refinement of anesthetic and analgesic protocols is required. In addition, humane euthanasia practices must be further refined and harmonized globally.

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CHAPTER

35

Health Surveillance Programs

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Introduction

Scientific research should be conducted in a responsible manner using consistent, objective evaluations, and detailed documentation of experimental design, processes, and results. The research should be demonstrated as reproducible for testing a given hypothesis. In biomedical research, there is an even greater ethical goal to ensure that animals are used responsibly, and determination and reporting of animal health status are of paramount importance to ensure adequate transparency of research results. Both the Institute for Laboratory Animal Research (ILAR) Guidance for the Description of Animal Research in Scientific Publications and the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines emphasize the importance of reporting pathogen status for animals in studies, including a description of equipment and procedures utilized to maintain a consistent microbial status during a study (ILAR, 2011; Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010). The presence of pathogens can increase overall animal morbidity and mortality, or decrease fecundity or larval survival, thereby requiring the use of more animals. In addition, the presence of infectious diseases has been demonstrated to influence research practices and research results in several species, including zebrafish (Baker, 1998; Franklin, 2006; Kent, Harper, & Wolf, 2012). For example, some *Mycobacterial* spp. have been demonstrated to alter gene expression and upregulate various inflammatory cytokines (Parikka et al., 2012; Van der Vaart, Spaink, & Meijer et al., 2012). Infection with *Pseudoloma neurophilia* has been shown to influence results of behavioral research (Spagnoli, Xue, & Kent, 2015a, Spagnoli, Xue, Murray, Chow, & Kent, 2015b, Spagnoli, Sanders & Kent, 2017), and the presence of *Pseudocapillaria tomentosa* altered the rate of tumorigenesis in a chemical mutagen study (Kent, Bishop-Stewart, Matthews, Spitsbergen, 2002, Kent, Harper, Wolf, 2012). More examples are referenced in Table 35.1. When a disease is detected, it is important to communicate this information with research staff so that they are aware of the possible impact of the disease on their research and so that this information can be included when they report the results of their study.

The ethical responsibility to determine and report the health status of zebrafish is also important for reducing risks during collaborations that involve "sharing" of fish between zebrafish laboratories. When the health status of fish is not known or shared by individuals involved in these collaborations, there is increased potential for the transmission of pathogens to other facilities. The risk of disease from imports, as well as processes to mitigate the risk, should be determined prior to the importation of fish to minimize risk to the conditioned colony. Therefore, it is important for each facility, regardless of size, to know the pathogen status of their fish and to share this when distributing fish to other institutions.

Knowledge of the presence of disease can become even more important and difficult to manage when fish are housed in a recirculating water system because of the potentially rapid transmission of pathogens through the water, especially if filtration methods are inadequate. Timely recognition of infectious agents will be of assistance in the appropriate management of these conditions. In addition, zebrafish will demonstrate clinical signs of illness as a result of deviations to environmental factors, including water quality (i.e., elevated levels of nitrogenous waste, fluctuations in pH) and light cycles, and it is important to differentiate these conditions from infectious causes.

Although much is still unknown regarding infectious diseases in zebrafish, there are known pathogens with zoonotic significance. Consistent inhabitants of aquatic environments include several *Mycobacterium*

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TABLE 35.1 Examples of clinical impact and research impact of select infectious organisms.

Agent	Clinical impact	Interference with research	References
Pseudoloma neurophilia	Subclinical or variable morbidity and mortality; skeletal deformities; reduced growth; emaciation; decreased fecundity; lethargy; decreased larval survival	Histologic alterations of involved tissue; myonecrosis and autofluorescence interferes with muscular development and imaging studies; altered behavioral studies, including startle response and shoaling behavior; increased mortality following gamma irradiation	Kent et al., 2012; Ramsay et al., 2009b; West et al., 2014; Spagnoli et al., 2015 and 2016a and 2016b
Pseudocapillaria tomentosa	Variable morbidity and mortality; diffuse granulomatous peritonitis; emaciation; decreased growth rates and fecundity	Histologic alterations of involved tissue; hematologic and gastrointestinal eosinophilia; altered results of carcinogenesis studies	Kent et al., 2002; Balla et al., 2010; Spitsbergen, Buhler, & Peterson, 2012; Murray, & Peterson, 2015
M. marinum	Variable morbidity and mortality (exacerbated by stress); dermal lesions; granuloma formation; zoonotic potential; coelomic edema (dropsy), zoonotic potential	Activation of innate immune response; alterations of gene expression; tumor promoter (Medaka); reactivation of latent infection by gamma irradiation	Ramsay et al., 2009a; Parikka et al., 2012; Meijer et al., 2005; Van der Vaart, Spaink, & Meijer, 2012; Cronan et al., 2014; Broussard et al., 2009
M. haemophilum	Moderate to significant morbidity/mortality, emaciation, lethargy, dropsy, dermal lesions, granuloma formation, zoonotic potential	Tumor promoter evokes chronic inflammation with alteration if inflammatory mediated pathways, granulomatous nephritis and hepatitis may impact kidney and liver function	Kent et al., 2004; Whipps, Dougan, & Kent, 2007; Spitsbergen et al., 2012
M. chelonae	Typically subclinical; aerocystitis; zoonotic potential	Autofluorescence interferes with imaging studies	Kent, Watral, Kirchoff, Spagnoli, & Sharpton, 2016; Whipps et al., 2014; Kent et al., 2004; Whipps et al., 2008
Pleistophora hyphessobryconis	Lethargy, morbidity, mortality	Severe necrosis and expansion of myofibers; muscle deformity	Sanders et al., 2010
Edwardsiella ictaluri	High mortality epizootics	Necrotizing inflammation in various visceral organs	Hawke et al., 2013

spp., and other species of bacteria, fungi, and protozoa that are either opportunistic pathogens (*Aeromonas hydrophilia, Saprolegnia* spp.) or pathogenic to humans (i.e., *M. marinum*). It is important to be aware of the presence of these and other potential zoonotic pathogens to ensure the safety of individuals that are working with these animals.

Development of a Health Surveillance Program

The development and implementation of a health surveillance program present several challenges. The first challenge is that there is limited published data on the diagnosis, treatment, and transmission of all diseases that are known to impact zebrafish. In addition, the diagnosis of many of the known pathogens often requires lethal testing methods. Therefore, health surveillance programs require periodic reassessment as further research is published regarding the transmission and treatment of diseases and as new and improved diagnostic testing becomes available. The second challenge is that standard housing methods typically involve a holding system consisting of individual tanks, holding between 20 and 90 fish each, that are supplied with filtered and treated recirculated water. As a result, the risk of tank to tank transmission of pathogens is inherent and may progress quickly if filtration and water treatment methods (e.g., ultraviolet light) are not maintained adequately or are insufficient to eliminate all zebrafish pathogens present. Although cryopreservation of zebrafish spermatozoa is possible, it is not commonly practiced; thus, the introduction of a disease can be devastating to a research program and result in the loss of unique research models. A third challenge is that the sporadic availability of colony health information from zebrafish sources results in a high risk for pathogen exposure with each import. A fourth challenge is that although expertise in this area is growing, animal care staff are often more familiar with recognizing clinical signs of disease in mammalian species, and individuals with expertise in aquatic laboratory animal husbandry are often more difficult to find.

A well-designed health surveillance program should ideally track the health status of a colony over time, as well as rapidly identify a breach of biosecurity or the

presence of a new pathogen to allow for rapid biocontainment, treatment, and prevention of transmission of disease. Each program must be designed specifically for the individual facility, taking into consideration the size and diversity of aquatic populations therein, the agents to be excluded, the types of housing systems in use and available for use, the frequency of importation of new animals, the skill level of staff, the diagnostic capabilities and resources available, and the types of research conducted. Financial limitations of each facility, especially for smaller facilities, must also be taken into consideration and balanced with the intellectual and financial costs of losing an entire colony due to disease. Several of these conditions and available options will be discussed below.

Selection of Agents

For an existing facility, the first step in developing a health surveillance program is to determine what pathogens are already present. Once this information is known, the animal care program personnel, in collaboration with investigative staff, must determine if pathogens should be treated (if possible), or if the agent(s) will be considered acceptable, and therefore, not be excluded from that facility and not warrant frequent testing. Further determination of pathogens to be excluded from the facility and the frequency of testing needed within the conditioned colony should be based on the direct impact of pathogens on animal health (i.e., associated morbidity and mortality), impacts on research, modes of transmission, prevalence of disease, availability of treatments and diagnostic tests, host specificity, and potential zoonosis. For example, pathogens known to be highly transmissible with high levels of morbidity or mortality (such as *Edwardsiella ictaluri* and in some situations, *P. tomentosa)* and external parasites could warrant exclusion and frequent testing (Hawke et al. 2013; Murray & Peterson, 2015). Immunodeficient fish and those undergoing immunosuppressive procedures, such as irradiation, have an increased susceptibility to disease. In these scenarios, known pathogens and some opportunistic pathogens may need to be excluded and be included in surveillance testing (Parikka et al., 2012; Spagnoli, Sanders, Watral, & Kent, 2016). Facilities supporting toxicology or infectious disease research may need to limit infectious agents as confounding factors necessitating the use of Specific Pathogen Free (SPF) fish and more frequent and extensive testing for a wide range of agents. Finally, an immediate action plan should be outlined for instances when an excluded pathogen is detected in either quarantine or the conditioned colony.

Testing Frequency

There are several known pathogens, as well as several opportunistic organisms to be considered when determining agents to be excluded and testing frequency. Some of the more commonly reported zebrafish pathogens will be discussed here. For more detailed information on these organisms and others, please see the chapters of this book describing the specific diseases.

Pseudoloma neurophilia and *Pleistophora hyphessobryconis* are two microsporidian pathogens that have been identified in zebrafish. There are no known treatments for either agent. Of the two, *P. neurophilia* is more common and is frequently reported as an endemic pathogen in laboratory zebrafish facilities (Lawrence, Eisen, & Varga, 2016; Sanders, Watral, & Kent, 2012). Reports of *P. hyphessobryconis* are more sporadic and are typically associated with importation of fish from pet stores, aquarium suppliers, or other atypical sources (Sanders et al., 2010). Because *P. neurophilia* is more common, much more is known about its transmission and the impact of infection. *P. neurophilia* preferentially infects neural tissue, but severe infections may become more disseminated. Infection can be subclinical or can cause clinical disease characterized by emaciation and spinal deformities. Severe infections can result in death. This disease can impact research as a result of decreased fecundity and can interfere with imaging and behavioral studies (Spagnoli et al., 2015a, 2015b; Spagnoli et al., 2017; West, Miles, Kent, & Frazer, 2014). This agent can be transmitted vertically, as well as horizontally, and there are no known embryo surface disinfection protocols that are safe and effective for elimination of this organism (Ferguson, Watral, Schwindt, & Kent, 2007). Spawning events and cannibalism contribute to the spread of the pathogen within a facility (Murray et al., 2011b; Sanders, Watral, Clarkson, & Kent, 2013; Peneyra et al., 2018). Transmission studies have demonstrated that naïve fish can become infected in less than 2 months under certain conditions (Kent et al., 2009), but allowing fish to be exposed to the pathogen for 3–6 months or longer will likely increase chances of detection. Older fish are more likely to be infected with this pathogen. For these reasons, quarterly testing of fish potentially exposed to this pathogen for 6 months or longer would allow the best chance for detection of this pathogen. However, if this pathogen is endemic in a colony and is not excluded, frequent testing for its presence may not be warranted. *P. hyphessobryconis* primarily targets skeletal muscle, resulting in severe necrosis and associated deformities, lethargy, and sometimes death. Although it is currently reported to be much less prevalent in laboratory zebrafish colonies than *P. neurophilia*,

this organism is suspected to behave similarly to *P. neurophilia* in several ways, and thus, testing frequency recommendations are similar (Sanders et al., 2010; Murray et al., 2011b).

Mycobacterium spp. are commonly found in aquatic habitats, and the virulence of disease is dependent upon the particular mycobacteria species present, the immune status of the exposed fish, and the experimental history of exposed fish (Parikka et al., 2012). Additionally, some *Mycobacterium* spp., can result in zoonotic infection of fish handlers or caretakers. Several different species have been reported to be potentially pathogenic in zebrafish, including *M. marinum, M. haemophilum, M. fortuitum, M. abscessus, M.* pere*grinum, M. saopaulensce, and M. chelonae* (Nogueira et al., 2015; Whipps, Lieggi, & Wagner, 2012). *M. marinum and M. haemophilum* are commonly associated with widespread granuloma formation, and associated clinical signs with moderate to high levels of mortality, whereas, variable morbidity and mortality are reported with other species. *M. chelonae* is a frequent inhabitant of biofilms, and infections in zebrafish are frequently subclinical with low mortality (Meritet, Mulrooney, Kent, & Löhr, 2017); thus, detection of this pathogen is difficult without targeted screening. The efficacy of embryo surface disinfection procedures varies by mycobacteria species. Maintaining a facility free of all mycobacterial species for an extended period of time can be challenging and cost-prohibitive and will only be realistic in such situations where germ-free or SPF fish are imperative for meeting specific research goals (Collymore et al., 2016; Pham, Kanther, Semova, & Rawis, 2008). As such, a more realistic goal for most facilities would be to identify the species of *Mycobacterium* that will be excluded and establish exclusion and testing criteria based on the infectious profile of those species. Greater numbers of bacterial organisms are associated with more chronic infections, and thus, older fish or fish with greater exposure periods, are likely to be more useful for detecting infection.

Pseudocapillaria tomentosa is a parasitic nematode that is reported in zebrafish facilities with relative regularity. Infections can be subclinical or can be associated with emaciation, lethargy, and even death. Zebrafish strains may vary in susceptibility to infection, and some fish may develop a tolerance to infection (Murray & Peterson, 2015). Transmissions can occur quickly, as soon as 2-4 weeks following exposure in adult fish and as early as 12 h post exposure in larvae (Kent & Bishop-Stewart, 2003; Sanders, Peterson, & Kent, 2014; Murray & Peterson, 2015; Collymore et al., 2014b; Gaulke et al., 2016). Standard embryo surface disinfection procedures are not effective for killing *P. tomentosa* eggs (Martins, Watral, Rodrigues-Soares and Kent, 2017); however, the rinsing of embryos is likely to mechanically remove the parasite eggs that have adhered to the chorion (Crim et al., 2017). Due to its rapid transmission rate, the presence of this disease can ideally be identified and treated, or routinely treated prophylactically, during quarantine as large scale treatment of a large conditioned colony is often labor-intensive and may be associated with reproductive side effects (Collymore et al., 2014b). More frequent monitoring for this agent within the conditioned colony and quarantine is recommended to allow for rapid recognition and containment. Exposure of sentinel fish to effluent water for as little as 3 months should be sufficient for detection of this pathogen, if present.

Edwardsiella ictaluri is a gram-negative bacteria associated with severe systemic infection and high mortality. Effective transmission occurs through a variety of routes, including oral intake and direct contact with infected water and/or feces; therefore, the infection can spread rapidly through recirculating water systems. Due to the rapid and detrimental effects of infection, this pathogen is often identified in the quarantine period when significant morbidity and mortality become evident. Several other gram-negative bacteria have the potential to be primary or opportunistic pathogens of zebrafish. These include *Aeromonas hydrophilia,* other species of *Edwardsiella,* and some *Flavobacterium* spp. The desire to exclude these agents and their testing frequency should be based on experimental goals and the immune status of colony fish.

External parasites are commonly associated with fish obtained from pet stores, can result in significant morbidity and mortality, and should be excluded from research colonies. These types of infections are infrequently found in laboratory fish and have known treatments; thus, their identification in quarantine is imperative. Less frequent testing for these pathogens in the main colony may be warranted if importation into the colony is infrequent and/or if fish are obtained from reliable sources.

Little is known about viral infections in zebrafish. A novel picornavirus, which appears to have a relatively high prevalence with worldwide distribution in zebrafish, has recently been discovered. As of this writing, this virus has not been associated with clinical disease (Altan et al., 2019). Natural infections of zebrafish with spleen and kidney necrosis virus and viral nervous necrosis virus have occurred, with associated morbidity and mortality (Bermudez, 2018, Binesh, 2013). The most likely source for these infections is the ornamental or pet fish trade, reinforcing the need to use extreme caution when importing fish from or associated with these sources. Several other viruses have been experimentally induced without any reported natural infections. The relevance of routine testing for these viruses is unclear at this time due to limited knowledge of

disease transmission, maintenance, and effects on research and animal health. Testing may be justified if receiving fish from a questionable source. In addition, routine testing, or testing prior to shipment may be necessary for compliance with import/export regulations for some countries, and this may determine the need for testing and testing frequency.

Health Surveillance Sampling Sources

In general, health surveillance sampling sources can be divided into two categories: live animals (adults, fry, embryos) and environmental samples. Each method has its advantages and disadvantages, with the most obvious advantage of environmental sampling being the conservation of animals. However, the presence of an organism within an environment does not necessarily translate into an infection of animals, and some pathogens may not be readily detectable in the environment. The ideal program would incorporate both testing methodologies (Collymore et al., 2016; Mocho, 2016). The use of both live animal and environmental sources will be discussed below.

Live Animals

Daily health monitoring, performed by individuals knowledgeable in the normal appearance and behavior of zebrafish, is an important component of health surveillance and disease prevention. All zebrafish should be evaluated at least once daily and observed for both physical and behavioral abnormalities. Physical abnormalities associated with sick fish may include frayed fins or gills, skin lesions, the presence of excessive mucus, thin body condition (Clark, 2018), a change or alteration in body color, coelomic distension, abnormal body conformation or size when compared to tank mates, altered respiratory rate, altered gill color, or abnormal eye coloration or size (e.g., exophthalmos). Individual sick or dead fish should be removed from recirculating systems in a timely manner, and diagnostic evaluation of live, sick fish pursued if possible. Quickly removing sick or dead fish from the holding system can help decrease the potential for transmission of disease, especially for those that are transmitted orally (e.g., *P. neurophilia* and *Mycobacterium* spp.). Documentation of sick and dead fish should be maintained to include details such as the location of affected fish, associated clinical signs, genotype, and age; and these records should be reviewed regularly and evaluated for trends. Large numbers of fish demonstrating the same clinical presentation could also indicate a systemic water quality issue, and thus, should prompt immediate evaluation of water parameters.

The behavior of zebrafish can often be indicative of a health or environmental condition; therefore, it is important that the personnel responsible for performing daily observations of fish are familiar with normal behavior so that abnormal behavior is easily recognized. Zebrafish are a shoaling species and typically swim together in groups. Any fish that appears to be isolated from the shoal should be more closely evaluated. Zebrafish typically swim throughout the tank in even, fluid movements, and darting or other fast irregular movements within the tank can be indicative of stress or disease. Position within the water column for both individual fish and entire tanks of fish should be closely monitored. The inability of an individual fish to maintain adequate buoyancy and spend equal amounts of time throughout the water column is abnormal. Whole shoals of fish remaining at the top of the tank near the air/water interface could indicate inadequate oxygen levels in the tanks, whereas whole shoals remaining in the lower portions of the tank could indicate a gas supersaturation condition. These situations should prompt an immediate evaluation of these water quality parameters. Abnormal body conformation could be associated with infectious disease, genetic background, nutritional deficiency, toxins, trauma, egg retention, or advancing age. Fish that exhibit a decreased appetite compared with other fish in the tank should also be evaluated for disease.

Routine monitoring of growth rate and reproductive parameters can be used to evaluate the overall health of a colony. A decline in reproduction can potentially identify issues with water quality or infectious disease prior to the observation of clinical signs. Consistent documentation and evaluation of spawning and reproductive success of the most commonly used background strains can be used for this purpose. This baseline information can also be helpful when investigating potential causes for poor breeding success in investigator bred fish.

In addition to the daily health monitoring described above, a formal health surveillance program is recommended to evaluate and document the health status of a colony. Each distinct housing system should be evaluated separately as the health status of each may differ (Marancik, 2019). Evaluation of sufficient numbers of randomly selected animals of a uniform genetic background and disease distribution for a given colony would provide the best information regarding colony health. Table 35.2 provides an example of the sample size of fish required to achieve statistical significance for a disease at an estimated prevalence for a given population size (Kent et al., 2009; Simon & Schtll, 1984). In most laboratory research facilities, however, adherence

TABLE 35.2 Sample sizes required for a population (N) to detect at least one infected animal assuming prevalence (P), with 95% confidence[a].

	P											
N	**0.5**	**0.4**	**0.3**	**0.25**	**0.2**	**0.15**	**0.1**	**0.05**	**0.02**	**0.01**	**0.005**	**0.001**
10	4	5	6	7	8	9	10	10	10	10	10	10
20	5	6	8	9	10	13	16	20	20	20	20	20
50	5	7	9	10	13	16	22	35	48	50	50	50
100	5	7	9	11	14	18	26	45	78	96	100	100
200	5	7	9	11	14	19	27	51	105	156	191	200
300	5	7	9	11	14	19	28	54	118	190	260	300
400	5	7	9	11	14	19	28	55	125	211	311	400
500	5	7	9	11	14	19	29	56	129	225	349	500
600	5	7	9	11	14	19	29	57	132	236	379	597
700	5	7	9	11	14	19	29	57	134	243	403	691
800	5	7	9	11	14	19	29	57	136	250	422	782
900	5	7	9	11	14	19	29	58	138	255	437	868
1000	5	7	9	11	14	19	29	58	139	259	451	951

[a]Adapted from *Simon et al., 1984*.

to this formula is not practical for several reasons. First, many research colonies are likely to consist of a nonuniform population in terms of genetic background, age, pathogen exposure, and experimental use. Second, high numbers of animals are required to detect a low prevalence pathogen, but the direct use of high numbers of animals in a research colony is often not possible due to research needs, limited availability for testing of some fish with a unique genetic background, and cost limitations of large-scale testing. In lieu of sufficient numbers of randomly selected animals from a colony, a combination of several different methodologies can be used to increase the chances of detecting the presence of disease. This includes the submission of sick colony animals, aged zebrafish (>12 months), fish found in the system sumps with exposure to effluent water, or purposefully placed sentinel zebrafish. Each of these sample types has advantages and disadvantages and can be utilized to provide information about colony health.

Sentinel Fish

Sentinel animals are purposefully placed into an animal colony to aid in the detection of disease. The use of sentinel fish in zebrafish facilities utilizing recirculating housing systems is analogous to the use of "dirty bedding" sentinels in rodent facilities. Sentinel zebrafish are exposed to unfiltered, effluent wastewater from holding tanks from a specific rack, room, or facility, and therefore, have a much higher chance of being exposed to pathogens than colony fish in individual tanks on the system. As a result, fewer sentinel fish would be required for evaluation to detect disease at a given prevalence. The sensitivity for pathogen detection will vary based on the pathogen being evaluated, pathogen prevalence, age and strain of the fish tested, water quality, and other factors. For example, some pathogens, such as some *Mycobacterium* spp., are likely to be more prevalent in older fish, and thus may not lend itself to detection if using younger sentinel fish (Sasaki 2013; Marancik, 2019). Each independent holding system should have its own sentinel fish. The exposure of fish to effluent water can be accomplished by collecting fish housed in effluent "sumps" of the system, or by utilizing fish that are purposefully placed in tanks that are fed with effluent water from a specific sump, or a larger area of that system.

The use of fish from the effluent sump(s) has both advantages and disadvantages. Many effluent sumps of large recirculating systems will become populated with fish originating from the holding system as a normal occurrence. System design and appropriate placement of baffles and handling of tanks will likely influence how many fry or fish are found in the sump. These fish are continuously exposed to large amounts of effluent water containing uneaten food, eggs, debris, and feces from all tanks draining to that sump. These fish are, thus, potentially exposed to high levels of any organisms present without using special equipment and/or fish produced or procured for this purpose. These fish are also being housed in an environment with sub-optimal water quality, which may make them more susceptible or more likely to develop clinical signs of disease. As a result, fish exposed to unfiltered effluent water are more likely to demonstrate histopathologic changes associated with decreased water quality, such as branchial hyperplasia (Marancik, 2019). Fish that reach the sump will no longer be available for research and should be removed routinely as standard practice to avoid the propagation of any diseases they may be carrying; and thus, are available for diagnostic testing. It has been documented that zebrafish of different genotypes may be more susceptible to certain pathogens (Whipps, Matthews, & Kent, 2008), and the genetic background of sump fish will be unknown. The genotype of fish present in a sump is likely heterogeneous, similar to that of the fish on the system; therefore, the population of sump fish may be more representative of the overall susceptibility of the represented colony fish to disease as opposed to using any one strain of wild type fish for testing. However, there may be an unequal distribution of genotypes if, for example, a

user of one line of fish more frequently has fish escaping into the sump. In addition, any previous experimental history, duration of exposure to effluent water, and age of the fish in the sump will be unknown.

The advantages of using effluent sump fish should be balanced with the advantages of using sentinel fish that are purposefully placed in tanks fed with effluent water. Use of sentinel tanks with designated sentinel fish has several advantages to the use of colony fish, or sump fish, including sentinel fish that (1) are not actively being used for research, (2) can be exposed to effluent water from the larger overall holding system, albeit likely at a lower overall volume than fish housed in a sump, (3) are a known age and have had a known exposure period to effluent water, (4) can be easily observed on a daily basis and can be removed from the system quickly if there is evidence of illness, (5) are easy to remove from the system for testing, and (6) are of a known genotype. In addition, historical sentinel data comparisons can be made when sentinels come from the same source. Sentinel fish should be of a known pathogen status with susceptibility to the pathogens of interest and should be placed into the system in which they will be used as sentinels during the larval stage. This minimizes the risk of the introduction of new pathogens associated with the addition of adult fish from another source and allows environmental exposure to be evaluated for the maximum amount of time. A male sex bias toward susceptibility to *P. neurophilia* has been described (Chow, Xue, & Kent, 2016) and might be a consideration if excluding this pathogen; however, similar information is not yet available for other pathogens. The housing of all female fish in one tank is discouraged as this will result in the accumulation of eggs in the females, predisposing them to develop egg-associated inflammation. As mentioned previously, genotype may influence susceptibility to disease, for example, Tübingen (TU) fish appear to be more susceptible to infection from *M.chelonae* (Murray et al., 2011a) and fish that have alterations to their immune system may be more sensitive to several infectious agents. Because the influence of genetic background and susceptibility to disease in zebrafish is still not well defined, the use of sentinel fish that are similar in genetic background to most of the fish used in the particular research colony (often a wild type strain, such as AB or TU) may be preferable. For most zebrafish pathogens, a minimum duration of system water exposure of 3–6 months is recommended to ensure the best chances of pathogen detection, although longer exposure often increases the chance of detecting certain diseases. Frequency of testing for each agent is discussed elsewhere, but will likely be determined based upon expected outcomes (i.e., transmission rate, availability of treatments, associated morbidity/mortality), and financial considerations.

Sentinels exposed to the same filtered system water as colony fish (postfiltration sentinels) can be used to evaluate the efficacy of the filtration and UV disinfection when results are compared with those from sentinel fish exposed to effluent water from that system. Similarly, sentinel fish placed on the quarantine system can be compared with results from both pre- and post filtration sentinels to evaluate the efficacy of quarantine procedures and to detect pathogens that have entered the quarantine system so that additional actions can be taken, if necessary, to prevent entry of the pathogen(s) into the conditioned colony (Martins et al., 2016).

The use of contact sentinel fish, in which fish for sampling are purposefully cohoused in tanks also containing colony fish, has not been used extensively in zebrafish health surveillance programs. To utilize this strategy, zebrafish of a different phenotype can be cohoused with colony animals and subsequently sampled to evaluate the presence of disease in the conditioned colony animals. The number of fish required for evaluation is dependent upon the expected prevalence for each disease and the size of the colony, as these fish would not have greater potential pathogen exposure than the other fish within the colony, in contrast to fish exposed to system effluent water on a grander scale. Even so, this method allows testing without requiring the use of valuable research animals. It can be advantageous in this case to use one of the several phenotypically distinct zebrafish as contact sentinels, including those with hypopigmentation (Lister, Robertson, Lepage, Johnson, & Raible, 1999; White et al., 2008), variations in fin length (Iovine & Johnson, 2000), or altered pigmentation patterns, such as the leopard mutant (Watanabe et al., 2006). Current limitations of utilizing this strategy include limited vendor sources of specific pathogen-free fish and limited knowledge regarding the influence of genetic background on susceptibility to disease.

Colony Fish

Direct sampling of clinically sick colony animals allows for the identification of pathogens which result in clinical disease and can be used to address concerns regarding morbidity and mortality within a colony. In these situations, a greater chance of disease detection may be achieved by sampling these animals, rather than a random sampling of colony animals (Collymore et al., 2016; Crim et al., 2017; Marancik, 2019). Clinical signs, location within the facility (room, holding system, rack, tank location, etc.), genotype, age, and experimental history should be recorded. Best results can be achieved when sick animals are submitted when they are still alive as tissues autolyze quickly following the

death of a fish. This is a reliable way to identify pathogens with a high prevalence without the need to purchase and sacrifice additional animals for screening; however, pathogens of a lower prevalence or that result in subclinical infections may be missed in this approach.

The submission of aged (>12 months) colony zebrafish allows for evaluation of animals with prolonged duration of exposure to pathogens which may be present on a holding system. There are several pathogens, which are more easily detected with prolonged exposure, and which, often cause subclinical infections that would not be detected if only clinically ill fish are evaluated. For example, *P. neurophilia* is more prevalent in zebrafish over 1 year of age (Ramsay, Watral, Schreck, & Kent, 2009b). Older zebrafish are also more likely to be carriers of chronic subclinical or latent infections with *Mycobacterium* spp., and so may harbor greater numbers of bacterial organisms, making pathogen detection easier (Meritet et al., 2017). Removal of aged fish from a system is good practice from a biosecurity standpoint since they may be a reservoir for pathogens, and utilizing these fish as part of a health surveillance program provides a valuable use for them (Kent et al., 2009; Sasaki & Kishi, 2013).

Embryos

Testing of embryos has been used as an adjunct method for detection of disease. The efficacy of this method of evaluation varies by the pathogen being evaluated, pathogen load, the spawning success of the adults utilized to generate the embryos, as well as the proportionate number of embryos from a clutch sampled. There are several flaws with the assumption that negative test results from embryos ensure that the spawning pair that produced those embryos is negative, or that all embryos in the same clutch are negative, as some pathogens may be spread intermittently, and therefore, may not be passed on to all offspring or even to some offspring at each spawning (Crim et al., 2017). For example, *P. neurophilia* is an obligate intracellular pathogen, and therefore, is only capable of growing and reproducing inside the cells of a host, although spores remain viable for long periods of time in the water. Therefore, *P. neurophilia* will be detected in embryos only if there is active external shedding of the organism during the spawning event allowing for extraovum transmission to embryos or rarely, true vertical (intraovum) transmission (Sanders et al. 2013). Other pathogens may be shed intermittently, or may be shed only if the animal is clinically ill. Less healthy animals are not as likely to participate in spawning or to produce sufficient embryos for evaluation, decreasing the sensitivity of this sampling source.

Environmental Samples

Environmental samples can have diagnostic value for some zebrafish diseases. The sensitivity of environmental samples is likely to vary based on pathogen prevalence, exposure levels of the sample being evaluated, and the biology and life cycle of the agent. For example, the collection of tank detritus containing fish feces for double centrifugation with a saturated sugar solution is effective for the isolation and identification of *P. tomentosa* eggs (Murray & Peterson, 2015). However, the sensitivity of detection will likely vary based on the prevalence of the organism and the most prevalent life stage represented in the sample taken. In the case of *P. tomentosa*, for example, adult worms are found in the fish, and eggs in the environment, therefore, the life stage and numbers of the worms present will influence the numbers of eggs released into the environment that could be detected. The concentration of effluent water by filtration through a filter membrane followed by extraction of DNA for molecular diagnostic analysis allows a large volume of water to be evaluated as a single sample but may not be effective at detecting all pathogens. For example, real-time PCR of water concentrated by filtration has been shown to be useful for the detection of several *Mycobacterium* spp., but inconsistently detects other pathogens, such as *Pseudoloma neurophilia* and *Pseudocapillaria tomentosa*.

Biofilms from tanks, gutters, sumps, or water lines on the system may be more useful for the evaluation of environmental bacteria, including *Mycobacterium* spp., or fungal pathogens having the potential to result in disease (Whipps et al., 2012). Areas exposed to high flows of effluent are likely to be of greater value, such as debris from the bottom of a specific tank or sump or from filter pads receiving effluent water. Evaluation of detritus has been shown to be sensitive for the detection of some *Mycobacterium* spp., and *Pseudocapillaria tomentosa*, with the evaluation of feces, also being sensitive for the detection of *Pseudocapillaria tomentosa* (Crim et al., 2017). This provides useful information which can be incorporated into the design of a health surveillance program. Interpretation of environmental results must be evaluated with knowledge and additional testing methodologies (e.g., histopathology) regarding the potential for the specific organism to cause disease and the particular risk to the colony based on the immune status of the fish and research goals. For example, the presence of environmental bacteria does not necessarily reflect infection of the fish on that system. In contrast, negative results may not be reflecting the presence of infection in fish for organisms that will only be present in the environment following the shedding of spores or eggs (i.e., *Pseudoloma neurophilia, Pseudocapillaria tomentosa).*

Testing Methods

Testing methods and commercial diagnostic services for zebrafish have become more accessible in recent years, and both environmental and in vivo testing methodologies are available for some, but not all agents. Unfortunately, the most comprehensive method for detecting the presence of disease in zebrafish requires the inclusion of lethal testing methodologies. Several testing methods will be discussed briefly here as more comprehensive details regarding diagnostic procedures can be found elsewhere in this book.

External parasites can be diagnosed in fish utilizing nonlethal methods, such as examination of external slide impressions or fin clip samples taken from anesthetized fish. A more comprehensive evaluation, however, can be conducted by utilizing fin clips, gill clips, or cutaneous wet mounts together when the entire body is available for diagnostic evaluation. When screening for *Pseudoloma neurophilia* exclusively, a simple brain prep can be utilized (Matthews et al., 2001).

Gross necropsy incorporating external examination for external parasites, followed by internal necropsy with sterile culture and subsequent harvest of all organs for histopathology, is a method for a comprehensive evaluation of animal health and allows for detection of both infectious and noninfectious diseases. Noninfectious diseases that could be detected utilizing these testing methods include neoplasia, egg-associated inflammation, degenerative lesions, or other changes which may be indicative of poor water quality or the presence of toxins or nutritional deficiencies. Although complete histopathologic evaluation allows for a comprehensive evaluation, it may not always be the most sensitive method for all agents, and molecular diagnostic methods (i.e., polymerase chain reaction) may be more sensitive. Gross necropsy is best conducted immediately after euthanasia to avoid rapid autolysis of tissues. A dissecting microscope is often helpful for collection of samples and gross evaluation of organs. Histopathology results of animals found dead are often nonconclusive because of autolysis.

Sterile samples may be cultured for identification of some pathogens as part of a necropsy. The collection of sterile culture samples requires advanced planning and technical skills to decrease sample contamination and to ensure diagnostic value. The potential bacterial species of interest should be considered before samples are collected to allow for the selection of the appropriate culture media and incubation temperature. Attempts to collect samples for bacterial culture in zebrafish should only occur immediately after euthanasia as the bacterial species present can change during postmortem autolysis of the fish. Before the fish is opened for culture, its surface should be disinfected with 70% ethanol. Sterile instruments must be used to ensure sample integrity, and nonpowdered moistened gloves should be used to avoid contamination of samples with substances on the gloves. Organ cultures from the spleen and liver may allow isolation of *Mycobacterium* spp., and organ culture from the kidney (from either a dorsal or ventral approach) may be used for isolation of other organisms (Whipps et al., 2008; Zebrafish International Resource Center, 2016). Although culture may be diagnostic for known pathogenic organisms, positive results may be of questionable value unless evaluated in context with histopathologic findings or clinical disease.

Molecular diagnostic methods, such as real-time polymerase chain reaction (PCR), conventional PCR, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) are available for several known zebrafish pathogens. These testing methods can be used to evaluate live and dead animals, embryos, and environmental samples. PCR can be used to evaluate biofilms, detritus, food (both live and processed diets), feces, filter material, water, surface swabs, sperm, and microbial cultures. When used for the evaluation of live animals, there are several advantages to PCR. When compared with histopathology, PCR has greater sensitivity for small pathogenic burdens, as with some P. neurophilia infections, which may not be seen via histopathology (Kent et al., 2009; Murray et al., 2011b). Histopathology alone cannot be used to identify specific *Mycobacterium spp*., or the specific agent involved in other bacterial or fungal infections (Astrofsky, Schrenzel, Bullis, Smolowitz, & Fox, 2000). That is, a specific histological lesion may suggest an agent, but species-level identification requires molecular testing in most cases. Many fish can be pooled for molecular diagnostic evaluation, allowing for large numbers of fish to be sampled at once; and often allowing for a faster turnaround time compared to histopathology, standard microbial culture, or culturing of slow-growing bacteria (i.e., *Mycobacterium* spp.). Submission of a fresh-frozen whole fish is ideal to ensure representation of all organ systems for testing and to provide the most comprehensive information, especially for the evaluation of pathogens, such as *Mycobacterium* spp., which have a high limit of detection in PCR assays. However, fish that are found dead can be submitted for PCR analysis when histopathology would not provide any diagnostic value due to rapid tissue autolysis. Due to the small size of the zebrafish, it is often not practical to perform both histopathology and PCR on the same fish without decreasing the diagnostic sensitivity of both due to insufficient sample evaluation. However, a moderate level of sensitivity has been demonstrated when

evaluating formalin-fixed paraffin-embedded samples for some *Mycobacterium* spp. (Meritet et al., 2017) and may assist with speciation if acid-fast bacteria are noted during a histological evaluation.

In summary, the testing methods utilized will often be based on the facility exclusion list and the best testing methodology available for the pathogens of greatest importance. A combination of gross necropsy with pathology and molecular diagnostic techniques, in addition to environmental testing, can likely provide the complete picture of the health status of a colony. The specific testing strategies used becomes increasingly more important if a facility wishes to exclude pathogens, which will not be eliminated with embryo surface disinfection.

Health Report

A health surveillance report should be available for all zebrafish colonies and should include a summary of recent tests, test results, as well as significant historical information. A housing and husbandry report may also be created to describe standard housing and husbandry procedures, including an overview of the quarantine and health surveillance programs and a description of acceptable and nonacceptable pathogens for that facility. Please see Table 35.3 for an example of a health surveillance report. An example of a housing and husbandry report has also been published (Collymore et al., 2016).

Health reports should be made available to all researchers utilizing the facility. Pathogen status should be included when reporting experimental results in publications and should be critically evaluated by researchers to determine what influence, if any, pathogen status may have on their studies.

In addition, health information should be disclosed whenever zebrafish are transferred between facilities. If not readily provided when arrangements for the transfer of fish are made, the receiving institution should request a health surveillance report prior to receiving the fish. It is imperative to disclose all positive test results because the presence of some diseases, especially those that have no treatments and are not prevented by embryo surface disinfection, can have a devastating effect on both animal health and research programs.

Disease Prevention

An adequate health surveillance program, the provision of a stable aquatic environment, and adherence to appropriate quarantine and biosecurity practices are all important aspects of disease prevention. Adequate detection of disease is required in order to quickly identify and remove sick fish and minimize transmission of disease. Similarly, there are other practices which can help minimize the transmission of new or endemic diseases.

All efforts should be made to maintain breeding stock that is as free as possible from diseases. This can be achieved by replacing the breeding stock with fish of a known health status every 6–12 months. Ideally, fish used for spawning are only crossed with fish from one other tank or group of tanks. Because the rate of transmission for some pathogens, such as *P. neurophilia*, increases during spawning events, spawning tanks or mass spawning chambers should be disinfected using a validated method, after each spawning event (Peneyra et al., 2018).

Older fish are more likely to be infected with pathogens, such as *P. neurophilia* or *Mycobacterium* spp., and are more likely to succumb to illness from disease and/or to potentially transmit disease as they age or are exposed to experimental stress (Keller & Murtha, 2004; Parikka et al., 2012; Spitsbergen & Kent, 2003). Colony culling of all fish older than 1–1.5 years of age has been recommended. Alternatively, these older fish may be moved to quarantine (Borges et al., 2016; Collymore et al., 2016) if they are valuable as aged fish for experimental reasons. If maintained in the colony for longer periods of time, these fish are likely to serve as reservoirs of infection. They may also eventually die in their tank and subsequently expose their tank mates to disease after oral ingestion of spores or other infectious particles from their carcass occurs (Murray et al., 2011b). As mentioned earlier, these culled fish may also be useful for health surveillance purposes.

Routine monitoring of system water quality parameters ensures a stable environment for zebrafish and has a direct influence on zebrafish health. Alterations in water quality can result in stress and subsequent expression of or susceptibility to disease. Water quality monitoring should include confirmation of adequate water exchanges in recirculating systems, confirmation of adequate gross particle filtration, and frequent monitoring and verification of values for important parameters including pH, temperature, conductivity, hardness/alkalinity, levels of nitrogenous waste, and contaminants, such as chlorine. Stocking density can also impact water quality. Recirculating water systems should include adequately sized UV disinfection units to assist with the prevention of tank to tank transmission of pathogens. The level of UV irradiation required to eliminate aquatic pathogens has been published (Harper & Lawrence, 2011). The functioning of the UV lights should be verified on a frequent basis, and the UV light bulbs and associated quartz sleeves should be replaced as per the manufacturer's recommendations.

One of the most important aspects of biosecurity involves the prevention of pathogen entry into a facility.

TABLE 35.3 Example of a health surveillance report[a].

Health Surveillance Report for Zebrafish											
Institution:								**Date:**			
Building								Re-circulating			
Room								Flow through			
System/Rack Identification								Static			
Contact person: (name, e-mail, phone)								Other			
								Recent testing		**Historical results**	
	Tested Subjet (sentinel, colony fish, sump fish, environmental)	**Sampling Location** (pre-filtration, post-filtration, main colony, quarantine)	**Age of fish**	**Exposure time**	**Testing Frequency**	**Testing Method**	**Testing Laboratory**	**Sampling Date**	**# positives/ # tested**	**Sampling Date**	**Collected over ___ months # positives/ # tested**
Bacteria											
Aeromonas hydrophila											
Edwardsiella ictaluri											
Flavobacterium columnare											
Mycobacterium spp.											
Mycobacterium abscessus											
Mycobacterium chelonae											
Mycobacterium fortuitum											
Mycobacterium haemophilum											
Mycobacterium marinum											
Mycobacterium peregrinum											
Microsporidia											
Pseudoloma neurophilia											
Pleistophora hyphessobryconis											
Protozoa											
Ichthyophthirius multifilis											
Piscinoodinum pillulare											
Fungi											
Saprolegnia brachydanis											
Parasites											
Pseudocapillaria tomentosa											
Additional Agents											
Infectious spleen and kidney necrosis virus (ISKNV)											
Spring Viremia of Carp Virus (SVCV)											
Pathology:											
Additonal comments:											

[a] *Adapted with modifications from Collymore et al., (2016).*

Limitation of the entry of pathogens associated with live fish and embryos is managed with an appropriately designed import and quarantine program. This is discussed in detail in another chapter of this book. Limitation of the entry of pathogens by individuals handling and caring for the fish must also be considered. Individuals that provide care to fish at home or at other locations (e.g., pet stores, schools) should be counseled regarding necessary disease prevention precautions, which may include not handling other aquatic species in the morning prior to entering the zebrafish facility. Personal Protective Equipment

(PPE) requirements should be determined for all areas where zebrafish are manipulated, including laboratories, holding rooms, and quarantine. To avoid the transfer of water from room to room, disinfection mats or baths, shoe covers or dedicated shoes may be used. Due to the possibility of exposure to zoonotic pathogens, such as *Mycobacterium* spp., the use of gloves and thorough washing of hands and arms when leaving the aquatic facilities is recommended.

Limiting the entry of pathogens in the feed provided to animals is another important aspect of biosecurity and also must be considered during disease evaluation. Most dry diets utilize a fish protein source and information regarding measures taken to ensure that the protein source does not contain pathogenic organisms. The quality assurance methods utilized by the vendor providing the feed should be critically evaluated. For example, does the vendor test for aquatic pathogens or autoclave or irradiate the diet? Irradiation is one method for preventing the transmission of disease in animal diets (Reuter, Livingston, & Leblanc, 2011; Watson, 2013). In the absence of defined quality assurance for feed, testing for the presence of pathogens, via PCR is an option. Similarly, the feeding of a live diet, such as paramecium, *Artemia* (brine shrimp) or rotifers (i.e., *Brachionus* spp.), carries an inherent risk. It has been demonstrated that paramecium, rotifers, and *Artemia* can be used as a vector for the transmission of *Mycobacterium* and other bacteria, including *Aeromonas* spp. (Cantas, Sørby, Alestrom, & Sørum, 2012; Mason et al., 2016; Peterson et al., 2013). *Artemia* are nonselective filter feeders and can also be infected with microsporidia and other parasites (Hoj, Bourne, & Hall, 2009; Méndez-Hermida, 2007). Crustaceans have been documented to pass microsporidian spores through their digestive tract, experimentally resulting in heavier infections than fish infected through direct IP injection (Solter, 2014) and have been suggested as a vector for cryptosporidiosis in other fish (Méndez-Hermida, Gómez-Couso, & Parasitol Res, 2007). *Artemia* is commonly sourced from natural lakes with varying levels of lead, arsenic, chromium, and other metal contaminants or pollutants with limited quality control (Peterson & Gustin, 2008; Tye, 2018). This results in a food source with varying nutritional content that can potentially introduce pathogens or other nonbiological contaminants.

Workflow for feeding and other procedures should begin with provision of care to embryos and larvae first since they are less likely to be infected with a disease, then to adult fish, then aged fish, and finally to quarantined fish. Because of the risk for water splashing to tanks below during removal of tanks or other manipulations, it has been recommended that younger fish be placed above older fish on racks when possible and that the younger fish be fed first (Mason et al., 2016). Ideally, the staff providing care to quarantine areas are distinct from those providing care to the conditioned colony. If staff providing care to zebrafish also provide care to other animal species, an appropriate workflow and PPE procedures for these individuals must be determined based on the health status and transmissibility of pathogens of all animal species being cared for.

Prevention of tank to tank transmission of disease within a facility is closely associated with the proper handling of tanks and the use of validated cleaning methods. Nets and other supplies should be used for only one tank and should be adequately disinfected and thoroughly dried, using a validated method, prior to being used in another tank. Similarly, feeding implements ideally should not come into contact with the lids of multiple tanks during a feeding session. Cleaning methods for tanks, baffles, lids, and enrichment devices should all be validated to be effective and should utilize methods that are either nontoxic to the fish or that incorporate appropriate rinsing procedures to avoid the possibility of chemical residues (Martins et al., 2016). Regular cleaning and sanitization of holding tanks will allow for adequate visualization of the fish during health monitoring and also will prevent the accumulation of bryozoan and biofilms. Sanitization methods may be evaluated for efficacy by utilizing ATP-based monitoring systems, cultures, or PCR (Collymore, Porelli, Lieggi, & Lipman, 2014; Garcia & Sanders, 2011) and for safety utilizing chemical detection kits or embryo toxicity studies. Tank biofilms colonized with *Mycobacterium*spp. have been associated with transmission of this disease (Chang, 2019), emphasizing the need for regular sanitization of holding tanks, and any enrichment in the holding tanks, utilizing validated methods.

The impact on fish health must be considered if adding plants or other structural enrichment devices into an aquatic environment. The release of chemical components (e.g., phthalates, Bisphenol A) may have direct or indirect biologic effects on physiology, pharmacokinetics, behavior, and neoplasia (Lawrence & Mason, 2012; Mathieu-Denoncourt et al., 2015). These structures also increase the surface area for microbial growth which can impact water chemistry and algal and bacterial growth. The structure may also provide a good location for the harboring of pathogens (i.e., parasites, *Mycobacterium* spp.). As such, all enrichment devices require adequate sanitization, by a validated method, prior to being moved into a different tank. These devices may also provide additional surface area for chemical binding of cleaning agents, so if a chemical method of disinfection is chosen, care should be taken to ensure no residual chemical remains. Other considerations include the physical presence of structural

devices and any associated potential to cause physical harm (i.e., skin damage leading to secondary infections, choking, etc.) and finally any behavioral impact (alteration in swimming patterns, territory aggression, etc.).

Pest control in a zebrafish facility must take into account aquatic, terrestrial, and airborne parasites. Due to the sensitivity of zebrafish to chemicals in their environment, prevention by maintaining a clean facility is of paramount importance. This includes minimizing the amount of food and other organic material available to pests, by maintaining food in closed containers, maintaining lids on tanks and sumps, minimizing food on lids of tanks, avoiding standing water, and flushing floor and sink drains daily. The maintenance of snails, algae-eating fish, or other organisms within a zebrafish holding system should be avoided as these animals often have an unknown health status and have the potential to transmit disease either directly (i.e., *P. hyphessobryconis*, *P.neurophilia)* or indirectly (i.e., *P. tomentosa*, *Transversotrem* spp.) (Sanders et al. 2010, 2016; Womble, Cox-Gardiner, Cribb, & Bullard, 2015).

As with other animal species, adequate cleanliness of the facility, verification of appropriate lighting (levels, cycling, wavelength), and development of an emergency response plan are all important for providing adequate care.

In summary, health surveillance of laboratory zebrafish colonies is necessary to ensure animal welfare and responsible use of animals. Timely recognition of the presence of infectious agents will be of assistance in the appropriate management of these conditions and will allow for safe collaboration, including the sharing of different zebrafish lines, with other scientists. In addition, several zebrafish pathogens result in pathology that may influence research, therefore, reporting the presence of these pathogens in scientific publications is of paramount importance to ensure adequate transparency of research results.

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CHAPTER

36

Importation and Quarantine

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Introduction

The importation of new zebrafish increases the risk of colony animals being exposed to pathogens if the imported fish are not managed appropriately. In an existing facility, it is important to identify any pathogens already present and determine the ones that will be excluded from the conditioned colony. Determination of excluded pathogens should be based on the direct impact of the pathogens on animal health (i.e., associated morbidity, mortality) and research results, their zoonotic potential, and financial resources available. An importation and quarantine program should be designed to minimize risk to the existing colony, while still allowing for scientific collaboration, sharing of fish lines, and adherence to regulations that require tracking numbers of vertebrate animals used in research.

Importation Request and Approval

Defined animal requisition and importation procedures should be agreed upon between the researchers, veterinarians, zebrafish facility staff, and the Institutional Animal Care and Use Committee (IACUC) to ensure that all aspects of importation and regulatory control are met. Currently, commercial vendors dedicated to the production of Specific Pathogen Free (SPF) zebrafish for research do not exist. As a result, most fish obtained from outside sources are obtained through a zebrafish repository, such as the Zebrafish International Resources Center (ZIRC; University of Oregon, Eugene, OR, United States of America) or the European Zebrafish Resource Center (Karlsruhe Institute of Technology, Institute of Toxicology and Genetics, Eggenstein-Leopoldshafen, Germany); other research labs around the world that can provide SPF fish (SARL Facility, Oregon State University, Corvallis, OR, United States of America); or other laboratories that may not be able to provide health status. As a result, the importation of zebrafish may best be handled like that for rodents or other species from noncommercial sources. A centralized animal ordering program is preferable to achieve the greatest control over the process. Importation requests should be cross-checked, following the institution's standard animal ordering procedures, to confirm that the number and genetic line of zebrafish requested are described in an approved IACUC protocol and that the number of animals (including embryos and larvae) imported are deducted accurately from that protocol.

If the receiving institution wishes to exclude specific pathogens, the health status of the exporting colony should be evaluated, ideally by a veterinarian, to determine if the shipment should be accepted, if special accommodations are needed for pathogen control, or if it should be rejected. For example, fish requested from a colony infected, or potentially infected, with an excluded pathogen may be accepted with a requirement for the administration of a specific treatment prior to shipment or upon arrival (e.g., surface disinfection of embryos, anthelminthic treatment, etc.). Alternatively, such a shipment may require further testing upon arrival, or standard quarantine procedures may be sufficient to prevent transmission of that specific pathogen, or unknown pathogens, to the conditioned colony. Some sources may be designated as "approved vendors." Granting this status to a vendor generally requires the exporting facility to conduct routine colony health monitoring and to accurately disclose those results, which, at the time of this writing, is not well standardized and is inconsistently done for zebrafish. Even if a health report is available, the information must be reviewed with the understanding that results are always

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retrospective and may not reflect the current health status of the colony and thus, an understanding of the biosecurity practices of the exporting facility is also required. If a detailed health history and testing results are not available, the importing institution may attempt to retrieve details regarding the import, quarantine, and health monitoring procedures of the exporting institution to better analyze risk, or may request additional animals to be shipped to allow for more stringent quarantine strategies, including diagnostic testing, to be implemented. When receiving additional animals for testing upon arrival, it should be recognized that these fish will only be representative of a small group of fish from the colony and will likely only be valuable for detecting disease conditions that are highly prevalent in the source colony. Therefore, diagnostic testing on these animals would best be used to evaluate the health status of offspring generated from these specific animals, rather than used to extrapolate the health status of the originating colony. Some of these strategies will be discussed more thoroughly in the quarantine section below. Once the responsible facility administrator has reviewed all information provided on colony health status and determined the most appropriate location or handling procedures to be used upon arrival of the imported fish, it should be verified that there is adequate space in the quarantine area to accommodate them. Even if embryos or larvae are being received, accommodations should be planned for adult fish in the conditioned colony for the eventual housing of larvae derived from surface disinfected embryos.

Receipt

Zebrafish are typically exported as either embryos or adults, and there are advantages and disadvantages to each method. Embryos may be surface disinfected prior to shipping to decrease potential contaminants that can impact water quality during shipping and decrease the risk of transmission of some pathogens to the receiving facilities, and a relatively large number of embryos can be held in a small container. Embryos do not require an external source of nutrition and do not generate large amounts of waste during transport. When imported embryos/larvae are received they are typically maintained on the quarantine system until they are able to produce embryos on their own, which can then be surface disinfected for placement into the main conditioned colony; this process may take 2–3 months.

Additional considerations are required when shipping adults to alleviate the adverse effects of nitrogenous waste production and decreased water oxygenation. Shipments of adult fish must be limited to smaller numbers in a given volume of water, when compared to embryos, to allow for safe shipping. Please refer to the Exportation and Transport chapter for more details regarding regulations and methods for shipping embryos and adults.

Receipt procedures will differ based on the life stage of animals; however, the first step for all life stages is to inspect the shipping container for damage before it is opened for removal of animals. Upon receipt of embryos, the container should first be evaluated for integrity and location of embryos; it will be easier to remove embryos from the container if they are suspended in water. Embryos should be removed from the shipping container as soon as possible upon receipt. The embryos should be transferred to a clean container filled with embryo media or autoclaved system water, and the shipping water should be discarded down the drain. The easiest way to do this is to pour the contents of the shipping container through a nonabrasive strainer to catch the embryos, which are then gently rinsed, while still in the strainer, with embryo media or autoclaved system water. No more than 50–100 embryos should then be transferred into a standard 90 mm Petri dish filled with embryo media or autoclaved system water. It is important to limit the density of embryos and to use a clean media to minimize fungal, protozoan, and bacterial contamination and to provide sufficient aeration (Nüsslein-Volhard and Dahm, 2002, chap. 1). The embryos should be evaluated and any nonviable embryos (see Fig. 36.1) or debris removed, as this will contribute to the deterioration of water quality within the Petri dish. The embryos are then placed in a dedicated quarantine incubator and maintained at $28.5 \pm 2^{\circ}C$. If embryos were not surface disinfected prior to shipping, facility quarantine policies would dictate if surface disinfection of embryos upon arrival is required. However, embryos must not be disinfected once they begin to emerge from the chorion as this may lead to deformities or death of embryos/larvae.

Adult zebrafish should be inspected as soon as possible upon arrival to evaluate their overall condition and health status. Fish that appear to be stressed will require an accelerated acclimation process to get them into better water conditions more quickly. From the time adult fish are placed into shipping containers of static water, conditions of the water begin to change. Fish consume oxygen and release carbon dioxide, which combines with water forming a weak acid and decreasing pH. Because nitrogenous waste is also released by fish during transport, the decreased pH is somewhat beneficial because it decreases the ratio of toxic, un-ionized ammonia (NH_3) to nontoxic, ionized

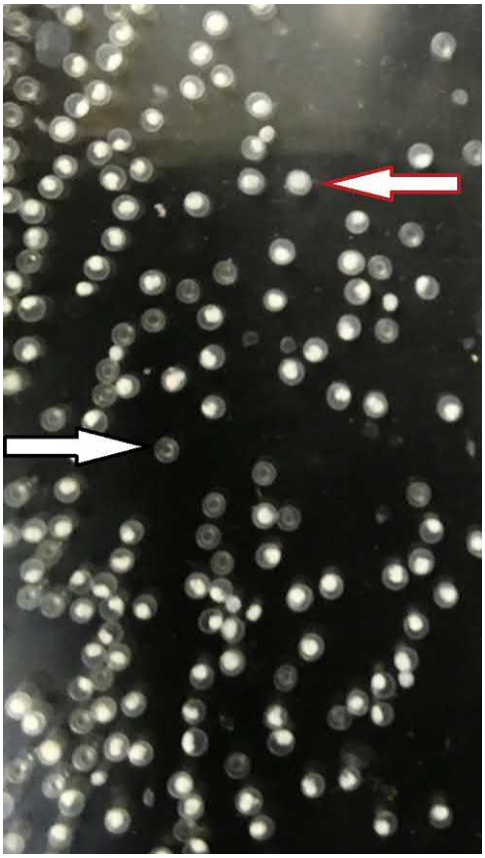

FIGURE 36.1 No magnification. The chorion of a viable developing embryo will be transparent (*white arrow* with black outline), whereas nonviable embryos will appear opaque (*white arrow* with red outline). This clutch demonstrated a high level of embryo death following exposure to 100 ppm sodium hypochlorite for 10 min.

ammonia (NH_4^+), also known as ammonium. Once the shipping bag is opened and exposed to the environment, the carbon dioxide is released into the environment, resulting in a quick increase in pH of the water, shifting the balance toward a higher level of toxic ammonia. Therefore, the shipping bag holding fish should not be opened until fish are ready to be transferred out of the bag (Harper and Lawrence, 2011). It is also important to remember that the water inside and on the surface of the bag maybe contaminated with unwanted organisms; transfer of these organisms to the receiving facility should be avoided. Prior to removing fish from the bag, they should be acclimated to the temperature on the holding system. To achieve this while minimizing contamination risk, a static tank can be filled with water at the same temperature as that of the holding system, and the shipping bag can be placed into this tank to allow the water in the bag to acclimate (Fig. 36.2). It is estimated that acclimation of small volumes of water requires about 5 min per degree of temperature difference.

FIGURE 36.2 Without opening the bag, the shipping bag containing imported fish is placed in a static tank or other container filled with water that is the same temperature as the holding system to allow the water in the bag to acclimate.

When temperature acclimation is satisfactory, cut a hole in the bag and then pour the water and fish through this hole into a perforated strainer or breeding tank insert to separate the fish from the shipping water (Figs. 36.3 and 36.4). Then quickly place the fish into their new holding tank without placing the net inside the new tank, and discard shipping water down the drain (Fig. 36.5). Any equipment exposed to the shipping water should not contact the system water or facility equipment until after it has been adequately disinfected. Continue to evaluate the fish after the transfer, and allow fish to acclimate to their new environment for at least 24 h prior to feeding and 2–5 weeks prior to breeding.

It is important to minimize the stress of animals during transportation and receipt and to provide a stable environment once animals are received into a new facility. Stress is known to result in several physiologic changes in fish, including cortisol release (Ramsay et al., 2006, 2009a; Schreck, 1996), which can lead to immunosuppression, allowing a previously subclinical infection to exert clinical effects and result in shedding of organisms into the environment (Dror et al., 2006; Ramsay, Watral, Schreck, & Kent, 2009b). Because of this, it is important to ensure that the receipt of animals is as stress free as possible, and handling of animals, once they have arrived, should be minimized to allow them to acclimate to their new environment. For this reason, it is recommended that fish are simply observed for overall health status for the first 2–5 weeks after arrival before breeding or any other manipulations begin.

FIGURE 36.3 Example of equipment preparation for the transfer of imported adult fish onto a quarantine holding system following water temperature acclimation. There is a tank and a net to be used for separating fish from the shipping water and a labeled tank filled with system water that will be used to house new fish in the quarantine holding system.

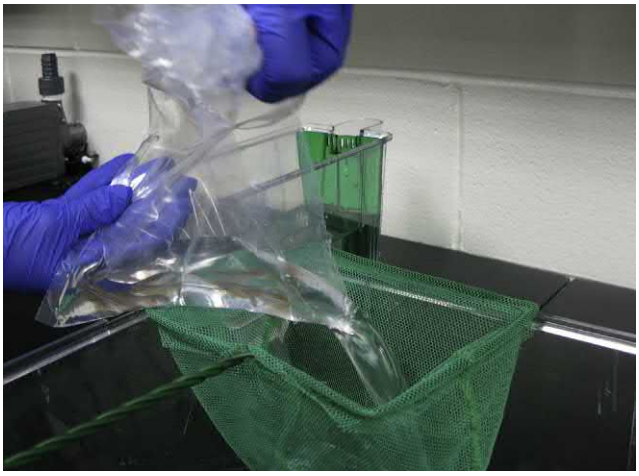

FIGURE 36.4 Following water temperature acclimation, the fish are separated from the shipping water and moved into a holding tank. A hole is cut in the bag, and the contents of the shipping bag are poured through a net. The captured shipping water is then disposed of down the drain.

FIGURE 36.5 Fish are quickly placed into their new holding tank without putting the net inside the new tank.

Quarantine Goals

The establishment of defined quarantine procedures is the first step in ensuring biosecurity during the importation of fish from outside sources. Because there are currently limited commercial sources of zebrafish guaranteed to be free of known pathogens, quarantine of incoming fish is extremely important. In the case of zebrafish, the purposes of quarantine are to allow the import of fish from colonies that may have an undefined health status, provide the opportunity to evaluate these for the presence of disease, prevent disease from incoming fish to be transferred to the main colony, and allow for acclimation of incoming fish. When the main conditioned colony is shared by several different laboratories, the importance of quarantine increases as any break in biosecurity will have the potential to affect a large number of users. This risk is amplified further when the conditioned colony is maintained on a recirculating holding system.

General Quarantine Practices

The most universally utilized quarantine concept used by zebrafish facilities is an "eggs only" policy, meaning that only larvae derived from surface disinfected embryos are allowed to be placed on the housing system used for the conditioned colony. Therefore, all imported embryos, larvae, or adults are initially placed into the quarantine system to undergo treatments, procedures, or evaluations. All embryos generated in quarantine are (at minimum) surface disinfected prior to being placed as larvae into the designated nursery area within the conditioned colony. Once the imported line is established on the main system, the imported fish on the quarantine system should be culled. Imported fish should not be allowed to remain on the quarantine

system longer than necessary to decrease the amount of fish that are potentially exposed to the disease each time a new import is added, and to allow for adequate space when new imports are requested. Generally, the entire process for an import should be completed in <6 months.

Close visual evaluation of fish for the presence/absence of disease within the first 1–2 weeks of arrival is an important aspect of biosecurity. Shipping can be stressful for adult fish, and this can result in the development or expression of disease. The environment of the receiving facility likely varies from the originating facility, and differences in husbandry and the microflora of the new facility may alter the microbiome of the fish and/or disease expression (Breen et.al., 2019). There have been reports of zebrafish that appear clinically healthy upon arrival, but then begin showing signs of illness or begin shedding disease during the quarantine period at a new facility. One such example involves epizootics of Edwardsiellosis, in which fish appeared healthy or demonstrated minor evidence of shipping stress upon arrival, but high levels of morbidity were noted 24 h to 3 weeks after arrival (Hawke et al., 2013). Similarly, fish of different strains or at different facilities may be more or less susceptible to parasites, such as *Pseudocapillaria tomentosa* (*P. tomentosa)* (Murray and Peterson, 2015). In addition to clinical observations, whole fish or environmental samples associated with imported animals may be evaluated for the presence of disease.

Quarantine Strategies

The quarantine strategy utilized must be determined based on each facility's uses and goals. The goals should be agreed upon by all users of the facility. This requires all users to be educated regarding the currently known pathogens of zebrafish and the impacts of these diseases on the health of their fish and their research. It must then be determined which pathogens, if any, are acceptable and which should be excluded so that the quarantine program can be designed to provide the best chances of preventing entry of these excluded organism(s) into the main colony while minimizing unnecessary delays in the use of newly imported fish. Restriction of pathogens that are not easily diagnosed or treated, such as the microsporidian pathogens, will require vigorous and possibly lengthy quarantine procedures.

The first step of importation involves obtaining a health history and, if possible, a health report from the exporting institution. If the exporting institution lacks sufficient quarantine procedures or reports the presence of a pathogen that the importing institution wishes to exclude, then treatments, off-system or static tank housing, and/or additional testing may be required. The importation of zebrafish from nonconventional sources, such as pet stores, or pet store suppliers, requires extra caution. External parasitic infections, such as *Ichthyophthirius multifilis,* are common in these environments and the introduction of *Pleistophora hyphessobryconis* (*P. hyphessobryconis*) to laboratory zebrafish has been associated with the importation of zebrafish from these sources (Sanders et al., 2010).

The most basic, and currently the most common import strategy, involves placement of imported larvae or adult fish on a quarantine system, followed by visual evaluation of fish for the presence/absence of disease. If no clinical signs of disease are noted, fish are bred, embryos are surface disinfected, and larvae hatched from those embryos are placed on the main holding system. To be most effective, evaluation for clinical disease must happen before surface disinfected embryos are placed on the recirculating holding system with the conditioned colony. Recommendations for time of observation vary from 1 to 5 weeks, with the longer recommendations based on the reported appearance of mycobacteriosis-associated ulcerative lesions as late as 4 weeks after import (Westerfield, 2007; Murray, Varga, & Kent, 2016; Geisler, Borel, Ferg, Maier, & Strähle, 2016; Mason et al., 2016). This strategy is only useful in eliminating the introduction of pathogens that are susceptible to embryo surface disinfection via the chosen surface disinfection method. It should be noted that the efficacy of surface disinfection for prevention of transmission of several zebrafish pathogens is not yet known. When larvae or embryos are imported utilizing this system, there may be a 45–90 day waiting period from the time of arrival until sexual maturation and generation of embryos. Some surface disinfection methods may result in toxicity or decreased survival of embryos, which could be a concern if small numbers of adults are imported or if newly imported adults are not breeding well. In these cases, adults will need to be maintained in quarantine for a longer interval, and multiple spawning events may be needed to generate sufficient numbers of the imported line. For some research conducted on earlier life stages, studies may be performed in quarantine, which is a biosecurity risk if research staff enter quarantine frequently while also working with fish in the conditioned colony. In cases where embryos are imported prior to hatching, some facilities may allow embryos to be surface disinfected upon arrival,

bypassing quarantine, followed by placement on the system. This practice does not allow imports to be isolated and visually evaluated for the expression of disease following import, or the opportunity for the diagnostic evaluation of clinical signs that could manifest prior to exposure to the conditioned colony. If chlorine surface disinfection is used, it should be noted that chlorine toxicity is more likely to affect zebrafish embryos at later time points postfertilization (≥24 h) than at earlier time points; thus, chlorine surface disinfection of imported embryos after arrival may result in small numbers of surviving larvae (Kent, Buchner, Barton, & Tanguay, 2014). The overall potential for the introduction of disease to the facility utilizing this importing strategy is high because the importing institution is reliant on receiving accurate health information from the exporting institution. However, this approach may be necessary if a quarantine facility is not available.

Several variations of this basic import strategy can be used to decrease risk, including the use of a "high risk" and a "low risk" importation scheme, utilizing alternating quarantine racks to allow for their periodic disinfection, or utilizing a two-stage quarantine system. The use of a separate "high risk" quarantine holding system allows researchers to import animals with an unknown health status for evaluation and conditioning on a "high risk" holding system, while minimizing risk to fish imported with known pathogen status, or larvae derived from bleached embryos, separately maintained on a "low risk" system (Martins et al., 2016). If a highly contagious or lethal pathogen is imported on the high-risk system, valuable imports on the low-risk system will be spared. Alternatively, if two quarantine racks are available, another strategy is to use one rack to house all imports arriving over a period of 6–12 months. As the census in the first rack begins to decrease as disinfected embryos are moved into the conditioned colony, imported fish can start being placed on the second holding system. Once the first holding system is empty, that holding system can be surface disinfected, decreasing potential cumulative pathogen load, and preparing it for the next import cycle. Finally, two holding systems can be used as a two-stage quarantine system. Imported larvae or adults are imported into the first holding system, and embryos generated on that system are surface disinfected and placed on the second stage quarantine system. Embryos generated on the second holding system are surface disinfected for placement onto the holding system of the conditioned colony. Other variations have been published with various advantages and disadvantages (Liu et al., 2016).

A method to decrease the potential pathogen load of imported fish is to import only surface disinfected embryos. This theoretically decreases the load of pathogenic organisms brought into the quarantine facility due to the decrease in pathogen load from contact with the disinfectant, along with dislodgement of pathogens from the chorion by mechanical rinsing during the disinfection procedure. The acceptance of embryos that have been surface disinfected by the exporting institution for placement with the conditioned colony is discouraged as the receiving institution will have little ability to monitor that surface disinfection has been carried out properly and because surface disinfection methods vary by institution. The surface disinfected imported embryos or larvae are imported into the quarantine facility upon arrival, raised to sexual maturity, and then spawned as described above. The potential for the introduction of disease utilizing this strategy is lower than when importing adult fish, or nondisinfected embryos/larvae, directly from another facility's conditioned colony. A disadvantage with this method is the need to wait for the imported embryos/larvae to become sexually mature and produce embryos, which can then be disinfected for placement in the conditioned colony.

A more refined method of importing fish will be required if the exclusion of pathogens whose transmission cannot be prevented with embryo surface disinfection is desired. This involves a two or possibly three-step process and requires diagnostic testing but achieves the highest degree of biosecurity. In this process, a minimum of two isolated holding systems, ideally in two physically separate locations or rooms, is needed. Adults are received onto a dedicated stage 1 quarantine holding system and observed for 2–5 weeks for clinical signs of disease. If adults appear healthy, they are spawned; and embryos are surface disinfected and moved onto the second stage of quarantine on the second isolated holding system. The adults, and possibly a subset of embryos are then evaluated for the presence of disease, utilizing diagnostic techniques, such as histopathology or PCR, singly or in combination. It should, however, be noted that negative results from a subset of embryos are not necessarily reflective of the pathogen status of the adult fish or the remainder of the clutch. Lethal evaluation of adult fish appears to be the most sensitive diagnostic sample for evaluating the broadest range of zebrafish pathogens, although environmental testing may be equally or more sensitive to detect the presence of some pathogens (Crim et al., 2017). If results are positive for any excluded pathogens, the steps are repeated with any remaining imported fish until SPF

adults have been identified. This strategy has been used to successfully develop a stable SPF zebrafish facility (Barton, Johnson, & Tanguay, 2016; Kent et al., 2011). When results are negative for excluded pathogens in an adult pair or pairs, larvae on the second stage of quarantine are bred when sexually mature, and their surface disinfected embryos are moved into the conditioned colony. Although not ideal, if two isolated systems are not available, modifications may be made to adapt these concepts to a system that incorporates flow-through tanks or off-system housing for new imports until the time of euthanasia and testing. In this scenario, surface disinfected embryos obtained from spawning events can be moved onto a quarantine rack or can be held off-system until diagnostic testing has been completed and they are cleared for movement into the conditioned colony.

Unlike with rodents, it is currently not a common practice to prophylactically treat zebrafish for diseases in quarantine. Limitations include the lack of effective, convenient, and safe treatments for common zebrafish diseases, such as microsporidiosis. Treatment strategies have been described for infection with the nematode, *P. tomentosa* (Collymore et al., 2014; Maley, Laird, Rinkwitz, Thomas, & Becker, 2013; Samaee et al., 2015) and *Mycobacteriosis* (Chang, Doerr, & Whipps, 2017; Chang and Whipps, 2015; Whipps et al., 2012). Prophylactic treatment for mycobacteriosis is not recommended due to the potential for the development of antibacterial resistance; however, prophylactic treatment of all newly imported fish for *P. tomentosa* utilizing one of several published methods can be considered. When administering medication to zebrafish, it is important to do so off the system or in a flow-through system because other fish on the system and the biofilter can be impacted by the treatment.

Environmental Testing

The use of environmental samples during quarantine may be a useful adjunct to test for the presence of some pathogens; however, environmental testing has not been shown to be equally sensitive for the detection of all zebrafish pathogens. Successful application of these testing methods will vary by pathogen prevalence, pathogen type, and sampling method used; therefore, negative test results should not result in bypassing quarantine and placement of imported fish directly into the conditioned colony. In contrast to colony health monitoring, environmental testing in the quarantine setting is primarily directed toward pathogen detection in small groups of imported animals rather than the entire colony, as sampling is generally limited to system surfaces, detritus, feces, or water from individual tanks or groups of tanks. For example, collection of feces from tanks housing an imported cohort, followed by double centrifugation of the sample with a saturated sugar solution, is effective for the identification of *P. tomentosa* eggs (Murray, et al., 2015). However, the sensitivity of detection will likely vary based on the prevalence and life cycle stage of the worms present. Molecular diagnostic assays (real-time PCR) of water concentrated by filtration has been shown to be useful for detecting several *Mycobacterium* species, but this method inconsistently detects other pathogens, such as *P. tomentosa and Pseudoloma neurophilia (P.neurophilia).* Evaluation of detritus and feces, however, is sensitive for the detection of *P. tomentosa* (Crim et al., 2017). This provides useful information, which can be incorporated into the design of a quarantine program. For example, if *P. tomentosa* is an excluded pathogen, detection of this pathogen during quarantine could prompt treatment of the imported cohort (if utilizing static or flow-through systems) or the entire quarantine system.

Embryo Surface Disinfection

Embryo surface disinfection is currently the most commonly used method to prevent the transmission of some aquatic diseases when importing fish from another facility. This method is easy to apply, requires minimal training or financial investment, and takes advantage of the presence of the protective embryonic chorion. Embryo surface disinfection, used commonly in the commercial aquaculture industry, involves using both mechanical agitation and exposure to chemical disinfectants and rinse baths for a prescribed period to remove and destroy infectious organisms that may be transmitted from the parental fish to the surface of the embryos (Fig. 36.6). In general, regardless of the disinfectant used, surface disinfection should not be conducted once the embryo begins to hatch to avoid

FIGURE 36.6 Example of embryo surface disinfection procedure. A large bowl with a strainer is used to isolate the embryos. The strainer holding the embryos is then used to move the embryos through the various rinse and disinfectant solutions for determined amounts of time. The embryos should be agitated slightly to allow for uniform distribution of the disinfectant.

the introduction of the chemical disinfectant directly to the developing larvae (Nüsslein-Volhard and Dahm, 2002). It is generally recommended that surface disinfection be conducted between 6 and 24 h postfertilization (hpf), noting that earlier time points are associated with less chemical toxicity to the embryo for several disinfectants (Chang, Amack, & Whipps, 2016; Kent et al., 2014). The most obvious limitation to the use of embryo surface disinfection is that it is not effective for preventing diseases transmitted vertically. It has been demonstrated that *P. neurophilia* can be transmitted vertically by both extraovum and intraovum routes. Surface disinfection may help to lower extraovum transmission but will have no impact on intraovum transmission (Sanders, Watral, Clarkson, & Kent, 2013). It is not yet known if other microsporidia in zebrafish (i.e., *P. hyphessobryconis*) can be transmitted vertically; however, several microsporidian species can be transmitted by this route in other hosts (Sanders et al., 2010, 2012). The type of disinfectant used, its required contact time, its efficacy, and its safety are other factors to be considered. There is not currently an embryo surface disinfection protocol proven safe and effective against all known horizontally transmitted zebrafish pathogens.

Sodium hypochlorite is currently the most common disinfectant used for surface disinfection of zebrafish embryos. There are several variables to consider when utilizing this method, including the concentration of the chlorine solution, exposure time, pH of the disinfectant solution, and age of the embryos. The concentration and dose most commonly used is 25–50 ppm for 5–10 min (Westerfield, 2007; Harper and Lawrence, 2011). Although this regimen should be effective at reducing transmission of most Gram-negative bacteria (Goñi-Urriza et al., 2000) and trophozoite stages of protozoa (Vaerewijck et al., 2012), several studies have shown this to be an incomplete method for safely preventing transmission of some known extraovum pathogens in zebrafish. For example, it has been demonstrated that 100 ppm is not efficacious in preventing larvation of the nematode *P. tomentosa* (Martins et al., 2017). Studies evaluating the germicidal properties of chlorine have demonstrated that higher pH levels reduce the efficacy of sodium hypochlorite as a disinfectant, and this has been proven for the efficacy of chlorine against *P. neurophilia* spores (Clark, Member, Eleanor, & Hoff, 1989). The disadvantage, however, is that buffered chlorine solutions have also been shown to have a more narrow safety margin in zebrafish (Ferguson et al., 2007; Kent et al., 2014). Exposure of *P. neurophilia* spores to an unbuffered chlorine solution of 25, 50, and 100 ppm resulted in the death of only 40%, 60%, and 83% of the spores of this microsporidium, respectively (Ferguson et al., 2007). Studies have also demonstrated that sensitivity associated with chlorine varies by age, with 6 hpf embryos being less sensitive to toxic effects (based on malformations and death) when compared with 24 hpf embryos exposed to the same disinfection protocol. Conclusions of that study suggest that the best chlorine-based protocol to minimize toxic effects, and still maximize lethality to *P. neurophilia* spores, would require treatment of 6 hpf embryos for 10 min and 24 hpf embryos for 5 min with an unbuffered chlorine solution at 100 ppm. Strain variability to toxicity sensitivity has also been suggested, with AB fish being more sensitive than 5Ds (Kent et al., 2014). The sensitivity of the other microsporidian found in zebrafish, *P. hyphessobryconis*, has not been evaluated. Gram-negative bacteria are generally sensitive to chlorine disinfection; however, the sensitivity of mycobacteria varies by species. For example, some of the more commonly found environmental species (e.g., *Mycobacterium chelonae* and *fortuitum*) are more resistant than some pathogenic species, such as *Mycobacterium marinum* (Bardouniotis, Ceri, & Olson, 2003; Mainous & Smith, 2005). A recent in vitro study concluded that the use of an unbuffered 100 ppm bleach solution for 10 min resulted in a decrease in mycobacterial survival (2.94%–28.36%), which varied by species, but was not 100% effective against any of the species tested (Chang et al., 2015).

Other surface disinfection methods that have been evaluated utilized povidone iodine (PI), commonly used in the salmonid industry, or hydrogen peroxide, commonly used for catfish. Germicidal efficacy of hydrogen peroxide (15,000 and 30,000 ppm) has been shown to be poor against four *Mycobacterial* species commonly found in zebrafish systems when evaluated in vitro. In comparison, treatment with 25ppm PI resulted in less than 1% average survival in vitro for the same four species (Chang et al., 2015). The related in vivo studies demonstrated <0.1% survival of *M. chelonae* and *M. marinum* when embryos were exposed to 12.5–50 ppm buffered PI with a contact time of 2 min. In contrast to chlorine disinfection, unbuffered solutions of PI resulted in a higher level of toxic effects, but embryos were less sensitive to toxic effects when treated at 50 ppm for 2 min at 6 hpf versus 24 hpf. This is similar to the age-related sensitivity difference noted with chlorine treatment (Chang et al., 2016). However, the efficacy of PI against other pathogens has not been evaluated.

Embryo surface disinfection assists with mechanical disruption and reduction of pathogen load and is an essential component of biosecurity. For example, although sodium hypochlorite concentrations that are typically used are not efficacious at preventing larvation of the nematode *P. tomentosa*, implementation of surface disinfection procedures have been used successfully to prevent the introduction of the pathogen from known

infected populations (personal communications), possibly from mechanical effects. Regardless of the disinfectant used, surface disinfection can be most effective if solutions are prepared fresh before use and their appropriate concentrations verified prior to use. Embryos should be rinsed first to remove organic debris and should be disinfected for an appropriate time to decrease toxic effects. All solutions used should be free of pathogens (i.e., do not use holding system water), and disinfected embryos must be placed in appropriate solutions, such as clean embryo media, after disinfection. Avoid keeping stock volumes of disinfection solutions near the holding systems, as inadvertent contamination of the system could result in high levels of toxicity to zebrafish.

FIGURE 36.7 Example of a door sign used for a zebrafish quarantine facility in a dedicated room. Only specifically trained staff are allowed into the facility, and the room is entered after all work with the conditioned colony has been completed.

Quarantine Facility Design

The ability to contain or isolate imported pathogens is dependent upon the design (use of flow-through or static tanks, the efficacy of UV disinfection, etc.) and workflow of the quarantine facility, as well as adherence to quarantine procedures by facility staff. The ideal zebrafish quarantine facility allows for a physical separation of imported fish from the main conditioned colony. Basic concepts of quarantine facility design used for other species also apply to zebrafish. The ideal quarantine facility would be located in a physically separate building or room, with a separate entrance from that serving the main conditioned colony. Access of individuals to the quarantine facility should be limited to aquatics facility staff or limited individuals trained to work appropriately in the quarantine facility; this is best done utilizing a customizable, secure entry system (Figs. 36.7 and 36.8). The quarantine facility should have the ability to hold one or more self-contained holding systems provided with the same quality of water that is available to the conditioned colony. All effluent and recirculation pathways should be isolated from that of the conditioned colony holding systems. A wide variety of holding systems can be useful depending upon the frequency of importation, quarantine procedures, available space, and resources. For small facilities with infrequent imports, one or two small aquariums that function as static, recirculating or flow-through tanks may be sufficient. For moderate-sized facilities, a self-contained holding rack in a separate building, room, or isolation cubicle, would be sufficient (Fig. 36.9). Larger facilities with frequent imports will likely need multiple independent racks and could utilize a combination of both recirculating and flow-through water supplies, depending on quarantine strategy used. Facilities that require the use of fish that are SPF for pathogens not prevented by embryo surface disinfection may need multiple separate

FIGURE 36.8 The use of an ID activated controlled entry system for a zebrafish quarantine area. The black box on the wall is a key card reader used to control an electronic lock on the door to the facility; only those granted key card access will be able to open the door by scanning their key card.

FIGURE 36.9 Example of a self-contained single rack, which could be used for quarantine. Compact racks are also available that can be placed in small areas, such as an isolation cubicle or on a benchtop.

rooms and/or independent holding systems. The ability to house imported fish on flow-through tanks, or in a static tank, rather than on a recirculating system with other fish is useful for several reasons. If fish are being imported with unknown health status, or from a high-risk source (e.g., pet store), fish can be isolated until diagnostic testing is completed prior to releasing fish onto a recirculating system with other fish. If fish are imported with a known pathogen that is amenable to treatment (e.g., *P. tomentosa)*, or if prophylactic treatment is administered to all imports, flow-through tanks would allow for fish to receive medication without the risk of excess and excreted medication influencing other fish on the system or the biological filtration.

The quarantine facility should be fully equipped with all necessary equipment and supplies so that there is no need to bring anything into or out of the quarantine facility. For example, adequate workspace and equipment (e.g., microscopes, incubators) should be provided for investigative staff to work with animals in quarantine without the need to utilize the same equipment that is used for fish from the main conditioned colony. Nets, buckets, tanks, and other supplies must be fully stocked at all times. Supplies for euthanasia, including an ice machine if hypothermia is used, and a location for carcass storage, should be readily available. Tanks and other supplies should be sanitized separately from equipment from the conditioned colony. For this reason, it is often helpful to have an undercounter dishwasher in quarantine. No equipment should be taken from the quarantine room into the main room.

Facility Workflow

Ideally, the staff providing husbandry to the quarantine facility are not the same as those providing husbandry to the main facility, however, this is often difficult to achieve in smaller facilities; and cross-contamination is minimized by only accessing the quarantine facility after working with the conditioned colony and/or by utilizing additional personal protective equipment (PPE). If there are several zebrafish facilities with varying health statuses, a room entry order should be determined and posted. All feeding, health evaluation, and maintenance tasks should be completed in the quarantine facility only after these tasks have been completed with the conditioned colony. It is easier to limit staff access with a separate entrance, having a different coded entry than that of the main facility. This may also provide the option for timed entry so that any individual that enters the quarantine facility is not allowed to enter the main facility until a certain duration of time has passed.

In addition to limiting the flow of people in and out of quarantine, the traffic flow of equipment and supplies must be controlled to prevent unwanted contamination. Ideally, food used in quarantine is stored inside the quarantine facility. If live food is prepared in a central location, it should be transported to the quarantine facility in a disposable container that is not carried back to the main food preparation area after it has been in quarantine. PPE requirements may differ from that of the main facility. Limiting the transfer of potentially contaminated water outside of the quarantine facility is the most important aspect to keep in mind when determining these requirements. Water is most likely to be transferred on the bottom of shoes, impermeable surfaces, or on equipment, so cross-contamination by these routes should be the focus of biosecurity practices. For preventing the transfer of water on shoes, dedicated shoes maintained in the quarantine facility, shoe covers, or footbaths may be used. Due to the higher risk of zoonotic disease in imported fish, gloves should always be worn. Facilities may also wish to include a type of body covering to enforce the concept of quarantine.

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CHAPTER

37

Export and Transportation of Zebrafish

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Over the past 30 years, zebrafish have become a very important animal model in biomedical research, including the creation of a growing number of genetically modified lines. The Zebrafish International Resource Center (ZIRC), a central resource and repository for zebrafish, has nearly 30,000 different strains donated by researchers for distribution (Zebrafish Lines at ZIRC, 2017). This is likely a very small fraction of the total number of lines created, many of which are shared among labs worldwide. For facilitating this distribution, researchers and the research animal facilities that support them, need to understand the requirements for safely and legally shipping fish around the world.

The goal of this chapter is to provide a generalized resource for those shipping zebrafish. Due to the incredible diversity of requirements for importing and exporting zebrafish globally, the exporting institution should use a reputable shipping company to ensure that the shipment has the necessary documentation to clear customs in the country receiving the shipment. The cost of shipping zebrafish in this manner can be expensive, but it is important to consider the cost of shipping the zebrafish a second time if the first shipment has not been successful. This not only includes the additional shipping costs, but the labor and materials involved in obtaining and packaging the fish, embryos, or sperm a second time.

The remainder of the chapter will provide additional details on the different types of documents that may be required to accompany the shipment, as well as the packaging and labeling needed for the different types of zebrafish specimens shipped: adults, embryos, or cryopreserved sperm. Regardless of the type of specimen shipped, it is important to take into consideration the weather conditions that the shipment may encounter and the duration of the shipment, as this will influence how the package is assembled for shipment. This not only includes the weather conditions at the origin and destination of the shipment but also along the entire transportation route, especially any location where the shipment has a layover.

Documentation

When shipping any type of animal or animal specimen, there is often a requirement for a specific type of documentation to accompany the shipment. The type of documentation needed will depend on the type and destination of the shipment. This section provides examples of the types of documentation that may be required, but it is critical to work with a reputable shipping company to ensure that the documents meet all requirements. Typically, the shipping company will request copies of the documentation prior to scheduling the shipment and will ensure that the documents will be accepted. Once the documents are approved, the company will schedule a date and time for pick up. In addition to the documents that accompany the shipment of fish, many institutions require material transfer agreements (MTA), if the strain was created at the exporting facility. If the strain was received from another institution, it is important to verify if the fish to be sent are currently covered by an MTA, which may prohibit further distribution.

For shipments from one institution to another within the United States, in addition to the shipper's waybill, it is recommended to have a health certificate (Fig. 37.1), as described below, to accompany the shipment.

The documentation required for an international shipment depends on the laws and regulations of the importing country. Due to the variety and changing

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AQUATIC ANIMAL HEALTH CERTIFICATE

Identification

Contents of Shipment:	Live fish adults
Species:	*Danio rerio*
Common name:	Zebrafish
Description:	This is a tropical, freshwater aquarium species commonly used as a model organism for genetic and biomedical research.
Age:	Adults (X months)
Quantity:	XXX

Place of Origin		Destination	
Aquaculture Facility:	XXX	Name:	XXX
Address:	XXX	Address:	XXX

Shipping Company: XXX

Declaration

I, the undersigned, certify that the live fish in the present consignment were reared from embryos and have been housed in a scientific aquaculture facility that maintains *Danio rerio* exclusively, accepts additions only from other scientific institutions, and has been subjected to continual fish health surveillance. At least semi-annually, pre- and post-filtration sentinel fish are submitted for PCR evaluation (for *E. ictaluri, M. abscessus, M. chelonae, M. fortuitum, M. haemophilum, M. marinum, M. peregrinum, M. spp., P. tomentosa*, and *P. neurophilia*) and histopathologic evaluation of the brain, epidermis, gills, heart, intestine, kidney, liver, reproductive organs, skeletal muscle, spinal cord, swim bladder, vertebral column, and general sections (as a mycobacterial screen).

Date of Issue:	XXX
Certifying Official:	XXX, D.V.M. (Attending Veterinarian)

Signature: _____ Date: _____
XXX, D.V.M.

FIGURE 37.1 Sample health certificate – provided by Diana Baumann.

nature of each country's requirements, utilizing a reputable shipping company that can provide advice on the documentation required is recommended. Most countries will require at least two documents: an international health certificate (Fig. 37.1), as described below, and a customs invoice.

A customs invoice (Fig. 37.2) provides the importing country with the information needed to demonstrate the value and use of what is being shipped. Items specifically required on the customs invoice are as follows:

- Name, address, and phone number of the shipper, typically the head of the lab responsible for the fish
- Name, address, and phone number of the recipient
- A description of the contents of the shipment, including age, quantity, and scientific name of the animals
- Reason for sending the shipment
- The estimated value of the shipment
- Country of origin
- Weight of the shipment
- Signature of the shipper; the head of the lab responsible for the fish

Other documents that may be required include a country-specific importation form; a country-specific importation permit, obtained and provided by the receiving institution; and a packing slip. If a packing slip is required, this should describe the species, strain (wild-type, mutant, or genetically modified strain name), date of birth, and quantity.

Regardless of the documentation needed, the original paperwork should be provided to the shipping company upon pickup. It is recommended to attach copies of the documentation to each shipping container and maintain a copy for your institution's records.

Health Certificate

Receiving fish from outside sources brings the potential for introducing pathogens into an existing colony. Ideally, origin facilities would provide information on their current animal health status, allowing the recipient facility to make informed decisions on whether to accept

CUSTOMS INVOICE

Date of Export:	
Shipper:	HEAD OF LAB NAME INSTITUTION NAME ADDRESS COUNTRY PHONE NUMBER
Recipient:	HEAD OF LAB NAME INSTITUTION NAME ADDRESS COUNTRY PHONE NUMBER
Contents:	Non-hazardous biological specimens for research purposes only. Lab Bred tropical fish (*Danio rerio*). Adults (QUANTITY) Embryos (QUANTITY)
Reason for sending:	The contents are intended for scientific research and are a gift from Shipper to Recipient.
Declared Value:	VALUE OF SAMPLES PLUS CURRENCY
Country of Origin:	COUNTRY
Total Weight:	WEIGHT OF PACKAGE

These commodities are licensed for the ultimate destination shown. I, the undersigned, do certify that this statement is both true and correct.

Shipper Name: SIGNATURE OF HEAD OF LAB

Date:

FIGURE 37.2 Sample customs invoice – provided by James Cox.

the fish. However, there are no accepted standards of health status for zebrafish, and some facilities may not have health information due to the lack of a sentinel program. For more information, see the chapter in this book on health surveillance.

In general, a health certificate (Fig. 37.1) for shipment of fish within the United States should include the following information:

- A description of the shipment, including the quantity and age of fish in the shipment
- The name and address of the origin of the shipment
- The name, address, and phone number of the receiving institution
- A statement of the health status, indicating the infectious agents for which the production colony is known to be both negative and positive
- A signature of the veterinarian confirming this health status

For international shipping, different countries have differing requirements for health documentation or certification. When working with a commercial shipping company, company representatives should be able to provide the requirements for the recipient country. It is worth noting that frequently, changes happen as countries refine their processes, so information used for previous shipments may well be out-of-date.

The World Organization for Animal Health (OIE) Aquatic Animal Health Code (The Aquatic Code, 2017) sets out standards for safe international trade in aquatic animals and their products. It provides a model health certificate for international trade in live aquatic animals and gametes, which is required in some import/export situations. The Aquatic Code offers guidance on completing the health certificates and lists 10 diseases of concern, although these are mostly not applicable to zebrafish.

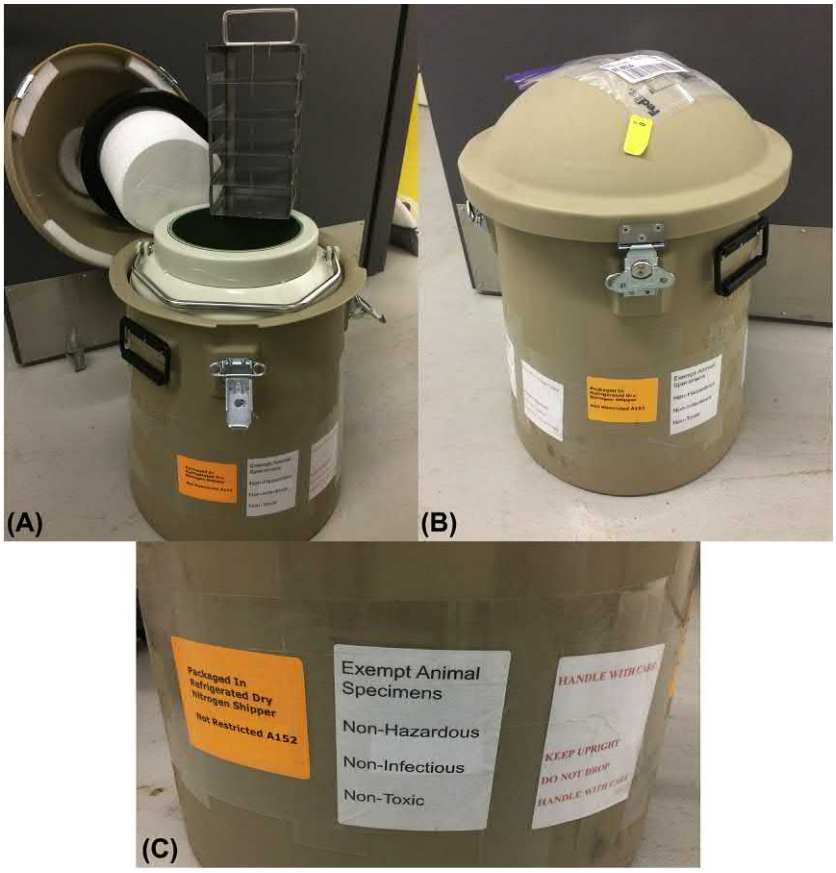

FIGURE 37.3 Dry shipper with labeling – Provided by Carrie Carmichael, This image shows a common dry shipper that is used when shipping cryopreserved zebrafish sperm. (A) The rack that contains the samples and is placed inside the chamber; (B) A closed container showing the placement of one of three sets of labels around the outside of the dry shipper; (C) The three required labels, which must be placed in triplicate on the outside of the chamber.

Where a USDA endorsed health certificate is required, communication with the local area office is recommended. A USDA accredited veterinarian must inspect the animals, sign, and date the health certificate, which is then sent to the USDA/APHIS local area office along with the applicable fees for endorsement. This health certificate is then valid only for a limited period of time, so coordination with the shipping company is critical.

Transportation

Materials and Methods for Shipping Cryopreserved Sperm

Cryopreserved sperm are shipped to share lines with colleagues, transfer to a storage repository, or for relocating a laboratory. The critical issue is maintaining the ultracold temperatures necessary to maintain the integrity of the samples. Although samples are stored in either the liquid or the vapor phase of liquid nitrogen, they are shipped in the vapor phase. Liquid nitrogen is classified as "Dangerous Goods" by the United States Department of Transportation (DOT); however, the DOT has determined that the use of liquid nitrogen-charged "dry shipper" containers for the shipment of samples falls within the regulation exceptions (Cryogenic liquids; exceptions, 2003). Dry shippers are typically vacuum-insulated Dewar style containers with an internal material designed to absorb liquid nitrogen, leaving no free liquid in the container (Fig. 37.3). This facilitates moving frozen biological samples in liquid nitrogen vapor.

It is important to select a dry shipper of a suitable size for the size and numbers of samples to be shipped and to use the appropriate racks for the type of storage containers containing the sperm (boxes, vials, or straws). Other preferred characteristics of shippers are a locking lid and optional presence of a data logger to record temperature during the transport.

Dry shippers must be tested and charged prior to shipment following the manufacturer's instructions for the shipper. The testing procedure generally involves

filling the container with liquid nitrogen and then measuring the container's weight several times over a given time interval. During this period, it is important to check the outside of the shipper for any signs of frost or sweating, which would indicate vacuum loss. To charge a dry shipper, place the racks inside the chamber and fill it with liquid nitrogen. Allow the shipper to stand for 24 h after filling, and top it off with additional liquid nitrogen as needed to keep it full during that time. All excess liquid must be poured off prior to shipping. Once the shipper is charged, samples may be placed into the chamber. The Dewar is then packaged and shipped overnight to the destination. Usual precautions of working with liquid nitrogen apply and appropriate safety attire should be used.

Dry shippers are available for purchase, or there are specialized courier companies offering a comprehensive service. For particularly valuable samples, consideration should be given to the additional cost, but reduced risk, of splitting the shipment and sending it on two separate occasions.

Labels on the package (Fig. 37.3) need to indicate its origin, destination, and contents, as well as any pertinent safety requirements. It is very important that shippers remain upright during shipment, so an indication of direction is needed on the package. Warnings of the need for careful handling and the possible occurrence of exposure to ultra-cold temperatures and nitrogen vapors that can deplete oxygen in the air are included on dry shippers.

The outside plastic jacket, or shipping carton, should be labeled to indicate the nonhazardous, noninfectious, and nontoxic character of the biological specimens, in addition to DOT exception labeling. Three identical sets of labels should be placed equally at 120-degree intervals around the shipper. The shipping bill is placed on top of the outside jacket.

Materials and Methods for Shipping Adult Zebrafish

Many domestic couriers will not accept live fish or embryo shipments without preapproval of packaging materials. Preapproval typically involves the courier conducting a stress test on the proposed packaging to ensure the shipping materials used are appropriate to withstand the rigors of the shipping process. During this process, a mock shipment is sent to a testing facility and will undergo a series of mechanical stress tests that can include dropping, compression, and exposure to extended periods of vibration. Package testing is a one-time event, and once approval has been attained, subsequent shipments are allowed without additional testing as long as the same packaging method is used. Some couriers will allow independent assessment through the International Safe Transit Association (ISTA) prior to shipment, as an alternative to internal package testing, while other couriers may simply provide you with specific instructions on the materials to use. Because requirements for shipping live animals can vary depending on the courier, it is important to confirm these details with the courier of choice prior to submitting animals for transport. All packaging, regardless of the courier, must meet standards set by the International Air Transport Association (IATA) for live animal transport (Live Animal Regulations, 2017).

Exterior Packaging

The packaging (Fig. 37.4) required to safely ship zebrafish is comprised of multiple components. The external container should be designed for insulated shipping and made of expanded polystyrene; this container should fit well into a corrugated cardboard box. The Styrofoam insert should have a minimum wall thickness of 1.5–2 inches for optimal strength and insulating capability. These two-part insulated shipping boxes are readily available from a number of scientific and shipping supply companies. Most couriers require shipping containers used for transporting live fish to meet specific requirements for edge crush test and burst strength. Some couriers require testing of the packaging materials prior to shipping live zebrafish.

Primary and Secondary Containment of Fish

Traditionally, fish are shipped in three or four mil polyethylene bags for primary containment, with an additional bag of the same thickness for secondary containment in the event the primary containment fails. It is important to note that all bags should have a square or single seam bottom (Fig. 37.5) to avoid fish becoming trapped in layers of plastic during transit. Bags should be tied off using a secure knot with an additional fastener, such as a heavy-duty rubber band, or a bag staple.

An alternative approach for primary containment is the use of a collapsible cubitainer insert (Fig. 37.6). Cubitainers are polyethylene cubes with a screw cap lid that can be extended to create a spout; they come in sizes ranging between one and 20 L. Because of their square shape, they nest into polystyrene boxes efficiently and have a higher strength rating than four mil plastic bags. Cubitainers are available from many foodservice and laboratory supply companies and are fairly inexpensive. To achieve secondary containment, nest the cubitainer inside a plastic bag and seal as described above.

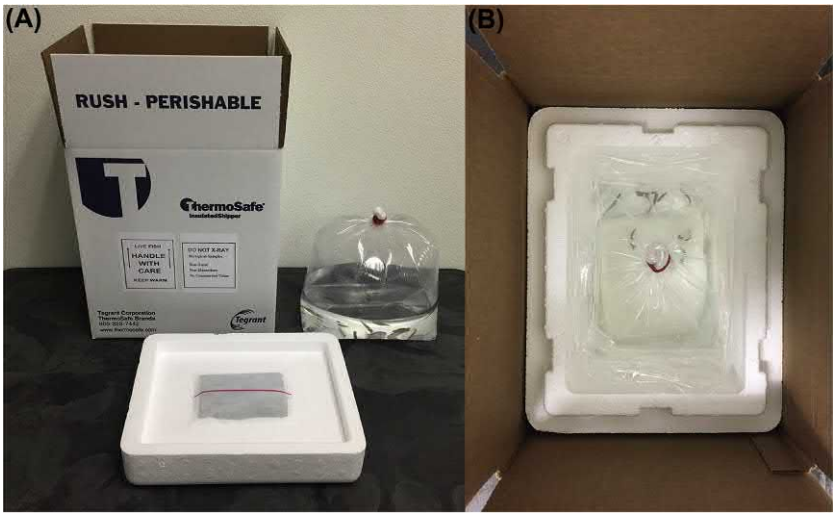

FIGURE 37.4 Picture of shipping box for adult fish — Provided by Carrie Barton, This is typical packaging for shipping adult zebrafish, but it can also be used for shipping flasks of embryos. (A) Cardboard box with appropriate labels indicating the contents of the shipment. Notice the heat pack that is taped to the lid of the internal Styrofoam container. This keeps it away from the water in which the fish are shipped in, to prevent overheating of the water. (B) Internal view of the box showing the bag of adult fish placed inside the Styrofoam container.

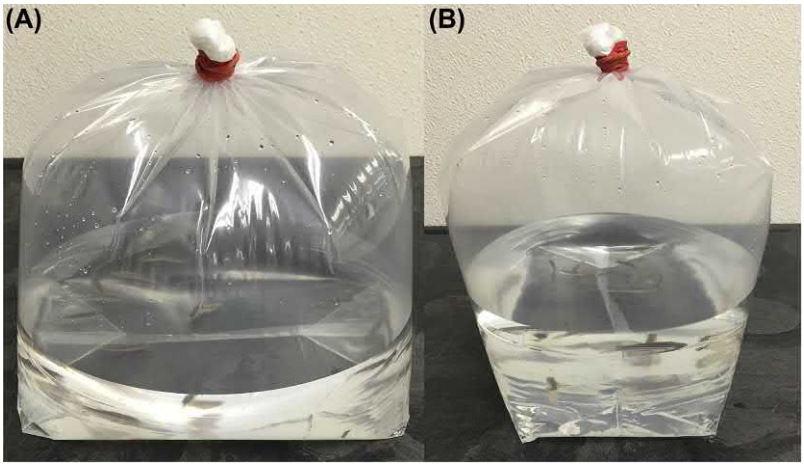

FIGURE 37.5 Picture of plastic bag with fish — Provided by Carrie Barton, Adult zebrafish are often shipped in plastic bags, which must be prepared to prevent fish from getting stuck in the corners. The corners of the bag are folded and taped to the bottom of the bag to create a "square" bag. (A) Front of the bag; (B) Side of the bag.

FIGURE 37.6 Picture of cubitainer with fish — Provided by Carrie Barton, Adult zebrafish packed in a cubitainer, an alternative to plastic bags.

Absorbent Material

Some couriers require that absorbent material be placed within the exterior packaging to soak up any water in the event of complete or partial failure of both the primary and secondary containment system. Absorption capacity should be equal to or exceed the volume of liquid contained within the shipment. The absorbent bench or spill pads are inexpensive and compact materials that meet this requirement when it is requested.

Filler

Bubble wrap, packing peanuts, air packs, or any other water-resistant packing material can be used to fill unused space within the box. For international shipments where fish may be in transit for multiple days, additional primary containers filled with warm water can also be used to fill the dead space and create an additional thermal mass to help keep temperatures at optimal levels.

Temperature Control and Monitoring

During cold months, shipping warmers or "heat packs" should be placed inside the shipping container to maintain favorable conditions for fish during transit. Rapid rusting heat packs are safe for use in live animal shipments and come in a variety of durations depending on the need. It is important to note that heat packs should never be placed in direct contact with the primary container or water. Heat packs should be affixed to the underside of the Styrofoam lid of the shipping box (Fig. 37.4). Additionally, heat packs require a constant supply of oxygen to continue working properly. To achieve this without impacting the thermal retention of the Styrofoam box, drill a small hole in the lid, directly in the center of where the heat pack will sit. This will allow air to come in contact with the heat pack throughout the duration of transport. If temperature data is required or requested by the recipient, one-time-use disposable data loggers can be used. Place these units in direct contact with the outside of the primary container to achieve the most accurate results. Some couriers will provide these upon request for an additional fee.

Labels

Properly labeled boxes are vital for the safe transport of zebrafish, and many couriers require a declaration on the package stating it contains live animals. Labels should be clear, concise, and visible on all sides of the box. Labels should include statements, such as (Westerfield, 2007):

- Live Fish
- Keep at Room Temperature
- Handle with Care
- Do Not X-ray
- Nontoxic
- Nonhazardous
- No Commercial Value

Water, Air and Shipping Densities

Maintaining optimal water quality during transit is the most challenging aspect of shipping live fish. During metabolism, fish consume oxygen and produce ammonia and carbon dioxide. The closed shipping environment can be problematic due to the prolonged consumption of oxygen and production of waste products by the fish. Potentially unfavorable water conditions can be mitigated by ensuring that stocking densities are kept low, so that, excess waste products are not introduced into the shipping container with the fish. Fresh fish water should be used for packaging fish. Adult zebrafish should be packaged at a density of approximately five fish per liter. If shipping time will exceed 24 h, as is often the case with international shipments, a lower density of fish to water is recommended. Withholding feed for at least 24 h prior to the shipment can also decrease the amount of waste released into the water during transit. Water conditioning agents, such as ammonia binders can be used to further reduce the potential for developing dangerous water conditions. Dissolved oxygen levels can be maintained by ensuring the water to air ration is at least 1:3 in each primary container, and the containers are full of either air or pure oxygen if available.

Methods and Materials for Shipping Zebrafish Embryos

Embryos are shipped in a very similar manner to adult zebrafish. All materials used for exterior packaging, the addition of absorbent material within the box, the use of water-resistant packaging filler, temperature control/monitoring, and labels are all the same, regardless of developmental stage. Embryos do require different primary containment and shipping media, as well as preshipment surface disinfection.

Primary Containment of Embryonic Zebrafish

Much like adults, embryonic zebrafish require both primary and secondary containment within the shipping box. Unlike the bags or cubitainers used to hold adults, zebrafish embryos should be shipped in smaller rigid containers, such as tissue culture flasks with a solid sealed screw cap (Fig. 37.7). A good example would be a standard 250 mL tissue culture flask filled to a level no higher than 200 mL to leave an approximately 50 mL headspace (25% total volume) of air. As with packaging

FIGURE 37.7 Picture of embryo shipping materials – Provided by Carrie Barton, Zebrafish embryos were transferred into 250 mL plastic flasks. These flasks were placed in a plastic bag with absorbent material in case the flasks break or leak.

adults, a flask can be placed within a polyethylene bag to serve as secondary containment. Multiple tissue culture flasks can be nested within a single bag.

Water and Stocking Density for Embryonic Zebrafish

Embryos should be shipped in sterile embryo medium (E2), as described in the chapter on larviculture in this book. Stocking density should be one embryo to ~1.5 mL embryo medium. Multiple smaller containers holding a smaller number of embryos (i.e., four 200 mL flasks, each holding 100 embryos) is preferred over larger containers holding higher numbers of embryos (one 800 mL container holding 400 embryos). Should any embryos die in transit, they can have a negative impact on water quality and put the surviving embryos at risk. Using multiple containers will decrease the risk of a single embryo negatively impacting the entire shipment.

Surface Disinfection

Shipping success is increased when embryos are surface disinfected prior to transport as the chorion can have detritus, bacteria, fungus, protozoans, or parasites present. It is important to remove these potential contaminants as they can often impact water quality, resulting in increased mortality during shipping. Refer to the chapter on importation and quarantine for information on surface disinfection procedures using sodium hypochlorite and other disinfectants.

Shipping zebrafish to other institutions in the United States and internationally is a very common procedure and requires knowledge of required documents, packaging of the fish, and labeling of the shipping boxes to ensure a successful shipment. Working with reputable shipping companies and utilizing the information and resources provided in this chapter will help ensure that the shipments of zebrafish adults, embryos, and cryopreserved sperm, will arrive quickly and safely at the receiving institutions. This is beneficial to both the research and the health and welfare of the fish.

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CHAPTER

38

Regulations, Policies and Guidelines Pertaining to the Use of Zebrafish in Biomedical Research

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Introduction

The use of zebrafish as a biomedical research model of human and animal diseases has steadily increased since the first published report in 1951. Projects utilizing zebrafish are very diverse with the top five published subject areas being developmental biology (27%), biochemistry/molecular biology (18%), cell biology (14%), neurosciences/neurology (11%), and genetics (10%) (Kinth, Mahesh, & Panwar, 2013). In recent years, research using zebrafish has expanded to include using this experimental system to model many human and animal diseases (cardiovascular, cancer, obesity). The National Institutes of Health (NIH) has ranked zebrafish as the second most important animal model behind the mouse (Goldsmith & Solari, 2003). Following NIH's 1998 program announcement "The Zebrafish as an Animal Model for Development and Disease Research," the number of zebrafish publications in Clarivate Analytics' Science Citation Index Expanded database rose by 149% between 1997 and 2006 compared to an increase of only 2.7% for animal research in general. And between 2005 and 2010 zebrafish research saw a sixfold increase in NIH funding and almost a fourfold increase in publications, with over 800 zebrafish research publications in 2010 representing almost $354 million in NIH funding alone (Dietrich, Ankeny, & Chen, 2014). The Zebrafish Information Network (ZFIN) Website maintains a database of laboratories using zebrafish. As of this writing, the list is well over 1000 (Howe DG et al., 2013).

Some reasons zebrafish have become such an increasingly used biomedical model are that its eggs are laid and fertilized externally, embryos are transparent, and development of organs is rapid and may be observed in vitro. Zebrafish are especially useful in genetic research as one breeding pair can produce over 100 embryos from a single mating and techniques for their genetic manipulation are well described (Harper & Lawrence, 2011; Westerfield, 2007). Zebrafish are also appreciated for containing research cost as they can be housed in large numbers, in less space, and more economically than mammalian animal models.

This chapter will briefly review the applicable regulatory oversight of the use of zebrafish in biomedical research in the United States. Some of the challenges for institutional oversight of zebrafish research will be discussed.

Laws, Policies, and Guidelines (Anderson 2002; Bayne & Anderson, 2015, 2017)

Researchers, funding agencies, and the general public expect that animals are cared for and used humanely and responsibly. The required regulations and oversight for vertebrate species contribute to the standardization of procedures and care, and thus, promote the production of reliable and consistent data. They also serve to ensure the humane care and use of research animals. As the use of the zebrafish model has become more common, several governmental agencies and nonprofit organizations have published documents to provide guidance for the specific and appropriate care and use of this animal.

United States Department of Agriculture (USDA)

One of the most notable laws governing the use of animals in biomedical research in the United States is the

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Animal Welfare Act (AWA) signed into law in 1966. This law authorized the USDA to write and enforce the Animal Welfare Regulations (AWR). While these are often the most recognized by the general public due to their importance for the use of dogs, cats, nonhuman primates, and other warm-blooded animals (excluding birds and the genera Mus and Rattus), the AWR do not apply to the use of zebrafish. However, institutions receiving funds from the Department of Health and Human Services (DHHS) for research using the AWR covered species must comply with these regulations. So while the AWR do not apply to zebrafish, failure to follow AWR for other species could impact zebrafish research at an institution should program-wide restrictions/punishments be imposed for noncompliance.

Department of Health and Human Services (DHHS)

In 1985, the U.S. Congress passed the Health Research Extension Act that mandated the Secretary of DHHS, acting through the director of the NIH, to establish policy for the proper care and treatment of animals used in biomedical and behavioral research. Much of the animal research performed in the United States is funded through the DHHS, including the Public Health Service (PHS) and NIH. Any institution receiving funds for animal research, training, biological testing, or animal-related activities from the DHHS must comply with the PHS *Policy on Humane Care and Use of Laboratory Animals* and the *US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training* (Table 38.1) (OLAW 2015).

The NIH Office of Laboratory Animal Welfare (OLAW) enforces the PHS policy. NIH awardees must negotiate a letter of assurance with OLAW assuring that they meet the PHS Policy and that they use *The Guide for the Care and Use of Laboratory Animals* (*The Guide*) as the basis of their animal program development and management (Garber, Barbee et al. 2011). *The Guide* applies to all vertebrate animals, including zebrafish.

One of the key requirements of the PHS Policy is that all animal research facilities must establish an Institutional Animal Care and Use Committee (IACUC) and that this committee includes members with specific qualifications. This is described in detail later in this chapter. The IACUC is responsible for reviewing all aspects of the institutional animal care and use program and assuring that all activities are conducted according to PHS Policy and *The Guide*.

Another important NIH office with oversight of NIH funded research is the NIH Office of Science Policy (OSP). The OSP oversees a wide range of biomedical research areas, including biosafety, biosecurity, and emerging biotechnology. All NIH funded research involving the genetic manipulation of living organisms must meet the *NIH Guidelines* for Research Involving Recombinant or Synthetic Nucleic Acid Molecules (*NIH Guidelines*). The Guidelines require an Institutional Biosafety Committee (IBC) review and approval of any genetic additions, deletions, or changes done by recombinant engineering. Most IBC's have broader purview, including the review and oversight of all biological hazard research involving infectious agents. The Center for Disease Control and Prevention (CDC), which publishes the *Biosafety in Microbiological and Biomedical Laboratories*, presumes that the institution (IBC and/or biological safety officer) conducts a biological risk assessment

TABLE 38.1 U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training.

I.	The transportation, care, and use of animals should be in accordance with the Animal Welfare Act (7 U.S.C. 2131 et. seq.) and other applicable Federal laws, guidelines, and policies.
II.	Procedures involving animals should be designed and performed with due consideration of their relevance to human or animal health, the advancement of knowledge, or the good of society.
III.	The animals selected for a procedure should be of an appropriate species and quality and the minimum number required to obtain valid results. Methods, such as mathematical models, computer simulation, and in vitro biological systems should be considered.
IV.	Proper use of animals, including the avoidance or minimization of discomfort, distress, and pain when consistent with sound scientific practices, is imperative. Unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings may cause pain or distress in other animals.
V.	Procedures with animals that may cause more than momentary or slight pain or distress should be performed with appropriate sedation, analgesia, or anesthesia. Surgical or other painful procedures should not be performed on unanesthetized animals paralyzed by chemical agents.
VI.	Animals that would otherwise suffer severe or chronic pain or distress that cannot be relieved should be painlessly killed at the end of the procedure, or if appropriate, during the procedure.
VII.	The living conditions of animals should be appropriate for their species and contribute to their health and comfort. Normally, the housing, feeding, and care of all animals used for biomedical purposes must be directed by a veterinarian or other scientist trained and experienced in the proper care, handling, and use of the species being maintained or studied. In any case, veterinary care shall be provided as indicated.
VIII.	Investigators and other personnel shall be appropriately qualified and experienced for conducting procedures on living animals. Adequate arrangements shall be made for their in-service training, including the proper and humane care and use of laboratory animals.
IX.	Where exceptions are required in relation to the provisions of these Principles, the decisions should not rest with the investigators directly concerned but should be made, with due regard to Principle II, by an appropriate review group, such as an institutional animal care and use committee. Such exceptions should not be made solely for the purposes of teaching or demonstration.

and reviews and approves experiments with infectious agents, including viral vectors and pathogens.

Institute of Laboratory Animal Research (ILAR)

The Guide was published in 1963 and has been revised seven times through committees of ILAR, which report to the National Research Council of the National Academies. The eighth edition (2011) introduced expanded sections on aquatic animal care. Because the needs of fish and other aquatic or semiaquatic animals are diverse and species-specific, these expanded sections provide broad guidelines for the management of aquatic animal systems. Specific recommendations are made for environmental parameters, such as water quality, temperature, humidity, ventilation, lighting, noise, and vibration and for documenting monitoring and maintenance of appropriate water quality. *The Guide* also describes recommendations for appropriate housing and feeding; food preparation and storage; veterinary care, including health monitoring, anesthesia, and euthanasia; and colony and life system management, including social housing and environmental enrichment. Specifics from *The Guide* are described in later sections of this chapter.

Association of Assessment and Accreditation of Laboratory Animal Care International (AAALACi)

Many institutions seek to attain the highest standards in animal care and use and voluntarily participate in an accreditation program administered by the AAALACi. AAALACi accreditation is widely recognized as the gold standard for animal care and use programs. AAALACi is not associated with any governmental agency; however, it is recognized by OLAW as evidence of achieving a high standard of care. Accreditation is obtained after an animal care and use program is reviewed by professionals from accredited peer institutions for adherence to the standards of *The Guide* and other applicable regulations and guidelines. These professionals are appointed by AAALACi to carry out such reviews as a site visit. AAALACi accredited programs must undergo this extensive review at least every 3 years to maintain their accreditation. Since AAALACi accreditation is based on successful adherence to PHS Policy, *The Guide,* AWR, *NIH Guidelines*, AVMA Guidelines, and other regulations, the maintenance of AAALACi accreditation is interpreted by the funding agencies and others as evidence that the institution is meeting all requirements to receive PHS funding. OLAW assigns those institutions with AAALACi accreditation category 1. Such institutions do not have to submit their semiannual program assessment reports directly to OLAW, unlike unaccredited institutions, which are assigned category two and must submit the semiannual program assessments directly to OLAW.

Institutions with DHHS funding may have other sources of funding that recognize OLAW assurance and AAALACi accreditation, such as the National Science Foundation and Department of Veterans Affairs. Many of these agencies have a memorandum of understanding (MOU) with OLAW that states that OLAW will apply PHS Policy and require PHS assurance for their funded projects. The MOU allows these projects using vertebrate animals to come under the purview of OLAW oversight.

American Veterinary Medical Association (AVMA)

The AVMA is a nonprofit organization representing more than 91,000 veterinarians working in private, government, industry, academia, and uniformed services primarily in the United States (AVMA Website). The AVMA provides information resources, continuing education opportunities, and publications, and lobbies for animal-friendly legislation. The AVMA also produces policies in response to member requests and stakeholder interests. These policies are based on the best available scientific evidence. One of these policies is the AVMA Guidelines on the Euthanasia of Animals, intended for use by members of the veterinary profession who carry out or oversee the euthanasia of animals (Leary, Underwood et al. 2013). The overriding commitment of this policy is to provide veterinarians guidance in relieving pain and suffering of animals that are to be euthanized. Specific guidelines for the euthanasia of fish and other aquatic species are defined, and most regulatory bodies discussed in this chapter require recognition and adherence to this publication for euthanasia of zebrafish used in biomedical research.

Occupational Safety and Health Administration (OSHA) and Other Responsible Organizations

Zebrafish facilities should comply with local and state regulations regarding workplace safety, general biosafety, environmental compliance, and waste management. For occupational hazards, some U.S. states regulate workplace safety at the state level, with the others falling under the Occupational Safety and Health Administration (OSHA). Laboratory and research safety differ from other workplace chemical safety concerns in several ways; this is why the United States has a separate OSHA standard for laboratories and research workplaces. Laboratory chemical safety in zebrafish research facilities falls under OSHA's Occupational

Exposure to Hazardous Chemicals in Laboratories standard (29 CFR 1910.1450) for OSHA-regulated states. State equivalents apply elsewhere: 8 CCR 5191 addresses laboratory chemical safety in the California Code of Regulations (OSHA 1990). Medical sharps or human blood or tissues that are used in the zebrafish facility may also be regulated under OSHA's Bloodborne Pathogens standard (29 CFR 1910.1030) or a similar local equivalent (OSHA 1991).

Testing laboratories should follow the Organization for Economic Cooperation and Development Guidelines for the Testing of Chemicals. The National Research Council's Prudent Practices in the Laboratory: Handling and Management of Chemical Hazards, Updated Version (2011) addresses laboratory chemical safety. This applies to everything from pH balancing chemicals to mutagens and teratogens used on zebrafish embryos (NRC 2011). Imaging or other biokinetics work with radioisotopes must meet local or federal, As Low As Reasonably Achievable (ALARA) principles using time, distance, and shielding in addition to complying with applicable state regulations, or Nuclear Regulatory Commission regulations in 10 CFR 20 for facilities that are not regulated by the 37 "Agreement State" regulations.

The CDC and NIH *Biosafety in Microbiological and Biomedical Laboratories* (*BMBL*), provides guidance for work at biosafety levels (BSL) and animal biosafety levels (ABSL) one to four (CDC 2007). The American Society for Microbiology (ASM) "Biological Safety: Principles and Practices," fifth edition, and ASM "Guidelines for Biosafety in Teaching Laboratories" also provide laboratory biosafety guidance (Emmert, Byrd, Gyure, Hartman, & White, 2012; Wooley & Byers, 2017).

In addition to normal laboratory waste, which may be either solid waste or listed hazardous waste, zebrafish facilities may also encounter wastewater and medical waste disposal constraints based on local, state, or federal environmental regulations. For example, most larger zebrafish facilities in the United States will fall under the National Pollutant Discharge Elimination System (NPDES) point source regulations and will need to coordinate with the local wastewater publicly owned treatment works (POTW) for discharge pH and any possible additional chemicals in the discharge. Different cities and towns have different wastewater treatment capabilities, so this often varies by the sewer system.

Local medical waste regulations apply to the handling and disposal of potentially infectious waste, which may include viral vectors for recombinant work or pathogens used in zebrafish. In the United States, these regulations vary by state but fall under the state EPA equivalent, with additional guidance by CDC, OSHA, and U.S. Food and Drug Administration (FDA). For ground transportation of biomedical waste, the United States. regulates untreated biomedical waste and samples as Hazard Class 6, Division 6.2 infectious substances under the U.S. Hazardous Materials Regulations (49 CFR 173.134), and air transportation of samples fall under the International Air Transport Association (IATA).

Hazardous Materials or Dangerous Goods Regulations (DGR) require medical wastes that may be Class 6, Division 6.2, Category A infectious material (defined as an "infectious substance in a form capable of causing permanent disability or life-threatening or fatal disease in otherwise healthy humans or animals") to be treated before transport, typically either by autoclaving or chemical sterilization. Note that medical waste vendors frequently require zebrafish facility staff to complete IATA training every 2 years, or U.S. DOT hazmat training every 3 years.

Those institutions receiving DHHS funding and creating, maintaining, and disposing of genetically manipulated zebrafish must meet the *NIH Guidelines* (see Section IIb NIH OSP) (RAC NIH OSP 2016). The *NIH Guidelines* require that the institutional IBC review and approve the creation of genetically modified zebrafish. Regulations differ between recombinant zebrafish and recombinant rodents; the *NIH Guidelines* exempt purchase, transfer, and certain types of recombinant rodent breeding in Section III-D-4-c. But the *NIH Guidelines* otherwise regulate recombinant animals, including zebrafish, under Section III-D-4, *Experiments Involving Whole Animals*. Researchers must coordinate with their IBC prior to creating knockout or transgenic zebrafish.

Institutional Responsibilities for Zebrafish Oversight

Scientists

The primary responsibility of any scientist conducting animal research is to assure the integrity and quality of the research performed and the welfare of the animals involved. The integrity of the research conducted begins in the planning stages of the study. Well-founded research designs and methods are key to the responsible conduct of animal studies and are supported and strengthened by extensive literature reviews and interactions with peers. Maintaining compliance throughout the study by adhering to approved study protocols and funding agreements can assure compliance with animal welfare regulations, laws, and standards. It is also important to apply ethical and responsible practices to both the conduct of research activities, and the collection, analysis, and presentation of data. Performing all aspects of the animal study with a conscientious regard for animal welfare and scientific integrity is key to the successful outcome of any animal research study. Due to the specialized nature of zebrafish care and the relative novelty of the model,

primary care of the animals and management of the housing system or facility may fall partially or in whole to the investigator. In these circumstances, the investigator, who may possess unique knowledge and expertise, could be responsible for the day-to-day management of the animal model but should work closely with the program veterinary and care staff, as well as the IACUC, to assure that the needs of the animals, the research, and the program are aligned and being consistently met.

Veterinary Care and Animal Care Staff

The health and well-being of research animals is the responsibility of all research staff but is the primary concern for veterinarians and animal care staff providing daily animal care and management. The best way to assure proper care and management of animals is to establish and adhere to daily care practices that support their physical and psychological health. These practices and standards should be designed to comply with the required standards and industry best practices, as well as to meet the individual needs of the institution's research program. Practices should provide for the animals' general care, and include methods for identifying, treating, and following-up on health issues and medical cases, as well as maintaining accurate and complete records documenting animal care. Beyond the immediate care of the animals, the veterinarians and care personnel are responsible for managing and maintaining a suitable environment for research animals and the facilities that support research activities. Facilities and established care practices should also be designed to promote and protect personnel safety to ensure a research program that provides consideration of animal welfare and the health and well-being of staff involved in animal care. In some instances, the investigator's knowledge of proper care and management of the animals and their housing may exceed that of the program staff. In these cases, the veterinary and care staff are responsible for working closely with the researcher to assure the care of the animals supports their welfare and is in line with the industry-standard practices.

Institutional Official

The authority to assure an institution's compliance with PHS Policy falls primarily to the Institutional Official (IO). The IO is the administrator responsible, with the authority, to sign the institution's Assurance with OLAW making a commitment that the requirements of the PHS Policy will be met. The IACUC, often the IBC, and other safety committees report to the IO. The IO must have authority to allocate resources to support the research program's ongoing compliance with ever-changing regulations and standards and evolving institutional needs. The IO must have a working knowledge of established safety committees, IACUC, and animal care activities, and the processes to promote and support the efforts of the research program. Frequent communications between the IO, the IACUC, and other unit administrators and staff about the needs and challenges of the program are crucial for preventing and managing noncompliance. Noncompliance incidents are reported to OLAW and other regulatory agencies through the IO. It is important for the IO to maintain open lines of communication internally to address any vulnerable or ineffectual areas of the program in order to avoid or minimize noncompliance, and to assure a culture of compliance within the institution.

Institutional Animal Care and Use Committee (IACUC) (Garber, Barbee et al. 2011, OLAW 2015)

The IACUC is responsible for overseeing all aspects of the animal care and use program to ensure compliance with animal welfare regulations, standards, and recommendations. The committee represents the interests and needs of the animals in balance with the needs of the researchers and the institution, in accordance with all applicable laws and requirements. The IACUC is required to oversee the use of zebrafish by the PHS Policy, and *The Guide*. The committee must be appointed by the Chief Executive Officer and include at least five members. The members must have sufficient knowledge, experience, and ability to assess animal care, treatment, and practices in experimental research and represent society's concerns regarding the welfare of the animals. The IACUC must include one member who is a doctor of veterinary medicine, one practicing scientist, one member whose primary concerns are in a nonscientific area, and one member who is neither affiliated in any way with the research facility nor an immediate family member of the research staff. One person can serve both roles of the nonscientific and unaffiliated members. However, the minimum number of members must be met, and not more than three voting members should be from the same administrative unit of the facility. The IACUC is responsible for reviewing all aspects of the institutional animal care and use the program, including specific research proposals, animal housing and procedures, facilities, personnel training and medicine programs. The IACUC must review program procedures and facilities at least every 6 months and report to the research facility's IO. Before any animal research can be performed, a written proposal must be submitted, reviewed, and approved by the committee.

The IACUC is authorized to require modifications of the proposal and suspend a research activity if it is determined that the activity is not being conducted according to the approved protocol. The research proposal must include the following information: (1) the species and approximate number animals to be used; (2) a rationale for the use of animals and justification of the species and number of animals to be used; (3) a description of the proposed use and procedures to be conducted with animals; (4) a description of procedures to minimize pain and discomfort; and (5) a description of any euthanasia method to be used.

Disaster Response Planning

All research facilities either registered with the USDA, have assurance with OLAW and/or accredited by AAALACi are required to have a disaster response plan. Aquatic facilities are especially vulnerable to power outages and other support system failures. Please see the chapter on emergencies for important details to consider.

Personnel Qualifications and Training

Principle VIII of the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training (Table 38.1) requires all researchers and other personnel to be appropriately qualified and experienced for conducting procedures on living animals. Housing facilities for aquatic species, especially high volume zebrafish facilities can be challenging for personnel not accustomed to working in such environments. Institutions should take advantage of training opportunities for staff to obtain high-quality instruction and experience.

Specific Challenges for Oversight of Zebrafish Research

Many factors affect the applicability of the various regulations that may govern zebrafish use in research or teaching.

Knowledgeable IACUC Members

Unless the institution has an established aquatics research program, it can be challenging to find IACUC members with the knowledge of issues and/or experience specific to the use of zebrafish to apply performance-based standards during project review or to assess the program and facilities. This is especially an issue when the pool of IACUC members with relevant knowledge and experience is limited to those researchers or instructors who are also submitting their own proposals; therefore, leaving none or only a few individuals who do not have a potential or perceived conflict of interest. It may be possible to address this problem by using ad hoc reviewers from other institutions or providing additional training to IACUC members who do not have expertise with aquatic models in general or zebrafish in particular.

Performance Based Standards

Zebrafish are relatively new animal models compared to mice, and the number of investigators and institutions using this novel model remains comparatively low to those using mice. Thus, the information about zebrafish husbandry in a research setting is sometimes extrapolated from other species or from commercial aquatic management practices. *The Guide* makes specific recommendations for the microenvironment, which includes anything with which the fish has immediate contact. Water quality is essential to the well-being of aquatic animals, and routine measurements of its characteristics are essential. Some important characteristics to monitor include pH, salinity, and levels of ammonium, nitrate, and nitrite. Monitoring should be documented with recorded data.

Transportation

Many concerns about transporting terrestrial animals also apply to the shipping of zebrafish. These include the availability of commercial carriers, security provided by the transport containers, environmental control of transport vehicles and any loading or holding facilities, and time in transit. Transporting fish involves additional concerns of assuring appropriate oxygen levels are present in the water and avoiding any deleterious buildup of ammonia or other contaminants. In general, the longer the transport distance, the more serious the concern. There is also a need for care during intrainstitutional transportation between animal rooms and to or from procedure space, laboratories, or facilities. Please see additional information in the Chapter on Import/Export and Transportation of Zebrafish.

Animal Numbers

IACUCs typically oversee and regulate the number of animals approved for use by requiring animal acquisitions from vendors, other institutions, or other investigators to be handled and recorded centrally. When animals are transferred between investigators within the institution, between protocols, or produced in breeding

colonies, self-reporting, or other procedures for census monitoring are needed. It is relatively easy to count the number of adult fish present at any time or to estimate it based on the number and size of aquaria present and the average number of fish per aquaria of a specific size (density); however, it may be necessary to estimate the number of eggs collected and their average survival time to estimate the total number of animals used.

Counting of Zebrafish Onto Protocols and Pain Category Assignment

OLAW provides guidance for accounting of larval forms of fish. Zebrafish hatch at approximately 3 days postfertilization (dpf) and must be included in an approved protocol at this stage and oversight provided by the IACUC (Bartlett & Silk, 2016). While fish at four to 7 dpf may require inclusion in a protocol, studies suggest that during this phase of development, the animals do not yet feel pain or distress. Thus, zebrafish do not need to be assigned a pain category until older than 7 dpf (NIH, 2016). Juvenile and adult fish, 8 dpf and older, must be assigned a pain category based on the proposed procedures. Institutions that are accredited through AAALACi must also follow recommendations for animal care and the oversight and guidance for aquatic housing and care, as outlined in *The Guide*. While *The Guide* does not refer to developmental stages in its application, it is generally accepted that *The Guide* standards be applied to zebrafish at and beyond the larval stage (>3 dpf).

Pain, Distress and Discomfort

The rule of thumb for assessing whether a procedure is painful has generally been "If a procedure is painful to a human, it is likely to be painful to a fish." It is also important to consider what factors or conditions might be potentially distressful for a fish during its use. For instance, fish are social animals, and so it may be distressful for a fish to be housed in isolation. If it is necessary to house fish singly in a tank, the tank should be placed so that the single fish inside it can see other fish group housed nearby. In these circumstances, the singly housed fish may be seen to shoal in unison with the group in the adjacent tank, thus exhibiting a natural group behavior.

Centralization of Zebrafish Facilities

Comparisons of the benefits of centralized versus decentralized facilities address many of the same considerations for zebrafish as for nonaquatic species. Centralized aquaculture facilities may cost less to build and maintain (ventilation, humidity, temperature, water treatment, sanitation), reduce duplication of equipment, provide more efficient use of animal care personnel, and reduce transportation of animals. Decentralized facilities offer greater convenience to individual research teams. Decentralized facilities offer redundancy and may provide backup resources if there is a planned or unplanned disruption of support services in a facility. Populations and studies can also be isolated to address disease concerns or research needs.

Environmental Enrichment

The Guide recommends that all animals be given the opportunity to express species-specific behaviors, and this may be accommodated through the provision of environmental enrichment. For fish, this can be met in several ways. Fish are social animals and will school if housed in groups. If zebrafish must be individually housed for experimental reasons, they may be given enrichment, such as plastic plants to accommodate their being alone. Live food is often offered to zebrafish because of its nutritional advantages, but this provision also serves as environmental enrichment because it allows zebrafish to exhibit their natural predatory feeding behavior.

Primary Housing: Housing Density and Sanitation

For many species, *The Guide* offers very specific information on the minimal acceptable amount of space allowed for each animal based on size and age. For fish, *The Guide* states that the number of animals in a location can vary based on the type and size of tank and age of fish, but the recommendation for adult zebrafish is a maximum of five zebrafish per liter. Many institutions have used performance standards to justify higher housing densities, which is allowable under PHS Policy if approved by the IACUC. There are also specific requirements for sanitation of tanks. This is in part based on the type of housing used, type of system used (static or recirculating), and water quality. Accumulation of debris or algae that would block the ability to view and monitor fish is not acceptable and should be removed and/or reduced. Fish tank lids may need to be cleaned regularly for removal of food debris.

Environmental Monitoring

The macro environment is the physical environment of the secondary enclosure, generally the room in which an aquatic housing system is located. These conditions must also be regularly monitored and documented. Room humidity is closely monitored for other species,

but is obviously not as critical for fish immersed in water and is often based on what is safe and comfortable for the staff. Similarly, the rate of air exchange in a room is not as crucial in fish facilities and may even be purposely lower than that required in rooms housing terrestrial animals in order to reduce evaporation from tanks. Some rooms housing aquatic species are kept at a similar temperature to that of the tank water to minimize water temperature fluctuations and to accommodate fish housed off the recirculating system in static tanks in the room. Illumination is also important, and because breeding is strongly affected by the room light cycle, the room photoperiod must be carefully regulated; the natural stimulus for breeding in zebrafish is the onset of daylight. Fish rooms should be maintained so that there is total darkness during the dark cycle, and the presence of even partial light during the dark period may disrupt breeding subsequently for days or even weeks.

Veterinary Care

Zebrafish colony health should be regularly monitored and the monitoring documented. *The Guide* requires that trained personnel observe all animals for signs of illness, injury, or abnormal behavior at least daily. Professional judgment should be used to ensure that the frequency and character of the observations minimize risk to the individual animals and do not compromise the research. Unexpected deviations from the normal should be reported promptly to assure timely delivery of appropriate intervention when needed. Procedures for disease prevention, diagnosis, and therapy should be those currently accepted in veterinary and laboratory animal practice. Additionally, appropriate procedures should be in place to allow colony disease surveillance, diagnosis, and management. Health monitoring programs may include regularly scheduled submission of sentinel fish. Fish found in sump tanks may serve as sentinels. Alternatively, sentinel fish placed into the facility from a known specific-pathogen-free source and exposed to colony animals and subsequent diagnostic evaluation may be used. Sentinels may be placed in various locations, including on the system, in a separate tank, or into the sump or waste stream of a system. Sentinel fish should be submitted to a laboratory with aquatic disease diagnostic experience and capabilities. *The Guide* states that health monitoring programs should be designed based on the size and complexity of the facility, the species involved, and the institutional research focus. In addition, records of morbidity or sickness and mortality or death should be kept as part of the monitoring program.

Anesthesia, analgesia, and euthanasia of zebrafish are discussed in more detail in other chapters, but it is discussed briefly here, as it relates to PHS policy and *The Guide*. Anesthesia is necessary to minimize pain and distress to fish during procedures, such as for the collection of tissue samples for DNA isolation and genotyping, often done by fin clipping. Anesthesia may be used for other surgical procedures and to allow manual gamete removal for in vitro work. The most commonly used anesthetic technique is immersion in tricaine methanesulfonate (MS 222, Finquel). Investigators may discuss appropriate anesthetics and analgesics, dose and route of administration with veterinarians experienced with zebrafish research. *The Guide* encourages the use of pharmaceutical-grade drugs and chemicals whenever possible in order to prevent adverse effects caused by impurities, instability, or toxicity of compounds. The IACUC may approve the use of agents unavailable in pharmaceutical grade after appropriate consideration of purity, sterility, efficacy, and other relevant characteristics. Euthanasia of zebrafish used in research must comply with the regulatory requirements. In the United States these requirements are the AVMA Guidelines for Euthanasia 2013 (Leary, Underwood et al. 2013). Commonly used methods are immersion in the recommended concentrations of buffered MS 222, and rapid chilling in an ice water bath with the appropriate holding times. Embryonic and larval life stages <7 d postfertilization may be euthanized by immersion in 10% sodium hypochlorite solutions. Dead fish should be disposed of as medical waste. Water solutions contaminated with chemicals may require treatment before entering the public wastewater stream. Some states may require an alternative disposal method.

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SECTION IV

Diseases

CHAPTER

39

Water Quality and Idiopathic Diseases of Laboratory Zebrafish

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Introduction

Diagnosis of non-infectious diseases often involves reviewing a history of dietary, environmental, and water parameters, assessing gross and behavioral clinical signs, and evaluating microanatomic changes in tissue sections. A thorough evaluation of these parameters can also be important in assessing whether identified infectious agents are in fact opportunistic and secondary to environmental or physiological changes.

Suboptimal water quality can induce a range of signs from subclinical to severe morbidity and mortality with acute to chronic effects. Many laboratory zebrafish facilities monitor water parameters electronically and utilize handheld meters and assays for back-up and periodic confirmation of computerized readouts. The ability to do on-demand testing of water parameters at different locations on and off the water system is important because the parameters to which the fish are exposed can vary depending on multiple factors. On a recirculating water system parameters may be affected by time of day, demand for water on the system, and whether water is flowing through new equipment or areas that have been stagnant for long periods of time. Water parameters on a flow through system may vary with fluctuations at the water source, especially seasonally. Microenvironments like Petri dishes, glass bowls, shipping bags, and spawning tanks should also be considered when investigating pathologies associated with suboptimal environmental parameters.

In this chapter, we review the most frequently encountered non-infectious diseases of laboratory zebrafish. Diseases of water quality include ammonia and nitrite toxicity, chlorine and chloramine exposure, heavy metal toxicities, supersaturation, and nephrocalcinosis. Idiopathic diseases and those with multiple potential non-infectious etiologies are also discussed. These include egg-associated inflammation, spinal deformities, operculum malformations, hepatic megalocytosis, cardiac pathologies, tissue hyperplasia, and fin lesions.

Diseases of Water Quality

Ammonia Toxicity

Description. Ammonia is released into the aquatic environment during the breakdown of organic material and as a waste product of fish metabolism. In water, ammonia exists as NH_4^+ and NH_3. The unionized form predominates at higher pH and temperature and is considered most toxic to fish, as it more readily crosses the gills. However, once it has crossed the gills, NH_3 is converted to NH_4^+, which is more toxic *in vivo*, damaging cellular structures, and metabolism (Rosenberg, 2012). Elevated ammonia results in increased tissue consumption of oxygen coupled with decreased transport of oxygen to the tissues (Schwedler, Tucker, & Beleau, 1985).

Pathobiology and Clinical Signs. Clinical signs of acute ammonia toxicity in fish include hyperventilating, or piping, at the surface, being in lateral recumbency on the bottom of a tank, rapid and erratic swimming, anorexia, and mortality (Daoust & Ferguson, 1984). Convulsions have also been described in trout (Smart, 1978). Chronic exposure to elevated ammonia can cause immunosuppression and impede growth (Colt & Armstrong, 1979; Walters & Plumb, 1980). Prolonged exposure is often associated with gill epithelial hypertrophy and hyperplasia with the fusion of lamellae in

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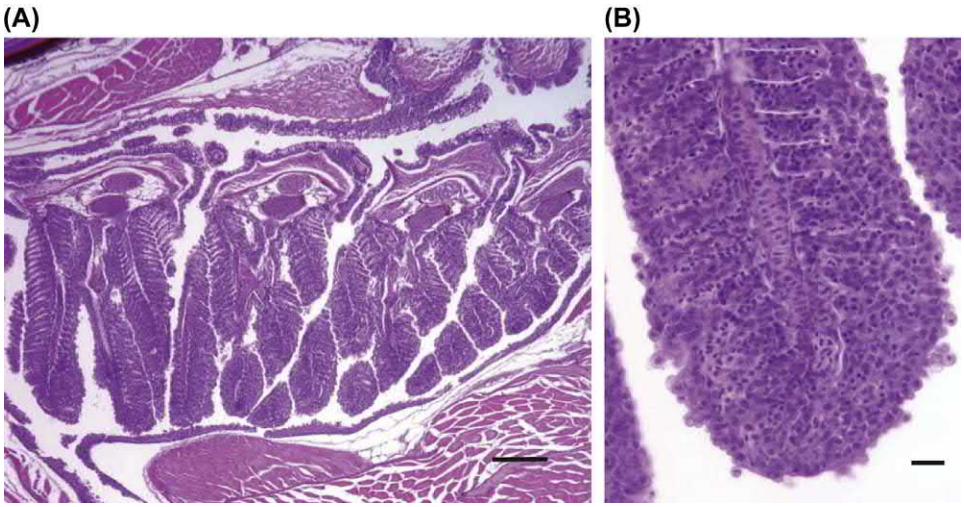

FIGURE 39.1 Gill epithelial hypertrophy and hyperplasia with the fusion of secondary lamellae. Scale bar is 200 μm in A and 20 μm in B. Sagittal section with H&E stain.

severe cases (Fig. 39.1). However, this gill lesion has not been reproduced in studies that selectively expose fish to high levels of ammonia (Braun, Steele, & Perry, 2009; Daoust & Ferguson, 1984), suggesting the epithelial change is a nonspecific effect of poor water quality, of which elevated ammonia is often a component.

Diagnosis. Ammonia toxicity is diagnosed by measuring unionized ammonia in system, tank, or microenvironment water. Many test kits simply report total ammonia nitrogen (TAN), but the amount of NH_3 can be calculated if pH and temperature are known (see Table 29.1 in Water Quality for Zebrafish Culture chapter). Ideally, zero ammonia should be measured in the system water. Even 0.02 mg/L of NH_3 can be harmful and greater than 1.0 mg/L is usually lethal (Meade, 1985).

Control and Treatment. Treatment should involve immediate efforts to reduce measured ammonia and correcting the source of the problem. Reducing unionized ammonia can be achieved by increasing the frequency of water changes, adding an ammonia adsorbent (e.g., zeolite), and using ammonia-neutralizing agents (e.g., ClorAm-X). The percentage of total ammonia in the toxic unionized form will decrease with reductions in pH and temperature and with higher salinity (Wedemeyer, 1996). However, changes in other parameters should be made cautiously and gradually as rapid changes can result in stress, immunosuppression, and even death. Long-term reduction of ammonia may entail improving biological filtration and evaluation of stocking densities, live food preparations, decomposition of uneaten food, and static water locations.

Nitrite Toxicity

Description. During nitrogen cycling, nitrites rise in a water system following an ammonia peak. Therefore, nitrite toxicity may occur alone or with ammonia toxicity. Chloride cells transport nitrite across the gills (Bath & Eddy, 1980) to the bloodstream where it oxidizes the ferrous iron (Fe^{+2}) in hemoglobin to ferric iron (Fe^{+3}), forming methemoglobin, which does not bind oxygen. Furthermore, the presence of methemoglobin also increases the affinity of the remaining hemoglobin for oxygen, reducing its ability to release and deliver oxygen to tissues.

Pathobiology and Clinical Signs. Nitrite toxicity, or methemoglobinemia, is also referred to as brown blood disease because the conversion of red hemoglobin to brown-colored methemoglobin can give blood and gills a brown appearance. Clinical signs associated with nitrite toxicity are typical of hypoxia. Fish may be lethargic, remain near the water inlet, and hyperventilate in well-oxygenated water (Kroupova, Machova, & Svobodova, 2005; Lewis & Morris, 1986). If ammonia toxicity is concurrent, gills are likely to have been damaged, impairing oxygen absorption and exacerbating general hypoxia. Nitrites can also induce hypertrophy and increased turnover of chloride cells, possibly to maintain normal body chloride levels in spite of competition by nitrites for uptake (Gaino, Arillo, & Mensi, 1984). Long-term exposure to nitrites can impair growth rates in zebrafish (Voslarova, Pistekova, Svobodova, & Bedanova, 2008). Mortalities are typically attributed to tissue hypoxia. Arillo, Gaino, Margiocco, Mensi, and Schenone, (1984) proposed that liver hypoxia and dysfunction are central to nitrite-induced mortality. As in other fish, zebrafish larvae are less sensitive to higher nitrite concentrations than older fish, which is likely a function of gill development and nitrite uptake (Voslarova, Pistekova, & Svobodova, 2006). Similar to ammonia toxicity, the poor water quality associated with elevated nitrites may result in hypertrophy and hyperplasia of gill epithelial cells (Wedemeyer & Yasutake, 1978).

Diagnosis. Diagnosis is made by observation of clinical signs of methemoglobinemia and measuring elevated nitrites in the water. Nitrites should be kept below 0.1 mg/L (Wedemeyer, 1996).

Control and Treatment. Nitrite toxicity is primarily a problem of recirculating water systems where biological filtration facilitates nitrogen cycling. Nitrites can be reduced by increasing water changes and adding chloride, which will compete with nitrite for uptake by the gills. Bowser, Falls, Vanzandt, Collier, and Phillips, (1983) recommend 3 mg of chloride to 1 mg of nitrite for treating channel catfish. Correcting the instigating cause is equally important. Improving biological filtration and examining stocking densities and feeding protocols should be considered. Once nitrites are removed, methemoglobin is reduced to nearly normal levels of hemoglobin in 24 h (Huey, Simco, & Criswell, 1980).

Chlorine and Chloramine Toxicity

Description. In zebrafish facilities, a dilution of household bleach (sodium hypochlorite) is commonly utilized to surface disinfect embryos, and higher concentrations are used to disinfect equipment. Chlorine is also used as a disinfectant in some municipal water supplies. When bleach is added to water, hypochlorous acid (HOCl) and hypochlorite ion (OCl^-) are formed. HOCl predominates in zebrafish system water, where the pH range favors the unionized form.

Pathobiology and Clinical Signs. Both HOCl and OCl^- are strong oxidizers that cross cell membranes and damage structures, enzymes, and nucleic acids, ultimately destroying the gills (Wedemeyer, 1996). Since HOCl is neutral, it crosses cell membranes more freely than OCl^- and is, therefore, more toxic. Chlorine also reacts with ammonia and nitrogenous compounds to form chloramines, which are more toxic than chlorine and also induce gill necrosis. Clinical signs of chlorine and chloramine exposure include respiratory distress and mortality.

Diagnosis. Diagnosis of chlorine toxicity is made by measuring chlorine in the water. Chlorine is acutely toxic and should be undetectable in zebrafish water systems.

Control and Treatment. Chlorine from municipal water can enter a zebrafish water system if carbon filters are damaged, expired, or improperly installed. Fish may also be exposed to chlorine if bleach used for embryo and equipment disinfection is not properly neutralized. If chlorine toxicity is suspected, fish should be immediately moved to chlorine-free water. Sulfur compounds can be utilized to neutralize chlorine. Sodium thiosulfate should be used at a ratio of 7.4 to 1 ppm chlorine (Jensen, 1989). However, this treatment will also release ammonia from chloramines, and therefore, concurrent ammonia treatment may be warranted. Some ammonia binders, e.g., ClorAm-X, will also bind chlorine and chloramines. Activated carbon will convert chlorine and chloramines to carbon dioxide and ammonium salts (Wedemeyer, 1996). Chlorine will also dissipate naturally, although this process is slow and 20 h should be allowed per mg/l of chlorine. Vigorous aeration can speed dissipation of chlorine, but not chloramines.

Heavy Metal Toxicity

Description. Zebrafish may be exposed to deleterious amounts of heavy metals through water or feed. A common route is by leaching from copper, lead, and zinc-coated (galvanized) plumbing components. Heavy metals are more toxic in low alkalinity, soft water, due to increased solubility. High temperature and low pH also increase metal solubility. Cadmium, zinc, copper, and nickel are likely absorbed via calcium pathways (Alsop & Wood, 2011; Hogstrand, Verbost, Bonga, & Wood, 1996; Niyogi & Wood, 2004). Although metal exposure reduces the uptake of Ca^{2+}, acute metal toxicity seems to be from loss of total body Na^+ (Alsop & Wood, 2011). Chowdhury, Girgis, and Wood, (2016) showed that copper decreases Na^+ influx and increases Na^+ efflux across the gills in rainbow trout by inhibiting branchial Na^+, K^+ ATPase activity.

Pathobiology and Clinical Signs. Numerous nonspecific clinical signs have been associated with metal toxicities. Gill pathologies include increased mucus production and epithelial changes involving separation from pillar cells, hyperplasia, swelling, necrosis, and desquamation (Authman, Zaki, Khallaf, & Abbas, 2015; Farrell, Ackerman, & Iwama, 2010). Copper exposure has been associated with an increase in gill chloride cells and decrease in mucous cells (Baker, 1969), as well as degeneration of chemoreceptors and mechanoreceptors (Gardner & Laroche, 1973). Gill alterations associated with metal toxicities result in decreased surface area for gas-exchange and osmotic dysregulation, leading to respiratory distress. Lead and mercury decrease acetylcholinesterase activity in zebrafish brains after 48 h exposure, but activity returns to normal by 30 days of chronic exposure (Richetti et al., 2011). Unlike other metals, iron toxicity is due to a direct effect of iron oxide precipitation on the gill surface (Abbas, Zaghloul, & Mousa, 2002; Peuranen, Vuorinen, Vuorinen, & Hollender, 1994). Other organs affected by metal toxicity include kidney, liver, and muscle. Reproduction, growth, and immune response may be compromised (Authman et al., 2015). In zebrafish, metal exposure has been shown to decrease embryo survival and hatching (Dave & Xiu, 1991).

Zebrafish exposed to harmful levels of chromium through contaminated nonhatching, decapsulated Artemia cysts (decaps) produced orange embryos that

had reduced survival rates (Tye, Montgomery, Hobbs, Vanpelt and Masino, 2018). Hexavalent chromium (Cr^{6+}) induces toxicity as a powerful oxidant that crosses cell membranes. Toxic effects are observed in gills, liver, and kidney (reviewed in Authman et al., 2015).

Diagnosis. Heavy metal toxicosis is diagnosed by measuring toxic levels of metal in system water, feed, or fish tissue. Toxicity will vary depending on the hardness, alkalinity, and amount of organic material in the water. Submitting fish tissue for metal testing may be especially useful as water and feed levels can vary and could be within normal limits during sampling. Samples should be submitted to specialized laboratories with experience in this kind of testing.

Control and Treatment. Treatment involves identifying and removing the source of the metal contaminants. For removing circulating metals, ion exchange filters and metal chelators can be utilized. However, they will also bind Ca^{2+} and Mg^{2+}, which are essential to fish survival and may need to be added back during water treatment. Ultraviolet sterilizers may break down metal-chelate complexes if they are recirculated. Reverse osmosis and carbon filters will also remove heavy metals from the water supply. Environmental factors that impact metal toxicity, like water hardness, temperature, and pH, should also be considered. Increasing calcium hardness significantly decreased copper toxicity in juvenile channel catfish (Chowdhury et al., 2016; Perschbacher & Wurts, 1999). In rainbow trout, the effects of calcium were independent of alkalinity, suggesting that the protective effect is from calcium competing for binding sites rather than a decrease in copper bioavailability (Chowdhury et al., 2016). We observed a similar protective effect of calcium at the Zebrafish International Resource Center, where the addition of a water softener in a flow-through quarantine room lowered dissolved calcium, which significantly increased the toxicity of copper leaching from pipes.

Supersaturation and Gas Bubble Disease

Description. Water is supersaturated with gas when the total pressure of dissolved gases exceeds atmospheric pressure. Supersaturation can occur when saturated cold water, which holds more dissolved gas than warm water, is pumped into a sealed system and warmed under pressure. System water can also become supersaturated if there is a leak on the suction side of a centrifugal pump, which will draw air into a pressurized system. Air trapped in distribution plumbing can also dissolve as water sits in the pipes and pressure builds. When the water in an aquaculture system becomes supersaturated, it can be deleterious to fish.

Pathobiology and Clinical Signs. Gas bubble disease refers to the tissue changes that can occur in fish exposed to supersaturated water. Fish blood and tissues quickly equilibrate with the partial pressures of dissolved gases in the surrounding water and become supersaturated as well. Once exposed to atmospheric pressure, gases come out of solution, forming bubbles on tank surfaces and in fish tissues. Gas emboli in the vasculature can result in occlusion, tissue necrosis, and mortality (Smith, 1988). In acute stages, bubbles form in well-perfused tissues, like the brain, gills, and vasculature (Fig. 39.2). However, gas can also be lost more easily from these tissues. In chronic stages, bubbles are more likely to be found in poorly perfused tissues like fat, where bubbles are slower to develop and resolve (Machado, Garling, Kevern, Trapp, & Bell, 1987; Strauss, 1979). While gas bubble disease is a population-level problem, not all fish in a supersaturated tank will develop clinical signs. There can be great variability in disease, especially at low levels of supersaturation. The reaction to supersaturation can differ for fish of different size and age. But even within and between tanks of the same age and size, some fish will develop gross lesions

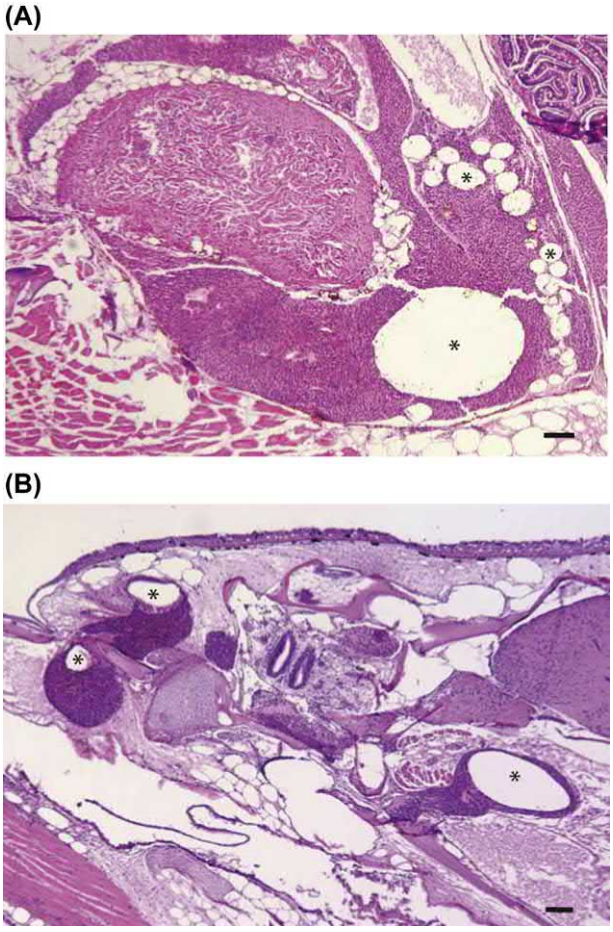

FIGURE 39.2 Gas bubble disease. Bubbles (*) in the heart (A) and the head vasculature (B). Scale bars are 100 μm. Sagittal sections with H&E stain.

while others appear unperturbed (Bouck, 1980; Weitkamp & Katz, 1980). The level of supersaturation and exposure time will influence the degree of associated fish morbidity and mortality. In a recirculating aquaculture system this may be influenced by the time of day and demand on the water system. Water that sits under pressure for extended time periods may contain more dissolved gases than when water demand is high and consistently flowing through the system. We have seen gas bubble disease in zebrafish exposed to water that had been sitting, unused, in a pipe for several weeks. Under pressure and over time, the water in the pipe may have become supersaturated by pockets of trapped air.

The formation of bubbles in the tissue will alter the buoyancy of fry and may drive them to the water surface. Juvenile and adult fish may exhibit lethargy, altered buoyancy, disequilibrium, and reduced feeding. Adult zebrafish with gas bubble disease generally exhibit rapid respiration and hover at the bottom of the tank, in contrast to incidences of oxygen deprivation, which drive fish to the surface. Unilateral and bilateral exophthalmia and bubbles within the tissues may be grossly visible, particularly in the eyes and fins (Lund & Heggberget, 1985; Machado et al., 1987). Mortalities may occur several days after exposure to supersaturated water and may transpire without accompanying gross lesions. Long-term effects of gas bubble disease include decreased growth rate (Batzios, Fotis, & Gavriilidou, 1998) and predisposition to secondary infections due to stress and tissue damage (Speare, 1991).

Diagnosis. The occurrence of gas bubbles in fish tissues is considered pathognomonic. However, supersaturation in zebrafish is frequently not associated with obvious bubbles in the tissues either on gross or microscopic postmortem examination. Therefore, history and clinical signs should be carefully evaluated. Bubble formation on tank surfaces is an important warning sign. Saturometers can be used to measure the total concentration of dissolved gas in the water. Measurements should be taken at several locations on the water system and at multiple times during the day. Fish species vary in their sensitivity to supersaturation. In general, 110% saturation is considered dangerous, but even 102% can be deleterious to salmonids (Krise & Meade, 1988).

Control and Treatment. Treatment will involve identifying and correcting the source of excess dissolved gas. Removing excess gas can be accomplished by aeration and allowing water to equilibrate with atmospheric pressure before it is sent to fish tanks. Vacuum and passive degassers can also be used. Water and air should be bled out of pipes that have been out of commission for an extended period before filling fish tanks.

Nephrocalcinosis

Description. Nephrocalcinosis is the result of deposition of calcium precipitate in renal tubules and ducts. High dissolved carbon dioxide and low oxygen, magnesium deficiency (Cowey, Knox, Adron, George, & Pirie, 1977), high selenium (Hilton, Hodson, & Slinger, 1980), and arsenic (Cockell, Hilton, & Bettger, 1991) have all been associated with nephrocalcinosis. High dissolved CO_2 seems to be the most important factor in the pathogenesis. Deviations in Mg, Se, and As would likely have additional nonrenal effects (Dabrowska, Meyerburgdorff, & Gunther, 1991; Sorensen, 1991). In zebrafish, nephrocalcinosis is commonly diagnosed on microscopic postmortem examination in the absence of an identifiable inciting cause and many cases may be idiopathic.

Pathobiology and Clinical Signs. In zebrafish, the effects of nephrocalcinosis are generally subclinical. Lesions range from a few small deposits to large accumulations of calcareous material resulting in extensive dilation of surrounding structures (Fig. 39.3). In other fish, nephrocalcinosis has been associated with decreased growth, impaired renal function (Lall, 2010), and mortalities when combined with stressful events (Wedemeyer, 1996). Growth rate and renal function have not been assessed in zebrafish with background nephrocalcinosis, although severe cases would likely negatively impact physiology and growth. Many cases of nephrocalcinosis diagnosed on postmortem examination are unlikely to be the primary cause of disease in these fish. As in mammals, mineral deposition within the tubules in these cases is likely the result of altered renal function secondary to systemic disease or dehydration.

Diagnosis. Diagnosis is made by histology and the observation of basophilic deposits in the renal tubules and/or collecting ducts. Not uncommonly, granulomas will form around large calcium deposits, and acid-fast staining should be performed in these cases to rule out renal mycobacteriosis.

Control and Treatment. Lowering fish stocking density and ensuring good water exchange and aeration are important for maintaining an appropriate level of dissolved CO_2. Chen, Wooster, Getchell, Bowser, and Timmons, (2001) saw a reduction in severe nephrocalcinosis in Nile tilapia after switching from $CaCO_3$, in the form of agricultural-grade lime, to $NaHCO_3$ to buffer the water. However, these fish were reared in a situation of high stocking density and feed rates, which typically pushed the pH below 7. On a zebrafish system, $CaCO_3$ in the form of aragonite can be advantageous because it is slow to dissolve and will increase pH and both general and carbonate hardness. Therefore, treatment should first focus on optimizing fish density, water exchange, and aeration.

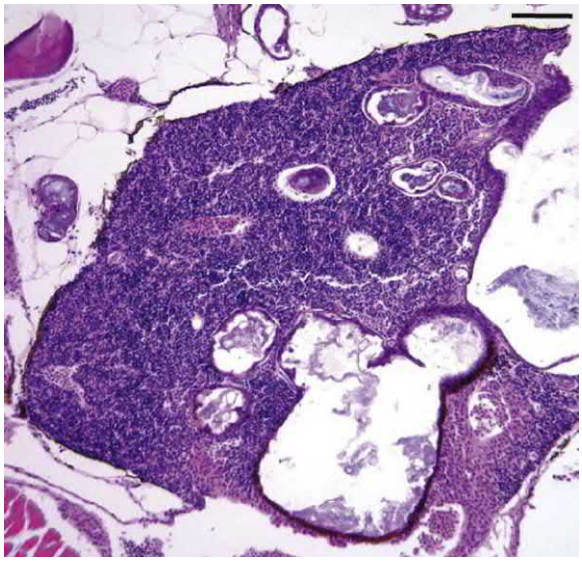

FIGURE 39.3 Nephrocalcinosis. Renal tubules are variably dilated with calcareous debris. Scale bar is 100 μm. Sagittal section with H&E stain.

Idiopathic Diseases

Egg-associated Inflammation

Description. It is common to find inflamed tissue associated with degenerating egg material in the ovaries of zebrafish. Lesions range from small foci of degenerating material with a mild inflammatory response to extensive, severely inflamed areas with fibroplasia and large rafts of degenerating egg debris (Fig. 39.4). External ulcers have been observed secondary to severe egg-associated inflammation and adhesions to the body wall (Kent et al., 2016). Granulomas may or may not be present. Lesions are usually sterile, with no evidence of infectious agents.

Pathobiology and Clinical Signs. The pathogenesis of egg-associated inflammation is believed to begin with egg retention, degeneration of egg material within the ovary, and a subsequent inflammatory response. Depending on the size of the lesion, zebrafish may present with distended abdomens. Similarly, fecundity may be reduced in accordance with the size of the lesion. Rossteuscher, Schmidt-Posthaus, Schafers, Teigeler, and Segner, (2008) observed spontaneous degenerative and inflammatory lesions in the ovaries of 78% (n = 46) of control zebrafish in a toxicity study. They noted that

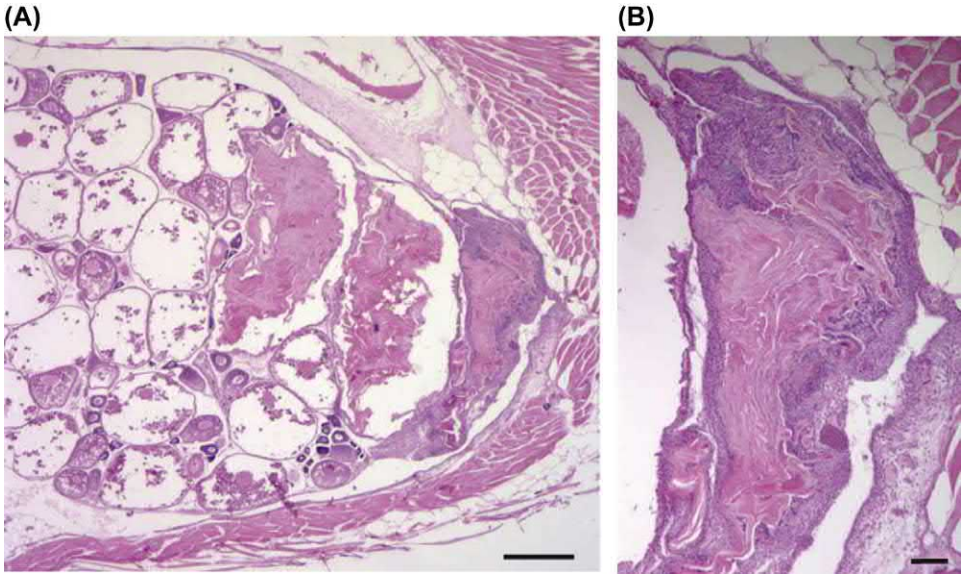

FIGURE 39.4 Egg-associated inflammation. (A) Eosinophilic, proteinaceous debris and inflammation in the caudal ovary. (B) Higher magnification of the caudal pole of the ovary. Scale bars are 0.5 mm in A and 100 μm in B. Sagittal section with H&E stain.

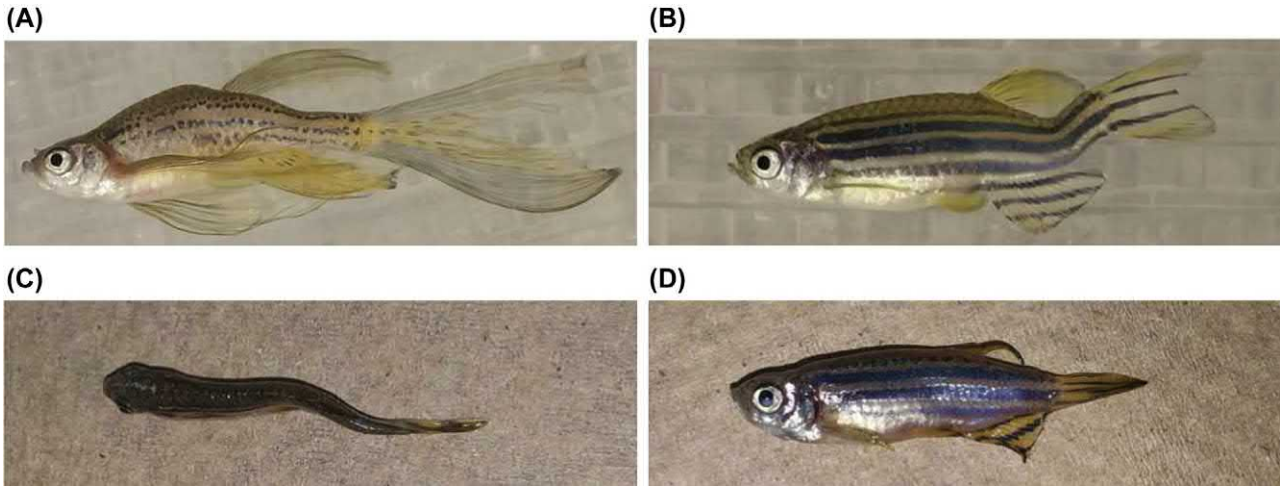

FIGURE 39.5 Spinal deformities. (A) Kyphosis, (B) lordosis, (C) scoliosis, and (D) platyspondyly.

gonad histopathology is often an endpoint in toxicity experiments, and these spontaneous lesions should be considered when determining appropriate sample size and evaluating potential treatment-induced alterations.

Diagnosis. Egg-associated inflammation is diagnosed by observation of inflammation in the ovary associated with degenerating egg material in histological sections. Inflammation may be mild to severe and granulomatous. Occasionally acid-fast bacilli (i.e., *Mycobacterium* spp.) are observed within granulomas, but it may represent secondary infection of the altered tissue. Lesions may be focal or locally extensive with inflammation extending from the ovary to adjacent tissues.

Control and Treatment. Housing male and female fish together and regular spawning may prevent egg retention, which is believed to be the underlying cause.

Spinal Deformities

Description. Spinal deformities include abnormal curvature and shortening from compressed vertebrae (platyspondyly). Curvature can occur in ventral (lordosis), dorsal (kyphosis), and lateral (scoliosis) directions. Since bone is metabolically active and is continually being remodeled, factors affecting bone development, growth, and repair can precipitate bone deformities at all ages. Still, ossification of bone during larval stages may make that stage especially sensitive (Nüsslein-Volhard & Dahm, 2002). Environmental, dietary, and genetic factors can all influence the development of spinal deformities. In particular, temperature, toxins, and infectious agents have all been linked to spinal deformities (Kent et al., 2016; Pohl, 1990; Sfakianakis, Georgakopoulou, Papadakis, Divanach, Kentouri, & Koumoundouros, 2006; Stickland, White, Mescall, Crook, & Thorpe, 1988). Several nutrients are important for skeletal development and pathology including calcium, phosphorous, manganese, zinc, selenium, and vitamins A, D, C, E, and K (Cahu, Infante, & Takeuchi, 2003; Lall & Lewis-McCrea, 2007). Vitamin C, ascorbic acid, is a cofactor in the biosynthesis of collagen and often mentioned in the pathogenesis of skeletal deformities. Fish do not synthesize vitamin C so it must be supplied as a dietary nutrient. Traumatic events during fish handling can also result in spinal changes.

Pathobiology and Clinical Signs. Spinal deformities are commonly observed in laboratory zebrafish colonies (Fig. 39.5). Curvatures may be noted as individual occurrences in mature fish or affecting large numbers of larval or juvenile fish. In aged zebrafish, spinal deformities have been attributed to degenerative changes including vertebral dislocations, thickening of bone at vertebral joints, fractures, and remodeling of vertebral bone and cartilage (Hayes et al., 2013). The authors have observed vertebral deformities with subsequent impingement of the spinal cord and nerve tracts in zebrafish that exhibited altered swimming behavior.

Diagnosis. Diagnosis is made by gross and microscopic examination of spinal curvature, compression, or alteration.

Control and Treatment. Multiple factors have been implicated in the pathogenesis of spinal deformities in fish. Environmental, nutritional, infectious, and genetic causes should be considered. An assessment of the onset of clinical signs, affected ages, and distribution within the population may help narrow down possible etiologies.

Operculum Malformations

Description. Operculum deformities have been described in multiple intensively reared fish species with changes including outward flaring, inward curling,

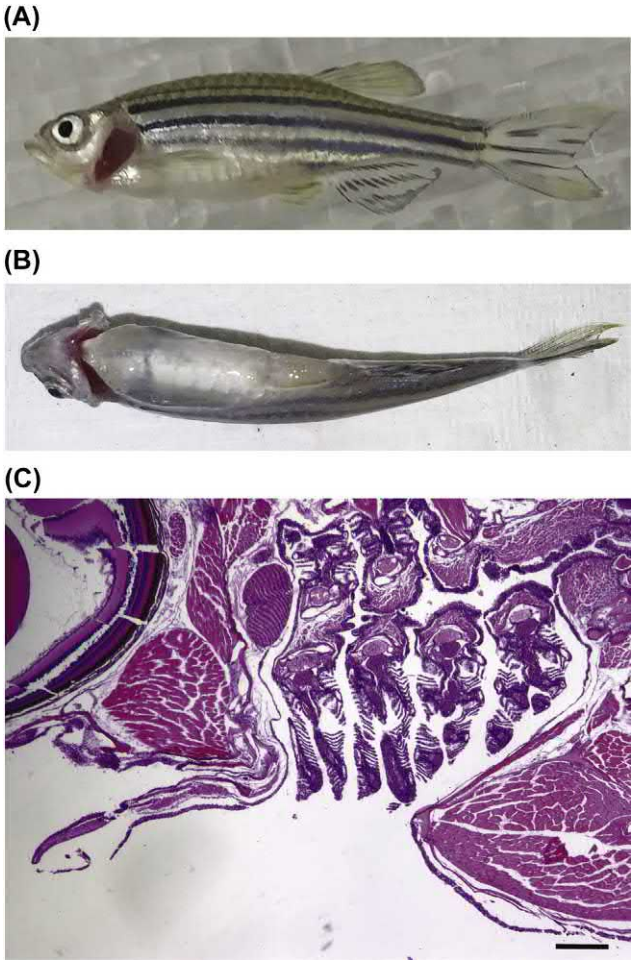

FIGURE 39.6 Operculum malformation. (A) Lateral and (B) ventral views of everted opercula. (C) Sagittal section with H&E stain. The ventral aspect of the operculum is everted, exposing the gill cavity. Scale bar is 20 mm.

and shortening (Beraldo, Pinosa, Tibaldi, & Canavese, 2003; Hilomen-Garcia, 1997; Lindesjoo, Thulin, Bengtsson, & Tjarnlund, 1994).

Pathobiology and Clinical Signs. Operculum malformations are common in laboratory zebrafish and almost always involve outward curling or eversion of the opercular flap (Fig. 39.6). The deformity is usually bilateral, but unilateral changes can occur. Gills are often visible as the malformation prevents complete coverage of the gill cavity. Within a zebrafish facility, the prevalence of malformed opercula may be quite high in particular lines and absent in others.

During genetic screens, Harris, Henke, Hawkins, and Witten, (2014) recovered zebrafish with opercular defects with Mendelian inheritance. Some of these mutants had general effects on skeletal development and differentiation and were not specific to the opercula. At the Zebrafish International Resource Center, we consistently see outward curling of the opercula without other grossly apparent skeletal changes in particular wild-type lines. We performed in-crosses and out-crosses to lines with normal opercula and did not observe simple Mendelian inheritance, suggesting multiple genes are involved and/or partial penetrance. Substantial recovery of unilateral inward curling of opercula was observed in gilthead sea bream (*Sparus aurata* L.) over a 16-month observation period (Beraldo & Canavese, 2010). Reversal of phenotype has not been documented in zebrafish. Histologically, the operculum malformations are not accompanied by degenerative or inflammatory changes. Importantly, deformations of the operculum may decrease growth rate, and the inability to cover the gill cavity may negatively affect the ability of a fish to handle situations of poor water quality and respiratory distress (Koumoundouros, Oran, Divanach, Stefanakis, & Kentouri, 1997).

Diagnosis. Diagnosis is made by gross examination and visualization of the gills where they should be covered by the operculum. Mild malformations may only be visible by histology.

Control and Treatment. There is likely a strong heritable component to the outward curling of opercula observed in laboratory zebrafish. Particular lines may have a high prevalence of the malformation compared to unaffected lines exposed to the same environmental and husbandry parameters. Breeding strategies to minimize the malformation should be considered, although efforts can be confounded by the fact that the lesions appear to progress as fish mature and mild curling may not be visible until after fish have started spawning. Delaying propagation of a line until phenotypes seem fixed may be advisable. Environmental causes have been linked to operculum deformities in other fish (Hilomen-Garcia, 1997; Lindesjoo et al., 1994), and should be considered along with nutritional and metabolic causes in the case of widespread operculum deformities in laboratory zebrafish.

Hepatic Megalocytosis

Description. Hepatic megalocytosis refers to the presence of greatly enlarged hepatocellular nuclei and cytoplasm components (Fig. 39.7).

Pathobiology and Clinical Signs

It is not uncommon for hepatic megalocytes to be multi-nucleate, presumably due to mitotic failure. Hepatic megalocytosis and nuclear pleomorphism are considered early lesions in the progression of hepatic neoplasia in English sole exposed to polycyclic aromatic hydrocarbons (Myers, Johnson, & Collier, 2003). In laboratory zebrafish, however, no link between

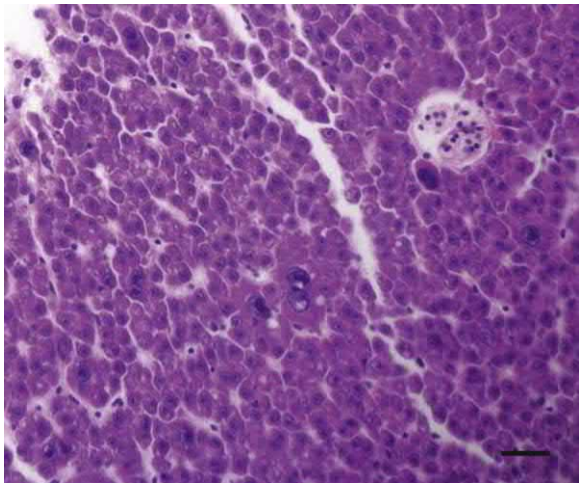

FIGURE 39.7 Hepatic megalocytosis. In scattered hepatocytes nuclei and cytoplasm are greatly enlarged. Binucleate megalocytes are present. Scale bar is 20 μm. H&E stain.

TABLE 39.1 Percentage of 8-month wild-type fish sampled at ZIRC between January 2016 and November 2017 with hepatic megalocytosis (HM).

Line	Total fish	Fish with HM	% With HM
AB	1281	453	35
WIK	91	1	1
TL	80	0	0
TU	70	0	0
SAT	110	0	0
TAB-14	45	0	0
NHGRI-1	70	0	0

megalocytosis and neoplasia has been elucidated. Hepatic megalocytosis has been described in chinook salmon from apparently unpolluted water in British Columbia (Stephen, Kent, & Dawe, 1993). Other liver and organ lesions were not present, and the prevalence of hepatic megalocytosis in wild chinook was greater than in chinook farmed in seawater netpens. Hepatic megalocytosis has frequently been associated with exposure to xenobiotics, although these cases also describe morbidity and additional microanatomic lesions. In rainbow trout, pyrrolizidine alkaloids induced hepatic megalocytosis, fibrosis, and necrosis along with venoocclusive and renal changes (Hendricks, Sinnhuber, Henderson, & Buhler, 1981). A water-borne or natural food toxin was presumed to be the cause of severe liver disease in Atlantic salmon that included hepatic megalocytosis, hydropic degeneration, necrosis, gross liver changes, and mortality (Kent, Myers, Hinton, Eaton, & Elston, 1988). In netpen-reared Atlantic salmon in coastal British Columbia, microcystin toxicity was the presumed cause of hepatic megalocytosis, severe necrosis, and hydropic degeneration (Andersen et al., 1993). In contrast, hepatic megalocytosis frequently occurs in apparently normal laboratory zebrafish with no accompanying gross, behavioral, or other microanatomic changes. An association has been drawn between the occurrence of hepatic megalocytosis in zebrafish and recirculating water systems with fluidized sand filters (Spitsbergen, Buhler, & Peterson, 2012; Spitsbergen & Kent, 2003). However, these system parameters alone are insufficient for lesion induction as not all facilities with recirculating systems and fluidized sand filters have zebrafish with hepatic megalocytosis. The lesion was observed in moribund zebrafish in a new facility, where it was presumed to be associated with a toxin leaching from new plastics on the system (Kent et al., 2011). Spitsbergen et al. (2012) postulated that tumor promoters and carcinogens in recirculating systems fluctuate over time resulting in cohort variations in phenotypes, including hepatic megalocytosis and liver and gastrointestinal neoplasias.

The Zebrafish International Resource Center operates on recirculating water systems with fluidized sand filters. We have noticed a particularly high prevalence of hepatic megalocytosis in the AB line compared to other wild-type lines exposed to the same water and husbandry conditions, suggesting a possible genetic component to susceptibility (Table 39.1). The effects of megalocytosis on liver metabolism and physiology are unknown; however, the possible variations in line susceptibility are worth considering when choosing a line for toxicology studies. Importantly, the observation of this abnormality in such studies should be interpreted very carefully with a close examination of and comparison to control and background livers.

Diagnosis. Hepatic megalocytosis is diagnosed by histological examination of the liver and observation of hepatocytes with greatly enlarged nuclei and cytoplasm. Cells may be multi-nucleate. In zebrafish, the megalocytes are usually randomly distributed throughout the hepatic parenchyma but may be focally clustered.

Control and Treatment. Toxin exposure should be considered, especially if morbidity, mortality, or other microanatomic changes are noted along with hepatic megalocytosis. When hepatic megalocytosis was associated with leaching plastics in a new zebrafish facility, installation of a carbon filter was preventative (Kent et al., 2011).

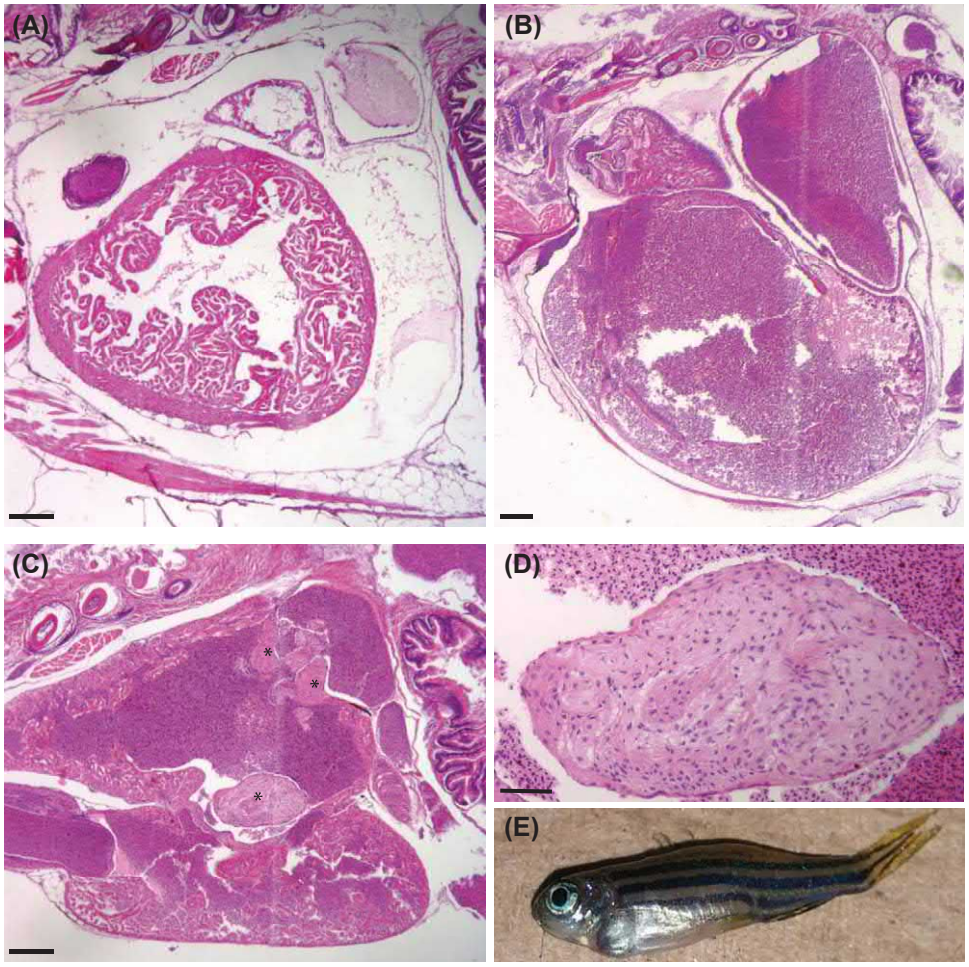

FIGURE 39.8 Cardiac pathologies. Sagittal sections with H&E stain of (A) pericardial effusion, (B) dilated cardiomyopathy, and (C) and (D) myxomatous heart valves (*). (D) Higher magnification of the atrioventricular valve. (E) Grossly visible swelling of the cardiac area. Scale bars are 0.2 mm in A, B, and C and 50 μm in D.

Cardiac Pathologies

Description. Pericardial effusion, dilated cardiomyopathies, and degenerative cardiac valves are occasionally observed in laboratory zebrafish.

Pathobiology and Clinical Signs. Cardiac pathologies may result in grossly visible lesions, including swelling of the cardiac area and raised scales from secondary scale pocket edema. Accumulation of fluid in the pericardial space, dilation of one or more chambers of the heart (sinus venosus, atrium, ventricle, bulbous arteriosis), and myxomatous valvular changes may be apparent in histological sections (Fig. 39.8). Varying degrees of secondary edema may be observed in the coelomic cavity and scale pockets.

Diagnosis. Diagnosis is made by examination of cardiac structures in histological sections. Sagittal sections can provide good visualization of multiple chambers, although multiple sections may be needed to see the valves. Mild dilation of one or more cardiac chambers in tissue sections is not necessarily diagnostic for dilated cardiac disease and may instead reflect changes at euthanasia or sectioning parameters. Additional changes, like gross swelling of the cardiac area, valvular abnormalities, or scale pocket edema, may indicate that the observed dilation is pathological.

Control and Treatment. Cardiac pathologies in zebrafish are generally sporadic and infrequent. If they are regularly identified in a facility, an investigation into affected stocks, ages, and distribution should be considered along with possible genetic, environmental, and nutritional factors.

Organ and Tissue Hyperplasia

Description, Pathobiology, and Clinical signs. Hyperplasia of certain tissues is not an uncommon finding during routine evaluation of histological sections of zebrafish. Testis, ultimobranchial gland, biliary tree, and thyroid are most frequently affected. It is worth noting that ectopic thyroid tissue outside of the pharynx is occasionally

observed in zebrafish. Therefore, distinguishing between metastatic thyroid neoplasia and hyperplasia of ectopic tissue is a particular diagnostic challenge.

Diagnosis. Diagnosis is made by histology and observation of increased size or amount of normal organ or tissue structures. Cellular characteristics should be carefully evaluated to distinguish between hyperplastic, dysplastic, and neoplastic growth. These parameters are discussed in detail in chapter 43, Nonexperimentally Induced Neoplastic and Proliferative Lesions in Laboratory Zebrafish.

Control and Treatment. Genetic and environmental factors may influence the development and progression of hyperplastic growth. A high prevalence of bile duct hyperplasia has been noted in TL zebrafish relative to other wild-type lines (Spitsbergen et al., 2012). Calcium and iodine availability and metabolism should be considered when multiple fish exhibit ultimobranchial and thyroid hyperplasia, respectively. Spontaneous occurrence is also possible.

Fin Lesions/Erosion

Description. Fin lesions that do not have a primary infectious etiology are often termed fin erosion, to distinguish them from fin rot. Fin erosion encompasses dermal and epidermal damage resulting in fin splitting, fraying, reduced length, nodularity, necrosis, and epithelial hyperplasia (Latremouille, 2003; Turnbull, Richards, & Robertson, 1996). Fin erosion is a common problem in intensive aquaculture. Fortunately, these lesions are not common in laboratory zebrafish. We include a description of them here because the Zebrafish International Resource Center (ZIRC) has experienced two episodes of widespread fin erosion, indicating that zebrafish populations are susceptible and preventive protocols should be considered.

Pathobiology and Clinical Signs. Fin erosion is a multifactorial problem (reviewed in Ellis et al., 2008; Latremouille, 2003). Interfish aggression, abrasion from environmental surfaces, and contact from handling and transportation have all been identified as primary causes of fin erosion. Secondary factors, like water quality, opportunistic infections, and diet, can inhibit healing and regeneration. Stress is frequently believed to be a contributing factor as cortisol is a notorious inhibitor of regeneration and repair (Iger, Balm, Jenner, & Bonga, 1995; Roubal & Bullock, 1988). Genetics may also play a role in the propensity for aggressive behavior and immune response. Damage to epidermal club cells results in the release of alarm substance, a pheromone that induces anxiogenic behavior and increased cortisol in conspecific zebrafish (Abreu, Giacomini, Koakoski, Piato, & Barcellos, 2017; Egan et al., 2009; Pfeiffer, 1977; Waldman, 1982). The compound effects of injury, stress, and altered behavior should, therefore, be considered when evaluating the prevalence of fin lesions and the roles of primary and secondary factors.

Zebrafish fins have a robust regenerative capacity (Azevedo, Grotek, Jacinto, Weidinger, & Saude, 2011). Complete regeneration of the caudal fin takes approximately 2 weeks under optimal circumstances. However, if a fin ray is removed to the base, it may not regrow (Goss and Stagg., 1957). The ZIRC noted a high prevalence of fin lesions, including splitting, fraying, missing bony rays, and severe truncation in the year following a fire in the building (Fig. 39.9). Postfire recovery and reconstruction involved significant and random variations in sound, vibration, and light intensity in the fish room. Tanks were covered in opaque plastic sheeting during ceiling work, which affected tank illumination, feeding schedules, and regular observation of the fish. Six to 8 months after the fire, fin lesions developed in several lines. The most severe lesions occurred in the pelvic fins, which were almost completely truncated in some cases. Although pelvic fin lesions were the most dramatic, they were also the most difficult to visualize and required anesthesia for an accurate assessment. Lesions were also routinely observed in dorsal, anal, and caudal fins. Water and fire-related fine black debris were negative for common toxins. Histological sections of fish with lesions were evaluated, and no infectious agents or lesions besides those on the fins were identified. There was no change in diet source, expiration date, quantity, or content. Lesions occurred in several different wild-type, mutant, and transgenic lines, ruling out a simple genetic cause. Color mutants were the most severely affected stocks. Interestingly, albino zebrafish have previously been described as high-anxiety compared to other zebrafish lines (Egan et al., 2009). Aggressive nipping was observed in some tanks, but aggression did not seem consistently elevated in all tanks. In 20-gallon (75.7-L) tanks, strands of green netting were suspended from floating tubes to give fish a hiding place or distraction. Healing was observed in lesions that did not involve complete removal of a bony ray, and the lesions eventually resolved as operations and room parameters normalized. We concluded that the stress associated with fire recovery and reconstruction played a major role in lesion development. A second incidence of fin lesions occurred 2 years later. The elevated noise and vibration that accompanied installation of a cage washer adjacent to the fish room may have played a role.

Diagnosis. Diagnosis is made by gross visualization of fin lesions in multiple fish in a tank or population. Fins may be split, frayed, reduced in length, or nodular. Histologically, necrosis and epithelial hyperplasia may also be observed. Both dermis and epidermis are affected. Histological sections should be evaluated to

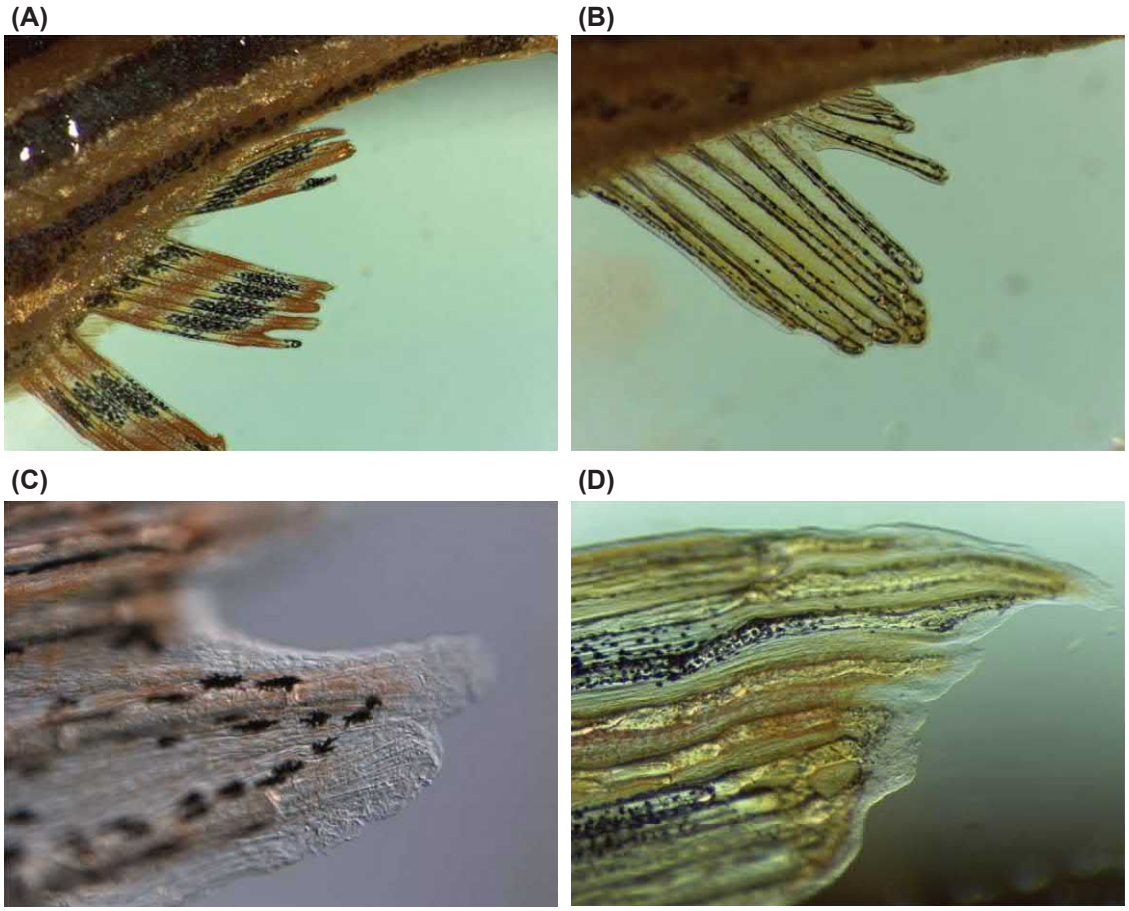

FIGURE 39.9 Fin lesions observed at the Zebrafish International Resource Center. (A) Missing fin rays, (B) split fin, (C) truncated fin, and (D) healing fin.

rule out a primary infectious cause for the lesions. Bacterial and fungal cultures may be useful, but should be interpreted cautiously and in addition to histology as the culture of external surfaces are easily contaminated by normal environmental flora and opportunistic secondary invaders.

Control and Treatment. The etiology of fin lesions at a population level can be multifactorial. Therefore, control efforts should include ruling out infectious, genetic, diet, toxic, and water quality-related causes and minimizing environmental and mechanical stress. Methods to reduce aggression should be considered. Feeding protocols should be evaluated to determine if fish are fed to satiety, if all fish have access to food and whether the location and/or method of food delivery potentiates competition and/or stress. The role of tank dynamics, stocking densities, and environmental enrichment should also be assessed. Spawning tank aggression may be mitigated by limiting the amount of time males and females are mixed, providing hiding places or distracting material (e.g., fake grass), and assuring optimal water quality. Facility alterations in light, sound, and vibration should also be considered and mitigated to the greatest possible extent.

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CHAPTER

40

Important Parasites of Zebrafish in Research Facilities

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This chapter describes the parasites of importance to zebrafish maintained and used in laboratory settings. Parasites emphasized are those capable of spreading and proliferating in research facilities. As with most wild fishes, zebrafish have been reported to be hosts for a variety of helminth parasites (Smith et al., 2011). However, parasites that have only been reported from wild zebrafish are not addressed in this chapter, as most have complex life cycles requiring other hosts, and hence, would not proliferate in a typical zebrafish facility.

It may be surprising that parasites are actually common in zebrafish facilities, given the confined environment and the parasite-free water sources used in most facilities. However, laboratories usually exchange fish among themselves with no knowledge of prior disease history (Lawrence et al., 2012). Moreover, many researchers still use zebrafish procured from pet fish dealers. Fish from this source is of particular concern, perhaps even more so than wild-caught zebrafish, as they are reared in water systems that are shared with numerous fish species that originated from a variety of geographical locations around the world. Many pet fish are reared in outdoor ponds, which is another likely source of parasites native to fishes of that particular region. Moreover, the development and adoption of strategies to avoid parasitic infections lag far behind those used for rodent models and food fish aquaculture. This is likely because zebrafish researchers often do not recognize the level of impact on their research caused by using fish with underlying diseases. For example, up until about 5 years ago, the ZIRC diagnostic services conducted disease evaluations on approximately 5% of the zebrafish facilities listed in ZFIN (Lawrence et al., 2012).

For this chapter, we review the following parasites: *Pseudoloma neurophilia* and *Pleistophora hyphessobryconis* (Microsporidia), *Piscinoodinium pillulare* (a parasitic dinoflagellate), *Ichthyophthirius multifiliis* (Ciliata), *Myxidium streisingeri* (Myxozoa), *Pseudocapillaria tomentosa* (a capillarid nematode), *Transversotrema patialense* (Digenea), and encysted metacercariae (Table 40.1).

Microsporidia

The Microsporidia are a group ofx single-celled, obligate intracellular parasites with a complex taxonomic history. Originally considered to be a member of the now defunct Phylum Microspora, they are currently considered to be either sister to or within the Fungi. However, their nomenclature is still administered according to the International Code of Zoological Nomenclature, and clinically, they have many characteristics of protists rather than fungi.

Microsporidian parasites are among the most common pathogens of fishes, with over 120 species and about 15 genera described from numerous fish species (Kent, Shaw, & Sanders, 2014; Lom, 2002). Relatively few species have been described from laboratory fishes (Kent & Fournie, 2007), and two are recognized as significant causes of disease in laboratory-reared zebrafish: *Pseudoloma neurophilia* and *Pleistophora hyphessobryconis*.

TABLE 40.1 Important parasites in zebrafish research facilities.

Parasite group	Species	References
Dinoflagellate	*Piscinoodinium pillulare*	Westerfield, 2000
Ciliate	*Ichthyophthirius multifiliis*	Matthews, 2004
Nematode	*Pseudocapillaria tomentosa*	Kent et al., 2002
Myxozoan	*Myxidium streisingeri*	Whipps et al., 2015
Digenea	Metacercariae of undetermined species	Smith et al., 2011; Kent & Fournie, 2007
Digenea	*Transversotrema patialense*	Womble et al., 2015
Microsporidia	*Pseudoloma neurophilia*	de Kinelin, 1980; Matthews et al., 2001
Microsporidia	*Pleistophora hyphessobryconis*	Sanders et al., 2010

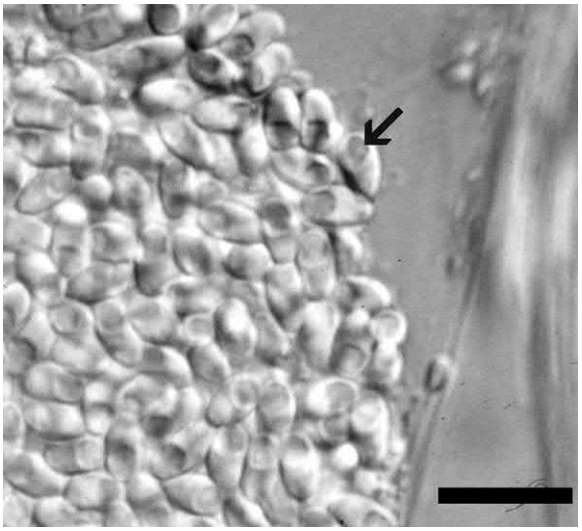

FIGURE 40.1 *Pseudoloma neurophilia*. Wet mount, Nomarksi phase interference. *Arrow* = posterior vacuole. Bar = 10 μm.

Pseudoloma Neurophilia

Description. The first report of a microsporidium infecting zebrafish was by de Kinkelin in fish purchased from a pet store for use in a toxicological study over 30 years ago (De Kinkelin, 1980). Subsequently, *Pseudoloma neurophilia* was described by Matthews et al. (2001) from a large zebrafish facility. Since then, awareness of *Pseudoloma neurophilia* infections in laboratory zebrafish has grown dramatically.

In 2011, infections of zebrafish by *P. neurophilia* were reported in 74% of zebrafish facilities examined through the ZIRC diagnostic service (Murray et al., 2011) and the concern of researchers for potential nonprotocol induced variation (Kent, Harper, & Wolf, 2012) in studies utilizing zebrafish with chronic microsporidian infection led to the derivation of a zebrafish colony, which is specific-pathogen-free (SPF) for *P. neurophilia* (Kent et al., 2011). In addition to providing SPF fish for toxicological research, this colony also provides the opportunity to study transmission of *P. neurophilia* using fish, which are known to be *P. neurophilia* free prior to experimental exposure.

As the name implies, the primary site of infection of *P. neurophilia* is usually neural tissue of adult fish, generally centered around the spinal cord and hindbrain and the parasite can often be observed to track along myelinated nerve fibers (Spagnoli, Xue, & Kent, 2015; Spagnoli, Xue, Murray, & Chow, 2015). However, the ultrastructure of *P. neurophilia* developmental stages has been described within the sarcoplasm of somatic muscle cells in larval fish (Cali, Kent, Sanders, Pau, & Takvorian, 2012), and its early development has been described in many other organs including kidney, and most notably, ovarian stroma and within developing oocytes (Sanders, Peterson, & Kent, 2014).

Spores of *P. neurophilia* are pyriform with an average length of about 5 μm and width of 3 μm (Fig. 40.1). They contain a large posterior vacuole, which is characteristic for the spores of microsporidia. As with most microsporidian spores, these are extremely tough structures meant to withstand long periods in the environment and possess a thick, chitinous endospore requiring special methods to disrupt them (Sanders & Kent, 2011).

Host Range. Sanders, Watral, Stidworthy, and Kent (2016) experimentally infected fishes that are also used in research, including giant danio, *Devario aequipinnatus*, fathead minnow *Pimephales promelas*, Japanese medaka *Oryzias latipes*, betta *Betta splendens*, goldfish *Carassius auratus*, platyfish *Xiphophorus maculatus*, as well as neon tetra *Paracheirodon innesi*. These hosts represent members of five different families of fishes. Infections in these fishes appeared similar to those seen in zebrafish, with aggregates of spores in the CNS. In addition, they documented a natural outbreak of *P. neurophilia* in fathead minnows from a laboratory population in England.

Life Cycle and Modes of Transmission. Cali et al. (2012) and Sanders et al. (2014) described the sequential development of *P. neurophilia* based on experimental infections of zebrafish using electron microscopy and light microscopy with an in situ probe, respectively. The infection is initiated by ingestion of intact spores, which inject their sporoplasm into the cytoplasm of an intestinal epithelial cell. The development of *P. neurophilia* appears to be relatively rapid with proliferative stages and sporonts observable at 4–5 days postexposure and all stages, including mature spores, present after about 1 week. Histological examination using a *P. neurophilia*-specific oligonucleotide probe revealed that the parasite

initially infects a wide variety of organs, including the liver. Later in the infection, the parasite is restricted mostly to the CNS, skeletal muscle, and ovaries in immunocompetent fish (Kent & Bishop-Stewart, 2003).

As with many other microsporidia, the transmission of *Pseudoloma neurophilia* is more complex than originally thought. Elucidation of the transmission characteristics of *P. neurophilia* has been important not only to the control of infections in laboratory zebrafish colonies but also to the understanding of the basic biology of microsporidian parasites. Our early studies showed that *P. neurophilia* is transmitted horizontally, ostensibly per os, by exposing adult zebrafish to inocula prepared from the spinal cords of infected fish (Kent & Bishop-Stewart, 2003). These results suggested that the cannibalism of dead tank mates serves to transmit the infection to naïve hosts. Spores are also shed into the water by live fish during spawning (Sanders & Kent, 2011), and fish cohabitating with infected fish become infected at a high rate.

Whereas *P. neurophilia* is clearly horizontally transmitted, the shedding of spores during spawning and the presence of spores in the ovarian tissue and within developing oocytes suggests the possibility of vertical transmission of *P. neurophilia*, and Sanders and Kent, 2013 verified that vertical transmission, both intraovum and extraovum, occurs. The low prevalence of intraovum transmission observed in single spawning events in this study (~1%) suggests that this mode of transmission plays only a minor role in the maintenance of *P. neurophilia* in a zebrafish population. However, it is important to note that zebrafish spawn continuously and the cumulative effect of even a low frequency of intraovum infections can result in a widespread infection, especially in populations where horizontal transmission is controlled. In addition, extraovum transmission (i.e., the shedding of spores during spawning), coupled with the high susceptibility of larval zebrafish, provides further opportunities for the parasite to be transmitted.

Clinical Signs and Pathobiology. Fish with acute microsporidiosis attributed to *P. neurophilia* will often appear emaciated, and less frequently they may exhibit spinal deformations such as lordosis, kyphosis, and scoliosis (Fig. 40.2) (Matthews et al., 2001; Spagnoli, Xue, & Kent, 2015; Spagnoli, Xue, Murray, et al., 2015). However, the majority of fish infected with *P. neurophilia* show no overt signs of infection. Typically, low levels of mortality and some reduced fecundity may be present in fish populations with chronic infections (Matthews et al., 2001; Ramsay, Watral, Schreck, & Kent, 2009).

FIGURE 40.2 Emaciated zebrafish with clinical microsporidiosis.

Zebrafish are increasingly used in experiments measuring behavioral endpoints (Fetcho et al., see Research Chapter 40). *P. neurophilia* is a potential confounding factor in studies using behavioral endpoints, which is of particular concern given that the infection is common and infected fish often do not exhibit obvious clinical changes. Chronic neural infections by *P. neurophilia* have been demonstrated to result in significant behavioral changes attributed to a hyper-vigilante phenotype, leading to behavior associated with higher anxiety, such as the formation of tighter shoals and a lack of habituation to startling stimulus (Spagnoli, Xue, & Kent, 2015; Spagnoli, Xue, Murray, et al., 2015; Spagnoli, Sanders, & Kent, 2016). Consistent with this change, in a study by Spagnoli, Xue, and Kent (2015); Spagnoli, Xue, Murray, et al. (2015) infected fish were less likely to be caught by netting compared to uninfected fish, which should be considered when sampling populations for the presence of the infection.

The primary site of *P. neurophilia* infection is the central nervous system (Fig. 40.3). Spagnoli, Xue, and Kent, (2015); Spagnoli, Xue, Murray, et al. (2015) provide an extensive description of the histopathology and precise distribution of the infection based on the retrospective analysis of hundreds of infected zebrafish from diagnostic cases. Parasite clusters (PCs) generally occur in distinct axonal swellings, frequently with no associated inflammation. Inflammation is observed in viable cell bodies distant from PCs. Multiple PCs occasionally occur within a single axon, suggesting axonal transport. PCs occur most frequently in the spinal cord ventral white matter and the spinal nerve roots. Within the rhombencephalon, PCs are common in the primary descending white matter tracts. Within the rhombencephalon gray matter, PCs occurred most frequently in the reticular formation and the griseum centrale. The occurrence of high numbers of PCs within the brain and spinal cord structures that are associated startle responses and anxiety is consistent with the behavior changes seen in infected fish (Murray, Varga, & Kent, 2013; Spagnoli, Xue, & Kent, 2015; Spagnoli, Xue, Murray, et al., 2015).

Inflammation associated with *P. neurophilia* infection is common, and infections in the CNS are associated with encephalitis/myelitis when present in the neuropil of the brain or spinal cord, or meninxitis (corresponding to meningitis in mammals) when it is associated with the membranes surrounding the central nervous system (Fig. 40.3C) Muscle infections occur in about 10%–15% of the cases, and present as a distinctive pattern characterized by multifocal to coalescing areas of granulocytic to granulomatous inflammation with concurrent myodegeneration (Fig. 40.3B). Only a few free spores or PCs are usually detected within the usually extensive muscle lesions (Sanders, Watral, & Kent, 2012). The ovary is another common site of infection, with spores occurring

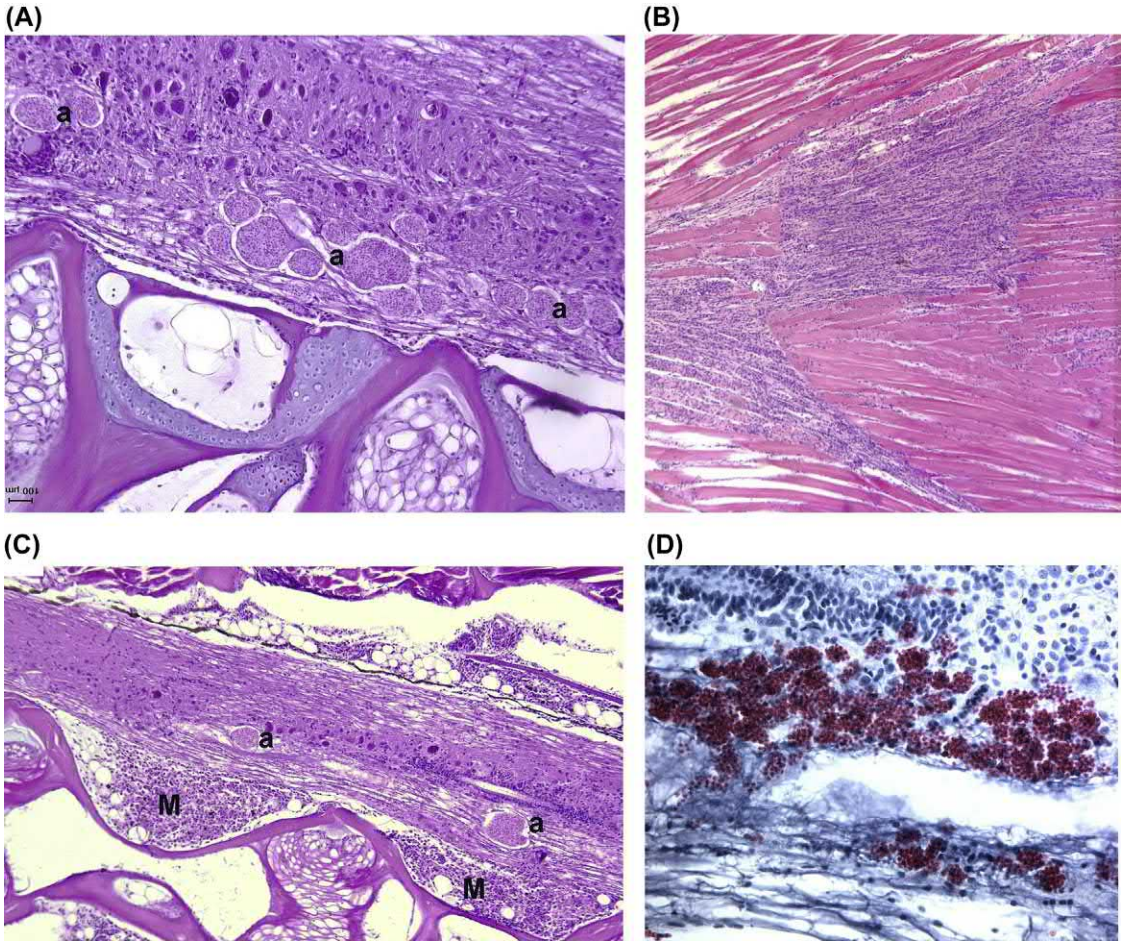

FIGURE 40.3 Histology of *Pseudoloma neurophilia*. (A) numerous aggregates of spores (a) in the spinal cord. (B) severe, chronic myositis associated with the infection. (C) Chronic meninxitis (M) associated with the infection a = aggregates of spores. (D) Aggregates of spores in the spinal cord, stained red with the Luna stain.

in the stroma and occasionally within developing oocytes (Sanders et al., 2012). Disseminated infections can occur in other organs (Kent & Bishop-Stewart, 2003), particularly in fish that are immunosuppressed (Spagnoli et al., 2016).

Diagnosis. The spore stages of microsporidia are generally visible by light microscopy using wet mounts. As with other species, the spores of *P. neurophilia* are oval to pyriform shaped, highly retractile, and contain a distinct posterior vacuole. Aggregates of spores are detected in the spinal cord or hindbrain by carefully dissecting these tissues and preparing wet mounts. The chitin-binding fungal stains, such as Fungi-Fluor, Calcofluor, and Uvitex 2B, can be added to wet mounts and visualized using fluorescence microscopy with a DAPI filter to aid in identification (Kent & Bishop-Stewart, 2003).

In standard hematoxylin and eosin (H&E) stains on histological sections, spores in heavy infections are relatively easy to detect. However, light infections can easily be missed, as spores do not stain well with H&E. There are several other staining methods that increase the sensitivity of detecting microsporidian infection in histological sections, such as acid-fast, Gram stain, and the Luna stain (Peterson, Spitsbergen, Feist, & Kent, 2011). We have found the latter to be particularly useful for detecting *P. neurophilia* spores as the stain is simple, consistent between labs and specimens, spores usually stain intensely, and the background is stained with iron hematoxylin, allowing for easy determination of the location of the infections.

Several molecular diagnostic assays have been developed for *P. neurophilia* using both conventional (Whipps and Kent, 2006; Murray et al., 2011) and quantitative PCR (Sanders & Kent, 2011). These are typically used for postmortem detection of *P. neurophilia* DNA in anterior brain and spinal tissue of adult fish; however, the use of membrane filtration, acetone dissolution of the membrane, and spore disruption by sonication enables the detection of small numbers of spores from water (Sanders & Kent, 2011, Sanders and Kent, 2013). This method can be a particularly effective way to screen fish by examining spawn water as the parasite is often shed during spawning. ZIRC and private veterinary

diagnostic laboratories provide PCR testing for this parasite.

Control and Treatment. Various therapeutic drugs, particularly oral fumagillin, have been effectively used to treat several microsporidian infections in fishes (Kent et al., 2014). To date, none have been tested on *P. neurophilia*. Therefore, the infection is best controlled by avoidance. Kent et al. (2011) developed a successful rederivation program for establishing new populations of zebrafish that entailed screening brood fish and their progeny. Reducing or eliminating the infection once established in large facilities is more challenging given that the parasite can survive chlorine levels used to disinfect zebrafish eggs (Ferguson, Watral, Schwindt, & Kent, 2007), and the ability of the parasite to be transmitted within eggs.

In a large, established facility, control of *P. neurophilia* can be far more complicated. Murray et al. (2011, 2013) have successfully reduced the levels of *P. neurophilia* infections at the Zebrafish International Resource Center (ZIRC) by diligent screening of moribund and healthy fish throughout the facility, with subsequent euthanasia of infected populations and they provided several recommendations for reducing the infection after it has been established in a large facility, where depopulation of the entire laboratory zebrafish stock is not possible. First, all imported fish should be reared and bred in quarantine. After spawning, embryos should be surface-sanitized with bleach before being introduced into the main fish facility to reduce the numbers of infectious spores (Ferguson et al., 2007). Second, maintaining effective UV sterilization as a component of the filtration system is very important to prevent the spread of the parasite throughout an entire facility as microsporidian spores are quite susceptible to the levels used in most zebrafish facilities (e.g., >35,000 μWsec/cm^2). Third, sick and old fish may be reservoirs for the infection. Fourth, given the potential for transmission of microsporidiosis during spawning (Sanders and Kent, 2013), fish that are used for crossing should not be spawned with fish derived from more than one other tank. Finally, once a tank of fish has been identified as positive for microsporidiosis, efforts should be made to spawn and replace the stock as soon as possible. Our data show that the infection spreads rapidly through a tank population. Many of these approaches are used for control of pathogens in general and are also discussed in the chapter on Special Procedures (Kent et al., 2020) and Quarantine and Importation (Lieggi, 2018).

Pleistophora Hyphessobryconis

Description. *Pleistophora hyphessobryconis* is a common microsporidian infection of aquarium fishes, and it has been detected in a few research facilities (Sanders et al., 2012). This parasite, the cause of neon tetra disease, infects a wide variety of aquarium fishes, including tetras, barbs, and goldfish. The infection can result in massive numbers of developing parasites and spores throughout the skeletal muscle, particularly in immunosuppressed fish, and spores are found in other organs within macrophages. Wet mount preparations of skeletal muscle from infected fish often reveal numerous sporophorous vesicles, many containing fully developed spores. Spores in wet mounts are approximately somewhat larger than those of *P. neurophilia* (about 7 μm by 4 μm), and contain a prominent posterior vacuole measuring approximately 3 μm by 3 μm (Figs. 40.4 and 40.5).

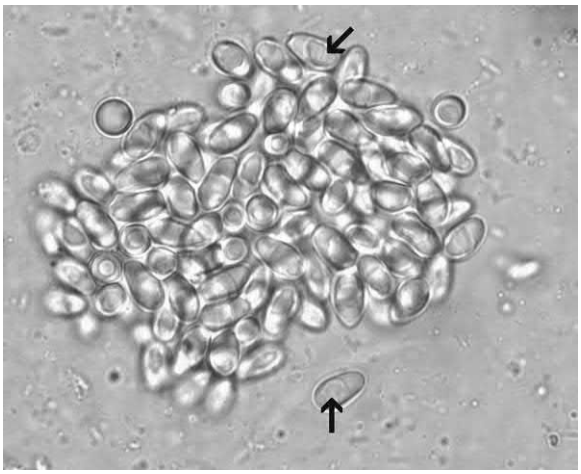

FIGURE 40.4 *Pleistophora hyphessobryconis* spores. Wet mount, Nomarski phase interference. *Arrow* = posterior vacuole.

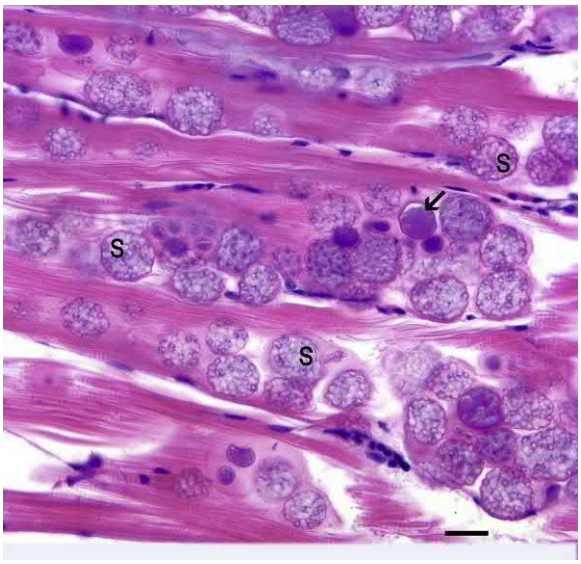

FIGURE 40.5 Histological section of zebrafish muscle with infected with *Pleistophora hyphessobryconis* Numerous aggregates of spores (S) and sporonts (*arrows*) within myofibers. Bar = 20 μm.

FIGURE 40.6 *Piscinoodinium pillulare*, trophonts and dinospore (*arrow*). *Courtesy of Mauricio L. Martins.*

Host Range. This microsporidium shows broad host specificity and has been reported from many species of aquarium fishes in several families (Characidae, Cyprinidae, Cyprinodontidae, Poecilidae, Cichlidae), including *Danio rerio* and *D. nigrofasciatus* (Steffens, 1962).

Life Cycle and Modes of Transmission. Whereas the parasite apparently does not infect developing oocytes, the massive numbers of spores occasionally observed in the ovarian tissues support Schäperclaus (1941) suggestion regarding the possible risk of maternal transmission.

Clinical Signs and Pathobiology. As is the case with *P. neurophilia* infections, most *P. hyphessobryconis* infections in immunocompetent adult fish are subclinical. However, heavily infected fish may appear emaciated and lethargic, and infections may lead to death. The infection targets the skeletal muscle, which will appear more opaque through the skin, particularly near the dorsal fin. Removal of the skin reveals extensive, coalescing opaque regions throughout the muscle.

Histological examination reveals numerous spores and developmental stages within myocytes (Fig. 40.6). The muscle is the primary site of infection for *Pleistophora hyphessobryconis*. Massive infection by proliferative stages and spores occupy the myocyte, with inflammatory changes occurring after infections become so severe that the myocytes rupture. Spores of this parasite can also be observed in the kidney, spleen, intestine, and ovaries in heavier infections (Sanders et al., 2010).

The pattern of infection in zebrafish is consistent with reports from neon tetras and other species (reviewed by Schäperclaus, 1991; Kent et al., 2014). The most remarkable pathological feature of *P. hyphessobryconis* is the severe intensity of infection in the somatic muscle, with well over half of the myofibers containing numerous spores and developmental stages in some fish. Parasites within intact myocytes are generally not associated with inflammation. However, mature spores released from degenerated myofibers into interstitial spaces are consistently associated with inflammation, and these spores are often engulfed by macrophages. As with *P. neurophilia* and some other microsporidia of fish, spores elicit significant inflammation only after they are released from their intracellular environment. With *P. hyphessobryconis,* there is minimal inflammation, while the spores remain within intact myofibers. Inflammation follows rupture of myofibers, but spores remain intact within phagocytes, and are thus, often transported to other organs.

Diagnosis. The occurrence of prominent opacities throughout the muscle is a presumptive diagnosis, and visualization of the spores in either wet mount preparations or histological sections allows for confirmation. As with other microsporidia, spores of *P. hyphessobryconis* are Gram or Luna positive (Peterson et al., 2011; Sanders et al., 2010) and these stains are particularly useful for visualizing individual spores within visceral organs. Private veterinary diagnostic laboratories provide PCR testing for this parasite.

Control and Treatment. As with *P. neurophilia*, fumagillin and other drugs have not been evaluated for treating *P. hyphessobryconis* infections. The strategies for avoidance of *P. neurophilia* provide a useful guide for reducing or eliminating this microsporidium in zebrafish facilities. Given the large numbers of spores in the ovary, the risk of maternal transmission should be considered. We, therefore, recommend employing similar strategies as used for *P. neurophilia* to avoid transmission between generations (Kent et al., 2011).

Piscinoodinium Pillulare

Piscinoodinium pillulare, the cause of velvet disease, is a yellowish, parasitic dinoflagellate in which one stage of the life cycle infects the skin and gills of fish. The parasite can multiply very rapidly in aquaria and will rapidly kill fish. This is a common pathogen of ornamental fishes and infections are occasionally seen in zebrafish, particularly in those fish that are acquired from pet stores. With proper quarantine procedures, the infection is often detected before the fish are introduced to the main facility (Lieggi, 2018).

Description. The nonmotile trophont of *Piscinoodinium pillulare* measures about $9–12 \times 40–90$ μm, is pear-shaped and possesses an attachment disc from which, numerous rod-like organelles called *rhizocysts* radiate. These structures penetrate into and are firmly embedded in the host epithelial cells. The trophont is covered by a theca and contains well-developed chloroplasts, starch granules, but no digestive vacuoles, and appears brown

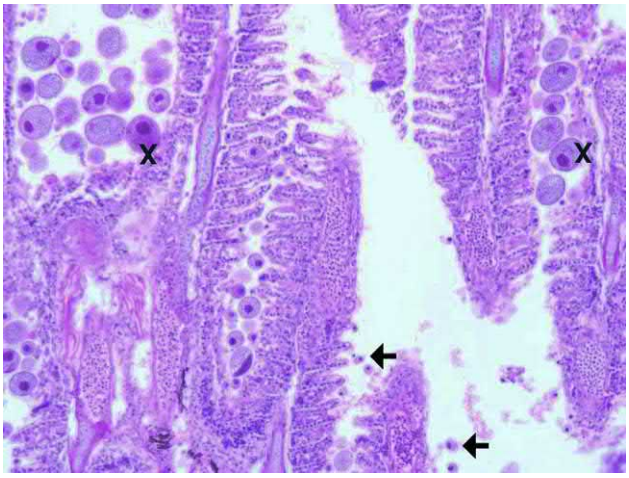

FIGURE 40.7 Histological section of zebrafish with heavy *Piscinoodinium pillulare* infection. Trophonts (X) are characterized by a large, subspherical nucleus and refractile, cytoplasmic granules. *Arrows* = early, infective stages (dinospores?). Note relatively minimal tissue reaction given the severity of infection.

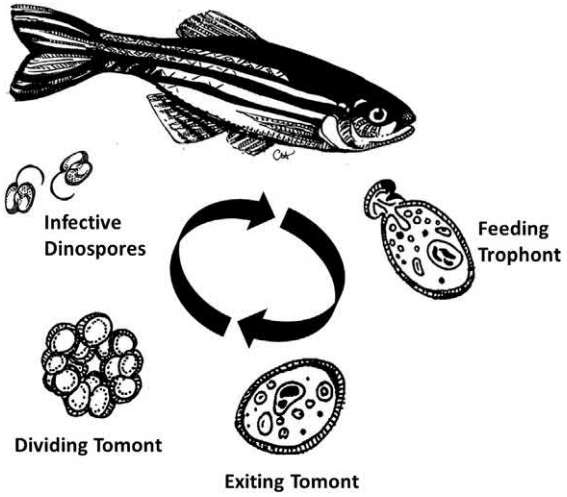

FIGURE 40.8 Life cycle *of Piscinoodinium pillulare*. *Courtsey of Claire Howard.*

to golden in wet mount preparations (Fig. 40.6). They also have a single, prominent, spherical nucleus that contains large dinoflagellate chromosomes. The trophont cytoplasm possesses a spherical nucleus, digestive vacuoles, and starch grains, but lacks chloroplasts. These starch granules appear as refractile particles throughout the cytoplasm in both wet mount preparations and histological sections (Figs. 40.6 and 40.7).

Host Range. Essentially any freshwater fish that is maintained above 17–20°C is susceptible to the infection.

Life Cycle and Modes of Transmission. The life cycle involves an off-host stage, in which the parasite proliferates in a cyst stage (tomont) and actively swimming dinospores are released to infect new fish hosts (Fig. 40.8). After approximately 6 days of growth at 25°C, the developed trophont of *P. pillulare* drops off the host, sinks to the substratum, rounds up, and becomes a tomont. It undergoes a series of successive divisions, resulting in the production of 256 tomites which differentiate into motile dinospore resembling other dinoflagellates.

Pathobiology and Clinical Signs. *Piscinoodinium pillulare* is the causative agent of velvet disease and is called as such because infected fish may exhibit a golden, velvety hue on the body surface. Clinical signs include the appearance of discomfort, with infected fish scratching or rubbing against objects in the water (flashing). Heavily infested fish are lethargic, may hang near the surface of the water, and exhibit labored breathing. There may also be excess mucus, darkening of the skin, and petechial hemorrhages. The disease progresses rapidly, and high mortality occurs if fish are not treated.

Histopathologic changes in the gills of fish infested with *P. pillulare* range from separation of the respiratory epithelium to lamellar fusion, and prominent epithelial hyperplasia, which may involve entire gill filaments. However, from zebrafish cases that we have seen, there may be large numbers of parasites on the gills with relatively little tissue reaction (Fig. 40.8).

Diagnosis. *Piscinoodinium pillulare* infestations are diagnosed by observation of the brown to golden, subspherical to pyriform shaped trophonts in either wet mounts or histological sections on fish gills or skin. For the former, *P. pillulare* is distinguished from many other surface-infesting protists by its lack of motility. The presence of numerous retractile granules in the cytoplasm and large central nucleus are key identifying features. Commercial veterinary diagnostic laboratories provide PCR testing for this parasite.

Control and Treatment. Copper has been suggested as a treatment, but its unpredictable toxicity in soft, acid freshwater often makes it dangerous to use in aquaria. Copper sulfate is used at 0.3–0.5 mg/L for 10 days or formalin at 100–200 mg/L for 10 days.

The presence of off-host developmental stages should be considered when implementing a treatment regime.

Raising water temperatures to 33–34°C has also been reported to control infestations by shortening the time for trophont and tomont development so the motile infective stages can be exposed to the chemicals in the water. Healthy zebrafish can tolerate this temperature, but this approach should be used with caution as the fishes oxygen demand is increased due to increased activity and metabolic rate, and at the same time maximum dissolved oxygen in the water is decreased, and the respiratory abilities of the fish would likely be compromised by the infection. This freshwater parasite

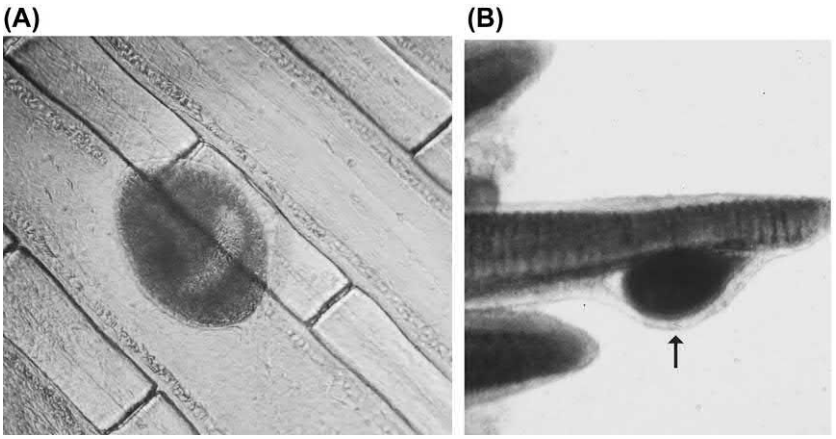

FIGURE 40.9 Wet mount of fish with trophont of *Ichthyophthirius multifiliis*. Note prominent, curved macronucleus. *Courtesy of Claire Howard. B. Ich parasite* (arrow) *under gill epithelium.*

is susceptible to hyperosmotic conditions, and Noga (2010) describes various therapies using NaCl, including short-term baths, 1–3 min baths in full-strength seawater (35 ppt) to dislodge attached parasites or more prolonged treatments at teaspoon salt (5.7 g)/5 gallon (285 ppm). Falanghe, Laterca Martins, and Criscuolo-Urbinati (2002) recommended 6 g/L NaCl for 4 h. Although a freshwater fish, zebrafish can tolerate relatively high levels of NaCl (e.g., 25 ppt) for short periods (Uliano et al., 2010), and hence, the use of NaCl for treated external parasites in zebrafish is a viable option. Noga (2000) and Noga and Levy, 1995 provide comprehensive reviews of treatments for *P. pilluare.*

Avoid exposure of fish and water systems to infected fish, water, nets, or any other items that have come in contact with infected systems. Quarantine of fish and prophylactically treating them with a salt bath prior to introduction into a laboratory system will also help prevent the introduction of this parasitic dinoflagellate into the main facilities. Treatment of aquaria and other holding tanks with chlorine bleach, followed by thorough washing with fresh water, and then drying of the systems for several days before reuse, should provide additional assurance for not spreading the parasite to newly introduced fishes.

Ichthyophthirius Multifiliis

The ciliate *Ichthyophthirius multifiliisis* is very common in freshwater aquarium fishes, and given its very broad host specificity, it is a risk to zebrafish in research facilities.

Description. *Ichthyophthirius multifiliis* is a holotrichous ciliate and is the largest parasitic protozoan affecting fishes. Adult trophonts are oval to round, reach up to more than 1 mm in size, are uniformly ciliated, and possess a characteristic horseshoe-shaped macronucleus (Fig. 40.9).

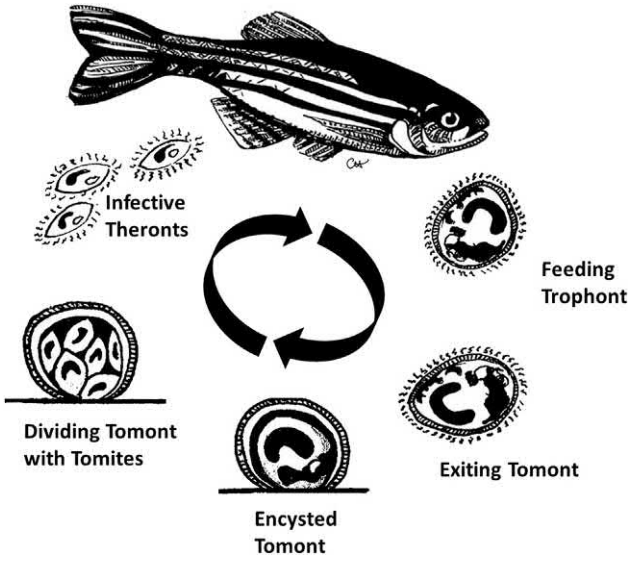

FIGURE 40.10 Life cycle of *Ichthyophthirius multifiliis. Courtesy of Claire Howard.*

Life cycle. The life cycle of *I. multifiliis* is direct but does include a free-living developmental stage (Fig. 40.10). After a period of growth, large trophonts leave the host tissues, settle on the substratum, and secrete a gelatinous cyst. This nonmotile tomont resorbs its buccal apparatus and divides successively by binary fission. One tomont may produce from 250 to 2000 small tomites (Lom & Dyková, 1992, p. 316). After the last division, the buccal apparatus is restored, and the tomites break through the cyst wall and become theronts. Susceptible fish are infected by free-swimming theronts. Theronts develop to the trophont stage under the epithelium of the skin or gills, where they expand in size (up to about 1 mm) and occasionally divide. Development is directly linked to temperature; for example, the growth period of the trophont on the fish host ranges from 7 d at 20°C to 20 d at 7°C.

Hosts. *Ichthyophthirius multifiliis* is the most important parasite affecting freshwater aquarium fishes. The parasite is also a problem in food fishes reared in captivity, such as catfish and salmonids, and has even caused epizootics in wild fishes. Virtually all freshwater fishes are susceptible. In the laboratory setting, these include swordtails, platys, sticklebacks, and trout (when reared at warmer temperatures). Interestingly, we have heard of only a few reports of this infection in zebrafish. Cherry (2003) showed that zebrafish are more resistant than channel catfish to the infection, and perhaps zebrafish are more resistant than most aquarium fishes in general(von Gersdorff Jørgensen, 2016). In addition, the infrequency of natural infections in zebrafish may be because most laboratories rear zebrafish at 28°C, which is only a few degrees lower than the lethal temperature for free-swimming stages of the parasite (Dickerson, 2006).

Pathobiology. *Ichthyophthirius multifiliis* is highly pathogenic, reproduces rapidly in confined systems, and shows broad host specificity. Because of these features, this parasite has the potential to cause serious disease in laboratory fishes. Tissue irritation caused by *I. multifiliis* results in epithelial hyperplasia and excessive secretion of mucus on the body surfaces and gills. Lesions produced by *I. multifiliis* may lead to secondary bacterial or fungal microbial infections.

Ichthyophthirius multifiliis causes a condition known as "Ich," or "white spot disease." Infected fishes exhibit characteristic white spots on the body surfaces, which result from the ciliates being encysted within the host's epithelium (Figs. 40.9 and 40.11). Fish with heavy infections also exhibit excessive mucus production, labored breathing/rapid gilling, lethargy, and flashing (scraping their body on surfaces of the aquarium). Histological examination reveals the parasite on the epithelium with extensive hyperplasia associated with the infection, often resulting in the fusion of gill lamellae even with relatively few parasites (Fig. 40.12).

FIGURE 40.11 White focal nodules in the skin of a zebrafish with *Ichthyophthirius multifiliis*. These represent parasites and associated focal hyperplasia of the epithelium. *Courtesy of Marcus Crim.*

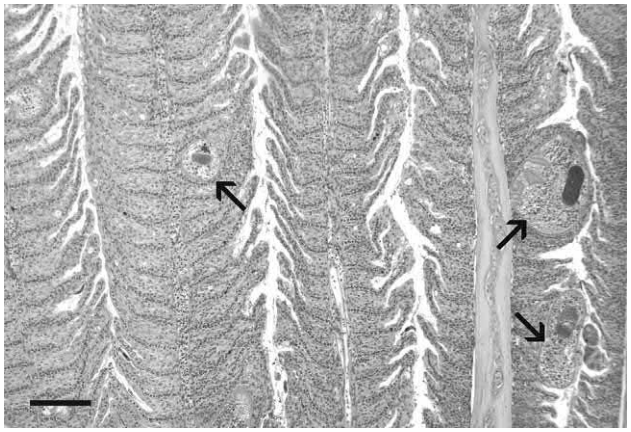

FIGURE 40.12 *Ichthyophthirius multifiliis* in gills (*arrow*) associated with severe epithelial hyperplasia, resulting in complete fusion of the secondary gill lamellae. Bar = 100 μm.

Diagnosis. Identification of active ciliates of varying sizes in wet mounts of the skin or gills offers a good presumptive diagnosis. Definitive diagnosis is achieved by observing the distinctive horseshoe-shaped macronucleus, and by observing parasites under the epithelium of the gills or skin. Commercial veterinary diagnostic laboratories provide PCR testing for this parasite.

Control and Treatment. Considerable information is available on control strategies for infection with *I. multifiliis* as it is an important disease in both aquaculture and ornamental fishes. The most common treatment is an external bath with formalin (Noga, 2000). Usually fish are treated with formalin at about 1:4000 to 5000 ppm formalin for about 1 h. Each fish species responds differently to formalin, and thus, a few fish should be tested before applying the treatment to large numbers. Moreover, formalin depletes dissolved oxygen, and hence, it is important to aerate the water during treatment. Multiple daily treatments are necessary to eliminate the stages under the skin, which are somewhat protected from external baths. The entire aquarium system should also be treated to destroy the free-living stages of the parasite. Unfortunately, treating the entire system may be detrimental to biological filters in recirculating systems.

The life cycle of *I. multifiliis* is affected by the temperature, with warmer temperatures accelerating development. However, temperatures above 29–30°C are lethal to theronts. Raising water temperatures above 29°C for several days is an effective method for eradicating the infection. At higher temperatures, it is particularly important that the water is well aerated because oxygen saturation in water is inversely correlated with temperature; fish at higher temperatures have higher

oxygen demands, and the gills (and thus, gas exchange) are impaired when they are infected. Sodium chloride may also be effective. Concentrations of NaCl at 7–20 ppt have been used in pond culture, so this option should be considered for zebrafish as they can tolerate this salinity (Uliano et al., 2010).

As with other common pet fish parasites, infections in the main facility are prevented by quarantine of new fish before introduction, prophylactic treatment with external parasiticides if the fish comes from a dubious source (e.g., pet fish industry) and implementation of the "eggs only" policy (see Collymore, Crim, & Lieggi, 2016; Kent et al., 2009). This is particularly important because of the difficulty in eradicating free-living stages in recirculating systems. There are no commercial vaccines against infection with *I. multifiliis*, but recovered fish, including zebrafish, show resistance to reinfection (von Gersdorff Jørgensen, 2016). Immobilizing ciliary antigens against the parasite could ultimately serve as a vaccine candidate (Dickerson & Clark, 1998; Maki & Dickerson, 2003).

Myxidium Streisingeri (Myxozoa)

Myxozoan parasites (phylum Myxozoa, class Myxosporea) are common parasites of cold-blooded vertebrates, particularly fishes (Kent et al., 2001). Traditionally the Myxozoa have been classified with the Protozoa, but it is now clear that they are primitive metazoans most closely related to Cnidaria (Okamura, Gruhl & Bartholomew, 2015). There are over 2200 described species of myxozoans within about some 64 genera (Fiala, Bartošová, & Whipps, 2015), and most are relatively nonpathogenic. Those that infect the lumina of organs are called *coelozoic* species, and these are usually less pathogenic than those that infect the tissues (histozoic). The former are commonly found in the gall bladder, bile ducts, or urinary tract.

Some undescribed histozoic myxozoans (*Myxobolus* and *Thelohanellus* spp.) have been observed in wild-caught zebrafish (Smith et al., 2011), but *Myxidium streisingeri* is the only myxozoan described from zebrafish in research facilities (Whipps, Murray, & Kent, 2015). It is actually quite common and has been found in about one-fourth of the laboratories that have submitted fish to the ZIRC diagnostic program. It is a coelozoic myxozoan, infecting the renal tubules and mesonephric ducts (Fig. 40.13).

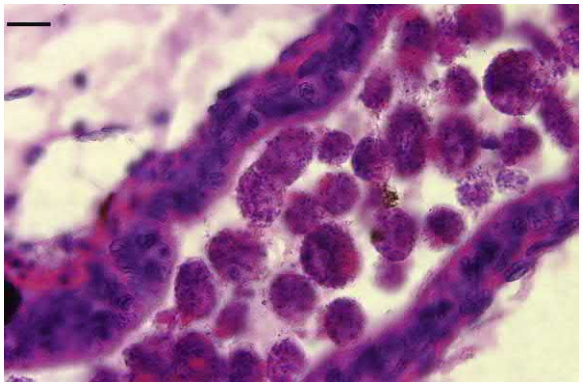

FIGURE 40.13 Multinucleate plasmodia of *Myxidium steisingeri* within the mesospheric duct. H&E. Bar = 10 μm.

The organism seen in zebrafish is comprised of a multicellular trophozoite (plasmodium) in which careful examination reveals myxospores inside. The hallmark identifying the character of myxospores is the presence of polar capsules, which can be better visualized with special stains, such as methylene blue or Giemsa (Fig. 40.14A). The genera *Myxidium* or *Zschokkella* have members with polar capsules located on the opposite end of the spores, observable in either histological sections or in wet mount preparations (Fig. 40.14B).

Life Cycle. Myxozoans are heteroxenous and require alternate development between the vertebrate host and an aquatic annelid to complete their development, except a few that use bryozoans. The life cycle of *M. streisingeri* is unknown, but based on other myxozoans it probably uses an oligochaete worm as an alternate host. The wide distribution of *M. streisingeri* is somewhat surprising given the assumption that the parasite is heteroxenous and requires an oligochaete worm to complete its life cycle. However, oligochaetes are often found within the filters or sumps in recirculating systems from zebrafish laboratories.

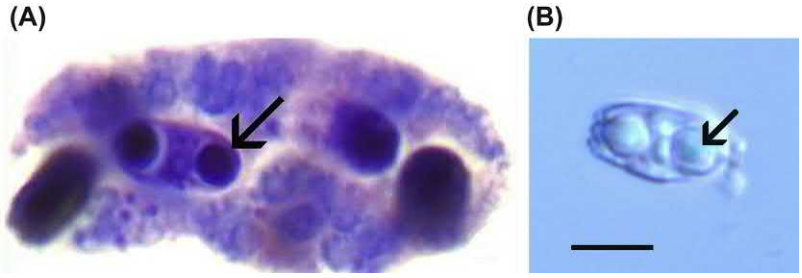

FIGURE 40.14 Spores *Myxidium stresingeri*, note the polar capsules at the opposing ends of the spores (*arrows*). (A) Giemsa, showing multiple spores within a plasmodium. (B) Wet mount, Normaski phase interference. Bar = 5 μm.

The most complete information on the life cycle of a myxozoan is based on *Myxobolus cerebralis* . Multicellular "myxospores" are released from infected fish following death, or are discharged in body fluids. Oligochaetes (annelid worms), ingest the myxospores and serve as alternate hosts. They are not considered intermediate hosts because sexual reproduction of myxozoans may actually occur in this host (Okamura, Gruhl, & Reft, 2015). In the oligochaete host, reproduction is followed by sporogony, resulting in the formation of another spore type "actinospores". Actinospores are released from the oligochaete, and upon contacting the surface epithelium of a fish, release sporoplasms, which penetrate the skin of the fish. Shortly thereafter, clusters of dividing myxosporean cells are found, sometimes intracellularly, within the epithelium. The parasite then migrates to its final target tissue, where development continues, ultimately forming myxospores.

Host Range. To date, the parasite has only been reported from zebrafish in research facilities. As may be the case for *Pseudoloma neurophilia*, the primary host may be a different ornamental fish species.

Pathobiology and Clinical Signs. To date, we have not associated the clinical disease with the infection. Histological sections of infected fish reveal the parasite in the common mesonephric ducts and occasionally in the lumen of the kidney tubules. Infections may be heavy but are seldom associated with significant histological changes.

Diagnosis. The infection is diagnosed by observation of the parasite plasmodia in histological sections of the urinary tract. Multinucleate plasmodia with retractile granules are observed in the lumen in the mesonephric, and occasionally, the renal tubules (Fig. 40.13). A Giemsa stain is useful for demonstrating the spores within plasmodia. Spores are elongated and have two polar capsules at the opposing ends of the spore (Fig. 40.14). Wet mount preparations of the ureters reveal numerous plasmodia free in the lumen or attached to the epithelium. Myxospores, which are often difficult to find, contain two polar capsules at the opposing ends of the spore. They are approximately 5 × 8 μm and have ridges on the spore walls (Fig. 40.14).

Control and Treatment. Infections in a zebrafish facility indicate that the oligochaete host is present in the water system (e.g., tanks or filters). Small oligochaetes frequently colonize sumps and filters in zebrafish systems In addition, oligochaetes, used to feed fish (e.g., black worms, red worms), may be sources of myxozoan infections to fishes held in aquaria (Hallett, Atkinson, Erséus, & El-Matbouli, 2005). Fumagillin and a few other drugs show variable efficacy for treating myxozoan infections. Treatment of the *M. streisingeri* would probably not be warranted as it has not been associated with the disease. However, we have not excluded the possibility that these subclinical infections may produce nonprotocol-induced variation in research.

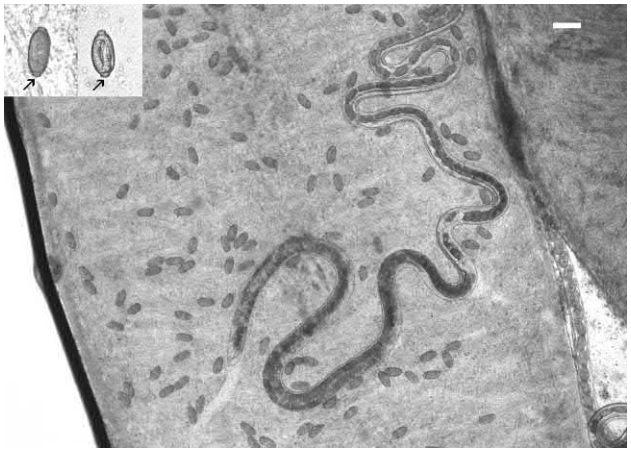

FIGURE 40.15 *Pseudocapillaria tomentosa*. Wet mount of intestine with adult female and numerous eggs. Inset is higher magnification of fresh egg (left) and larvated egg after 6 d (right). Note plugs on opposing ends of eggs (*arrows*). Bar = 100 μm.

Pseudocapillaria Tomentosa

Description. Females of *Pseudocapillaria tomentosa* are long, thin, white worms ranging from 4 to 12 mm long that infect the intestine of zebrafish (Fig. 40.15). Gravid female worms are replete with the distinctive eggs bearing the bipolar plugs typical of capillarid nematodes. As with many nematodes, males are smaller, about 4–7 mm in length. The anterior end of mature worms reveals stichosomes with a distinct banding pattern.

Hosts. *Pseudocapillaria tomentosa* has a broad host range, infecting many species of cyprinids. Fishes in the orders Anguilliformes (eels), Gadiformes (cod fish), Salmoniformes (salmon), and Siluriformes (catfish) are also susceptible (Moravec, 1987). *Pseudocapillaria brevispicula* is considered a junior synonym of *P. tomentosa* (Moravec, Wolter, & Körting, 1999).

Life Cycle. Lomankin and Trofimenko (1982) showed that oligochaetes (e.g., *Tubifex tubifex*) can serve as paratenic hosts for *P. tomentosa* in laboratory transmission studies. We and others have demonstrated direct transmission in the absence of adult worms (Kent et al., 2001; Collymore et al. 2014). This observation explains why *P. tomentosa* can easily spread in zebrafish research facilities. Eggs larvate and are infectious about 5 d at 28°C after release from female worms (Martins, Watral, Rodrigues-Soares & Kent, 2017). Larvae are detected in the mucosa shortly after an infection, and gravid females are found about 2–3 wk postexposure (Collymore et al., 2014; Gaulke et al., 2019). Based on our observations, the infection persists at high

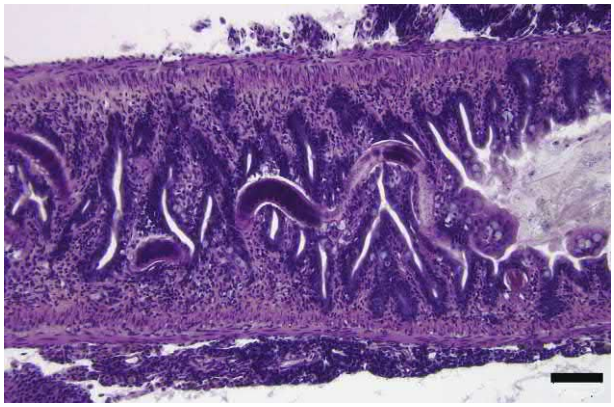

FIGURE 40.16 Zebrafish intestine infected *Pseudocapillaria tomentosa*. Note worm penetrates mucosa and is associated with chronic inflammation. Bar = 50 μm.

prevalence within a fish colony for several months after initial infection.

Pathologic Effects and Clinical Disease. The parasite induces a chronic disease, often with high mortality. The hallmark macroscopic change is emaciation. Histological examination of infected fish reveals profound chronic enteritis and occasionally coelomitis (Kent, Bishop-Stewart, Matthews, & Spitsbergen, 2002; Murray & Peterson, 2015) (Figs. 40.16 and 40.17). The worms are usually confined to the epithelium, but occasionally penetrate through all layers of the intestine and can be found within the coelom. Balla et al. (2010) showed that infected fish had significantly increased eosinophils. Spitsbergen et al. (2000) found that infected zebrafish that were exposed to a low dose of the carcinogen dimethylbenze[a]antracene had a higher incidence of gastrointestinal tumors than similarly exposed fish that were not infected. Recently, we showed that the infection in zebrafish was also associated with early intestinal cancer development, as well as significant changes in the intestinal microbiome (Gaulke et al., 2019). Most of the fish appeared clinically normal, and hence, the infection should be a concern even in normal-appearing fish used in research, as it may cause nonprotocol-induced variation (Kent et al., 2012).

Diagnosis. Diagnosis is made by observing the worms in wet mount preparations or histological sections of the intestine. Identification of capillarid nematodes is confirmed observing the eggs with distinctive bipolar plugs, in either wet mounts or histological sections of the intestine, or observing eggs in the feces or tank sediments These characteristic eggs are easily visualized in wet mount preparations of the gut and can be detected in sediment and feces using fecal flotation methods (Murray & Peterson, 2015). Martins et al. (2017) used a sugar centrifugation method to concentrate eggs from tank sediment. Precise identification of the species level requires careful examination of the male sexual organs, which are rather diminutive in *Pseudocapillaria* spp. However, to date, the only nematode infection identified in zebrafish from research facilities is *P. tomentosa*. Commercial diagnostic laboratories provide PCR tests for this parasite.

Control and Treatment. The first report of treating *P. tomentosa* in zebrafish was by Pack, Belak, Boggs, Fishman, and Driever (1995), where fish were treated with a mixture of trichlorfon and mebendazole (Fluke-Tabs, Aquarium Products, Glen Burnie, MD). We demonstrated that both emamectin and ivermectin showed efficacy for significantly reducing infections with the nematode, but the latter had toxic side effects (Collymore et al., 2014). While effective against fish parasites, ivermectin is more toxic to fish than mammals (Johnson, Kent, Whitaker, & Margolis, 1993). In contrast, emamectin shows less toxicity and has been used as the active ingredient of SLICE (Merck Animal Health, Summit, NJ), which is used for treating parasitic copepods in farmed salmon (Roy, Sutherland, Rodger, & Varma, 2000). We recently repeated the efficacy trials with emamectin with an increased dose of emamectin (0.35 mg/kg/d for 2 wk), and observed no apparent toxic side effects and significant efficacy.

Maley et al. (2013) orally treated the infection in a large facility with fenbendazole by adding the drug (2 mg/L) to artemia cultures, which were then fed to fish. Although no untreated controls were used in this

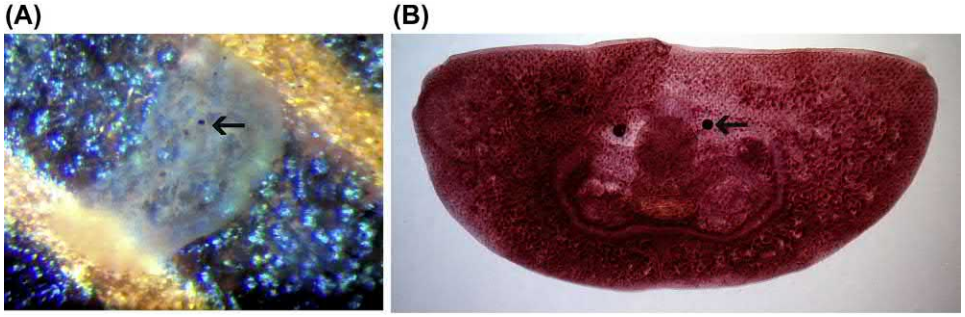

FIGURE 40.17 *Transversotrema patialense* from zebrafish. (A) Worm on skin, note prominent eye spots. (B) Whole mount, preserved and stained with a mixture of Van Cleave's and Ehrlich's hematoxylins. *Courtesy of Ash Bullard.*

study, they observed a substantial decline in infection and mortality following treatment. Samaee (2015) evaluated several anthelminthics for treating *P. tomentosa* infecitons by adding the drugs to water. Using the presence of eggs or larvae in feces as endpoints, they reported that ivermectin, fenbendazole, albendazole, mebendazole, and even praziquantel, was efficacious. The latter finding is interesting as praziquantel is usually used to treat cestode or trematode, but not nematode, infections.

Another method for control is eliminating eggs in the system environment, which is challenging in large recirculating systems. Martins et al. (2017) evaluated the efficacy of chlorine and heat to kill eggs and observed that lower doses of chlorine (i.e., 500 or 1000 ppm) actually enhanced larvation compared to controls, and 3000 to 6000 ppm was required to kill the eggs. Many terrestrial nematodes are heat resistant, but given that *P. tomentosa* would seldom experience temperatures greater than 35−40°C, we investigated the efficacy of heat to kill its eggs. Exposing eggs to 45−50°C for only 1 h was very effective for killing the eggs, while exposure at 40°C required at least 8 h to be very effective.

Cryopreservation is a common method used to preserve the zebrafish sperm. As with other animals, there is a possibility of paternal pathogen transmission through sperm. Eggs of *P. tomentosa* showed no survival based on larvation when subjected to either freezing method with or without cryopreservant (Norris, Watral, & Kent, 2018).

Oligochaete worms may be a source of the infection, and thus, should be avoided as food, particularly if their source is unknown. There is a risk of maternal transmission as the parasite eggs are resistant to chlorine at the levels used for disinfecting zebrafish embryos. Therefore, infected broodstock should not be used for generating F1s for introduction to the main facility. Murray et al. (2013) Murray et al. (2013) provided a flow chart for responding to different biosecurity risks posed by cryopreserved sperm. For fish infected with *P. tomentosa*, cryopreserved sperm may be used for in vitro fertilization, but resultant progeny should be kept isolated and the tank debris screened for the presence of eggs multiple times.

Transversotrema Patialense

In contrast to fish in the wild and aquaculture, infections by digenean trematodes are relatively rare in fishes reared in research laboratories (Kent and Fournie 2007). However, one unusual trematode, *Transversotrema patialense* was observed in zebrafish reared for research purposes (Womble, Cox-Gardiner, Cribb, & Bullard, 2015). Unlike most other trematodes, which infect the gastrointestinal tract as adults, adult *T. patialense* infects the scale pockets of various freshwater fishes.

Description. The worms have a rather unusual shape for an adult trematode in that they are very broad (about 750 um) and is about twice that in length. They also have two eye spots. Otherwise, they exhibit typical features with a prominent ventral sucker and a rather small oral sucker (Fig. 40.17).

Life Cycle and Modes of Transmission. The first intermediate host of this trematode is the red-rimmed melania, *Melanoides tuberculata*, a snail that has been imported to the United States with the aquarium trade. Members of this genus do not have second intermediate hosts, and cercariae that emerge from snails directly infect fish.

Pathobiology and Clinical Signs. No significant lesions or clinical disease are associated with the infection with natural infections (Womble et al., 2015). Mills, (1979) induced experimental infections in zebrafish have reported mild skin lesions at the site of parasite attachment.

Host Range. Reported from many freshwater fishes in the families Cyprinidae, Clupeidae, Poecilidae, Osphronemidae, etc. from the Philipines, Thailand, Israel, and India. Fish hosts that may occur in laboratories include tilapia species *Poecilia latipinna* (mollies), and other *Danio* spp (Womble et al., 2015).

Diagnosis. The infection can be diagnosed by observing trematodes on the skin, with eye spots. In contrast to monogeneans, which also infect the skin, *T. patialense* width is considerably greater than its length.

Control and Treatment. Zebrafish should not be housed with the first intermediate host, *Melanoides tuberculata*. Moreover, aquatic snails, in general, should be avoided in research systems, as they are frequently the first intermediate host for a variety of trematodes that infect fishes. We are not aware of reports regarding the treatment of fishes infected with *Transversotrema patialense.* Perhaps, those used for monogeneans, which are also external parasites fishes, would be effective. These would include formalin or praziquantel baths (Noga, 2000).

Metacercariae of Digenea

As with other helminths, metacercariae have been reported in wild-caught zebrafish (Smith et al. 2011). Encysted metacercariae are occasionally observed in zebrafish, particularly those that originate from pet fish suppliers. We occasionally see these infections submitted to the ZIRC diagnostic service.

Description. As with adult trematodes, metacercariae have a soft tegument, blind gut, and two suckers (oral suck and acetabulum). Metacercariae occur within prominent cysts. They range in size but are usually spherical to subspherical and are about 100−300 μm. They could be identified in wet mounts, but those that we have detected, thus far, are in histological sections (Fig. 40.18).

Pathobiology and Clinical Signs. No significant lesions or clinical disease are associated with the infection

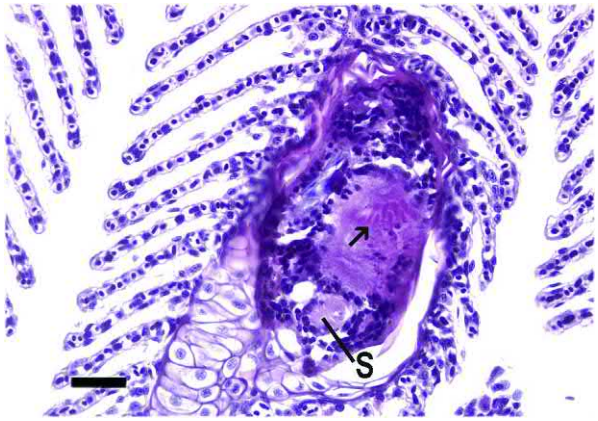

FIGURE 40.18 Metacercaria in gills of zebrafish. *Arrow* = oral sucker with a prominent spiny head collar suggestive of a member of the family Echinostomatidae. S = ventral sucker. Bar = 50 μm.

with natural infections. Nevertheless, the presence of these infections should be considered as a possible variable in research involving immunologic endpoints.

Life Cycle and Transmission. The first intermediate host of most trematodes are aquatic snails. Asexual reproduction occurs in the snail host, ultimately releasing hundreds to thousands of cercariae into the water. These cercariae usually swim through the water and penetrate the skin or gills of the fish hosts, and migrate to specific sites for development into an encysted metacercariae. This stage then remains in a quiescent state until it is ingested by a piscivorous bird or mammal, which is the definitive host. Here worms develop into adults in the gastrointestinal tract, mate, and eggs are released into the environment in the feces.

Host Range. There is a rather narrow host specificity for the first intermediate hosts (i.e., snails) for most trematodes. In contrast, a wide range of fish hosts can serve as hosts for metacercariae with trematode species that use fish as their second intermediate host.

Diagnosis. Bruno, Nowak, and Elliott (2006) provide an extensive review of the identification of parasites in histological sections. Larval stages of nematodes and cestodes also commonly encyst in tissues. Metacercariae can be distinguished from larval cestodes as the latter has basophilic structures (calcareous corpuscles) and no intestinal tract. Both metacercariae and nematodes both have an intestinal tract, but nematodes have a much more prominent cuticle. Observation of a section also aids in the identification of metacercariae in tissue sections (Fig. 40.18).

Control and Treatment. Given that snails, birds or mammals are required to complete the life cycles of these metacercariae, the infection can easily be avoided by using zebrafish that are not reared in outdoor tanks or ponds.

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CHAPTER

41

Bacterial and Fungal Diseases of Zebrafish

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Introduction

Zebrafish were recognized early as a potential model to study bacterial infectious diseases (Herbomel, Thisse, & Thisse, 1999; Pullium, Dillehay, & Webb, 1999; Rougier et al., 1992, 1996). It was not until after the turn of the recent century that the first publications reported on diseases of zebrafish for their own sake, with Astrofsky (2000), describing mycobacterial infections, and Dykstra et al. (2001) describing fungal infections caused by *Lecythophora mutabilis*. These reports and many that came later emphasized the concern for such infections impacting fish, whether by causing mortalities or impacting experiments.

The Zebrafish International Resource Center (ZIRC) diagnostic service, provides data on the diagnosis of pathogens in submitted fish from 2006 to 16 (https://zebrafish.org/wiki/_media/health/submission/submissions_summary_2006-2016.pdf). Over these 10 years, bacterial infections are divided into three categories; (1) *Mycobacterium* spp., (2) nonacid-fast bacteria, and (3) *Edwardsiella ictaluri*. Mycobacteria are much more commonly diagnosed, with 39% of submitting facilities being positive (33%–48%), and an overall prevalence of 6% in submitted fish over 10 years. The nonacid-fast bacteria represent 8% of submitting facilities and overall prevalence in fish of 0.5%. *E. ictaluri* is rarely diagnosed, with positive cases occurring in only 1 year, but the pathogen can be so devastating that it warrants specific attention. Overwhelmingly, mycobacteria are the most common bacterial infections in zebrafish.

The conditions associated with a closed zebrafish aquaculture system can be a benefit for biosecurity, as many infectious diseases commonly found in wild fish are not found in zebrafish. Those infections that do occur range in virulence and impact, and although some are pathogens regardless of conditions, others are more likely opportunists. Thus, certain husbandry conditions may result in an opportunity for an infectious agent, or the increased severity of what was previously subclinical disease. For example, Stress and overcrowding were attributed to *Aeromonas* disease (Pullium et al., 1999), and generally, many of the nonmycobacterial infections are considered opportunists. Thus, some bacteria (*Aeromonas* spp., *Pseudomonas* spp.) may more likely be opportunists, and only in suboptimal environmental conditions does disease occur.

Several *Mycobacterium* species are known to infect zebrafish, with some more virulent than others (Watral & Kent, 2007; Whipps, Matthews, & Kent, 2008). *Mycobacterium haemophilum* and *M. marinum*, in particular, are highly virulent, resulting in devastating outbreaks (Whipps, Lieggi, & Wagner, 2012; Mason et al., 2016). Other species (*M. chelonae*, *M. fortuitum*, etc.), are less virulent and are more likely to cause chronic disease that could become worse under stressful conditions. Experimental studies on mycobacterial infections and handling stress found more disease in stressed fish as compared to controls (Ramsay, Watral, Schreck, & Kent, 2009). Regardless, mycobacteria are common in zebrafish, and fish in general, warranting specific attention. *E.ictaluri* is not a common finding in zebrafish, but the high virulence of this species can be devastating to a colony (Hawke et al. 2013).

Recognition of these infections in zebrafish is fairly well established at this point, with a broad range of tools available (histology, culture, PCR diagnostics). Control of infection has largely focused on biosecurity, which is of utmost importance, but advances in disinfection (Chang, Colicino, DiPaola, Al-Hasnawi, & Whipps, 2015; Whipps et al., 2012) and antibiotic treatment

(Chang, Doerr, & Whipps, 2017) are being made. Furthermore, for some aspects, there is a rich literature on the control of such infections in other fishes; for example, *E. ictaluri* in catfish (Hawke et al., 2013).

Fungi and water molds are only occasionally reported in zebrafish colonies, and most infections are likely opportunistic. Water molds from the genera *Saprolegnia* and *Aphanomyces* are common in aquatic systems, but fish are reasonably resistant under typical husbandry conditions (Ekblom, 2017). When fish are stressed, injured, or immunocompromised, these infections could become a problem. Eggs and embryos are potentially at the greatest risk of impacts by molds and fungi, and Matthews (2009, pp. 321–346) has reported such species from egg dishes and embryo shipping containers. Signs of infection vary but may include lesions with hair-like projections (hyphae). Diagnosis is typically accomplished via wet mount or with histological sections with a PAS stain. Control focuses on biosecurity to prevent introductions and improve husbandry conditions where opportunistic agents may have established themselves in stressed fish. Due to their widespread and opportunistic nature, the diversity of fungi that can infect zebrafish is likely great. Those that are virulent pathogens appear to be few.

The goal of this chapter is to describe the known bacterial and fungal agents in zebrafish, including their diagnosis and control.

Bacterial Diseases

Mycobacterium Species

Background

Mycobacteriosis in wild and captive fishes is a common occurrence and has been reviewed by several authors (Decostere, Hermans, & Haesebrouck, 2004; Frerichs, 1993; Gauthier & Rhodes, 2009; Parisot, 1958). In zebrafish, Astrofsky, Schrenzel, Bullis, Smolowitz, and Fox (2000) first described these infections, reporting zebrafish exhibiting decreased survival and reproductive output. Kent et al. (2004) further characterized mycobacteriosis in zebrafish, describing infections in several facilities. The topic has been covered by Kent et al. (2009) in their article on recommendations for control of zebrafish pathogens, and thoroughly reviewed by Whipps et al. (2012).

Mycobacteriosis presents a challenge for monitoring and control because there is no single agent of the disease. Instead, many *Mycobacterium* species have been characterized by zebrafish. Historically, most cases of mycobacteriosis in fishes have been attributed to three species: *M. chelonae*, *M. fortuitum*, and *M. marinum* (Frerichs, 1993). Indeed, these species are found infecting zebrafish, but several others have also been identified, including *M. haemophilum*, *M. peregrinum*, *M. saopaulense* (Nogueira et al., 2015; Whipps et al., 2012). This list of species will likely grow with the expansion of research into these infections, and refinements in diagnostic techniques (i.e., DNA sequencing). In addition, different strains within species may have different characteristics relevant to virulence, monitoring, and control (Ostland et al., 2008).

Mycobacteriosis ranges from acute to chronic, with infections causing severe outbreaks to those that are unrecognized (Astrofsky et al., 2000; Kent et al., 2004; Whipps et al. 2012). In general, *M. haemophilum* and *M. marinum* are associated with severe acute to chronic infections with mortalities. The other species are associated with less severe chronic infections (Whipps et al., 2012). Many times, infections will be subclinical, with no obvious signs of infection (Kent et al., 2004; Whipps, Dougan, & Kent, 2007). In all cases, there is cause for concern because of: (1) direct losses due to mortality, (2) impact of underlying infections on survival, reproduction, or experimental endpoints, and (3) in the case of *M. marinum* the safety of employees because the zoonotic potential for this species is well documented (Ang, Rattana-Apiromyakij, & Goh, 2000; Aubry, Chosidow, Caumes, Robert, & Cambau, 2002) including a report of these infections in an employee at a zebrafish facility (Mason et al., 2016).

Role of Surface Biofilms

Mycobacteria are generally considered to be ubiquitous in aquatic systems, readily surviving in the environment, either planktonically or in surface biofilms (Beran, Matlova, Dvorska, Svastova, & Palik, 2006; Whipps et al., 2008). The salient point here is that this environmental reservoir of infection makes avoidance or elimination of these pathogens very difficult. Mycobacteria are very hydrophobic, and their ability to persist in low nutrient environments (Falkinham, 2009; Falkinham, Norton, & LeChevallier, 2001) makes them well adapted to persist in aquaria. There are many more species of *Mycobacterium* that can be isolated from biofilms than are found in fish (Whipps et al., 2012). However, identical strains of some mycobacteria have been found in both fish and biofilms (Whipps et al., 2008; Yanong, Pouder, & Falkinham, 2010). Thus, surface biofilms are a potential source of infection, as has been suggested when zebrafish living in the system sump had only biofilm material to eat (Whipps et al., 2012). Chang, Lewis, and Whipps (2019) found that in an experimental system, fish can acquire infections from biofilms in as little as 2 weeks. Elimination of mycobacteria from biofilms is not likely possible without extreme measures. However, it may be possible to influence the particular species that are present.

For example, Whipps et al. (2012) describe a case from a facility where *M. haemophilum* was found in both fish and biofilms. By disinfecting the facility, and repopulating with strict biosecurity measures, *M. haemophilum* was eliminated from this facility, although other mycobacteria were subsequently detected in the biofilms. It is possible that sampling surface biofilms, tank detritus, and water could be useful for system monitoring, particularly for the more pathogenic species (Crim et al., 2017; Whipps et al., 2008, 2012), keeping in mind that the tests need to be specific because many nonpathogenic mycobacteria could yield a positive in a general diagnostic test.

Transmission

Transmission of mycobacteria to zebrafish is likely primarily through ingestion. This has been demonstrated in other fishes through the consumption of infected tissues (Ross, 1970, pp. 279–283). Harriff, Bermudez, and Kent (2007) identified the intestine as the primary route of exposure specifically in zebrafish, and feeding fish mycobacteria embedded in gelatin feed has been effective for establishing infections (Chang, Lewis, et al., 2019b). Involvement of the swim bladder (aerocystitis) is often reported (Whipps et al., 2008), and may be an additional site of invasion because the zebrafish swim bladder is directly connected to the gastrointestinal tract via by a pneumatic duct. By histology, bacteria may be observed in the intestinal epithelium and within the lumen (Whipps et al., 2007), suggestive of a point of invasion or shedding, or both. In fact, many cases from the ZIRC database show infections only in the swim bladder.

Given the oral route of transmission, there is a risk of exposure through consumption of infected tissues, biofilms, and feed. Zebrafish may cannibalize dead tank mates and become infected, highlighting the importance of monitoring tanks for dead and moribund fish and removing them. Food is a potential source of infection, especially live feeds. For example, Beran et al. (2006) reported mycobacteria from brine shrimp eggs, a commonly used feed for zebrafish. It has been suggested that passage through an organism, such as is used in live feeds might enhance virulence. Peterson et al. (2013) tested this by feeding fish paramecia containing *M. marinum*, and found higher prevalence and severity in groups in these groups relative to controls. More recently, Chang, Benedict, Whipps, (2019) reported that infections can be established in zebrafish when fed live feeds (paramecia, brine shrimp, and rotifers) containing mycobacteria, but levels of infection were not significantly different from controls fed mycobacteria without a vector.

Infected fish are a ready source of exposure for tank mates. Bacteria may be shed from skin lesions or the intestine (Noga, 2010), providing a continuous source of mycobacteria. Testing of tank detritus, feces, or water from tanks reliably yields positive PCR results when infected fish are present (Crim et al., 2017) further supporting the idea that these are easily shed.

Mycobacterium species are potentially zoonotic, which is a concern for husbandry staff. It is also a concern for introductions of new mycobacteria into the facility, as many are considered ubiquitous in the environment, and it is easy to envision bacteria being carried in on skin, clothing, etc. *Mycobacetrium marinum* infections in zebrafish staff have been reported (Mason et al., 2016), and it is likely others are unreported in the literature. *Mycobacterium marinum* infections in humans are known to be associated with aquaria or handling fish, with about 84% cases associated with home aquaria (Ang et al., 2000; Aubry et al., 2002). Furthermore, Ostland et al. (2008) found the same strains of *M. marinum* in fish and human (skin infection) based on genetic analysis. Other mycobacteria are considered as potential opportunistic human pathogens (Brown-Elliott & Wallace, 2002; Whipps et al., 2007), but such infections are not typically associated with aquaria, and none have been reported to occur in zebrafish husbandry staff.

Clinical Signs and Pathological Changes

The manifestation of mycobacteriosis in zebrafish is broad-ranging (Astrofsky et al., 2000; Kent et al., 2004). Fish may present with nonspecific dermal lesions (Fig. 41.1A), have raised scales, or have swollen abdomens. Emaciation may occur and is often observed associated with *M. haemophilum* infection (Whipps et al., 2007). Fish may swim erratically or be lethargic. Animals may show no external signs of disease at all (Kent et al., 2004; Whipps et al., 2007). Internally, granulomas may be visible throughout all tissues (Fig. 41.1B and C), but primarily in the spleen, kidney, and liver. Granulomas are not always observed, in the case of diffuse systemic infections that have been reported for *M. haemophilum* (Whipps et al., 2007) and *M. marinum* (Ramsay et al., 2009). As mentioned above, the swim bladder is also a common site of infection. Infections vary from simple colonization of the lumen with a "biofilm" of mycobacteria lining an apparently normal swim bladder epithelium (Fig. 41.1E) to invasion into the swim bladder with severe, chronic aerocystis and granulomatous lesions extending into the coelomic cavity (Fig. 41.1D). Bacteria and granulomas may exhibit autofluorescence when observed with an epifluorescent microscope, as has been reported for *M. chelonae* (Whipps et al., 2014). Bacteria and granulomas are often observed in the ovaries of female fish, suggesting the potential for contamination of offspring (Kent et al., 2004). It is important to distinguish mycobacterial granulomas using acid-fast staining, from similar structures formed due to the degradation of eggs.

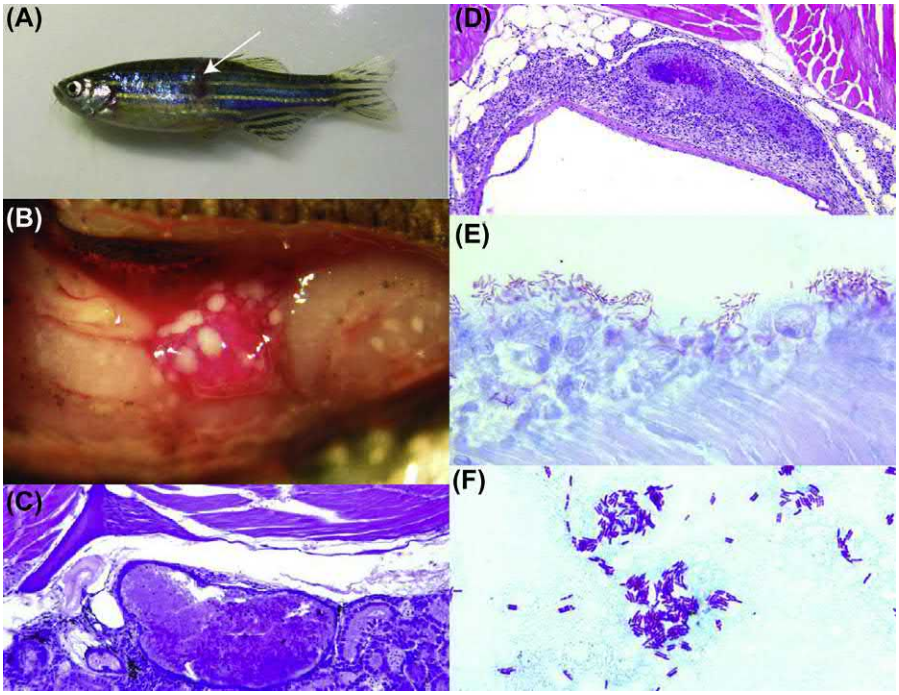

FIGURE 41.1 Mycobacteriosis. (A) Skin lesion associated with *M. marinum* infection. (B) Severe granuloma formation in the spleen and viscera of a fish infected with *M. marinum*. (C) Histological appearance of large *M. chelonae* granuloma in the kidney. (D) Granuloma associated with swim bladder inflammation. (E) Colonization of swim bladder epithelium by acid-fast bacilli (*Mycobacterium* sp.) (F) Acid-fast rods of mycobacteria in a stained tissue imprint.

Diagnosis

Often mycobacteriosis is diagnosed by the presence of acid-fast bacteria in histological sections (Kent et al., 2004; Whipps et al., 2008). Acid-fast staining may not be routine for screening but is often called for following the observation of granulomas consistent with mycobacterial infection. Because granulomas may not always be present with mycobacteriosis (Whipps et al., 2008), and sectioning may miss granulomas, it may be prudent to routinely use acid-fast staining for sentinel screening (or other fish used for monitoring), to identify diffuse and low-level infections. Histology has the advantage of observing whole fish sections, but cannot determine the species of *Mycobacterium* present. Molecular diagnostics can be used on preserved tissues and blocks (Meritet, Mulrooney, Kent, & Löhr, 2017; Peterson et al., 2013; Zerihun, Hjortaas, Falk, & Colquhoun, 2011) to identify species, but depending on how the tissue was processed, these are not as useful as starting with fresh, frozen or ethanol preserved tissues.

Bacterial culture and biochemical assays for identification of mycobacteria are well described (Kent & Kubica, 1985), but can be time-consuming. Specialized media is necessary, with most growing on Middlebrook 7H10 agar plates or Löwenstein–Jensen slants. These contain supplements known to facilitate mycobacterial growth and inhibit the growth of other bacteria and fungi. Where *M. haemophilum* is suspected, the medium must be supplemented with iron. Species considered rapid-growers take approximately 5–7 days before colonies are noticeable on plates, and the slow-growers can take anywhere from 2 to 8 weeks, or more before growth is observed. Strains isolated from zebrafish typically grow well at 29°C, but if an incubator is not available, they will grow at room temperature, but possibly more slowly. Some major advantages of culturing the organism are that it can be evaluated using common strain typing techniques for which pure cultures are required, and it allows for subsequent virulence testing. For long-term storage, cultures can be frozen down at −80°C in glycerol, or lyophilized.

Detection of mycobacteria by PCR has become commonplace. It compares well to both histology and cultures for diagnosing infection (Whipps et al., 2008). In addition, PCR is more rapid than other diagnostic tests; can be used on preserved tissues or directly on infected tissues and to test environmental samples, and allows for direct identification of species by DNA sequencing. Disadvantages of PCR are that if done on infected tissues it may not allow for subsequent strain typing techniques that require isolated cultures, and a PCR positive sample does not indicate the clinical significance of the result like histology. Screening water, biofilm, and feces samples may be an option for nonlethal monitoring of a system for mycobacteria (Crim et al., 2017; Whipps et al., 2008, 2012). However, because

mycobacteria are common in aquatic systems, it is important that PCR tests are very specific. As a consequence, PCR may be very useful for initial screening, but follow-up with a secondary method like histology will relate the biological significance.

Many PCR tests have been developed either by commercial diagnostic services or by researchers for use in-house (Meritet et al., 2017; Zerihun et al., 2011). Tests may be general to any mycobacteria or directed to a certain species. Identification of PCR positives or confirmation of identity in specific tests can be accomplished through DNA sequencing of PCR products. A commonly used target for species identification is *hsp65* gene, using DNA sequencing or restriction fragment length polymorphism (Kim et al., 2005; McNabb, Adie, Rodrigues, Black, & Isaac-Renton, 2006; Ringuet et al., 1999; Whipps et al., 2008). In some cases, the DNA sequencing results will allow for assignment to a species, and other times only to a species complex. A phylogenetic analysis or other methods (biochemical tests) may need to be performed for very closely related species (Kent et al., 2004; Noguiera et al., 2015). Direct PCR and sequencing can be used on tissues (mix of bacterial and host DNA), environmental samples, and preserved samples. These can also be used directly on cultures.

Control and Treatment

Surface Disinfection Eggs

A recommendation for the avoidance of pathogens, and hence control of diseases in zebrafish facilities, is to surface-disinfect eggs using bleach, as fish are bred into the facility (Kent et al., 2009; Lawrence, 2007; Westerfield, 2000). This likely works well on many potential pathogens, but mycobacteria tend to be resistant to chlorine bleach at the levels typically used to disinfect eggs, and different *Mycobacterium* species show different susceptibilities to chlorine (Chang et al., 2015). Increased bleach concentrations have a greater germicidal effect, but these tend to negatively impact development and survival of embryos (Kent, Buchner, Barton, & Tanguay, 2014). Iodine, on the other hand, shows the greater germicidal effect at relatively low concentrations (Chang et al., 2015). Based on this, Chang, Amack, and Whipps (2016) tested iodine on embryos and found that a buffered iodine solution (Ovadine) had the least impact on fish relative to unbuffered iodine and that these levels also resulted in a high germicidal effect on mycobacteria. As such, iodine may be a better option for disinfection over chlorine, and it is recommended that embryos be disinfected for 2 min with a buffered iodine solution at 12.5–25 ppm. As with chlorine solutions, it is important to note that iodine solutions must be prepared fresh because free iodine decreases dramatically in as little as 24 h (Chang et al., 2015).

Antibiotic Treatment

Antibiotic treatment of mycobacteriosis in food fish aquaculture is usually impractical because of the high costs of antibiotics, long treatment regimens, and concerns about the use of these powerful human drugs in fish destine for human consumption. Still, antibiotic treatment of zebrafish may be called for with subclinical infections in valuable lines of zebrafish to remove this confounding factor from the experiments or to minimize the chances of maternal transmission when breeding new fish for research. From clinical isolates, rifampin and rifabutin are known to be effective against *M. marinum* (Aubry, Jarlier, Escolano, Truffot-Pernot, & Cambau, 2000), and amikacin and clarithromycin effective against *M. chelonae* and other rapid growers (Brown-Elliott & Wallace, 2002). There are a few reports of successful treatment of mycobacteriosis in aquaculture. Kanamycin added to water was reported to successfully treat *M. marinum* in guppies (Conroy & Conroy, 1999) and gouramis (Santacana, Conroy, Mujica, Marin, & De Lopez, 1982). Kawakami and Kusuda (1990) reported that rifampin, streptomycin, and erythromycin were effective for reducing mortalities in cultured yellowtail with only two doses within 24 h. In contrast, Hedrick, McDowell, and Groff (1987) found rifampin treatment ineffective against *M. marinum* in striped bass. Most of these studies focused on *M. marinum* and little is known about treating rapid growers like *M. chelonae* in fish.

Treatment of nontuberculosis *Mycobacterium* infections in humans is commonplace (Griffith et al., 2007; Wu et al., 2012), but the application of these same treatments for infections in fish has only been investigated sporadically. Antibiotic treatment of mycobacteriosis in food fish aquaculture is usually impractical because of the high costs of antibiotics, long treatment regimens, and concerns about the use of these powerful human drugs in fish destined for human consumption (Whipps et al., 2012). Few studies have investigated the antibiotic treatment of fish, with results highly variable. Some report decreases in disease, others do not, but the approaches are difficult to compare (i.e., different doses, different means of administration, different duration, different endpoints) (Boos, Schmidt, Ritter, & Manz, 1995; Colorni et al., 1998; Conroy & Conroy, 1999; Hedrick et al., 1987; Kawakami & Kusuda, 1990; Santacana et al., 1982; Strike, Feltrer, Flach, Macgregor, & Guillaume, 2017). For mycobacteria isolated from zebrafish, Chang and Whipps (2015) tested several antibiotics and identified tigecycline and clarithromycin as most effective against the greatest numbers on strains in culture. These were subsequently tested in fish (Chang et al., 2017) against experimental infections with *M. chelonae*, and tigecycline (at 1 μg/g) and

clarithromycin (at 4 μg/g), for either 14 or 30 days, there was a decrease in severity of disease as compared to controls, but no decrease in prevalence. Antibiotics were administered orally by mixing in a gelatin-based feed. Antibiotics were tolerated well by zebrafish. It may be that higher doses or longer durations of treatment may prove curative. It is important to emphasize that widespread use of these antibiotics to treat fish is not recommended. However, this approach could be useful for the treatment of valuable or rare fish, or in certain broodstock known to be infected, prior to breeding. Adults could be treated, eggs surface disinfected, and then the lines rederived.

Specific Recommendations for Employee Safety

From a human health perspective, the species found in fish are categorized as nontuberculosis mycobacteria (NTM). All the species reported from zebrafish have also been reported in humans (Aubry et al., 2002; DeGroot & Huitt 2006; Noguiera et al., 2015; van Coppenraet, Kuijper, Lindeboom, Prins, & Claas, 2005); however, it is important to make clear that most of these infections are not linked with handling fish, and likely represent different strains. An exception to this are *M. marinum* infections, which are well known to be associated with aquaria or handling fish (Aubry et al., 2000, 2002). Indeed, Ostland et al., (2008) reported genetically identical strains of *M. marinum* in fish, and from a hand infection in a fish handler. Conversely, Ucko and Colorni (2005) found that isolates of *M. marinum* from fish and human clinical isolates were distinct from one another. This suggests that some infections are acquired from fish and some from other sources.

Although most mycobacteria found in zebrafish likely represent a low risk of infection for facility employees, *M. marinum* infections are linked to handling fish, and precautions should be taken if identified in a facility. Animal biosafety level 2 is recommended and generally require restricted access to the facility (common for most vivaria) and appropriate personal protective equipment and training of all personnel (see Chosewood & Wilson, 2009 for complete details). Hand infections occurred in staff at zebrafish facility suffering an outbreak in their fish (Mason et al., 2016). This facility then developed 14 new strategies and protocols to reduce the infection in their fish and protect their staff. These included personal protective equipment (particularly gloves) and enhanced training for staff working with fish, and posting signs on the risk of *M. marinum* infections. Regarding reducing the exposure of fish, they developed an environmental surveillance program using a PCR test for *M. marinum*, which included screening live feeds, tanks, equipment, and floors. Other strategies they included were removing dead, moribund, or elderly fish and disposal of water from spawn tanks.

Edwardsiella Ictaluri

Background

E.ictaluri is a Gram-negative bacterium, first described by Hawke, McWhorter, Steigerwalt, and Brenner (1981) from catfish. The bacterium is best known for causing enteric septicemia of catfish (ESC) (see review by Evans, Klesius, Plumb, & Shoemaker, 2011). *E.ictaluri* has been reported from fishes other than catfish (Evans et al., 2011), including ornamental species, such as the glass knifefish (*Eigenmannia virescens*) (Kent & Lyons, 1982), the rosy barb (*Pethia conchonius*) (Humphrey, Lancaster, Gudkovs, & McDonald, 1986), and the Bengal danio (*Danio devario*) (Blazer, Shotts, & Waltman, 1985; Waltman, Shotts, & Blazer, 1985). Although infections occur in fishes from a wide range of fish families, the specificity is patchy, i.e., only some species are susceptible. Moreover, the isolate from zebrafish and other aquarium fishes is distinct from those infecting catfish and tilapia (Griffin et al., 2016).

In experimental exposure studies, Petrie-Hanson et al. (2007) evaluated zebrafish as a potential model for ESC in catfish and found them to be susceptible by either injection or bath exposure, with mortalities occurring within 6 days. Then in 2013, Hawke et al. described the first outbreaks of *E.ictaluri* in zebrafish colonies, suggesting that not only are zebrafish susceptible in experimental studies, but this is also a pathogen that should be monitored for in a zebrafish facility. Outbreaks of *E.ictaluri* are uncommon, with the records from the ZIRC health service showing only three facilities submitting positive cases over 10 years, all in 2011 (Kent et al., 2016). While these infections may be rare, they can be devastating with many mortalities (Hawke et al., 2013). In experimental transmission studies with zebrafish, Petrie-Hanson et al. (2007) found that even when exposed to a low dose of *E.ictaluri* (1×10^2 CFU/g), fish began exhibiting signs of disease (abnormal swimming behavior) at 12 days postinjection. Hawke et al. (2013) evaluated the impact of *E.ictaluri* on zebrafish using a strain isolated from zebrafish. A bath exposure of 1×10^7 CFU/mL for 30 min resulted in 100% mortality by 10 days postexposure.

Known routes of exposure for *E.ictaluri* in catfish are through the nasal opening and olfactory system (Morrison & Plumb, 1994), across the intestinal wall (Shotts, Blazer, & Waltman, 1986), and over the gills (Plumb, 1999). In catfish, the liver and spleen are most severely

impacted by the infection, which is similar to infections in zebrafish (Hawke et al., 2013). Other tissues affected include the intestine, heart, liver, nervous tissues, and skin (Evans et al., 2011; Petrie-Hanson et al., 2007). Skin lesions may be particularly striking as fish develop raised scales, hemorrhages, and ulcerations (Hawke et al., 2013). Fish may also exhibit abnormal or lethargic swimming even before outward signs of infection are present (Petrie-Hanson et al., 2007).

E.ictaluri is considered to be an obligate pathogen, surviving poorly outside of a host (Plumb, 1999). This may be partly due to *E.ictaluri* being a poor competitor for resources outside of the host when other microbes are present (Earlix, 1995). However, Plumb and Quinlan (1986) found that *E.ictaluri* survived over 90 days in sterilized mud held at 25°C. This ability to persist in the environment when there are few competitors may be important for zebrafish systems, which may have surface biofilms and microbial biofilters but are likely microbially less diverse compared to the mud of a catfish pond. The environmental persistence likely matters little for fish in the same tank, as *E.ictaluri* can be transmitted directly through water (Klesius, 1994), typically shed in feces of infected fish. Dead fish may also be a source of infection (Earlix, 1995), highlighting the importance of any zebrafish health monitoring program to remove dead and moribund fish.

Clinical Signs and Pathological Changes

Typical of septicemic infections in fish with Gram-negative bacteria, fish usually exhibit reddening of the skin (diffuse erythema). Severe necrosis is common in visceral organs, such as the spleen and kidneys. In addition, severe necrosis of the forebrain, olfactory nerves, and nares is often observed. This is consistent with the nares being a possible point of invasion for *E.ictaluri* (Plumb, 1999). In histological sections, many Gram-negative rods can be observed in lesions, often in large aggregates within macrophages (Fig. 41.2). High mortality is common, but survivors may become carriers, as has been described in catfish (Plumb, 1999).

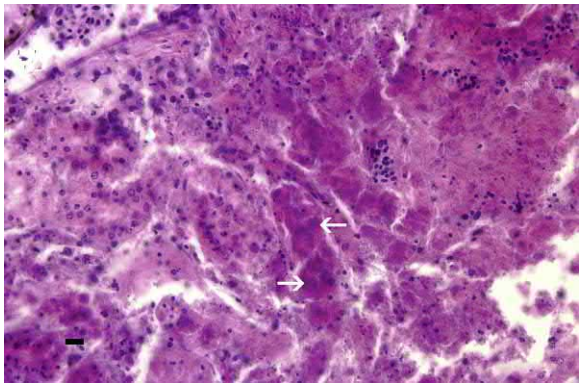

FIGURE 41.2 Severe necrosis of zebrafish kidney infected with *Edwardsiella ictaluri*. *Arrows* = basophilic colonies of bacteria within the lesion. Bar = 10 μm.

Diagnosis

In cases where high mortalities are observed, and reddening of the skin is associated with these mortalities, *E.ictaluri* would be suspected. An initial diagnosis can be made from H&E stained sections, where bacilli are observed to be associated with lesions in the forebrain, nerves, nares, and viscera. A confirmatory diagnosis would typically be made by plate culture and biochemical identification (Hawke et al., 1981; Waltman, Shotts, & Hsu, 1986), or by PCR (Bilodeau, Waldbieser, Terhune, Wise, & Wolters, 2003). Agar media, such as blood agar and brain-heart-infusion agar at 25–30C for five to seven, are appropriate for *E.ictaluri* (Hawke et al., 2013). *Aeromonas hyrdophila* and other rapidly growing Gram-negative bacteria will also grow on these media, and so, if coinfections occur (Nusbaum & Morrison, 2002), *E.ictaluri* may be overgrown. Therefore, it is useful for inoculating several plates with dilute inocula, and wait for many days for the smaller, slower growing, colonies of *E.ictaluri* to emerge.

Control and Treatment

The onset of disease in fish exposed to *E.ictaluri* is typically rapid (Hawke et al., 2013; Petrie-Hanson et al., 2007). As such, this pathogen, in particular, highlights the importance of quarantine. Even when fish were exposed to a low dose of *E.ictaluri*, signs of disease were apparent within 2 weeks (Petrie-Hanson et al., 2007). By holding fish for at least 2 weeks, the presence of the pathogen will likely become obvious, and introduction into the main facility avoided. In the cases described from zebrafish, the infections were recognized in quarantine (Hawke et al., 2013). Were the bacterium to get into the main facility, the results could be disastrous. Mass mortalities would be expected, and given that survivors are often carriers, euthanasia of infected stocks is recommended.

Antibiotics effective for the treatment of catfish include oxytetracycline or ormetoprim-sulfadimethoxine. Other drugs that have been tested in vitro include kanamycin, streptomycin, and oxolinic acid (Noga, 2010). Zebrafish isolates have been found susceptible to Romet, oxytetracycline, florfenicol, and enrofloxacin (Hawke et al., 2013), but these have yet to be tested in vivo.

Other Bacterial Infections

Background

Reports of other bacteria in zebrafish are few. The ZIRC disease manual (Kent et al., 2016) identifies cases of gliding bacteria (i.e., *Flavobacterium*, *Flexibacter*, or *Cytophaga* spp.), *Aeromonas*, and *Pseudomonas* species. The gliding bacteria have been associated with bacterial gill disease and *Aeromonas* species with infections of the swim bladder (Kent et al., 2016). Pulliam et al. (1999) reported high mortalities in zebrafish with *Aeromonas* infections, associated with high nitrite levels in the water. All are poorly characterized in zebrafish but are well known in other fishes (Cipriano & Austin, 2011; Starliper & Schill, 2011). Zebrafish have been used as a model for infections with *Flavobacterium* species (Guz, Puk, Walczak, Oniszczuk, & Oniszczuk, 2014; Moyer & Hunnicutt, 2007; Olivares-Fuster, Bullard, McElwain, Llosa, & Arias, 2011; Solís et al., 2015) and *Aeromonas* species (Hu et al., 2017; Saraceni, Romero, Figueras, & Novoa, 2016), so they are certainly susceptible to these infections. Their low prevalence in zebrafish systems supports the assumption that these infections are opportunistic in fish compromised by other diseases or poor water quality. *Flavobacterium columnare* is recognized as a primary pathogen in freshwater fishes, but zebrafish only become infected following the damage to their skin (Moyer & Hunnicutt, 2007).

Clinical Signs and Pathological Changes

Skin infections with gliding bacteria (*Flavobacterium* spp.) can cause erosion of the body surface and tail epithelium, resulting in the tail or fin rotting (Fig. 41.3). Wet mounts reveal long filamentous rods (Fig. 41.4). Bacterial gill disease exclusively affects the gills, with signs typical of respiratory distress, such as lethargy and flaring of opercula (Noga, 2010). Wet mount or histological examination of gills reveals hyperplasia and fusion of secondary lamellae (Fig. 41.5). In severe cases, entire primary lamellae may fuse (Noga, 2010).

FIGURE 41.3 Posterior skin and tail lesions associated with filamentous bacteria.

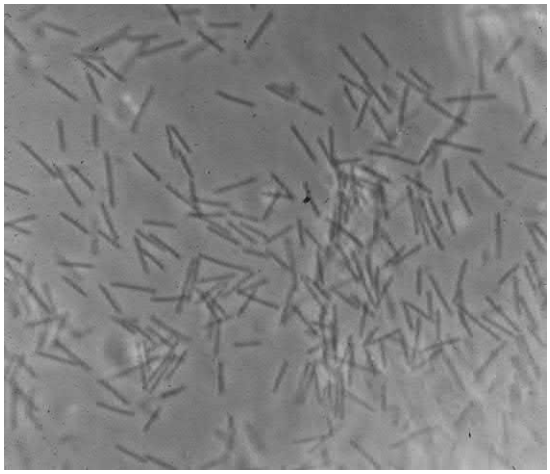

FIGURE 41.4 Filamentous (gliding) bacteria from skin scraping.

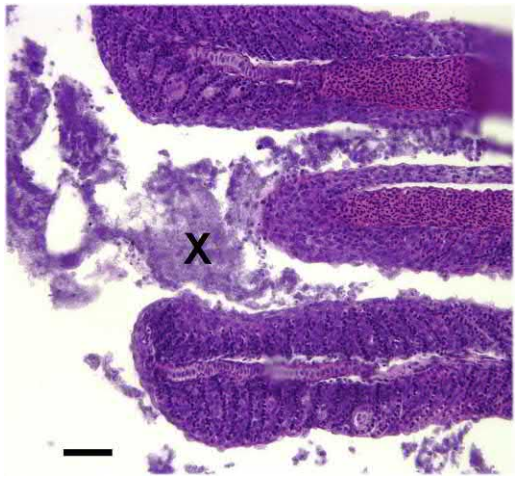

FIGURE 41.5 Bacterial gill disease in histological section (H&E). Severe, diffuse epithelial hyperplasia associated with large mats of bacteria (X). Note complete loss of interlamellar spaces. Bar = 50 μm.

Signs of *Aeromonas* species and other Gram-negative infections are more wide-ranging, from external lesions on skin and fins to septicemia (Cipriano & Austin, 2011). External lesions of the skin vary in size and may penetrate deep into the muscle or develop into ulcerations (Noga, 2010). Internally, there may be fluid accumulation, petechial hemorrhaging, and inflammation and hemorrhage of the intestine. Histologically, skin lesions may be observed with adjacent myositis, necrosis of the hematopoietic tissues, and other tissues (Cipriano & Austin, 2011).

Diagnosis

Gliding bacteria (*Flavobacterium* spp.) infections can be presumptively diagnosed by the wet mount of gill

tissue or skin scraping, with the observation of numerous, long filamentous rods (Fig. 41.4). Infections can be diagnosed by histology as clinical infections involve massive numbers of bacteria associated with the lesions. Culture of *F. columnare* requires a low nutrient medium, such as tryptone yeast extract salts agar (TYES), cytophaga agar, Hsu-Shotts agar, or modified Shieh agar (DeClerq et al., 2013; LaFrentz & Klesius, 2009). Cultures are incubated at 20–30°C for 1–3 days, with the formation of flat, rhizoid, yellow colonies with irregular margins. Immunofluorescence assays and PCR tests are available for *F. columnare* (DeClerq et al., 2013), but it is likely that other members of *Flavobacterium* or related genera infect zebrafish. Given that these infections are not well characterized in zebrafish, it may be prudent to attempt both culture and PCR using general bacterial primers, so that other possible agents of the disease may be detected if present.

For a definitive diagnosis of *Aeromonas* infections, bacteria can be cultured on TSA or brain-heart infusion agar followed by isolation on a more selective medium, such as RS medium Cipriano, Bullock, & Pyle, 1984, p. 134; Shotts & Rimler, 1973). Biochemical assays are expected to yield the following results (Shotts, 2012): arginine dihydrolase positive, ornithine decarboxylase negative, lysine decarboxylase variable, 0/129 resistant, novobiocin resistant, and motile.

Control and Treatment

Largely considered to be opportunistic, associated with suboptimal water quality or husbandry conditions. The stress associated with these conditions allows for the establishment of infection or worsening of existing infections. *Aeromonas* infections have been associated with elevated water temperatures, increases in ammonia, and carbon dioxide (Cipriano et al., 1984, p. 134). For *Flavobacterium* infection, Decostere, Haesebrouck, Turnbull, and Charlier (1999) reported higher titers of bacteria on fish gills with decreasing water quality and increasing nitrite. Infections reported by ZIRC (Kent et al., 2016) have been associated with suboptimal water quality conditions during the shipping of zebrafish. Thus, maintaining good quality water likely reduces the chances of infection by these bacteria.

Flavobacterium columnare infections can be treated by bath exposure to antimicrobial agents or through oral administration of antibiotics for more advanced infections (Declercq, Haesebrouck, Van den Broeck, Bossier, & Decostere, 2013). Various chemical bath treatments have been explored in other fishes, including copper sulfate and potassium permanganate (Declercq et al., 2013). *Aeromonas* infections can be treated with oxytetracycline (Cipriano & Austin, 2011), but some strains may exhibit resistance (Shotts, Vanderwork, & Campbell, 1976). Improving water quality often allows infections to resolve themselves (Noga, 2010), so antibiotic treatment may not be necessary. None of these have been evaluated in zebrafish with regards to sensitivity of strains isolated from zebrafish, nor the efficacy of treatments by oral or bath exposure or otherwise.

Mycoplasma Species

Background

Mycoplasma species are not typically thought of as fish pathogens but are familiar to those working on fish diseases as being associated with contamination of fish cell culture lines (Uphoff & Drexler, 2014). Kirchhoff and Rosengarten (1984) appear to be the first to report isolating a *Mycoplasma* species from fish gills and subsequently described as *Mycoplasma mobile* (Kirchhoff et al., 1987). This species has been used as a model to study infection by *Mycoplasma* species with pathological changes occurring in both rodents (Stadtländer & Kirchhoff, 1990) and fish Stadtländer, Lotz, Körting, & Kirchhoff, 1995) following exposure. More studies have reported *Mycoplasma* species from fish based on data of intestinal microbiota studies (Holben, Williams, Saarinen, Särkilahti, & Apajalahti, 2002; Karlsen et al., 2017; Song et al., 2016; Suhanova et al., 2011). Recently, Burns et al. (2018) reported a species genetically similar to *Mycoplasma penetrans*, identified during the analysis of intestinal microbiota. One particular strain/species was associated with a transmissible small-cell carcinoma of the intestine. This association was further strengthened in another experiment, again showing that *Mycoplasma* spp., including the strain described by Burns et al. (2018) was associated with intestinal cancers in zebrafish (Gaulke et al., 2019).

Clinical Signs and Pathological Changes

Mycoplasma species are rarely reported in fish, and therefore, little is known about signs and pathology of infection. *Mycoplasma mobile* was isolated from a tench (*Tinca tinca*), which Stadtländer et al. (1995) described as having "red disease," although other bacteria were also isolated from this fish (*Aeromonas* spp.). Stadtländer et al. (1995) exposed tench (*Tinca tinca*) to *M. mobile* via multiple methods (gills, skin, and intraperitoneal injection), and reported gill epithelial necrosis. The *Mycoplasma* species associated with intestinal neoplasms reported by Burns et al. (2018) is also associated with epithelial hyperplasia, dysplasia, and chronic inflammation of the anterior intestine.

Diagnosis

Although apparently uncommon in zebrafish, and fish in general, it is possible that these infections are

underdiagnosed. Standard plate culture techniques and microscopy would not likely identify infections.

Appearances of viral etiology or neoplastic lesions in routine histological monitoring might warrant further investigation. Specialized culture media can be used. For example, *M. mobile* can be cultured on modified Hayflick medium containing horse serum (Kirchhoff et al., 1987), but the utility of this medium for all *Mycoplasma* species is not known. Transmission electron microscopy can be used to identify the flask-like shaped cells of *Mycoplasma* species (Stadtländer et al., 1995). More rapid methods like DNA typing can identify a *Mycoplasma* species if present (Burns et al., 2018), but occurrence does not necessarily mean the identified species is a pathogen.

Control and Treatment

The association of neoplastic lesions and *Mycoplasma* species reported by Burns et al. (2018) suggests that if identified, these infections should be controlled. Burns et al. (2018) suggested a fecal-oral route of transmission, which is logical for a gut-inhabiting bacterium, but otherwise, little is known about transmission. There are no known treatments for *Mycoplasma* infections in fish; however, several antibiotics have been tested in cell culture (Drexler & Uphoff, 2002). The intestinal lesions generally occur in fish older than 9–12 mo (Paquette et al., 2013), so removing older fish from populations should reduce the occurrence of the intestinal lesions.

Water Molds and Mycotic Diseases

Background

Fungi and water molds are well characterized in other fishes (Roberts, 2012), but few reports come from zebrafish colonies. The Zebrafish International Resource Center (ZIRC) diagnostic service, provides data on the diagnosis of pathogens in submitted fish from 2006 to 2016 (https://zebrafish.org/wiki/_media/health/submission/submissions_summary_2006-2016.pdf), and fungal infections were observed in only 11 of some 10,000 fish over that time period. It is worthwhile noting that the water molds, or oomycetes, are not fungi but protists. They have been historically grouped with fungal fish pathogens due to their fungus-like appearance. A key differentiating feature is the presence of septa (septate) in fungi, versus the absence of septa (aseptate) in water molds. Fungi and water molds are usually considered opportunistic pathogens, so their presence may be indicative of some other process, like a different disease outbreak or substandard husbandry conditions.

Water molds from the genera *Saprolegnia* and *Aphanomyces* are among those that might be found in aquatic systems. Matthews (2009, pp. 321–346) indicated that *Saprolegnia* could be found in egg dishes or in embryo shipping containers. Ekblom (2017) evaluated the susceptibility of zebrafish embryos to *Saprolegnia parasitica*, and found that under standard conditions, fish are reasonably resistant. However, at lower temperatures, the fish were more greatly impacted. In catfish, problems with *Saprolegnia* are associated with temperature drops and other stressors (Howe, Rach, & Olson, 1998). Dykstra et al. (2001) reported finding an *Aphanomyces* species in a zebrafish system, isolated from the body surface of fish.

True fungi have been reported from zebrafish facilities, but like the water molds, these are not commonly reported and are likely opportunists. Dykstra et al. (2001) described mass mortality even associated with *Lecythophora mutabilis*, with anywhere from 10% to 100% of juvenile fish infected in a given tank. Kulatunga et al. (2017) isolated *Fusarium oxysporum* from zebrafish tanks, where mortalities were observed in larvae (50%) and adults (1%). Healthy adult fish seem to be less impacted by the infection, but injecting *F. oxysporum* caused notable damage within 72 h. Although this is not a natural route of exposure, it does highlight the potential for these fungi to be pathogenic, perhaps in stressful conditions.

Zebrafish have a physostomus swim bladder, with a direct connection to the intestinal tract. Hence, both bacteria and undescribed fungi are often associated with severe necrosis and inflammation of swim bladder (aerocystitis) (Fig. 41.6). We also occasionally observe undefined fungal organisms within granulomas in other organs, such as the kidney and muscle (Fig. 41.7).

Clinical Signs and Pathological Changes

Fungal or water mold infection on the surface of fish may present with a cotton or wool-like appearance, hairy, or with long thin strands on the surface of the fish. Lesions may also be present and can expand leaving necrotic tissue at the center with fungi at the periphery or entirely covering the lesion. *Aphanomyces* can invade deep within the tissues causing ulcerative necrotic lesions on the skin (Yanong, 2003). Eggs and larvae may be particularly susceptible to infection with any of these species. Fungal infections of the swim bladder are associated with severe acute necrosis and occasionally granulomatous inflammation.

Diagnosis

Wet mount preparations of skin scrapings will reveal fungal hyphae and spores (and similar structures in the

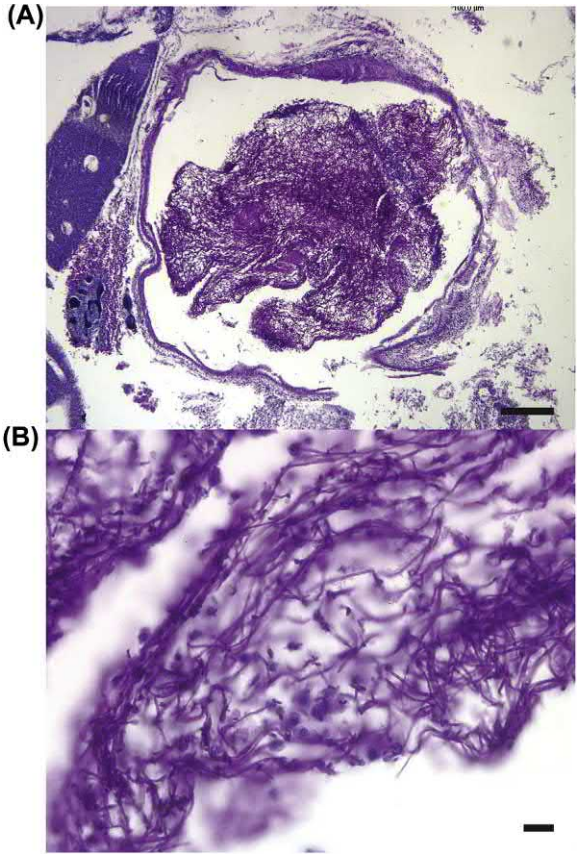

FIGURE 41.6 Colonization of anterior swim bladder by a septate fungus. PAS. (A) Low magnification. Bar = 200 μm (B) High magnification showing septate and branching hyphae. Bar = 10 μm.

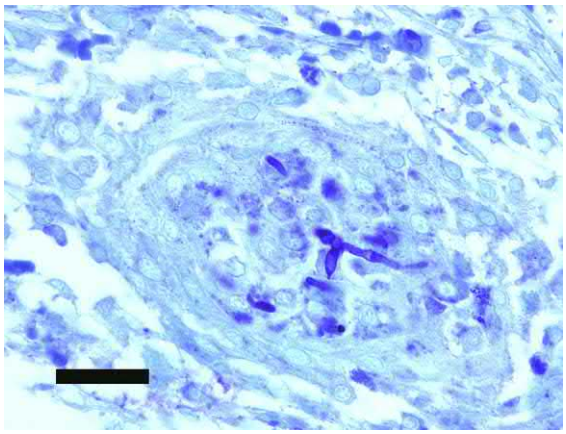

FIGURE 41.7 Granuloma in the kidney with pleomorphic aseptate fungi. Lesions extended through the muscle and communicated with the skin. PAS. Bar = 20 μm.

water molds), consistent with fungal infections. Infected tissues or whole fish can be sectioned and stained with PAS. This stain or other fungal stains are particularly useful for distinguishing between granulomas caused by fungi versus mycobacteria and other agents. Observation of septate hyphae is indicative of true fungi, whereas aseptate hyphae are indicative of the water molds. A definitive diagnosis requires fungal culture, and techniques are specific to each species.

Control

As many water molds and fungi may be common in aquatic systems, completely eliminating them may not be possible. However, some species may be more pathogenic than others, so good biosecurity is still important. Quarantine and surface disinfection of eggs will help to limit introductions. Bleaching eggs/embryos and using sterile embryo media are recommended (Matthews, 2009, pp. 321–346), but this has not been specifically tested. Iodine treatment of catfish eggs was less effective at killing *Saprolegnia* than formalin treatment (Walser & Phelps, 1994). Once fungi are established, treating fish and cleaning the system may be required. Dykstra et al. (2001) reported that cleaning their system and sanitizing area where uneaten food collected, greatly decreased *L. mutabilis*. Malachite green is very effective for killing water molds and fungi, but its use is often avoided because of its toxicity (Noga, 2010). Salt baths, formalin, and peroxide can be effective for surface infections (Yanong, 2003). For systemic infections, no treatments have been tested on zebrafish, but Dykstra et al. (2001) reported *L. mutabilis* from zebrafish was susceptible to amphotericin B, 5-fluorocytosine, fluconazole, and itraconazole. Kulatunga et al. (2017) reported that *F. oxysporum* was susceptible to nystatin. Finally, because these infections are often opportunistic, evaluating husbandry conditions and improving these simultaneously to any other control measures being implemented is important.

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CHAPTER

42

Viral Diseases

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Introduction

Considering the dramatic rise in importance of the laboratory zebrafish for a broad range of biomedical research areas, perhaps the most striking aspect of health management for zebrafish colonies is the lack of information available with respect to naturally occurring viral infections in this species. Importantly, this deficiency reflects minimal investigation in this area, rather than an inability of viruses to infect zebrafish (Crim & Riley, 2012; Kent et al., 2009).

The zebrafish has been utilized as a developmental model organism since the 1930s (Creaser, 1934; Goodrich & Nichols, 1931; Roosen-Runge, 1938), was later popularized as a developmental genetics model by George Streisinger (Eisen, 2019), and has since radiated into many different fields of biomedical research. The zebrafish is an exceptional model organism for many fields because it meets nearly all animal model selection criteria, which include financial feasibility, experimental history, unusual biological characteristics that can be exploited, and advanced imaging and molecular techniques (Ericsson, Crim, & Franklin, 2013). In many laboratories, adult zebrafish are still maintained exclusively as breeding stocks for embryo production, as most or all of the experimental data collection occurs during the first few days postfertilization. In these laboratories, there may be less concern for the confounding effects of unrecognized subclinical disease in breeding stocks unless there is a significant drop in fecundity or the quality of embryos produced. The extraordinary fecundity of zebrafish may have even contributed to the lack of information about viral infections in this species, as zebrafish often produce adequate numbers of viable embryos for study, even when harboring subclinical infections (Crim & Riley, 2012). A lack of concern regarding subclinical viral infections is not advisable, however, as many subclinical viral infections in other species are known to be vertically transmitted, reduce fecundity, alter future immune responses to other pathogens, and/or alter developmental processes.

Undiagnosed viral infections in zebrafish also have the potential to expose other investigator groups to increasing risk as aquatics facilities are increasingly centralized with shared systems and husbandry personnel to accommodate more investigators (Crim & Riley, 2012; Kent et al., 2009). Biosecurity in multiinvestigator zebrafish facilities is extremely problematic, as most zebrafish are currently housed in large, centralized recirculating aquaculture systems (RAS) that cannot be easily compartmentalized, taken offline, or thoroughly disinfected (Collymore, Crim, & Lieggi, 2016). Moreover, it is increasingly common to house other fish species, such as other *Danio* spp., Japanese medaka (*Oryzias latipes*), Mexican tetra/cavefish (*Astyanax mexicanus*), killifish (*Nothobranchius furzeri*), platyfish (*Xiphophorus* spp.), fathead minnows (*Pimephales promelas*), guppies (*Poecilia reticulata*), and *Betta* spp. in centralized aquatics facilities near zebrafish, or even on the same system as zebrafish, presenting additional risk for the introduction of new diseases (Collymore, Crim, & Lieggi 2016; Crim, Lawrence, Livingston, Rakitin, Hurley, & Riley, 2017; Lawrence, Adatto, Best, James, & Maloney, 2012), including viral infections. This challenge is further complicated by the development of immunocompromized zebrafish lines, used for hematopoiesis, tumorigenesis, infection, and immunity experiments, which are often housed on the same RAS as wild-type fish (Crim & Riley, 2012). Immunocompromized fish lines are likely to be more susceptible to viral diseases than wild-type zebrafish (Crim & Riley, 2012) and thus may display more severe lesions, higher mortality, and shed higher numbers of infectious virions back into the RAS.

The susceptibility of zebrafish to viral infections is reflected in an epizootic of naturally occurring lethal viral nervous necrosis (VNN) in zebrafish (Binesh, 2013), natural infections of zebrafish with infectious spleen and kidney necrosis virus (ISKNV) (Bermudez et al., 2018),

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natural infections of zebrafish with a novel picornavirus (Altan et al., 2019), endogenous viral elements (EVEs) in the zebrafish genome (Basta, Buzak, & McClure, 2007; Shen & Steiner, 2004), and many experimental infection experiments with a wide range of fish and mammalian viruses (Table 42.1). This chapter includes a brief review of VNN, infectious spleen and kidney necrosis, zebrafish picornavirus-1 (ZfPV-1), experimental susceptibility of zebrafish to DNA and RNA viruses, and a general discussion of detection, diagnosis, risk assessment, and restrictions on zebrafish movement.

Naturally Occurring Oiral Infections

Importance of Natural Viral Infections in Zebrafish to Biomedical Research

Importance for developmental biology: Even for developmental biologists, the potential exists for unrecognized viral infections to confound experimental data. It is likely that zebrafish embryos can be infected by viruses as a result of vertical transmission, as a number of viruses are known to be vertically transmitted in other species (Breuil, Pepin, Boscher, & Thiery, 2002), and intraovum vertical transmission has already been demonstrated for at least one zebrafish pathogen, *Pseudoloma neurophilia* (Sanders, Watral, Clarkson, & Kent, 2013). Embryos and larvae are also potentially susceptible to viral infection by horizontal transmission. Experimental infection of zebrafish embryos and larvae with various pathogens, including *Betanodavirus*, has been shown to significantly alter gene transcription, including the transcription of cytokine genes in the developing embryos or larvae (Kim, Hancock, Del Cid, Bermudez, Traver, & Doran, 2015; Morick & Saragovi, 2017; Ordas et al., 2011; Saraceni, Romero, Figueras, & Novoa, 2016). Importantly, cytokines play important developmental roles in addition to their roles in immunity, which is a critically important consideration for development models. In addition, the innate immune system of zebrafish larvae develops rapidly, with primitive macrophages at 22 h postfertilization (hpf), primitive neutrophils at 33 hpf, and active neutrophils with the ability to migrate and phagocytize pathogens by 52 hpf (Kanther & Rawls, 2010). Altered cytokine levels adversely impact numerous developmental processes in many systems, including the central nervous system (CNS) (Cui, Eauclaire, & Matthews, 2013; Deverman & Patterson, 2009).

Importance for other areas of biomedical research: The zebrafish model has expanded into other research areas, including aging, behavior, degenerative diseases, immunology, infection, neoplasia, and toxicology, and the probability of confounding experimental data is increased for these types of studies. These research areas often include experimentation using adult fish, maintenance of adult fish for longer periods, and collection of histopathologic data that can be difficult to interpret when background lesions are evident. Important

TABLE 42.1 Viral experimental infection studies conducted in zebrafish.

Virus	Family	Natural host	Viral genome	Reference(s)
Infectious pancreatic necrosis virus (IPNV)	Birnaviridae	Fish	dsRNA	LaPatra et al., 2000; Seeley et al., 1977
Herpes simplex virus type 1 (HSV-1)	Herpesviridae	Human	dsDNA	Burgos et al., 2008
European sheatfish virus (ESV)	Iridoviridae	Fish	dsDNA	Martín et al., 2015
Infectious spleen and kidney necrosis virus (ISKNV)	Iridoviridae	Fish	dsDNA	Xu et al., 2008
Malabar grouper nervous necrosis virus (MGNNV)	Nodaviridae	Fish	(+)ssRNA	Lu et al., 2008
Redspotted grouper nervous necrosis virus (RGNNV)	Nodaviridae	Fish	(+)ssRNA	Furusawa et al., 2007
Influenza A virus	Orthomyxoviridae	Human	(−)ssRNA	Gabor et al., 2014
Infectious hematopoietic necrosis virus (IHNV)	Rhabdoviridae	Fish	(−)ssRNA	Ludwig et al., 2011
Snakehead rhabdovirus (SHRV)	Rhabdoviridae	Fish	(−)ssRNA	Phelan et al., 2005
Spring viremia of carp virus (SVCV)	Rhabdoviridae	Fish	(−)ssRNA	Lopez-Munoz et al., 2010; Sanders et al., 2003
Vesicular stomatitis virus (VSV)	Rhabdoviridae	Mammalian	(−)ssRNA	Guerra-Varela et al., 2018
Viral hemorrhagic septicemia virus (VHSV)	Rhabdoviridae	Fish	(−)ssRNA	Novoa et al., 2006
Chikungunya virus (CHIKV)	Togaviridae	Human	(+)ssRNA	Palha et al., 2013
Sindbis virus (SINV)	Togaviridae	Human	(+)ssRNA	Passoni et al., 2017

confounding factors resulting from unrecognized viral infections could include increases in colony mortality, chronic inflammation, cellular damage, hyperplasia and neoplasia, and tissue remodeling. Notably, when subclinical disease processes negatively impact research, they are rarely reported (Kent, Harper, & Wolf, 2012).

Oncogenic viruses: Spontaneous neoplasia is fairly common in zebrafish, and a wide variety of tumors have been described. Thus, it is possible that some neoplasms are caused by unrecognized oncogenic viruses. Viruses associated with tumorigenesis have been reported in other fish species, including angelfish (Francis-Floyd, Bolon, Fraser, & Reed, 1993), common carp (Sano, Morita, Shima, & Akimoto, 1991), and yellow perch (Bowser et al., 2005). The prevalence of certain tumor types in zebrafish varies according to population, including cutaneous papillomas (Beckwith, Moore, Tsao-Wu, Harshbarger, & Cheng, 2000) and gastrointestinal tumors (Paquette et al., 2013). N-nitroso-N-ethylurea–treated wild-type zebrafish displayed 100% incidence of cutaneous papillomas in one study, but cutaneous papillomas were not observed in similar studies conducted at other institutions (Kent et al., 2009). Neoplasms that vary by population further suggest the possibility of unrecognized oncogenic viruses (Crim & Riley, 2012).

Evidence from coinfection studies: Subclinical viral infections have been shown to dramatically alter subsequent immune responses in other teleosts, which have important implications for the use of zebrafish as an infection and immunological model for human and aquaculture pathogens. Coinfections have the potential to alter both the course and the severity of infection, and can involve either priming the immune system or suppressing it (Kotob, Menanteau-Ledouble, Kumar, Abdelzaher, & El-Matbouli, 2016). In synergistic coinfections, one pathogen hinders the immune response to subsequent infections. Antagonistic interactions may mean that one pathogen modulates the immune system such that immune responses are more effective against subsequent infection(s) (Kotob et al., 2016). In other antagonistic coinfections, pathogens may compete for one or more resources provided by the host (Andrews, Petney, & Bull, 1982).

There are many examples of coinfections in fish that illustrate the potential confounding effects of unrecognized viral infections on the use of zebrafish as an infection or immunology model (Crim & Riley, 2012; Kotob et al., 2016). Subclinical or unrecognized viral infections can alter simultaneous or subsequent viral infections, potentially including host susceptibility, disease progression, morbidity, shedding, and/or mortality. For example, rainbow trout simultaneously infected with both infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV) exhibited approximately 50% of the mortality displayed by trout infected with either virus individually (Alonso, Rodriguez Saint-Jean, & Perez-Prieto, 2003). In another study, trout that were exposed to IPNV before being infected with IHNV displayed 2% mortality, in contrast to 72% mortality in trout infected with IHNV alone (Byrne, Castric, Lamour, Cabon, & Quentel, 2008). The tissue distribution of IHNV is reduced in trout coinfected with IPNV and IHNV when compared with that of trout only infected with IHNV (Brudeseth, Castric, & Evensen, 2002). Exposure to cutthroat trout virus was also shown to be protective against IHNV exposure in rainbow trout for up to 4 weeks (Hedrick et al., 1994). Similarly, infection of Atlantic salmon with IPNV conferred resistance to subsequent infections with infectious salmon anemia virus (ISAV), compared with Atlantic salmon infected with ISAV only (Johansen & Sommer, 2001). Flounder birnavirus–infected olive flounder were more resistant to viral hemorrhagic septicemia virus (VHSV) than negative control fish (Pakingking et al., 2003). In a separate experiment, olive flounder infected with flounder birnavirus displayed significant protection when subsequently exposed to VHSV at 3, 7, or 14 days following birnavirus exposure, but exhibited mortality that was not significantly different than controls when VHSV exposure occurred 21 days after birnavirus infection (Pakingking, 2004). Infection with flounder birnavirus also protected sevenband grouper that was subsequently infected with redspotted grouper nervous necrosis virus (RGNNV), whereas RGNNV-infected fish that had not been previously infected with flounder birnavirus exhibited 80% mortality (Pakingking et al., 2005).

Subclinical or unrecognized viral infections can also potentially alter simultaneous or subsequent infections with bacteria, fungi, oomycetes, or parasites. This phenomenon is best documented for viral infections followed by bacterial infection. For example, flounder birnavirus–infected olive flounder were more susceptible to infection with either *Edwardsiella tarda* or *Streptococcus iniae* (Pakingking et al., 2003). Similarly, IPNV-infected Atlantic salmon exhibited higher mortality when subsequently challenged with *Aeromonas salmonicida* or *Vibrio salmonicida* (Johansen, Eggset, & Sommer, 2009; Johansen & Sommer, 2001).

Redspotted Grouper Nervous Necrosis Virus

RGNNV, a *Betanodavirus*, was the first naturally occurring viral infection reported for zebrafish (Binesh, 2013). *Betanodavirus* spp. exhibit an extremely broad host range and cause clinical and subclinical neurological infections, as well as high mortality epizootics, especially in young fish.

Description: The Nodaviridae family consists of nonenveloped, single-stranded RNA viruses with an icosahedral capsid composed of 32 capsomers and enclosing a linear, positive-sense, bisegmented RNA genome comprising two segments, RNA1 and RNA2 (Mori, Nakai, Muroga, Arimoto, Mushiake, & Furusawa, 1992). The Nodaviridae family contains two genera, *Alphanodavirus*, which includes viruses of arthropods, fishes, and mammals, and *Betanodavirus*, which includes four species (and a number of other unassigned genotypes) that primarily infect marine and some freshwater fishes, causing a disease known as VNN or as viral encephalopathy and retinopathy (VER). The four recognized species in the *Betanodavirus* genus, based on phylogenetic analysis of RNA2, are Striped Jack nervous necrosis virus (SJNNV), Barfin flounder nervous necrosis virus, RGNNV, and Tiger puffer nervous necrosis virus (TPNNV) (Doan, Vandeputte, Chatain, Morin, & Allal, 2017; Nishizawa, Furuhashi, Nagai, Nakai, & Muroga, 1997). Genetic sequence analysis of the variable region of RNA2 indicates that the *Betanodavirus* associated with VNN and mortality in zebrafish falls within the RGNNV cluster (Binesh, 2013). RGNNV is known to infect a wide variety of species and has an optimal growth temperature of 25–30°C (Doan et al., 2017), matching the temperature range in which most laboratory zebrafish are routinely maintained. In contrast, the three other *Betanodavirus* species grow better at cooler temperatures (Doan et al., 2017).

Host range: Betanodavirus spp. are known to infect more than 120 species of fishes and aquatic invertebrates (Costa & Thompson, 2016). RGNNV also has a very broad host range (Table 42.2) and infects at least 32 species of fish (Doan et al., 2017), including zebrafish and several other species that are used in biomedical research, such as goldfish (Binesh, 2013), guppies (Hegde, Teh, Lam, & Sin, 2003) and Japanese medaka (Furusawa, Okinaka, & Nakai, 2006).

Although one study reported that zebrafish were not susceptible to infection by RGNNV (Furusawa, Okinaka, Uematsu, & Nakai, 2007), subsequent experimental infections using a field strain of *Betanodavirus* isolated from Malabar grouper (*Epinephelus malabaricus*) in Taiwan were successful in establishing subclinical infections in adult zebrafish and high mortality in zebrafish larvae (Lu et al., 2008). Clinical signs of VNN, including erratic swimming and high mortality (up to 32%), were later observed in a group of zebrafish and goldfish (*Carassius auratus*) that had been obtained from ornamental pet stores and then maintained in a laboratory for 2 weeks (Binesh, 2013). An investigation of this epizootic confirmed natural susceptibility to RGNNV infection by reverse transcription PCR (RT-PCR) designed to amplify the variable region of RNA2, amplicon sequence analysis, and histopathology (Binesh, 2013). A subsequent experiment comparing subclinically infected zebrafish and naïve zebrafish housed at different densities and temperatures revealed that cumulative mortality was significantly greater when infected fish were housed at higher temperatures and at greater housing densities (Binesh, 2014).

Life cycle and modes of transmission: The specifics of viral transmission, dissemination within the host, and viral cell entry are poorly understood (Costa & Thompson, 2016). The virus is waterborne and stable in the water column. Thus, it is likely that there is more than one route for horizontal transmission. Cell entry is believed to occur by endocytosis when viral-like particles bind to sialic acid on the cell surface (Liu et al., 2005). Heat shock cognate protein 70 (GHSC70) interacts with the *Betanodavirus* capsid protein and has been suggested to act as a receptor or coreceptor (Chang & Chi, 2015).

Evidence from laboratory experiments and VER epizootics suggests that *Betanodavirus* spp., which have been identified in sperm, ovary, oocytes, embryos, and larvae, can also be vertically transmitted (Breuil et al.,

TABLE 42.2 Selected freshwater fishes susceptible to redspotted grouper nervous necrosis virus (RGNNV) infection.

Scientific name	Common name	Family	Infection	Reference(s)
Betta splendens	Siamese fighting fish	Osphronemidae	Experimental	Furusawa et al., 2007
Carassius auratus	Goldfish	Cyprinidae	Natural	Binesh, 2013
Danio rerio	Zebrafish	Cyprinidae	Natural and experimental	Binesh, 2013; Binesh, 2014; Morick et al., 2015
Iriatherina werneri	Threadfin rainbowfish	Melanotaeniidae	Experimental	Furusawa et al., 2007
Melanotaenia praecox	Dwarf rainbowfish	Melanotaeniidae	Experimental	Furusawa et al., 2007
Oryzias latipes	Japanese medaka	Adrianichthyidae	Experimental	Furusawa et al., 2006; Furusawa et al., 2007
Poecilia reticulata	Guppy	Poeciliidae	Experimental	Furusawa et al., 2007
Pterophyllum scalare	Freshwater angelfish	Cichlidae	Experimental	Furusawa et al., 2007
Trichogaster lalius/Colisa lalia	Dwarf gourami	Osphronemidae	Experimental	Furusawa et al., 2007

2002; Costa & Thompson, 2016). It has been suggested by some authors that the stress of spawning permits viral replication in nonneuronal tissues including the gonads, with subsequent viral infection of sperm and oocytes (Valero et al., 2015).

Clinical signs and pathobiology: *Betanodavirus* epizootics are commonly associated with significant larval or juvenile mortality in many species, although significant mortalities can also occur among adult fish (Munday, Kwang, & Moody, 2002). In some species, mortality in epizootics of VNN among young fish can be as high as 100% (Costa & Thompson, 2016). In general, the level of mortality and morbidity attributed to VNN is greatest in larval fish, followed by juvenile fish, and then adults (Munday et al., 2002). An isolate from a white grouper (*Epinephelus aeneus*) that was 99% similar to SGNNV was more virulent when 4 dpf zebrafish larvae were infected than when older larvae were infected (Morick et al., 2015) (Fig. 42.1).

Other physical and behavioral clinical signs include erratic swimming, which may present as darting or spiral swimming, changes in coloration, anorexia, weight loss, and loss of swim bladder control (Munday et al., 2002). Infected fish may also be lethargic, resting on the bottom or drifting upside down at the surface (Costa & Thompson, 2016).

Only one brief description of a naturally occurring outbreak of VER has been described in zebrafish to date; therefore, much of the pathologic changes described below reflect VER pathology in other fishes. Host specificity of *Betanodavirus* spp. is conferred by differences in the variable region of RNA2 (Ito et al.,

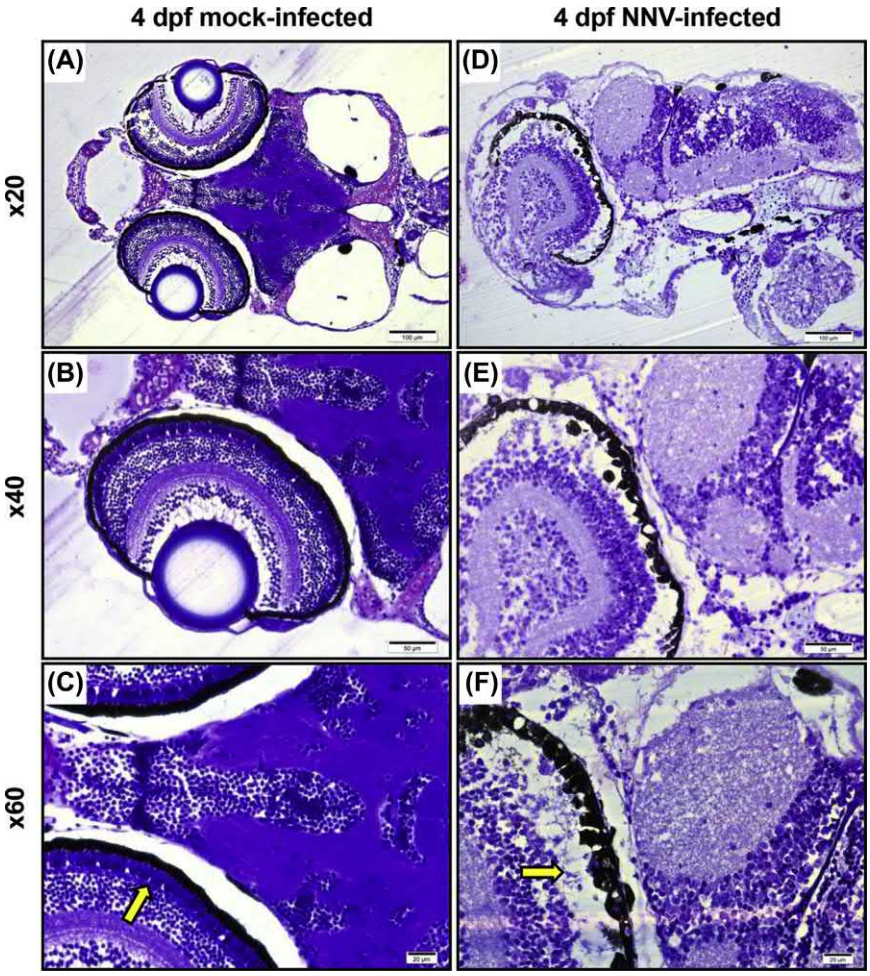

FIGURE 42.1 Brain and retinal lesions in a zebrafish larva experimentally infected with Betanodavirus (SGNNV). Methacrylate-embedded sections of zebrafish larvae at 4 dpf stained with toluidine blue. (A–C) Control mock–infected larvae; (D–F) NNV-infected larvae showing marked neuropil vacuolation, as well as a relative paleness of neurons involving both the brain and retina. The most prominent injury appears to be in the photoreceptor layer (yellow arrows) of the retina, with apparent nearly total lysis of the photoreceptors. dpf, days postfertilization. *Image from Morick, D., Faigenbaum, O., Smirnov, M., Fellig, Y., Inbal, A., & Kotler, M. (2015). Mortality caused by bath exposure of zebrafish (*Danio rerio*) larvae to nervous necrosis virus is limited to the fourth day postfertilization.* Applied and Environmental Microbiology, 81*(10), 3280–3287. http://ds.doi:10.1128/AEM.04175-14. Copyright 2015 by the American Society for Microbiology. Reprinted with permission.*

2008; Iwamoto et al., 2004). Detailed descriptions of brain lesions have portrayed differences with respect to the species of *Betanodavirus* and age and species of fish infected (Grotmol, Totland, Thorud, & Hjeltnes, 1997; Le Breton, Grisez, Sweetman, & Ollevier, 1997; Yoshikoshi & Inoue, 1990). VER is characterized by extensive necrosis and vacuolation of the brain and retinae (Fig. 42.1), and the course of infection proceeds rapidly in the host (Su et al., 2015).

RGNNV is neurotropic, and infection does not generally appear to cause pathology in tissues outside of the nervous system (Mori et al., 1991), except for evidence of viral replication in the testis of gilthead seabream and European seabass (Valero et al., 2015) and one report including endocarditis, gill lesions, and viral-like particles in gill pillar cells of infected juvenile Atlantic halibut (Grotmol et al., 1997). Both pyknosis and cell lysis are frequently observed; pyknotic cells contain densely packed cytoplasmic viral particles, whereas cell lysis predominates and is closely associated with vacuolation (Yoshikoshi & Inoue, 1990). In many cases, vacuolation due to *Betanodavirus* infection is more severe in the anterior aspect of the brain compared with the hindbrain and spinal cord (Munday et al., 2002), although other authors emphasize neuronal degeneration and vacuolation of the mid- and hindbrain (Costa & Thompson, 2016). Basophilic cytoplasmic inclusion bodies have been reported by several authors in multiple fish species (Yoshikoshi & Inoue, 1990), and electron microscopy (EM) demonstrates numerous cytoplasmic viral particles in affected neurons with margination of nuclear chromatin (Glazebrook, Heasman, & Beer, 1990). Vacuolation in the retinae involves the bipolar and ganglionic nuclear layers (Munday, Langdon, Hyatt, & Humphrey, 1992) and the rod and cone layer (Munday et al., 2002). In retinal neurons, EM studies revealed separation of the nuclear membrane, disintegration of mitochondrial cristae, and that virions may be free in the cytoplasm or attached to the endoplasmic reticulum (Glazebrook et al., 1990).

Diagnosis: Definitive diagnosis in zebrafish is based on positive results of RT-PCR analysis, positive viral culture, or clinical signs confirmed by the observation of specific lesions consistent with VNN, including vacuolation in both the brain and retinae and the presence of basophilic cytoplasmic inclusion bodies. Although *Betanodavirus* is a neurotropic agent, viral RNA can be detected by molecular techniques in many other tissues, including the fins, gastrointestinal tract, gills, gonads, heart, kidney, liver, muscle, and spleen (Lopez-Jimena et al., 2011; Su et al., 2015).

Control and treatment: No treatment is available for RGNNV infection in zebrafish. Vaccines against RGNNV and other *Betanodavirus* spp. have been shown to be protective for other fish species (Kai & Chi, 2008; Liu, Hsu, Chang, Chen, & Lin, 2006; Pakingking, Bautista, de Jesus-Ayson, & Reyes, 2010; Pakingking, et al., 2009; Thiery, Cozien, Cabon, Lamour, Baud, & Schneemann 2006) but have not been applied to zebrafish.

The most effective biosecurity measure is exclusion of new pathogens from a system (Collymore et al., 2016). Prevalence of RGNNV in zebrafish research colonies is unknown because colony screening by sensitive assays is rare. Undetected introduction of RGNNV is possible, however, for several reasons, including its broad host range, ability to cause only subclinical infections in adult zebrafish, the possibility of vertical transmission, and the reality that some laboratory zebrafish colonies still have links to the ornamental fish trade. Exclusion is especially important for zebrafish colonies, as most colony zebrafish are housed on large recirculating systems with centralized filtration that are not designed to be compartmentalized, making containment and disinfection far more difficult (Collymore et al., 2016). Moreover, *Betanodavirus* is particularly difficult to eliminate from aquaculture systems because it is environmentally stable (Costa & Thompson, 2016). Spawning imported zebrafish in quarantine is routine at many institutions and reduces colony risk, as larval fish are the most susceptible to infection and the most likely to display clinical signs and mortality. Sodium hypochlorite surface disinfection of zebrafish embryos is a common practice that reduces the risk of introducing *Betanodavirus* into zebrafish colonies.

Infectious Spleen and Kidney Necrosis Virus

ISKNV, a *Megalocytivirus*, has an extremely broad host range and was the second virus reported to cause natural infections in zebrafish, resulting in clinical disease although mortality was not reported (Bermudez et al., 2018).

Description: Iridoviridae is a Group I (dsDNA) family of viruses that includes five genera recognized by the Eighth Report of the International Committee on the Taxonomy of Viruses (ICTV): *Chloriridovirus*, *Iridovirus Lymphocystivirus*, *Megalocytivirus*, and *Ranavirus* (Chinchar, Essbauer, & He, 2005; Xu et al., 2008). Three of these genera, *Lymphocystivirus*, *Megalocytivirus*, and *Ranavirus*, are known to infect fishes. Iridoviruses are unique among eukaryotic vertebrates in possessing a circularly permuted and terminally redundant genome (Darai et al., 1983, 1985). The iridoviruses that infect fishes exhibit highly methylated genomes (Wagner et al., 1985; Willis & Granoff, 1980).

Megalocytivirus spp. are very large (150−250 nm) icosahedral DNA viruses with a large linear double-stranded DNA genome (Song et al., 2008). Along with

red sea bream iridovirus (RSIV) and Turbot reddish body iridovirus (TRBIV), ISKNV is one of the three genotypic groups based on the sequence of the major capsid protein gene (*MCP*) within the genus *Megalocytivirus*. The ISKNV genome has been completely sequenced (He et al., 2001), and ISKNV isolated from the freshwater mandarinfish (*Siniperca chuatsi*) (He, Zeng, Weng, & Chan, 2002) serves as the type species for the genus. Isolates belonging to the ISKNV genogroup are variably referred to as strains of ISKNV (Rimmer et al., 2015), ISKNV-like viruses (Rimmer, Whittington, Tweedie, & Becker, 2017; Wang et al., 2007), or *Megalocytivirus*/ISKNV genotype II (Fu et al., 2011). Named isolates from different fish species in the ISKNV genotypic group include African lampeye virus (Sudthongkong, Miyata, & Miyazaki, 2002), Banggai cardinalfish iridovirus (Weber et al., 2009), and dwarf gourami iridovirus (Go, Lancaster, Deece, Dhungyel, & Whittington, 2006; Sudthongkong et al., 2002).

Host range: *Megalocytivirus* spp. are important pathogens with broad host ranges among cultured and wild fish stocks. For example, in one PCR-based survey of marine fishes of the South China Sea, 13 cultured fish species and 39 wild fish species were identified as hosts (Wang et al., 2007). Of the 52 marine species that tested positive by PCR, 47 species were subclinically infected, whereas 5 species displayed acute infections evidenced by clinical signs (Wang et al., 2007).

ISKNV infects a broad range of fishes (Table 42.3). In contrast to RSIV and TRBIV, which are predominantly marine pathogens, ISKNV also infects a diverse array of freshwater fishes, although transmission of megalocytiviruses from freshwater fish to marine species and vice versa can also occur (Jeong et al., 2008). Importantly, the host range of ISKNV includes not only zebrafish but also other small freshwater species commonly used as research models, such as the southern platy (*Xiphophorus maculatus*) and the guppy (*P. reticulata*) (Anneke E Rimmer et al., 2015), in addition to species important for commercial aquaculture and the ornamental fish trade (Table 42.3). Risks for zebrafish colonies and multispecies biomedical fish core facilities are discussed below under "Control and Treatment."

Life cycle and modes of transmission: Naïve fish are believed to be infected by exposure to contaminated water, cannibalism, or consumption of infected fish tissues. Some authors have also suggested that intraspecific

TABLE 42.3 Selected freshwater fishes susceptible to infectious spleen and kidney necrosis virus (ISKNV) infection.

Scientific name	Common name	Family	Infection	Reference(s)
Aplocheilichthys normani	African lampeye	Poeciliidae	Natural	Sudthongkong et al., 2002; Fu et al., 2011
Astronotus ocellatus	Oscar	Cichlidae	Natural	Rimmer et al., 2015
Danio rerio	Zebrafish	Cyprinidae	Natural and experimental	Bermudez et al., 2018; Xu et al., 2008; Li et al., 2010
Helostoma temminkii	Kissing gourami	Helostomatidae	Natural	Rimmer et al., 2015
Maccullochella peeli	Murray cod	Percichthyidae	Natural and experimental	Fu et al., 2011; Rimmer et al., 2017
Mikrogeophagus ramirezi	Blue ram	Cichlidae	Natural	Rimmer et al., 2015
Micropterus salmoides	Largemouth bass	Centrarchidae	Experimental	He et al., 2002
Poecilia latipinna	Sailfin molly	Poeciliidae	Natural	Rimmer et al., 2015
Poecilia reticulata	Guppy	Poeciliidae	Natural	Rimmer et al., 2015
Poecilia sphenops	Common molly	Poeciliidae	Natural	Rimmer et al., 2015
Pterophyllum scalare	Freshwater angelfish	Cichlidae	Natural	Rimmer et al., 2015
Siniperca chuatsi	Mandarin fish	Percichthyidae	Natural	Fu et al., 2011
Trichogaster labiosa	Thick-lipped gourami	Osphronemidae	Natural	Rimmer et al., 2015
Trichogaster lalius/Colisa lalia	Dwarf gourami	Osphronemidae	Natural	Fu et al., 2011; Rimmer et al., 2015
Trichogaster microlepis	Silver/moonlight gourami	Osphronemidae	Natural	Rimmer et al., 2015
Trichopodus leerii	Pearl gourami	Osphronemidae	Natural	Rimmer et al., 2015
Trichopodus trichopterus	Blue/gold gourami	Osphronemidae	Natural	Rimmer et al., 2015
Xiphophorus maculatus	Southern platyfish	Poeciliidae	Natural	Rimmer et al., 2015

aggression may play an important role in viral transmission in some host species (Rimmer et al., 2017).

Clinical signs and pathobiology: Susceptibility to ISKNV infection appears to be temperature-dependent with disease outbreaks occurring at temperatures over 20°C (He et al., 2002). Disease progression and severity vary among reports and by host species. In laboratory zebrafish, a natural outbreak of ISKNV was associated with a variety of clinical signs, including edema, petechial hemorrhage, and pale or hyperemic gills (Fig. 42.2) (Bermudez et al., 2018), accompanied by lethargy, loss of appetite, abnormal swimming, and respiratory distress. Histopathology revealed a number of changes, most prominently the presence of widespread hypertrophied virus-infected cells, or megalocytes (Fig. 42.3) with enlarged nuclei displaying marginated chromatin and basophilic, often granular cytoplasm in the kidney (Fig. 42.4), spleen, dermis, and lamina propria of the intestine, as well as in various other tissues including gills (Fig. 42.5) (Bermudez et al., 2018). Necrosis, inflammation, and degenerative lesions were also observed, including granulomatous polyserositis, hepatic granulomatous inflammatory infiltrates, lymphohistiocytic dermatitis, and small areas of renal necrosis and hemorrhage (Bermudez et al., 2018).

Clinical signs resulting from natural infections in other fish species are diverse and can include anorexia, flared, pale, or swollen gills, dark or light skin coloration, lethargy, an open mouth, petechiae, ulcers and broken fin rays, uncoordinated movements, and mortality (Jung-Schroers et al., 2016; Rimmer et al., 2017). Gross lesions may include enlarged and brown kidney tissue, a

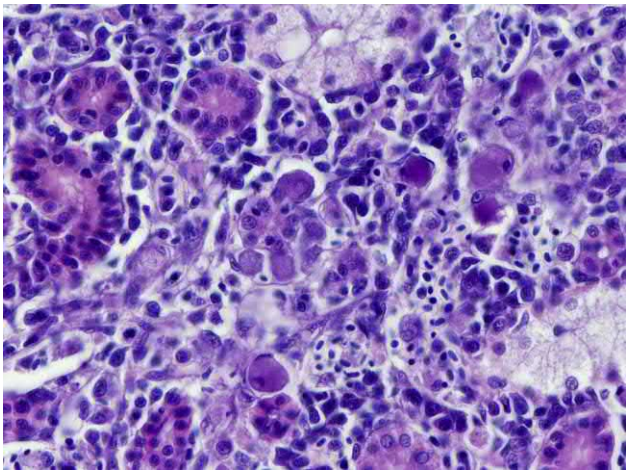

FIGURE 42.4 Zebrafish kidney, displaying many hypertrophied infectious spleen and kidney necrosis virus (ISKNV)–infected zebrafish cells (megalocytes). *Image courtesy of Roberto Bermúdez, DVM, Ph.D., Dipl. ECAAH.*

FIGURE 42.2 Euthanized zebrafish naturally infected with infectious spleen and kidney necrosis virus (ISKNV) displaying prominent scale protrusion and petechial hemorrhages. *Image courtesy of Roberto Bermúdez, DVM, Ph.D., Dipl. ECAAH.*

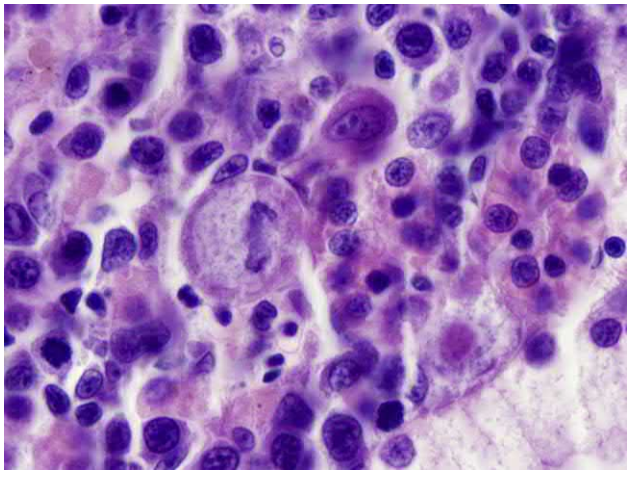

FIGURE 42.3 Photomicrograph of a hypertrophied infectious spleen and kidney necrosis virus (ISKNV)–infected zebrafish cell (megalocyte). *Image courtesy of Roberto Bermúdez, DVM, Ph.D., Dipl. ECAAH.*

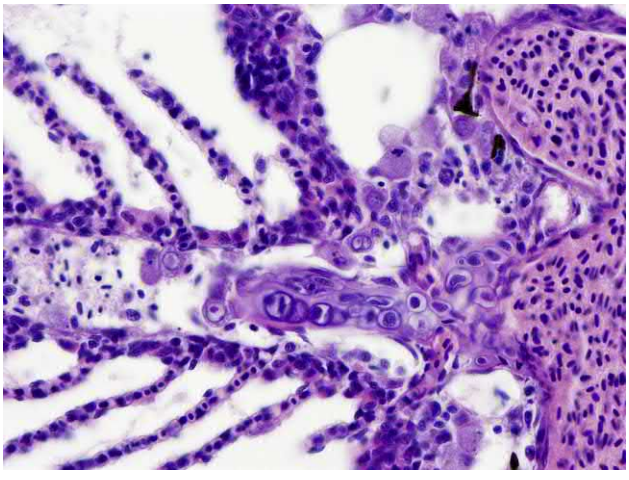

FIGURE 42.5 Zebrafish gill tissue displaying many hypertrophied infectious spleen and kidney necrosis virus (ISKNV)–infected zebrafish cells (megalocytes). *Image courtesy of Roberto Bermúdez, DVM, Ph.D., Dipl. ECAAH.*

pale heart, an enlarged liver with petechiae, and splenomegaly (He et al., 2002). Histopathologic findings include diffuse necrosis of the renal hematopoietic tissue and spleen, as well as the presence of large, basophilic hypertrophied virus–infected cells (megalocytes) in multiple tissues, including the cranial connective tissues, endocardium, kidney, and spleen (He et al., 2002).

Diagnosis: The most sensitive diagnostic platform for the detection of ISKNV is real-time PCR. Historically, diagnosis of ISKNV was based on clinical history and histopathologic evaluation and confirmed by EM (He et al., 2002). ISKNV can also be detected in zebrafish tissues using immunohistochemistry based on immunolabeling with the monoclonal antibody M10, previously employed against RSIV (Bermudez et al., 2018).

Control and treatment. Zebrafish are susceptible to natural ISKNV infection, exhibiting clinical signs including edema with scale protrusion, lethargy, and multifocal petechial hemorrhage (Bermudez et al., 2018). Experimental infections exhibited viral replication, similar clinical signs, and high mortality (Xu et al., 2008). Exclusion from entire colonies is the most effective measure for pathogen control (Collymore et al., 2016), and ISKNV poses more risk to laboratory zebrafish colonies than many other viruses due to its extremely broad host range (Table 42.3) and prevalence in the ornamental fish trade. Moreover, the capacity of ISKNV to jump among species and between the ornamental fish trade and commercial aquaculture has already been documented (Go et al., 2006; Jeong et al., 2008). Thus, ISKNV poses an increased risk to zebrafish laboratories that import wild-type zebrafish from high-risk sources, defined as any sources providing zebrafish that are not purpose-bred for biomedical research, are coreared with other fish species, or have not been maintained in an adequately biosecure laboratory environment. High-risk vendors may include ornamental wholesalers, biological supply companies, pet shops, laboratories working with wild-caught zebrafish, or multispecies commercial aquaculture facilities. Some zebrafish colonies are at greater risk because many investigators and even some zebrafish core facilities still obtain zebrafish from biological supply companies, multispecies aquaculture facilities, pet shops, and wholesalers who lack the necessary biosecurity practices to prevent exposure of zebrafish destined for biomedical research laboratories (Collymore et al., 2016). In some cases, zebrafish are coreared with other tropical freshwater ornamental species that have similar husbandry requirements and are susceptible to ISKNV. Susceptible tropical freshwater ornamental species include dwarf gouramis (*Trichogaster lalius*), other gouramis (Luciocephalinae), guppies (*P.reticulata*), other mollies (*Poecilia* spp.), platies (*Xiphophorus* spp.), and others (Rimmer et al., 2015). ISKNV epizootics have been documented in freshwater aquaculture and ornamental fish stocks in Asia, Australia, Europe, and North America (He et al., 2002; Jeong et al., 2008; Jung-Schroers et al., 2016; McGrogan, Ostland, Byrne, & Ferguson, 1998; Rimmer et al., 2015). The international movement of ornamental fishes is therefore a major concern regarding the spread of ISKNV, which adversely impacts aquaculture stocks, biomedical research stocks, natural fisheries, and ornamental stocks (Rimmer et al., 2015).

No treatment is available for ISKNV infection in zebrafish. The zebrafish has been suggested as a model for ISKNV vaccine development (Xu et al., 2008). Although killed vaccine preparations are reportedly protective for other fishes (Dong et al., 2013; Fu et al., 2012; Fu et al., 2015; Huang et al., 2012; Li et al., 2015), they have not been applied to zebrafish.

Zebrafish Picornavirus

ZfPV-1 is a highly divergent novel virus in the family Picornaviridae that was recently discovered by viral metagenomic analysis of laboratory zebrafish intestine and intestinal contents (Altan et al., 2019). Based on phylogenetic analysis of the viral genome, ZfPV-1 has been proposed as the type strain for a new species, *Cyprivirus A*, in a new genus, *Cyprivirus* (Altan et al., 2019).

Description: The family Picornaviridae includes more than 45 ICTV-recognized genera. Viruses in the family Picornaviridae are nonenveloped, and virions consist of a single molecule of positive-sense single-stranded RNA enclosed in a small (approximately 30–32 nm in diameter) 60-protomer icosahedral viral capsid.

Host range: Picornaviruses are often specific to a single host species or a few closely related host species; however, there are notable exceptions, including encephalomyocarditis virus (Carocci & Bakkali-Kassimi, 2012) and foot-and-mouth disease virus (Brito, Rodriguez, Hammond, Pinto, & Perez, 2017). The host range of ZfPV-1 beyond zebrafish is currently unknown. As multispecies aquatic facilities become increasingly common, surveys and experimental infection studies may be warranted to define the host range.

Life cycle and modes of transmission: In situ RNA hybridization studies of infected zebrafish demonstrated viral replication that was confined to enterocytes and scattered subjacent cells in the lamina propria (Fig. 42.6 and 42.7) (Altan et al., 2019). Positive signal was also apparent along the apical surface of enterocytes and admixed with mucus in the intestinal lumen (Figs. 42.6 and 42.7) (Altan et al., 2019). These findings strongly support an enteric tropism and fecal–oral mode of transmission.

Clinical signs and pathobiology: ZfPV-1 was frequently detected by real-time RT-PCR in clinically normal as

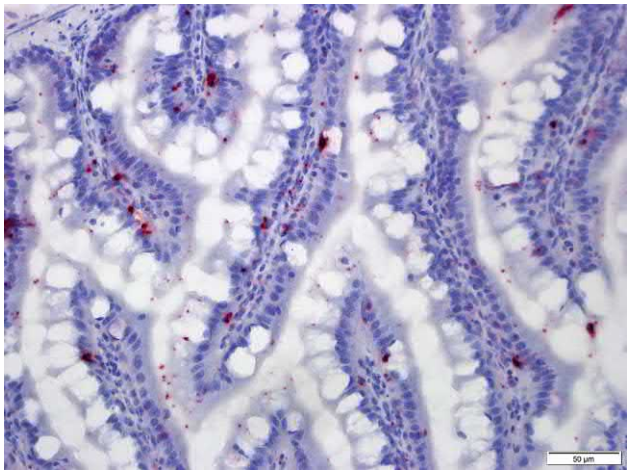

FIGURE 42.6 RNA in situ hybridization of zebrafish picornavirus-1 (ZfPV-1) infected zebrafish intestine showing discrete, punctate red staining of virus in enterocytes, along the apical surface of enterocytes, and scattered cells in the lamina propria. *Image courtesy of Steven Kubiski, DVM, Ph.D., Dipl. ACVP.*

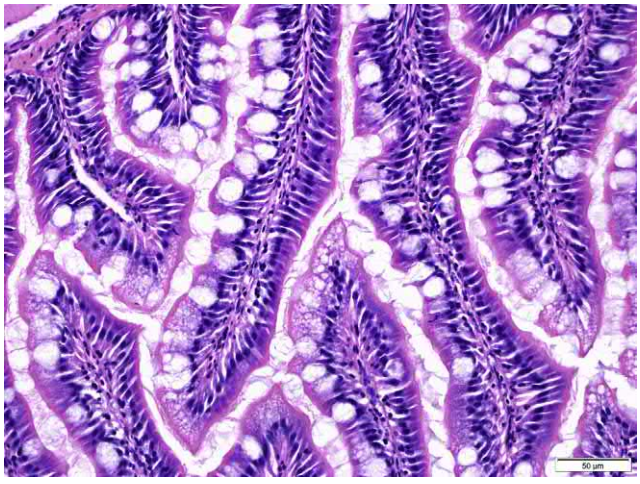

FIGURE 42.7 Section of the same segment of zebrafish picornavirus-1 (ZfPV-1)–infected zebrafish intestine as Fig. 42.6, showing an apparently normal enteric mucosa without inflammatory infiltrates. *Image courtesy of Steven Kubiski, DVM, Ph.D., Dipl. ACVP.*

well as diseased zebrafish. Thus, ZfPV-1 infection is not known to cause clinical disease in zebrafish, and the impact of infection on the zebrafish gut, immune system, microbiome, coinfections, and experimental outcomes have not yet been determined. The discovery of this novel virus that is widespread in zebrafish colonies and to date is not associated with morbidity or histologic changes presents the same challenges that researchers have experienced with subclinical novel viral infections in mice. Although the virus appears to be benign, it is possible it could cause disease in immunocompromized fish, affect research endpoints, or be pathogenic when it occurs as a coinfection. Subclinical viral infections can have a variety of impacts on the host, such as altering tissue-specific gene expression and modulation of the immune system, and these changes have the potential to influence research endpoints. If the impacts of infection on the host are minor, there may be no or limited adverse effects on research, or the impact may vary according to the type of research being conducted. The distribution and prevalence of ZfPV-1 infections in a model organism that is used in such a wide array of research areas necessitates detailed investigation into the epizootiology, shedding and transmission, time-course, and pathophysiology of viral infection in immunocompetent and immunocompromized zebrafish lines.

Diagnosis: Real-time RT-PCR is used to identify ZfPV-1 infection. No histopathologic lesions are associated with ZfPV-1 infection in zebrafish.

Control and treatment: Zebrafish picornavirus is widespread and prevalent in laboratory zebrafish colonies. The virus was detected by real-time RT-PCR in pooled zebrafish samples from 23 of 41 (56%) of research institutions tested, including institutions in Canada, Europe, Southeast Asia, the United Kingdom, and the United States (Altan et al., 2019). As with other infectious agents, exclusion of ZfPV-1 from zebrafish colonies is the most effective control measure available. Additional studies are warranted to evaluate the efficacy of other biosecurity measures, including surface disinfection of zebrafish embryos, ultraviolet irradiation of recirculated water, and chemical disinfection of surfaces in the elimination of ZfPV-1. Picornaviruses are hydrophilic nonenveloped viruses, which, according to the Klein–Deforest scheme, are among the least sensitive viruses to chemical disinfectants (Campagna et al., 2016), although the sensitivity of ZfPV-1 to chemical disinfection has not been evaluated. Zebrafish picornavirus has not yet been shown to cause any clinical disease, and no treatment is available.

Endogenous Viral Elements

EVEs are viral sequences (DNA or cDNA) that have become integrated into the host genome in germ cells, permitting vertical transmission of the viral sequence as genomic DNA and allowing the possibility of the viral sequence to ultimately become fixed in the host population (Feschotte & Gilbert, 2012). Although many other kinds of viruses can also be endogenized, endogenous retroviruses (ERVs) were the first to be described and comprise the great majority of known EVE (Feschotte & Gilbert, 2012). At least 1514 ERV sequences have been identified in the zebrafish genome that together comprise approximately 0.89% of the zebrafish genome (Hayward, Cornwallis, & Jern, 2015).

Zebrafish endogenous retrovirus (*ZFERV*): ZFERV is an Epsilon-line endogenous retrovirus that is phylogenetically related to the salmon swim bladder sarcoma virus

(Naville & Volff, 2016). The provirus, located on chromosome 19, is approximately 11.2 kb and is transcribed predominantly in the thymus in both adult zebrafish and larvae (Naville & Volff, 2016). Importantly, ZFERV is the only intact endogenous retrovirus identified in teleosts, with intact open reading frames for the *gag*, *pol*, and *env* genes and LTR sequences (Shi, Zhang, Gong, & Xiao, 2015). Two additional groupings of Epsilon-like sequences have also been identified, which are phylogenetically distinct from ZFERV-related elements (Naville & Volff, 2016). A total of 36 Epsilon-like sequences have been reported for the zebrafish genome (Hayward et al., 2015).

Endogenous foamy virus (*Spumaretroviridae*) *sequences:* The zebrafish genome, like that of several other fish species, includes endogenous foamy virus sequences, even though exogenous foamy virus infections have not yet been described for any teleost species (Naville & Volff, 2016; Ruboyianes & Worobey, 2016). *Danio rerio* Foamy Virus Type 1 is considered a spumaretrovirus but is phylogenetically intermediate among other LTR retroelement families and Retroviridae (Llorens, Munoz-Pomer, Bernad, Botella, & Moya, 2009). *Danio rerio* Foamy Virus Type 2 displays identical flanking LTR sequences and is believed to have become endogenized in zebrafish more recently (Ruboyianes & Worobey, 2016). *Danio rerio* Foamy Virus Type 3 is recombinant and shares a 5′ flanking sequence with at least six ERV fragments (Ruboyianes & Worobey, 2016).

Snakehead fish retrovirus (*SnRV*)*—like sequences:* SnRV—like sequences are recently described primitive ERV with an evolutionary branch point near to that of the Spuma clade (Hayward et al., 2015) and can be separated into two phylogenetic groups (Naville & Volff, 2016). Nine SnRV-like sequences have been identified in the zebrafish genome (Hayward et al., 2015).

Experimental Susceptibility to Viral Infections

The Zebrafish as a Viral Infection Model

The zebrafish is a very attractive model organism for the study of viral infection and host immunity for many reasons, including the ability to study different components of the immune system at various developmental stages, functional similarity to the immune systems of mammals as well as other teleosts, the availability of zebrafish cell lines, experimental susceptibility to a wide range of viruses, extensive molecular and genetic tools, fluorescence and live imaging techniques, amenability to eurythermal maintenance and temperature-shift experiments, whole-animal histopathology and immunohistochemistry, and low relative cost of experimentation, among others (Crim & Riley, 2012). Importantly, zebrafish display innate immunity very early in development, but the adaptive immune system is not functional until later, allowing study of innate components independently from the adaptive immune system (Kanther & Rawls, 2010). Innate immunity in zebrafish has many similarities to that of mammals, and includes homologues for mammalian toll-like receptors, adaptor proteins for signal transduction, cytokines, and many elements of the complement system (Crim & Riley, 2012). Zebrafish type 1 interferon induces expression of the myxovirus resistance gene (*Mx*), which has antiviral activity against viruses from several families, as in mammals (Haller, Kochs, & Weber, 2007). Juvenile and adult zebrafish exhibit both adaptive and innate immunity, and leukocytes including tissue macrophages, circulating monocytes, B and T lymphocytes, neutrophils, and eosinophils (Grzelak et al., 2017), as well as dendritic antigen-presenting cells (Lugo-Villarino et al., 2010). Similarly, the adaptive immune system also bears resemblance to that of mammals, and B and T lymphocytes in zebrafish function similarly to those of other species. T-lymphocyte receptor genes and B-lymphocyte receptor genes display V(D)J recombination (Haire, Rast, Litman, & Litman, 2000), and zebrafish produce IgD, IgM, IgZ, and IgZ-2 immunoglobulins (Danilova, Bussmann, Jekosch, & Steiner, 2005; Hu, Xiang, & Shao, 2010). Zebrafish neutrophils resemble those of other species with myeloperoxidase-positive cytoplasmic granules (Bennett et al., 2001) and round, band, or segmented nuclei (Grzelak et al., 2017). Eosinophils are larger and display prominent cytoplasmic granules and eccentric nuclei (Grzelak et al., 2017). These similarities between the zebrafish and mammalian immune systems offer great utility for zebrafish models of viral infection and viral immunity that can be applied to both human health and aquaculture. It should be noted, however, that experimental infections are often produced under unnatural conditions, such as by utilizing intracoelomic injection, intravenous injection, mutant or recombinant viruses, etc., and thus altering many aspects of infection. Moreover, infection studies with viruses (that do not naturally infect zebrafish) do not necessarily demonstrate the same types of host—pathogen interactions that would be evident when those viruses naturally infect coevolved host species.

Experimental Infection Studies with Fish Viruses

Experimental infection studies in zebrafish using viruses of commercially important aquaculture finfish demonstrate the experimental susceptibility of zebrafish to viruses of several families, including both DNA and RNA viruses. The susceptibility of laboratory zebrafish to these viruses, together with naturally occurring

Betanodavirus, *Cyprivirus*, and *Megalocytivirus* infections, suggests that other, undescribed naturally occurring viral infections also occur in research colonies. Table 42.1 summarizes the viruses of fish and humans that have been utilized for zebrafish experimental infection studies.

DNA viruses: Zebrafish are experimentally susceptible to at least two viruses in the *Iridoviridae* family, European sheatfish virus (ESV) (Martín et al., 2015) and ISKNV (Li et al., 2010; Xu et al., 2008). ESV is an amphibian-like ranavirus that is phylogenetically related to epizootic hematopoietic necrosis virus of rainbow trout. ESV experimentally infects zebrafish, producing morbidity and mortality and thus permitting use of the zebrafish as a genetically tractable ranavirus infection model (Martín et al., 2015). There is a well-established model for experimental infection of zebrafish with ISKNV (Li et al., 2010; Xiang et al., 2010; Xiong et al., 2010; Xu et al., 2008). In the first ISKNV experimental infection study using zebrafish, intracoelomic injection produced a wide range of clinical signs, including erratic swimming, hovering near the surface, multifocal petechial hemorrhage, and scale protrusion. Initial infection of zebrafish using ISKNV filtrates obtained from infected mandarinfish produced 80% mortality (male) and 65% mortality (female); however, subsequent passages in zebrafish resulted in mortality that ranged widely, from 0% to > 70% (Xu et al., 2008). Experimental infections with ISKNV in other fish species have also resulted in high morbidity and mortality. For example, in one experimental infection study, all (n = 40) mandarinfish (*S. chuatsi*) maintained over 20°C and exposed by intracoelomic injection died in less than 25 days, with average time to death for each treatment group ranging from 9 to 15 days (He et al., 2002). Importantly, however, these experimental infections were achieved via intracoelomic injection, which is not the natural route of infection.

RNA viruses: A greater diversity of RNA viruses of fish has been experimentally evaluated in the zebrafish model, including representatives of three families: Birnaviridae, Nodaviridae, and Rhabdoviridae. Zebrafish have been experimentally infected with IPNV (LaPatra, Barone, Jones, & Zon, 2000; Seeley, Perlmutter, & Seeley, 1977), and *Betanodavirus* spp., including Malabar grouper nervous necrosis virus (Lu et al., 2008), RGNNV (Furusawa et al., 2007), and SGNNV (Morick et al., 2015). Zebrafish are experimentally susceptible to infection with at least four viral species that infect fish from the family Rhabdoviridae: IHNV (Ludwig et al., 2011), snakehead rhabdovirus (Phelan et al., 2005), Spring viremia of carp virus (SVCV) (Lopez-Munoz, Roca, Sepulcre, Meseguer, & Mulero, 2010; Sanders, Batts, & Winton, 2003), and VHSV (Novoa et al., 2006). Experimental infection of zebrafish with IHNV demonstrated the utility of the zebrafish model for temperature-shift experiments (Ludwig et al., 2011). As zebrafish are poikilothermic and can survive a wide range of temperatures, the temperature at which infected embryos or larvae are maintained can be shifted several degrees to stop viral replication at various time points to better characterize the course of infection (Ludwig et al., 2011).

Experimental Infection Studies with Mammalian Viruses

Zebrafish have also been used as a model organism to study several viruses that infect humans and other mammals. Not all human or mammalian viruses are capable of infecting zebrafish, however. Several important factors include the breadth of each viral host range, the expression of zebrafish orthologs of known viral receptors, and the necessary viral incubation temperature (Goody, Sullivan, & Kim, 2014). Some authors have noted that because zebrafish are commonly housed at 28°C, the utility of the zebrafish model for human infections might be limited (Lieschke & Currie, 2007). However, not all human viruses need to be maintained at 37°C for viral replication. Moreover, zebrafish can be slowly acclimated to a wide range of temperatures and have been maintained successfully at 37°C to facilitate the study of mammalian pathogens (Sanders et al., 2015).

DNA viruses: Herpes simplex virus type (HSV)-1 is a member of the family Herpesviridae and is closely related to HSV-2 and varicella zoster virus. HSV-1 was the first human virus used to experimentally infect zebrafish in a study that demonstrated the reduction of viral load in response to the antiviral acyclovir in contrast to increased viral loads and mortality when treated with cyclophosphamide (Burgos, Ripoll-Gomez, Alfaro, Sastre, & Valdivieso, 2008).

RNA viruses: Mammalian RNA viruses that have been studied in zebrafish are chikungunya virus (CHIKV) (Palha et al., 2013), sindbis virus (SINV) (Passoni et al., 2017), influenza A virus (IAV) (Gabor et al., 2014), and vesicular stomatitis virus (VSV) (Guerra-Varela et al., 2018). The zebrafish model is attractive for research using these viruses because other animal models do not permit efficient visualization of host−pathogen interactions. SINV and CHIKV are arboviruses in the genus *Alphavirus* in the Togaviridae family. SINV is spread by *Culex* mosquitos, whereas CHIKV is spread by *Aedes* mosquitoes. IAV is in the Orthomyxoviridae family. Experimental infection using a fluorescent IAV demonstrated a pattern of vascular endothelial infection and visualization of a reduction in fluorescence in infected larvae treated with an antiviral compound, illustrating the utility of the zebrafish model for antiviral screens (Gabor et al., 2014). VSV rarely causes zoonotic

infections and also infects insect vectors, but is primarily known as a virus causing economic losses among livestock. Zebrafish embryos were highly susceptible when infected by microinjection into the yolk, with rapid viral spread to the CNS followed by other tissues just before death (Guerra-Varela et al., 2018).

Comments on Detection, Diagnosis, Risk Assessment, and Decision-making

Husbandry and health monitoring practices for zebrafish are underdeveloped relative to rodent biomedical research models. To date, three naturally occurring viral infections have been reported in zebrafish (Altan et al., 2019; Bermudez et al., 2018; Binesh, 2013), and no zebrafish suppliers currently offer stocks that are guaranteed to be virus-free. It is important for the biomedical research community to identify and characterize naturally occurring zebrafish viruses and move toward the use of zebrafish that are free of adventitious viruses. The identification of viral pathogens in zebrafish will improve the utility of zebrafish as a model organism by improving zebrafish welfare, reducing confounding experimental variability, and providing insight regarding the most effective biosecurity measures in aquatic research facilities.

Detection and Diagnosis

As viral pathogens are identified in zebrafish, monitoring should be put into place for producers, resource centers, established research colonies, and zebrafish quarantine units. As has been suggested for rodent colonies, prospective sampling according to a prearranged schedule permits effective health monitoring, and sampling can be increased in response to suspicion or evidence of viral infection (Suckow, Weisbroth, & Franklin, 2005). Many of the biosecurity practices that are widely practiced in rodent research facilities are only beginning to become recognized as critically important for zebrafish research, such as the use of vendor lists, purpose-bred pathogen-free animals, entry quarantine, pathogen exclusion lists, routine sentinel health monitoring, and environmental monitoring; however, widespread application of these practices will facilitate the exclusion and containment of infectious agents from zebrafish colonies (Crim et al., 2017).

Risk Assessment and Decision-making

Biosecurity considerations: When a previously unknown viral infection is detected in a zebrafish colony as the result of a break in biosecurity, the next steps include an assessment, followed by actions to contain the outbreak, interrupt viral transmission to naïve fish, reduce the viral burden of the system, and reduce the impact of infection on research objectives. There are a number of important factors to consider during the initial and subsequent assessments to evaluate the options for control and/or elimination. These include any threat to human or animal health, the impact of infection on research objectives, the potential for spread to other systems, rooms, or institutions, the efficacy of possible measures, the potential disruption of any corrective measures to research, and the time and expense required. Importantly, many of these considerations require some understanding of viral stability and mode of transmission, in addition to virulence and shedding in different age groups of immunocompetent and immunocompromized zebrafish.

Restrictions on Zebrafish Movement

SVCV, a commercially important viral pathogen of common carp and other fishes, has been studied experimentally using the zebrafish as a model (Encinas et al., 2013; Lopez-Munoz et al., 2010; Sanders et al., 2003; Wang et al., 2017). Because laboratory zebrafish were shown to be experimentally susceptible to infection with SVCV under experimental conditions, zebrafish were controversially added to the list of SVCV-susceptible species in the World Organization for Animal Health (OIE) Aquatic Manual, which was issued to support the implementation of the OIE Aquatic Animal Health Code (Hanwell et al., 2016). Zebrafish have been experimentally infected by both intracoelomic injection and immersion challenges (Encinas et al., 2013; Lopez-Munoz et al., 2010; Ruyra et al., 2014; Sanders et al., 2003). As a result of SVCV experimental infection studies using zebrafish, the Canadian Food Inspection Agency placed restrictions on the importation of zebrafish models into Canada, increasing the difficulty of sharing genetically engineered zebrafish lines internationally, and therefore adversely impacting the Canadian biomedical research community (Hanwell et al., 2016).

Conclusions

Zebrafish are susceptible to viral infections, as demonstrated by a naturally occurring outbreak of VER among zebrafish obtained from the ornamental fish trade for research purposes, an outbreak of clinical disease due to natural ISKNV infection in laboratory zebrafish (Bermudez et al., 2018), the recent discovery of a widely distributed and prevalent novel enteric picornavirus, ZfPV-1 (Altan et al., 2019), and experimental susceptibility to both fish and mammalian viruses. The relative lack of information available with

respect to naturally occurring viral infections in zebrafish is surprising, considering the widespread use of zebrafish as both a model organism and an ornamental species. Many facilities now only allow the introduction of surface-disinfected embryos into main systems to exclude new or unknown pathogens (Kent et al., 2009). However, zebrafish were shared among investigators at various institutions for many years before this practice was common, and viruses that are vertically transmitted might not be eliminated by surface disinfection. The conditions of zebrafish culture in rack systems with tanks plumbed in parallel also reduce pathogen transmission among fish (Crim et al., 2017). The lack of information available about naturally occurring viral infections primarily reflects limited diagnostic data and scientific investigation in this area (Crim & Riley, 2012). Historically, only a small number of research institutions implemented health monitoring programs for zebrafish colonies, with minimal diagnostic investigation of colony morbidity and mortality at most institutions. Moreover, the existing programs primarily relied on histopathology, which lacks sensitivity for viral infections, as viral inclusion bodies or pathognomonic lesions are not evident for many viral infections. Importantly, as the increasing use of metagenomic approaches will likely result in the discovery of novel viruses in zebrafish, the experimental investigation of viral epizootiology, pathogenesis, and transmission in immunocompetent and immunocompromized zebrafish lines will be extremely important to inform biosecurity practices in zebrafish facilities. Husbandry and health monitoring practices for zebrafish are currently immature relative to those for rodent models, although that is beginning to change. As the zebrafish continues to grow in importance as a model organism, greater investigation into naturally occurring viral diseases and reliance on evidence-based biosecurity measures will be necessary and critical for protecting zebrafish health, welfare, and data integrity.

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CHAPTER

43

Nonexperimentally Induced Neoplastic and Proliferative Lesions in Laboratory Zebrafish

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Introduction

When considering neoplasia in any species, it is best to begin by examining the cell populations most likely to become neoplastic. In mammals, we are broadly familiar with common neoplasms arising from rapidly dividing cell populations: Lymphosarcoma from replicating lymphocytes, squamous cell carcinomas from high-turnover epithelial cells, and intestinal adenocarcinomas from the labile intestinal epithelium. By applying these basic principles correlating neoplasia risk with rapidly replicating cell populations, it might be assumed that we can make reasonable predictions concerning the types of neoplasms we might observe in any species.

However, because of the wide spectrum of factors including environmental influences, genetic predispositions, and the role of chance in the development of neoplasms, it is nearly impossible to predict the types and frequencies of neoplasms that may develop between different species. For example, mast cell tumors, which are common in dogs, are exceedingly rare in humans. While all species can develop teratomas and nephroblastomas, frequencies vary between species. Furthermore, embryonic neoplasias and choristomas (the growth of normal tissue in abnormal places) of certain types can be common in some species but unheard-of in others.

In human medicine, we tend to think of the development of cancers secondary to exposure to certain environmental factors, such as toxins that can act as initiators and promoters. However, viruses like hepatitis and human papillomaviruses are responsible for many cases of neoplasia, particularly in the developing world. In the realm of veterinary medicine, viral infections are known to cause a wide range of neoplasms. Bovine leukemia virus, avian lymphoid leucosis, equine sarcoid, and plasmacytoid leukemia are just a few examples of neoplasms associated with viral infections (Truyen & Lochelt, 2006). Nonviral infectious entities, such as gastric carcinomas in humans caused by *Helicobacter pylori* (Correa & Blanca Piazuelo, 2011), should also be considered.

In the following chapter, we will discuss both common and uncommon zebrafish tumors observed in zebrafish research facilities, focusing on pathogenesis and comparative pathology. Since zebrafish are a relatively recent introduction to the lab animal stable, we are continually discovering new trends and disease entities. Therefore, this chapter is written with the support of peer-reviewed data where possible, but we also introduce newer, previously unpublished entities that the authors have observed during their tenures as researchers and diagnosticians.

Diagnosis of neoplasia in zebrafish relies primarily on microscopic or histological techniques because grossly visible lesions and readily identifiable clinical signs are generally nonspecific. The most common clinical signs of neoplasia in zebrafish are emaciation with or without scoliosis, both of which are nonspecific. Similarly, a distended abdomen could indicate a coelomic tumor; however, this is the primary differential only in males. In females, it is more likely that this represents egg-binding (egg-associated inflammation with fibroplasia) or simple gravidity. In continuing with the trend, exophthalmia can be due to etiologies that include neoplasia, granulomatous inflammation, or gas bubble disease (See chapter on Water Quality and Idiopathic Diseases of Laboratory Zebrafish Murray et al.). Even mass-like lesions have multiple possible etiologies. White or pink dermal masses can be neoplasms but

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are just as likely to be granulomas due to mycobacterial infection, parasitic infections, or transdermal migration of egg-associated inflammatory debris. The only relatively reliable gross signs of neoplasia in zebrafish are dark-colored external masses, which tend to be diagnosed as melanomas, and ventral mandibular lesions appearing in multiple fish with thyroid lesions (either hyperplasia or neoplasia). Although these clinical presentations are more likely to be neoplastic than inflammatory, histology is still needed for confirmation.

Unlike mammalian neoplasms, naturally occurring malignancies in zebrafish rarely metastasize. Intestinal adenocarcinomas can spread through the coelom by carcinomatosis, and thyroid neoplasia may occur as a solitary mass with additional coelomic foci of proliferative follicles, but it is unclear whether this represents metastasis or activation of ectopic tissue (Spitsbergen, Buhler, & Peterson, 2012; Fournie, Wolfe, Wolf, Courtney, Johnson, & Hawkins, 2005). Hematopoietic tumors, such as lymphosarcoma, can spread to multiple organs, but are commonly considered multicentric, rather than metastatic. In general, spontaneous tumors in zebrafish can result in severe organ compression and displacement and aggressive local invasion but for most tumors, vascular metastasis has not been confirmed.

Because of the immense utility of zebrafish for studies of genetic manipulation, toxicology, and xenotransplantation, the number of potential neoplasms in these animals is effectively infinite (Langenau, 2016). Therefore, this chapter will only focus on neoplasms that we consider to be "naturally occurring" in cultivated zebrafish strains used for experimental work, that is, neoplasms that have arisen in multiple fish from multiple laboratories over time in the absence of any consistent experimental manipulation. Where information is available about potential infectious or environmental etiologies, we include a section on methods for control and treatment. However, as most tumors identified outside of carcinogenesis studies arise spontaneously, no methods of prevention or treatment in laboratory zebrafish are currently known. Although a complete experimental history is often not provided, it is important to consider the previous genetic manipulations that may give rise to unexpected lesions and presentations.

The images in this chapter, as well as most of the information presented, have been gleaned from the authors' careers along with the ZIRC zebrafish diagnostic service's archives, representing 18 years' worth of cases from around the world. We would like to recognize, in this chapter, the contributions of Jan Spitsbergen to the field with her seminal paper on zebrafish neoplasia (Spitsbergen et al., 2012). Please refer to Table 43.1 for details regarding the types and relative frequencies of tumors identified by the ZIRC diagnostic service.

TABLE 43.1 Tumors, hyperplastic, and dysplastic lesions diagnosed at the ZIRC zebrafish diagnostic service between 2006 and 2016. Total number of fish examined by histology: 16,169 (Includes fish from ZIRC quarantine and husbandry rooms, internal sick fish, and nonclinical sentinels).

Intestinal epithelial hyperplasia, dysplasia, dysplasia, or neoplasia	322
Seminoma	182
Biliary or pancreatic duct hyperplasia	169
Ultimobranchial gland hyperplasia, adenoma, or adenocarcinoma	82
Thyroid hyperplasia, adenoma, or adenocarcinoma	52
Melanoma	5
Chordoma	20
Hepatocellular carcinoma	17
Peripheral nerve sheath tumor	52
Spindle cell tumor (nonspecific)	11
Lymphoma/lymphosarcoma	14
Nephroblastoma/ependymoma	10
Hemangioma	7

Seminomas

Description

The most common neoplasm reported in zebrafish is the seminoma (Spitsbergen et al., 2012). In mammals, seminomas are derived from primitive male germ cells and appear as disorganized sheets of spherical spermatogonia-like cells with marked variation in cell and nucleus size. Normal cellular architecture is generally obliterated, and the absence of mature spermatozoa in the masses likely indicates that these tumors are nonfunctional—that is, no fertile sperm is produced. Mitotic rates are generally high, and these neoplasms have the potential for local infiltration, as well as distant metastasis (Hayes & Sass, 2012). In contrast, in zebrafish, most of the putative seminomas that we have observed exhibit minimal deviation from normal testis tissue at a histological level, presenting a challenge for their diagnosis as a true neoplasm (Figs. 43.1–43.4).

Pathobiology and Clinical Signs

Seminomas, when evident macroscopically, usually present as swollen abdomens in males. When opened, the coelom is filled by a pink, fleshy mass. Larger masses with more mature sperm appear less pink. Many seminomas in zebrafish, however, are incidental and are

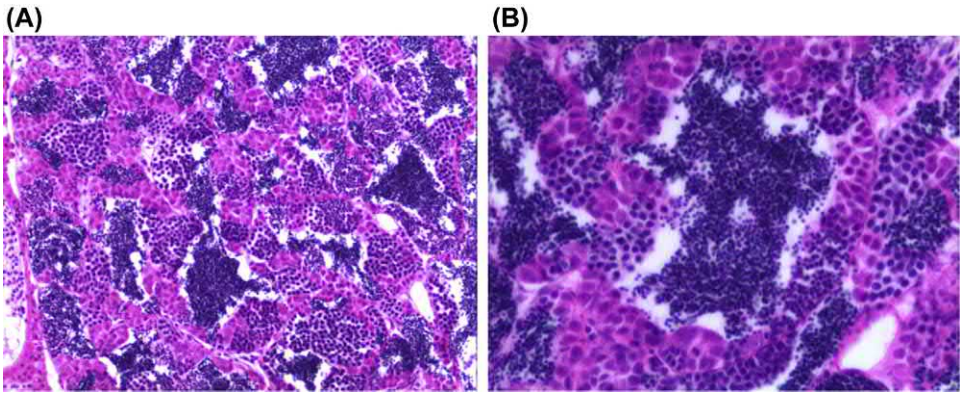

FIGURE 43.1 Normal testis H&E. (A) 200× magnification. (B) 400× magnification.

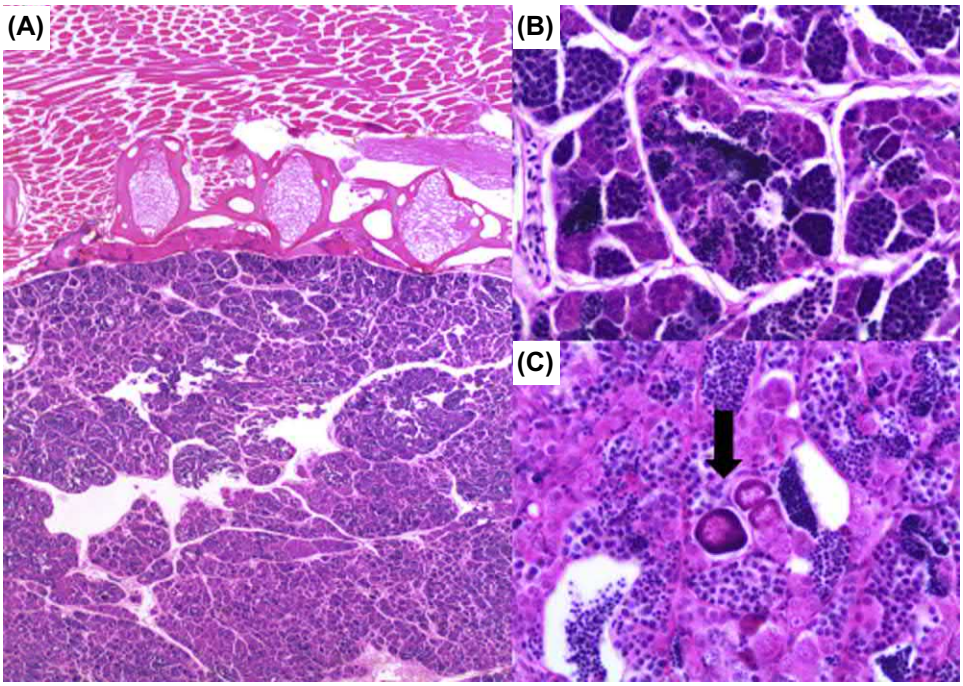

FIGURE 43.2 Common well-differentiated seminoma. (A and B) While the structure of the mass appears similar to that of the normal testis (Fig. 43.1), it can often take up the majority of the coelomic cavity. H&E. 20×. (B) H&E. 200×. (C) Seminomas not uncommonly contain female germ cells at various primordial stages of development (*Arrow*). Vitellogenic oocytes are rare to nonexistent in these tumors. H&E 400×.

discovered on microscopic evaluation of clinically normal fish. It is unclear whether these tumors represent functional testicular tissue, and their effect on fecundity has not been measured. However, the masses are composed of all normal cell types, including mature sperm. At the Zebrafish International Resource Center (ZIRC) we occasionally see presumptive seminomas during testis dissection for sperm cryopreservation, and these testes yield greater than normal amounts of sperm. However, the sperm from these presumptive seminomas has not been individually assayed for activity and viability in in vitro fertilization as ZIRC pools sperm samples from several fishes to cryopreserving a line.

A distinct etiology for spontaneously occurring 2tumors of this type has not been identified; however, hormonal influences are likely contributors. The ZIRC diagnostic service typically diagnoses seminomas in 1%−2% of the submitted fish per year. To date, there is no documented zebrafish line predisposition. Although the masses can be quite large, filling the majority of the coelomic cavity, they are not typically associated with loss of body condition or signs of compromise to other organs.

Diagnosis

Diagnosis is made based on postmortem histology. As many of the extremely enlarged testes appear essentially normal at a microscopic/tissue level, it raises the question of whether or not these are true neoplasms. A

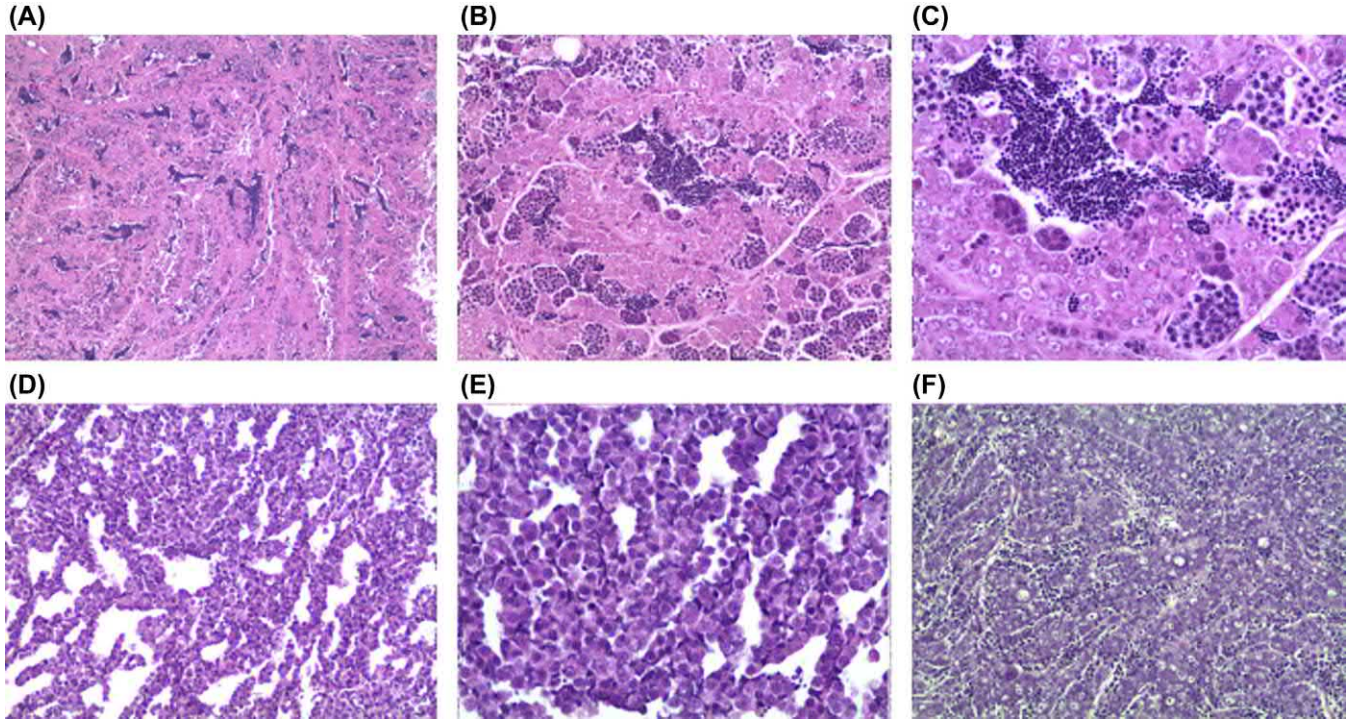

FIGURE 43.3 (A–D) Some well-differentiated seminoma variants have complete sperm maturation, but earlier stages of spermatogenesis make up the majority of the tumor. (A) H&E 40×. (B) H&E 200×. (C) H&E 400×. (D and E) Other variants lack maturation beyond early germ cell stages, and no spermatozoa are present. (D) H&E 200×. (E) H&E 400×. (F) Ovarian dysgerminoma. H&E 200×.

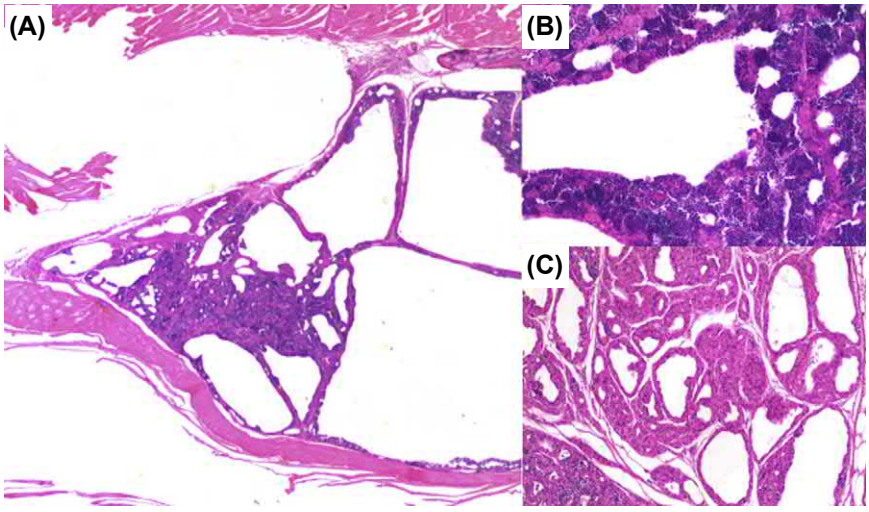

FIGURE 43.4 Cystic seminomas. Even within a single seminoma, different areas can have variable degrees of spermatogenic maturation. (A) H&E 20×. (B) H&E 40x. (C) H&E 40×.

neoplasm is defined as a pathological overgrowth of abnormal cells, while hyperplasia is defined as either a physiologic or pathologic overgrowth of normal cells. As stated previously, there is a fine line between benign neoplasia and hyperplasia, particularly because hyperplasia generally predisposes a cell line to the development of neoplasia. While we currently call this particular entity a seminoma, the presence of normal, albeit enlarged, testicular tissue could indicate that, rather than a neoplastic process producing a benign tumor of testicular tissue, it is, in fact, diffuse hyperplasia of the testis in these animals.

Conversely, evidence that these are neoplasms (i.e., well-differentiated benign neoplasia) is supported by the fact that they grow into large, ovoid, multinodular, expansive masses in which the overall architecture of

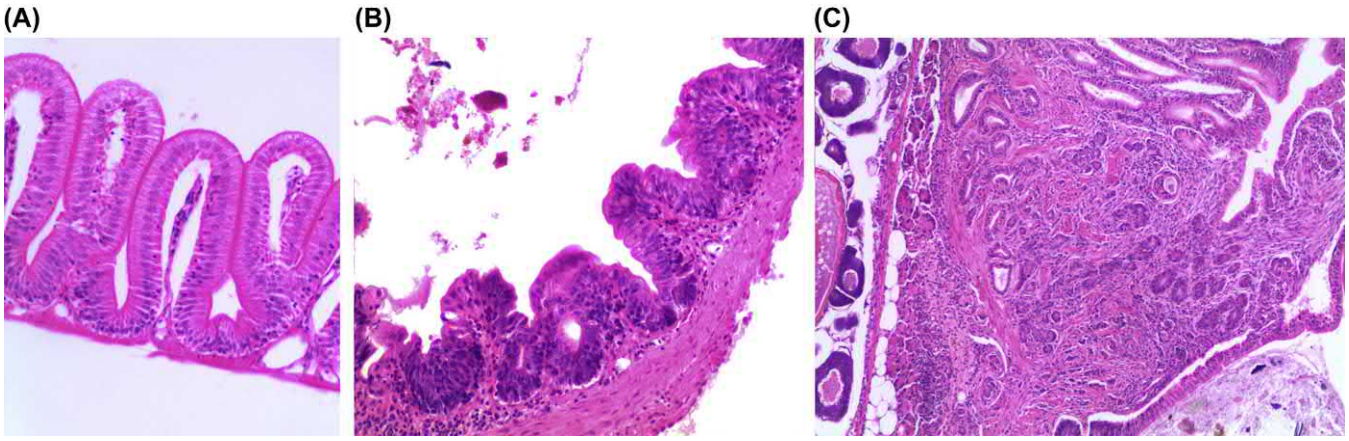

FIGURE 43.5 Intestine. (A) Normal intestine is lined by a single layer of columnar epithelial cells. Unlike the mammalian intestine, the zebrafish intestine has an extremely limited submucosal layer and very few resident leukocytes H&E 200×. (B) Enteritis with intestinal hyperplasia. As opposed to the smooth profile of intestinal folds in (A), the mucosal surface is thickened and irregular, and there is "stacking" of nuclei. The submucosa is expanded by moderate numbers of leukocytes. H&E 200×. (C) This intestinal adenocarcinoma presents as a severely infiltrative mass composed of duct- and tubule-like structures lined by epithelial cells embedded in a scirrhous and inflamed stroma. H&E 100x.

the testis is markedly distorted (although there is spermatogenic progression and some tubule-like structures occasionally remain). In Japanese medaka, similar well-differentiated testicular lesions do metastasize (J. Wolf, EPL, pers. comm.), an indicator of malignancy. Finally, hyperplastic lesions theoretically regress following the removal of the stimulus for proliferation. To date, regression has not been documented, but this would admittedly be hindered by the fact that many of these masses are diagnosed by postmortem histology on clinically normal zebrafish.

Less common than the "hyperplastic-type" seminomas are a group of seminomas that are composed primarily of earlier sperm developmental stages. These can either have a solid or a cystic appearance, and portions of these tumors may undergo differentiation into normal-appearing testicular tissue with the patchy appearance of mature spermatozoa. It is also not uncommon to find various stages of oocyte development in seminomas, which also contributes to the argument against a hyperplastic process.

Dysgerminomas, primitive tumors of oocytes, have been observed in female zebrafish, although they are far less common than seminomas in males. These appear similar to mammalian dysgerminomas: They are infiltrative, aggressive tumors composed of pleomorphic spherical cells in contrast to more well-organized zebrafish seminomas.

Control and Treatment

It is unknown whether or not well-differentiated testicular masses are responding to some type of hormonal stimulation. If multiple seminomas are diagnosed in a tank or location, exposure to an external stimulus should be considered. Spitsbergen et al. noted that seminomas are one of the most frequently diagnosed tumors associated with carcinogen exposure (Spitsbergen et al., 2012).

Intestinal Carcinomas

Description

No neoplastic entity in these animals demonstrates the progression from inflammation to hyperplasia to neoplasia better than the intestinal carcinoma. While some intestinal carcinomas may arise spontaneously, a distinct linkage between chronic inflammation and the development of intestinal carcinomas has been observed in zebrafish. Furthermore, it has been shown that these tumors, or rather, their as-yet-unidentified tumor-causing agent is transmissible, and animals in the transmission study showed distinct stages of chronic enteritis, epithelial hyperplasia, dysplasia, and finally, neoplasia (Burns et al., 2018; Paquette et al., 2015).

Burns et al. (2018) showed that these tumors are caused by a transmissible agent. They also found a single 16S rRNA 30 sequence, which was most similar to *Mycoplasma penetrans*, to be highly enriched in the donors and recipients compared to disease-free controls using general microbiome evaluations. Furthermore, the original donor fish and exposed fish populations were positive for the *Mycoplasma* sp. using a targeted PCR test, while corresponding unexposed control fish were negative. Whereas this *Mycoplasma* sp. was directly associated to the neoplasm, a direct cause and effect link has yet to be verified. Paquette et al. (2013) showed that the occurrence of these intestinal tumors was not

directly related to diet or zebrafish strain, and they usually occur in fish older than 1 year. The intestinal nematode *Pseudocapillaria tomentosa* is another transmissible cause of intestinal cancer resulting from chronic inflammatory processes rather than direct change to the genome as with viruses (Kent, Bishop-Stewart, Matthews, & Spitsbergen, 2002; Spitsbergen et al., 2012). There does not appear to be any recognizable difference morphologically between *Mycoplasma*-associated, *Pseudocapillaria*-associated, and spontaneously arising intestinal neoplasms (Figs. 43.5–43.7).

Intestinal carcinomas in zebrafish are associated with general malaise, including lethargy and emaciation (Burns et al., 2018), but the large retrospective survey conducted by Paquette et al. (2013) showed that the tumors also frequently occur in subclinical fish. There are no obvious macroscopic changes in affected fish, even during necropsy when the intestines are closely examined.

Diagnosis

Diagnosis of intestinal carcinomas in zebrafish can be difficult as the most common type of intestinal carcinoma is considered the "small cell" carcinoma (Spitsbergen et al., 2012). These neoplasms tend to manifest subgrossly as thickenings in the wall of the gut with loss of intestinal folds. Upon close examination, one can observe small nests of one to three cells invading transmurally, often infiltrating the coelomic cavity and spreading along serosal surfaces (carcinomatosis). Paquette et al. (2015) provided evidence that the tumors are of epithelial origin based on immunohistochemistry using epithelial and neural mammalian antibodies. Frequently, these neoplasms are associated with intestinal perforation. The difficulty of identifying small cell intestinal carcinomas is threefold: neoplastic cells are usually severely atypical and bear little resemblance to normal intestinal epithelium, severe inflammation and chronic fibrosis can obscure neoplastic cells, and the distortion and thickening of the gut wall secondary to the desmoplastic

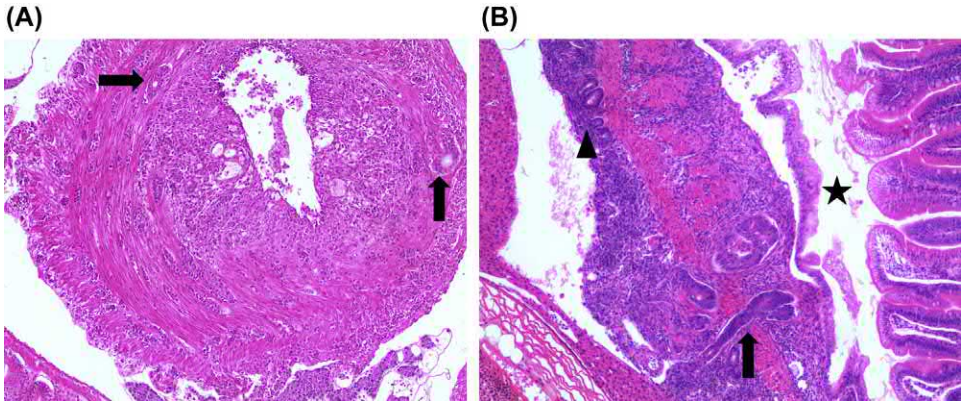

FIGURE 43.6 Transmural invasion of intestinal adenocarcinoma. (A) Intestinal folds have been replaced by an infiltrative neoplasm. Tubular structures lined by epithelium are observed within the tunica muscularis (*arrows*). H&E 100×. (B) Intestinal folds are blunted and effaced by the infiltrative neoplasm lined by a layer of hyperplastic epithelium (star). Tubular structures extend through the tunica muscularis (*arrow*) and into the serosal surface (arrowhead). The separation of the overlying epithelium from the lamina propria in this image is likely artifactual. H&E 200×.

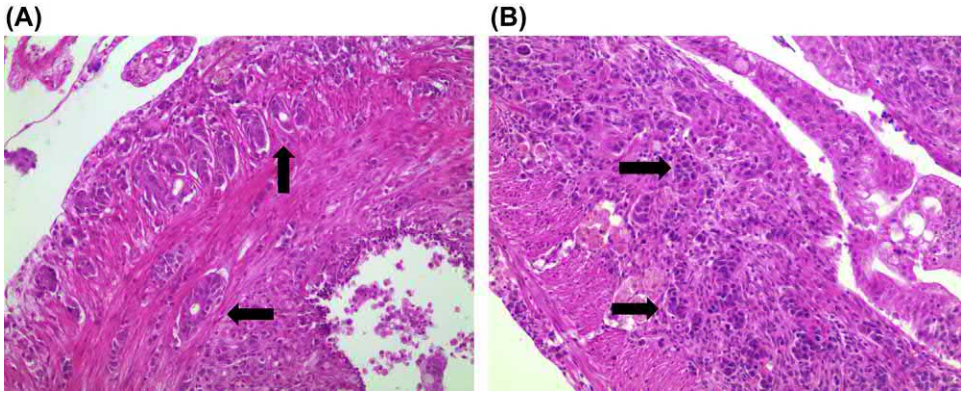

FIGURE 43.7 Duct-like versus small cell adenocarcinomas. (A) Duct-like adenocarcinomas continue to form tubular/duct-like structures with distinct lumens (*arrow*). H&E 400x. (B) Small cell adenocarcinomas no longer form duct-like structures but form infiltrative clusters of polygonal cells (*arrow*) that could be mistaken for inflammatory cells. H&E 400×.

response can present a tangential face to the histologic section. That is, rather than receiving a full transverse section of gut, which is either circular or ovoid with a lumen present, the microtome "grazes" the edge of the thickened gut which shows portions of muscularis and lamina propria with no readily apparent lumen. In this way, it becomes difficult to identify the characteristic features of the tumor. It is therefore important to consider deeper recuts of samples that contain suspect carcinomas. There exist more adenomatous-type intestinal carcinomas, which appear similar to the intestinal epithelium and recapitulate intestinal fold structure. They are less common, but just as invasive as small cell carcinomas (Burns et al., 2018). Differentiation of hyperplastic lesions from neoplastic lesions is based on invasion of neoplastic cells into the tunica muscularis, as preneoplastic atypical hyperplastic cells generally remain within the lamina propria.

Control and Treatment

Intestinal neoplasms can arise spontaneously, in which case no control measures exist. If enteritis is observed in multiple fish with occasional neoplasms, *P. tomentosa* should be ruled-out. Treatments with ivermectin, emamectin, and fenbendazole have been described for *P. tomentosa* infections (Collymore et al., 2014; Maley, Laird, Rinkwitz, & Becker, 2013). If multiple fish in a tank are diagnosed with preneoplastic and neoplastic lesions of the gut, transmissible tumors should be considered when *P. tomentosa* has been eliminated as a differential. This is an active area of research, and currently, no treatment is recommended. Although a particular16S rRNA sequence similar to *M. penetrans* has been associated with the tumors (Burns et al., 2018), it is unknown whether the identified *Mycoplasma* sp. is always pathogenic or if there are particular strains of *Mycoplasma*, some of which are pathogenic and some of which may be considered normal flora (Fig. 43.8).

Ultimobranchial Gland

Description

The ultimobranchial gland in cyprinids is located just ventral to the musculature of the pharynx. It produces calcitonin, and it functions in osmotic regulation and calcium homeostasis.

Pathobiology and Linical Signs

Ultimobranchial hyperplasia, adenomas, and adenocarcinomas occur in laboratory zebrafish. Presently we have not identified a specific line or age of zebrafish that is predisposed to the lesions. Zebrafish in the wild in India live in water with a wide range of conductivity levels, 10−271 μS (Engeszer, Patterson, & Parichy, 2007) compared to those routinely used in zebrafish facilities (Chapter 29). The main action of calcitonin, which is produced by the ultimobranchial organ, is to increase bone calcium content and decrease the blood calcium level when it rises above normal concentrations. Hence, while no distinct etiology has been proven, electrolyte and osmotic imbalances are possible stimulants for hyperplasia based on the organ's function. Chronic hyperplasia is a risk factor for neoplasia. Hyperplastic and neoplastic changes to the ultimobranchial gland are not typically visible during postmortem examination and are not generally associated with clinical disease.

Diagnosis

Ultimobranchial gland lesions are typically diagnosed by evaluation of histological sections. In paramedian sections of bisected animals, the gland is frequently small enough that it is out of the plane of section and therefore cannot be evaluated. It can, however, become enlarged up to three times normal size (up to roughly 50 μm), revealing a number of ducts or acini with central lumens lined by columnar epithelium. These lesions are another important example of the fine line between hyperplasia and neoplasia: Often, an ultimobranchial gland adenoma has the appearance of a diffusely enlarged, well-differentiated gland. In other words, much like the common hyperplastic-type seminoma, there is rarely a distinct area, where one can differentiate normal from tumor tissue. What may be considered a neoplastic process may actually be a hyperplastic process in response to some external environmental stimulus. As with seminomas, it is not known if the lesions regress with the removal of environmental stimuli. Lesions that are obviously anaplastic with a high mitotic index are appropriately diagnosed as adenomas. A diagnosis of adenocarcinoma can be made if neoplastic cells invade surrounding tissues.

Control and Treatment

If ultimobranchial gland pathology is routinely diagnosed in a fish population, it may be appropriate to consider environmental stimuli that could initiate these changes. While it has not been definitively proven, it is possible that one cause of nephrocalcinosis could be aberrant calcium metabolism. It may be prudent to monitor for coincidences of ultimobranchial gland pathology and nephrocalcinosis.

Ultimobranchial hypertrophy has been associated with migration and spawning in the Atlantic salmon (Deville & Lopez, 1970). We have not noticed a predilection for ultimobranchial changes associated with any particular line, sex, or the age of laboratory zebrafish. However, in the laboratory, conditions are kept relatively constant, and fish are spawned throughout the year.

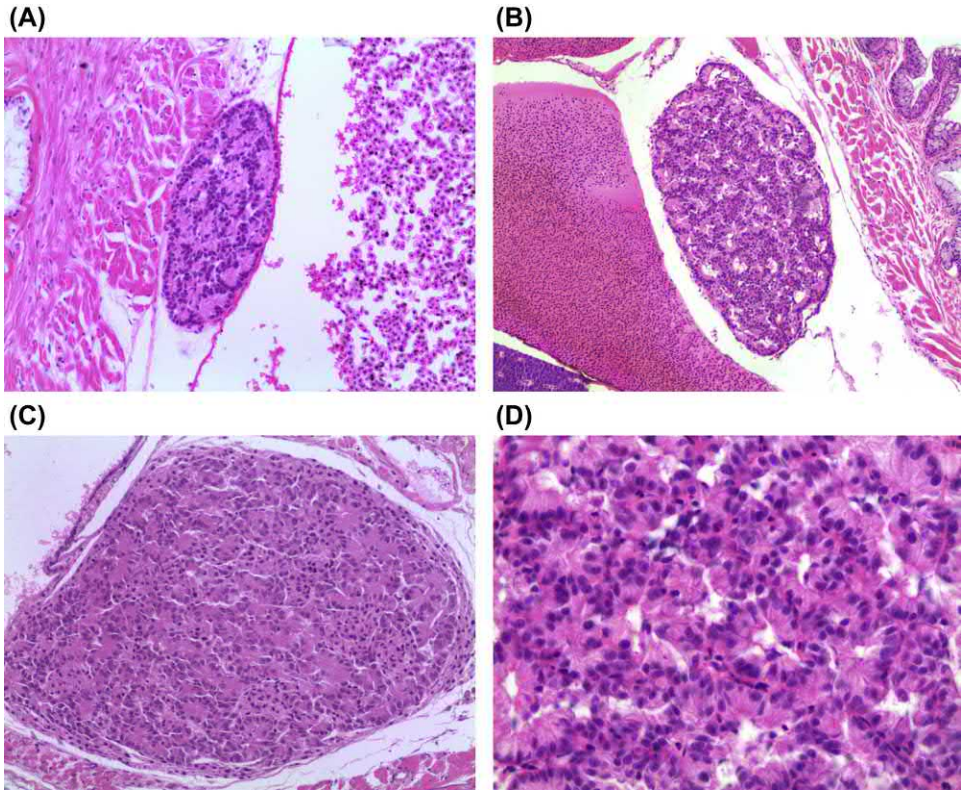

FIGURE 43.8 Ultimobranchial gland lesions. (A) Normal gland. H&E 200×. (B) Hyperplastic ultimobranchial gland. Note the increased size compared to the gland in (A) along with the irregular margins. H&E 100×. (C) Ultimobranchial gland adenoma. Note the extremely large size and irregular appearance of cells. H&E 200×. (D) Ultimobranchial gland adenoma. Simple to pseudostratified columnar epithelial cells surround small tubular lumens. The columnar cells have basilar nuclei and slightly flocculent pale eosinophilic cytoplasm. H&E 400×.

Whether ultimobranchial gland anatomy and physiology fluctuates in zebrafish in the wild has not been reported (Fig. 43.9).

Lymphosarcoma and Other Hematopoietic Tumors

Description

Lymphoid tumors have been reported in numerous wild and captive fishes. As with mammals and birds, at least some of these neoplasms are caused by oncogenic viruses (Coffee, Casey, & Bowser, 2013). Zebrafish have proven to be useful models of hematopoietic neoplasias (Langenau et al., 2003; Rasighaemi, Basheer, Liongue, & Ward, 2015; Zhuravleva et al., 2008). Infectious agents have yet to be associated with naturally occurring lymphoid neoplasms in zebrafish.

Pathobiology and Clinical Signs

Lymphosarcoma in zebrafish is a highly aggressive neoplasm derived from lymphocytes. T lymphocytes originate in the thymus, while B lymphocytes, along with other erythroid and myeloid cells, are produced in the kidney. Fish with lymphosarcoma most commonly present with emaciation and nonspecific signs of illness. When lesions are grossly visible, they can present as discrete masses, or diffuse epidermal invasion can result in skin pallor. A common gross manifestation of dermal lymphosarcoma is infiltration of the tissues surrounding the head, resulting in a "lionhead" appearance. Epidermal infiltration can also have an appearance similar to scale pocket edema, which makes the fish present as if it had "dropsy." Exophthalmia may result a from retro-orbital accumulation of malignant cells, although this is a nonspecific finding as any retro-orbital, space-occupying lesion, including granulomas, can result in exophthalmia.

Diagnosis

This neoplasm is composed of leukocytes that do not recapitulate any particular tissue structure. Therefore, the most common appearance is that of an infiltrative process wherein sheets of cells with the appearance of lymphocytes invade, expand, and destroy normal tissues. Diagnosis is usually confirmed with postmortem histology. Impression smears of externally presenting masses may reveal sheets of monomorphic round cells with a high nuclear to cytoplasmic ratio. These cells generally have medium-sized nuclei and very little cytoplasm, making H&E stained tissues appear dark blue when viewed

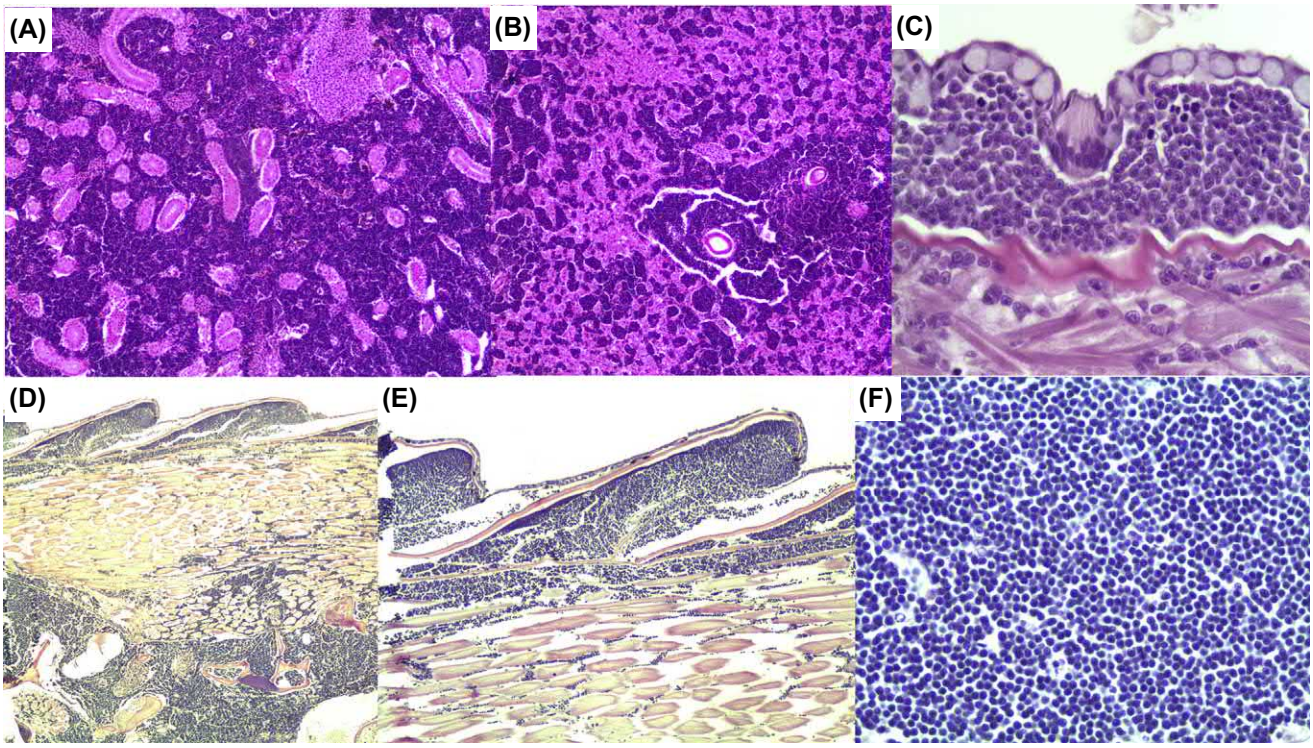

FIGURE 43.9 Lymphosarcoma. (A) Lymphosarcoma in the renal interstitium. The only leukocytes present are neoplastic lymphocytes which separate renal tubules. H&E 100x. (B) Lymphosarcoma in the liver. Sheets of neoplastic lymphocytes fill sinusoids and compress adjacent hepatic cords. H&E 100x. (C) Oral cavity. Sheets of neoplastic lymphocytes infiltrate just under the mucosal epithelium. H&E 400×. (D) Whole body and skin. Neoplastic cells fill the coelom (bottom of the frame) and infiltrate the musculature just under the scales (top). H&E 40x. (E) Epidermis and dermis. Neoplastic lymphocytes invade beneath the scales and cause them to lift. H&E 100x. (F) Neoplastic lymphocytes arranged in sheets. Giemsa 400×.

subgrossly. Many of these neoplasms likely arise from the renal interstitium, where most of the zebrafish hematopoiesis occurs. Microscopically, the renal interstitium is very commonly affected. In these cases, most to all other hematopoietic precursors have been replaced by neoplastic lymphocytes that expand the interstitial space and separate renal tubules. It is possible that lymphosarcoma that infiltrates the gills and the head arises from the thymus, but many fish with these lesions also have kidney lesions, and so a specific origin is unclear.

While neoplastic lymphocytes can invade any and all organs of the zebrafish's body, many of them appear to have a particular tropism for the epithelium, infiltrating just beneath the basement membrane of the epidermis, oral mucosal epithelium, and even the gills. Unlike the mammalian counterpart of epitheliotrophic lymphoma, however, neoplastic cells do not generally infiltrate between epithelial cells, and so-called *Pautrier's abscesses* have not been observed.

Other hematopoietic tumors have been observed in zebrafish during the authors' tenures as diagnosticians. These neoplasms frequently appear as sheets of hematopoietic round cells, morphologically unlike lymphocytes, that have a similar appearance to hematopoietic precursor cells, usually of the myeloid lineage. They are generally localized to the kidney and spleen, although neoplastic cells can be observed in vascular profiles, as in leukemias. Unfortunately, in the absence of a the distinct identity of a known genetic knockout and/or appropriate immunohistochemical stains, it is difficult to identify the particular cell type responsible for these neoplasms. In general, it helps to think of the renal interstitium as bone marrow. In a bone marrow biopsy, if adipocytes and the various stages of hematopoiesis are replaced by a single monomorphic population of round cells, a hematopoietic tumor is likely to be present. The same is true for the renal interstitium with the added benefit that one can also identify expansion by separation of renal tubules. Caution is warranted, in these cases, however, since interstitial expansion or hyperplasia can occur physiologically as a response to inflammation, causing tubules to be separated by a mixed population of normal hematopoietic cells. Differentiation between a hyperplastic and neoplastic hematopoietic population is made based on the presence of all hematopoietic stages (hyperplasia) or the presence of a monomorphic population of cells in a single stage (neoplasia).

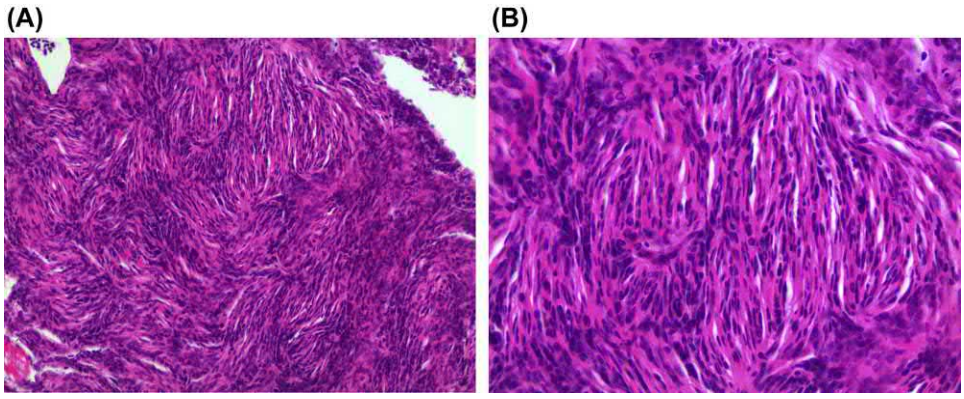

FIGURE 43.10 Peripheral nerve sheath tumor. (A) These tumors are composed of short, intersecting streams and bundles of spindle-shaped cells. H&E 200×. (B) Spindle cells are stacked in a storiform pattern similar to those observed in mammals. H&E 400×.

Control and Treatment

To date, infectious agents have not been associated with lymphoid neoplasms in laboratory zebrafish. Although, it should be considered as a possible etiology if multiple fish are affected since tumors have been associated with retroviruses in other fishes (Coffee et al., 2013). The genetic background should also be considered, as lines have been engineered to recapitulate hematopoietic neoplasias (Rasighaemi et al., 2015) (Fig. 43.10).

Soft Tissue Srcoma/Peripheral Nerve Sheath Tumors

Soft tissue sarcomas are one of the more common neoplasms diagnosed in laboratory zebrafish. These tumors can occur in any part of the body, and clinical presentation varies with affected tissues.

Pathobiology and Clinical Signs

These tumors can present as single or multiple swellings externally. Tumors that arise in the retrobulbar region cause massive, unilateral exophthalmia. Clinical signs can be absent or nonspecific and include emaciation and general malaise. If tumors are internally invasive, they can appear as amorphous white masses that surround and adhere to organs upon dissection.

Diagnosis

In zebrafish, the most common soft tissue sarcoma is the peripheral nerve sheath tumor, which generally appears as a solid, highly infiltrative, densely cellular mass composed of short streams and bundles of spindle-shaped cells. It is very similar in appearance to mammalian peripheral nerve sheath tumors, and most tumors bear a distinct storiform pattern. These neoplasms can be highly aggressive, invading, and effacing large amounts of normal tissue both in the exterior and the interior of the animal (Spitsbergen et al., 2012). While zebrafish can develop other types of soft tissue sarcomas, those morphologically similar to mammalian peripheral nerve sheath tumors are, by far, the most common and best described. When other soft tissue sarcomas are identified that do not fit this morphological pattern, comparison to similar tumors in other cyprinids is warranted. Again, due to the lack of validated immunohistochemical stains in zebrafish, identification of specific cells of origin for soft tissue sarcomas, including peripheral nerve sheath tumors, is difficult and diagnoses at this point in time are made based solely on morphology in histological sections. It is entirely possible that soft tissue sarcomas in zebrafish represent a broad range of cell types beyond the common "peripheral nerve sheath tumor." Overall, we tend to refer to nonspecific soft tissue sarcomas as "spindle cell sarcomas" of course, providing that the primary cell type is spindle-shaped.

Control and Treatment

A distinct etiology for these tumors in zebrafish is unknown, and they have not correlated these tumors with a particular wild-type line or sex. However, we often diagnose soft tissue sarcomas in zebrafish with mutations in the *p53* gene (Fig. 43.11).

Optic Pathway Tumor

Description

An interesting tumor that does not seem common in other laboratory animal species is a neural tumor with multiple areas of differentiation that resemble various layers of the retina. The closest description in the literature is found in Stolin et al. (2014), wherein they called the tumors simply "optic pathway tumors" and found that they were likely of glial origin—most likely the multipotent Muller glia—based on molecular and cellular characteristics.

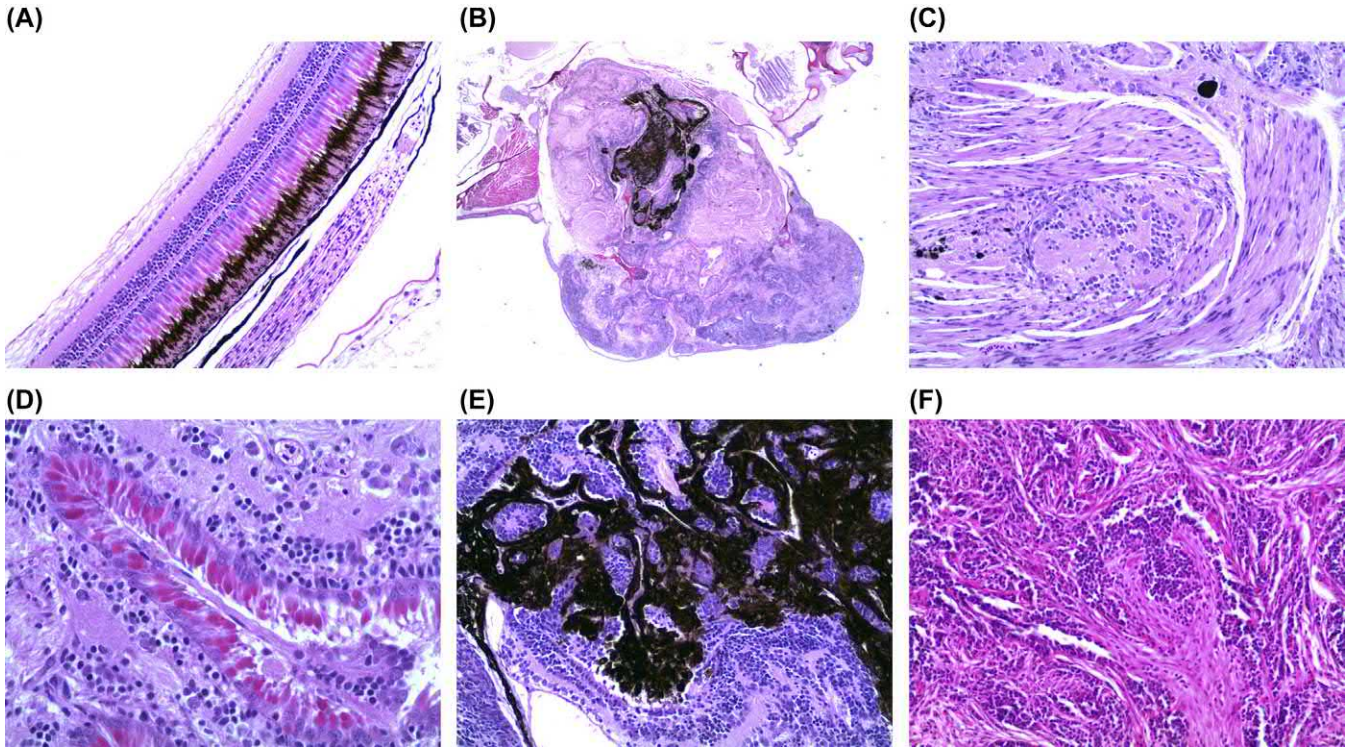

FIGURE 43.11 (A) Normal retina 200x. (B) Ocular pathway tumor with multiple areas of varied retinal differentiation. H&E 20×. (C–F) demonstrate these varying areas. (C) Ganglion cells and nerve fibers. H&E 200×. (D) Photoreceptor layer. H&E 400×. (E) Retinal pigmented epithelium. H&E 200×. (F) Plexiform and nuclear layers. H&E 200×

Pathobiology and Clinical Signs

Exophthalmia is the most common lesion associated with these tumors. They can appear as pink, fleshy masses behind the eye although this is nonspecific since any mass effect in orbit would have a similar appearance.

Diagnosis

Diagnosis is made via postmortem histology. These tumors can be highly variable in appearance based on the proportions of retinal layers represented in each lesion. These are considered distinct from retinoblastomas because there is no identified abnormality in *rb1*, and morphologically, the degree of differentiation into cells representing different retinal layers is far more advanced than the simple rosettes observed in well-differentiated retinoblastomas. Also, to the authors' knowledge, there have been no confirmed diagnoses of spontaneously arising retinoblastomas in zebrafish.

Control and Treatment

In the Stolin study (2014), these tumors developed in heterozygous *Tg(flk1:RFP)is18* transgenic adult fish. While this means these tumors are likely caused by genetic abnormalities, a distinct etiology for spontaneously occurring tumors of this type has not been identified. Therefore, no methods of control or prevention are known; however, culling of animals with this lesion may prevent transmission of tumor-associated genes to offspring (Fig. 43.12).

Melanoma

Description

Zebrafish can develop spontaneous melanomas. Although not as well known for these lesions as their *Xiphophorus* counterparts (Meierjohann & Schartl, 2006), zebrafish are also utilized for studies of melanoma biology. Several lines of zebrafish have been established with mutations in conserved genes affecting specification, differentiation, and function of melanocytes (Ceol, Houvras, White, & Zon, 2008).

Pathobiology and Clinical Signs

A distinct etiology for spontaneously occurring tumors of this type has not been identified. Ultraviolet light is a possible contributor, as in mammals, although this has not been definitively shown in zebrafish. Mitchell, Paniker, and Douki, (2009) showed that early life stages of a *Xiphophorus* backcross hybrid developed melanomas following UVB exposure. Zebrafish studies may elucidate some of the outstanding genetic questions in melanoma biology (Ceol et al., 2008).

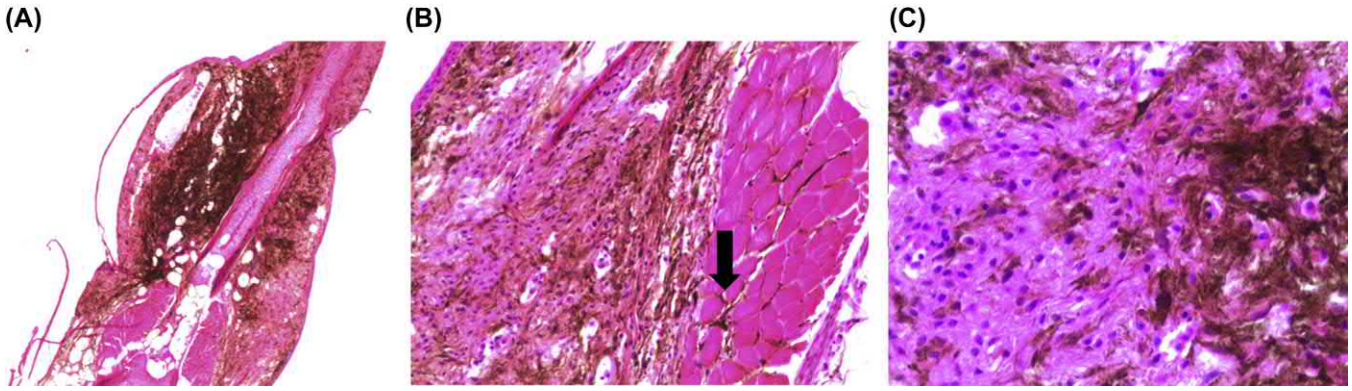

FIGURE 43.12 Melanoma. (A) Dermal melanomas present subgrossly as a variably pigmented subdermal mass. H&E. 20×. (B) The neoplasm is moderately invasive into the adjacent musculature (*arrow*). H&E. 100×. (C) The neoplasm is composed of intersecting streams of spindle-shaped to stellate cells containing variable amounts of intracytoplasmic brown pigment granules.

These tumors can present anywhere inside or outside the body, usually as pigmented masses. Amelanotic melanomas may also occur, but their diagnosis is difficult and will be discussed later. Disruption of internal organs could result in physiological alterations that present as nonspecific morbidity, e.g., edema, lethargy, reduced spawning activity, and inanition. While locally aggressive, metastasis has rarely been confirmed outside of experimental conditions.

Diagnosis

Diagnosis is made on postmortem histology. As in mammals, neoplastic melanocytes can take on a number of appearances, but generally, these tumors appear as sheets of irregular, spindle-shaped to stellate and occasionally rounded cells containing variable amounts of intracytoplasmic melanin pigment. These tumors are generally locally infiltrative and may occasionally show neuroendocrine-type "packeting." It is important to differentiate melanomas from other melanistic lesions, such as those associated with chronic inflammation. These lesions are generally composed of clusters of melanomacrophages, which are not always easy to differentiate from neoplastic melanocytes. Generally, melanomacrophages contain intracytoplasmic vacuoles—presumably phagolysosomes containing degenerate melanin. Because these are the products of phagocytosis and digestion, pigmented granules in macrophages are relatively variable in size and shape, while granules in melanocytes are small and uniform in size. Also, while melanomacrophages can have a moderate amount of pleomorphism based on their ameboid characteristics, more frequently, they tend to have round to polygonal appearance with less pleomorphism than is usually observed in melanomas. Furthermore, the margins of inflammatory melanistic lesions will be relatively well demarcated or at least bounded by resident anatomical structures. This differs from melanocytic neoplasms, which tend to infiltrate between the cells of surrounding anatomic structures. Of course, due to the variable appearance of both lesions, there is likely to be some morphological overlap. Fontana-Masson staining can help in highlighting the shape of melanocytic granules, which may aid in differentiation.

Amelanotic melanomas presumably occur spontaneously in zebrafish, although without a robust array of immunohistochemical stains, it is difficult to confirm these diagnoses. A presumptive diagnosis may be made based on the irregular appearance of a mesenchymal neoplasm that does not quite conform to any particular cell type morphologically. Neuroendocrine packeting may aid in this presumption, and Fontana-Masson staining may reveal rare melanin granules. Due to the notorious variability of melanoma morphologies, it is possible that a large number of amelanotic melanomas in zebrafish may not be confirmed (Fig. 43.13).

Extraspinal Chordoma

Description

Like most teleosts, zebrafish retain prominent notochord remnants within their vertebrae for their entire lives. In mammals, the notochord remnant is reduced to the nucleus pulposa of the intervertebral disks. Chordomas are tumors of notochord remnants that most commonly affect the axial skeleton in humans, and in terms of domestic animal species, the tails of ferrets. Zebrafish are unique in that chordomas most frequently occur in the intestinal tract, although axial skeleton chordomas have also been reported (Cooper, Murray, Spagnoli, & Spitsbergen, 2015).

Pathobiology and Clinical Signs

While extraskeletal soft tissue chordomas are exceedingly rare in humans and mammals, they are considered to be a background lesion in aged zebrafish. Although not exceedingly common, extraspinal soft tissue chordomas

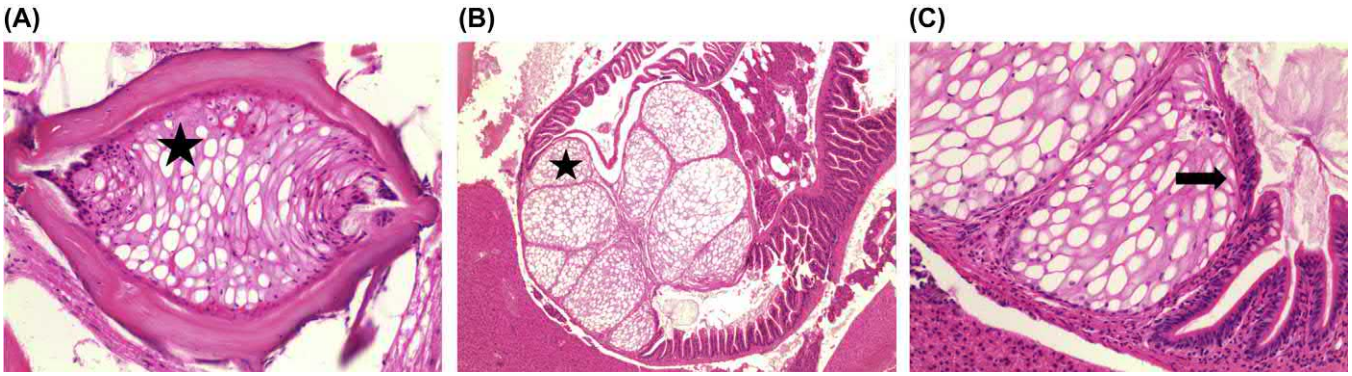

FIGURE 43.13 Chordomas. (A) Normal vertebral body with notochord remnant (star). H&E 200×. (B) Intestinal chordoma (star). H&E 40×. (C) The chordoma arising in the lamina propria is composed of physaliphorous cells and elevates the overlying mucosal epithelium (*arrow*). H&E. 200×.

in the zebrafish occur most frequently in the intestine (Cooper & Spitsbergen, 2016). Extraspinal chordomas generally do not elicit clinical symptoms. They are usually too small to be observed on gross postmortem examination. Whether or not the masses are large enough to disrupt digestion and feeding and potentially contribute to weight loss or other nonspecific signs associated with aging, is not known. A distinct etiology for spontaneously occurring tumors of this type has not been identified. Spitsbergen et al. speculated that *sonic hedgehog b* mutations could be involved in tumor development in the notochord and intestine (Cooper et al., 2015).

Diagnosis

Diagnosis is made on postmortem histology. Chordomas are multilobulated and contain large, vacuolated polygonal cells with fine cytoplasmic processes (physaliphorous cells). They are similar in appearance to the notochord remnants in the vertebrae (Fig. 43.14).

Hepatocellular Tumors

Description

Hepatocellular tumors have been described from countless wild fishes, particularly associated with polluted waters. Whereas viruses are important causes of liver tumors in humans and other mammals, they have only been linked to anthropogenic toxicants (Myers, Landal, Krahn, & McCain, 1991) or specific natural hepatotoxins, such as aflatoxin (Sinnhuber, Hedricks, Walse, & Putnam, 1997) in fish. They have also been experimentally induced in numerous laboratory studies, including those with zebrafish (Spitsbergen et al., 2012).

Pathobiology and Clinical Signs

These tumors arise from hepatocytes and can be discrete, focal masses in the liver. The degree of demarcation is highly variable, and often low-magnification evaluation of the liver as a whole is the best method of initial identification. On necropsy, these tumors may appear as soft tissue swellings on the liver, but they are generally too small to be identified grossly.

Diagnosis

Diagnosis is made based on postmortem histology. Hepatocellular tumors in zebrafish can have a number of appearances, but are generally, poorly demarcated and composed of sheets of disorganized polygonal cells similar in appearance to, but smaller or larger than, adjacent hepatocytes. They can sometimes be difficult to differentiate from normal adjacent liver tissue, particularly in emaciated animals with generalized reduced hepatocellular cytoplasm. They are frequently associated with hemorrhage and severely vacuolated hepatocytes, and it can be difficult to discern characteristic neoplastic hepatocytes in the midst of hemorrhage and degenerate cells. Small patches of shrunken hepatocytes or vacuolated hepatocytes have also been observed within nonneoplastic livers, and it is unclear whether these represent degenerate changes or preneoplastic lesions. As mentioned in the Water Quality and Idiopathic Disease of Laboratory Zebrafish chapter (Murray et al. This book.), the relationship between hepatocellular karyomegaly and neoplasia is unclear. In mammals, it is extremely difficult to differentiate between hepatocellular adenomas and hepatocellular carcinomas, since they can have similar microscopic morphologies. Differentiation between benign and malignant tumors generally depends on the size and clinical behavior: Malignant neoplasms, which tend to be larger, metastasize while benign neoplasms do not. Since metastases in zebrafish tumors are rare in all tumor types, we cannot rely on clinical behavior for a diagnosis.

Examining for characteristics of malignancy may, therefore, be the best way to distinguish between hepatocellular adenomas and carcinomas. Tumors with severe cellular and nuclear pleomorphism, high mitotic

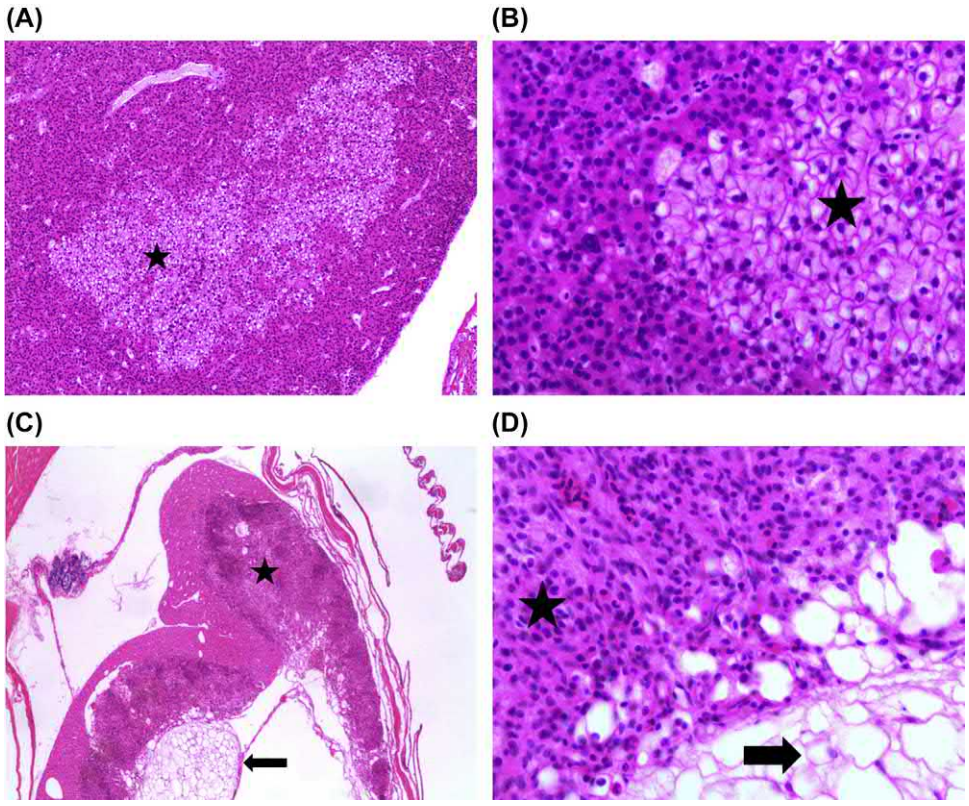

FIGURE 43.14 (A) Hepatocellular adenoma (star). The mass is an irregular aggregate of vacuolated hepatocytes paler than the adjacent normal tissue. H&E. 100×. (B) Hepatocellular adenoma. Hepatocytes have vacuolated cytoplasm with moderate nuclear pleomorphism (star). H&E. 400×. (C) Hepatocellular carcinoma. The mass is locally destructive and contains large areas of hemorrhage. H&E. 20×. (D) Hepatocellular carcinoma. Hepatocytes display marked nuclear and cellular atypia (star). There are frequently multifocal regions of lipid-laden hepatocytes throughout these tumors (*arrow*). H&E 400×.

rates, necrosis, and infiltration of the surrounding tissue, along with multicentric intrahepatic lesions and/or carcinomatosis are best-termed carcinomas. Small, discrete masses with cells similar in appearance to normal hepatic tissue are best-termed adenomas.

The identification of benign hepatic lesions may be further complicated by an entity, common in aged canines, known as nodular hyperplasia. This is usually a benign, focal, or multifocal overgrowth of normal hepatic tissue with loss of portal architecture. In a case where multiple nodules of normally appearing hepatocytes are present, this would best be termed *nodular hyperplasia*. Distinguishing focal nodular hyperplasia from a hepatocellular adenoma is difficult and should be made based on the altered appearance of cells in the mass. To the authors' knowledge, spontaneously occurring nodular hyperplasia has not been specifically identified in zebrafish.

As it a laboratory animal, it is worth discussing the appearance of preneoplastic "altered foci," particularly in the light of carcinogenesis studies. While basophilic, eosinophilic, clear cell, and mixed cell foci, are well described in mice, they are uncommon in zebrafish. Naturally occurring altered foci have been observed anecdotally by the authors, but they have not been reported in the literature, nor is it clear if these lesions are preneoplastic in zebrafish.

Control and Treatment

Hepatocellular neoplasms, including those of fishes, are often caused by anthropogenic toxicants. However, it is well recognized that natural toxins, such as aflatoxin, cause such tumors in fish as well (Myers et al., 1991; Sinnhuber et al., 1997). A distinct etiology for spontaneously occurring tumors of this type in zebrafish has not been identified. Carcinogens most frequently affect the liver in both wild-type and mutant zebrafish (Spitsbergen et al., 2012) (Fig. 43.15).

Biliary and Pancreatic Ductal Lesions

Description

Liver and pancreatic tumors arising from ductal structures occur in laboratory zebrafish. These tumors must be differentiated from ductal hyperplasia, particularly in the context of toxicant research with regard to carcinogenesis.

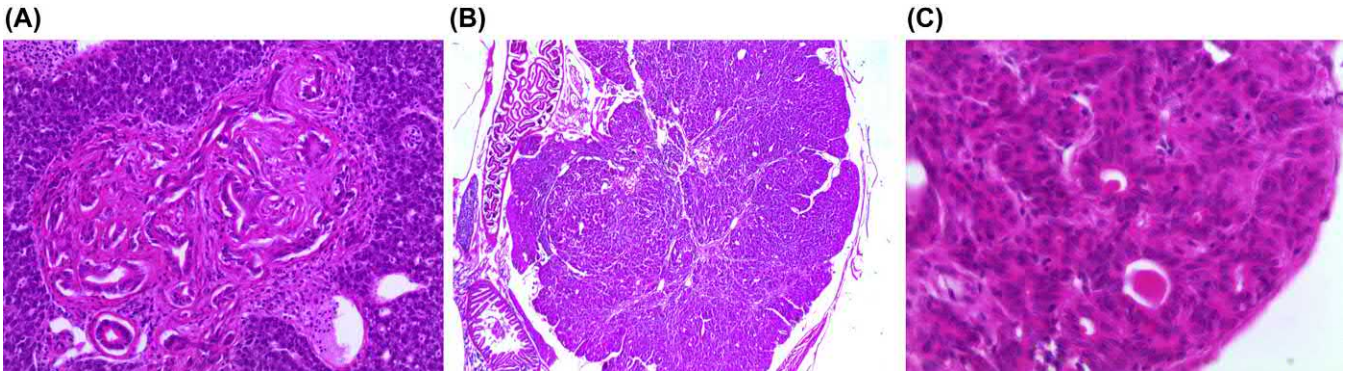

FIGURE 43.15 (A) Bile duct adenoma. Focally within the liver, there is a mass composed of tortuous ducts embedded in a dense collagenous stroma. H&E 200x. (B) Bile duct adenocarcinoma. The liver is severely expanded and infiltrated by a mass composed of tortuous dysplastic bile ducts, giving the liver a bulging, rounded appearance. H&E 20×. (C) Biliary adenocarcinoma. Incomplete bile ducts are lined by atypical epithelial cells, often forming ducts filled with proteinaceous material. H&E 400×.

Pathobiology and Clinical Signs

Biliary and pancreatic ducts normally course through the parenchyma of their respective organs to the intestinal tract. Therefore, tumors arising from these ducts can involve any combination of the liver, pancreas, and intestine. These can rarely appear as masses that protrude above the liver capsule. Carcinomatosis is also possible. Clinical signs are generally nonspecific for emaciation and general malaise.

Diagnosis

Diagnosis is made based on postmortem histology. As in mammals, it is extremely difficult to differentiate biliary from pancreatic ductal tumors based on appearance alone. It is even more difficult in zebrafish due to the anatomical proximity of the pancreas and the liver, and so unless it is very clear that the tumor "belongs" to one organ or the other, it may be practical to lump the two entities together into simply a "ductal" tumor. These neoplasms are generally composed of tortuous, variably sized ducts lined by squamous to columnar epithelium and embedded in variable amounts of loosely organized fibrous connective tissue. They can be highly invasive.

To further complicate matters, there is an entity in zebrafish wherein there is diffuse, presumably benign, hyperplasia of the biliary tree in which most of the ducts observed throughout the liver are tortuous, reduced in diameter, and lined by squamous to low cuboidal epithelium. They are generally associated with a moderate amount of fibrous connective tissue. They are frequently associated with granulomas that contain pigmented acellular material, presumably bile, although the material does not stain with Hall's bile stain and is distinct in color and character from melanin (Spagnoli, Xue, & Kent, 2015). Rather than forming distinct masses with infiltration into the surrounding tissue, as is typical of ductal tumors, bile duct hyperplasia occurs throughout the entire biliary tree and atypical cellular features are generally absent. These hyperplastic populations may be preneoplastic, although this has not been demonstrated. Most lesions of diffuse biliary hyperplasia tend to occur in clinically normal fish submitted for sentinel screening.

While biliary adenomas and carcinomas are focal to multicentric lesions that are either well demarcated or locally infiltrative, biliary hyperplasia occurs diffusely throughout the biliary tree without forming discrete nodules. Furthermore, bile ducts in biliary hyperplasia generally lack cellular atypia, mitotic figures, and necrosis.

Control and Treatment

A distinct etiology for spontaneously occurring tumors of this type has not been identified. However, TL line fish appear to be particularly predisposed to bile duct hyperplasia (Spitsbergen et al., 2012). This should be considered when choosing a line for toxicology and carcinogenesis studies where the liver is expected to be affected (Fig. 43.16).

Nephroblastoma/Ependymoma

Description

Nephroblastomas have been reported in a number of fish species and after lymphoma, are the second most common primary renal neoplasms in fish (Lombardini, Hard, & Harshbarger, 2014). These tumors have been associated with water or environmental factors in Japanese eel (Masahito, Ishikawa, Okamoto, & Sugano, 1992) and carcinogen exposure in trout (Bailey, Williams, & Hendricks, 1996). Given the described response in rainbow trout, Spitsbergen et al. noted the remarkable lack of nephroblastomas in zebrafish exposed to carcinogens (Spitsbergen et al., 2000).

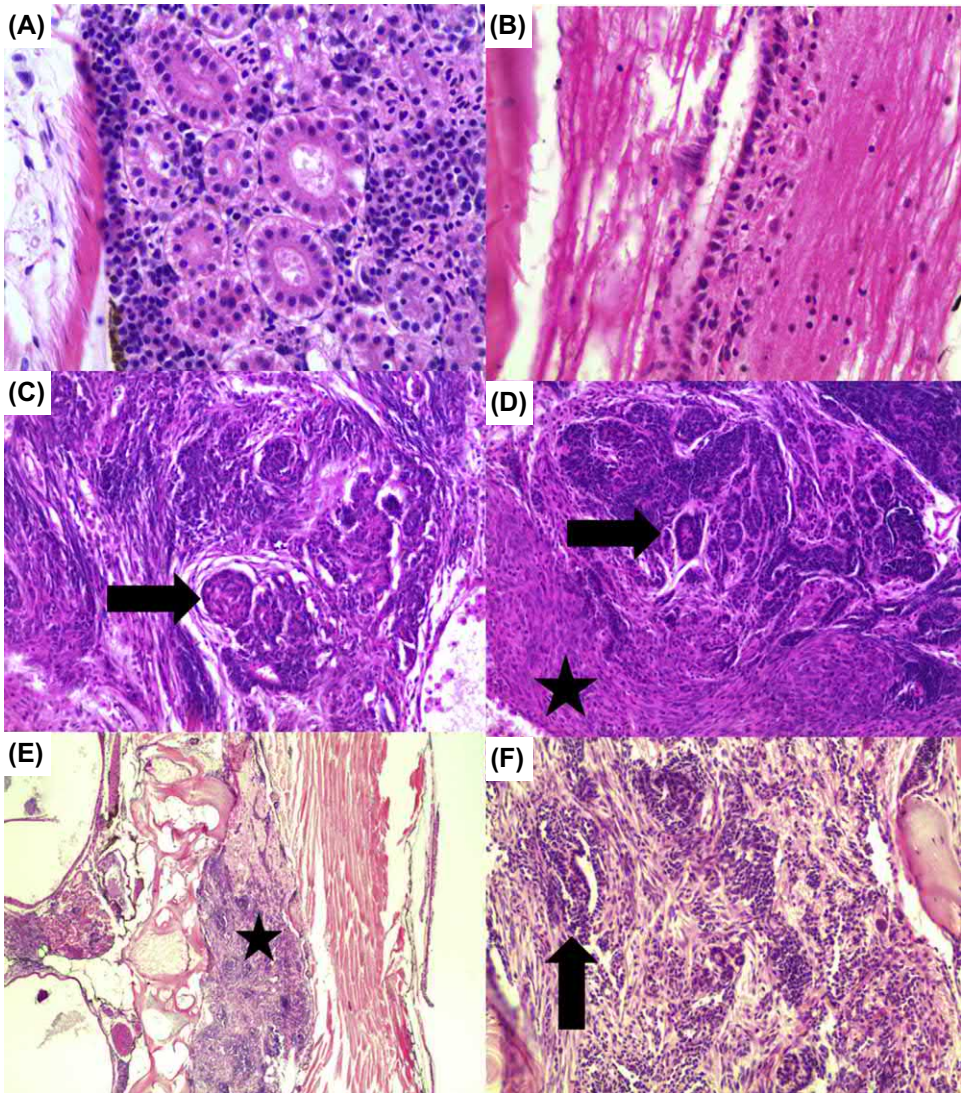

FIGURE 43.16 (A) Normal kidney tubules. H&E 400×. (B) Normal spinal cord with central canal lined with ependymal cells. H&E 400×. (C) Nephroblastoma. Note the primitive glomerular structures embedded in the mesenchymal cell population (*arrow*). H&E 200×. (D) The majority of the tumor is composed of epithelium-lined tubules (*arrow*) embedded in dense primitive mesenchyme (blastema, star). (E) Ependymoma. A highly infiltrative mass effaces the spinal cord and invades surrounding tissue. H&E 40×. (F) The neoplasm is composed of tubules recapitulating the central canal (*arrow*) embedded in a matrix that resembles neuropil. H&E 400×.

Pathobiology and Clinical Signs

These tumors can cause scoliosis, kyphosis, or lordosis. Otherwise, clinical signs are generally nonspecific for emaciation and general malaise. A distinct etiology for spontaneously occurring tumors of this type has not been identified. Presently, we have not recognized any predilection for these tumors related to sex or a particular line of zebrafish. Mutations in *WT1*, encoding Wilms tumor protein, results in pediatric renal cancer in humans. Two paralogs, *wt1a* and *wt1b*, have been identified in zebrafish and activation experiments indicate that they have different functions in renal development compared to their human counterparts (Perner, Englert, & Bollig, 2007).

Diagnosis

Diagnosis is made based on postmortem histology. These tumors can exceedingly difficult to differentiate from each other, due to the proximity of the spinal cord and kidney as well as their similar appearances. It is for this reason that they are discussed in a combined category here. Generally, these tumors contain both mesenchymal and epithelial components that have the appearance of tubules lined by high cuboidal epithelium embedded in sheets of poorly differentiated spindle-shaped cells. If glomerular structures are observed in the tumor, then it can definitively be called a *nephroblastoma*. However, these structures can be difficult to observe microscopically in normal zebrafish, and their

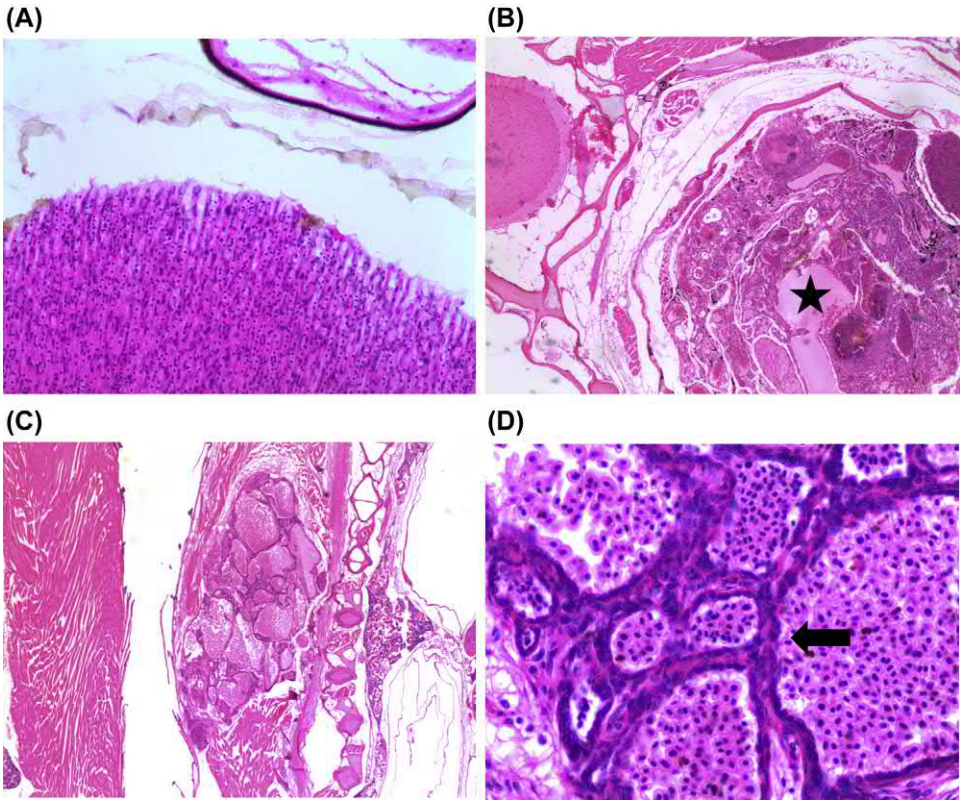

FIGURE 43.17 (A) Normal periocular vascular rete. H&E 200×. (B) Periocular hemangioma (star). H&E 40×. (C) Intramuscular hemangioma (star). H&E 20×. (D) Hemangioma composed of variably sized, blood-filled cavities separated by thin connective tissue trabeculae lined by flattened endothelial cells (*arrow*). H&E 400×.

absence does not necessarily rule out nephroblastoma. Neoplastic tubules in nephroblastomas are also generally surrounded by primitive blastema which has a densely packed appearance compared to ependymomas, which may contain components similar to neuropil. Ependymomas may also form rosettes. Both of these neoplasms are highly aggressive and can invade and efface adjacent kidney, musculature, spinal cord, and vertebrae.

Hemangioma

Description

Hemangiomas are benign tumors of endothelial structures. Carcinogen-associated and spontaneous hemangiomas have been described in several different fishes, including zebrafish (Couch, 1995; Fournie, Herman, Couch, & Howse, 1998; O'Hagan & Raidal, 2006; Spitsbergen et al., 2000).

Pathobiology and Clinical Signs

Hemangiomas arise from endothelial cells. They have been described in the heart, gills, liver, and in the retrobulbar space (Couch, 1995; Grizzle & Thiyagarajah, 1988; O'Hagan & Raidal, 2006), but presumably can occur in any vascularized tissue. These tumors generally do not cause clinical signs. Rarely, they may appear as soft, red masses anywhere in the body, although most are too small to be observed grossly.

Diagnosis

Diagnosis is made based on postmortem histology. Generally, these tumors are quite well organized and composed of well structured, but irregularly sized and tortuous capillaries lined by flattened to slightly plump endothelial cells. Most are intramuscular but some can occur within the vascular rete caudal to the eye. Because of their relatively well-organized appearance, an argument can be made that some of these represent a more benign hamartomatous lesion, such as angiomatosis or vascular hamartoma. However, they can be locally aggressive with severe tissue destruction despite the benign appearance of the endothelium, which argues against a benign, hamartomatous lesion. The distinction between benign and malignant lesions should be made based largely on an evaluation of adjacent tissue destruction first, and if present, features of malignancy.

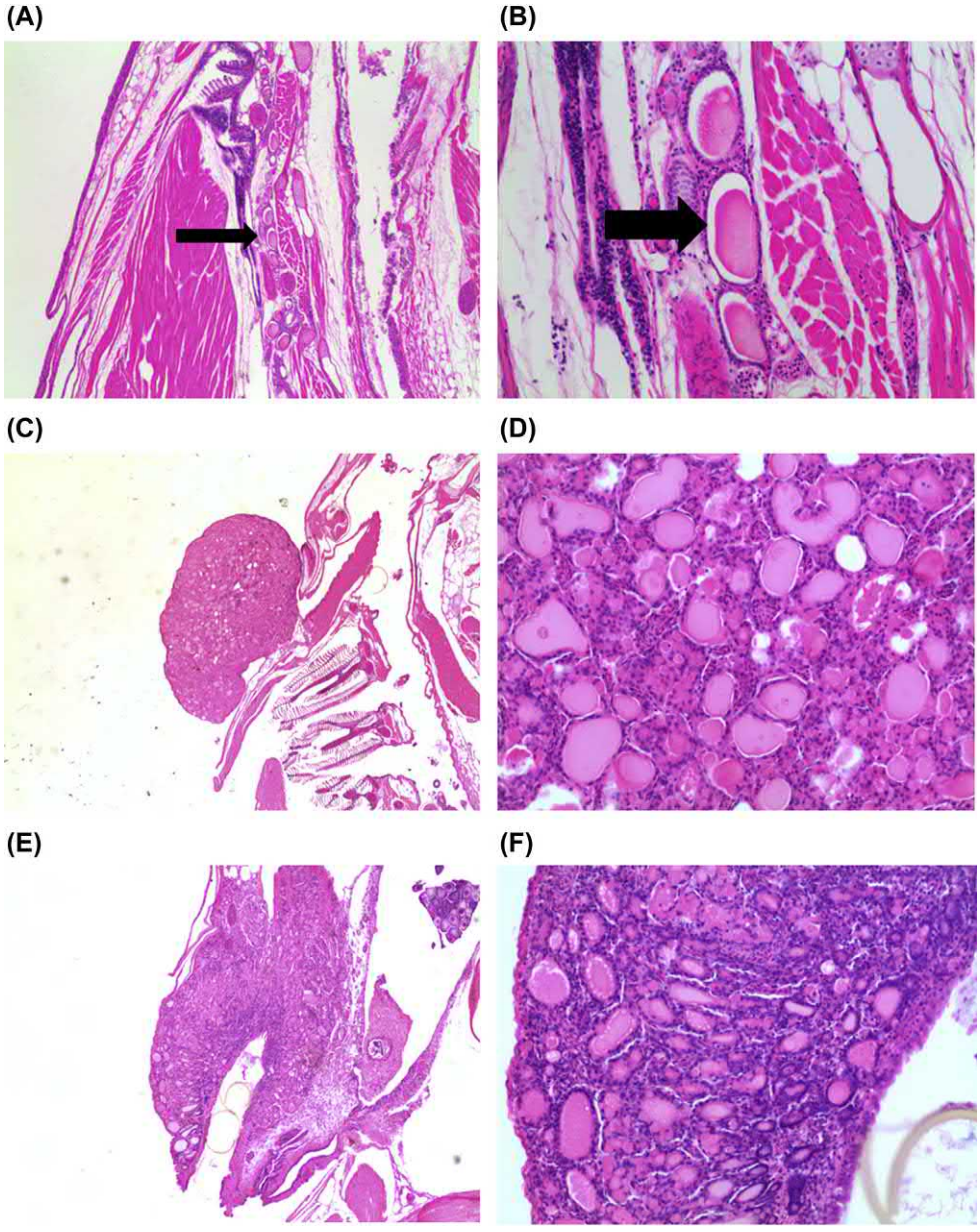

FIGURE 43.18 Thyroid tumors/hyperplasia. (A) Anatomic location of "normal" thyroid tissue (*arrow*). H&E 20×. (B) Normal thyroid follicle lined by flattened epithelial cells and filled with colloid (*arrow*). H&E 200×. (C) Thyroid adenoma. The mass, arising ventral to the gills and visible externally, is pedunculated. H&E 20×. (D) Thyroid adenoma. Frequently, epithelial cells fail to form follicles, producing clusters of polygonal cells with minimal cellular atypia (*arrowhead*). H&E 200×. (E) Thyroid carcinoma. The mass appears highly infiltrative and destructive to the surrounding tissue. (H&E 20×). (F) Follicles are frequently lined by more than one layer of high cuboidal epithelium (*arrows*). Mitotic figures are frequent. (*arrowheads*). H&E 200×.

Control and Treatment

Couch described a high incidence of hemangioendothelial neoplasms in *Rivulus marmoratus* (Couch, 1995). He postulated a number of potential etiologies, including avian sarcoma virus associated with a chicken liver diet, latent sarcoma virus activation, general activation of oncogenes and downregulation of tumor suppressor genes, and carcinogen exposure. Hemangiomas are not common in laboratory zebrafish, and to date, we have not identified cohorts with a high prevalence of this tumor that might indicate any of these etiologies (Fig. 43.17).

Thyroid Tumors

Description

The thyroid gland in zebrafish is unencapsulated, and generally, follicles of various sizes run along the ventral

aorta, from the bulbous arteriosus to the first gill arch (Wendl et al., 2002). The occurrence of thyroid follicles in ectopic locations is common in some species, but only occasionally recognized in zebrafish. Single cases of thyroid tumors can occur, but the diagnosis of these tumors typically involves multiple fish in a facility or tank in response to extrinsic factors, especially iodine deficient conditions. As these conditions generally instigate hyperplasia of thyroid tissue, cases of multiple tumors should be carefully evaluated, considering environmental conditions and genetic backgrounds of the affected stocks. Specific criteria for the microscopic distinction between hyperplasia and neoplasia are provided.

Pathobiology and Clinical Signs

Thyroid tumors generally present as reddened masses on the ventral mandible. Masses have also been observed extending dorsally to the snout area and at the vent. Macroscopic masses may be the only clinical sign observed. However, perturbations in thyroid hormone levels could result in other abnormalities. Thyroid hormone is involved in masculinization of larval zebrafish, and hypothyroid females can give rise to female-skewed progeny (Mukhi, Torres, & Patino, 2007; Sharma, Tang, Mayer, & Patino, 2016).

Diagnosis

Diagnosis of thyroid tumors is made by histological examination. Fournie et al. described a range of thyroid proliferative lesions, highlighting the importance of adhering to specific criteria and standardized nomenclaons, Zimmermann and Galetti concluded that iodine deficiency can act as a tumor promoter and is a risk factor for thyroid cancer in people (Zimmermann & Galetti, 2015). Therefore, cases of multiple thyroid lesions should be carefully evaluated using histologic criteria to determine whether true neoplastic changes are present. Genetic background of the affected stocks should also be considered, as the effects of iodine deficiency in a mutant or transgenic line that is prone to tumors may increase the likelihood of follicular cell neoplastic changes. The diagnosis of multiple thyroid proliferative lesions should be followed by an assessment of food and water parameters and correction of any nutrient imbalances, particularly iodine. Significant regression and recovery of lesions following these adjustments may be evidence for observed masses being a result of hyperplasia. Conversely, these findings could indicate a high hormone or nutrient responsive neoplasm in which removal of the inciting stimulus causes a reduction in the size of neoplastic cells to the point, where they can no longer be identified in 5 μm sections (Fig. 43.18).

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CHAPTER

44

Special Procedures for Zebrafish Diagnostics

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Environmental Samples

Water quality-related diseases are one of the major causes of morbidity in zebrafish (See Hammer (2020), Chapter 30): Water Quality Chapter; Murray, Lains, & Spagnoli, 2020, Chapter 45). Inappropriate water chemistry parameters or the presence of certain toxicants in the water are the main causes of acute mortality. Therefore, all disease investigation should include documentation of the most common water quality parameters (i.e., temperature, pH, ammonia, nitrate, conductivity, and alkalinity). Depending on the system, data on chlorine, copper, etc. should be obtained. All of these data are usually available from the facility records, but if not, should be obtained by the diagnostician using the appropriate water quality testing kits or meters. Testing of pathogens in the environment is discussed below under "Nonlethal Testing."

Clinical examination

Fish should initially be evaluated while in their home environment. Behavior within the tank may be useful for the indication of an emerging disease, and some behavioral changes are useful for presumptive diagnoses. For example, if fish are flashing (rubbing on the sides of tanks), this may indicate that they are infected with external parasites. Other indications of the disease include cessation of feeding, lethargy, and abnormal position in the aquarium. Fish with impaired gills or other respiratory problems will often have rapid opercular movements and be gathered near the water surface, whereas fish suffering supersaturation will also demonstrate respiratory distress, but will congregate at the bottom of the tank. Macroscopic changes are seldom pathognomonic with zebrafish diseases, but they do provide useful information. Erythema is a common presentation with sick zebrafish but can be caused by both water quality problems and infectious agents. Distinct ulcers on the flanks can be caused by both infectious agents, such as *Mycobacterium marinum* or by a noninfectious inflammatory process, such as egg-associated inflammation (Murray et al., 2020, Chapter 45). Emaciation is associated with most of the common infectious diseases in zebrafish; *Pseudoloma neurophilia*, *Pseudocapillaria tomentosa* (see Kent and Sanders, 2020, Chapter 43) or mycobacteriosis (Whipps & Kent, 2020, Chapter 44).

Selecting Fish for Diagnostic Evaluation

Examinations should be conducted on diseased fish that are collected while still alive (moribund) as dead fish left in water autolyze extremely rapidly, hence many histological changes are obliterated by postmortem autolysis. However, dead fish may be suitable for some parasitological examinations and for observing obvious macroscopic pathological changes. Additional information can be obtained from textbooks that provide general guidelines for conducting health and disease investigations and necropsies on fish (e.g., Noga, 2010), and some that are specifically directed to zebrafish (Westerfield, 2000; Harper & Lawrence, 2011; Kent et al., 2009 [ZIRC online manual]).

The Zebrafish in Biomedical Research
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External Examination and Gross Necropsy

Although their small size presents some necropsy challenges, general procedures used with larger fishes can be employed with some modifications. With patience and practice necropsies can be efficiently accomplished with the aid of a stereo dissecting microscope and small, fine dissecting instruments (particularly high-quality fine tip forceps). Fish may be euthanized by two approved methods, either rapid hypothermia (icing) or by an overdose of tricaine methanesulfonate (MS-222). Collymore (2020) provides a review of euthanasia using these methods. Note surface abnormalities (e.g., frayed fins, cloudy eyes, ulcers, skin discolorations, parasites, and tumors). Prepare wet mounts of the skin mucus and a few scales by scraping the surface of the fish with a coverslip and placing the coverslip on a glass slide. Some water may be added to the preparation, so that the area between the slide and the coverslip is completely filled with liquid. Examine the wet mount with a compound microscope, starting with low power, for detecting external parasites and bacteria, such as gliding bacteria *Flavobacterium* spp. Reducing the light and lowering the condenser will produce higher contrast, which will make the microscopic parasites and other pathogens more visible. Remove the operculum (Fig. 44.1). Note the color of the gills (pale gills usually indicate anemia). Check for parasites, cysts, excessive mucus, and hemorrhages with a dissecting microscope. Prepare a wet mount by removing the entire gill arch with attached filaments with scissors, then place the arch in a large drop of freshwater on a glass slide. The cartilaginous arch is too thick for high magnification examination, and hence, should be separated from the filaments with a scalpel blade and discarded. Then carefully overlay the preparation with a coverslip. Examine for small parasites, fungi, and bacteria using a compound microscope.

Open the visceral cavity as described in Fig. 44.1, revealing the visceral organs. The fish should be opened with the right side down for better exposure of the liver (Fig. 44.2). Note if ascites, hemorrhages, enlarged spleen, or other abnormalities are present. Multiple, whitish cysts in the visceral organs are suggestive of granulomas (e.g., *Mycobacterium* spp. infections). Expose the kidney by removing the swim bladder and note any kidney abnormalities. Many diseases cause enlargement or discoloration of the kidney, but this may be difficult to see in zebrafish due to their small size. Examine the heart for any abnormalities. Examine squash preparations of organs with a compound microscope to detect encysted parasites, fungi or microscopic granulomas. Squash preparations are made by removing a small piece of tissue, and gently squashing it between a slide and coverslip so that a thin preparation suitable for examination with a compound microscope or dissecting microscope is made.

Leishman's Giemsa or Diff-Quik (Dade Behring AG, Newark, DE) stained imprints of the kidney or other affected organs are useful for detection of protozoa and bacteria. Remove a piece of tissue, blot it on a clean paper towel to remove most of the blood, and lightly touch the cut surface of the tissue on a clean glass slide. Several imprints from the same piece of tissue can be made on one slide. Air-dry the preparation for approximately 1/2 h. Fix the slide for 5–10 min in absolute methanol for Giemsa stains or the fixative provided with the Diff-Quik kit. The slide can then be stained with Giemsa or Diff-Quik, or shipped to a diagnostic laboratory. For *Mycobacterium* spp., imprints and tissue smears are stained with acid-fast stains.

Pseudoloma neurophilia, the most common parasite of zebrafish, can be detected by carefully removing the cranial cap (Fig. 44.1 – Step 5), removing the hindbrain

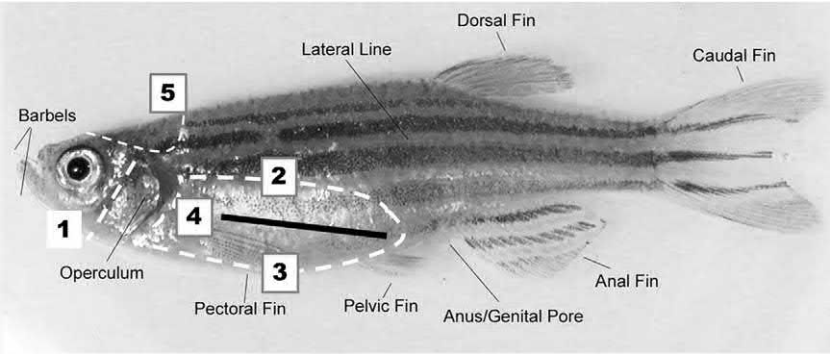

FIGURE 44.1 Dissection of a zebrafish. White lines designated demark location of incisions. (1) remove operculum. (2) Make an incision starting at dorsal posterior of opercular cavity and extend toward anus. (3) Connect incision with a cut starting at the ventral posterior of opercular cavity. (4) Cut dorsally to connect with Cut 2 and remove abdominal flank. (5) Remove cranial cap to access neural tissues for wet mounts. Black line = incision prior to preservation to assure proper fixation of visceral organs.

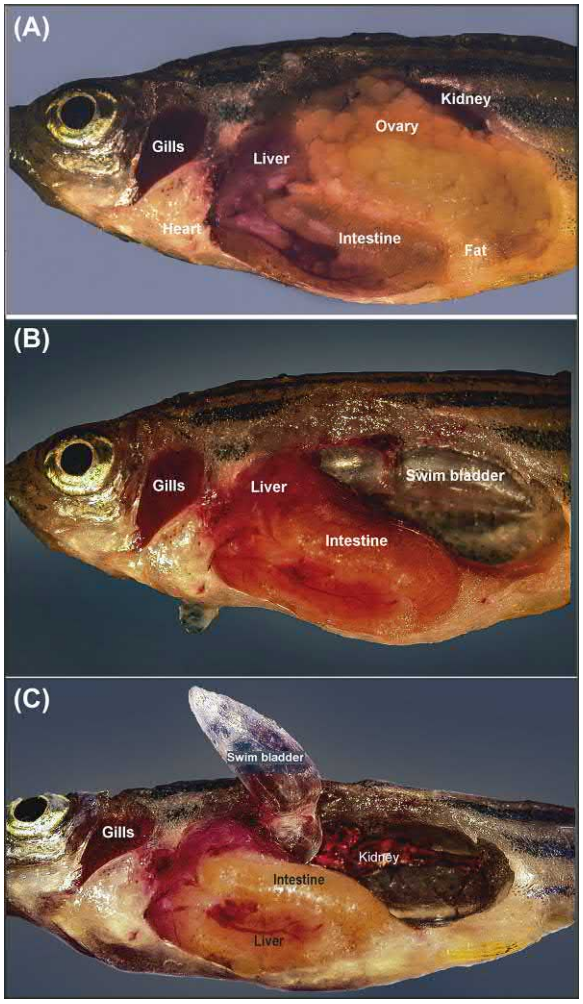

FIGURE 44.2 Internal anatomy of zebrafish. (A) Female (B) Male. (C) Swim bladder lifted to show kidney, which is dorsal to swim bladder. *Images courtesy of Dr. Jennifer Matthews, Zebrafish Internal Resource Center. Modified by Robert Pianavilla.*

and pieces of the anterior spinal cord, and preparing a wet mount. Avoid bone fragments, as these will prevent optimal thin preparations for microscopy. Spores occur in aggregates and appear more opaque than neural tissue (Fig. 44.3).

Histopathology

Histopathology is often the primary or first-line diagnostic test for zebrafish (Collymore, Crim, & Lieggi, 2016; Murray, Varga, & Kent, 2016). A major strength of histopathology is that it facilitates the documentation of changes in a variety of tissues and organs with no a priori assumptions relating to a specific disease. It allows for examination of all tissues for infectious pathogens, changes related to suboptimal water quality, neoplasia, and numerous other noninfectious pathologies. In fact, the initial diagnosis and descriptions of many of the major pathogens affecting zebrafish in research facilities, including *P. neurophilia* (Matthews, Brown, Larison, Bishop-Stewart, Rogers, & Kent, 2001), *M. chelonae* (Whipps, Matthews, & Kent, 2008), *Pleistophora hyphessobryconis* (Sanders et al., 2010), *Edwardsiella ictaluri* (Hawke et al., 2013), and *Myxidium streisingeri* (Whipps, Murray, & Kent, 2015) were made by screening histological sections of moribund zebrafish submitted to diagnostic services, such as the Zebrafish International Resource Center Diagnostic Pathology Service (https://zebrafish.org/health/index.php). If possible, withhold feed to fish for 2–3 days before they are euthanized. This reduces postmortem autolysis of the intestine and liver and reduces the likelihood of ingesta from entering the gill cavity and causing postmortem changes. Also, hard food items in the intestine cause difficulties (e.g., chatter) when preparing sections. It is critical to preserve fish for histology as soon as possible after fish are dead to avoid postmortem changes. If possible, do not use dead fish because significant autolytic changes may occur in 15–20 min after death. Fish are preserved in formalin-based fixatives. Davidson's or Dietrich's solutions are preferred because these fixatives also contain acidic acid and ethanol, and thus

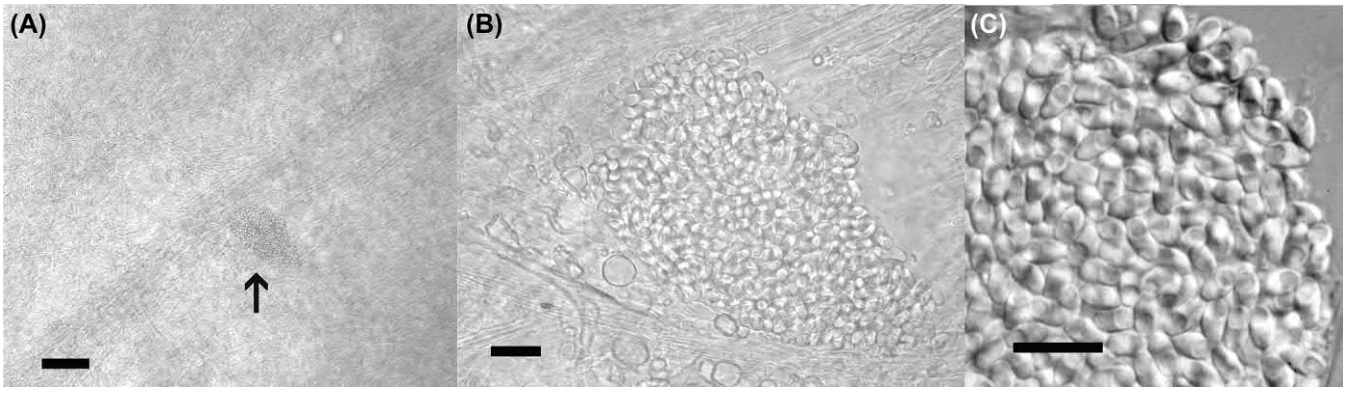

FIGURE 44.3 Wet mount preparation of neural tissue revealing *Pseudoloma neurophilia*, showing progressively higher magnifications (A–C). At lower magnification, aggregates of spores appear more opaque than surrounding tissues. Higher magnifications (B and C) (e.g., X 400 or 1000) demonstrates the typical oval microsporidian spore morphology with a posterior vacuole. (A), Bar = 100 μm, (B and C). Bar = 10 μm.

provide better penetration and fixation of all organs, good cellular detail and some decalcification of bone. Tissue preserved in formalin-based fixatives are generally considered less suitable for PCR from tissue blocks, but we found that there was no difference in success with mycobacteria PCR tests on zebrafish preserved in 10% formalin compared to Dietrich's (Peterson, Kent, Ferguson, Watral, & Whipps, 2013).

Most zebrafish submitted for histopathology evaluation are euthanized, fixed, and the entire fish submitted for histological processing. The visceral cavity of the fish is opened by cutting an incision starting at the posterior part of the opercular cavity and extending it to about 2/3 the length of the coelomic cavity. Then the whole fish is placed in fixative at approximately 1:20 (v/v) tissue to fixative. Injection of fixative into the oral cavity and coelom is another effective method to assure proper fixation (G. Sanders, University of Washington, pers. comm.). Fixative is injected into the coelomic cavity until it is extended but not ruptured. This volume will vary greatly with the size of the fish but on average ranges from less than 0.5 mL to up to ~1 mL for zebrafish. A 3 mL syringe and a 23 or 21 gauge needle can be used. Multiple needles will be required if multiple fish are being processed at once. When utilizing the oral injection procedure, account for at least 0.5 mL because fish are injected after needle placement until fixative backs up into the mouth and out the down side gill operculum. The fish is then place in fixative as described above. Fixation should be adequate as long as the fish are then placed in enough fixative (i.e., 20:1, fixative to fish carcass).

The entire fish can be processed (usually cut in sagittal sections) and representative organs can be visualized in multiple sections on one or two slides (Fig. 44.4). Preparation of high-quality histological slides from whole, small fish requires some additional steps and attention to detail compared to processing homogenous pieces of organs. Some general considerations are (1) Decalcification: whereas zebrafish are small, the skeleton of adult fish is ossified, and hence, often requires decalcification for optimal section. Cal-Ex II (formic acid) (Fisher Chemical) is recommended as more aggressive solutions, such as those containing HCl may inhibit acid-fast staining of *Mycobacterium* spp. in sections (Kent et al. 2006). (2) Whole-body sagittal sections: most often, the preserved fish is bisected, and both halves processed "cut side down." The spinal cord should be included in sections for detection of *Pseudoloma neurophilia*. Obtaining good sections of the spinal cord can be challenging. Double-edged razor blades are cut into two pieces with scissors and used for bisecting the fish. A scalpel blade or single-edge razor blade is unacceptable due to the thickness of the blades. Grasp the fish with forceps with the head facing to the left and place the blade on the other side of the fish slightly off of the spinal cord and make a clean cut through the fish. By firmly grasping the fish with the forceps, it may be possible to straighten out the fish with spinal deformities prior to bisecting. Fish lacking pigment (e.g., Caspers) can be particularly difficult, as it is nearly impossible to visualize the centerline. Cassettes of fish that are brittle are placed in a jar of dedicated Cal-ex II for about 4–8 h before processing. (3) Facing the block to obtain spinal cord in the section requires careful embedding, and it is important to press the fish to the bottom of the mold starting with the half of the fish that does not contain the spinal cord. Clean the embedded cassettes of any extra paraffin, especially the part of the cassette that touches the back of the microtome. A dissecting microscope with a light source angled from the side is set up near the microtome to enhance visualization.

The cassettes are faced by taking a few turns at 10 μm, stopping, and examining at the cut surface under the microscope and resuming until the spinal cord comes into view and the coelomic organs are exposed. The spinal cord may appear before the organs. In this case, cut sections and then face deeper to obtain the coelomic organs. Use charged adhesion slides and dry the slides thoroughly before staining. Fish within the blocks may be dry and should soak on a cold tray with water for at least 30 min after facing and prior to sectioning. A few squirts of a 5% solution of Downey brand fabric softener can be added to the cold tray water to enhance the softening but should be judiciously used, as it can interfere with forming a sectioning ribbon.

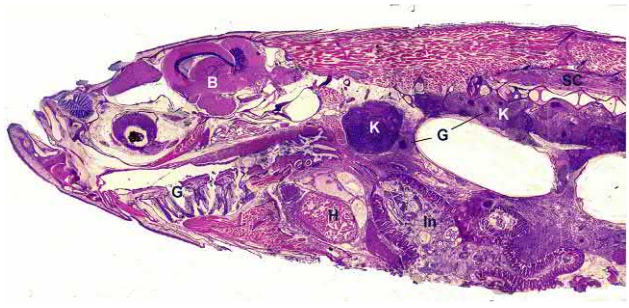

FIGURE 44.4 Sagittal histological section of a male zebrafish showing major organs. K = kidney, G = Gills, B = Brain, SC = spinal cord, H = heart, In = intestine, G = granulomas due to mycobacteriosis.

Immunohistochemistry and Diagnostic Antibodies

The whole-body sagittal section provides the ability to have the important organs on one slide, and therefore, provides an excellent format for positive and negative controls within the same section. Antibodies directed specifically to zebrafish antigens are becoming more

available. For example, as of 2017, Genetex list some 450 antibodies that cross-react with zebrafish. Antibodies developed against epitopes in human and mouse proteins often have useful cross-reactivity with conserved proteins in zebrafish. Hence, tissue or cell type-specific antibodies can be used to characterize -neoplasms to elucidate tissue or cell of origin (Paquette et al., 2015). Nevertheless, antibodies not specifically developed against zebrafish epitopes must be carefully validated to avoid overinterpretation of findings (Ramos-Vara, 2005; Bordeaux et al., 2010). Some companies, such as AbCam (Eugene, Oregon) also offers trials where an antibody is purchased, evaluated, and when results are reported back to the company (e.g., results with zebrafish), the researcher is credited the cost of the antibody. This strategy allows for the zebrafish community to contribute to a growing list of mammalian antibodies with known cross-reactivity (do or do not work) in zebrafish. Quality of immunohistochemistry stains can be negatively impacted by improper fixation. Inappropriate fixation times can result in poor epitope preservation and antibody binding.

Bacteriology

Bacteriology can be performed as part of the initial diagnostic evaluation, or when bacteria are observed via histopathology in conjunction with pathologic lesions and clinical morbidity or mortality is present in other fish within the population. Some bacterial infections, such as surface gliding bacteria and *Mycobacterium* spp., can be identified to the genus or family level using simple Gram or acid-fast stains (e.g., Ziehl Neelsen) or distinct histological presentations. However, a more precise diagnosis of other bacterial infections often requires isolation of the bacteria in culture.

Only freshly euthanized fish should be used for bacteriological examinations as dead fish in the water are essentially worthless due to invasion of tissues by non-pathogenic bacteria. The optimal organs for isolating systemic bacteria are the kidney and spleen. Aerocystitis is commonly associated with bacterial infections, and hence, the culture of the swim bladder is often useful. The surface of the fish should be disinfected with 70% ethanol, and dissecting instruments should be sterilized. Two methods are used for obtaining inocula from the kidney or swim bladder, the dorsal approach, or through the coelomic cavity. Dorsal: Make an incision anterior to the dorsal fin, extending through the muscle and spinal cord, into the kidney or even more ventral into the swim bladder (Fig. 44.5A and B). Small inoculating loops are used to obtain samples, which are streaked on blood agar for Gram-negative bacteria, and specialized media for mycobacteria (See Whipps and Kent (2020), Chapter 44). Plates are then delivered to the appropriate diagnostic laboratory for further diagnosis. Placing inocula in liquid transport media is not recommended because it is difficult to avoid all extraneous, nonpathogenic bacteria. And these can quickly overgrow slower growing bacteria, such as *Mycobacterium* spp. and *Edwardsiella ictaluri*.

Mycobacteria are often slow-growers and require special media. However, they have characteristics (i.e., thick, waxy cell wall) that provide selective advantages for culture and isolations. Tissues are often decontaminated overnight in 1% cetyl pyridinium chloride (Whipps, Dougan, & Kent, 2007). Cultures are usually grown on Middlebrook (MB) 7H10 and Lowenstein–Jensen (LJ), and *M. haemophilum* requires 7H10 agar supplemented with OADC and 60 mM hemin (Whipps et al. 2007; Whipps & Kent, 2020, Chapter 44). For gliding bacteria (*Cytophaga*, *Flexibacter* and *Flavobacterium* spp.) are usually cultured from skin or gills, and *Cytophaga* Medium (Anacker & Ordal, 1959) is recommended. Although the lesions may exhibit massive numbers of gliding bacteria, other bacteria (e.g., *Aeromonas* spp.) may overgrow the former. Therefore, the best approach to obtain pure cultures of gliding bacteria is to homogenize the infected tissue (e.g., gills, skin, and muscle) in sterile water, and inoculate plates in serial log dilutions.

Gram-stained preparations may reveal bacteria when they are numerous in infected tissue. Smear or imprint suspect tissues thinly on a glass slide, air dry and fix the slide by gently heating the slide over an open flame

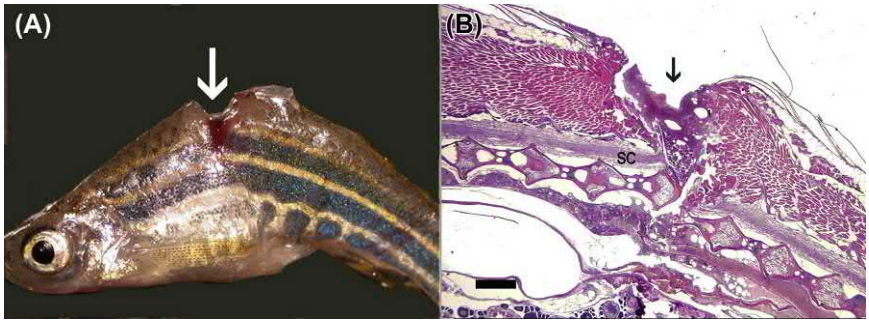

FIGURE 44.5 Culture for bacteria from kidney, dorsal approach. (A) Arrow = location of the incision to access the kidney. (B) Histological section showing the path of incision, through dorsal muscle, spinal cord (SC) and vertebrae to access the kidney (*arrow*). Bar = 50 μm.

for 3–5 s. Gram stain kits are available from scientific supply houses and include instructions for their use. Likewise, mycobacteria can be visualized in imprints stained with acid fast stains.

Bacteriology With Histopathology

Bacterial culture and histopathology can be performed on the same fish. This is particularly useful because of the risk of bacterial contamination of internal structures, and histology provides useful information to discern if the bacteria actually represent an infection. False-negative culture results may occur with an inaccurate sampling of focal infections or with slow-growing and difficult to culture bacteria (such as *Mycobacterium haemophilum*). False-positive cultures may occur with skin or gut contamination of samples, which is common with small fish, and in mixed bacterial infections, where the easily cultured bacteria are not responsible for the observed fish morbidity and mortality.

For Gram negative septicemia and aerocystis, we usually use the dorsal approach to perform aseptic cultures of the kidney and swim bladder (Fig. 44.5). For mycobacteria, we recommend removing a large portion of the spleen and part of the liver. These can then be examined by culture, acid-fast imprints or preserved in ethanol or frozen for PCR analysis. The remaining carcasses are fixed in Dietrich's fixative and processed for histological evaluation. We have found that both of the methods to obtain samples for bacteriology do not significantly compromise histological evaluations on the same fish.

Virology

Zebrafish are susceptible to numerous fish viruses, and even some human viruses. However, we are aware of two documented natural outbreaks of viral diseases in zebrafish (See Crim (2020), Chapter 42). As with bacterial diseases, isolation of viruses in culture may be required to diagnose a viral disease, and culture is best conducted on tissues collected from freshly killed fish. If this is not practical, the fish should be refrigerated for no longer than 24 h before examination. As a last resort, fish for virus examination can be frozen. The specimens are then transported to a qualified fish virology laboratory. As has been the case with other relatively new forms of fish culture, as fish health researchers thoroughly investigate outbreaks and incorporate tissue culture in their examinations, it is very likely that "new" viruses and viral diseases will be recognized in zebrafish.

Molecular Diagnositic Tests

Traditional or qPCR tests have been developed for several zebrafish pathogens for examining fish tissues, which include *P. neurophilia* (Sanders & Kent, 2013; Whipps & Kent, 2006), *Mycobacterium marinum*, *M. chelonae* and *M. haemophilum* (Meritet, Mulrooney, Kent, & Loehr, 2017), and *Edwardsiella ictaluri* (Griffin et al., 2016). Crim, Lawrence, Livingston, Rakitin, Hurley, & Riley (2017) described PCR tests for *Mycobacterium* spp., *Pseudoloma neurophilia*, and *Pseudocapillaria tomentosa*, and evaluated them for testing fish tissues versus environmental samples (see Nonlethal Testing). There are countless species of *Mycobacterium* that occur in the aquatic environment, and many are nonpathogenic or only potential opportunists. Therefore, molecular tests require the use of well-designed specific primers utilizing a variety of genes. With zebrafish mycobacteria, these have most often been the *hsp 70* gene (Meritet et al., 2017; Whipps et al., 2007). DNA fingerprint methods can also be employed to further characterize variations in strains within an outbreak (Ostland et al., 2008; Whipps et al., 2008).

For bacteria, molecular identification can be performed directly on fish tissues (Whipps et al., 2007; 2008) or on isolated colonies from cultures. The former has certain advantages as culture, as stated above, may be misleading. For example, a population of wild mollies and swordtails suffered an epidemic of mycobacteriosis based on the presence of granulomas. Here *M. chelonae* was cultured from a few fish, but the actual etiologic agent was an *M. triplex*-like organism that was never cultured (Poort, Whipps, Watral, Font, & Kent, 2006).

In recent years methods for retrival of DNA sequences from formalin preserved tissues, including those in paraffin blocks have significantly improved. Usually, it is more difficult to retrieve useful DNA from tissues that have been stored in formalin for extended periods of time. However, Peterson et al. (2013) were able to obtain diagnostic sequences of mycobacteria from about 50% of zebrafish in paraffin blocks in which tissues were stored in either Dietrich's or 10% formalin for up to 45 days before processing. Alternatively, DNA can be extracted from cores made in tissue blocks, allowing for precise targeting of bacteria within lesions (Meritet et al., 2017) (Fig. 44.6). This approach is particularly useful with mycobacteria as nonpathogenic *Mycobacterium* spp. often colonize the intestine, and thus, may confused diagnoses with whole-body sections. Conversely, it may be more difficult to extract DNA from dense, solid cores that thin tissue sections. Although about half of the paraffin blocks samples do not yield mycobacterial sequences, most cases involve submitting multiple fish. And this approach was useful

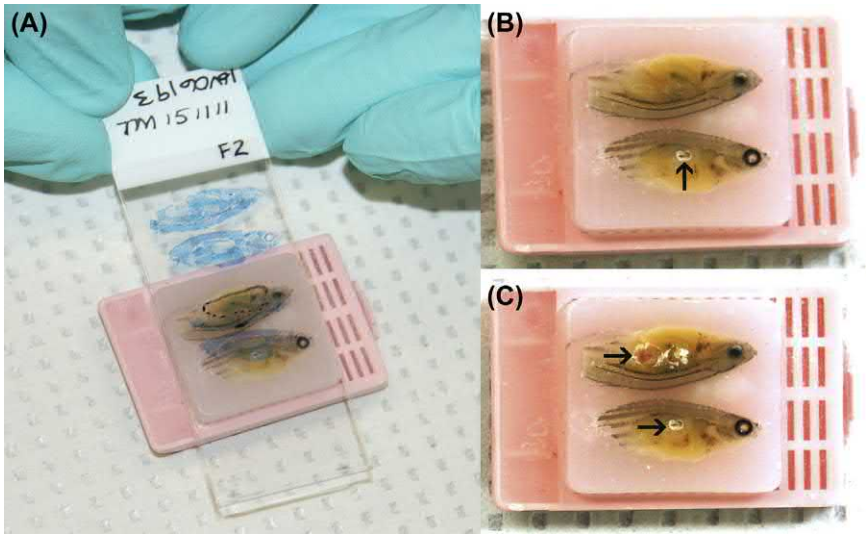

FIGURE 44.6 Coring blocks to target granulomas with acid fast bacteria for PCR analysis. (A) Granulomas with bacteria are matched to blocks. (B and C) are whole in blocks, with arrows demarking location of cores.

for conducting a retrospective study on the first occurrence of *M. marinum* in a large facility (Mason et al., 2016).

Nonlethal Testing

Some mutants or transgenic lines are available in only very small numbers, and therefore euthanasia of enough fish to obtain sufficient confidence that negative results are correct for the presence of a particular pathogen is often not practical. However, the small, confined tanks and frequent spawning of zebrafish provide advantages for developing such tests based on water, biofilms, feces, gametes, or spawn water. This is consistent with the concept of screening for the presence of pathogens in mouse colonies using feces or bedding (Bauer et al., 2016; Livingston & Riley, 2003).

Mycobacterial pathogens zebrafish occur both in the feces and water, and as biofilms on solid surfaces within tanks (Whipps et al., 2007; 2008; Mason et al., 2016). Accordingly, Crim et al. (2017) found that mycobacteria could readily be detected in feces, detritus, and water. Detritus and feces were useful for detecting *P. tomentosa*, which is consistent with the life cycle involving releasing of eggs in feces. Sugar centrifugation tests to detect parasite eggs in feces are fundamental in veterinary diagnostics, and can be employed to detect eggs of *Pseudocapillaria tomentosa* in feces collected from zebrafish tanks (Martins, Watral, Rodrigues-Soares, & Kent, 2017; Murray & Peterson, 2015). This is particularly useful, as the eggs of this nematode have the distinctive capillarid morphology with bipolar plugs, and hence can be easily differentiated from other nematode eggs and other organisms found in tank detritus.

Crim et al. (2017) found that environmental samples, including water, feces, and detritus, were not useful for detecting the microsporidium *Pseudoloma neurophilia*. In contrast, water collected following a spawning (e.g., from spawning tanks) is useful for PCR testing for *P. neurophilia* as the parasite is common in eggs and other material in the ovary (Sanders & Kent, 2011). This study found that very few eggs are positive by PCR, which agrees with Crim et al. (2017). PCR methods have recently been developed that have improved the detection of DNA from environmental samples (eDNA). One of these is droplet digital PCR (ddPCR) (Rothrock et al., 2013). We are using this format to detect zebrafish pathogens in water. This approach partitions the sample into hundreds of millions of water-in-oil droplets before thermal cycling; essentially running thousands of PCR reactions, and it thus greatly reduces inhibition with nontarget DNA and other environmental inhibitors. At this point, we have improved detection of *P. neurophilia* in tank water and can detect *P. tomentosa* DNA in water long before eggs are released in feces from adult female worms. Most diagnostic laboratories do not use the droplet digital system, but with its accuracy and sensitivity, it provides a very useful foundation for developing other PCR water tests.

Blood can be used for nonlethal testing for pathogens from larger fish (Whipps et al., 2005). Although challenging due to their small size, blood can be nonlethally collected from blood using special techniques (Zang, Shimada, Nishimura, Tanaka, & Nishimura, 2015). Presently there are no reported blood tests for zebrafish pathogens.

Treatment

Details for treating specific infectious and noninfectious diseases are provided in other chapters (Kent & Sanders, 2020, Chapter 43; Whipps & Kent, 2020, Chapter 44; Murray et al., 2020, Chapter 45). Here we provide a general overview of common methods used for administering antibiotics and antiparasitic compounds. There are many texts that review treating infectious diseases in food and ornamental fishes (Treves-Brown, 2000; Noga, 2010). These approaches and methods can generally be applied to zebrafish, but laboratory zebrafish provide some unique challenges. First, concerns about nonprotocol induced variation should be considered when administering therapuetants to fish being used in experiments. Second, many therapeutants are administered by adding them directly to water, particularly for external parasites (Kent & Sanders, 2020, Chapter 43), and some of these may be detrimental to beneficial bacteria in biological filters (Pedersen, 2009). Last, some drugs are administered by injection, and this is often impractical for many zebrafish laboratories.

Treatments Delivered in Water

Application of chemotherpeutants directly to water to control external parasites and bacterial infections is a common practice in aquaculture. Bath treatments, most often formalin, for about 1 hour or extended periods are effective for treating external parasites (See Kent & Sanders, 2020, Chapter 43). Formalin reduces dissolved oxygen levels, so it is important to maintain active aeration of the treated water. Sodium chloride baths are routinely used for reducing parasite burdens (Noga, 2010), and they offer an additional benefit in that fish with skin and gill infections often have reduced osmoregulatory capabilities, and hence adding salt at the appropriate level will reduce hypoosmotic stress.

Emamectin benzoate, an analog of ivermectin, is a macrocyclic lactone. This class of drugs is well recognized for its efficacy for treating intestinal nematodes, including by external delivery. A commercial product of emamectin benzoate (Lice-Solve) is available for treating external copepods by adding the product to water. We recently showed that this product is very effective for treating *Pseudocapillaria tomentosa* infections using bath treatments (Kent, Watral, Gaulke, & Sharpton, 2019).

Oral Treatments

Treatment of feed is now the most common approach in large aquaculture facilities, and more recently, public aquariums are employing this approach. With aquaculture, large quantities of drugs are incorporated into the diet as a premix by feed manufactures, or the diet is top coated with drug mixtures after pellets are made (Burridge, Weiss, Cabeelos, Pizzarro, & Bostick, 2010; Noga, 2010). This approach has been used for treating *P. tomentosa* infection with emamectin benzoate and ivermectin in zebrafish (Collymore et al., 2014; Kent et al., 2019). Gelatin-based diets containing antibiotics are often used in public aquariums, and this approach has been used to deliver antibiotics for treating mycobacteriosis in zebrafish (Chang, Doerr, & Whipps, 2017).

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SECTION V

Scientific Research

CHAPTER

45

Zebrafish as a Model to Understand Vertebrate Development

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Introduction to Embryology

Developmental Stages and Conservation

Chordates, more colloquially referred to as vertebrates, cleave the zygote into thousands of cells with each daughter cell half the size of its parent. These cleavages continue until cells start to move, grow, and specialize as different types of cells during gastrulation. A seemingly chaotic amount of tissue morphogenesis occurs over the course of gastrulation that precisely positions the three earliest germ tissue types: ectoderm, endoderm, and mesoderm. Ectodermal cells give rise to the nervous system located toward the back or dorsal-most region of the embryo, as well as contribute to the epidermis that covers the entire embryo. A primary outcome of these gastrulation movements is the proper positioning of both the endodermal and mesodermal derivatives within the embryo along all the basic axes (See also reviews on early development Hasley, Chavez, Danilchik, Wühr, & Pelegri, 2017; O'Farrell, 2015; Stower & Bertocchini, 2017). Importantly, the unifying feature of chordates is the notochord, which is formed at the midline of the gastrula during the initiation of mesoderm development (Annona, Holland, & D'Aniello, 2015).

The central nervous system, which is the first major organ system to develop following gastrulation, forms from the creation of a rudimentary tube sitting atop the notochord. This *neural tube* ultimately creates the brain and spinal cord along the full length of the anterior to the posterior axis (O'Connell, 2013). Soon after the onset of neural tube formation the entire vertebrate trunk elongates, portions of which become sequentially segmented into similarly sized blocks of cells known as somites (Bénazéraf & Pourquié, 2013; Hubaud & Pourquié, 2014; Maroto, Bone, & Dale, 2012; Rashid et al., 2014; Resende, Andrade, & Palmeirim, 2014). Differentiation of somites will contribute to the development of skeletal muscle, portions of the circulatory system, and most notably the bones and cartilage of the vertebrae. The embryonic period culminates with organogenesis, which marks the time when an array of different organ types likes the heart, gut, kidney, liver, pancreas, and others are established throughout the embryo (Miquerol & Kelly, 2013; Zorn & Wells, 2009).

The last moments of embryogenesis are known as the "pharyngula" stage, a stage when all vertebrates physically look the most similar (Collazo, 2000). Generally speaking, each embryonic part from the regions of the brain and eyes to the heart and segmented trunk are all similarly located and structurally resemble each other to such a degree that the conservation of their development is visibly undeniable. It is this biological conservation that enables researchers to use different vertebrate species as proxies or "model systems" to gain valuable insight into how humans may even develop (Dietrich, Ankeny, & Chen, 2014).

Genes and Development

Cleavage, gastrulation, neurulation, somitogenesis, and organogenesis are the progressive stages of embryonic development that are made possible through the precisely timed regulation of gene expression. With the sole exception of the gametes (sex cells), all the zygote-derived cells that make up an individual organism possess identical genomes (for instance, the DNA sequences in one's gut epithelial cell is identical to the

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DNA sequences found within the photoreceptor cell of their eye). This fact presents a profound realization of one of the key mechanisms of embryonic development, that of *differential gene expression*. Cell differentiation is based on the different ways cells use the genome to turn on (express) or off (repress) specific genes. The resulting composition of genes expressed will lead to the repertoire of proteins for a given cell that will provide its unique structural and functional properties. Therefore, embryonic development is foundationally governed by controlling which genes are expressed at different times and places. Most recently, zebrafish researchers have advanced the use of single-cell RNA sequencing to characterize *all* the transcripts (mRNAs) present in *all* the cells of embryos at different embryonic stages (reviewed in Harland, 2018). Analyses of this enormous amount of data with nearest-neighbor computational approaches and cross-referencing with known gene functions has enabled the visualization of "developmental trees"—3-Dimensional representations of the transcriptomic identity of each cell as it matures over the course of embryogenesis (Farrell et al. 2018; Wagner et al. 2018). See "movie S1" from Farrell et al., 2018 to see the trajectories of cell specification over early embryogenesis.

Due to the importance that a cell's transcriptome plays in defining its identity, the field of developmental biology has been ever captivated with determining how cells acquire these patterns of differential gene expression. Over the course of the blastula and gastrula stages, cells appear visibly similar and will, however, gradually become spatially distinct across the embryo. The environment cells experience in different regions of the embryo can profoundly influence the genes those cells ultimately express and consequently, the cell fates they adopt. Much research in developmental biology has been devoted to identifying and characterizing the myriad of regionally restricted signals present throughout embryogenesis and how such signals influence gene expression, cell differentiation, morphogenesis, and other developmental outcomes (Perrimon, Pitsouli, & Shilo, 2012; Wilcockson, Sutcliffe, & Ashe, 2017).

Several families of signaling proteins have been found to be essential for embryonic development, these include, but are not limited to, the superfamily of Transforming Growth Factors (TGFs, like bone morphogenic proteins or BMPs), and families of Wnts, Fibroblast Growth Factors (Fgf), and Hedgehogs (like Sonic hedgehog, Shh). Secretion of these proteins into the extracellular environment or their active transport can be received and interpreted by receptors in a cell's outer (plasma) membrane, which triggers progressive changes inside the cell resulting in a diversity of responses like alterations in gene expression, remodeling of the cytoskeleton to influence cell shape and movement, or promotion of cell division. Much attention has been placed on deciphering the array of downstream transcription factor proteins that these signaling systems effect, efforts that are providing insights into the whole gene regulatory networks responsible for specific tissue type development (Ettensohn, 2013; Ferg et al., 2014; Nowick & Stubbs, 2010; Peter, 2017; Peter and Davidson, 2009, 2017). Use of the zebrafish model system has greatly advanced our understanding of the genetic, molecular, and cellular mechanisms governing each stage of vertebrate embryogenesis.

The Emergence of Zebrafish as a Versatile Vertebrate Model System to Understand Embryology

From the fruit fly to the mouse, different model systems have provided unique advantages to dissecting the developmental mechanisms of embryogenesis. Among the many diverse advantages the zebrafish model system has to offer, it was initially adopted for research primarily because of its strong applicability to the study of embryonic development (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). For practical reasons the small size of the adult zebrafish enables many thousands of fish to be housed in a relatively small laboratory space making the zebrafish significantly more cost-effective than other vertebrate model systems. Most importantly, a single female and male zebrafish can produce hundreds of embryos a week. Being able to house thousands of adult fish and pairs of which producing hundreds of embryos all together makes genetic analyses feasible. At this time, it is clear that the numerous forward genetic screens conducted on zebrafish have yielded the field's greatest contributions to our understanding of embryogenesis. Moreover, the last 2 decades of zebrafish research has also amassed an ever-growing battery of powerful genetic and molecular approaches to both see and manipulate specific genes, cells, tissues, and whole physiological systems. Namely the creation of robust transgenic tools for cell-type specific reporter fish lines to visualize phenotypes even in live embryos, and for gene inducible techniques to answer both gain- or loss-of-function questions (as exemplified and reviewed in Halpern et al., 2008; Higashijima, Hotta, & Okamoto, 2000; Moro et al., 2013; Weber & Köster, 2013). Lastly, the ever-evolving approaches to capture and interrogate the zebrafish genome are providing novel insights from the specification of cell lineages to the comparative evolution of fish and humans (Gehrke et al., 2015; Harland, 2018; McCluskey & Postlethwait, 2015; McKenna et al., 2016; Pan et al., 2013; Pasquier et al., 2017).

Critical to making any gene or embryological manipulation effective is the ability to see or assess changes in embryonic morphology and progression. The accessibility of the zebrafish's external development, paired with the embryo's near transparency permits easy and direct microscopic visualization of each major embryonic structure. Additionally, this unique visual accessibility, combined with its rapid embryogenesis over a 24-hour period, allows researchers to quite simply watch development over time (Karlstrom & Kane, 1996). Today this advantage is being exploited to even higher degrees of resolution with the advent of more sophisticated live embryo imaging techniques, like laser-scanning confocal, two-photon, and light-sheet microscopy (Keller, 2013; Keller, Schmidt, Wittbrodt, & Stelzer, 2008; Royer et al., 2016). Researchers have also taken advantage of the visible accessibility of zebrafish by perfecting the use of physical manipulatives, such as, but not limited to, the bathing of embryos with specific compounds, chemicals, and other environmental alterations, the microinjection of dyes, DNA and RNA, or even the transplantation of cells from one embryo to another. Thus, the zebrafish model system offers many advantages that have dramatically helped to reveal the developmental mechanisms that are driving the vertebrate embryogenesis.

Zebrafish Research Advances Our Understanding of the Mechanisms Governing Development

Early Development In a "Fish-Shell"

Cleavages and Maternal Contributions: Externally developing vertebrates like zebrafish have provided a remarkable advantage to studying the mechanisms of early development from conception through gastrulation (See Fig. 45.1). Whether glaring though the outer protective chorion shell or experimentally observing a dechorionated embryo, simply being able to watch each cell division during blastula development has demonstrated some of the defining features of embryonic cleavages in vertebrates. Zebrafish cleavage is incomplete or meroblastic such that a blastoderm (cells of the embryo proper) is built with each cell division in the animal pole, while a single yolk cell is retained in the vegetal hemisphere and an outermost enveloping layer (EVL) develops over the embryo. These animal pole restricted cleavages occur as near synchronous waves of mitoses that pass across the entire blastoderm, each time resulting in an equal halving of the cell size. As the amount of cytoplasm to DNA approaches more equal proportions, cell movement becomes visible which marks the moment when a blastula begins gastrulation (Kane, Warga, & Kimmel, 1992; Kane & Kimmel, 1993).

Prior to conception critical preparatory events occur during oogenesis, namely the storing of maternal mRNA (transcripts) and protein contributions in the oocyte. These maternal contributions are themselves sufficient to drive all the mechanics of cell division during cleavage stages. For instance, *futile cycle* mutants fail to undergo pronuclear fusion leading to only 2 cells of the blastula actually possessing DNA containing nuclei, and despite this lack of DNA all *futile cycle* cells still undergo normal cleavages (Dekens, Pelegri, Maischein, & Nüsslein-Volhard, 2003). However, maternal factors alone are not sufficient for continued development past the blastula stage, and active transcription from the "zygotic genome" is required (Lee et al., 2013). This moment of shifting from maternal to zygotic control is known as the "midblastula transition" or "maternal-zygotic transition" (MZT) (Lee, Bonneau, & Giraldez, 2014; Miccoli, Dalla Valle, & Carnevali, 2017; Wragg & Müller, 2016).

Investigations into the zebrafish MZT have provided important insights into the role that microRNAs (miRNAs) play in degrading the maternal RNA stores to facilitate the rapid transition of developmental control to the zygotic genome (Lee et al., 2014). miRNAs do not code for a protein but rather can function in the RNA-induced silencing complex (RISC) as a sequence-specific guide to target the complex's ability to degrade RNA transcripts. More specifically, miR-430 has been demonstrated to be essential for the MZT as it is responsible for both translational repression and the targeted decay of hundreds of maternally provided transcripts (Bazzini, Lee, & Giraldez, 2012; Giraldez et al., 2006). As these maternal mRNAs diminish over the course of cleavages, the expression of zygotic mRNAs ramps up to regulate the rest of embryogenesis.

Gastrulation and Axis Determination: The zebrafish blastula culminates into a perfect sphere with nearly all of the animal pole composed of embryonic cells and the vegetal pole occupied by the yolk (See Fig. 45.1). Gastrulation is first visibly evident by the asymmetric collection of cells at the margin, which resembles a small bulge at the surface equidistant between the poles - as if this very early embryo were holding a *shield.* As a result of this physical feature, the start of zebrafish gastrulation is commonly referred to as the "shield" stage. Important gastrulating cell behaviors occur prior to the visible shield, namely the movements of radial intercalation that drive epiboly. Deeper blastula cells of the epiblast move radially outward to join more superficially positioned layers of the epiblast. Such cell intercalation causes the entire population of blastula cells to flatten and consequently spread outward toward the vegetal pole. This is the gastrulation movement called *epiboly*, and it will assist in powering the complete coverage of the yolk with

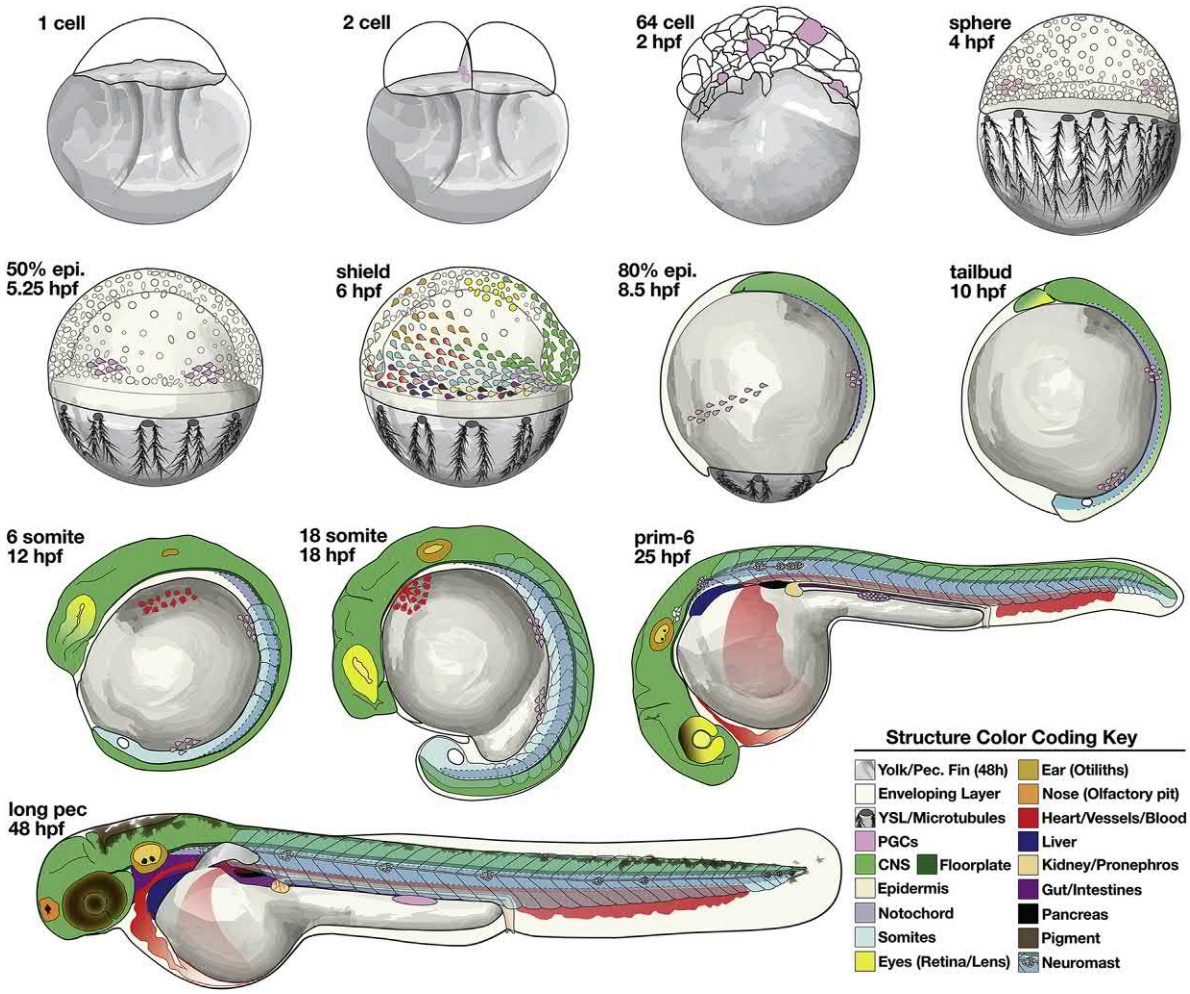

FIGURE 45.1 **An Illustrative overview of zebrafish embryonic development.** This developmental series progresses left to right and from top to bottom. The first four illustrations are representative of the cleavage period during which cells get smaller upon every division. Primordial germplasm is evident at the two-cell stage along the division plane (pink), while four discrete primordial germ cells (PGC) can be seen at the 64 cell stage that establishes four multicell clusters by the *sphere* stage (two shown). At the *sphere* stage, the enveloping layer covers the animal hemisphere stretching down to the yolk syncytial layer (YSL) where yolk syncytial nuclei and microtubules extend toward the vegetal pole. Next, gastrulation commences, as many mechanisms like contraction along these microtubules draws down the embryonic cells from 50% epiboly to 80% and finally full closure of the blastopore at the tailbud stage. The first indication of dorsal and ventral axes is visible at the shield stage, where the concentrated convergence of cells upon the midline produces a physical budge of cells. The most dorsal cells are destined to contribute to the development of the nervous system (green). Dorsal is to the right of each embryo shown from shield to tailbud. Individual representative cells are mapped at the shield stage in locations and colors indicative of their future fates (see structure color coding key). The pointed shape of these illustrated cells also denotes their directional migration/movement during gastrulation. Kupffer's vesicle is an early sign of posterior growth and left-right patterning (white circular structure in the tail from tailbud to 18 hpf). Mesoderm induction during gastrulation will give rise to the axial mesodermal structure of the notochord (light mauve color delineated with *dotted lines*), and as the tail extends the paraxial mesodermal structures of the somites (light blue block/chevron-shaped structures transparently illustrated) will sequentially form from anterior to posterior over time. The PGCs complete their long migratory journey around 24 hpf and develop into the gonads. Placodal structures in the ectoderm (neural and epidermal tissues) build the eye (yellow), ear (honey color) and nose (orange) organs. Development of the heart and circulatory system begins following the tailbud with bilaterally positioned cells that migrate over to the midline and converge to form the heart (only one population of cells shown, red), after which an elaborate vasculature system is created (only the dorsal aorta and vena cava with posterior blood island shown; high to low red gradient represents flow directionality). Development of the gut, kidney, liver, and pancreas are all illustrated in the approximate positions (see key). Vascularization of the glomerulus (kidney) is shown at 48 hpf. Key for staging embryos between 24 hpf and day 2 is the position of the lateral line primordium, which migrates from the brain to the tail atop of the middle of somites depositing neuromasts along the way (see key). 48 hpf is known as the long pec stage due to the presence of the extended pectoral fin tissue (show above the yolk, gray pattern). Although pigment can be first seen at 24.5 hpf in the dorsal region of the retina (eye, brown), body pigment increasingly appears between days 1 and 2 (only the dorsal most melanocytes are shown, 48 hpf). Illustration by Michael J.F. Barresi ©2018.

ectodermal cells by 10 h postfertilization (hpf) or the tailbud stage (Bruce, 2016).

Differential expression of the types and amounts of cell surface adhesion receptors, such as from the cadherin family, have been shown to be responsible for these radial intercalation movements. Loss of *E-cadherin* in the *half-baked* zebrafish mutant causes it to fail to progress beyond 70% epiboly and ultimately mutants arrest in development (Kane, McFarland, & Warga, 2005; McFarland, Warga, & Kane, 2005). In addition, epiboly progression in zebrafish is also influenced by the yolk syncytial layer that forms at the vegetal-most edge of the blastoderm. Of most significant relevance is the elaborate cytoskeletal networks affiliated with the external yolk syncytial nuclei (eYSN) (See Fig. 45.1 Bruce, 2016). The eYSN (and their centrosomes) project an expansive network of longitudinal microtubules toward the vegetal pole, as well as organize an actomyosin contractile ring at the blastoderm edge. By exposing zebrafish gastrulae to cytoskeletal blocking drugs applied to their growth media or using targeted laser ablation methods, researchers have shown that both the microtubules and actomyosin elements are in fact required for completion of blastopore closure and the full enwrapping of the embryo by the external enveloping layer (Behrndt et al., 2012; Solnica-Krezel & Driever, 1994; Strähle & Jesuthasan, 1993).

As epiboly pulls the blastoderm toward the vegetal pole, novel cell behaviors are simultaneously occurring at the location of the "shield." The midline positioned shield is akin to the dorsal blastopore lip (DBL) in frogs and salamanders or the node in mouse and chick (Thisse & Thisse, 2015). The shield represents the presumptive *dorsal* side of the embryo, as well as the location of mesendodermal induction (Heisenberg & Tada, 2002; Rohde & Heisenberg, 2007). Recently, researchers using light-sheet microscopy have recorded the position, movements and division patterns of every individual cell through the entire process of zebrafish gastrulation. This imaging has revealed a slowing in cell division specifically in the presumptive shield prior to any visible thickening, as well as quantifying the cell dynamics driving the primary internalization of the hypoblast by involution (Keller, 2013; Keller et al., 2008). This level of quantification from a single cell to the whole organism is unparalleled for the analysis of vertebrate embryogenesis. The tissue thickening that happens at the shield is due to the opposed mediolateral intercalation of cells on both sides of the midline. This midline convergence of cell movement produces a buildup of cells at the presumptive shield location that provides the cellular infrastructure to support early neural keel development (beginnings of the brain and spinal cord) development, as well as powering the involuting movement of presumptive hypoblast cells. Involution is the inward folding of the blastoderm that is first initiated at the shield and will create a second layer of cells underlying the epiblast called the *hypoblast*. As the hypoblast forms, it is rapidly patterned into dorsal and ventral endodermal and mesodermal precursor cells. Importantly, chordamesoderm, the predecessor of the notochord, is also derived from the cells of the involuting shield (Concha & Adams, 1998; Cooper & D'amico, 1996; D'Amico & Cooper, 2001; Warga & Kimmel, 1990).

The notochord is the defining feature of chordates and possesses important structural and regulatory roles throughout embryonic development (Annona et al., 2015; Lawson & Harfe, 2017). The notochord is formed at the quintessential midline through the bilateral convergence of hypoblast cells upon this location (See Fig. 45.1 Shindo, 2018). Chordamesoderm cells uniquely form a fluid-filled vacuole and a thick extracellular matrix that together build its characteristic rod-shaped morphology, which helps to provide skeletal-like support for the elongation of the embryonic axis (Ellis, Bagwell, & Bagnat, 2013a; 2013b). Moreover, the notochord will function as a central source of patterning signals to all surrounding tissues, such as the overlying neural keel, the bilaterally positioned paraxial mesoderm, as well as ventral mesendoderm derivatives like the gut and vasculature (Anderson & Stern, 2016). Interestingly, forward genetic screens using zebrafish identified many key transcription factors regulating notochord development (exemplified in Odenthal et al., 1996). Loss of *floating-head (Xnot)* and *no tail (brachyury)* homeodomain transcription factors result in a complete absence of the notochord (Fig. 45.2A–D). However, due to differences in the resulting cell fates that occupy the now vacant midline in these mutants (muscle or mesenchyme for *floating-head* and *no tail* respectively), researchers were able to decipher the cell lineages that *floating-head* and *no tail* were normally preventing by the promotion of notochord development (Amacher, Draper, Summers, & Kimmel, 2002; Halpern, Ho, Walker, & Kimmel, 1993; Melby, Kimelman, & Kimmel, 1997; Schulte-Merker, van Eeden, Halpern, Kimmel, & Nüsslein-Volhard, 1994; Talbot et al., 1995). Characterization of these genes illustrated the fundamental regulatory roles that transcription factors play on whole gene networks to control cell fate decisions during development.

Lastly, both forward and reverse genetic approaches have identified many essential genes for the correct patterning of germ layer cell fates across all axes of the developing gastrula. This work has demonstrated a prominent role for morphogenetic signaling proteins in regulating differential gene expression and cell fate induction during early development (For more extensive reviews see Thisse & Thisse, 2015; Zinski, Tajer, & Mullins, 2017a). In fact, complex spatial and temporal models

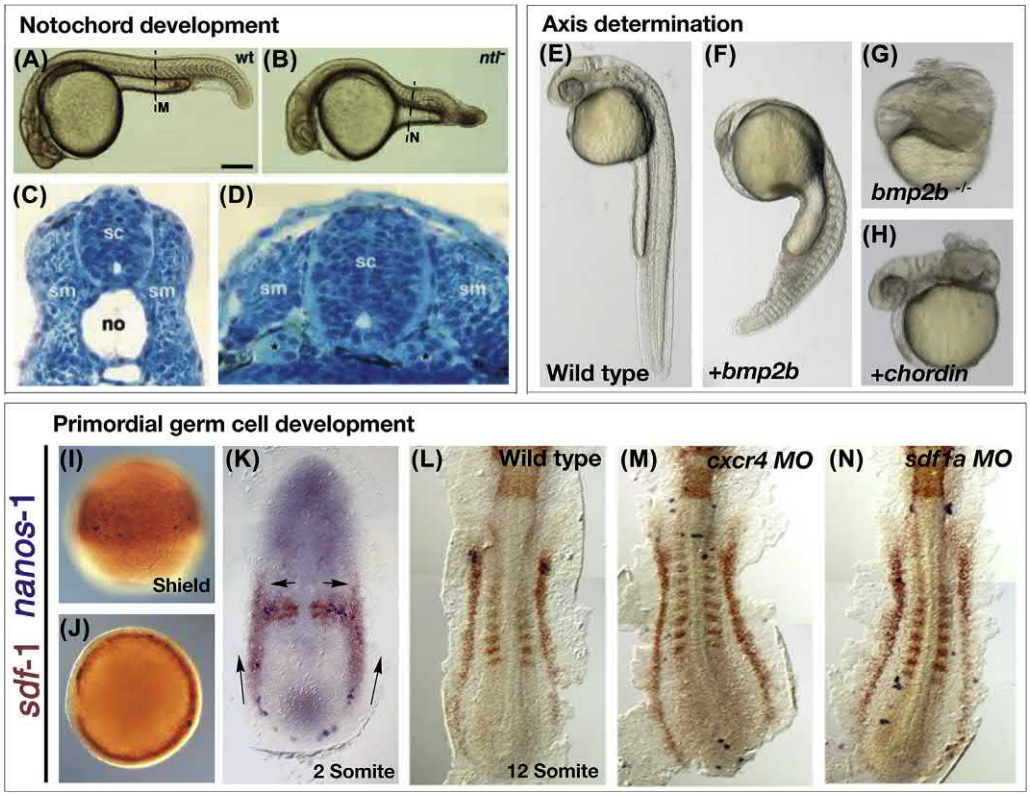

FIGURE 45.2 **Early development.** (A-D) *no tail (brachyury)* is required for the development of the notochord (no) as the notochord is completely lost in *ntl* mutants seen from lateral (A,B) and cross-sectional (C,D) views (Amacher et al., 2002). (E–H) Lateral views of 30 hpf embryos showing the ventralizing effects of *bmp2b* misexpression and the dorsalizing effects of a loss of *bmp2b* or misexpression of *chordin* (Pomreinke et al., 2017). (I–N) *nanos-1* expressing primordial germ cells overlap specifically with *sdf-1* expression patterns during PGC migration unless *sdf1a* or its receptor *cxcr4* are knocked down by morpholino (MO) (Doitsidou et al., 2002).

of signaling systems involving the Bmp, Nodal, Wnts, and Fgf pathways have all been implicated in early axis determination (as demonstrated in Zinski et al., 2017). For example, a loss of the signals encoded by *bmp4/7* or their downstream responsive transcription factors *smad1/5* result in a failure to develop more ventral structures like the tail, whereas loss of the Bmp antagonist, Chordin, causes the reciprocal effect of a reduction in head and neural tissues that are representative of more dorsal fates (Fig. 45.2E–H Pomreinke et al., 2017).

Primordial Germ Cells: Independent of the three germ layers, there is yet another important cell type developing during these early stages of embryogenesis, the *germ* cells. The germ cells referred to here will go on to form the gametes or sex cells within the gonads (ovaries or testes). Amazingly, known germ cell-specific transcripts (mRNA) can be detected in the two-cell stage embryo, and over the next few cleavages, they form four asymmetrically positioned clumps localized to plasma membrane borders (See Fig. 45.1 Braat, Zandbergen, van de Water, Goos, & Zivkovic, 1999). These clumps contain not only RNAs but proteins and mitochondria that together are known as the *germplasm*. Prior to the 1000-cell stage, these four clumps of germplasm are inherited into four separate cells at which point are considered the primordial germ cells (PGCs). The first four PGCs are relatively equally dispersed across the blastula, which is followed by an elaborate process of cell migration, ultimately to the site of gonadal differentiation (Paksa & Raz, 2015).

PGC migration is arguably one of the most remarkable developmental feats. Over the course of several divisions, the PGC population reaches near 50 cells in total. These cells actively migrate from their four different starting points to the presumptive gonads, which is a journey through the remaining cleavages and all of the chaotic movements of gastrulation and segmentation until they finally reside in two bilaterally positioned cell clusters just ventral to somites 8–10 (See Fig. 45.1 Raz, 2003). Analysis of PGC development in zebrafish has provided great insight into the mechanisms regulating their guidance to the presumptive gonad. Specifically, being able to watch PGCs in the live embryos with specific transgenic reporters has led to a "tumble and run" model of PGC migratory behaviors (Blaser et al., 2005; Reichman-Fried, Minina, & Raz, 2004). In this model, PGCs migrate briefly in one direction and then pause to reevaluate their new

location before migrating again. PGCs are following precisely positioned guidance cues in the embryonic environment, which PGC specific transmembrane receptors recognize as attractive or repulsive (Paksa & Raz, 2015). One guidance system that is evolutionarily conserved is the secreted protein Stromal Derived Factor 1 (Sdf1a/1b) and its PGC expressed receptor CXCR4 (Fig. 45.2I–N**)**. Based on both the expression pattern of Sdf1a/1b and loss of function testing for both *sdf* and *cxcr4*, the Sdf1 ligands function as attractive, migratory promoting cues for which the Cxcr4 receptor interprets (Doitsidou et al., 2002). In this way, PGCs will "run" toward the greatest concentrations of Sdf1a/1b, which is displayed in a gradient of expression leading directly to the gonadal precursor cell domain.

Kupffer's vesicle—Determinant of organ laterality: Unpaired internal organs of zebrafish, such as the liver, gut, pancreas, and heart display a characteristic asymmetry in their position either to the left or right side of the midline. This asymmetry has been determined to be regulated by the transient, spherical cyst-like structure termed *Kupffer's vesicle* (KV), which encloses a fluid-filled lumen. Functionally, KV is equivalent to the embryonic node of mammals (Nonaka et al., 1998) and can be observed in zebrafish embryos between 4 and 15 somite stages underneath the notochord and posterior to the yolk tube extension (See Fig. 45.1 Essner et al., 2002). The KV is formed of epithelial cells, each projecting a single cilium from its apical surface. Higher columnar shape and density of ciliated cells in the more anterior-dorsal section of the KV creates an overall leftward flow within the lumen fluid (Wang, Manning, & Amack, 2012). This induces preferential leftward expression of components in the Nodal-Lefty signaling pathway that instructs unpaired organs to form in an asymmetric manner, which can be visualized by uneven symmetry in the expression of genes, such as *southpaw* and *pitx* (reviewed in Joseph Yost, 1999). Gene mutations that affect the motility of KV cilia have also been shown to perturb cardiac laterality during zebrafish development (Choksi, Babu, Lau, Yu, & Roy, 2014; Kramer-Zucker, 2005).

The progenitors of KV originate from a specialized cluster of approximately 25 dorsal forerunner cells (DFC) that emerge around the shield stage (Melby, Warga, & Kimmel, 1996). The DFCs do not involute during gastrulation, but instead, migrate toward the leading edge of the blastoderm margin, move deeper and then undergo mesenchyme to epithelial transformations (Cooper & D'amico, 1996). KV architecture defects seen upon chemical inhibition of Nodal signaling and in mutants of nodal interacting proteins, such as *gdf3* suggests that the nodal pathway is also necessary for proper patterning of the KV during its early development (Pelliccia, Jindal, & Burdine, 2017). The development of KV also depends upon the T-box transcription factor encoding genes *no-tail (brachyury)* and *tbx16*, which regulate KV's mesenchymal to epithelial cellular transition (MET) (Amack, Wang, & Yost, 2007). Although the specific signal that induces leftward patterns of gene expression remains unclear and debated, many mutations that perturb left-right (L-R) asymmetry during zebrafish development have been traced to cilia genes. Further investigation of these cilia genes, as well as the observed correlations of asymmetric patterns in membrane potential, are promising areas for *development*.

Development of the Central Nervous System

Neurulation: The nervous system, which is the first organ system to develop, is *centrally* located at the dorsal midline. The earliest morphogenesis and specification of neural ectoderm occurs at the beginning of gastrulation, and this tissue will develop into the presumptive brain and spinal cord. Prior to the visible elaboration of the brain into its fore-, mid- and hindbrain regions and the spinal cord with its extended motor and sensory nerves, a rudimentary tube first needs to be constructed. From Sea squirt to primates all chordates develop an embryonic neural tube through a process called *neurulation*. Evolutionarily related to the invertebrate "nerve chord," the overall mechanisms for nervous system development across species are highly conserved. Although careful comparison of cell and tissue dynamics underlying neurulation between anamniotes (fish and frogs) and amniotes (birds and mammals) reveals some important differences in the ways by which such similar tubes are formed (Harrington, Hong, & Brewster, 2009; Korzh, 2014; Lowery & Sive, 2004).

All neurulation begins with the pseudo-epithelialization of the neural ectoderm into a plate. Interestingly, how teleosts like zebrafish change this plate into a tube is the primary difference from some other vertebrate species (Cearns, Escuin, Alexandre, Greene, & Copp, 2016). For instance, the neural plates of mouse and chick physically bend at discrete points parallel to the rostral-caudal axis, which serves to bring the folded edges in direct opposition to each other leading to their fusion and consequent separation from the presumptive surface epithelium (reviewed in Massarwa, Ray, & Niswander, 2014). The zebrafish neural plate does not undergo this type of folding morphogenesis. Rather, the bilateral convergent-extension movements during late gastrulation continue to force the stacking of pseudostratified epithelial cells upon the midline directly above the developing notochord. This mounting compaction of centrally positioned cells forms a precursor structure called the *neural keel*, which will continue to compress into a similarly epithelialized neural rod.

The functioning adult nervous system requires a central ventricle or lumen in which cerebral spinal fluid can flow. Amniotes maintain the continuity of apical to basal polarity throughout folding and fusion of the neural plate, which establishes the central ventricle upon closure. In contrast, many of the cells of the zebrafish neural rod cross the imaginary plane of the midline with some cells even maintaining contact to both lateral edges of the rod (Cearns et al., 2016). For creating a lumen through the full extent of the neural rod (formally referred to as the neurocoel), the midline needs to gain apical polarity. This is the step during zebrafish neurulation known as *cavitation*, during which the subcellular recruitment of apical junctions is targeted to the absolute midline of the neural rod. Polarized cell divisions connected to these new apical junctions establish an overall apical to basal polarity within each half of the developing neural tube, which creates the neurocoel and the completion of neurulation (Buckley et al., 2013).

Not surprisingly, adhesion proteins like N-Cadherin and E-Cadherin are required for the proper morphogenesis of the neural tube in all vertebrate species tested (Biswas, Emond, & Jontes, 2010; Detrick, Dickey, & Kintner, 1990; Hong & Brewster, 2006; Lele et al., 2002; Morita et al., 2010; Radice et al., 1997). More recently, researchers have used the zebrafish as a model to understand better the mechanisms governing the establishment of apical to the basal polarity that is suggested to be necessary for neurocoel formation. By both visualizing and directly testing the requirements of key apically restricted proteins like Partitioning defective 3 (Pard3), we now know that these apical proteins are first shuttled along microtubules within a cell to a position aligned with the neural rod's midline center (Buckley et al., 2013; Tawk et al., 2007). Remarkably, such positioning of these apical-defining proteins will occur at any asymmetric location as long as it is aligned at the midline, which ultimately coordinates cell division upon this plane, creating two symmetrical daughter cells. Loss of function of Pard3 or other related apical proteins causes a failure of an organized apical-basal axis, as well as a lack of lumen development (Buckley et al., 2013).

Neural tube patterning: The adult central nervous system (CNS) functions through the precise organization of an enormous diversity of different neuronal networks from the forebrain to the most posterior portion of the spinal cord. Neurons extend long, cell processes called *axons* to a target cell where a synapse is formed, and the electrical to the chemical transfer of neuronal signaling takes place. Moreover, the CNS is equally occupied by different types of glial cells that function to provide the insulating myelin sheath around axons (oligodendrocytes), support synapse signaling, provide structural support, establish the blood-brain barrier (astrocytes or astroglia), and offer immune responses (microglia). Lastly, neural stem cells reside in both the developing and adult brain. Radial glial cells (considered a type of astroglia) serve as the neural stem cell throughout embryogenesis and fetal development in all vertebrates; however, while radial glia maintain this role in the adult zebrafish brain they are transformed into a different neural stem cell morphology called *B-cells* in the adult mammalian brain (Johnson et al., 2016; Paridaen & Huttner, 2014; Taverna, Götz, & Huttner, 2014). How is the rich diversity of cell types and patterns of circuits established in the developing CNS? The greater simplicity of the zebrafish embryonic brain paired with its transparency for microscopic observation and amenable experimental tools have made it a favorite model system to explore the regulatory mechanisms governing nervous system development and disease (Schmidt, Strähle, & Scholpp, 2013).

Similarly, across all vertebrates, organization of the zebrafish CNS can be defined as a multisegmented structure along both the anterior to posterior and dorsal to ventral axes. Most obvious is the clear segmentation of the brain into its forebrain, midbrain and hindbrain portions, of which the hindbrain is further subdivided into seven successive regions called *rhombomeres* (Gahtan & Baier, 2004; Schilling & Knight, 2001; Wilson, Brand, & Eisen, 2002). These rhombomere segments house specific sets of neuronal lineages many constituting the branchiomotor circuitry of the cranial nerves, among others (Chandrasekhar, 2004). As with organisms across phyla, differential expression of the *Hox* family of homeodomain-containing transcription factors serve to set in motion the specification of the cell types from head to tail, including cell differentiation within the CNS. Prior to the visible morphological characteristics of rhombomere segmentation, different combinations of *hox* gene expression prefigure these presumptive rhombomere regions, and such segmented *hox* expression in the hindbrain has been shown to be conserved even to the phylogenetic base of vertebrate species like the lamprey (Krumlauf, 2016; Parker & Krumlauf, 2017). It should be referenced that the lineage leading to teleosts experienced an additional genomic duplication as compared to other vertebrate lineages, and as such, for instance, zebrafish do possess substantially more *hox* genes than mouse (Kuraku & Meyer, 2009; Postlethwait, 2006, 2007). This kind of genetic overlap in *hox* genes has demonstrated low penetrance to single gene loss experiments. When the entire paralog group 1 (*hoxb1a* and *hoxb1b*) was knocked out in zebrafish; however, specific deficits in the neuronal identities in the hindbrain were revealed, like the loss of the large Mauthner neurons found in rhombomere 4 (Fig. 45.3A–F Weicksel, Gupta, Zannino, Wolfe, & Sagerström, 2014).

Because of the importance that *hox* genes have in regulating the activity of whole-cell type determining gene regulatory networks, much research has focused on addressing how the correct pattern of *hox* gene expression is first established across the embryo. Recall that cell position within the embryo matters. Morphogenetic signaling is one critical mechanism known to influence the differential expression of *hox* genes along both the anterior to posterior and dorsoventral axes. One essential inducer of *hox* genes is retinoic acid, which is dispersed in a gradient with its highest expression in the anterior embryo. Interestingly retinoic acid can enter a cell and function directly as a transcription factor to regulate the expression of a variety of genes that include those that encode many *Hox* transcription factors. Loss of retinoic acid signaling in pathway-specific zebrafish mutants, gene-targeted morpholinos, or pharmacological inhibitors all cause changes in the pattern of *hox* gene expression in the hindbrain with predictable losses in the differentiation of specific neuronal types and rhombomere identity (Alexandre et al., 1996; Emoto, Wada, Okamoto, Kudo, & Imai, 2005; Hernandez, Putzke, Myers, Margaretha, & Moens, 2007; Lee & Skromne, 2014; Maves & Kimmel, 2005; Moens & Prince, 2002; Parker, Bronner, & Krumlauf, 2014; Shimizu, Bae, & Hibi, 2006).

Some of the greatest advances in our understanding of the mechanisms governing neural tube patterning have come from an analysis of cell fate determination along the dorsal to the ventral axis of the developing spinal cord. As found in all vertebrates, opposing dorsal and ventral gradients of morphogenetic signals establish the pattern of cell fates along this axis (Sagner & Briscoe, 2017). In the zebrafish spinal cord, sensory neurons occupy the most dorsal locations, interneurons and later forming oligodendroglia within the middle third of the spinal cord, and motor neurons sit just above the ventral-most structure, the floorplate (Lewis & Eisen, 2003). The floorplate is an important location, where many commissural neurons send their axons across the midline to connect the two hemispheres of the central nervous system (See floorplate in Fig. 45.1), while the sensory and motor neurons project their axons out of the spinal cord to innervate their nonneural target cells. This vertically stepped organization of the spinal cord is first patterned by the opposing gradients of Bmps and Wnts from the dorsal neural tube and overlaying surface ectoderm and Sonic hedgehog (Shh) secreted from ventrally situated structures of the floorplate and underlying notochord (Le Dréau & Martí, 2012). Cells of the neural tube that experience higher levels of Shh will adopt more ventral fates like motorneurons, whereas those cells exposed to higher concentrations of Bmp/Wnts will mature into more dorsally positioned neurons. Importantly, it is the combined balance of these overlapping morphogens that establish the correct pattern of cell fates along the dorsoventral axis of the neural tube (Sagner & Briscoe, 2017). Shh and Bmp/Wnt signaling function to antagonize one another by regulating specific downstream transcription factors that promote a given cell identity while also cross-repressing the transcription factors required for alternative fates. Many mutants of the Hedgehog pathway were discovered in the first forward genetic screens using zebrafish, like *shh (sonic-you), smoothened (slow-muscle-omitted* and *smo), you (scube2), gli2 (you-too), iguana (Dzip1),* and *hedgehog interacting protein (hip1)* to name some (Barresi, Stickney, & Devoto, 2000; Chen, Burgess, & Hopkins, 2001; Karlstrom, Talbot, & Schier, 1999; Koudijs et al., 2005; Schauerte et al., 1998; Sekimizu et al., 2004; Varga et al., 2001; Woods & Talbot, 2005). Predictably, loss of Hedgehog signaling leads to an expansion of dorsal cell types at the expense of more ventral cell types, while its misexpression replaces dorsal domains with more ventral cell type identities (as exemplified by Guner & Karlstrom, 2007; Park, Shin, & Appel, 2004; Ravanelli & Appel, 2015). Thus, morphogens along both the anterior to posterior and dorsal to ventral axes play major roles in determining the cell fates across the entire developing neural tube.

Neural crest development: The last hallmark of neural tube formation is the development of a transient, multipotent stem cell population known as neural crest cells. Neuroepithelial cells located at the dorsal-most region of the forming neural tube undergo a characteristic epithelial to mesenchymal transition (EMT). During this EMT the adherent epithelial cells atop the neural tube reduce their cell junctions and actively migrate away into peripheral tissues (Klymkowsky, Rossi, & Artinger, 2010; Kwak et al., 2013; Theveneau & Mayor, 2012). Neural crest cells exiting the neural tube along the spinal cord (known as trunk neural crest) will follow guidance cues outside the CNS expressed on the surface of cells or laden within the extracellular matrix. Through a ligand-receptor recognition system, cues like ephrin-ephs, semaphorin-neuropilins, and homodimerizing cadherin receptors serve to influence the trajectory of neural crest cell migration through attraction, repulsion, or adherence (reviewed in Shellard & Mayor, 2016 and exemplified by Berndt & Halloran, 2006; Yu & Moens, 2005).

Aside from their migratory behavior, one of the most uniquely important features of neural crest cells is that they are multipotent stem cells, and are thus able to contribute to several disparate types of lineages. The cell type generated by a neural crest cell progenitor depends on their specification within the neural tube, as well as the signals they experience along their journey and at their final destination (Kalcheim & Kumar, 2017; Kelsh & Raible, 2002; Martik & Bronner, 2017). Trunk neural crest has been shown to give rise to sensory

neurons of the dorsal root ganglion, the Schwann glial cells that myelinate peripheral nerves, and most noticeably pigment cells like melanocytes. The neural crest cells that emanate from more cephalic regions (known as the cranial neural crest) contribute to pharyngeal arch development, the cartilage and bone of the skull and jaw, the cranial nerves, and neurons of the enteric nervous system. Lastly, those cephalic crest cells that contribute to portions of the heart are called *cardiac neural crest*.

The generation of neural crest labeling transgenic lines *(sox10* being a common driver*)* has enabled remarkable live-cell imaging in zebrafish that is continually revealing new insights into the mechanisms of neural crest migration and differentiation (Antonellis et al., 2008; Dougherty et al., 2012; Gfrerer, Dougherty, & Liao, 2013; Rodrigues, Doughton, Yang, & Kelsh, 2012). For instance, trunk neural crest demonstrates a "follow-the-leader" sort of migratory streaming that laser ablation analyses have shown require cell-to-cell interactions. In contrast, cranial neural crest cells move as a whole population through a process known as "collective migration" (Carmona-Fontaine et al., 2011; Richardson et al., 2016). The types of neural crest and their associated mechanisms of migration behaviors appear to be largely conserved across vertebrate species (Kee, Hwang, Sternberg, & Bronner-Fraser, 2007). The processes regulating neural crest progenitor specification toward different lineages remains a major focus of the investigation, for which the zebrafish is proving to be a worthy model. The proneural transcription factor Neurogenin1 has been demonstrated to promote differentiation into dorsal root ganglion cells as opposed to the glia that wraps the dorsal root ganglion axons (Fig. 45.3G–H McGraw, Nechiporuk, & Raible, 2008). Interestingly, loss of *sox10* expression in the *colorless* mutant is necessary for these same glial progenitor cells to develop into chromatophores and contribute to the melanocyte lineage (Fig. 45.4A,B Dutton et al., 2001; Kelsh & Eisen, 2000; Kelsh et al., 1996). Work has even progressed to a fine charting of the precise combinations

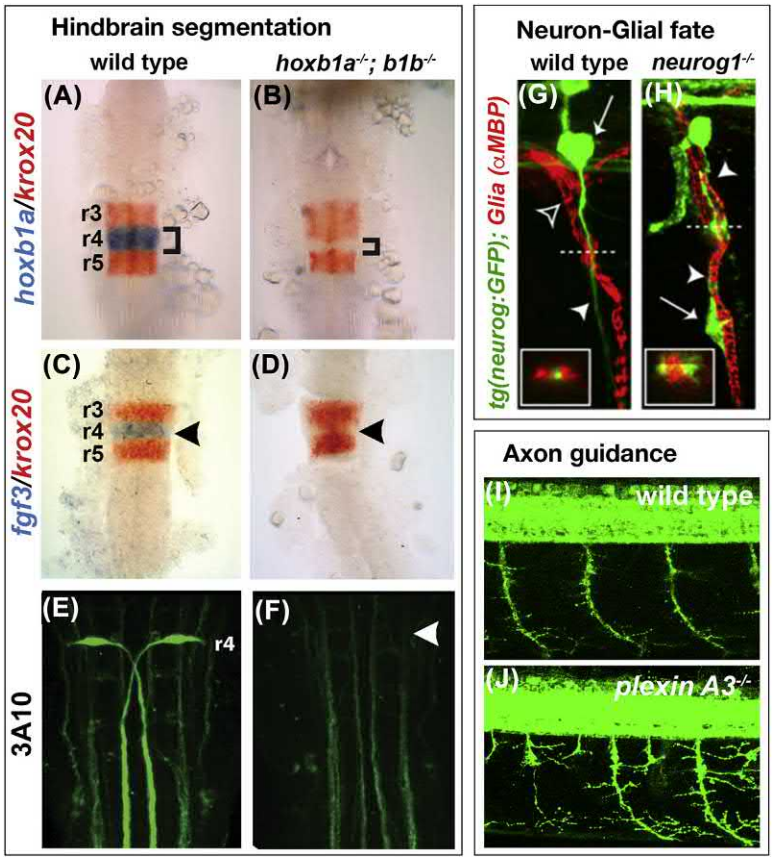

FIGURE 45.3 **Building a Brain.** (A–F) Early segmentation of the hindbrain is seen by the discrete banding expression of *hoxb1a, krox20,* and *fgf3.* Rhombomere four identity requires both *hoxb1a* and *hoxb1b* transcription factors as seen by the loss of segment boundaries (B,D) and loss of Mauthner neurons (3A10 antibody labeling); (F) in the double mutants (Weicksel et al., 2014). (G,H) Dorsal root ganglion precursor cells require *neurogenin1* for neural development as opposed to the glial, Schwann cell identity. The green transgenic reporter labeling of DRG precursors show their expression of glial markers in *neurogenin1* mutants (McGraw et al., 2008). (I,J) Lateral view of axon labeling in and out of the spinal cord with the Znp1 antibody at 48 hpf. Loss of the receptor *plexin A3* causes significant pathfinding errors of motor axons as they leave the spinal cord (Palaisa & Granato, 2007).

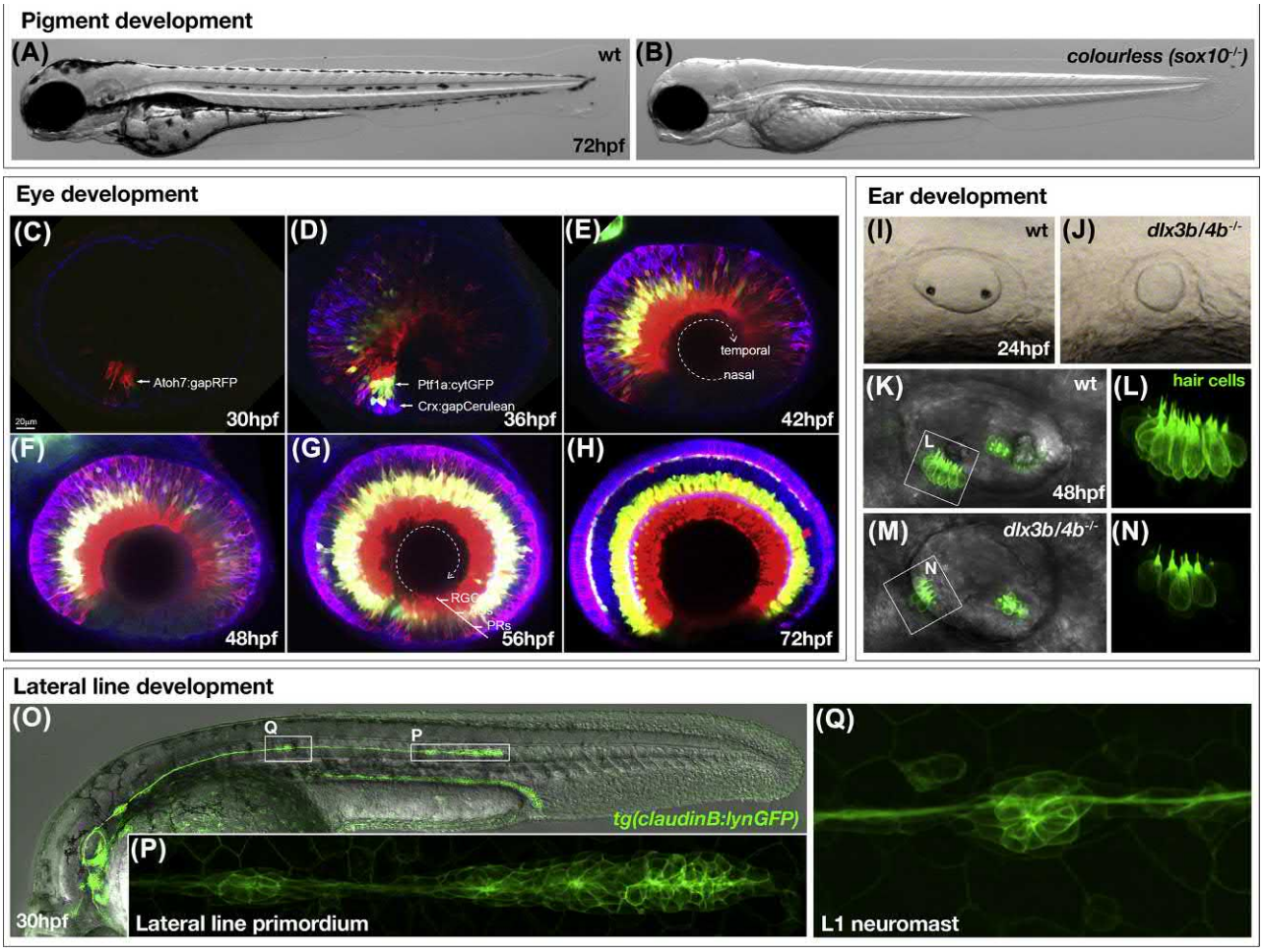

FIGURE 45.4 **Building with Ectoderm.** (A,B) *sox10* is a master regulatory transcription factor required for melanocyte (pigment) cell fate throughout the body but not pigmented epithelium of the retina as evident in the *colorless (sox10)* mutant (B) Data generously provided by Ellie Melancon and Judith Eisen; see also (Dutton et al., 2001). (C–H) Radial progression of cell development in the retina over time (Almeida et al., 2014). (I–N) The transcription factors *dlx3b* and *dlx4b* are required for the whole generation of hair cells (*Tg(pou4f3:GAP-GFP)+*) and otoliths during inner ear development (Schwarzer et al., 2017). (O–Q) The lateral line primordium (P) demonstrates collective migration upon the horizontal myoseptum of the somites as it progresses from the hindbrain to the tip of the tail depositing neuromasts along the way (Q). Data generously provided by Damian Dalle Nogare and Ajay Chitnis.

of gene expression changes that occur over the course of neural crest cell differentiation from progenitor cell to enteric neuron (Taylor, Montagne, Eisen, & Ganz, 2016).

The delaminating cell behaviors displayed by neural crest cells during their emergence from the dorsal neural tube are routinely compared to the behavioral characteristics of metastasizing cancers cells in the adult (Gallik et al., 2017). Recently, the well-known neural crest marker *crestin* was found to be induced in melanomas in the adult zebrafish, and forced overexpression of *sox10* in melanocytes was capable of triggering melanoma formation (Kaufman et al., 2016). Therefore, the study of neural crest development in zebrafish may very well be directly preparing us to understand better, cancer in those tissues whose cells originated from neural crest in the embryo.

Connecting up the nervous system- Axon guidance: The neuronal "wiring" of an adult organism is organized in a stereotypical pattern for which a scaffold of pioneering axons first laid down during embryogenesis (Lewis & Eisen, 2003; Wilson, Ross, Parrett, & Easter, 1990). Whether it is a dorsal root ganglion neuron adjacent to the somite, a motor neuron in the spinal cord or even a retinal ganglion cell neuron in the eye, once a neuron is born it must establish a physical connection or synapse with its target cell that may be located great distances away. A newly born neuron will extend a long, membranous process called an *axon* that is led by a highly motile and dynamic growth cone tip (Pacheco & Gallo, 2016). This growth cone houses a specific repertoire of chemical-sensing receptors capable of relaying the guidance information of environmental cues to the

cytoskeletal motors within the growth cone. Such cues like netrins, slits, semaphorins, and ephrins that we described previously in the guidance of migrating neural crest cells also function to attract or repel pathfinding axons (Morales & Kania, 2017; Seiradake, Jones, & Klein, 2016).

In zebrafish, axon guidance mechanisms have and continue to be defined for many different pathways, such as those for the spinal motor nerves, optic nerves, commissural pathways, and sensory efferent nerves to name a few. For instance, the first commissural axons to cross the midline of the central nervous system (CNS) occurs in the diencephalon of the forebrain by postoptic commissural axons at about 24 hpf. This commissure is soon followed by the crossing of anterior commissural axons in the telencephalon and then by retinal ganglion cell axons that form the optic chiasm also at the diencephalic midline. Prior to and during commissure formation, a midline spanning population of astroglial cells appears to provide a supportive substrate for pathfinding across the midline. In addition, the surrounding expressions of Slit2 and Slit3 function to repel all three of these axon types by serving to channel axons across the midline and prevent inappropriate axon wandering (Barresi, Hutson, Chien, & Karlstrom, 2005). Loss of the slit receptor *roundabout2* causes optic nerves to be led "astray," crossing the midline multiple times in aberrant positions (Hutson & Chien, 2002). Many more guidance cues and pathfinding behaviors have been identified and first characterized in the zebrafish embryo. For instance, the semaphorin receptor Plexin A3 was shown to be required for the proper exit positioning of motor nerves out of the spinal cord (Fig. 45.3I,J; Palaisa & Granato, 2007). Moreover, it was by examining the live pathfinding of peripheral sensory axons in the zebrafish epidermis that identified how random repulsive interactions between axons can create a perfectly elaborated pattern of sensory arbors (Sagasti, Guido, Raible, & Schier, 2005).

Ectodermal Derivatives

Like all vertebrates, zebrafish sense their external environment through their eyes, ears, nose, and skin, as well as with a unique set of mechanosensory lateral line organs specialized to detect the flow of water (See Fig. 45.1). Interestingly, zebrafish larvae can respond to diverse sensory stimuli even before hatching, and thus, must form functional response circuits while their sensory organs and nervous system are still developing. The rapid, ex utero development and transparency of zebrafish has been instrumental in revealing close developmental links between the nervous system and sensory organs by precisely revealing the location, timing, and processes involved in the origins of specific cell types and the formation of their functional connections. Primordia of specialized sensory organs known as cranial placodes first appear in the early vertebrate embryo as focal thickenings of the ectoderm in the vicinity of the developing brain (Schlosser, 2014). Specific cranial placodes can generate all cell types within an individual sensory organ through a series of differentiation events or inductive interactions with cells or secreted factors of the neural ectoderm, neural crest, endoderm, and mesoderm. Studies in mouse, chick, and frogs have defined specific gene members of the Six, Pax, Eya, Id, Dlx, Iro and Fox transcription factor families as the identifying molecular markers for individual placodes (reviewed in Streit, 2004; 2018). Expression analysis of these markers in developing zebrafish has helped to trace the origins of all placode precursors to an arc-shaped preplacodal region (PPR), which surrounds anterior-lateral aspects of the developing neural tube and brackets cells of the prospective neural crest (Kozlowski, Murakami, Ho, & Weinberg, 1997; Solomon & Fritz, 2002; Whitlock & Westerfield, 2000). Early lineage tracing studies demonstrated a close spatial relationship between progenitors of the epidermis, cranial placodes, neural crest, and nervous system. During gastrulation, these progenitors differentiate from the ectoderm in response to graded concentrations of BMP, Wnt, and FGF morphogens found along the dorsoventral embryonic axis (Kudoh, Wilson, & Dawid, 2002; Reichert, Randall, & Hill, 2013).

Beginning with the epidermis, we describe the development of individual zebrafish sensory structures highlighting major transitions and morphogenic interactions that shape the ontogeny of diverse cell types within each organ.

Epidermal development: The natural habitats of zebrafish are water bodies ranging from very low salinity to brackish ones (Harper & Lawrence, 2016). Zebrafish can thrive in such diverse habitats in part due to adaptations of their epidermal cells, which can regulate skin permeability by directly sensing the presence of distinct organic and inorganic ions, and in response to acute changes in their concentrations. Zebrafish do not development dermal scales until 30 days postfertilization (dpf), and therefore, protective adaptations of the epidermis are particularly essential to prejuvenile zebrafish survival (Sire & Akimenko, 2003). The epidermis is an expansive sensory organ comprised of a loosely organized but diverse array of sensory ionocytes.

Unlike terrestrial vertebrates, the zebrafish epidermis exclusively consists of living cells and is not covered by moisture conserving keratinized layers. The epidermis is a pseudostratified epithelium, in which cells from adjacent layers connect to each other via desmosomes and to the basement membrane via hemidesmosomes

(Le Guellec, Morvan-Dubois, & Sire, 2003). Although the adult epidermis has three layers, it forms as a bilayer in the embryo, which first develops an outer or enveloping layer (EVL) from the surface blastoderm and later forms the inner epidermal blastoderm layer (EBL) during gastrulation. The EBL covers the entire surface of embryos at 90% epiboly in a single layer and begins to fuse at the embryonic midline at the 14 somite stage (Schmitz, Papan, & Campos-Ortega, 1993). Cell types in the mature epidermis of teleosts include keratinocytes, mucus-secreting cells, four types of ionocytes that regulate entry of specific cations, sensory cells, and alarm substance-secreting club cells (Henrikson & Matoltsy, 1967a; 1967b; 1967c). These diverse cell types do not randomly occur across the surface and width of the epidermis but localize on the basis of their ontogeny and local cellular interactions.

Keratinocytes predominantly occur in the outermost layer and have mechanical resistance due to actin-based surface microridges. Ionocytes, secretory cells, and undifferentiated cells occur in the middle or basal epidermal layers (Chang & Hwang, 2011; Hawkes, 1974; Le Guellec et al., 2003). Lineage tracing experiments in zebrafish show that all epidermal cell types originate from non-neural ectodermal cells located near the ventral axis of the early embryo (Kimmel, Warga, & Schilling, 1990; Sagerström, Gammill, Veale, & Sive, 2005). Consistent with high levels of BMP in this region, epidermal progenitors distinctly express the BMP-induced gene *ΔNp63* (Lee & Kimelman, 2002). Both keratinocytes and ionocytes differentiate from common progenitors under the influence of Delta-Notch and Foxi3a/b signaling proteins (Esaki et al., 2009; Hsiao et al., 2007; Jänicke, Carney, & Hammerschmidt, 2007). Distinct skin ionocytes resemble kidney cells both in morphology and in the expression of ion transporter proteins, and interestingly, their proliferation, differentiation, and turnover are regulated by environmental factors, such as acidity and cold temperatures (Chou et al., 2008; Horng, Lin, & Hwang, 2009; Hwang, 2009). While the characterization of epidermal defects in the *psoriasis* mutant indicates that secreted factors could influence the differentiation of keratinocytes (Webb, Driever, & Kimelman, 2008), little is known about the development of mucus or alarm substance-secreting cells in zebrafish skin. It has recently been shown that all mature epidermal cell types exhibit phagocytic behaviors, and this function may serve to trim axon termini or remove debris from apoptotic neurons that are densely interspersed between the epidermal layers (Rasmussen, Sack, Martin, & Sagasti, 2015).

Epidermis specifically refers to the outermost skin layer but is also a loose term for the entire skin. Thus, it is pertinent to consider how the zebrafish skin increases in mechanical strength and develops its characteristic pigment stripes. Zebrafish biologists are well aware that it is more difficult to penetrate larvae than embryos either with a microinjection needle, antibodies or riboprobes. This is mainly due to the presence of a hard cuticle over the skin, the bulk of which is composed of epidermal cell secretions and debris. The epidermis begins to secrete mucus after 24 hpf but does not develop a collagenous envelope until 72 hpf as it is secreted by the later developing hypodermis. Although the thickness of skin increases with time and higher degrees of collagen crosslinking, it takes about 30 days to get covered by dermal scales, which arise from epidermal placodes through epidermis-hypodermis interactions (Le Guellec et al., 2003). Interestingly, the defining pattern of horizontal blue-black lines interspersed on zebrafish skin is generated by xanthophore, iridophore and melanophore pigments derived from neural crest cells (reviewed in Kelsh, Harris, Colanesi, & Erickson, 2009). In contrast to other vertebrates, zebrafish pigment cells typically do not invade the epidermis, but rather migrate along specific paths namely between somites and the neural tube (medial path) or between somites and non-neural ectoderm (lateral path). Different chromatophores will migrate along different paths; such that, melanoblasts move along both medial and lateral paths, whereas iridophores are restricted to the medial path and xanthophores to the lateral path. Melanophores begin to appear after 24 hpf first in the cephalic region and progressively over the trunk where they occur in four stripes, dorsal, lateral, ventral and yolk syncytial (Hirata, Nakamura, & Kondo, 2005). The characterization of the zebrafish pigment cell specification, *colorless* (*sox10*) mutant, indicated that the identity of particular pigment cells is not defined by their migration paths but is rather determined at the time of specification itself (see Fig. 45.4A,B Dutton et al., 2001; Kelsh & Eisen, 2000; Kelsh et al., 1996). Intriguingly, pigment pattern defects in the *choker* mutant indicate that germ cell migration and axonal guidance cues like the Sdf-1 factor also appear to play a role in melanophore migration (Svetic et al., 2007).

With its ready accessibility, the zebrafish epithelium represents a powerful alternative to tissue culture models to understand the development, physiology, and regulation of epithelial cells. Interestingly, a zebrafish enhancer trap screen has identified stable but mosaic patterns in the epidermal expression of Gal4 transgenes indicating that epidermal cells from specific locations may have distinct identities based upon local tissue interactions (Eisenhoffer et al., 2017).

Placodes—The eye with its lens: The zebrafish eye is the first peripheral sensory organ to emerge toward the end of gastrulation and appears prominently large relative to the developing embryo. Zebrafish are tetrachromats

that can perceive light in the visible and ultraviolet spectrum; however, the overall structure of their eye is quite similar to that of mammals. It is a spherical chamber with a transparent lens in the center of its exposed surface surrounded by a protective, light-proof sclera that encloses a laminar sensory retina. The outermost retinal layer is comprised of pigmented epithelial cells that support rod and cone photoreceptors, as well as the inner layers of bipolar, horizontal and amacrine neurons, and Müller glia. Lastly, the essential retinal ganglion cells transmit light stimuli from the eye to the optic tectum within the forebrain (Gestri, Link, & Neuhauss, 2012).

During early embryogenesis, the eyes begin to develop from a central eye field or anlage within the most anterior and central section of the preplacodal region specified by the expression of transcription factors Six3, Rx3, and Pax6. This central eye anlage is split into bilateral retinal primordia by the anterior migration of cells from the adenohypophyseal placode triggered by factors secreted by the axial mesoderm that includes TGFβ, FGF, and Shh. Thus, zebrafish deficient in these factors either due to genetic mutations or exposure to chemical inhibitors form one central fused eye instead of two bilateral ones (reviewed in Sinn & Wittbrodt, 2013).

The first step during eye formation involves evagination of the epithelium in the forebrain region driven by migration of retinal precursor cells (see Fig. 45.1). This results in the formation of an optic vesicle whose molecular signaling interactions with the overlying ectoderm lead to the development of the lens placode around 16 hpf. Subsequently, the bilayered optic vesicle invaginates to form an optic cup that gives rise to the laminar neural retina surrounded by pigmented epithelium. Within the retina, the ganglion cells form as the eye's first neurons around 32 hpf followed by cells of the inner nuclear layer. Rods and cones do not form until 55 hpf. All cells of the retina form circumferentially in the germinal zone near the ciliary margin. Thus, the oldest retinal cells are found toward the center, whereas, the newest form around the periphery (Fig. 45.4C–H Almeida et al., 2014; Sinn & Wittbrodt, 2013).

Placodes—Olfactory: Zebrafish rely on the sense of smell to forage, find mates, and detect alarm signals released from injured individuals in a group. The odors are transduced to specific olfactory sensory neurons via nares that appear as pits situated above the mouth and underneath the eyes. Interestingly, zebrafish have evolved unique olfactory adaptations to rapidly detect trace amounts of odorant molecules diffused in surrounding water (Hansen & Eckart, 1998). Focusing on the early development of the olfactory organ, we outline salient sensory and epithelial cell specializations that occur within the olfactory placode.

Olfactory placodes are formed by the convergence of precursor cells from the anterolateral edges of the preplacodal region, which first appear around the 17–18 somite stage as transient but distinctive ectodermal thickenings expressing the olfactory marker *dlx3* (see Fig. 45.1 Whitlock & Westerfield, 2000). Around 24 hpf the periphery of olfactory placodes can be identified by a dense whorl of motile multicilia extending from columnar epithelial cells (Pathak, Obara, Mangos, Liu, & Drummond, 2007). The central portion of the olfactory placodes contains a rosette-like arrangement of neuroepithelial progenitor cells that differentiate into olfactory sensory neurons, support cells, glial cells, and neuroendocrine cells expressing Gonadotropin-releasing hormone (GnRH). At this stage, transient adendritic pioneer neurons can be observed connecting the olfactory epithelium with the olfactory bulb. Following programmed cell death of the pioneer neurons, three mature olfactory cell types emerge, ciliated, microvillus, and crypt neurons (reviewed in Whitfield, 2013). Olfactory neurons are distinguishable by the expression of olfactory marker protein and the expression of olfactory receptors for distinct odorant classes (Yoshihara, 2009). Photoconversion experiments in zebrafish have distinguished that the microvillar olfactory neurons actually originate from neural crest (Saxena, Peng, & Bronner, 2013).

Placodes—Ear and Lateral line organs: The ear enables vertebrates to hear and maintain positional equilibrium by sensing linear acceleration and rotational movement. Although the zebrafish ear is not prominently visible externally like our outer ear structures, its organization does resemble that of the semicircular canals in the mammalian inner ear. It comprises three orthogonally arranged vestibular structures known as the utricle, saccule, and lagena, which end in sensory epithelial patches or maculae formed by specialized hair cells. The apical surface of these hair cells projects mechanosensory kinocilia that also tether large crystalline structures termed *otoliths*, which amplify and transmit mechanical stimuli to the brain via bipolar neurons that form the statoacoustic ganglion (reviewed in Whitfield, Riley, Chiang, & Phillips, 2002). Zebrafish mutants that exhibit otolith or inner ear structural defects, vestibular dysfunction and cilia abnormalities associated with sensory hair cells are unable to maintain an upright balance, and as a result such ear mutants swim abnormally either on their sides, upside down or in circles (Granato et al., 1996; Schibler & Malicki, 2007; Stooke-Vaughan, Huang, Hammond, Schier, & Whitfield, 2012; Whitfield et al., 1996).

All cell types within the ear including the sensory epithelium, neurons and supporting cells originate from the otic placode, which prominently appears in a 16 hpf embryo as a thickening in the ectoderm posterior to the eyes and underneath the hindbrain rhombomeres 6 and 7 (See Fig. 45.1 Alsina & Whitfield, 2017). The otic

and epibranchial (cranial sensory nerves) placodes originate commonly from a posterior section of the preplacodal region showing enriched expression of the transcription factor Pax2 (McCarroll et al., 2012). Migrating cells from the neural crest later separate the otic placode into its distinct position as observed by the expression of transcription factors *dlx3b* and *dlx4b*. By 24 hpf the otic placode invaginates into an otic vesicle containing a large anterior otolith associated with the utricle and a smaller posterior otolith associated with the saccule. Zebrafish double mutants of the *dlx3b* and *dlx4b* genes result in a dramatically smaller otic vesicle with reduced sensory hair cell development and lack otoliths (Fig. 45.4I–N; Schwarzer, Spieß, Brand, & Hans, 2017). The hair cells differentiate from the columnar epithelial cells along the periphery of the otic vesicle at distinct times; the first ones emerge prior to otoliths and anchor them. The otoliths begin to form around 18–20 hpf as crystals of calcium carbonate that nucleate around a matrix of glycoproteins, both secreted by supporting epithelial cells within the otic placode (Wu, Freund, Fraser, & Vermot, 2011).

Zebrafish sense movement patterns in surrounding water via a series of special lateral line organs, which like the ear are mechanosensory. The lateral line organs are formed by a series of individual neuromasts distributed all along the trunk and around the eyes and ears (See Fig. 45.1). Each neuromast, in turn, consists of a central cluster of sensory epithelium formed by hair cells surrounded by a rosette of supporting epithelium and encircled by mantle cells (reviewed in Dalle Nogare & Chitnis, 2017). Neuromasts can be visualized in live, unstained zebrafish using microscopes equipped with Nomarski Differential Interference Contrast (DIC) microscopy or with fluorescent styryl dyes, such as DASPEI or FM 1–43 which are preferentially endocytosed by hair cells (Ou, Simon, Rubel, & Raible, 2012).

The early development of lateral line organs is understood mostly with regard to the posterior lateral line primordia (pLLp), which originates from the neural crest around 22 hpf as a placode formed by 140 cells that migrates caudally under the skin (reviewed in Dalle Nogare & Chitnis, 2017). Prior to beginning its migration, the pLLp differentiates bipolar sensory neurons, which extend an anterior axon into the hindbrain and a posterior axon into the migrating primordium (Fig. 45.4O–Q Hava et al., 2009; Knutsdottir et al., 2017; Nogare et al., 2017; Sarrazin et al., 2010; Dalle Nogare et al., 2014). The path of these migrating cells is guided by affinity-based interactions between Sdf-1 (also called *Cxcl12a*), a chemokine secreted by cells of the horizontal myoseptum and Cxcr receptors expressed by the migrating pLLp cells. As the cluster migrates, cells at the leading edge display a mesenchymal character and express Cxcr4b whereas those at the trailing edge begin to acquire apicobasal polarity typical of epithelial cells and the express Cxcr7b receptor (Dambly-Chaudière, Cubedo, & Ghysen, 2007; Haas & Gilmour, 2006; Sapède, Rossel, Dambly-Chaudière, & Ghysen, 2005; Valentin, Haas, & Gilmour, 2007). This epithelial transition occurs within a cluster of ~20 cells which stop migrating and begin to constrict at their apical ends to form rosettes described as protoneuromasts. The central cell within each protoneuromast differentiates into a progenitor of sensory hair cells. pLLp cells that stop migrating but are left out of the protoneuromasts instead deposit as interneuromasts, which can later proliferate to produce more neuromasts. Neuromasts continue to be sequentially deposited up to 48 hpf. The somite position reached by the leading edge of the pLLp is used to accurately stage the development of embryos after the first 24 h.

Neuromast formation is regulated by a limiting balance between Wnt and FGF signaling pathways (Ma & Raible, 2009). Initially, the entire pLLp expresses high levels of Wnts, which activates expression of both Wnt, as well as FGF ligands. However, FGF producing cells do not respond to Wnt activators and Wnt expressing cells produce inhibitors of FGF signaling which results in establishment of an FGF signaling center at the trailing edge (Aman, Nguyen, & Piotrowski, 2011; Aman & Piotrowski, 2008; Head, Gacioch, Pennisi, & Meyers, 2013; Kozlovskaja-Gumbrienė et al., 2017; Nikaido, Navajas Acedo, Hatta, & Piotrowski, 2017; Tang, Lin, He, Chai, & Li, 2016).

Germ Layer Integration to Build the Craniofacial Skeleton

The head skeleton is structurally essential for both its protection of the brain and its functional roles in eating and hearing, and in the case of a fish, even breathing. Therefore, it is not surprising that malformations in the morphogenesis of the craniofacial skeleton during embryonic development are associated with a large range of disorders in humans, such as cleft palate, DiGeorge syndrome, or Treacher-Collins syndrome to name a few (Machado & Eames, 2017). Although the boney fish's head has over two times as many bone elements as the mammalian head skeleton, much conservation in overall structure and function exists between the two classes; additionally, the larval zebrafish craniofacial skeleton represents a simple organization of discrete parts making it a conserved and tractable model for embryological and disease relevance (Medeiros & Crump, 2012; Mork & Crump, 2015).

For instance, bones of the fish jaw and viscerocranium are evolutionarily homologous to the bones making up the mammalian middle ear.

By the early larval stages, the head cartilage elements are forming and will eventually ossify into bone (Kimmel et al., 1998). As mentioned earlier, most of the cartilage and bone in the head is directly descendant from cranial neural crest cells (Knight & Schilling, 2006). From single-cell microinjection to cell transplantation and laser light-induced transgenic procedures, lineage tracing approaches have demonstrated that the location of cranial neural crest cells along the neural tube and the timing of their exit reproducibly contributes to cartilage of particular regions of specific elements (Mork & Crump, 2015). One of the most fascinating aspects of craniofacial development however, is the requirement to first form pharyngeal arches, which are transient embryonic structures bilaterally repeated along the rostral to caudal axis of the ventral half of the head that contribute to the gills, jaw and head skeleton among other tissues (Frisdal & Trainor, 2014). Although cranial neural crest cells primarily make up the pharyngeal arches, the entire morphogenesis of craniofacial development involves the direct interactions of cells from the mesoderm, endoderm, and ectoderm.

Beginning at 20 hpf and ventral to the otic placode (ear), seven- bilateral, pharyngeal arches extend further ventral to the locations of element-specific chondrogenesis on each side of the developing jaw. Imagine the pharyngeal segments as pairs of oars being extended into the water from seven rowers seated in line within a common canoe. As mentioned above, the pharyngeal arch is made up of several types of cells, and in extending this rowing analogy, the central core of the oar would be made of mesodermal cells surrounded by successive rings of endodermal cells, cranial neural crest cells, and a final circumferential ring of ectodermal cells. Exciting recent research into zebrafish pharyngeal development is starting to reveal how these multilayered arches are formed. Cells of the endodermal layer, called the *pharyngeal pouches*, play an important role in dividing the streams of cranial neural crest cells into the seven separate arches, whereas, the initially positioned ventral mesoderm functions to provide a guidance cue for the directionality of arch elongation (Mork & Crump, 2015). The Tbx1 transcription factor is expressed by the mesodermal cells, and it has been shown to regulate Wnt11r and Fgf8a in subpopulations of the mesoderm. Wnt11r and Fgf8, in turn, function to properly coordinate endodermal pouch morphogenesis and attract the collective migration of the cranial neural crest cells, respectively (Fig. 45.5 Choe & Crump, 2014). This is substantially relevant to human health as mutations in the *Tbx1* gene are known to be the cause of DiGeorge syndrome (Chieffo et al., 1997).

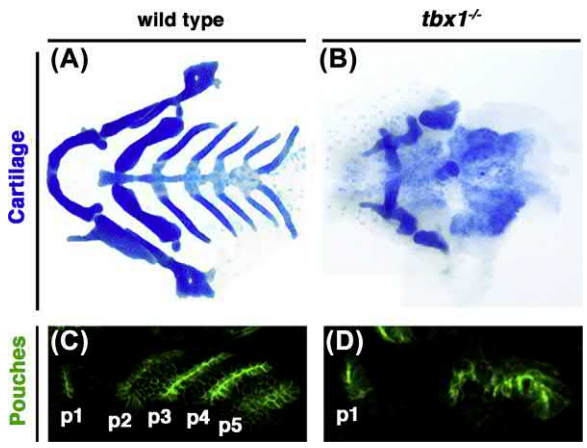

FIGURE 45.5 Building a Face. (A, B) Ventral view of Alcian Blue staining of cartilage elements in the jaw and branchial regions of 5-day old wild type (A) and *tbx1* mutant (B) larvae. Loss of *tbx1* leads to reduced and disorganized pharyngeal pouches at 34 hpf (C, D; Anti-Alcama labeling, green), which causes the ultimate loss of craniofacial elements later in development (B). Data generously provided by Gage Crump. See also (Choe & Crump, 2014).

Segmenting the Trunk for Muscle, Bone and Much More

The reach of hox genes: The stretch of *hox* gene influence on patterning anterior to posterior tissue identity does not stop at the hindbrain as described earlier, but is a robust mechanism for positional patterning from the olfactory placodes (nose) to the tips of caudal fin rays (tail) and all the parts in between. For instance, positioning of the pectoral fin is under the delineation of *hox* gene expression and its regulation homologous to the mechanisms of tetrapod forelimb development. Furthermore, patterning of the developing pectoral fin along its own anterior to the posterior axis is governed by highly conserved mechanisms involving *hox* genes among many others (Ahn & Ho, 2008). In particular, Sonic hedgehog morphogenetic signaling from the posterior fin bud and the antagonistic signals of Fgf8 and retinoic acid along the distal to proximal axes lead to the outgrowth of the fin, which is accomplished in part through the upregulation of progressively more 5′ *hox* genes over time that induce progressively more distal cell derivatives of the fin (Akimenko & Ekker, 1995; Hoffman, Miles, Avaron, Laforest, & Akimenko, 2002; Laforest et al., 1998; Noordermeer et al., 2014; Prykhozhij & Neumann, 2008; Sakamoto et al., 2009; Zhao et al., 2009).

One of the most obvious axial structures in the embryonic zebrafish is the repeated, chevron-shaped blocks of paraxial mesoderm running down the length of the trunk and tail. In the embryo, these segments are called *somites*, portions of which give rise to the myotome or skeletal muscle, to the sclerotome or cartilage and bone of the vertebrae, and to some vascular derivatives among others (Stickney, Barresi, & Devoto,

2000). As with all segmented organisms, the identity type of each segment is highly regulated by *hox* genes; such that, the anterior border of *hox* gene expression strongly influences the cell types that will develop in each segment (Iimura & Pourquié, 2007). As an example, *hoxc6* correlates with the cervical to thoracic transition in mouse, chick, and zebrafish; however, while the overall *hox*-mediated mechanisms of identity are conserved, there is a discord in the expression of certain *hox* genes at more posterior transition sites between boney fish and amniotes (Burke, Nelson, Morgan, & Tabin, 1995; Morin-Kensicki, Melancon, & Eisen, 2002). Importantly, the regulation of *hox* gene expression by the opposing gradients of and antagonistic signaling by retinoic acid from the anterior mesoderm and Fgf8 from the posterior mesoderm are highly conserved across vertebrate species, including zebrafish.

Somitogenesis and tail elongation: Although establishing different cell identities across the entire anterior to the posterior axis is critical, the boundaries of paraxial mesoderm segmentation represent a particularly unique physical feature that prepares this embryonic tissue for later differentiation. Somite formation or somitogenesis is the cyclical cutting of progressively more posterior paraxial mesoderm into roughly equal-sized blocks. A somite constitutes the mesoderm occupied between segment boundaries. Understanding the developmental underpinnings of somitogenesis have been part of intense research for many decades due to its common occurrence in all segmented creatures, its medical relevance to human disorders like scoliosis, as well as to biologist's pure phenomenological fascination (Boswell & Ciruna, 2017; Eckalbar, Fisher, Rawls, & Kusumi, 2012; Grimes et al., 2016). The rapid external development of zebrafish paired with its amenability to timelapse microscopy and cell tracking techniques have all garnered zebrafish as a superb model to study somite formation.

The zebrafish paraxial mesoderm is divided up into roughly 35 bilaterally positioned somites. Somite formation and maturation occur in remarkably regular order in a rostral to caudal direction over the trunk between 10 and 24 hpf (Fig. 45.6C and E). So standardized is somite development that a bilateral pair of somites is reliably formed every 30 min (at 28.5°C), and researchers use the total number of somites as a metric of embryonic age (e.g., 18 somites at 18 hpf, 22 somites at 20 hpf, 30 somites at 24 hpf) (See Fig. 45.1 Kimmel et al., 1995). Forming somites is a complex process involving at least four moving parts: one- the convergence of mesoderm toward the midline to both populate the presumptive anterior presegmental plate

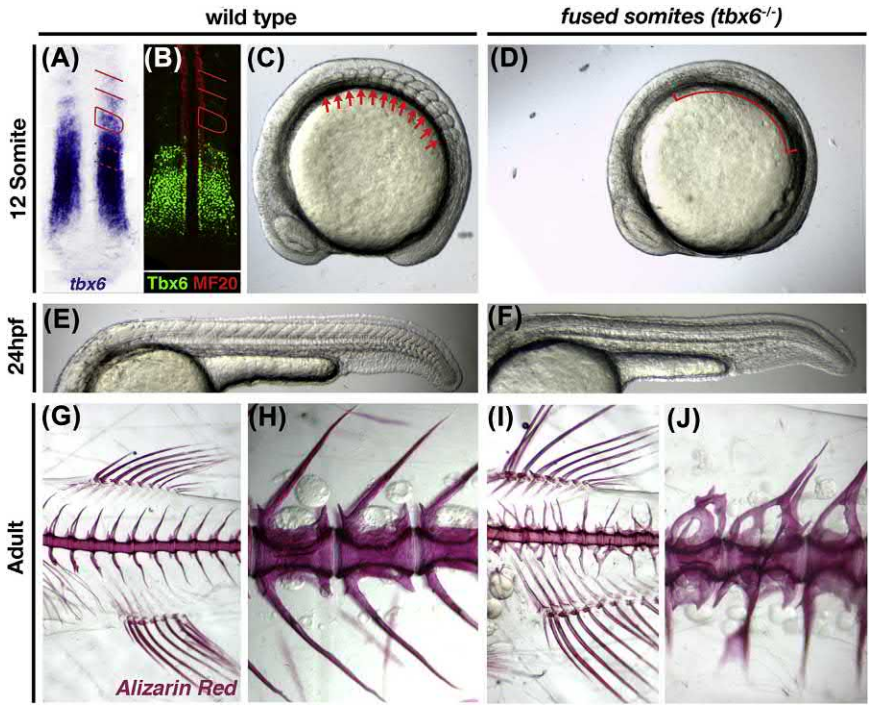

FIGURE 45.6 **Slicing up the segments.** (A–F) Dorsal (A, B) and lateral (C–F) views of 12- and 30-somite wild type (A–C,E) and *txb6* mutant (D,F) embryos. *Tbx6* transcript (A) and protein (B) are expressed within presomitic mesoderm that disappears as a somite matures. MF20 is a marker for skeletal muscle fiber differentiation. Somites fail to form in *fused somites (tbx6)* mutants (D, F). (G–J) Loss of proper segmentation in the *tbx6* mutant embryo leads to disorganization of the adult vertebral elements with many instances of vertebral fusions (I,J). Data shown in A–J was generously provided by Stephen H. Devoto.

and power the caudal extension of the trunk, two- the generation of new, migratory cells from the caudal progenitor domain at the tailbud to further drive growth of the posterior presegmental paraxial mesoderm, three- the inflation of chordamesoderm cells to elongate and harden the notochord further supporting posterior extension, and four- the sequential and targeted epithelialization of paraxial mesoderm cells at presumptive boundary locations during trunk extension. Together these different cell behaviors lead to the periodic segmentation of the paraxial mesoderm in an anterior to the posterior direction over the course of tail elongation (Yabe & Takada, 2016).

Despite the complexity of somitogenesis, the molecular components coordinating all of its moving parts are gaining clarity and found to be remarkably conserved across vertebrate species. Much insight has been garnered from mutagenesis screens that have identified many essential genes, which include but are not limited to T-box transcription factors, genes within the Notch and Fgf pathways, and cell adhesion receptors as in Eph-Ephrin signaling. One of the most dramatic phenotypes is seen when the *tbx6* transcription factor is lost in the *fused-somites* mutant, which results in a complete loss of any somitic furrows (Fig. 45.6A–F Nikaido et al., 2002; Windner, Bird, Patterson, Doris, & Devoto, 2012). It is hypothesized that Tbx6 functions to specify the anterior half of presumptive somites leading to its Eph-ephrin mediated epithelialization (Barrios et al., 2003). However, the timing of a somite boundary forming is dictated by the cycling expression (every 30 min) of Notch-Delta signaling factors from the tailbud through the paraxial mesoderm and culminating at the next segment to be created (Holley, Jülich, Rauch, Geisler, & Nüsslein-Volhard, 2002). Notch and Delta interact at the plasma membranes between presomitic paraxial mesoderm cells, which enables the transference of pathway activation like a wave from the tail to the last somite boundary. Simultaneously, the location of epithelialization is determined in part by the opposing antagonistic actions of Fgf8 from the tailbud and retinoic acid from more anterior, already segmented paraxial mesoderm. A caudally emanating gradient of Fgf8 morphogenetic signaling functions to repress epithelialization of the paraxial mesoderm, and as the tailbud continues to grow caudally, this Fgf8 gradient slowly releases cells from its repression (Reifers et al., 1998; Shimozono, Iimura, Kitaguchi, Higashijima, & Miyawaki, 2013). Paraxial mesoderm cells experiencing the right balance of retinoic acid stimulation, lack of Fgf8 repression, and the cresting wave of Notch-Delta activation will undergo a MET transition and form a somitic boundary (reviewed in Aulehla & Pourquié, 2010). Severe congenital disorders of the vertebral elements like spondylocostal dysostosis that cause many vertebrae to be fused and malformed are directly associated with mutations in genes of the Notch-Delta pathway (Penton, Leonard, & Spinner, 2012). The zebrafish provide a powerful model to better understand the developmental processes behind somitogenesis and potentially the causes of related human disorders.

Somite patterning to make skeletal muscle and vertebra: Failure to properly form somites in humans causes spine defects because the cells that generate the bones of vertebrae originate from somitic cells (Fig. 45.6G–J). The ordered segmentation of somites helps to guide the proper organization of the developing skeleton in the trunk, as well as guide the position of invading spinal motor nerves and the organized pattern of skeletal muscle fibers (Bernhardt, Goerlinger, Roos, & Schachner, 1998; Ward, Evans, & Stern, 2017; Zeller et al., 2002). Soon after a somite has formed it will begin to differentiate. The cartilage and bone precursors, called *sclerotome*, develop in the most ventral region of the somite, where they lose their epithelial constraints and migrate toward and around the notochord and neural tube. In an unintuitive manner, the sclerotome from the anterior half of one somite will fuse with the sclerotome of the posterior half of its neighboring somite to establish one vertebral element—a process known as *resegmentation* (Ward et al., 2017).

As the revered salmon steak would reveal, a vast majority of the remaining somite will develop into muscle. In an adult fish steak, the small, dark triangle of muscle at the outer edge represents a focus of slow-twitch muscle fibers, while the remainder of the body wall musculature is all fast-twitch muscle fibers (Devoto, Melançon, Eisen, & Westerfield, 1996). This is relevant because the slow-twitch muscle precursor cells are morphologically one of the first cell types to differentiate in the somite. These embryonic slow muscle precursor cells, named *adaxial cells* due to their direct adjacency to the notochord, will be the first to express myogenic genes-like *myoD*. Shortly after somite formation, these adaxial cells transform into fiber-like mesenchymal cells and complete a remarkable movement out to the superficial most edge of the somite, where they will differentiate into slow muscle fibers positioned in a near-perfect parallel monolayer. Fast-twitch muscle differentiation is initiated following the adaxial migration (Cortés et al., 2003; Daggett, Domingo, Currie, & Amacher, 2007; Devoto et al., 1996; Henry, McNulty, Durst, Munchel, & Amacher, 2005; Stellabotte, Dobbs-McAuliffe, Fernández, Feng, & Devoto, 2007). Interestingly, axon guidance cues are differentially expressed by slow muscle and fast muscle precursor cells to properly guide spinal motor axons out of the spinal cord and into the developing musculature (Rodino-Klapac & Beattie, 2004; Zhang, Lefebvre, Zhao, & Granato, 2004).

It has been well known that the amniote somite differentiates into a similar ventral sclerotomal region and a more dorsal myotome; still the myotome is derived from an outermost epithelial structure known as the dermomyotome. Only recently has the teleost dermomyotome been described (Devoto et al., 2006; Stellabotte & Devoto, 2007). The zebrafish dermomyotome is derived from the most anterior border cells (ABCs) of the newly formed somite, which will gradually become positioned to the outermost portion of the myotome, "external" even to the slow muscle monolayer. Similar to the amniote dermomyotome, these external cells function to support myotome growth (Feng, Adiarte, & Devoto, 2006; Stellabotte et al., 2007).

Research across vertebrate species has helped to identify the array of patterning signals derived from adjacent tissues, namely the notochord, neural tube, surface epidermis, and lateral plate mesoderm. Although characterization of these signals in zebrafish is an ongoing area of investigation, a huge wealth of important factors has been identified by noticing changes in the shape of the somite following gene specific loses. Mutants in genes of the hedgehog pathway were originally called *you-class* mutants due to the nonchevron, blocky, or "U"-shaped somite they exhibited (Amsterdam et al., 2004; Barresi et al., 2000; Chen et al., 2001; Schauerte et al., 1998; van Eeden et al., 1996); reviewed in (Stickney et al., 2000). As mentioned earlier, Sonic hedgehog is secreted from the notochord, which is required for the induction of adaxial cells into slow-twitch muscle fibers (Barresi et al., 2000, 2001; Du, Devoto, Westerfield, & Moon, 1997; Lewis et al., 1999). It is hypothesized that failure to form the superficial monolayer of slow muscle fibers contributes to the resulting U-shape of the somite.

Lastly, an emerging "area" where zebrafish are turning out to be an important model is occurring between the somites, at the myotendinous junction. Tendon development involves the sculpting of extracellular matrixes and elaborate connective machinery to muscle and bone, and in the case of the zebrafish myotendinous junction helping to connect the muscle fibers of one somite to another (Snow & Henry, 2009). A critical feature of somite boundary formation is the early deposition of extracellular matrix proteins like Fibronectin and Collagen. Of particular relevance is how defects in the composition or function of matrix factors in the zebrafish myotendinous junction resemble human muscular dystrophies (Charvet et al., 2013; Telfer, Busta, Bonnemann, Feldman, & Dowling, 2010). In fact, the sheer mass and direct accessibility of zebrafish muscle has made significant contributions to the modeling of human muscular dystrophies (Goody, Carter, Kilroy, Maves, & Henry, 2017; Li, Hromowyk, Amacher, & Currie, 2017).

Development of the Circulatory System

Zebrafish is a simple yet elegant vertebrate model that has yielded great insights into early cardiovascular system development. Using a stereomicroscope alone, one can view how the heart, vessels, and blood cells form in the externally developing zebrafish larva. Although the zebrafish heart has only two chambers, it has many structural features found in mammals. The fact that gas exchange through skin alone can suffice for zebrafish larvae to survive up to 5 days has allowed circulation defective mutants to reveal intermediate steps during cardiovascular development. Details regarding early morphogenesis of individual zebrafish cardiovascular organs have emerged mostly from analyses of cardiovascular cell type-specific gene expression and live tracing of fluorescent reporter transgenes (Fig. 45.7A–C; reviewed in Staudt & Stainier, 2012). In the discussion below, we highlight how and where distinct cells within individual circulatory organs originate, differentiate, and migrate in the embryo and how cellular processes and morphogenetic factors influence their assembly.

Heart development: The heart is the first circulatory organ that prominently appears as a contractile structure over the anteromedial aspect of yolk in a 24 h old zebrafish embryo (See Fig. 45.1). The early heart is a single, straight tube connected to vessel precursors of the cardinal vein and dorsal aorta. Heart assembly occurs in two discrete steps, first of which forms a tubular structure and the next separates it into ventricular and atrial compartments while adding cells for their growth.

The ontogeny of myocardial progenitors during distinct stages of heart assembly has been determined through the expression pattern of evolutionarily conserved transcription factors like *nkx2.5*. These studies have revealed that myocardial progenitors originate from bilateral fields near the blastula margins. In addition, expression analysis of the atrial cardiomyocyte-specific myosin gene *amhc* and the ventricular myocyte specific myosin gene *vmhc* show that progenitors of distinct heart chambers are spatially segregated in the blastula and occupy distinct positions in the Anterior Lateral Plate Mesoderm (ALPM) (reviewed in Bakkers, 2011; Staudt & Stainier, 2012).

Initial heart assembly occurs in the primary heart field area that is medially located behind the eyes. Around 20 somite stage, myocardial and endocardial progenitors from bilateral areas within the ALPM migrate into the primary heart field and fuse to form a cone-shaped cluster termed the *cardiac disk*. Later, cells within the cardiac disk involute to form the linear heart tube. Medial migration of myocardial progenitors is promoted by secreted Extracellular Matrix (ECM)

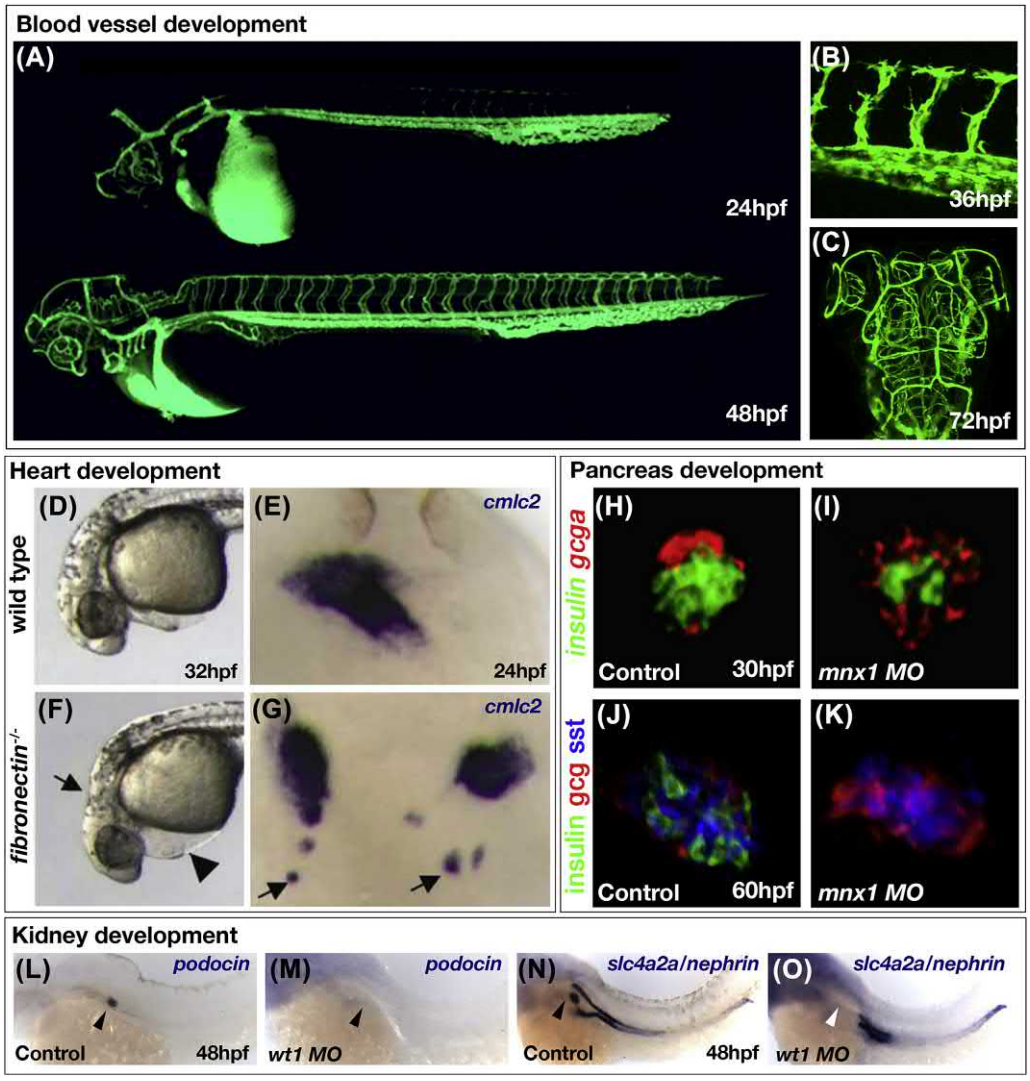

FIGURE 45.7 **Organogenesis.** (A–C) Development of the circulatory system has been observed with microangiography using fluorescent molecules (A,C) and endothelial cell-specific transgenic reporters (B, *tg(fli1a:GFP)*). A basic yet functional vasculature is present at 24 hpf that serves as a stereotyped foundation for the elaboration of a highly sophisticated system of capillaries and veins to develop throughout the entire zebrafish from 48 hpf onward (A). The intersegmental blood vessels and cerebral vasculature are visible in lateral (B) and dorsal (C) views, respectively. Data generously provided by Brant Weinstein. (D–G) Bilaterally positioned heart progenitor cells actively migrate to the midline to form the primary heart field that then becomes asymmetrically localized. Fibronectin is an extracellular matrix protein that is essential for successful heart progenitor cell migration and development of the primary heart field as seen by the pericardial edema (F, arrowhead) and sustained bilaterality with scattered progenitor cells in *natter (fibronectin)* mutants (F, G) (Trinh & Stainier, 2004). (H–K) Expression of glucagon, insulin, and somatostatin by the pancreatic alpha, beta, and delta cells, respectively (H,J). *mnx1* is required for insulin-secreting beta cells (I,K). Data generously provided by Gokhan Dalgin and Victoria Prince. See also (Dalgin et al., 2011). (L–O) The embryonic kidney is represented by the glomerulus (marked by *podocin* and *nephrin* expression) and the pronephric duct (also expressing *nephrin)* (L,N). Loss of *wt1* results in the specific loss of glomerulus formation (M,O) (Tomar, Mudumana, Pathak, Hukriede, & Drummond, 2014).

proteins, such as Fibronectin, whose deficiency in the *natter* mutant induces a cardia bifida-like phenotype (Fig. 45.7D–G Trinh & Stainier, 2004). As the heart matures around 48 hpf it loops to form a ventricle on the right side and atrium to the left with distinct and conspicuous outer and inner curvatures. From a dorsal view, the linear heart tube can be seen oriented toward the left eye of wildtype embryos around 27 hpf. This normal asymmetry in the embryonic heart position is regulated by expression of endoderm associated nodal related genes *southpaw* (spaw) and *pitx2* that begins around the Kupffer's vesicle (KV) and becomes progressively restricted to the anterior, left side. Heart laterality is reversed in the *spaw* gene mutant *straightforward* (Noël et al., 2013), as well as in many cilia gene mutants due to perturbation of KV signal sidedness (reviewed in Bakkers, Verhoeven, & Abdelilah-Seyfried, 2009).

Besides the outer myocardial layer, the early heart consists of an inner endocardium, which originates from endothelial progenitors. Endocardial progenitors

also originate in the ALPM but are located relatively anterior to the myocardial progenitors and migrate medially in response to Vegf (Reviewed in Haack & Abdelilah-Seyfried, 2016). The endocardium, in conjunction with hemodynamic forces, plays important roles in coordinating radial movements of myocardial cells and in regulating heart size (de Pater et al., 2009). The loss of endocardium and endothelial cells in *cloche* mutant embryos greatly increases the heart volume but reduces the thickness of its walls (Holtzman, Schoenebeck, Tsai, & Yelon, 2007). The AV valve separating the two chambers is also derived from the endocardium.

The heart grows substantially after 48 hpf and forms distinct internal divisions across the atrial and ventricular chambers. However, later growth of the heart does not occur by the proliferation of existing myocardial cells within the initial heart tube but by addition of differentiating mesenchymal cells from the secondary heart field (SHF) area. The SHF located in the pharyngeal mesoderm is identifiable by the expression of the *latent TGFβ binding protein 3 (ltbp3)* (Zhou et al., 2011). Late differentiating cardiomyocytes from the SHF add cells to the ventricle, atrium, and the outflow tract (de Pater et al., 2009).

The outermost protective heart layer or the pericardium is the last to form around 72 hpf and includes heterogeneous cells attracted from outside the heart field areas after these undergo an epithelial to mesenchymal transition (Peralta, González-Rosa, Marques, & Mercader, 2014; Serluca, 2008). Pericardial edema is a frequent phenotype associated with poor heart function and circulation caused by the expansion of space between the myocardium and pericardium due to fluid buildup.

Vascular development: Zebrafish larvae exhibit a stereotypic closed-loop pattern of vessels that circulate blood from the heart to peripheral tissues and back (See Figs. 45.1 and 45.7A–C). As the pattern of vessel network is reproducible across different zebrafish larvae, it is easy to detect perturbations induced by genetic or chemical manipulation of cellular processes. Significantly, the cellular origins of zebrafish arteries, veins, and capillaries (including those of lymph) are also similar to that of other vertebrates (Gore, Monzo, Cha, Pan, & Weinstein, 2012).

The cells that form blood vessels belong to a special category of endothelial cells known as angioblasts, whose identity can be detected by their expression of genes, such as *scl* or *flk-1*(Kabrun et al., 1997). In zebrafish, the earliest precursors of vascular endothelial cells have been traced to the ventral mesoderm in shield stage embryos. Around the 14–16 somite stage of development, clusters of endothelial cells specified to become angioblasts migrate toward the midline and in rostral and caudal directions. The rostral stream of migrating angioblasts reaches pharyngeal mesoderm and ALPM, whereas the caudal stream reaches the posterior lateral plate mesoderm (PLPM) and spreads along the trunk between the endoderm and hypochord (Bussmann, Bakkers, & Schulte-Merker, 2007). Around 24 hpf, the dorsal aorta (DA) and cardinal vein (CV) are the first major undivided vessels that emerge in the trunk. In contrast to other vertebrates, the DA and CV do not fuse together in zebrafish; however, steps in their formation have revealed significant insights into conserved cellular mechanisms that guide blood vessel formation in vertebrates (Isogai, Horiguchi, & Weinstein, 2001). Blood vessels form de novo via the process of vasculogenesis involving coalescence of adjacent angioblasts (Fouquet, Weinstein, Serluca, & Fishman, 1997). Interestingly, it has been demonstrated in zebrafish that the arterial and venous fates of major vessels are predetermined: regulated by Notch signaling pathway angioblasts committed to form arteries express genes encoding the membrane ligand EphrinB2 and components of Notch signaling pathway; whereas venous angioblasts express the *ephrinB4* gene encoding the receptor that recognizes EphrinB2 (Lawson et al., 2001).

In response to somite secreted VegF only the DA begins to sprout a set of uniformly spaced, perpendicular Intersegmental Vessels (ISV) from its dorsal aspect. ISV assembly represents a distinct process of angiogenesis that sprouts new vessels from preexisting ones. Studies with zebrafish *fli1a* transgenics show that during angiogenesis, the endothelial cells at specific locations within the walls of the DA divide and differentiate to sprout a distinct "tip cell" that extends filopodia in all directions. To begin migrating, the "tip cell" first divides asymmetrically to create itself and a new daughter "stalk cell" near its connection to the parent vessel. Extending dorsally, the ISVs stay confined between the somites in response to secreted chemorepellent cues. Upon reaching the level of the dorsal neural tube, the ISVs give out rostral and caudal branches that merge to form the dorsal lateral anastomotic vessel (DLAV) (Isogai et al., 2001). Initially the ISVs do not show arterial or venous characteristics but do so later in response to blood flow (Isogai, Lawson, Torrealday, Horiguchi, & Weinstein, 2003). Lastly, newly formed vessels do have a lumen which forms later as endothelial cells form vacuoles that fuse inside the vessel (Kamei et al., 2006).

Live imaging in zebrafish has shown that lymph vessels and capillaries form after cardiovascular development (Yaniv et al., 2006). Although lymph vessels are closely associated with those of blood, the two are not directly connected. Lymph endothelial cells are derived from venous endothelial cells but distinguishable via unique expression of the *vegf-c* or *prox-1* genes (Küchler et al., 2006). The trunk lymphatic vessels emerge from parachordal vessels, which are derived

from secondary sprouts of the posterior cardinal vein and migrate along arterial but not venous ISVs (Mulligan & Weinstein, 2014).

Hematopoesis: In developing zebrafish embryos, circulating blood cells first appear shortly after 24 hpf when the heart starts beating. Cells of blood like those of vessels originate from endothelial cells, presumably derive from a common precursor termed *hemangioblasts*. Zebrafish blood shows the presence of all major cell types of the erythroid and myeloid lineage, including granulocytes, macrophages, neutrophils, and platelets (reviewed in Carroll & North, 2014).

Zebrafish blood cells form in two waves, and these differ with respect to their origin in the embryos. Between 12 and 24 hpf an initial or primitive wave of hematopoiesis generates cells of the erythroid lineage from the inner mass of cells of the PLPM (Detrich et al., 1995) and macrophages from a rostral location of the ALM (Herbomel, Thisse, & Thisse, 1999; Lieschke et al., 2002). However, these cells are limited in their ability to meet the oxygen needs of growing embryos. Thus, similar to mammalian blood development, a second or definitive wave of hematopoiesis around 30 hpf generates multipotent hematopoietic stem cells (HSCs) from hemogenic endothelium in the ventral wall of the dorsal aorta designated as the aorta-gonadal-mesonephros (AGM) (Bertrand et al., 2010; Kissa & Herbomel, 2010). Zebrafish mutants of the *runx1* gene that is expressed in the AGM show severe loss of definitive blood cell lineages at 5 dpf (Kalev-Zylinska et al., 2002; Lam, Hall, Crosier, Crosier, & Flores, 2010). Around 36 hpf, most HSCs circulate in the blood and settle into supportive niches of the kidney or migrate to a posterior region in the tail termed *Caudal Hematopoietic Tissue* (CHT), which represent sites of continued hematopoiesis from 3–4 dpf through adulthood (Ma, Zhang, Lin, Italiano, & Handin, 2011).

Organogenesis—the Kidney

The pronephros: The kidney in zebrafish embryos or the pronephros is an anatomically simple structure comprising a pair of tubular nephrons (See Fig. 45.1). The zebrafish nephron is functionally similar to that of higher vertebrates, comprised of three functional segments, which, from the anterior to posterior, are the glomerulus, proximal tubule, and distal duct. The pronephros develops from intermediate mesoderm progenitors adjacent to those of the blood and distinguishable by the expression of transcription factors encoding genes *pax2, pax8, lhx1* and *hnf1ba* (reviewed in Drummond & Davidson, 2010). During somitogenesis, distinct pronephric segments form as cells of the intermediate mesoderm undergo mesenchyme to epithelial transition (MET) and differentiate under the influence of retinoic acid from the anterior paraxial mesoderm (Wingert et al., 2007).

The most anterior section of the pronephros or the glomerulus occupies a medial position in the embryo, posterior to the fin buds near the third somite. The glomerulus is essentially a network of leaky blood capillaries covered by a unique type of epithelial cells termed *podocytes* characterized by multiple apical foot processes. The interdigitating podocyte foot processes form a barrier around the capillary endothelial cells and filter blood by excluding cells and solutes on the basis of molecular size and charge. Pronephric cells within the glomerulus originate from a cluster of progenitors expressing paralogs of the Wt1 transcription factor (Majumdar & Drummond, 1999). The segments immediately posterior to the glomerulus include the pronephric neck and proximal tubule, which are highly endocytic and serve to reabsorb the bulk of leaked macromolecules (Anzenberger et al., 2006). These segments are distinguished by the expression of the transcription factor encoding gene *pax2a*. In zebrafish mutants of the *wt1, pax2,* and *hnf1* transcription factors, one kidney segment expands at the expense of the other (Fig. 45.7L–O Majumdar, Lun, Brand, & Drummond, 2000; Naylor, Przepiorski, Ren, Yu, & Davidson, 2013). Notch-dependent signaling regulates differentiation of pronephric epithelial cells into transporting or multiciliated cell types after 24 hpf (Liu, Pathak, Kramer-Zucker, & Drummond, 2007). Around 24 hpf, the anterior pronephros develops a characteristic lateral bend due to extension of the neck segment, which progressively contracts beyond 48 hpf due to fluid flow driven collective migration of proliferating cells (Vasilyev et al., 2009).

The very posterior pronephric segment, including the exterior tubular opening, is a highly proliferative zone distinguished by its expression of the oncogene *c-ret* (Vasilyev et al., 2009). The distal tubule of the kidney is closely associated with the corpuscle of Stannius. This is a cluster of cells marked by the T-box transcription factors Tbx2a/b that regulates calcium and phosphate levels (Drummond, Li, Marra, Cheng, & Wingert, 2017).

The zebrafish kidney begins to function around 48 hpf as the glomerular podocytes differentiate to filter blood. Physiological adaptations of kidney enable zebrafish to thrive in freshwater by wasting a high volume of water while reabsorbing essential solutes. The zebrafish kidney, unlike mammals, lacks smooth muscle and instead relies on highly motile multicilia that act as fluid pumps to continually expel urine. Embryonic kidney defects in zebrafish mutants prominently manifest phenotypes, such as glomerular cysts and/or severe pericardial or general edema. A majority of cystic kidney phenotypes have revealed the importance that cilia genes play in the cellular mechanisms associated

with complex human disorders of not only the kidney but across multiple organs.

In the adult zebrafish, the kidney appears as a dense bilateral network of, branched, pigmented tubules under the dorsal body wall between the gills and caudal fins. The growth of pronephric into adult mesonephric kidney occurs by the addition of more nephron units rather than proliferation within the initial nephron (Zhou, Boucher, Bollig, Englert, & Hildebrandt, 2010).

Organogenesis—Endoderm Derived Organs

The digestive system of zebrafish, which includes the intestinal tract, liver, and pancreas originates mainly from the embryonic endoderm. Its early development could potentially reveal what defects may underlie metabolic disorders, such as diabetes, and has thus, spurred excitement in the analysis of zebrafish endoderm mutants and transgenics (Field, Ober, Roeser, & Stainier, 2003). These efforts are defining a trajectory of specification and assembly of distinct digestive structures beginning as early as the blastula stage, although the digestive tract does not open from the mouth to anus until 5 days postfertilization. Severe defects in all endoderm derived structures in the zebrafish *cyclops* and *squint* mutants have revealed an essential role for the secreted morphogen Nodal and members of the FoxA transcription factor family in specifying distinct regions of the digestive tract (Dougan, Warga, Kane, Schier, & Talbot, 2003; Niu, Shi, & Peng, 2010). Consistently, *nodal* is expressed at high levels near the origins of endoderm within a few layers of circumferential cells near the blastula margin between the yolk and overlying cells of the embryo (Fauny, Thisse, & Thisse, 2009). With the onset of gastrulation these endodermal cells involute forming large, flat cells with bipolar filopodial extensions that eventually converge dorsally and distribute along the anteroposterior embryonic axis (Pézeron et al., 2008). With the onset of somitogenesis, these cells distribute sparsely along the most ventral aspect of the embryo and coalesce medially to form a rod-like structure along the midline of a 20 hpf embryo (Miles, Mizoguchi, Kikuchi, & Verkade, 2017). This endodermal rod specializes into anterior pharyngeal endoderm at the level of the first somite, and a posterior alimentary tract. In the following section, we summarize how the developing gut specializes into three functionally distinct segments; an anterior intestinal bulb, a mid-intestine, and posterior-intestine and gives rise to the liver and pancreas as outgrowths of the intestinal bulb.

Gut development: The distinct segments of zebrafish gut are lined by epithelial cells that in essence, become transformed from the endoderm into gut between 26 and 52 hpf (See Fig. 45.1). During this process, the rod-like endoderm grows and generates a bilayer of cells. Subsequently, this bilayered structure begins to form interspersed cavities as the cells form apical membranes. The cavities eventually join to form a continuous lumen, which by 76 hpf extends across its entire length (Ng et al., 2005). While no cell death occurs during the formation of intestinal cavities, the development of distinct membrane polarity needs to happen during a precise window of development. For instance, the delayed membrane polarization in the *heart and soul (has)* mutant produces multiple lumens in the intestinal bulb (Horne-Badovinac et al., 2001). The intestinal bulb, which forms the most anterior segment of the alimentary canal, has only one lumen but multiple folds directly connects the esophagus to the intestine. The intestinal bulb serves a similar role as the stomach, having highly proliferative secretory cells restricted to the basal layers. However, the intestinal bulb lacks the stomach's characteristic acid-secreting cells. The mid-intestine is comprised of enterocytes, as well as enteroendocrine cells interspersed with mucus-secreting goblet cells. Interestingly the same mid-intestinal enteroendocrine cells can secrete both glucagon and somatostatin, unlike cells of the pancreas, where these are secreted by distinct cell types (Ng et al., 2005). The posterior intestine is mainly comprised of cuboidal epithelium. While there are no folds in the middle and posterior intestine, the intestinal bulb develops multiple folds around 4 dpf, when the anus also opens to the outside. By 5 dpf, the entire intestine becomes enveloped by a thickened layer of mesenchyme, the yolk gets depleted, and the embryo begins to actively eat.

Liver development: The liver plays important roles in metabolism, detoxification, and fat digestion, and can be first distinguished by expression of differentiated liver markers, such as Ceruloplasmin (Cp) (Korzh, Emelyanov, & Korzh, 2001). *cp* expression at the 16 somite stage demonstrates the early left sidedness of the liver, located in the embryo near the junction of the intestinal bulb at about the first somite axial position (See Fig. 45.1). Cell fate analysis with gut-specific reporter lines shows that liver progenitors reside asymmetrically within late blastula stage embryos, in ventrolateral and dorsolateral positions along the left side and right sides respectively. Beside fluorescent transgenes, liver cells can be easily visualized by fluorescently labeled modified phospholipids. During the 24–28 hpf, the hepatocyte progenitors aggregate as a distinct projection within the intestinal primordium, near the spot where the prospective intestinal bulb also loops. Around 50 hpf as the liver buds and grows further, the tissue connecting it to the intestinal bulb becomes restricted to form the bile duct. The liver becomes vascularized by endothelial cells, which encapsulate it partially around 60 hpf with complete invasion by 72 hpf (Field et al., 2003).

Pancreas development: The zebrafish pancreas, like other vertebrates, is an important exocrine and endocrine gland associated with the intestine. In the developing zebrafish embryo, pancreas precursor cells can be distinguished by the expression of the *pdx1 (pancreatic and duodenal homeobox1)* homolog as a bilateral cluster adjacent to the midline around the 10 somite stage (Biemar et al., 2001). The initial pancreas appears to contain a single endocrine islet surrounded by exocrine tissue. These coalesce by the 18 somite stage to form a single domain located on the right side of the midline (See Fig. 45.1). The quintessential function of the pancreas is its production of hormones, including insulin. The pancreas is best known for its alpha, beta, and delta cell types that function to secrete glucagon, insulin, and somatostatin, respectively (Fig. 45.7H,J). While insulin expression can be observed within the pancreas as early as 12 hpf, somatostatin and glucagon expression appears later at 16 and 24 hpf respectively. The *mnx1* transcription factor plays a required role in promoting insulin-secreting beta-cell development at the expense of alpha cell fates while having no influence over delta cell development (Fig. 45.7I,K Dalgin et al., 2011). Moreover, the Hedgehog pathway plays an essential role in the development of the endocrine pancreas, which is lost in mutants like *sonic you (shh)* and *smoothened* (DiIorio, Moss, Sbrogna, Karlstrom, & Moss, 2002; Roy, Qiao, Wolff, & Ingham, 2001; Tehrani & Lin, 2011).

Summary

The multitude of experimental advantages from its size, accessibility, development speed, and visible tractability to its genetic, molecular, and cellular approaches have all made the zebrafish a powerful model system to uniquely piece together the complexities underlying vertebrate development. The conservation of these developmental mechanisms across phyla has provided great and often direct relevance of the investigations in zebrafish to human development and disease. An emergent theme in development is how only a limited array of signaling molecules is reiteratively used to induce distinct tissues by establishing different patterns of conserved, cell-type determining genes that predominantly vary only in their spatiotemporal expression within a given tissue. As new experimental tools and systems-level approaches improve, it will be fascinating to *watch* how our understanding of the complex interactions between cells, tissues, and even between the embryo and its environment function together to shape the vertebrate embryo—through the perspective of the zebrafish.

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CHAPTER

46

Zebrafish as a Model for Revealing the Neuronal Basis of Behavior*

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Introduction

Wh ile much of the original work using the zebrafish model in neuroscience was focused on developmental questions (Eaton, Farley et al., 1977; Eisen, 1991; Eisen, Pike, & Debu, 1989; Grunwald, Kimmel, Westerfield, Walker, & Streisinger, 1988; Kimmel, 1982; Kimmel, Sessions, & Kimmel, 1981; Myers, Eisen, & Westerfield, 1986; Streisinger, Coale, Taggart, Walker, & Grunwald, 1989), zebrafish offer major advantages for revealing how vertebrate brains produce behavior (Fetcho & Liu, 1998; Kimmel, Eaton, & Powell, 1980). This role for fish might not seem so obvious, but the advantages of small size, transparency, and genetic tools that lie at the heart of the power of the zebrafish model also catalyze its role in studies of brains and behavior. These studies are typically not directed toward understanding fish per se, but rather have as their goal the discovery of principles underlying brain function that apply to vertebrates broadly. This is possible because, to a first approximation, all vertebrate brains are the same (Butler & Hodos, 1996).

Vertebrate Brains Have Much in Common Across Species

Nearly every region in the nervous system, with the notable exception of the multilayered cerebral cortex in mammals, is present in all vertebrates, including such important regions as the olfactory bulbs, retina, telencephalon, hippocampus, amygdala, thalamus, hypothalamus, optic tectum, basal ganglia, hindbrain, cerebellum, spinal cord and the major dopaminergic, serotonergic, and noradrenergic neuromodulatory systems (Butler & Hodos, 1996). This is not too surprising because vertebrates evolved to do many of the same behaviors. They all use their senses (vision, hearing, smell, proprioception) to move about in coordinated ways to find food, water, shelter, and mates, and avoid becoming food. They learn about the external world through experience and store that information to produce adaptive behavior. While the execution (swimming vs. walking for example) might be different across animals, the brain regions and neural computations that process internal and external sensory information and produce appropriate outputs have much in common across species, allowing us to use zebrafish to inform us about how brains and spinal cords work across vertebrates generally.

The Challenge of Understanding How Brains Generate Behavior

Our understanding of the generation of behavior by vertebrate brains is still in its infancy; it is one of the greatest remaining biological puzzles. To set the stage for the power of zebrafish and its place along the path to revealing brain function, a short account of what is needed to explain how any particular behavior is

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produced by a brain will provide a useful context for discussion.

Behavior

The challenge begins with a proper definition of the behavior of interest. It could be a relatively simple motor behavior, such as the escape behavior that fish use to avoid predators or a seemingly simple decision to turn to the left or right. On the other hand, the behavior might be much more complex, such as learned avoidance of dangerous places, navigation through a complex environment (3D for fish), or chasing down a prey item. In either case, the behavior must be studied rigorously to define how it is shaped by sensory information from the environment and prior experience, and the details of the movements that underlie it. These transformations from sensation to decision and action, and the influence of behavioral history (learning and memory) and internal state (hunger, fear, sexual, or general arousal, etc.), are what must be explained at the level of the neurons and circuits in the brain and spinal cord.

Neurons

The next critical step is to reveal what neurons are involved by somehow monitoring their activity in the brain. Given that most brain regions are heterogeneous, with neurons of different function and connectivity, the ability to resolve the activity of individual cells is critical. Electrophysiological or imaging approaches are methods of choice. Electrophysiology, such as whole-cell patch recording from individual cells, can resolve the exact firing pattern of the neurons and even the synaptic inputs from other cell types. It also allows the detailed study of the electrical/ion channel properties of the cells, which influence how they respond to synaptic input from other neurons. The study of how these properties and circuit function can be modified by neuromodulators that can reshape activity without altering physical connections between neurons also demands electrophysiology (Lovett-Barron, Andalman et al., 2017). The limitations of electrophysiology are its invasiveness (surgical exposure and paralysis are typically needed) and an inability to record from more than a few neurons at once. Both of these limitations are overcome by using imaging, done most commonly with fluorescent calcium indicators, which change intensity when calcium levels in neurons rise (or fall) during activity. Here, the benefit of the transparency and small size of the larval fish is enormous, as large numbers of neurons, even deep in the brain, can be imaged with single-cell resolution to tie the activity of both individual neurons and neuronal populations to behavior. The benefit, however, comes at the cost of temporal resolution, as the millisecond and submillisecond resolutions of electrophysiology are impossible to achieve with the slow kinetics of calcium sensors and their indirect ties to the actual electrical activity. Fortunately, zebrafish are accessible both to electrophysiological and imaging approaches (Fetcho & O'Malley, 1995; Legendre & Korn, 1995), so one can obtain both high temporal resolution and population-level information about the ties between active neurons and the behavior of interest.

Wiring

The patterns of neuronal activity during a behavior are not sufficient to reveal how that behavior is produced by a brain because the brain uses connections/wiring between neurons to compute and transform external and internal information into appropriate action. The precise wiring is critical, although it sometimes does not get the attention it deserves. It is what defines the circuit! This is often missing from studies of zebrafish because it is one of the hardest bits of information to obtain. The most direct path to it is to record from pairs of neurons, then activate one and monitor the electrophysiological response in the other, to reveal the type (chemical, electrical, excitatory, inhibitory, neuromodulatory) of synapse, its strength, and the plasticity of the connection in response to different patterns of activation of the presynaptic cell. This pairwise (and even triple) recording can be done in zebrafish with optical targeting of electrodes to particular neurons (Koyama et al., 2016). It is hard, but feasible. Newer approaches to reveal connectivity combine optical activation of neurons with optical (or better, electrophysiological) monitoring of responses in potentially connected cells (Forster, Dal Maschio et al., 2017).

Another approach to connectivity that is just now reaching fruition in zebrafish is electron microscopic reconstruction of large regions of the brain, which reveals the pattern of connections of the neurons because both neuronal processes and their input and output synapses are visible at EM resolution. This does not provide direct functional information about connection strength or synaptic properties, but it is still critical information as it forms a ground plan for circuit wiring. Remarkably, there are still very few cases of any brain, in which, we know even how many output connections a single neuron has and how they are distributed because it is so difficult to obtain them with physiological methods. Still, this is critical for understanding circuit function and computations. EM provides this and can do so even for entire regions of the brain. In the small larval zebrafish, this opens access

to the baseline structural connectivity anywhere in the brain via whole-brain EM sectioning (Hildebrand et al., 2017; Wanner, Genoud, Masudi, Siksou, & Friedrich, 2016). The combination of light level imaging of structure, even at high resolution with expansion microscopy (Freifeld et al., 2017), and activity with EM level connectivity anywhere in the brain makes zebrafish unique among the vertebrates as a model for revealing how brains produce behavior.

Models

The complexity of the wiring in brains, the varying electrical properties of different neurons and the multitude of interactions among cells makes understanding how the brain generates behavior a challenge, even with activity and wiring in hand. We typically cannot look at the data and intuit how computation works. This demands models, grounded in the data, that simulate the neuronal interactions, so that we can see whether the information we have captures what is needed to produce observed patterns of activity appropriate for driving behavior (Koyama et al., 2016; Naumann et al., 2016).

Importantly, such models should have predictive power, since understanding and explanation in science is synonymous with predictive power. Indeed, even brains themselves are mostly concerned with predicting what to do next. Tests of the predictions assess model quality. Zebrafish are especially amenable for testing predictions because the model predictions often take the form of what happens to the behavioral output (and activity of other cells in the circuit) when particular neurons are removed or activated or inactivated. The transparency of the zebrafish allows for very specific optical and genetic perturbations, including application of light-activated proteins for activating or silencing neurons, discussed in later sections (Douglass, Kraves, Deisseroth, Schier, & Engert, 2008; Kimura, Satou et al., 2013).

The Goal

In the end, we can claim to understand the behavioral features under study when we have a model built upon known information about cell types, their electrical properties and activity, and their wiring that both produces the output seen during the behavior and that generates predictions that have been confirmed by empirical biological methods. The model need not include everything, and likely will not, as biological systems are complex at many levels. Predictions will eventually falter, but further biological studies will reveal critical features that are missing from the model and can be added to expand its predictive power. The goal is the simplest model that explains what is known, has large predictive value, and hopefully reveals features of the computational algorithms used to transform internal and external sensation and stored information into adaptive actions.

A History of Zebrafish as a Model for Understanding the Generation of Behavior

The Beginning

All of the steps toward revealing the neuronal basis of behavior summarized in the previous sections become easier if one can simply look to the brain of an animal and see everything that is happening all at once. Larval zebrafish are now close to offering that, but reaching the required level of sophistication took several decades of effort starting with the genetic studies of George Streisinger in the late 1970s and early 1980s. Working nearly alone, so as not to risk the careers of his students and postdocs, Streisinger pioneered methods for the induction and study of mutations in the zebrafish genome using gamma rays (Chakrabarti, Streisinger, Singer, & Walker, 1983; Stahl, 1994). He also developed methods of inducing haploidy in the progeny of mutants, which facilitated the identification of recessive lethal mutations, as the haploid zygotes can survive for several days after fertilization (Kimmel, 1989; Patton & Zon, 2001; Streisinger et al., 1989; Streisinger, Walker, Dower, Knauber, & Singer, 1981). These pioneering genetic methods, along with *in vivo* imaging of zebrafish during development (Eisen, Myers, & Westerfield, 1986; Liu & Westerfield, 1988), initial studies of motor innervation and function by the Oregon group (Liu & Westerfield, 1988), and their generosity in helping others with the model, catalyzed large-scale screens for mutations affecting embryogenesis in zebrafish in Boston (USA) and Tubingen (Germany), which identified roughly 2000 genes necessary for normal development (Driever, Solnica-Krezel et al., 1996; Haffter, Granato et al., 1996).

Mutagenesis and Behavior

Studies of genetic effects on behavior took a leap forward in zebrafish work because the big screens included behavior-based assays in the larvae that took advantage of the early development of motor behavior in order to identify mutations producing defects in motility and optomotor processing. In the screening by Granato et al (Granato, Van Eeden et al., 1996), mutagenized progeny were examined for motility defects in response to touch (which develops in normal embryos at around 24 h postfertilization). This screening

identified 166 mutations that resulted in motility defects and included mutations that affected muscle development, incapacitated proteins necessary for synaptic function at the neuromuscular junction or centrally, and genes necessary for correct wiring of the nervous system. Another interesting screen exploited the tendency of larval zebrafish to swim along and visually track moving gratings (termed the *optomotor response*). In an optomotor-based assay (Neuhauss, Biehlmaier et al., 1999), mutants from the Tubingen screen (450 lines) were exposed to a moving grating displayed on a monitor, and those that failed to accumulate at one end of a rectangular arena were examined for defects in visual anatomy and sensitivity. This screen identified 25 mutants, 13 of which exhibited retinal dystrophy (loss or degeneration of cell layers in the retina) and others that exhibited defects, such as lens degeneration, deficiency in the pigment melanin, miswiring of axons, and absence of retinal ganglion cells, among other defects.

The Quantitative Study of Behavior in Larval Zebrafish

Investigations into the neuronal basis of behavior in normal and mutant zebrafish depend on a critical foundation in the careful quantitative analysis of the behaviors. Early on, scientists were concerned with the relationship between activity in the giant Mauthner cell and the escape behavior it was thought to initiate in teleost fish (including the zebrafish), and sought to relate the activity of this cell to the behavior (Eaton & Farley, 1975). Because the escape response (C-start) elicited by this cell is so fast, there were early technological limitations in being able to record the movement of the animal with sufficient temporal resolution. In a C-start, fish make a characteristic fast bend to one side (so that the animal resembles a C) before a weaker bend to the other side followed by lower amplitude bends as the animal swims away (Kimmel, Patterson, & Kimmel, 1974). Kimmel et al. (Kimmel et al., 1974) were able to reveal the latency of this rapid response by using a photoresistor to detect breaks in a beam of light. This method determined that a fast C-start response develops after 4 days postfertilization (dpf) in zebrafish, although slower responses were present after 3 dpf. The C-start has a latency of 15 ms in the larvae, with similar latencies (and general movement patterns) in adult animals. Advances in video technology allowed higher rates of image acquisition. Eaton et al. (Eaton, Farley et al., 1977) filmed larval escapes to a probe touch at 133 frames per second and were able to obtain more detailed information about the series of movements larvae produce during a C-start escape. At 44–48 hpf and 88–95 hpf, analysis of these images revealed a response latency of 10.2 and 12.3 ms respectively. Video imaging of larvae at 1000 Hz with custom-written automated computer analysis (Liu & Fetcho, 1999), and more recently with software to analyze multiple animals simultaneously (Burgess & Granato, 2007), has refined this number even further; in response to an acoustic tone, 6 day old larvae produced either a short latency C-Start with a mean of 5.3 ms or a longer-latency C-start with similar movement kinematics as the fast one, but with a latency averaging 28.2 ms.

Although early behavioral studies focused on C-start escapes, the combination of high-speed imaging and detailed quantification they pioneered have provided the foundation for inquiries into circuits controlling other aspects of zebrafish behavior. Classification of slow and fast swimming behaviors, routine turns, and prey-capture behaviors by careful quantification of tail bend angles and frequencies, swim velocities, and other fin and eye movements, provided a valuable reference point for many studies of zebrafish neural circuits (Budick & O'Malley, 2000; Burgess & Granato, 2007).

The Advent of in vivo Functional Imaging in Zebrafish

Taking advantage of the transparency of zebrafish, researchers took a key step toward understanding the behavioral function of neurons by performing the first imaging of neuronal activity of single cells in the brain and spinal cord of an intact vertebrate (Fetcho & O'Malley, 1995; O'Malley, Kao, & Fetcho, 1996) (Figure 46.1A). Neurons were filled by injection of dextran tagged synthetic calcium indicators that are picked up by neuronal processes into the muscle or spinal cord and the labeled neurons were imaged with confocal microscopy in restrained fish embedded in agar. These indicators increase their fluorescence as calcium levels rise in electrically active neurons. The early experiments revealed the potential to simply watch the activity of cells even deep in a vertebrate brain. However, it was important to develop methods for filling neurons with indicators that did not depend on damaging their processes to facilitate indicator uptake. This was done initially by injecting calcium indicator into single-cell embryos and raising them to larval stages, at which time, cells throughout the body, including brain and spinal cord, were filled with an indicator that could be used to detect neuronal activity (Cox & Fetcho, 1996). A better way was on the horizon, however, with the demonstration of the ability to make transgenic fish with the beautiful expression of fluorescent proteins (Higashijima, Hotta, & Okamoto, 2000; Higashijima, Okamoto, Ueno,

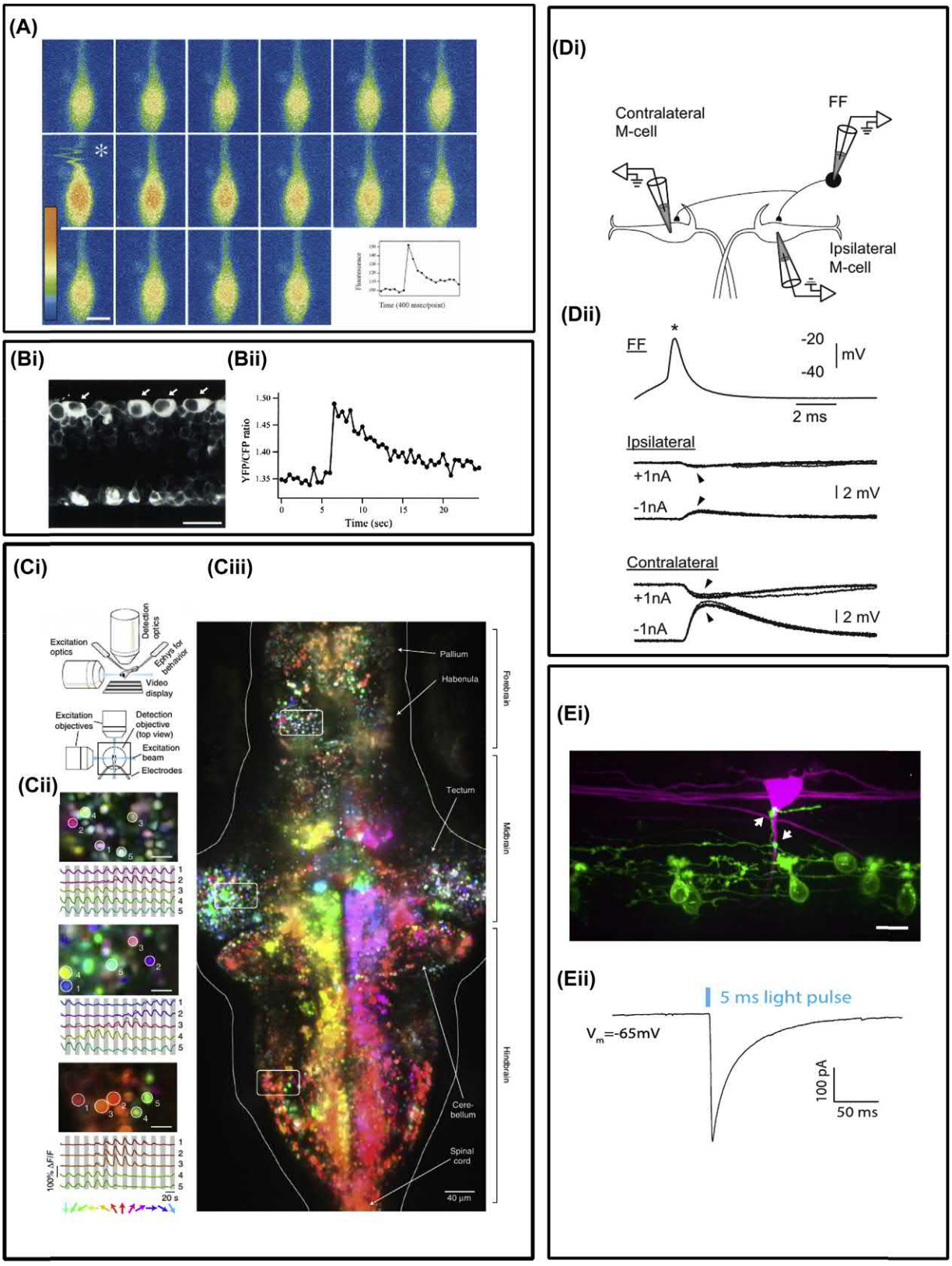

FIGURE 46.1 (A) Calcium green-dextran backfilled Mauthner neuron exhibits a dramatic increase of fluorescence during an escape response (hotter colors represent higher image intensities, see color bar lower left). Each image is 400 ms apart, and the scale bar is 10 microns. Inset in bottom right indicates the level of fluorescence in each image. Adapted from (O'Malley et al., 1996). (B) Cameleon expressed throughout the nervous system, and in particular, Rohon-Beard (RB) sensory neurons in the spinal cord (left, arrows). The RB neurons exhibit an increase in the YFP/CFP ratio in response to an electrical stimulus applied at 5 s. Adapted from (Higashijima et al., 2003). (C) Whole-brain light-sheet imaging during visual adaptation to moving gratings. Larval fish was suspended over a video display (top left) while the motor responses were monitored from spinal ventral roots. Tuning of individual neurons was assessed by their response to gratings moving in a particular direction (traces one to five, see arrows on bottom) and each neuron was color coded by its preferred direction (see bottom arrows). Each box on the left is a magnified region from the image on the right, corresponding to habenula, tectum, and hindbrain. The preferred tuning of all imaged neurons during the course of the experiment is shown by the color map on the left. Adapted from (Freeman, Vladimirov et al., 2014). (D) A triple patch recording showing the activity of the ipsi and contralateral Mauthner neuron after depolarization of a feedforward interneuron. Adapted from (Koyama et al., 2016). (E) Chr2-mCherry (green) expressed in spinal cSF neurons and GFP expressed in a sensory-related CoPA interneuron (top). Targeting the cSF neurons with blue light (bottom) causes current to flow into the CoPA neuron due to synaptic contact between the cells (arrows top). Adapted from (Hubbard et al., 2016).

Hotta, & Eguchi, 1997; Long et al., 1997). In parallel, the first genetically encoded fluorescent calcium sensor was engineered by adding a calcium-binding domain to a fluorescent protein (Miyawaki, Llopis et al., 1997). Subsequently, zebrafish were the first vertebrates with a transgenic fluorescent indicator of neuronal activity expressed in all neurons (Higashijima, Masino, Mandel, & Fetcho, 2003) (Figure 46.1B).

The early genetically encoded calcium sensors worked in fish but had relatively small signals, so there was a lag in their adoption after an initial successful demonstration of their functionality (Higashijima et al., 2003). Most early works applied various synthetic indicators, although there was some application of the early genetic indicators by the Friedrich lab (Li, Mack et al., 2005), which also did important early imaging work of exposed portions of adult zebrafish brains (Friedrich & Korsching, 1997). This has changed recently with the optimization of indicators that can produce robust calcium transients, in the best situations detecting calcium transients from even single action potentials (Chen, Wardill et al., 2013; Nagai, Yamada, Tominaga, Ichikawa, & Miyawaki, 2004; Tian, Hires et al., 2009). Many of these are based on an approach, in which, a calcium-sensitive protein linker (calmodulin) is placed in a split fluorescent protein such that the conformation changes upon calcium binding in a way that partially reconstitutes the normal structure of the fluorescent protein to produce a fluorescence increase (Nagai, Sawano, Park, & Miyawaki, 2001). Optimization of these so-called GCaMPs over the last decade has generated a variety of indicators with varying time-constants, sensitivity to changes in calcium concentrations, and improved brightness (Chen, Wardill et al., 2013; Nagai et al., 2004; Tian, Hires et al., 2009). Improved indicators expressed under the original pan-neuronal promoter produce robust signals in neurons throughout the brain in larval fish (Ahrens, Li et al., 2012; Ahrens, Orger et al., 2013). These new indicators, along with other technological developments including very sensitive, fast cameras and fast laser-based imaging with sheets of light swept through the brain, has led to the recent imaging of activity of nearly every neuron in the larval brain at about two brains a second as a fish restrained in agar attempted to move (Ahrens, Orger et al., 2013) (Figure 46.1C). In this case, the movements themselves can be observed by freeing portions of the body, as done initially in early functional imaging experiments (Ritter, Bhatt, & Fetcho, 2001), or motor activity monitored by recording from motor nerves in paralyzed fish (Masino & Fetcho, 2005). Active neurons can then be mapped onto a zebrafish brain atlas (Randlett, Wee et al., 2015), or they can be monitored in fish with known neuronal subtypes labeled in a different color than the calcium indicator to reveal more about the active neurons. Zebrafish is the only vertebrate in which simultaneous whole-brain imaging of neuronal activity with simultaneous behavioral monitoring is possible, after a history of innovations in the model.

Revealing circuits, however, depends on knowing more about the neurons than their activity. Fortunately, transgenic approaches have produced beautiful lines that label neurons with different neurotransmitter phenotypes and transcription factor identities (Bae, Kani et al., 2009; Higashijima, 2008; Kinkhabwala, Riley et al., 2011; Satou et al., 2013). Labeling fluorophores targeted to the membrane can also reveal the detailed projections of the cells (Forster, Arnold-Ammer et al., 2017; Pan, Freundlich et al., 2013). The combination of calcium imaging in one color with imaging of morphology or cell type markers in a different color provides the link between activity and some of the other key features that determine a neuron's role in a behavioral circuit.

This combination has been used in many ways, but one example is the combination of structural and functional imaging (Farrar, Kolkman, & Fetcho, 2018; Kimura, Satou et al., 2013; Kinkhabwala, Riley et al., 2011; Koyama, Kinkhabwala, Satou, Higashijima, & Fetcho, 2011) that revealed a striking pattern of alternating glycinergic and glutamatergic columns in the hindbrain, an organization that is less evident in adult vertebrates as neurons migrate (Higashijima, Schaefer, & Fetcho, 2004). This columnar patterning reflects a ground plan for circuit formation during hindbrain development. Mapping this ground plan in zebrafish revealed the transcription factors that define these columns, the morphologically different cell types localized to particular columns, the disposition of neurons for particular circuits within the columnar pattern, and the orderly recruitment of neurons during swimming by location/birth order in columns—patterns also evident in spinal cord (McLean, Fan, Higashijima, Hale, & Fetcho, 2007; McLean & Fetcho, 2009).

While much of the work with functional imaging has focused on calcium sensing, zebrafish offer the opportunity to look at fluorescently tagged synaptic markers *in vivo* (Chow, Zuchowski, & Fetcho, 2017; Niell, Meyer, & Smith, 2004), which has allowed studies of synapse formation during circuit construction in development (Niell et al., 2004), as well as the first *in vivo* imaging of the activity-dependent translocation of a kinase implicated in changes in synaptic strength to synaptic sites *in vivo* (Gleason et al., 2003). The possibilities for tying molecular dynamics to synaptic and circuit dynamics are enormous in zebrafish, but still largely untapped.

Electrophysiology in Zebrafish

Electrophysiology provides high temporal resolution information about neuronal activity that is missed by the slower calcium imaging. The giant Mauthner cell and its role in the initiation of escape from predators in fishes and amphibians discussed earlier was the focus of early physiological studies in fish (Diamond, 1971; Faber & Korn, 1978). The escape response can be elicited experimentally by mechanical or acoustic stimuli, such as touch or water pressure waves (Eaton, Bombardieri et al., 1977; Eaton & Farley, 1975; Eaton, Nissanov, & Wieland, 1984; Kimmel et al., 1974). The M-cell is much larger than other neurons, and as such, produces a noticeable change in the electric field in and around the M-cell and, possibly, even outside of the fish when an action potential occurs. These can be recorded utilizing extracellular electrodes. Early on, such recordings, along with a high-speed video of escaping zebrafish larvae, revealed that the response latency in larvae was much the same as in adult animals (Eaton & Farley, 1975). The M-cell could be detected responding to stimuli as early as 40 hpf, but with increases in response amplitude and decreases in escape duration up to 100 hpf (Eaton, Farley et al., 1977).

While extracellular recordings are useful, they often do not allow identification of the neuron being monitored, can suffer from low signal-to-noise, and may not permit satisfactory discrimination between action potentials generated by nearby neurons. Other studies, initially in goldfish, used sharp electrodes inserted into individual cells (Faber, Fetcho, & Korn, 1989; Korn & Faber, 2005) to reveal circuit connections of identified neurons. Single-cell electrophysiology from identified neurons in larval zebrafish, however, reached fruition with the application of patch electrophysiology, which was already being widely used in other animals, especially mammals. This approach was critical because larval neurons are smaller than mammalian cells (many are 5–10 um in diameter) and difficult to record with sharp electrodes, but amenable to patch recording, in which the recording electrode (usually a hollow glass pipette tapered to a polished tip filled with a physiological solution, a few microns in diameter) seals onto a patch of membrane that is then ruptured to give electrical access to the inside of the neuron. The neuron can then also be filled with dye via the recording electrode to reveal its structure. The earliest patch physiology in zebrafish was performed by Legendre and Korn (1994, 1995) and was used to study the quantal nature of the synaptic release of glycine (an inhibitory neurotransmitter) onto M-cell, and the voltage-dependence of the glycine receptor channel conductance. Patch recording from spinal neurons followed later (Drapeau, Ali, Buss, & Saint-Amant, 1999). Extending physiological work on the M-cell in zebrafish beyond studies of inhibitory conductances in larvae, Hatta and Korn used whole-cell patch recording to characterize the electrophysiological properties of the M-cell in adult zebrafish and compare its properties to the known ones of adult goldfish (Hatta & Korn, 1998). Physiological properties between the two species were very similar.

This early work in zebrafish larvae and adults, along with anatomical studies suggested that a class of interneurons called cranial relay neurons played a major role in Mauthner cell circuitry in zebrafish, as previously shown for goldfish (Faber et al., 1989; Korn & Faber, 2005). However, it would take simultaneous paired and triple neuron patch recordings a decade later to definitively show the circuit connectivity of cranial relay neurons and the M-cell in zebrafish larvae and to begin to reveal synaptic connectivity of neurons in the larval brain and spinal cord (Bhatt, McLean, Hale, & Fetcho, 2007; Koyama et al., 2011; Koyama et al., 2016; Satou, Kimura et al., 2009) (Figure 46.1D).

Whole-cell recordings allowed scientists to narrow down the defects present in some of the zebrafish mutants generated in the Tubingen screen. For example, a careful electrophysiological analysis of the *twitch once* mutant, whose body movements fatigue easily, revealed a previously undiscovered consequence of a mutation in the protein rapsyn, which was known to localize acetylcholine receptors on the surface of muscle fibers (Ono, Shcherbatko, Higashijima, Mandel, & Brehm, 2002). Such a mutation causes muscles to become less responsive to high-frequency stimulation, leading to quick fatigue in response to stimuli—an observation that presaged the identification of human patients with similar deficits. Similar physiology led to critical discoveries about synaptic transmission and the functional organization of nerve-muscle connections (Wang & Brehm, 2017; Wen et al., 2013; Wen, McGinley, Mandel, & Brehm, 2016).

Patch electrophysiology was also key to revealing the circuit properties of interneuron-motoneuron connections and network behavior in the spinal cord. Early work recording from single spinal motoneurons, red/white muscle fibers, and interneurons with characteristic morphology gave indications of the spinal motoneurons and interneurons active during locomotion in larvae (Drapeau et al., 1999; Saint-Amant & Drapeau, 2000) that complemented prior studies of motoneurons in adults (Liu & Westerfield, 1988). Paired recordings of a specific interneuron class (Chx10 CiDs) in the spinal cord and large primary motoneurons that innervate massive numbers of muscle fibers in a body segment revealed that the motoneurons receive excitatory input from this class (Bhatt et al., 2007). This circuit is active during fast swimming and escapes. However, during slow swimming, paired recordings (and functional

imaging of populations) revealed that a different class of commissural interneurons, the MCoDs, provide drive to the smaller secondary motoneurons that innervate fewer muscle fibers (McLean et al., 2007; McLean, Masino, Koh, Lindquist, & Fetcho, 2008; Ritter et al., 2001). Paired recordings between the CiDs and MCoDs reveal that when the CiD network is active, the MCoD network is inhibited (they are mutually exclusive in the activity), which revealed, as a general principle, that different interneuron networks are used in the spinal cord depending on locomotor speed and strength. The findings from these studies in larval zebrafish were later confirmed to operate across vertebrates moving at different speeds. Mutations of interneuron networks in both mice and horses walking, galloping or bounding revealed defects at certain speeds depending on which, interneurons were disrupted (Andersson, Larhammar et al., 2012; Crone, Zhong, Harris-Warrick, & Sharma, 2009; Kullander, Butt et al., 2003). Today, electrophysiological approaches continue to reveal with high temporal resolution the physiological activity of cells and the connectivity that underlies behavior.

Circuit Perturbations

Zebrafish offer powerful ways to perturb neuronal activity to test ideas and formal models of the role of those neurons in behavior. Early perturbations in larvae used laser ablation of specific neurons, followed by behavioral testing. Calcium imaging of activity within the reticulospinal neurons in hindbrain suggested that several large reticulospinal neurons (a class of neurons in the hindbrain, which projects into the spinal cord), along with the Mauthner cells, contributed to the fast escape response (O'Malley et al., 1996). To demonstrate their respective contributions, Liu & Fetcho (Liu & Fetcho, 1999) used a focused excitation laser to kill calcium-dye loaded neurons via a phototoxicity effect. These experiments revealed that two non-Mauthner reticulospinal neurons were important, along with the Mauther cell, in head-touch triggered escapes, whereas the Mauthner cell acted independently of them in tail-triggered escapes. The development of high-power long-wavelength lasers for multiphoton fluorescence imaging allowed for direct, specific ablation of neurons via heating effects, with the care necessary to avoid damaging the surrounding tissue. Even higher power lasers and multiphoton excitation can be used for ablation and cutting of processes *in vivo* with minimal heating (Koyama et al., 2016). Another method for cell ablations relies on introducing a nitroreductase enzyme into zebrafish neurons, which metabolizes the prodrug metronidazole into a cytotoxic compound (Curado et al., 2007). This method has the advantage of being genetically targeted to specific cell types and can be used to ablate larger populations of neurons that would be more time-consuming to achieve using laser ablations. For example, nitroreductase mediated ablation of serotonergic dorsal raphe neurons was used to demonstrate their role in visual sensitivity (Yokogawa, Hannan, & Burgess, 2012).

Until relatively recently, stimulation during single-cell electrophysiology was the only method available for affecting the activity of a specific neuron without ablation or chemical reagents. This changed with the advent of the field of optogenetics, in which, light-sensitive ion channels (e.g., channelrhodopsin from the alga *Chlamydomonas reinhardtii*), genetically expressed by neurons, may be used to excite or inhibit cells when they are exposed to specific wavelengths of light (Boyden, Zhang, Bamberg, Nagel, & Deisseroth, 2005). This approach was easy to implement and rapidly adopted in neuroscience in many models. In the earliest published use of optogenetics in zebrafish, mechanosensory neurons in the trigeminal nucleus and spinal cord were transgenically modified to express channelrhodopsin, and exposure to blue light was sufficient to trigger an escape response in 24 hpf embryos (Douglass et al., 2008)—with optogenetic activation of mechanosensory neurons simulating a noxious touch stimulus. Understandably, optogenetics has revolutionized the field of neuroscience, with impact on zebrafish neuroscience as well. Since the earliest days, a wide variety of more effective optogenetic constructs have been engineered from the original ion channels, and new tools specifically for zebrafish are available (Forster, Dal Maschio et al., 2017).

Optogenetics may be used to reveal the behavioral consequences of circuit activation. For example, expression of ChR2 and halorhodopsin specifically in Chx10 (a transcription factor) positive hindbrain neurons was used to conclusively link their activity with the initiation of (and stopping, in the case of halorhodopsin) swimming in the animal (Kimura, Satou et al., 2013). In other work, activating subsets of a group of spinally projecting midbrain cells (the nucleus of the medial longitudinal fasciculus) elicited smooth tail bending in a direction-specific manner, implicating these neurons in the generation of body posture and steering (Thiele, Donovan, & Baier, 2014).

Another application of optogenetics in zebrafish is to fill or supplement a role traditionally occupied by paired-patch electrophysiology—demonstrating the connectedness of neurons in a neural circuit. A recent example comes from studies of the cerebrospinal fluid contacting neurons (CSF-cNs), which are now known to be a type of proprioceptive sensory neuron in the spinal cord that modulate bending movements

(Hubbard, Böhm, Prendergast, Tseng, Newman, Stokes, & et al., 2016). Hubbard and colleagues demonstrated that these neurons connect both to a specific type of primary motoneuron (the CaP, an individually identifiable cell present in each repeating segment of the tail) and a sensory interneuron by recording the motoneurons or interneurons with traditional electrophysiology while using patterned light to stimulate a subset of CSF-cNs expressing channelrhodopsin (Figure 46.1E). A definitive advantage of optogenetics over electrophysiology for neuronal activation studies is the ability to stimulate multiple individual neurons in a class at once, or in any sequence, in a non-invasive manner. This allowed the authors of this study to examine the convergence of input from the CSF-cNs to motoneurons.

Functional imaging and optogenetics applied in zebrafish have also included studies of eye movements, where imaging of activity revealed how a neuronal integrator was implemented at the population level (Miri et al., 2011)—a discovery that informed work on primates (Joshua & Lisberger, 2015). Subsequent work applied optogenetics to push the network dynamics of the brain from one regime to another in order to test mathematical models of network function (Gonçalves, Arrenberg, Hablitzel, Baier, & Machens, 2014). In that work, none of the tested models fully predicted the results, and the experiments led to the creation of a new model that had not been anticipated.

Imaging and Perturbations in Freely Swimming Zebrafish

The vast majority of modern imaging and perturbation techniques only work well in restrained fish. However, there are a handful of methods developed for recording and perturbing neuronal activity in freely swimming zebrafish larvae. One system for recording the activity of unrestrained animals relies on bioluminescence using the jellyfish protein aequorin. Aequorin emits light upon a rise in calcium during neuronal activity in the absence of light stimulation, unlike fluorescent proteins (Naumann, Kampff, Prober, Schier, & Engert, 2010). Another approach conditionally converts a green fluorophore (CaMPARI) to red based on the activity level of neurons, when and only when they are in the presence of UV light. This allows scientists to reveal populations of neurons that were active specifically during a particular interval of a behavioral task at the time the UV light was applied (Fosque, Sun et al., 2015). Calcium responses can also be imaged in unrestrained fish at times when they are not moving (Muto & Kawakami, 2016), but imaging of cells in larvae while they are moving is a much greater challenge only now being attacked (Kim et al., 2017). Finally, a number of perturbation approaches have been developed and used in animal models to genetically render neurons sensitive to chemical compounds to which they are normally insensitive. The compounds can then be applied to activate (or silence) specific sets of neurons. This methodology relies on introducing chemically activated ion channels under genetic control. For example, zebrafish neurons are not normally sensitive to capsaicin or to menthol but can be made sensitive by the introduction of ligand-gated ion channels from other species (Chen, Chiu, McArthur, Fetcho, & Prober, 2016). These approaches, along with recent technological developments to perform high-resolution fluorescence imaging in moving animals, provide a path to studies of neuronal function in freely behaving animals (Kim et al., 2017; Symvoulidis, Lauri et al., 2017).

Advantages and Disadvantages of Zebrafish Compared to Other Model Organisms for the Study of the Neural Basis of Behavior

What is a Model Organism in the Context of Systems Neuroscience?

A model organism is one of a few species that have been chosen for extensive study by large communities of biologists. These few species typically: share a relatively short generation time, facilitating genetic approaches; share a wide range of available tools and techniques; and are amenable to genetic manipulation. The implicit assumption of studying model organisms is that the neurobiological principles gleaned from a small number of species will ultimately be widely applicable to many species (including humans) because of shared organizational features and shared behavioral problems that all species must solve. These are coupled with similar principles underlying the circuit level solutions of shared behavioral problems, such as finding food or mates, determining what is good or bad in the world, or making adaptive behavioral choices. Of course, much is also to be gleaned from studies of non-models with specialized behavioral abilities, ala Krogh's principle, which states "For a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied," especially as genetic engineering technology moves toward powerful methods like CRISPR, which allows access to genetic manipulation and labeling in species more broadly (Albadri, Del Bene, & Revenu, 2017; Kimura, Hisano, Kawahara, & Higashijima, 2014; Krogh, 1929; Liu & Westerfield, 1988).

Nematodes, Flies, and Mice Versus Zebrafish

The relative merits of zebrafish for behavioral work can best be appreciated through comparison with other model organisms used in neuroscience today. Here, we focus on nematode worms, fruit flies, zebrafish, and mice, due to their wide use and genetic accessibility.

The nematode roundworm *C. elegans* is quite small (about 1 mm in length) and exists in a male form and a hermaphrodite form. The development of the animal is highly stereotypical across individuals. As there are only 302 neurons in the adult animal, it was possible for developmental biologists to track the lineage of each cell from the single-cell stage (Sulston & Horvitz, 1977; Sulston, Schierenberg, White, & Thomson, 1983) and to identify specific cells across different individuals. Not only is every neuron individually identifiable, but the entire network of synaptic and electrical connectivity between neurons has been mapped with serial electron microscopy (White, Southgate, Thomson, & Brenner, 1986). *C. elegans* exhibits a number of interesting behaviors, such as movement toward sources of food, movement away from noxious stimuli, and mating behaviors (Bargmann, 1993). They are amenable to genetic manipulation, and all of the genetically expressed molecular tools known to work in zebrafish also work in *C. elegans*. Like the larval zebrafish, nematode worms are completely transparent, so it is possible to perform fluorescence microscopy anywhere in their bodies. However, the small scale of the animal and the internal pressurization needed to maintain its body integrity make electrophysiological recordings substantially more challenging than in fish (Goodman, Hall, Avery, & Lockery, 1998). Additionally, the neurons of the nematode worm typically function using graded potentials, unlike the action potentials utilized by most neurons in other animals (Lockery & Goodman, 2009). The main difference from other models, however, is that *C. elegans* manages to accomplish the challenges of survival and reproduction within its natural environment with many fewer neurons than more complex invertebrates and vertebrates. The distinction between nervous systems that solve behavioral problems with fewer neurons (as in *C. elegans*) versus many more neurons (as in zebrafish) gets to the heart of how neurons generate behavior.

In terms of nervous system complexity and behavioral repertoire, the fruit fly *Drosophila melanogaster* seems not wildly different from zebrafish. Larval zebrafish may exhibit less behavioral complexity than adult fruit flies because of the added requirements of flight and mating in the fly. Adult zebrafish, however, have additional behavioral complexity and cognitive abilities. Importantly, both free-swimming larval fish and adults must produce critical behaviors well enough for survival to live to reproduce; in that sense, understanding the behavior in a larval animal is just as important as in adults, something that is also true for larval and adult flies. The adult fruit fly brain has roughly 135,000 neurons (Alivisatos et al., 2012), similar in amount to that of a larval zebrafish. Also, like zebrafish, fruit flies have sensory organs for vision, olfaction, taste, and touch/proprioception, although sensitivity to auditory stimuli may be more limited (Albert & Göpfert, 2015; Gillespie & Walker, 2001; Vosshall & Stocker, 2007). The wealth of genetic and molecular tools available to label these systems in Drosophila is better than those available for zebrafish.

Differences in the organization between vertebrate and invertebrate brains provide some unique advantages (and disadvantages) for Drosophila. Invertebrate neurons may rely less on using large numbers of neurons to drive behavior (via what is called population coding) than vertebrate ones (Pearson, 1993), and as a result, smaller subsets of neurons may have a similar genetic and functional identity in Drosophila. It has been possible to isolate small populations of interneurons—in some cases, single interneurons—genetically in the fly (Fischbach & Dittrich, 1989; Jenett, Rubin, Ngo, Shepherd, Murphy, Dionne, & et al., 2012). Single neurons with big behavioral effects are more common in insects, such as Drosophila, than in vertebrates, with the notable exception of the Mauthner cell. However, differences between the fly brain and the brain of vertebrates also lead to disadvantages in flies. The small size of the neurons and axonal and dendritic processes distant from the cell body make electrophysiological recording of the relevant activity of the neurons (which may occur in fine processes of tiny cells) more challenging in flies, although this is also an issue in many larval zebrafish neurons. Even calcium imaging data may have some unresolvable ambiguity, as active regeneration of voltage changes happening in neuronal processes may not be reflected in the calcium levels measured at the cell body. Furthermore, insights gained from studying neural circuits in zebrafish are more likely to apply to the more closely related humans than work in flies, if the goal is simply to understand humans rather than reveal principles of the biological organization more generally. The fruit fly is not as optically transparent as larval fish. Fruit flies also pupate between maggot stage and adults, so it is difficult to follow the development of neural circuits in the same individuals. In contrast, the development and formation of neural circuits are accessible at any stage in larval zebrafish because of its transparency.

Mice are the most popular model organism for systems neuroscience because of their close phylogenetic relationship with humans. Mice, unlike zebrafish, possess a neocortex, which is the subject of many studies in systems neuroscience because of its role in complex

behavioral tasks. Mice do exhibit cognitively sophisticated behaviors that do not exist in zebrafish, such as acoustic communication, social bonding, and caring for young (although there are fish that have some of these) (Bass & Baker, 1990). Despite relatively long generation times compared to flies and worms, there are sophisticated molecular tools available to mouse researchers that do not have close analogs in other model systems. For example, it is possible to introduce transgenic constructs into the brains of mice by applying viruses that infect particular types of neurons (Warden, Selimbeyoglu et al., 2012). Other synaptic crossing viruses, such as rabies virus, have been engineered to jump from neuron to neuron, tracing out the circuit of connected cells involved in a particular behavior (Callaway & Luo, 2015). Furthermore, mice can be trained to perform complex tasks and conditioned to fear certain stimuli, and in this way, have been fundamental in developing theories of memory formation and learning at a network level (Betley, Xu et al., 2015; Harvey, Collman, Dombeck, & Tank, 2009; Redondo et al., 2014). Because of their young age, larval zebrafish seem to have a lesser capacity for learning in some, probably less biologically relevant tasks. These differences may be mitigated by using adult zebrafish for studies of learning and memory, where appropriate. The large scale of the mouse brain also provides some advantages for optogenetics. Because it is so large, it is easier to excite neurons using light in freely moving mice because miniscopes can be mounted on their heads. For the most part, however, the large, opaque brain and the skull are a disadvantage for optical imaging.

In sum, each model, as one might expect, has strengths and weaknesses. The special advantages for studying the neuronal basis of behavior in zebrafish are optical and electrophysiological access to neurons anywhere (and even everywhere at once, using optical whole-brain imaging) in an intact vertebrate brain in an animal model with established tools to genetically target neurons.

Major Avenues of Investigation in Zebrafish With a Couple of Case Studies

Because of its unique advantages, the zebrafish serves as a model system for the study of many neural circuits and behaviors. Zebrafish have been used to study a variety of visual behaviors (Helmbrecht, Dal Maschio, Donovan, Koutsouli, & Baier, 2018; Portugues & Engert, 2009), including the optokinetic (Beck, Gilland, Tank, & Baker, 2004; Chen, Bockisch et al., 2014; Emran et al., 2007; Kubo et al., 2014; Portugues, Feierstein, Engert, & Orger, 2014; Schoonheim, Arrenberg, Del Bene, & Baier, 2010) and optomotor (Ahrens, Huang et al., 2013; Maaswinkel & Li, 2003; Naumann et al., 2016; Portugues, Haesemeyer, Blum, & Engert, 2015; Quirin et al., 2016) responses to visual motion. Indeed, these visual responses have been used as behavioral assays in mutagenesis screens to identify genes involved in visual system development and function (Brockerhoff et al., 1995; Muto, Orger et al., 2005; Neuhauss, 2003). Zebrafish also serve as a model of gaze (Beck et al., 2004; Bianco, Ma et al., 2012; Easter & Nicola, 1997; Greaney, Privorotskiy, D'Elia, & Schoppik, 2017; Mo, Chen et al., 2010) and postural stabilization (Ehrlich & Schoppik, 2017; Hubbard et al., 2016; Migault, van der Plas et al., 2018; Roberts, Elsner, & Bagnall, 2017; Semmelhack et al., 2014), as well as spatial navigation strategies, such as phototaxis (Ahrens, Huang et al., 2013; Burgess, Schoch, & Granato, 2010; Guggiana-Nilo & Engert, 2016; Horstick, Bayleyen, Sinclair, & Burgess, 2017; Lee, Ferrari, Vallortigara, & Sovrano, 2015). The olfactory system also plays critical behavioral roles in both larval and adult fish (Friedrich & Korsching, 1997; Li, Mack et al., 2005; Yaksi, von Saint Paul, Niessing, Bundschuh, & Friedrich, 2009). Considerable work has focused on motor behaviors and their control by the brain, including swimming (Ahrens, Li et al., 2012; Bagnall & McLean, 2014; Bhatt et al., 2007; Bianco, Kampff, & Engert, 2011; Borla, Palecek, Budick, & O'Malley, 2002; Budick & O'Malley, 2000; Burgess & Granato, 2007; Fidelin, Djenoune et al., 2015; Gahtan, Tanger, & Baier, 2005; Granato, Van Eeden et al., 1996; Hubbard et al., 2016; Kimura, Satou et al., 2013; Kinkhabwala, Riley et al., 2011; Liu & Westerfield, 1988; McLean & Fetcho, 2009; McLean et al., 2007; McLean et al., 2008; Menelaou, VanDunk, & McLean, 2014; Montgomery, Wiggin, Rivera-Perez, Lillesaar, & Masino, 2016; Mu, Li, Zhang, & Du, 2012; Patterson, Abraham, MacIver, & McLean, 2013; Portugues et al., 2015; Ritter et al., 2001; Sankrithi & O'Malley, 2010; Satou, Kimura et al., 2009; Thiele et al., 2014; Trivedi & Bollmann, 2013; Warp, Agarwal et al., 2012; Wiggin, Peck, & Masino, 2014; Wyart, Del Bene et al., 2009), escape movements (Burgess & Granato, 2007; Dunn, Gebhardt et al., 2016; Eaton & Emberley, 1991; Eaton & Farley, 1975; Eaton et al., 1984; Eaton, Bombardieri et al., 1977; Eaton, Lavender, & Wieland, 1982; Fetcho & O'Malley, 1995; Kimmel et al., 1980; Kinkhabwala, Riley et al., 2011; Korn & Faber, 2005; Koyama et al., 2011; Koyama et al., 2016; Lacoste, Schoppik et al., 2015; Lambert, Bonkowsky, & Masino, 2012; Liu & Fetcho, 1999; Liu, Bailey, & Hale, 2012; McLean et al., 2007; Mu et al., 2012; O'Malley et al., 1996; Prugh, Kimmel, & Metcalfe, 1982; Pujala & Koyama 2019; Ritter et al., 2001; Satou, Kimura et al., 2009; Takahashi, Narushima, & Oda, 2002; Temizer, Donovan, Baier, & Semmelhack, 2015; Thorsen & Hale, 2005; Thorsen & Hale, 2007; Thorsen, Cassidy, & Hale, 2004; Trivedi &

Bollmann, 2013; Yao, Li et al., 2016), fin movements (Green & Hale, 2012; Green, Ho, & Hale, 2011; Hale, 2014; Hale, Katz, Peek, & Fremont, 2016), opercular movements(McArthur & Fetcho, 2017) and rheotaxis (Haehnel-Taguchi, Akanyeti, & Liao, 2014; Levi, Akanyeti, Ballo, & Liao, 2015; Liao & Haehnel, 2012; Olszewski, Haehnel, Taguchi, & Liao, 2012; Oteiza, Odstrcil, Lauder, Portugues, & Engert, 2017).

In the realm of more complex behaviors, zebrafish exhibit fear responses and learned fear conditioning (Agetsuma, Aizawa et al., 2010; Amo, Fredes et al., 2014; Duboue, Hong, Eldred, & Halpern, 2017; Okamoto, Agetsuma, & Aizawa, 2012), which can be used to study neural circuitry related to anxiety and associative learning. Zebrafish also show typical circadian activity patterns and have been used to study circuit mechanisms—and consequences—of vertebrate sleep (Gandhi, Mosser, Oikonomou, & Prober, 2015; Kaslin, Nystedt, Ostergard, Peitsaro, & Panula, 2004; Oikonomou & Prober, 2017; Prober, Rihel, Onah, Sung, & Schier, 2006; Yokogawa, Marin et al., 2007; Zhdanova, Wang, Leclair, & Danilova, 2001). Zebrafish have complex social behaviors like shoaling (Buske & Gerlai, 2012; Canzian, Fontana, Quadros, & Rosemberg, 2017; Hinz & de Polavieja, 2017; Saverino & Gerlai, 2008), which have been used as behavioral assays to study the developmental impact of early ethanol and nicotine exposure (Buske & Gerlai, 2011; Fernandes, Rampersad, & Gerlai, 2015; Miller, Greene, Dydinski, & Gerlai, 2013). These are a just sample of behavioral abilities - efforts are underway to categorize the many behaviors in this model (Cachat, Stewart et al., 2011; Kalueff, Gebhardt et al., 2013; Mu et al., 2019).

To illustrate how zebrafish and associated technologies have been used to investigate the neural basis of behavior, we present two case studies in which this animal model's unique advantages have been used to advance our understanding of sensorimotor processing. In each case, the scientific progression parallels (and depends upon) the course of methodological innovation. Furthermore, these two cases follow similar scientific trajectories: initial characterization of stereotyped behavior, followed by efforts to reveal the identity and activity patterns of the neurons involved, with subsequent utilization of that basic understanding to ask even more diverse and sophisticated questions. Regarding each type of behavior, we outline major findings about its neural underpinnings and suggest fundamental principles derived from these discoveries.

Short-Latency Escapes

Behavior in goldfish: Like many animals, fish respond to sudden aversive stimuli with a startle response—a quick, decisive maneuver that orients the animal away from a potentially threatening stimulus (Eaton & Emberley, 1991; Hale et al., 2016). An aversive visual, auditory, or tactile stimulus evokes a short-latency escape maneuver, typically comprised of a sharp C-shaped turn followed by a rapid acceleration away from the threat (Eaton & Emberley, 1991). This behavior was initially well-characterized in adult goldfish, and early work in goldfish identified one of the neurons responsible for driving it: the Mauthner cell (M-cell).

Identifying premotor neurons in goldfish: In normal fish, there is one Mauthner cell on each side of the hindbrain. This specialized reticulospinal neuron receives multisensory input onto its prominent ventral and lateral dendrites, and projects directly to primary motor neurons in the contralateral spinal cord (Korn & Faber, 2005; Zottoli & Faber, 2000). Electrophysiological recordings in goldfish demonstrated that the M-cell fires a single action potential in response to an aversive stimulus, which drives contraction of the body and tail muscles on the contralateral side (Fetcho & Faber, 1988; Prugh et al., 1982)—evoking the short-latency turn away from the threat. However, following M-cell ablation, goldfish could still generate some short-latency escapes (Eaton et al., 1982), indicating that other neurons must also be involved. Further, anatomical studies suggested additional candidates: two M-cell segmental homologs, MiD2 and MiD3 (Lee & Eaton, 1991; Lee, Eaton, & Zottoli, 1993).

Identifying premotor neurons in zebrafish: Like goldfish, larval zebrafish execute short-latency escapes away from threatening stimuli (Figure 46.2A). However, larval zebrafish provided better *in-vivo* accessibility than goldfish (in terms of size and optical transparency) to facilitate further progress in our understanding of escape circuitry. Using backfills with dextran-conjugated calcium indicators to monitor neuronal activity in larval zebrafish, O'Malley et al. (1996) demonstrated that the M-cell and its homologs (MiD2 and MiD3) are active during escapes in response to tactile stimuli to the head, whereas the M-cell alone is active during escapes in response to tail stimulation. This supported a prediction made by Foreman and Eaton (1993) that additional reticulospinal neurons participate along with the M-cell in driving stronger head-evoked escapes (vs. weaker, M-cell-dependent tail-evoked escapes). Further, by refining a non-invasive method for laser ablation of single neurons, Liu and Fetcho (1999) observed that short-latency escapes to head stimuli were robust to M-cell loss alone but were abolished by ablation of the M-cell along with MiD2 and MiD3 reticulospinal neurons—consistent with the original hypothesis.

Interneurons—action selection: The M-cell and its homologs provide the shortest synaptic pathway from an aversive sensory input to an escape behavior, but

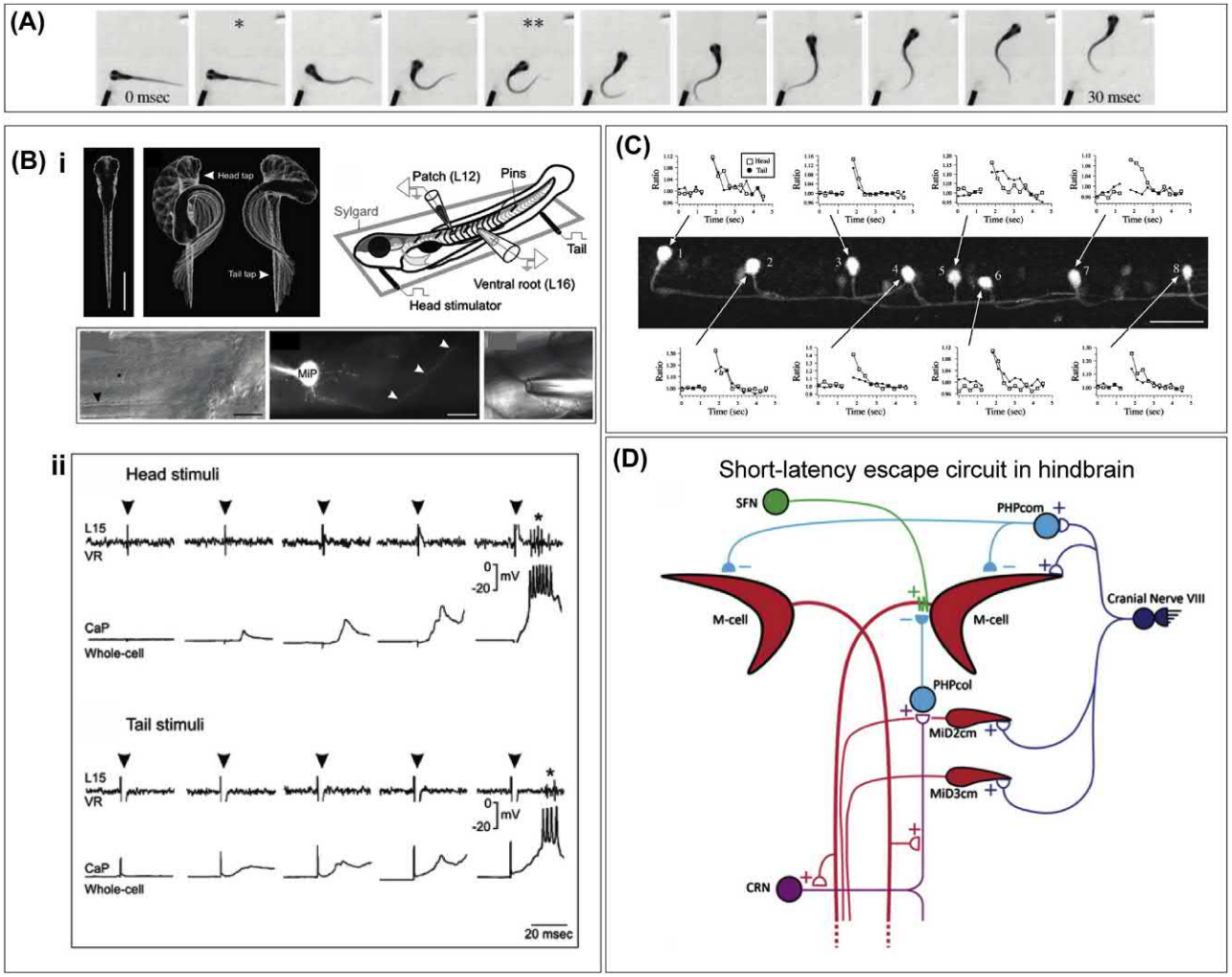

FIGURE 46.2 Early advances in the neural basis of short-latency escapes in larval zebrafish. (A) Example of an escape response to a unilateral water pulse delivered to the head. The single-asterisk marks the onset of the response; the double-asterisk marks the frame of the maximal C-bend. Images were collected at 1000 frames/s, and every third frame is shown. Modified from Liu and Fetcho (1999). (B) (i) Top left: Dorsal view of a larval zebrafish (4 dpf) and the kinematics of escape responses to head and tail stimuli, each compiled from frames captured at 1000 frames/s. Note the larger bend in response to a head stimulus. Scale bar = 1 mm. Top right: Schema showing experimental preparation used for whole-cell recordings from the spinal cord, with simultaneous extracellular recordings of tail motor activity. Bottom: DIC (left) and fluorescent (middle) views of a MiP motor neuron in the spinal cord, targeted for whole-cell recording and filled with dye. White arrows mark the MiP axon. Scale bars = 20 μm (right) DIC view of extracellular ventral root recording. (ii) Whole-cell recordings from a CaP motor neuron during electrical stimuli of increasing intensity, applied to the head or tail. Simultaneous recordings from the ventral root (VR) are also shown. Black arrows mark truncated stimulation artifacts. Modified from Bhatt et al. (2007). (C) Calcium activity from an array of CiD interneurons in the spinal cord during escapes elicited by head versus tail stimuli. The y axis is the ratio of calcium green to Alexa Fluor 647 divided by the mean ratio from 10 frames collected prestimulus. The escape movement occurs during the gap in the plot, when the cells move briefly out of the focal plane. Note that head and tail stimuli evoke responses in largely the same set of CiDs, consistent with changes in response amplitude being mediated by changes in the magnitude of interneuron activity—rather than the addition of more active neurons. Modified from Bhatt et al. (2007). (D) Summary of short-latency escape circuitry in the zebrafish hindbrain. Modified from Hale et al. (2016).

researchers had reason to suspect that other neuronal populations optimize the functionality of the escape and further shape motor output. For example, the M-cell fires a single action potential, an all-or-none response (Nissanov, Eaton, & Didomenico, 1990)—but the details of the motor output (though relatively stereotyped) vary based on the parameters of the stimulus. Further, early calcium imaging experiments revealed the influence of inhibitory networks in shaping M-cell activation (Takahashi et al., 2002). In addition, calcium imaging in zebrafish spinal cord indicated that both primary and secondary motor neurons in the tail respond strongly during escapes (Fetcho & O'Malley, 1995)—although the M-cell only provides direct input to the primary motoneurons. Thus, interneurons must be involved, at a minimum, in modulating motor output and distributing motor commands. Once again, neuronal candidates were identified based on

morphology (Hale, Ritter, & Fetcho, 2001) and backfilled with calcium indicator and imaged during behavior (Ritter et al., 2001). Two spinal interneuron subtypes (the ipsilateral descending CiD and the contralateral descending MCoD) were investigated, and Ritter et al. (2001) discovered that their activity is behavior-specific: CiDs are active during escapes but not spontaneous swims, and MCoDs are active during spontaneous swims but not escapes. This study provided evidence for an additional participant in the escape circuit, but it also demonstrated that different interneuronal populations can be deployed to generate distinct behaviors. Following the development of a fictive swimming preparation in paralyzed zebrafish (Masino & Fetcho, 2005), researchers used whole-cell patch-clamp recordings (shown for motor neurons in Figure 46.2B) to show that individual CiDs (active during escapes) increase their firing rate during stronger escape maneuvers (Bhatt et al., 2007). In the same study, simultaneous calcium imaging from multiple CiDs showed that stronger escapes did not recruit additional CiDs from the population (Figure 46.2C). These experiments, taken together, are consistent with increased motor output strength carried out by modulation of active interneurons' firing rates, rather than changes in the size of the active population.

Interneurons—visual stimulus selectivity: Since these early studies, the widespread adoption of classical approaches (such as electrophysiology) and the advent of transgenic methodologies for use in larval zebrafish (Higashijima et al., 2000)—with the subsequent explosion in the number and variety of available transgenic lines—has spurred further progress in our understanding of the neural circuitry underlying short-latency escapes. For example, the use of genetically expressed calcium indicators enabled deeper investigations of the sensory side of the escape behavior—including the responses of tactile sensory neurons (Higashijima et al., 2003) and of the visual circuits involved in generating an escape response to looming stimuli (Dunn, Gebhardt et al., 2016). Looming stimuli elicit C-bend escapes that are abolished in mutants lacking retinal ganglion cells and impaired in larvae with unilateral ablations of tectal neuropil (Temizer et al., 2015). Two-photon calcium imaging in restrained larvae identified looming-specific regions of tectum that might participate in these escapes (Dunn, Gebhardt et al., 2016; Temizer et al., 2015). A study from Yao, Li et al. (2016) used an impressive variety of approaches—including electrophysiology, pharmacology, and optogenetics—to reveal how dopaminergic inputs can act (via modulation of inhibitory glycinergic interneurons) to increase the specificity of visually evoked escapes by facilitating M-cell responses to looming stimuli and suppressing escape responses to other visual stimuli.

Interneurons—auditory/vestibular and tactile stimulus selectivity: Other studies have investigated the role of hindbrain interneurons in ensuring optimal function of the escape circuit. For example, spiral fiber neurons receive contralateral sensory inputs and wrap their axons around the axon hillock of the contralateral M-cell (Lacoste, Schoppik et al., 2015), where they form excitatory electrical and chemical synapses (Koyama et al., 2011). Based on studies of mutant fish lacking the hindbrain commissure formed by their axons (Lorent, Liu, Fetcho, & Granato, 2001), spiral fiber neurons have long been thought to play a role in short-latency escape circuitry. Lacoste, Schoppik et al. (2015) proposed that this indirect interneuron pathway ensures that brief, weak stimuli do not evoke strong short-latency escapes—consistent with their optogenetic experiments demonstrating that coincident activation of spiral fiber neurons enhances M-cell responses (and short-latency escape behavior) to weak stimuli or sensory noise. Thus, under ethological conditions, weak dendritic inputs will only elicit an M-cell response if they also excite the spiral fiber neurons (and persist long enough for the direct and indirect excitation to overlap in time).

Interneurons—laterality: Koyama et al. (2016) investigated another example of an interneuron motif that optimizes escape circuit function, focusing on feedforward inhibitory neurons located near the M-cell. These neurons receive ipsilateral sensory input and project to both the ipsilateral and contralateral M-cells (Korn & Faber, 1975)—though, importantly, each inhibits the contralateral M-cell more strongly (Koyama et al., 2016). In a study combining electrophysiology, calcium imaging, modeling, and behavior, Koyama et al. (2016) showed how these feedforward inhibitory neurons enhance short-latency escapes by ensuring a quick, lateralized response. Reciprocal inhibition of M-cells (and other feedforward inhibitory neurons) ensures that left and right sensory inputs compete to ultimately produce a strong movement away from a threat, even in the presence of ambiguous stimuli. This work provides a circuit-level account of how a simple, left/right decision is implemented in the brain (see also (Shimazaki, Tanimoto, Oda, & Higashijima, 2018) for a recent careful look at another inhibitory control mechanism in the M-cell network).

Circuit development: Because we understand something about the various interneurons participating in escapes, this circuit was used as a test case to determine if hindbrain interneurons are recruited into specific circuits in an orderly way, based on their time of differentiation and positioning during early development. In larval zebrafish, hindbrain interneurons are organized into stripes according to their expression of specific transcription factors (Kinkhabwala, Riley et al., 2011). The neurons in each stripe share the same neurotransmitter

identity and gross axonal morphology. Further, neurons are organized within a stripe according to relative age, with older neurons typically located in the most ventral portion of the stripe. Importantly, the dorsoventral position also correlates with neuronal excitability, suggesting that a neuron's position in a transcription factor stripe might predict its functional role in a motor circuit. Conversely, if you understand the functional role of a specific interneuron, you should be able to predict its location—which stripe it inhabits, as well as its dorsoventral position. Indeed, Koyama et al. (2011) used patch-clamp recordings and cell morphology to confirm this hypothesis for the interneuronal components of the M-cell escape circuit, including the spiral fiber neurons and feedforward inhibitory neurons.

Conclusion: Short-latency escape behavior in larval zebrafish has been used to establish basic principles of sensorimotor control, to reveal interneuron circuit motifs that support optimal behavioral output, including one used to implement two-alternative decisions, and to facilitate our understanding of how circuits recruit specific neurons during development (circuit summary in Figure 46.2D). Further, the pursuit of these objectives propelled many of the methodological innovations that make larval zebrafish such an attractive model for neurobiological study.

Prey Capture

Behavior: Soon after they acquire the ability to swim, larval zebrafish begin foraging for food and pursuing small prey, such as paramecia (Muto & Kawakami, 2013). Like short-latency escapes, prey capture maneuvers involve both sensory and motor processing—to select and transform appropriate sensory input into patterned muscle activity. An episode of prey capture behavior includes a series of slow swims and small-angle turns by which the larva approaches and re-orients itself relative to the prey, followed by a strike to engulf the prey (Budick & O'Malley, 2000; Borla et al., 2002; Patterson et al., 2013). Using a closed-loop virtual reality system to simulate prey capture in restrained larvae, researchers showed that the sequence of orienting turns (J-turns) is always preceded by binocular convergence of the eyes—not to direct the fish's gaze toward the prey, but to create an area of binocular overlap in front of the fish that facilitates prey tracking and capture (Bianco et al., 2011; Trivedi & Bollmann, 2013). In addition, studies in juvenile and adult zebrafish indicate that prey capture behaviors become more flexible and effective during development, associated with a transition from separate J-turns and approach swims to a single complex maneuver merging orientation and approach (Westphal & O'Malley, 2013).

Identifying premotor neurons: Early studies found that M-cell ablations did not affect prey capture (Borla et al., 2002), so researchers began investigating other reticulospinal neurons that might be involved in generating prey capture maneuvers. Because the behavior relies heavily on vision—and laser ablation of the retinotectal neuropil causes a prey capture deficit (Gahtan et al., 2005)—candidate reticulospinal neurons would need to receive visual input (directly or indirectly) from the tectum. In zebrafish, the nucleus of the medial longitudinal fasciculus (nMLF) includes two neurons (per side: MeLr and MeLc) that both extend dendrites into the deep output layers of the optic tectum and project to the spinal cord (Gahtan et al., 2005; Sankrithi & O'Malley, 2010). As assessed by a feeding assay, laser ablation of MeLr and MeLc decreases the number of successful prey captures without disrupting other motor behaviors. Further, a combined unilateral ablation strategy provided evidence that the tectum and the nMLF are part of the same pathway in the prey capture circuit (Gahtan et al., 2005). Thus, detection of visual prey stimuli by the optic tectum likely activates neurons in the nMLF to initiate or sustain prey capture maneuvers. Indeed, optogenetic activation of the anterior tectum can initiate J-turns (Fajardo, Zhu, & Friedrich, 2013). Consistent with these results, calcium imaging in freely swimming larvae indicates that anterior tectal activity precedes prey capture maneuvers (Muto, Ohkura, Abe, Nakai, & Kawakami, 2013), and quantitative analyses of tectal activity in restrained fish indicate that some neurons contain information about both sensory input and motor output (Bianco & Engert, 2015).

Sensory processing—size selectivity: In executing prey capture behaviors, it is important that zebrafish correctly identify potential prey. Larvae must discriminate between a small moving object (which might be suitable prey and should be approached) and a large moving object (which might be a predator and should be avoided). Thus, there should be some mechanism in the sensory circuitry for discriminating between small and large visual stimuli. Using genetically expressed calcium indicators, researchers discovered a region in the deep layers of optic tectum containing projection neurons that are more responsive to small stimuli than to large stimuli (Del Bene, Wyart et al., 2010). Using pharmacology, laser ablations, and transgenic strategies for blocking synaptic transmission in specific neuronal populations, researchers concluded that tectal inhibitory interneurons are necessary for establishing size filtering in deep layers of tectum—and that interfering with these microcircuits eliminates those projection neurons' size tuning (Barker & Baier, 2013). However, it is worth noting that other studies using calcium imaging have found evidence for prey stimulus selectivity in the axons of some retinal ganglion cells (Semmelhack et al., 2014),

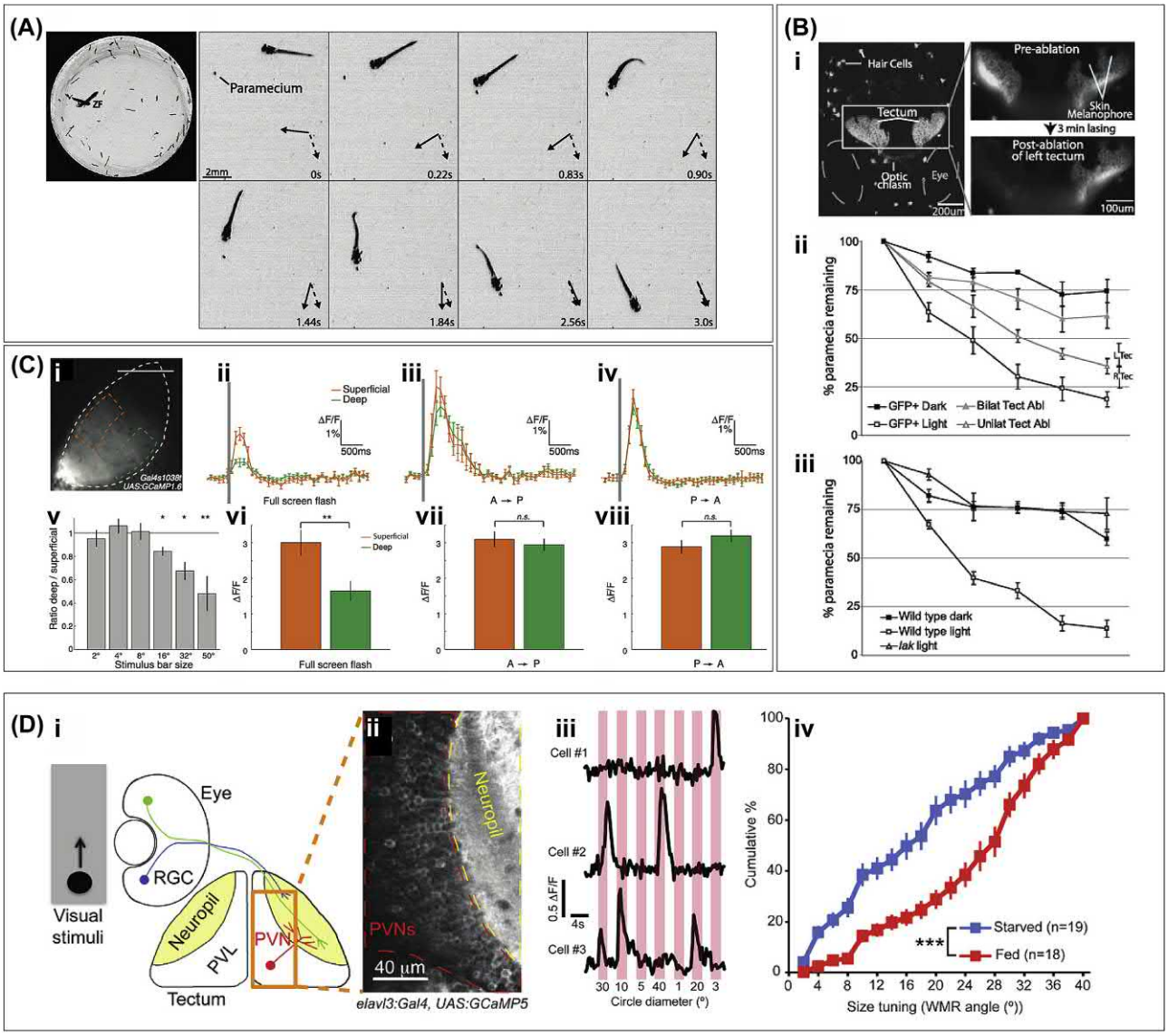

FIGURE 46.3 Advances in the neural basis of prey capture behavior in larval zebrafish. (A) Example of pursuit and capture of a paramecium by a zebrafish larva. Time projection is shown in the upper left panel, illustrating the travel paths of individual paramecia during 3.3s of imaging (200 frames total, imaged at 60Hz). Remaining panels show a single prey capture sequence (3s total), with frames chosen to highlight sequence components: approach swims (0−0.83s), pursuit swims (1.44−2.56s), and the final capture swim (2.56−3.0s). Dashed arrows indicate the heading of the paramecium, and solid arrows indicate the heading of the larva. Note that as the zebrafish pursues its prey, it makes orienting turns that bring its heading into alignment with the path of the paramecium. Adapted from Gahtan et al. (2005). (B) Prey capture relies on visual processing in the optic tectum. (i) Laser ablations were used to eliminate the retinotectal visual pathway. (ii) Prey capture behavior was evaluated by quantifying the fraction of available paramecia remaining over the course of 5h total feeding time. Note that darkness severely disrupts prey capture, and tectal lesions also cause dramatic impairment. (iii) Blind *lakritz* mutants normally cannot hunt, providing further evidence for the importance of visual input. Adapted from Gahtan et al. (2005). (C) Deep layers of the optic tectum neuropil exhibit selectivity for small, prey-like stimuli. (i) Regions of interest highlight superficial (orange) and deep (green) layers of tectal neuropil. (ii-iv) Calcium responses of tectal neuropil to three types of visual stimuli. (v) Ratios of maximum responses in deep and superficial tectal neuropil layers to bars of increasing width. (vi-viii) Average maximum responses of deep and superficial neuropil layers to three types of visual stimuli. Note that deep layers respond less than superficial layers to full-screen flashes and large bars, consistent with selectivity for smaller stimuli. Adapted from Del Bene et al. (2010). D) Feeding state affects visual processing in the tectum. (i) Schematic diagram of the zebrafish retinotectal pathway. (ii) Calcium indicator expression in the optic tectum was used to observe neuronal activity to prey-like stimuli. (iii) Examples of tectal neuron calcium signals in response to visual stimuli of different sizes. (iv) Comparison of the cumulative percentages of weighted mean response (WMR) angles for tectal neurons in starved and fed larvae. Note that starved larvae have more tectal neurons tuned for small, prey-like stimuli, consistent with a state-dependent modulation of visual processing favoring prey capture behaviors in starved larvae. Modified from Filosa et al. (2016).

raising the possibility that some stimulus filtering may occur before processing in the tectum.

Stimulus selectivity and behavioral state: Filosa, Barker, Dal Maschio, and Baier (2016) conducted an important study of how behavioral state might influence sensory processing (Figure 46.3D). They first observed that an ambiguous visual stimulus (intermediate in size) can evoke either prey capture or avoidance maneuvers, but starving the larvae increases the likelihood that they treat the stimulus as prey (and not a threat). Starved fish also have lower cortisol levels, suggesting the involvement of the hypothalamic-pituitary-interrenal (HPI) axis—previously shown to be involved in the regulation of food intake in fish (Bernier & Peter, 2001). Because the HPI axis can modulate serotonin levels (Fox & Lowry, 2013), they pursued a role for serotonin and found that the activity of serotonergic neurons in the raphe nucleus is elevated in starved fish (Filosa et al., 2016). Moreover, the increase in avoidance (in response to ambiguous stimuli) caused by prefeeding is abolished by selective serotonin reuptake inhibitors (SSRIs), and starved fish lacking serotonergic neurons (killed via nitroreductase ablation) behave like prefed fish. These results, taken together, are consistent with a model, wherein satiety activates the HPI axis, thereby decreasing serotonin release from neurons in the raphe nucleus. But what mediates the effect on behavior? The same study also used calcium imaging to observe the effect of starvation on size selectivity in the optic tectum, revealing that starvation induces a serotonin-mediated shift in the population-level tuning of interneurons and periventricular neurons—which may bias motor output in favor of prey capture in starved larvae. Thus, behavioral state modulates how sensory stimuli are represented in the brain and how they are transformed into motor output.

'*Conclusion:* Prey capture behavior has provided a window into the neural basis of ethologically relevant stimulus selection, motor sequence generation, and state-dependent sensorimotor processing. These studies capitalized on many of the methodological approaches first pioneered to study the escape circuitry, including electrophysiology, laser ablations, and imaging with genetically encoded calcium indicators. Further, because prey capture (unlike escapes) comprises a sequence of behaviors modified by the stimulus in real time, these studies have innovated in their use of closed-loop virtual reality arenas for calcium imaging in restrained zebrafish. Researchers investigating this behavior can now move to take advantage of increasingly sophisticated tools for the dissection of neuronal connectivity in larval zebrafish that subserve it.

The Future for Zebrafish in Studies of the Neuronal Basis of Behavior

In spite of the remarkable advances that make zebrafish such a powerful vertebrate model for revealing the neuronal basis of behavior, the glimpses we have provided are just the beginning. The number of labs using fish is growing rapidly, as are the tools that set zebrafish apart from other models. This last section looks to things on the horizon that ensure that the fish model is not going away any time soon as a path to revealing how vertebrate brains work.

EM Connectivity on Order

Several recent reports make strong use of electron microscopy (EM) to produce serial EM sectioning of the larval zebrafish brain, to reconstruct the connectome of the olfactory bulb, and to reconstruct the connectivity of neurons in the oculomotor system after a functional study by calcium imaging. These point to a fruitful interface between synaptic level EM morphology and functional studies of circuits in zebrafish (Hildebrand, Cicconet et al., 2017; Vishwanathan et al., 2017; Wanner et al., 2016). While a connectome of the entire larval nervous system remains a challenge, mostly because of limitations in automated tracing, it is less wildly out of reach than in other, larger vertebrate brains. The ability to reveal connectivity within regions is critical for constraining circuit models, so we can expect many regional reconstructions in zebrafish to become available soon. Even more fruitful in the short term, however, will be the ability to quickly reconstruct the projections of specific functionally identified neurons, to tie function to exact structural connectivity. As this becomes routine, we can expect that the future will offer the ability to compare the connectivity of identified neurons between animals with different experiences, such as exposure to a specific learning paradigm. This will provide an unprecedented view of how experience reshapes connectivity in neural circuits. If the EM processing and reconstruction becomes more automated and commercialized, it may be possible to simply send tissue and obtain connectivity data from individual animals, much like genomic data.

Transynaptic Mapping

Viruses that cross synaptic connections offer a potential path to revealing the set of inputs to a class of neurons without the need for an EM reconstruction. Rabies virus-based approaches modified to jump only one synapse already provide information in mammals

about the set of neurons connected directly to a target population (Kim, Jacobs, Ito-Cole, & Callaway, 2016). There remain limitations due to toxicity of the viruses, as well as lingering concerns about whether there is leakage of the labeling to adjacent but unconnected neurons, but the tools are improving substantially in mammals. Similar efforts using vesicular stomatitis virus (rVSV) are underway in zebrafish as part of an effort to extend the range of species available for viral tracing, but these efforts are still in progress (Beier, Mundell, Pan, & Cepko, 2016). As these reach fruition, they will provide a direct path to in vivo tracing in zebrafish. An ability to transynaptically express activity indicators or optogenetic constructs, when combined with the fish's transparency, will provide functional and behavioral information for specific components with known connectivity.

The Physiological Holy Grail—Genetically Encoded Voltage Imaging

The calcium indicators used to monitor neuronal activity in vivo are only indirectly coupled to the transmembrane electrical activity that actually contributes to behavior. Efforts to optically monitor membrane potential have a long history, with challenges centering around small signals and phototoxicity. Both issues are a result of the need for the indicators to be localized in the cell membrane, a small and vulnerable region of the neuron, unlike the cytosol where calcium indicators typically reside. Still, there are many efforts underway to improve genetically encoded indicators of voltage, with entire families of them now in existence (Ouzounov, Wang et al., 2017). Improvements are coming quickly, and single-cell resolution in vivo has been achieved in some species (Xu, Zou, & Cohen, 2017). While efforts in zebrafish have not yet been resoundingly successful (Kibat, Krishnan, Ramaswamy, Baker, & Jesuthasan, 2016), there is little doubt that the optical access of the model will lead to a burst of voltage imaging if reliable, easy-to-use voltage indicators become available, which seems imminent (Piatkevich et al., 2018; Adam et al., 2019; Abdelfattah et al., 2019). Still, the opposition of membranes of adjacent neurons will make separation of signals from adjacent cells difficult, and deeper imaging with the superior optical sectioning of multiphoton microscopy may not work for voltage indicators—because of the limited number of photons available from restricted regions of the membrane imaged at the kilohertz rates needed to detect action potentials that last a millisecond or so. The voltage indicators will likely prove most useful for sparsely labeled animals.

Direct to Circuits in Zebrafish

One promise of zebrafish that builds upon prior optical approaches to circuits involves a tool that is still under development but could be revolutionary when combined with the optical access to the larval brain. There are now several new approaches to activate genes using light with cellular specificity in culture, and hints of potential for *in-vivo* application (e.g., (Polstein & Gersbach, 2015)). This ability could prove very powerful when combined with whole-brain calcium imaging, as in a recent combination of whole-brain imaging and laser ablation (Vladimirov, Wang et al., 2018). One could imagine utilizing whole-brain calcium imaging to identify "interesting" candidate neurons, which participate in a particular behavior, and then use light-induced gene expression to cause those candidate neurons to express a genetically encoded label or control element (e.g., optogenetic constructs to turn them on or off, designer ligands for experiments in freely swimming animals, electron-dense markers for EM, fluorescent proteins, or voltage indicators). These candidate neurons may be ones that become active during the behavior or could even be neurons that change their activity after learning. The cells' structure could be revealed by the light-activated expression of membrane-targeted proteins. Inducing expression of optogenetic constructs would allow perturbations in neuronal activity, to test hypotheses about those neurons' specific role in behavior. Light induction of EM markers would even allow later reconstruction of those neurons' connectivity throughout the brain. This would offer the possibility of moving more directly from a specific behavior to formulating and testing predictions about the neural basis of that behavior(Dal Maschio, Donovan, Helmbrecht, & Baier, 2017).

Circuits and Behavior From Embryo to Adult

Most of the studies of brain and behavior in zebrafish focus on larval fish, typically less than 3 weeks of age. This is a practical choice, as the larvae are small and translucent, so optical tools and targeted recording are easy. The fish must survive on their own after hatching—meaning that they execute a wide variety of behaviors and simple forms of learning - so the larvae have a major place in understanding brains and behavior. A strong understanding of even simple behaviors, such as movement, visual-motor orientation, feeding, and simple forms of learning still elude us on the scale of the whole nervous system.

However, while larval behavior is just as consequential as any other behavior (dead larvae have no

TABLE 46.1 Advantages and disadvantages of zebrafish by comparison to other model systems for the study of the neural basis of behavior.

	C. elegans	*D. melanogaster*	*D. rerio (larvae)*	*M. musculus*
Number of neurons	302	~135,000	~130,000	~75 million
Body length	1 mm	3 mm	4 mm	7.5–10 cm
Time to sexual maturity	4 days	1 week	12 weeks	10 weeks
Example behaviors studied at the level of neural circuits	Locomotion, Taxis, foraging, mating	Flight, crawling (larvae), foraging, mating, Aggression, visual-motor reflexes	Swimming, escape, visual-motor reflexes, prey capture, phototaxis,	Aggression, mating, fear conditioning, spatial learning, contextual memory, foraging, Maternal care, communication
Optical accessibility	Transparent	Surgical window	Transparent	Surgical window
Electrophysiology	Difficult	Difficult	Feasible	OK
Unique advantages	Full connectome	Defined genetic access to small groups of individually identifiable neurons	Best vertebrate model for optical microscopy.	Most similar to humans. Synaptic tracing viruses. Has a cortex.
Disadvantages	Less behavioral complexity; graded neurons	Opaque cuticle; neuronal computations take place in dendrites	Limited capacity for learning and memory in the larval stage.	Has a cortex. Large scale and high complexity of brain.

offspring!), the fish brain and behavior does change as the fish grows to reach sexual maturity at about 3 months of age. For example, a very small cerebellum in larvae grows to a large and recognizably vertebrate cerebellum in adults. With growth and adulthood, comes more complex social interactions, seemingly more sophisticated motor control and learning, and new behaviors, such as mating. The advantages of the transparency are lost, however, which would seem to undermine the utility of the model later in life.

Recent innovations will likely circumvent that. The development of longer wavelength three-photon microscopy and the attendant ability of long wavelengths to more easily penetrate opaque tissue allows deeper imaging into previously inaccessible regions of adult brains (Ouzounov, Wang et al., 2017). The depth of imaging possible with three-photon microscopy (on the order of 1.5 mm) matches the thickness of an adult zebrafish brain. The combination of three-photon microscopy and some adaptive optical tools with the pigmentless *Casper* fish lines will likely allow optical access anywhere in the intact living adult zebrafish brain. This would make zebrafish the first vertebrate where brain function in behavior can be studied in the same animal from embryo to adult, opening up many questions of how brain circuits and behavior change with maturity and experience, and eventually degenerate with age. Table 46.1.

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CHAPTER

47

Zebrafish as a Model to Understand Human Genetic Diseases

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Zebrafish Versus Human: How Similar are They?

The completion of the human genome project and, more recently, the shift to Whole Exome Sequencing (WES) and Whole Genome Sequencing (WGS) has further accelerated the number of identified human disease genes. At the time of this writing, the Online Mendelian Inheritance in Man (OMIM) database lists over 5000 diseases for which the clinical phenotypes have a known molecular basis, with more confirmed disease genes being identified regularly. This wealth of new data accessible to clinicians, though, would not have been possible without the contributions of model organisms, such as the zebrafish in identifying and validating disease loci.

Since their original use as a vertebrate model (Streisinger, Walker, Dower, Knauber, & Singer, 1981), zebrafish have been illuminating and informing our knowledge of human development, genetics, and disease. Throughout the late 20th century, the previously segregated fields of evolution, development, and genetics increasingly converged, united by the common thread of molecular biology.

Despite the divergent evolutionary processes that have occurred in the teleost and human lineages over the past 450 million years, the ancestral vertebrate "operations manual" can be clearly identified in both organisms. Although the specifics of how a fertilized egg develops into a fish versus a human are obviously and importantly different, the patterns of cell movement, tissue differentiation, and organogenesis are strikingly conserved. The retained similarities extend even to the structure and function of their genomes. Analysis of the arrangements of genes on fish and human chromosomes reveals many regions where linear organization of the genes is preserved (Catchen et al., 2011), whereas analysis of the genes themselves highlight that zebrafish orthologs for 70% of all human genes, and a striking 84% of human disease genes, have been identified (Howe et al., 2013). This conservation indicates a close adherence to the development, structure, and function of our common vertebrate ancestor and strengthens the rationale for characterizing zebrafish, despite their divergent freshwater adaptations, as a legitimate tool for understanding the human genetic disease.

Moreover, even significant anatomical differences are often surmountable in terms of modeling human genetic disorders. For example, in humans, hematopoietic stem cells in the bone marrow give rise to the different classes of blood cells, several of which can be affected by genetic mutations leading to various diseases. Zebrafish lack bone marrow, but instead have a population of hematopoietic stem cells in the kidney, which gives rise to all of the analogous blood cells, and thus, have still contributed valuable insights into the mechanisms underlying blood cancers and other disorders (reviewed in Choudhuri, Fast, & Zon, 2017; Rissone & Burgess, 2018). Similarly, although the zebrafish telencephalon is rudimentary compared to the mammalian cerebrum, zebrafish neuronal connections and neurological functions, including learning, memory, social behavior, and anxiety, are genetically and physiologically influenced in ways similar to that of humans (Kalueff, Stewart, & Gerlai, 2014; Newman, Ebrahimie, & Lardelli, 2014; Matsui, 2017; Velkey, Boles, Betts, Kay, Henenlotter, Wiens, 2019).

Another potentially influential difference in disease pathology between zebrafish and humans is the regenerative capacity of many zebrafish tissues, which in principle could compensate for or mask defects precipitated by genetically regulated cell loss. This concern is

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largely resolved by the observation that numerous zebrafish models of degenerative disease have been characterized (Matsui & Takahashi, 2017; Sher, 2017; Sánchez, Azcona, & Paisán-Ruiz, 2018; Dona et al., 2018; Mishra et al., 2019) and further allayed by the availability of mutant backgrounds inhibiting the normal regeneration process (Pei et al., 2016) that could be used to unmask more completely the degenerative potential of a human disease gene under study.

An Expanding Toolkit for Generating Zebrafish Models of Human Genetic Disease

Mutagenesis Screens

As with many other genetic model organisms, parallel and often reciprocal progress in genetic analysis and genomics of the zebrafish has increasingly led to more tractability in the ways in which gene function can be analyzed and manipulated. Initial large-scale forward genetics screens were conducted by inducing random mutagenesis via chemical exposure or viral insertion, (Mullins, Hammerschmidt, Haffter, & Nüsslein-Volhard, 1994; Haffter et al., 1996; Amsterdam, Nissen, Sun, Swindell, Farrington, Hopkins, 2004), screening the progeny at embryonic or larval stages for phenotypes of interest, and characterizing the developmental or functional anomaly while working to map the genetic cause of the defect to a precise locus within the genome. Concomitant efforts to sequence the zebrafish genome provided an increasing catalog of genetic markers on each of the 25 zebrafish chromosomes to assist in the mapping efforts (Ransom & Zon, 1999; Shimoda et al., 1999; Kelly et al., 2000). The efforts of the global zebrafish community over the past 40 years have isolated and characterized the recessive loss of function mutations in scores of genes linked to inherited disease in humans.

Broadly, forward genetics screens center on observing phenotypic abnormalities in morphology and/or behavior. Evaluation of large pools of larvae from mutagenized parents for defects in the development of craniofacial skeletal elements, for example, led to the discovery of several alleles of the zebrafish mutation *jellyfish*. These animals exhibit profound and ultimately lethal malformations in the skeletal elements of the head and trunk. Further research to identify the causative gene revealed mutations in *sox9a*, a transcription factor with a varied and important function in cell-fate specification during embryonic development (Yan et al., 2002). The zebrafish phenotype of *sox9a* mutants bears a striking similarity to the clinical symptoms observed in Campomelic dysplasia, which in humans is caused by mutations in *SOX9* (OMIM #114290). The data gleaned from characterizing these mutants led not only to new insights on how cartilage forming cells organize during skeletal development, but further provided important information about a severe human genetic disorder.

Behavioral screens can detect anomalous phenotypes in embryos or larvae with mild or undetectable changes to their morphology. A screen for mutants with diminished visual function, assessed by the ability to track moving stripes known as the optokinetic response, netted a large number of distinct lines that shared this common behavioral defect despite a wide range of underlying genetic causes. One such mutant initially termed "no optokinetic response a" (*noa*) (Brockerhoff, Hurley, Janssen-Bienhold, Neuhauss, Driever, & Dowling, 1995), exhibited impaired visual function despite a histologically normal retina. Additionally noted phenotypes included reduced physical activity, poor feeding behavior, and early larval death. Using positional cloning methods, Brockerhoff and colleagues identified *dlat* as the affected gene in these mutants (Taylor, Hurley, Van Epps, & Brockerhoff, 2004). Dihydrolipoamide S-acetyltransferase (Dlat) is a subunit in the Pyruvate dehydrogenase (PDH) complex, a crucial component in the metabolic process that produces energy from carbohydrates to power a number of neurological functions. Defects in the PDH complex in humans cause neurological defects, failure to thrive, and premature death. The advent of an animal model in which to study defects in the pyruvate dehydrogenase pathway not only provided new information on the molecular basis of the disorder, but also an opportunity to explore ways to treat it. When zebrafish *dlat* mutants were provided with a ketogenic diet, their visual and neurological symptoms improved along with their survival. Up to this point, some PDH patients had been advised to consume a ketogenic diet with low carbohydrate, high fatty acid content, but the clinical evidence for this recommendation was limited. Analyzing the connection between diet modifications and metabolic function in the *dlat* mutants provided strong validation for this approach in the clinic, and more rigorous clinical trials have since demonstrated positive outcomes for PDH patients following this protocol (Sofou, Dahlin, Hallböök, Lindefeldt, Viggedal, Darin, 2017).

Far from obsolete, mutagenesis screens are still used to good effect, particular in the discovery of genes important for postdevelopmental or more specific behavioral functions (Pelegri & Mullins, 2016; Henke et al., 2017; Gerlai et al., 2017; Kegel et al., 2019; and see Table 47.1).

TABLE 47.1 Data samples provided by the Zebrafish Information Network (ZFIN), taken at three intervals between September 2017 and April 2019 reveal the growing number of published genetic disease models and the changing frequencies with which various genetic models are being used.

	2017	2018	2019	'17-'19 increase
Total zebrafish genetic disease models in published studies	1334	1447	1582	+19%
Forward mutagenesis screen approaches				
N-ethyl-N-nitrosourea	260	267	333	+ 28%
Viral insertion	102	119	124	+ 22%
Reverse Genetic approaches				
Morpholino	1217	1356	1396	+ 15%
TILLING	46	64	80	+ 74%
ZFN	29	30	40	+ 38%
TALENS	65	115	136	+109%
CRISPR	55	187	247	+349%

Morpholino Knockdown

Although undeniably powerful, the genetic screens described above are perennially limited by the "luck of the draw," especially because the nature of mutagenesis can be limited by areas within the genome that are more or less susceptible to disruption by chemical or insertional mutagenesis. As human genetic studies advanced through the late 20th century and more molecular identities were conferred on known hereditary diseases, the need for targeted disruption of disease genes in the zebrafish model was increasingly pressing. The methodology of using an injection of double-stranded RNA to knockdown candidate gene function that was tremendously powerful in fly and worm models proved to be ineffective in zebrafish (Zhao, Cao, Li, & Meng, 2001). Thus, the introduction of morpholino oligonucleotides (short, single-stranded base sequences arranged on a modified morpholine backbone to impart stability) as a means of temporarily targeting a chosen gene for disruption by blocking mRNA translation or pre-mRNA splicing (Nasevicius & Ekker, 2000; Draper et al., 2001) was heralded with justifiable enthusiasm by the zebrafish community. With this new tool in hand, "morphant" models of human disease burgeoned over the next decade. One significant benefit to human health was the ability to validate candidate disease genes quickly. Advances in the sensitivity and cost-effectiveness of genomic analysis in the clinical setting provided new potential answers to unsolved genetic disease riddles, but the high number of sequence variations in every human genome can reduce confidence in attributing symptoms to defects in a gene not previously identified as causative. The ability to deplete the function of the orthologous gene(s) in zebrafish and quickly validate or rule out candidates derived from sequence analysis, is a significant asset to genetic diagnosis.

Morpholino knockdown of candidate genes has been used in numerous collaborations between basic researchers and clinicians to vet novel genes identified in sequence analysis of patients. Hanno Bolz and colleagues (Beck et al., 2014) detected lesions in the *POC1* gene of individuals with severe symptoms indicative of defective primary cilia. Primary cilia are integral to the functions of numerous tissues throughout the body, including the retina, kidney, and brain. These cilia emanate from centrosome-like microtubule-organizing centers made up of dozens of proteins. Although many inherited ciliopathies are known to be caused by defects in the genes that encode these centrosomal proteins, there is also considerable redundancy in this system, to the extent that uncharacterized factors in this category cannot be automatically assumed to cause disease when mutated. Morpholino knockdown of *poc1b* in zebrafish produced defects in retinal and renal cilia, consistent with the patients' symptoms. These data combined with the family genotyping and in silico modeling of the predicted changes to the POC1B protein were sufficient to diagnose the families genetically and categorize other truncating mutations in *POC1B* as likely pathogenic.

In other cases, zebrafish morpholino models have been used to exclude certain alleles from pathogenic categorization. In another case investigated by Bolz & colleagues (Elsayed et al., 2015), siblings with genetically undiagnosed deafness were found to have a homozygous mutation in the ciliopathy gene, *AHI1.* Not only did these affected individuals show no sign of ciliopathy themselves, but other family members with normal hearing also carried the same homozygous mutation. The allele in question was predicted to produce a C-terminal truncation of the protein, but a known truncating mutation mapping downstream of the newly identified one had previously been linked to severe ciliopathy. Use of two morpholinos to 5′ and 3′ regions of the transcript showed distinct differences, with the earlier disruption causing widespread cilia dysfunction consistent with early truncating human alleles, whereas the downstream disruption showed no symptoms. This analysis underscores the importance of being able to validate pathogenicity so that accurate information

about disease-causing alleles can be provided to human geneticists and genetic counselors worldwide.

Although the discoveries netted with morpholino knockdown were undeniably valuable in the pursuit of understanding human disease genes, one limitation is the relatively short-lived efficacy period of morpholinos, which, in most cases, restricted analysis of the morphant phenotype to the first week of development or less. Off-target effects, giving strong and deleterious phenotypes unrelated to the targeted specific gene can confound interpretation of the results. In some cases, differences have been noted between morphants and genetic mutants (Kok et al., 2015; Novodvorsky et al., 2015), although even this initially dispiriting finding is leading to a deeper understanding of gene regulation in zebrafish and how transcript-mediated genetic buffering can compensate for loss of function mutations (Rossi et al., 2015; Tuladhar et al., 2019). Overall, morpholinos presented the zebrafish community with early and fruitful opportunities to leverage the power of candidate gene approaches to modeling human disease. Morpholinos remain a valuable tool in analyzing human disease genes in zebrafish, particularly in clinical collaborations due to the speed with which loss of function results can be obtained (Table 47.1).

Targeting Induced Local Lesions in Genomes (TILLING)

As morpholino knockdown efforts gained prominence in the zebrafish research community, a concerted effort to capitalize on the permanence of genetic mutation and to increase the library of zebrafish mutants was mounted, first in smaller collaborations (Moens, Donn, Wolf-Saxon, & Ma, 2008) and later by the Zebrafish Mutation Project undertaken by the Sanger Institute (Zebrafish Mutation Project (https://www.sanger.ac.uk/resources/zebrafish/zmp/).

Which sought to create a loss of function alleles for every protein-coding gene in the zebrafish genome. As in the classic forward genetic screens, random mutagenesis was initiated by chemical mutagen exposure. But this time, instead of screening offspring for phenotypes of interest, collections of mutagenized genomes were screened for deleterious mutations in genes of interest by DNA sequencing. These TILLING mutants were subsequently cataloged and made available for study through the Zebrafish International Resource Center (ZIRC) in Eugene, Oregon, USA. At the time of this writing, the Zebrafish Mutation Project has isolated 37,624 alleles with putative loss of function point mutations, many of which are available for distribution from both the ZIRC and the European Zebrafish Resource Center (EZRC) in Karlsruhe, Germany. Considering the overlap of disease genes in the respective genomes of human and zebrafish, this library of zebrafish mutants represents an important resource for modeling human disease. For example, careful analysis of several different Sanger alleles of the chromatin-modifying factor *kdm2aa* has illuminated a new genetic contributor to melanoma formation (Scahill et al., 2017), adding a valuable tool to the global research efforts addressing this challenging and often fatal form of skin cancer.

Gene Editing

Zebrafish studies of human disease genes gained increasing international attention through the early 21st century, and optimization of the methods described above has reduced the inherent limitations on each to an impressive degree. The limits of modeling human genetic disease in zebrafish have been attenuated even further by the introduction of targeted gene editing that uses sequence-specific guided nucleases to find and create disruptions in a chosen genetic location. The first success at this targeted mutagenesis in zebrafish was obtained by generating customized zinc-finger nucleases (ZFNs; Doyon et al., 2008; Foley et al., 2009). ZFNs are synthesized to recognize a specific DNA sequence and, via nuclease action, create breaks in the double helix. When DNA repair mechanisms are activated to re-join the broken ends of the DNA strand, small stretches of sequence can be randomly deleted or inserted, creating a shift in the reading frame of the genetic code. When introduced into zebrafish zygotes, these constructs create mutations that can be propagated throughout multiple cells of the developing embryo, including the germline cells, thus creating heritable changes in the genes of interest.

The exciting potential of this new tool was tempered only by the labor-intensive nature of generating custom ZFNs, but parallel efforts to discover more effective and efficacious ways of altering selected regions of DNA soon provided alternatives. Shortly after the advent of ZFNs, a breakthrough harnessed Transcription Activator Like Effector Nucleases (TALENs), derived from a bacterial pathogen of plants, to edit zebrafish genes (Huang, Xiao, Zhou, Zhu, Lin, Zhang, 2011; Sander et al., 2011). This system consists of customizable DNA binding subunits (TALEs), each recognizing a specific nucleotide base that can be assembled in any order to recognize specific sequences. When these custom protein sequences are attached to a nuclease, such as Fok1, the whole assembly can create double-stranded breaks in the DNA at the binding site.

Just on the heels of this methodological development, research to characterize Clustered Regularly

Interspersed Short Palindromic Repeats (CRISPR), originally found in bacterial DNA and associated with CRISPR-associated (Cas) DNA cutting enzymes, led to the discovery that these short palindromic repeats could be used along with gene-specific guide RNA to bring a DNA-cutting enzyme to a selected position on the chromosome (Qi et al., 2013; Cong et al., 2013). Zebrafish researchers quickly jumped on this new technique (Chang et al., 2013; Hwang et al., 2013; Jao, Wente, & Chen, 2013), and currently, CRISPR/Cas9 system is the most widely used of the three gene-editing methods, largely due to the ease and low cost with which the constructs can be generated (Table 43.1). A rapidly expanding list of zebrafish mutants derived from CRISPR/Cas-generated disruption includes new models of atrial fibrillation (Ahlberg et al., 2018), congenital neutropenia (Pazhakh, Clark, Keightley, & Lieschke, 2017), osteoporosis (Zhang et al., 2017), structural heart and renal defects (Ta-Shma et al., 2017), scoliosis (Gao et al., 2017), thrombocytopenia (Marconi et al., 2019), and Wolf-Hirshhorn Syndrome (Yu et al., 2017), to name only a few.

The ability to create frameshift mutations at precise locations within the genome conferred unprecedented power to the ongoing work to generate and learn from zebrafish models of human diseases. Along with the ability to "break" a gene, thereby disrupting function, these methods provide the opportunity to change the DNA code in more specific ways, using the event of a double-stranded DNA break to overwrite the native code. By providing a template strand of DNA containing both homologous sequences from the targeted region and customized new code to be incorporated, researchers now have the potential to change the gene sequence in even more refined ways, for example introducing an in-frame missense change to emulate a specific human allele with known or unknown disease-causing potential (Gopal et al., 2015; Armstrong, Liao, You, Lissouba, Chen, Drapeau, 2016; Boel et al., 2018; Prykhozhij et al., 2018;), or generating a reporter sequence (Hoshijima, Jurynec, & Grunwald, 2016; Kesavan, Chekuru, Machate, & Brand, 2017). With these technical advances, the further goal of using knock-in strategies to repair mutations without disrupting the reading frame, thus restoring the ability of the organism to make a wild-type protein product, is not far behind. The potential for exploring genetic variants of unknown significance (VUS) and for research into repairing genetic defects by overwriting them with better code has barely been tapped as of this writing, but as with the advent of every new tool described herein, the more broadly used the technique within the zebrafish community, the more potential there is for innovation and variation that will carry research forward.

Disease Models: What Genes do we Choose to Target?

The gene-targeting tools described above, combined with a superior array of transgenic lines, vital dyes, and other methods of visualizing molecular physiological processes in action, have set up the zebrafish as an undeniably powerful system in which to explore any known or suspected human disease gene. Zebrafish models have been used both to study and to screen potential therapies for common genetic diseases, such as cancers (Anelli et al., 2017; Lu et al., 2017; Oppel et al., 2019; Tulotta, Stefanescu, Chen, Torraca, Meijer, Snaar-Jagalska, 2019), cardiovascular and kidney disorders (Minchin & Rawls, 2017; Chang et al., 2017; Schenk, Müller-Deile, Kinast, & Schiffer, 2017; van Rooijen et al., 2018; Zhao, Zhang, Sips, & MacRae, 2019), and cystic fibrosis (Zhang, Liu, & Chen, 2018). But although defects in human genes are responsible collectively for many millions of clinical cases, some genetic disorders are vanishingly rare. A disease affecting fewer than 200,000 people at any given time is defined as "rare" in the United States, but a growing number of genetic disorders affecting far fewer than that number, sometimes as few as a single patient, are reported. The struggle for equitable research funding for rare and extremely rare diseases far exceeds the scope of this chapter, but it is fair to say that clinicians, researchers, advocacy groups, and affected individuals alike are hopeful that the advancements that have made zebrafish a more tractable and versatile disease model for human afflictions will benefit the rare, as well as the common among them. To this end, collaborative organizations, such as the Undiagnosed Diseases Network (Chao, Liu, & Bellen, 2017; Wangler et al., 2017) have placed special emphasis not only on enhanced clinical workups and genomic analysis for patients with rare genetic disorders, but on the development of a high-throughput animal model core to explore the function of novel sequence variants implicated in unusual human genetic disorders. CRISPR/Cas9 zebrafish models of candidate genes implicated in these rare presentations are providing crucial validation of the genotype-phenotype correlation required to make a genetic diagnosis (Ferreira et al., 2018; Burrage et al., 2019; Lahrouchi et al., 2019).

Modeling human disease "on demand" is one way in which zebrafish is being harnessed at the interface of basic and clinical research, with data from human patients directly influencing the creation and analysis of their zebrafish proxies. With increasing frequency, the current flows in the other direction as well, as research in zebrafish disease models informs new clinical directions. The generation and ongoing analysis of

zebrafish mutants has provided new therapeutic targets for the treatment of cancers (Guan et al., 2019) craniofacial and other disorders (Seda et al., 2019), hearing loss (He, Bao, & Li, 2017), Niemann-Pick disease (Tseng et al., 2018), and a range of other genetic disorders. The ease with which zebrafish can be subjected to panels of chemicals that might enhance or suppress an underlying genetic condition also feeds directly into the foundational research required before seeking approval for clinical trials. Beyond the broad array of published work, the widespread uptake of gene editing techniques, including work on knock-out and knock-in endpoints, has provided vital data on the advantages and challenges of tissue-specific methods for altering DNA sequences in individuals with genetic disorders. The reciprocal influence of zebrafish models on the human health issues they seek to illuminate and ameliorate is only beginning, and the potential impact that our small but powerful model organism can have on the vast field of human genetic disease is realized with each new insight that moves the entire community forward.

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CHAPTER

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Zebrafish as a Model for Investigating Animal–Microbe Interactions

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Introduction

Over the past decade, a boom in DNA sequencing studies has revealed that the bodies of humans and other animals are inhabited by an amazing diversity of microscopic wildlife, including bacteria, archaea, viruses, and eukaryotic microorganisms, such as fungi and protozoa (Blaser et al., 2016; McFall-Ngai et al., 2013). These microbial consortia are now widely recognized to have far-reaching impacts on animal biology, from promoting normal growth and development to inciting infection and disease, making them the focus of intense biomedical research. However, unraveling the mechanisms by which resident microbes live and interact with each other, and their animal hosts remains incredibly challenging due to their intricate and multifarious nature. Animal–microbe interactions play out over a range of temporal and spatial scales at various concealed locations across the host's body, for example, deep within the intestinal tract, and involve numerous cell and tissue types. For understanding these complex interactions, multiple complementary experimental models are needed. The laboratory mouse is often employed because of its anatomic and genetic similarity to humans, and thus, it has yielded many important insights (Kostic, Howitt, & Garrett, 2013). However, the mouse's relatively slow development, inaccessible internal tissues, and expensive husbandry make certain experimental approaches unfeasible with this model. Alternatively, highly amenable in vitro technologies have been developed that use cultured animal cells (typically human-derived) to generate so-called *organs-on-chips*, but the ability of these distilled experimental approaches to recapitulate higher-order interactions involving multiple organ systems, developmental processes, or behavior is severely limited (Benam et al., 2015). Offering a balance between these approaches is the zebrafish, *Danio rerio* (Burns & Guillemin, 2017). In this chapter, we describe the attributes that make this popular aquarium fish an attractive model organism for studying animal–microbe interactions and illustrate through several vignettes of cutting-edge research how it is propelling the field forward.

The Zebrafish: An Adaptable and Multifaceted Model Organism

In this section, we overview five attributes that make the zebrafish an ideal model organism for studying animal–microbe interactions. These attributes include a conserved vertebrate immune system, the ability to be associated with a wide range of microbes, high fecundity and small size, rapid ex-utero development, and amenability to live imaging. Our descriptions of these attributes are not exhaustive; rather, they are meant to serve as introductions to each topic. In addition to the utility of the zebrafish to model human infectious diseases caused by single pathogens, we also highlight the potential of the system to inform our understanding of biomedically relevant interactions between humans and their complex resident microbial communities, known as microbiota.

Vertebrate Immune System

Understanding how microbes influence human health and disease motivates a great deal of research on animal–microbe interactions. Because of this, model

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systems capable of capturing aspects of human biology have immediate biomedical relevance. The zebrafish meets this need through its conserved vertebrate physiology, which shares considerable overlap with humans despite the 450 million years of evolution that separates them (Rauta, Nayak, & Das, 2012; Renshaw & Trede, 2012; Sunyer, 2013; Trede, Langenau, Traver, Look, & Zon, 2004). What makes the zebrafish particularly useful in studying animal–microbe interactions is its immune system. The immune system plays a frontline role in mediating tolerance to a large number of normally benign and beneficial members of resident microbiota while allowing rapid detection and defense against those that are harmful. Importantly, as in humans, zebrafish have two interconnected arms of their immune system, referred to as innate and adaptive immunity. For the first 3–4 weeks of development zebrafish possess only innate immunity, which provides a unique opportunity to study the contributions of this system to animal–microbe interactions in isolation (Lam, Chua, Gong, Lam, & Sin, 2004). The zebrafish innate immune system comprises an array of protein receptors and signaling networks that are expressed by cells throughout a variety of tissues (Rauta et al., 2012; Trede et al., 2004). Innate immunity proteins that are widely studied in zebrafish, for which there are human homologs, include various pattern recognition receptors (e.g., Toll-like receptors) that recognize microbe-derived molecules, the cytokines TNFα and IL1β, the signaling protein MyD88, and the transcription factor NF-κB (Jault, Pichon, & Chluba, 2004; Trede et al., 2004). Additional conserved features of the zebrafish innate immune system include antimicrobial proteins (e.g., lysozyme, C-reactive protein, and complement) and phagocytic myeloid cells (e.g., neutrophils and macrophages) that serve as first responders to sites of infection and inflammation (Harvie & Huttenlocher, 2015; Li et al., 2007; Lieschke, Oates, Crowhurst, Ward, & Layton, 2001; Rauta et al., 2012). Characterization of the adaptive immune system in zebrafish is more limited, but adult fish are clearly capable of elaborating an adaptive immune response that is highly similar to that of humans and other mammals (Rauta et al., 2012; Sunyer, 2013; Trede et al., 2004). Generally, the adaptive immune system of teleost fish is made up of T and B lymphocyte lineages that express the T cell receptor (TCR) and a repertoire of immunoglobulins, respectively. Other fundamental components of teleost adaptive immunity include the major histocompatibility complex (MHC) and the recombination-activating genes (RAG) 1 and 2. MHC molecules are expressed on the surface of many different cell types and present self and nonself antigens to T cells, whereas RAG1 and 2 are critical for generating TCR and immunoglobulin diversity and ultimately, T and B lymphocyte function.

Natural and Surrogate Microbial Associations

Zebrafish naturally associate with a multitude of commensal, beneficial, and pathogenic microorganisms (Roeselers et al., 2011; Stephens et al., 2016). An exciting and rapidly growing area of research aims to understand the diverse community of microbes that thrive within the zebrafish intestine, particularly the bacteria. The intestinal microbiota of zebrafish is an appealing system to study for several reasons (Burns & Guillemin, 2017). Foremost, it contains hundreds of bacterial species that can interact with each other and influence vital aspects of zebrafish biology (Bates et al., 2006; Hill, Franzosa, Huttenhower, & Guillemin, 2016; Stephens et al., 2016). The complexity of the zebrafish microbiota falls in between that of the simple gut communities of invertebrate models, such as the fruit fly, which comprise tens of bacterial species, and that of mammalian models, such as mouse and human, which comprise hundreds to thousands of species (Kostic et al., 2013). The intermediate complexity of the zebrafish microbiota aids tractability while at the same time recapitulating the diversity found within microbial communities of high biomedical interest, such as those within the human intestine. In addition, as with the intestinal microbiota of humans, the zebrafish microbiota undergoes characteristic changes in the types of bacterial species it contains as the host develops and ages. The codevelopment of both zebrafish and humans with their resident gut microbes, is therefore dynamic, but in ways that appear to conform to a general ontogenetic pattern (Palmer, Bik, DiGiulio, Relman, & Brown, 2007; Stephens et al., 2016; Vangay, Ward, Gerber, & Knights, 2015).

Another notable feature of the zebrafish intestinal microbiota is that many of its bacterial members are related to lineages known to associate with and influence the health of humans (Roeselers et al., 2011). Bacteria belonging to the phylum Proteobacteria—particularly the genera *Vibrio*, *Aeromonas*, *Shewanella*, *Plesiomonas*, *Acinetobacter*, *Pseudomonas*, *Enterobacter*, and *Escherichia*—are frequent residents within the zebrafish gut (Rolig et al., 2017). Intriguingly, proteobacterial lineages are highly abundant in the human intestinal microbiota over the first 3 years of life and their presence during this time has been linked to decreased incidence of allergy and chronic disease (Palmer et al., 2007; Vangay et al., 2015; Vatanen et al., 2016). In a similar vein, some lineages of *Escherichia* can promote intestinal homeostasis, a phenomenon that has been observed in humans, mice, and zebrafish (Behnsen, Deriu, Sassone-Corsi, & Raffatellu, 2013; Deriu et al., 2013; Rolig et al., 2017). However, in stark contrast to their beneficial activities, proteobacterial lineages are widely recognized as some of the most notorious human and animal

pathogens. Moreover, increased Proteobacteria abundance has been shown to play a role in inflammatory bowel disease and is correlated with elevated mucosal inflammation during pregnancy (Gevers et al., 2014; Koren et al., 2012). Therefore, understanding how zebrafish interact with this interesting and highly relevant group of bacteria promises to help illuminate many animal–microbe relationships, especially those involving humans.

The zebrafish also allows microbial associations to be experimentally controlled, which is a particularly powerful feature of this model organism (Melancon et al., 2017). Zebrafish embryos can be derived germ-free fairly easily and raised in the presence of defined microbial communities. This is achieved by surface sterilizing embryos with a series of washes in embryo medium containing low amounts of bleach and iodine. After sterilization, animals can be kept in standard tissue culture flasks containing filtered embryo medium as larvae when they can use endogenous yoke stores for nutrients. Rearing older germ-free zebrafish requires more labor-intensive husbandry and thus has been employed less often in research (Phelps et al., 2017; Rendueles et al., 2012). These sterilized animals can be maintained germ-free or in association with specific microbes via inoculation into the water column. Microbial associations with zebrafish can be further controlled with genetically engineered microbes. Many of the bacterial lineages native to zebrafish are generally amenable to established genetic techniques, with new approaches continuing to be developed that further improve their genetic tractability (Wiles et al., 2018). A major benefit of using genetically engineered bacteria in animal–microbe interaction research is that it provides an opportunity to track bacteria, for example, via the expression fluorescent proteins, as they colonize host tissues (Schlomann, Wiles, Wall, Guillemin, & Parthasarathy, 2018; Wiles et al., 2016). There is also exciting potential to control bacterial behavior and the manner, in which, they interact with the host using inducible genetic switches that are triggered by small diffusible molecules added to the water.

Aside from native microbial associations, the zebrafish has been incredibly useful as a surrogate host for modeling a wide range of human–microbe interactions. Microbiota transplantation experiments have been performed with fecal samples from both mice and humans, demonstrating the possibility of reconstituting communities of mammalian gut-derived microbes in zebrafish (Rawls, Mahowald, Ley, & Gordon, 2006). Zebrafish are also used to study pathogenic interactions with individual human-derived bacterial isolates, including *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, and the fungus *Candida albicans* (Gratacap, Rawls, & Wheeler, 2013; Prajsnar, Cunliffe, Foster, & Renshaw, 2008; van der Sar et al., 2003; Wiles, Bower, Redd, & Mulvey, 2009). Larval zebrafish, which defend against infection solely through their innate immune system, are especially well-suited for dissecting the early stages of pathogenic encounters. Illustrating this point, a larval zebrafish infection model was used to test whether human isolates of extraintestinal pathogenic *E. coli* (ExPEC) use the pore-forming toxin, α-hemolysin, to evade killing by neutrophils and macrophages (Wiles et al., 2009). Such activity was hypothesized over a decade earlier, but an adequate model system to test this idea was not available (Wiles & Mulvey, 2013). An alternative hypothesis put forth at the time was that α--hemolysin is required for liberating nutrients critical for bacterial growth through the lysis of host cells. In zebrafish hosts, in which, neutrophils and macrophages were depleted via morpholino-mediated knockdown of the transcription factor PU.1, it was shown that attenuated ExPEC mutants lacking α-hemolysin expression were capable of causing a lethal infection. This result simultaneously refuted the hypothesis that α-hemolysin is required for ExPEC nutrient liberation and demonstrated that its function is required to overcome early innate immune defenses mediated by neutrophils and macrophages.

High Fecundity and Small Size

The high fecundity and relatively small size of zebrafish make them exceptionally amenable to high-throughput experimental schemes. In a single cross, a mating pair can produce hundreds of offspring that grow to be 3.5–4.5 mm in length as larvae and 20–25 mm as adults. These attributes make it possible to generate and maintain tens to thousands of animals for any given experiment, and thus, are advantageous in animal–microbe interaction research for three main reasons. First, it is feasible to carry out forward genetic screens for host genes involved in mediating animal–microbe interactions, which is an endeavor rarely undertaken in vertebrate organisms (Tobin et al., 2010; Trede et al., 2004). For example, and as further detailed below, chemical mutagenesis of zebrafish has been used to identify genetic loci important for combating *Mycobacterium marinum* infection (Tobin et al., 2010). Second, many different experimental conditions can be rapidly tested and analyzed in parallel. Notably, the small size of embryonic and larval zebrafish has led to the innovation of robotic injection systems capable of delivering bacteria into thousands of embryos per hour in combination with morpholino-based gene knockdown or drug treatments (Carvalho et al., 2011; Ordas et al., 2015; Pardo-Martin et al., 2010; Spaink et al., 2013; Takaki, Cosma, Troll, & Ramakrishnan, 2012). Lastly, zebrafish boost statistical power in experiments by allowing large sample sizes to

be used, which is crucial for addressing the highly variable and complex interactions that occur between animals and associated microbes (Rolig et al., 2017, 2015).

Rapid Ex-utero Development

It has become apparent that how humans and other animals grow and develop is intimately connected to the microbes they associate with. This expanded view of what governs animal development challenges the very concept of individuality. Consequently, the emerging idea is that to understand the fundamental biology of animals, the composite system of the animal and microbial cells and their functions—known as the "holobiont"—must be taken into account (Gilbert, Sapp, & Tauber, 2012; McFall-Ngai et al., 2013). Indeed, the links between animal development and resident microbes are being studied in a variety of organisms, including humans; however, much is still to be learned about the extent of these interactions and their mechanisms.

The rapid ex-utero development of the zebrafish—which can go from a fertilized egg to a multicellular animal with a beating heart within 1 day and a functional digestive system within four days—makes this model organism an incredibly sensitive system for studying animal–microbe interactions during vertebrate development (Kanther & Rawls, 2010). Several studies have revealed that bacteria profoundly influence gene expression, proliferation, and differentiation within the intestinal tract of developing zebrafish (Bates et al., 2006, 2007; Camp, Jazwa, Trent, & Rawls, 2012, 2014; Cheesman, Neal, Mittge, Seredick, & Guillemin, 2011; Davison et al., 2017; Kanther et al., 2011; Lickwar et al., 2017; Marjoram et al., 2015; Semova et al., 2012). It was found that bacteria trigger gene expression patterns involving nutrient metabolism and innate immunity within the zebrafish intestine that mirror patterns seen in mice (Lickwar et al., 2017). Intriguingly, it was recently discovered that zebrafish HNF4A, which is a member of an ancient metazoan family of hepatocyte nuclear factor 4 (HNF4) transcription factors, plays a central role in mediating microbiota-dependent gene expression changes within the intestinal epithelium (Davison et al., 2017). Intriguingly, sequence variants of human HNF4A have been linked to the incidence of inflammatory bowel diseases and colon cancer (Barrett et al., 2009; Chellappa et al., 2016). Therefore, the microbiota–HNF4A axis characterized in zebrafish may represent an evolutionarily conserved signaling conduit between animals and their resident bacteria (Davison et al., 2017).

Another key player in the dialogue between microbiota and the developing zebrafish intestine is the innate immunity protein MyD88 that functions as an adaptor for multiple innate immune receptors, including both Toll-like receptors and Interleukin-1 receptors. For example, MyD88 was found to be required for the induction intestinal alkaline phosphatase (Iap) by the bacterial cell wall component lipopolysaccharide (LPS), also known as endotoxin (Bates, Akerlund, Mittge, & Guillemin, 2007). LPS can elicit severe and even fatal inflammatory responses in animals. However, through upregulation of the LPS-detoxifying Iap enzyme, moderate amounts of LPS promote mucosal tolerance to resident bacteria within the intestine. MyD88 was also found to play a role in mediating host responses to resident microbiota that shape intestinal development and homeostasis. For example, MyD88, in combination with canonical Wnt signaling, is required for normal intestinal epithelial cell proliferation in response to the presence of secreted bacterial products (Cheesman et al., 2011). Similarly, MyD88 was found to mediate microbiota-derived cues that modulate Notch signaling and influence the balance of absorptive versus secretory cells in the intestine (Troll et al., 2018).

Bacteria can also influence developmental processes at sites outside of the intestine. It has been discovered that some bacterial members of the zebrafish intestinal microbiota encode a secreted protein capable of stimulating the expansion of insulin-producing pancreatic β cells that are crucial for controlling metabolic homeostasis (Hill et al., 2016). Intriguingly, this protein, which has been dubbed β cell expansion factor A (BefA), can be detected in the genomes of various bacteria that live within the human intestine. In addition, the zebrafish is emerging as a tractable model for studying the role of microbiota in neurobehavioral development, which because of the strong legacy of zebrafish in neurobiological research, promises to help uncover novel mechanisms that govern the so-called *gut–brain axis* (Phelps et al., 2017).

Optical Transparency and Amenability to Live Imaging

Conventional approaches for studying animal–microbe interactions largely neglect their spatial and temporal organization, yet these elements play defining roles in biological systems throughout nature. Offering a solution to this problem is the zebrafish, which has an optically transparent body during its larval and juvenile stages. This unique characteristic of the zebrafish, combined with its amenability to a variety of live imaging techniques and the expression of fluorescent proteins for tracking the activity of host and microbial cells, provides a literal window into the spatiotemporal organization of animal–microbe interactions within a living host (Jemielita et al., 2014; Parthasarathy, 2018).

For example, researchers used state-of-the-art live imaging to track zebrafish intestinal colonization patterns of two fluorescently marked bacterial symbionts, *Vibrio* and *Aeromonas* (Wiles et al., 2016). On their own in mono-association, each symbiont forms a distinct population architecture within a different region of the intestine from the other. The consequences of these colonization patterns became evident after *Vibrio* and *Aeromonas* were monitored together within the same host. *Vibrio* induces sudden and dramatic collapses in *Aeromonas* abundance that occasionally result in complete extinction events while *Vibrio* grows just as it does during mono-association. Closer inspection of the underlying mechanism driving this apparent competitive interaction revealed that *Aeromonas* lives as rigid multicellular aggregates that are highly sensitive to peristaltic flow. By contrast, *Vibrio* exists mostly as highly motile single cells that readily adapt to the expanding and contracting environment of the intestine. Remarkably, in hosts with a dysfunctional enteric nervous system and thus, altered peristaltic activity, the competitive interaction between *Vibrio* and *Aeromonas* is neutralized, and these two bacteria coexist. This study shows that the host can exert physical control over the microbes that inhabit the intestine and highlights that changing the physical mechanics of the intestine may be a strategy for microbiota engineering.

The capacity of the zebrafish to reveal the state of animal–microbe interactions in real time also greatly enhances the possibilities for screening and characterization of specific phenotypes. For example, high-throughput imaging of zebrafish using flow cell sorting systems, coupled with robotic manipulation technologies like those mentioned earlier, represent a powerful screening platform (Carvalho et al., 2011; Pardo-Martin et al., 2010; Spaink et al., 2013). However, "low-tech" approaches based on fluorescence stereomicroscopy and manual analysis have also proven robust and fruitful. For example, using transgenic zebrafish that have fluorescently marked neutrophils, numerous bacterial members of the zebrafish intestinal microbiota were screened for their ability to induce neutrophil recruitment, which is a readout of inflammation (Rolig, Parthasarathy, Burns, Bohannan, & Guillemin, 2015). This work led to two important observations. First, several different bacterial lineages exhibit antiinflammatory activity, suggesting that this trait may be common among intestinal symbionts and that resident microbiota are active manipulators of their environment. And second, minor community members can disproportionately modulate immune responses. In other words, symbiotic bacteria that are present at a relatively low abundance can still have a large effect on the immune system.

The Zebrafish: Forging and Overturning Paradigms in Animal–Microbe Interaction Research

As outlined in the section above, the zebrafish is a multifaceted and highly amenable model organism for studying animal–microbe interactions. In this section, we summarize two sets of studies that showcase the utility of the zebrafish and its ability to reveal novel aspects of animal–microbe systems.

Immunity and Interhost Dispersal of Intestinal Microbiota

Humans and other animals are associated with diverse communities of microbes throughout their lives that have the capacity to dramatically influence their health and development. Although these symbiotic relationships appear to follow general patterns, in terms of the way they assemble and the types of microbes they involve, there is still a great deal of variation that exists between individuals that cannot yet be explained. A pair of recent studies using zebrafish have now shown that dispersal of microbes between hosts appears to play a major role (Burns et al., 2017; Stagaman, Burns, Guillemin, & Bohannan, 2017). The zebrafish is an excellent animal model for addressing potential sources of microbiota variation, exemplifying three of the features discussed above: first, zebrafish innate and adaptive immunity can be experimentally distinguished and are known to act as microbial filters in mammals; second, the small size of zebrafish makes them amenable to multiple experimental housing conditions that, for example, differ in the extent of microbial dispersal, and third, the animals' fecundity allows many replicate zebrafish hosts to be used in a single experiment to adequately capture and quantify variation. In these two studies, intestinal microbiomes were profiled from wild-type, and immunocompromised zebrafish deficient for either innate or adaptive immunity (*myd88*$^{-/-}$ and *rag1*$^{-/-}$ respectively), and the hosts' immune genotypes were found to contribute to the composition of their intestinal microbiota, in agreement with previous observations made in mice. Unexpectedly, the cohousing conditions had a profound impact on the microbiota, with conditions that facilitated interhost dispersal of microbes almost entirely negating the filtering effects of the immune system. These studies indicate that the factors governing microbiota composition are not necessarily inherent to an individual host, but also shaped by the metacommunity of hosts and microorganisms with which each individual coexists. Similar patterns of interhost transmission of microbiomes are beginning to emerge from studies of human populations (Brito et al., 2019). Zebrafish promises to be a powerful system

for modeling the epidemiology and mechanisms of interhost transmission of the microbiota.

Fighting Mycobacterium Infection is an Inflammatory Balancing Act

Tuberculosis (TB) is a disease in humans that is caused by the bacterium *M. tuberculosis*. According to the World Health Organization, in 2017, 10 million people worldwide developed symptomatic TB, and 1.6 million died from the disease (World Health Organization, 2018). Despite over a 100 years of research on *M. tuberculosis* pathogenesis, it is still unclear what determines a person's susceptibility to TB and of those who fall ill, why some respond to treatment while others do not (Ramakrishnan, 2013). Shockingly, new therapeutic strategies for TB have not been implemented for several decades—the current vaccine is 90 years old, and the current treatment regimen is over 50 years old (Ramakrishnan, 2013). New insights into this devastating human disease come from research on a related disease in zebrafish caused by *M. marinum*. A hallmark of both zebrafish and human *Mycobacterium* infection is the formation of granulomas, which are immune cell complexes mostly made up macrophages that are typically thought to protect the host by walling off the pathogen and controlling its growth (Ramakrishnan, 2013). However, live imaging of granuloma formation and dynamics in zebrafish has called this deeply rooted paradigm into question. It was found that granulomas actually promote the growth of *M. marinum* and remarkably, *M. marinum* attracts new and permissive macrophages to established granulomas to spur further growth and dissemination (Davis & Ramakrishnan, 2009).

Not only has the zebrafish model challenged conventional wisdom about human TB pathogenesis, but it has also uncovered new leads into host genetic susceptibility to this pathogen. A forward genetic screen of hundreds of chemically mutagenized larval zebrafish hosts infected with fluorescently marked *M. marinum*, which allowed disease progression to be monitored in real time, identified the gene encoding leukotriene A4 hydrolase (LTA4H) as a critical player in the host's defense against *M. marinum* (Tobin et al., 2010). Interestingly, follow up studies revealed that both LTA4H deficiency, as well as excess, led to increased disease susceptibility (Roca & Ramakrishnan, 2013; Tobin et al., 2012). Ultimately, it was discovered that LTA4H is part of a delicate balancing act in the inflammatory response to *M. marinum*—not enough results in a lack of pathogen containment, whereas too much, results in collateral damage that cripples host defenses. Remarkably, these observations were found to mirror similar pathogenic interactions in humans infected with *M. tuberculosis*. Humans who are genetically predisposed to have either low or high levels of LTA4H are more susceptible to TB compared to those with intermediate levels (Tobin et al., 2012, 2010). This represents an exciting breakthrough in TB research because it uncovered the underlying intricacies of the disease while at the same time highlighting that new TB treatments may need to involve personalized regimens that take into account host genetics.

Outlook

Advancements in high-throughput DNA sequencing and omics technologies continue to expose new facets of animal–microbe interactions. A growing awareness that microbes play a more diverse and far-reaching role in animal biology than previously thought has transformed the field into a melting pot of collaborative and cross-disciplinary research. A major goal moving forward is to dissect the mechanisms that underlie the overwhelming number of newly discovered animal–microbe relationships. As described in this chapter, the laboratory zebrafish represents an ideal animal model for addressing this challenge. Not only is the zebrafish useful in uncovering the cellular and molecular details of animal–microbe interactions, it is also well-suited to tackle many emerging areas of interest. In particular, zebrafish facilitate the experimental interrogation of ecological and population-level interactions, as well as microbial impacts on animal development. In addition, the amenability of zebrafish to large-scale comparative studies will help identify unifying principles of animal–microbe interactions. With the continued innovation of genome editing tools and gnotobiotic husbandry techniques, the zebrafish is poised to make major contributions in the exploration of animals and their microbial associations.

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CHAPTER

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Targeted Editing of Zebrafish Genes to Understand Gene Function and Human Disease Pathology

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Background

Why Modify Gene Expression in Zebrafish?

The zebrafish model system is a particularly powerful tool to investigate gene function, and for more than 3 decades (Detrich, Westerfield, & Zon, 1999), researchers have used various techniques to modify the zebrafish genome, studying the genetic basis of organism development and homeostasis. In addition, zebrafish genome editing can be performed to generate zebrafish models recapitulating pathological processes displayed in human diseases (Langheinrich, 2003; Santoriello & Zon, 2012). A common strategy consists of the inactivation of the gene/genes homologous to the human genes associated with the disease. A "sick" zebrafish that recapitulates the aspects of the human disease pathology may aid the understanding of disease mechanisms and the development of new clinical treatments.

Gain Versus Loss of Gene Function

Genome editing procedures often involve the interference of gene expression to generate two types of gene changes: "loss-of-function" and "gain-of-function." In loss-of-function experiments, the target gene is necessary for a specific biological process if the complete removal of a functional protein encoded by that gene disrupts the process. Generating loss-of-function alleles in genes of interest is the most common type of genome modification experiment in zebrafish. Usually, a "nonsense" or "frame-shift" mutation introduced in the coding sequence presents a high probability of resulting in a "null allele," meaning the complete abolishment of protein function. Nonsense alleles have a premature signal to end translation of the protein (stop codons). Frame-shift alleles have a small insertion or deletion of bases that shifts the normal three base pair reading frame used by the cell. Insertions or deletions that are not multiples of three (*e.g.* a five base pair deletion or a two base pair insertion) will change the reading frame of the RNA, causing the protein to be "mistranslated," resulting in an incorrect and usually truncated protein.

Mutated alleles retaining some functionality are considered "hypomorphic," and they can be the result of nonsynonymous mutations inducing amino acid substitutions in the protein code or small in-frame deletions that do not completely inactivate gene function. In contrast, a gain-of-function experiment adds (usually unwanted) functionality to the genome, by typically expressing genes outside of their natural spatiotemporal window or at higher levels than wanted in the cell. When ectopic expression of a gene induces new biological outcomes in strange, often unwanted locations, it indicates a regulatory role for the gene in that process. In addition, gain-of-function strategies have been developed to modify the zebrafish genome solely for experimental observation of biological processes, typically by the addition of controlled expression of detectable markers, such as green fluorescent protein (GFP) that can be observed in a live animal. The ability to share these "transgenic" lines between zebrafish laboratories has significantly contributed to the scientific popularity

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of the zebrafish model system and enables researchers to compare the outcome of new experiments with previously published experiments without having to construct reporter lines over again in his or her laboratory (Udvadia & Linney, 2003).

Until recently, gain-of-function experiments to edit the zebrafish genome typically required the synthesis of a DNA construct that was randomly integrated in the zebrafish genome. They used a gene "promoter" sequence that drove gene expression tissue—specifically followed by the gene you want to be misexpressed. Notably, expression from randomly inserted constructs is strongly influenced by the region of insertion, and it is crucial to carefully evaluate the resulting expression pattern. Recently developed technology has opened up the exciting possibility that rather than relying on random insertion, it is now possible to guide insertion of DNA constructs to a specific location in the genome using programmable nucleases, which allows for more precise control of gene expression (Kimura, Hisano, Kawahara, & Higashijima, 2014) (discussed below in "Sequence integration" section).

Forward and Reverse Genetics (Historical and Conceptual Differences in Research Approach)

The experimental process for linking genotype to phenotype follows one of two major procedures: "forward" (classical) or "reverse" genetics. Forward genetics procedures involve random mutagenesis of the zebrafish genome by mutagenic chemicals or injected viruses, followed by isolation of mutant phenotypes (Lawson & Wolfe, 2011). In zebrafish, these techniques were introduced in the early 1990s, and they were spectacularly successful in identifying major genetic components of embryo development. However, the huge number of mutated fish required and the efforts necessary for the identification of the affected gene using positional cloning limited the use of this approach in smaller zebrafish labs.

In contrast, a reverse genetic experiment is designed to answer questions about the role/function of a specific protein by the introduction of targeted mutations followed by phenotypic characterization. Historically, in zebrafish, the first tool to be used for reverse genetic screening was "morpholinos" (MOs) (Nasevicius & Ekker, 2000). These were used to transiently block protein translation from the mRNA. Soon after, the TILLING (or Targeting Induced Local Lesions in Genome) system was developed in the early 2000s (Wienholds, Schulte-Merker, Walderich, & Plasterk, 2002). Although TILLING still required random mutagenesis, it allowed the screening of mutations in specific genes of interest bypassing the laborious positional cloning step, and for that reason, it quickly became popular until the advent of the targeted mutagenesis approaches in the late 2000s.

The first targeted mutagenesis tools for reverse genetics, based on genome-editing nucleases, such as Zinc Finger Nucleases (ZFNs) (Doyon et al., 2008; Meng, Noyes, Zhu, Lawson, & Wolfe, 2008) and Transcription Activator-Like Effector Nucleases (TALENs) (Bedell et al., 2012; Huang et al., 2011) developed quite recently (in 2008 and in 2011, respectively) in the zebrafish model system and were for a long time expensive and/or laborious. More recently (2013) the development of the Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR associated protein 9 (CRISPR/Cas9) system (discussed below) has made targeted genome editing simple and affordable for most if not all zebrafish laboratories (Hwang, Fu, Reyon, Maeder, Kaini, et al., 2013; Hwang, Fu, Reyon, Maeder, Tsai, et al., 2013; Jao, Wente, & Chen, 2013; Varshney, Pei, et al., 2015).

Transient Methods for Altering Gene Expression

The intrinsic features of the zebrafish model system make it useful in the manipulation of single or multiple target genes using transient gene knockdown and transgenic technology approaches.

Morpholinos

One of the most rapid, inexpensive, and consequently, popular tools for performing reverse genetic analysis in zebrafish is morpholinos (MOs). MOs are modified antisense oligonucleotides designed to bind target RNAs, creating a transient gene knockdown effect (Bill, Petzold, Clark, Schimmenti, & Ekker, 2009; Eisen & Smith, 2008; Timme-Laragy, Karchner, & Hahn, 2012). The binding of morpholinos to the matching mRNA targets at the translational start-site directly inhibits the protein synthesis by sterically blocking the translation. The splice blocking MOs, targeting specific exon-intron boundaries of the pre-mRNA, instead interfere with the correct splicing mechanism. More recently, MOs have also been used to block microRNA (miRNA) activity directly targeting the guide strand of the microRNA, or indirectly targeting the miRNA binding regions on mRNA targets (Flynt, Rao, & Patton, 2017) (see Table 49.1 for a comparison between gene knockdown and knockout approaches in zebrafish).

In zebrafish, MOs are delivered by direct injection into the fertilized embryos during the first stages of development (one- to the four-cell stage maximum). It is important to note that MO action is always dose dependent, and usually, it is limited to the first 3–5 days postfertilization (Timme-Laragy et al., 2012). In order to try to overcome this limitation, light-activated MOs containing a photosensitive subunit (photo-MOs) have been developed (Tallafuss et al., 2012). The major advantage of photo-MOs is represented

TABLE 49.1 Comparison between gene knock-down (left) and knock-out (right) approaches in zebrafish.

	Morpholino	ZFN/TALEN/CRISPR Mutants
Targets	pre-mRNA, mRNA or miRNA	genomic DNA
Delivery	Direct injection in the embryo (1–4 cell stage)	
Validation of activity on target sequences	1–2 weeks (RT-PCR or Western blot)	1 week (genotyping/sequencing)
Off-target effects	High probability (often mediated by activation of *tp53* pathway)	Relatively low probability (reduced with outcrossing)
Effects	Partial to full knock-down (the effects are dose dependent and temporally limited to 120 hpf max)	Partial to full knock-out depending on the selected mutation
Proving specificity	At least 2 unique MOs required plus several controls	Genotype-phenotype correlation
Time required	Rapid (3–4 weeks)	Slow (4–7 month)
Cost	Relatively low	Low to moderate (based on the system used)

by the spatiotemporal control of the target gene expression, although with certain embryonic target regions or stages, the correct photo-activation using confocal microscope could sometimes be challenging.

Since the early 2000s, MOs have been extensively used in studies of zebrafish development, to confirm the function of candidate genes and even to model human diseases. Nonetheless, lately, MO use has been criticized because of the induction of off-target effects, mostly mediated by p53 (Robu et al., 2007); as well as, in several cases, because they failed to replicate mutant phenotypes (Kok et al., 2015). However, in 2015, Rossi and colleagues (Rossi et al., 2015) showed that some MO-specific phenotypes observed in morphants are not present in the corresponding mutant animals because of a genetic compensation, reopening the long-standing controversial debate about the knockout versus knockdown approaches (Blum, De Robertis, Wallingford, & Niehrs, 2015; Schulte-Merker & Stainier, 2014;Stainier, Kontarakis, & Rossi, 2015). Although the debate is likely to continue, a general consensus has emerged that studies involving experiments with a new MO design should include a direct comparison with the corresponding genetic mutant phenotype (Stainier et al., 2017).

mRNA Injection and Pharmacological Approaches

The overexpression of a target gene (Prelich, 2012) to study its function represents another very popular tool among the zebrafish research community. Specific variants of the gene can be introduced in the embryos by microinjection of capped mRNAs or DNA plasmids. The use of DNA plasmids will result in a variable expression of the protein through the cells of the embryo generating a "mosaic" embryo, while the injection of capped mRNA usually produces a more homogenous but more temporally limited distribution. In this approach, the effects of the normal and the mutated variants of a transcript can be compared to understand their functions better during embryo development. This approach proved to be very helpful in confirming the effects of MO knockdowns and gene knockouts, as well as in testing the effects of specific mutated forms found in human patients. In some cases, the transcripts can be tagged with specific epitopes in order to visualize the protein localization during development. However, the limits of this approach are represented by the fact that the effect is transient, and often it results in a non-physiological ectopic expression of the protein. From this point of view, a pharmacological approach targeting elements of a defined pathway can directly or indirectly affect the gene expression of specific targets, and thus, could represent a reasonable alternative. Moreover, because chemicals can be applied and removed in a very precise time-dependent manner, a pharmacological agent may also represent a more physiologically relevant option (Skromne & Prince, 2008). A pharmacological compound typically affects entire families of gene products, which makes the phenotypic effects sometimes hard to link to the function of single genes. However, with knowledge of the spatiotemporal expression of individual members of gene families and partial biochemical characterization of the pharmacological reagent, it may be possible to attribute at least the phenotypic effects of the administration of pharmacological compounds to the subset of genes expressed in the area of the observed phenotype. This approach can be helpful to show, for example, a stage-specific effect of the target gene expression or to

reduce toxicity or potential off-target effects due to ectopic expression of the target protein. However, when using a pharmacological approach, it is critical to be aware of potential pleiotropic effects due to a concentration-dependent specificity, and then the concentration in embryos should be carefully titrated. Finally, the genetic and pharmacological approaches can be used together to sustain and confirm each other (Lieschke & Currie, 2007; Skromne & Prince, 2008).

Techniques for Genome Editing

Site-specific Nucleases

The access to complete genomic sequence for a growing number of species in association with the advent of better technologies for genome modification facilitated the gene function studies and opened the genome-editing era. Compared to older technologies, such as TILLING and random mutagenesis, the genome editing using ZFNs, TALENs, or CRISPR/Cas9 allowed direct targeting of specific genomic sequences and the easy selection of mutations, increasing the rate of identifying development and disease-related phenotypes (Varshney, Sood, & Burgess, 2015) (see Fig. 49.1). Moreover, the use of ZFNs, TALENs, and in particular the CRISPR/Cas9 system, reduces the problem of nonspecific mutations in the genome and repeated mutagenesis of the same gene, both common side effects of using random mutagenesis techniques.

Genome editing consists of the use of proteins able to induce modification of genome sequences in specific locations of the genomic DNA. The sequence specificity is typically obtained using different kinds of DNA-binding

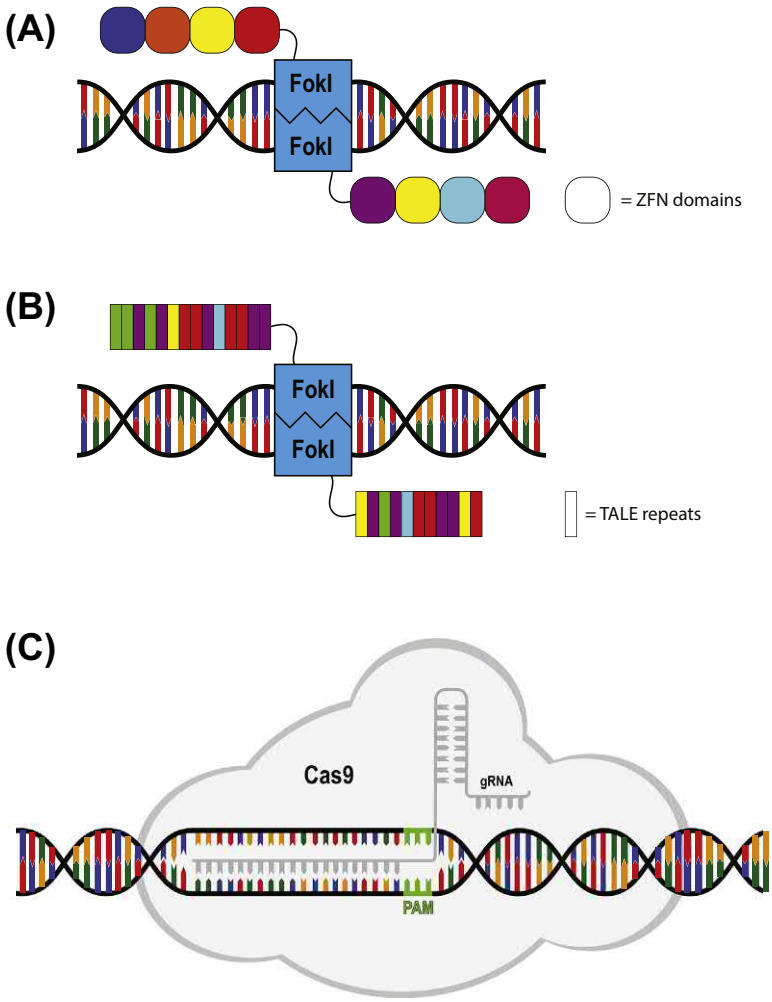

FIGURE 49.1 **Schematic representation of ZFNs, TALENs, and CRISPR/Cas9 genome editing systems.** Zinc Finger Nucleases (ZFNs) (A) and Transcription Activator-Like Effector Nucleases (TALENs) (B) technologies are based on fusing modified FokI nucleases able to cut the DNA only in the dimeric form to specific DNA binding motifs (ZFN domains or TALE domains, respectively). Different colors used for the DNA binding domains indicate different sequence specificities. (C) The Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR associated protein 9 (CRISPR/Cas9) system requires the base pairing of a guide RNA (gRNA) downstream of a protospacer adjacent motif (PAM) in order to locate and induce double-strand breaks.

TABLE 49.2 Comparison among different nuclease systems for genome editing.

	ZINC-FINGER nucleases	TALENs	CRISPR/Cas9
Recognition type	Protein-DNA	Protein-DNA	RNA-DNA
Cutting activity	Work as dimers	Work as dimers	Works as a monomer
Recognition site	9–12 nt/monomer	14-20 nt/monomer	~20 nt upstream a PAM site
Spacer	Required (5–7 nt)	Required (~14 nt)	No spacer required
Design	Limited (restrictions in triplets design)	Relatively flexible	Extremely flexible
Assembly	Laborious and time consuming	Moderately easy	Easy
Specificity	Usually high (but off target effect reported)	Usually high	Higher potential for off-target effects
Enzime	Coupling with non-specific nuclease FokI		Cas9
Mechanism of action	Induction of double strand brake in gDNA repaired by HDR or NHEJ mechanisms		
Induced Mutations	Insertions and/or deletions	Mostly deletions	Insertions and/or deletions
Mutagenic efficiency	Low	Moderate	High
Multiplexing	No	No	Yes
Cost	Moderate	Moderate	Low

domains or using guide RNA molecules (see Table 49.2 for comparison among different nuclease systems). In the most straightforward approach, the use of engineered enzymes (nucleases) induces a double-stranded break in the DNA of specific regions of interest. Following the DNA double-strand break (DSB), two different cellular DNA repair mechanisms are induced (Symington & Gautier, 2011): (a) Homology Dependent Repair (HDR), which is rare, and it uses a template DNA supplied by the researcher to repair the DNA break (see below), or (b) nonhomologous end joining (NHEJ), which is more frequent in vivo, but highly error-prone (see Fig. 49.2). As a consequence, NHEJ repair mechanism is often accompanied by loss or gain of small fragments of DNA (usually ranging from 1 bp to ~40 bps), which may induce frame-shift mutations in the coding sequence of the target gene impairing protein sequence and functionality. More recently, the use of different effector domains to perturb genome structure and function (such as recombinases, epigenetic modifiers, transcriptional repressors or activators, etc.) created a further level of versatility for the system (Hsu, Lander, & Zhang, 2014).

In zebrafish, one of the major problems of using it as a genetic model was the teleost-specific genome duplication event that occurred ~400 million years ago (Meyer & Van de Peer, 2005). Because of this ancient duplication event, zebrafish and other teleost species have a higher number of protein-coding genes (~26,000) compared to human, mouse, or chicken (Howe et al., 2013). Approximately 20% of all zebrafish genes have two copies per haploid genome instead of the usual one copy. Thus, for one in five genes, knocking out one copy is insufficient for seeing the effects of a null allele as the second copy of the gene could compensate some or all functions in the animal, making the genetic analysis more difficult. In general, after duplication, the gene duplicates tend to diverge using one of the following models: (a) one copy is lost via acquired genomic mutations and only one copy survives (nonfunctionalization); (b) subfunctionalization occurs when each duplicated gene retains a subset of the original ancestral function; (c) neofunctionalization is the process in which one or both of the duplicates gain new functions; finally, (d) subfunctionalization can be followed by neofunctionalization events increasing the overall functional divergence (Ohno, 1970; Postlethwait, Amores, Cresko, Singer, & Yan, 2004; Rastogi & Liberles, 2005). Although the duplication event is thought to be at the base of the evolutionary success of teleost fish, obviously, it represents a problem for genome editing analyses. In most of the cases, there is a risk of compensatory effects by the untargeted gene copy. Fortunately, the multiplexing approach available with most of the genome editing technologies in zebrafish helps to overcome this limitation, allowing, for example, the direct targeting of both the duplicates simultaneously or even different members of the same gene family.

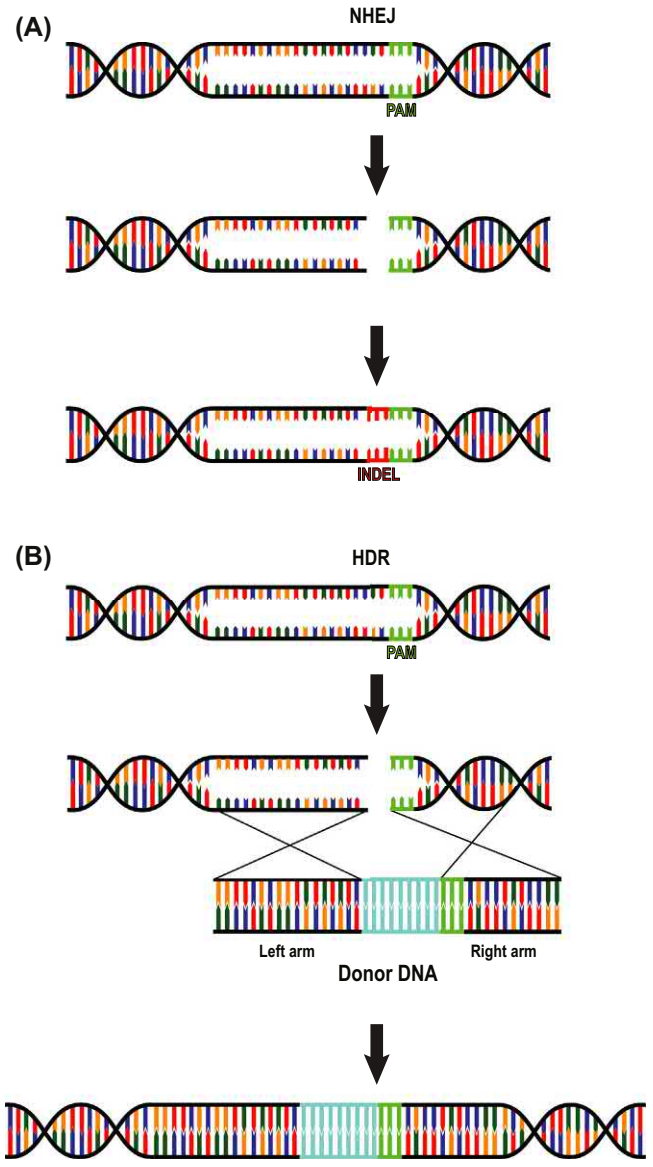

FIGURE 49.2 **Schematic representation of DNA repair mechanisms after DNA double-strand breaks.** DNA double-strand breaks induced by nuclease activity can be repaired by nonhomologous end joining (NHEJ) (A) or Homology Dependent Repair (HDR) (B) mechanisms. NHEJ is the most frequent mechanism, but it often introduces an insertion or a deletion of bases (indel) at the genomic target site. Instead, the HDR pathway requires the presence of a template DNA sequence (donor DNA) and consequently is more rare and precise. It can be used to introduce exogenous DNA into the zebrafish genome. PAM = protospacer adjacent motif.

ZFNs and TALENs

Zinc Finger Nucleases are chimeric proteins, including DNA-binding domains typical of zinc finger-containing transcription factors and a bacterial FokI endonuclease domain (Urnov, Rebar, Holmes, Zhang, & Gregory, 2010) (see Fig. 49.1A). Researchers showed that each individual "zinc finger" was able to recognize and bind to a specific sequence of three nucleotides (nt) in the major groove of DNA, and they developed a library of specific zinc-fingers. Combining several fingers in tandem allowed the targeting of unique 9–12 base pair sequences within the genome. Two different ZFN "arms" are created to recognize a 10–18 base pair target with a spacer of 5–7 nt between each half, which is where the FokI-induced double-strand break will occur. Researchers increased the specificity of the ZFNs system by the use of recombinant versions of FokI enzymes able to cut the DNA only when in dimeric form. The two different halves needed to be brought together (one on each side of the spacer) in order to cut at the locus.

ZFNs first became popular in cell culture systems, and eventually, in 2008, they became the first genome-editing tool to be used in zebrafish (Doyon et al., 2008; Meng et al., 2008). However, ZFNs adoption in zebrafish was limited by low efficiency and flexibility, the complex assembly of their arms, significant off-target activity, and lastly, by the advent of TALENs (2011) and CRISPR/Cas9 (2013) that represented more efficient, faster systems with higher mutagenic throughput.

After several years of ZFN use, TALENs emerged as a simpler-to-use alternative (Joung & Sander, 2013). TALEN technology is conceptually identical to ZFNs: the coupling of FokI nucleases to DNA binding motifs working as dimers to increase specificity (see Fig. 49.1B). In TALENs, the DNA binding motif is from the bacterial TALE repeats, which consist of 34 amino acids with the 12th and 13th residues consisting of a variable region (RVD) containing a simple code for binding to specific nucleotides. In contrast to ZFN domains, which bind to specific triplets of bases, each TALE repeat recognizes a single nucleotide. By changing only two amino acids in each RVD, researchers can generate a combination of motifs specific for any DNA sequence. While ZFN cutting induces insertion and deletion with the same frequency, TALENs rarely introduce insertions (Kim et al., 2013; Sood et al., 2013; Varshney, Pei, et al., 2015). Compared to ZFNs, TALEN technology represented a huge step forward in flexibility, efficiency, and design with similar, or potentially lower, off-target activity. However, compared to the CRISPR/Cas9 system, TALENs still present a reduced mutagenic throughput because of a time-consuming cloning process.

CRISPR

The development of the CRISPR/Cas9 as a genome-editing tool was the consequence of discovering Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) systems in prokaryotes (bacteria) (Wang, La Russa, & Qi, 2016). These unicellular organisms use a combination of short RNAs

and proteins to target invading DNA sequences for cleavage. In essence, CRISPR/Cas provides a primitive adaptive immune system against plasmids and bacteriophage infections. In contrast to ZFNs and TALENs where the targeting of a specific genomic site is obtained using DNA-binding motifs, the CRISPR/Cas9 technology is based on base pairing using a guide RNA (gRNA) (see Fig. 49.1C). Once the guide finds the matching DNA sequence in the genome, the Cas9 endonuclease induces a double-strand break that can be repaired by the cell using HDR or NHEJ. The CRISPR/Cas9 tool proved to be a very efficient, versatile, and easy-to-use system, which has been used in several different biological contexts, from human cells to different animal models, zebrafish included (Varshney, Sood, et al., 2015). One of the major advantages of this system compared to ZFNs and TALENs is the broad genomic target coverage: the only limitation is the requirement of a PAM (or protospacer adjacent motif) site sequence directly upstream, the target region bound by the gRNA (20 base pairs long). For the most commonly used Cas9 protein, the PAM is the nucleotide sequence NGG, which occurs on average approximately once in every eight base pairs in the genome (Varshney, Sood, et al., 2015). Changing the 20 nt protospacer of the gRNA changes the target site and can be easily accomplished by subcloning the nucleotide sequence into a gRNA plasmid backbone or into a DNA fragment used directly for in-vitro transcription (Varshney, Pei, et al., 2015).

In zebrafish, the Cas9 protein or mRNA encoding Cas9 and the gRNA (as a DNA cassette with a specific promoter or as an in-vitro synthetized gRNA of ~100 nt) are introduced by coinjection during the earliest stages of embryo development (one- to the four-cell stage). The injection of multiple gRNAs is usually well tolerated in zebrafish embryos allowing the targeting in parallel of multiple genes simultaneously (multiplexing). Multiplexing can be utilized for several different purposes. It can be used, for example, to engineer the deletion of large genomic regions (two guides, one targeting each side of the desired genomic deletion) or to target duplicated genes or multiple members of the same gene family simultaneously. Other examples could be the targeting of multiple genes involved in the same signaling pathway or biological activity or unrelated genes to obtain compound heterozygous fish to reduce the space and the number of fish needed in the animal facility.

Although most of the efforts so far have been focused on the targeting of gene coding sequences, recently some new studies are focusing on noncoding genomic regions, such as promoters, enhancers, and miRNA. In particular, Narayanan and colleagues (Narayanan et al., 2016) developed a multiplex approach to target miRNA gene families in vivo using zebrafish as the animal model. They generated inheritable mutations in multiple miRNA loci impairing the expression and activity of a miRNA family with negligible off-target effects (Narayanan et al., 2016).

Finally, another interesting use of CRISPR/Cas9 has been recently published by Ablain, Durand, Yang, Zhou, and Zon (2015); (Ablain & Zon, 2016). They took advantage of the Tol2 transposon (transgenic) technology (Kawakami, 2007) to induce random integrations in the zebrafish genome of a CRISPR-based vector system for tissue-specific knockout of genes. Inside the vector, a U6 (a small nuclear RNA gene) promoter induces the ubiquitous and continuous expression of the gRNA, while the spatiotemporal expression of Cas9 is determined by a tissue-specific promoter. In this way, the effects of CRISPR/Cas9 system are limited to specific territories or tissues, potentially avoiding the embryonic lethality of some gene knockouts and the resulting animal potentially represents a more informative disease model. Notably, adding a second or multiple U6:gRNA cassettes in the vector backbone can easily allow a multiplexing approach.

Compared to ZFNs and TALENs, one of the biggest concerns about the use of CRISPR/Cas9 is the increased potential for off-target effects induced using just 20 nt of sequence. So far, the data about nonspecific cleavage activity of CRISPR/Cas9 are limited. Moreover, the adoption of multiplexing approaches increases the concerns about the overall potential off-target effects of each injected gRNA. Varshney, Pei, et al. (2015) were able to detect off-target activity with germline transmission of the mutations in 1 of 25 predicted off-target-sites in exons, suggesting that the nonspecific activity of the system could be low but detectable. However, the potential off-target activity is always sequence-dependent and could vary based on the gRNA used for targeting the genome.

Several approaches were developed (Sander & Joung, 2014) to reduce the rate of off-target cleavages. One approach requires the use of an engineered version of Cas9 unable to cut the DNA (dead Cas9), fused with the same Fok1 endonuclease enzyme used in ZFN and TALEN systems. Similar to ZFNs and TALENs the increased specificity is obtained through targeting two different sequences to direct the Cas9-FokI fusion proteins to cut the same genomic site (Tsai et al., 2014) effectively doubling the length of the genomic targeting sequence. Another very efficient strategy used a mutant version of Cas9 (Cas9n or Cas9 nickase) able to induce only single-strand breaks in DNA and again using a pair of gRNAs targeting opposite DNA strands of the selected site. When the Cas9n cuts in off-target regions, the introduction of single-strand nicks can be efficiently repaired by the cells; however, the double nicks induced at the correct site create a double-stranded break that will be repaired by the more error-prone NHEJ system

(Ran et al., 2013). Although these methods can significantly reduce the off-target mutagenesis effects of CRISPR/Cas9 system, at the same time, they reduce its overall versatility, flexibility, and its multiplexing potential. However, in zebrafish, "out-crossing" the injected fish with wild-type strains, followed by identification of mutant "carriers" in the desired gene targets in most cases should be sufficient to segregate away any off-target activity.

Sequence Integration

Random Insertion of Exogenous DNA

In zebrafish, researchers have typically designed gain-of-function experiments by random insertion of promoter and coding sequences in the genome using DNA transposons (Tol2, Sleeping Beauty or Ac/Ds systems) or restriction enzyme-based methods (meganuclease) (Grabher & Wittbrodt, 2008). While efficient, the expression of a randomly inserted sequence might be strongly influenced by copy number variation or epigenetic regulation in the region of the insertion making it crucial to carefully evaluate each resulting expression pattern by the inserted construct. Moreover, the added complexity of the positional effects on the function of randomly integrated constructs makes the comparisons between experiments difficult. In addition, correctly identifying promoters that express in the desired tissue-specific expression pattern is often difficult, with some regulatory DNA sequences tens or hundreds of kilobases away from the regulated gene. Recently, some reports indicated that the high efficiency of the CRISPR/Cas9 system can be a very powerful tool to induce a locus-specific integration of expression constructs (Auer, Duroure, De Cian, Concordet, & Del Bene, 2014; Kesavan, Chekuru, Machate, & Brand, 2017; Kimura et al., 2014; Ota et al., 2016) which, consequently, are expressed under the control of endogenous native regulatory context in the zebrafish genome. Although the integration can impair the expression of the endogenous gene (Ota et al., 2016) or it can be outside the target site inducing the expression of the reporter in unrelated cells (Kimura et al., 2014), this new generation of zebrafish transgenic lines represents a big step forward in the real-time analysis of the gene expression, avoiding the aforementioned limits of the random integration systems.

Introduction of Exogenous DNA by Nucleases

Recent developments in editing technologies have inspired researchers using the zebrafish model to develop tools that induce site-specific insertion of DNA at the site of a double-stranded break in the zebrafish genome (Hisano et al., 2015). DNA integration directed by DSBs has been achieved by multiple groups by leveraging three different cellular mechanisms: Homology Dependent Repair (HDR), Microhomology Mediated End Joining (MMEJ) and Nonhomologous End Joining (NHEJ) (Symington & Gautier, 2011).

Knock-in by HDR and MMEJ Mechanisms

A cell can repair a DSB in a chromosome using homology dependent recombination where a DNA molecule with sequences matching both sides of the region of the DSB is used as a template (Sung & Klein, 2006) (see Fig. 49.2B). In zebrafish, exogenous DNA can be inserted by homology dependent recombination at the site of a DSB if the exogenous DNA is synthesized with long sequences identical to both sides of the DSB and the desired DNA insertion between the two sequence-matching "arms." Such identical sequences are commonly referred to as the homology arms, and typically they can be 1 Kb long or more (Hoshijima, Jurynec, & Grunwald, 2016; Shin, Chen, & Solnica-Krezel, 2014; Zhang, Huang, Zhang, & Lin, 2016; Zu et al., 2013). The recombination mechanism is typically error-free, and thus, well suited for integration into coding genes. However, the construction of donor DNA with long homology arms is relatively time consuming, which can limit the usefulness in zebrafish research projects where testing of several constructs is desired. In contrast, the MMEJ mechanism repairs DSBs by aligning microhomologous sequences internal to both ends of each side of a DSB. Several studies in zebrafish have successfully incorporated exogenous DNA at the site of a DSB using short homology arms (<50 bp) (Armstrong et al., 2016; Hisano et al., 2015; Hruscha et al., 2013; Hwang, Fu, Reyon, Maeder, Kaini, et al., 2013) and reported frequent addition of homology-independent insertion or deletion of bases (indels) at the integration sites, which would indicate the more error-prone MMEJ mechanism rather than a classical HDR mechanism. However, the precise molecular repair mechanisms implicated in these studies have not been extensively investigated.

Knock-in by Nonhomologous End Joining

The induction of DSBs in zebrafish in the presence of exogenous DNA lacking any sequence homology can result in sequence integration by NHEJ (Auer et al., 2014; Kimura et al., 2014) (see Fig. 49.2A). Proteins from the Ku family bind and protect DNA ends from degradation and DNA ligase IV catalyzes the end-joining at DSB (Sfeir & Symington, 2015), which at some frequency can incorporate the integration template which is provided in excess.

Compared to recombination or MMEJ, the NHEJ mechanism provides the significant advantage that the donor DNA does not need to be modified with homologous flanking sequences. Together with recent advances in CRISPR technology, NHEJ protocols would, for example, allow for high-throughput genome modifications using a single DNA construct, such as a fluorescent tag, but with many different CRISPR gRNAs. However, NHEJ-dependent sequence integration is a relatively rare and error-prone event. The integrations can be randomly oriented, and small insertions or deletions could alter the reading frame of the DNA resulting in only one over six integrated sequences in the correct orientation and reading frame (Auer et al., 2014). Notably, since the NHEJ mechanism repairs nuclease-induced DSBs and introduces insertions or deletions (indels) destroying the nuclease recognition sequence, it effectively inhibits the efforts to achieve DNA integration with homology arms. It is an interesting concept to block the activation of the NHEJ pathway, for example, using a morpholino against the DNA ligase IV (He et al., 2015) in order to favor MMEJ or HDR mechanisms, but no reports showing this have been published. It should be noted that, although the induction of precise DSB at any chosen position of the zebrafish genome is relatively straightforward (Varshney, Pei, et al., 2015), site-specific knock-in approaches present a very low efficiency and still pose a challenge for many labs. As discussed above, a number of successful experiments have been published, but many zebrafish researchers reported severe difficulties in performing analogous knock-in experiments at different genomic locations, potentially due to variation in chromatin structure and DNA modifications during development.

Although zebrafish null alleles (full inactivation) are powerful tools to investigate the full range of functions for a specific gene, many human diseases are linked to mutations predicted to cause single or multiple amino acid substitutions that partially inhibit gene function. A relevant zebrafish model requires the introduction of mutations that change the amino acid sequence in the zebrafish gene to match the human disease allele. Recently, Zhang and colleagues (Zhang et al., 2017) showed another interesting application of CRISPR/Cas9 system in zebrafish. Using a cytidine deaminase enzyme and an uracil-DNA glycosylase (UDG) inhibitor both fused to N-terminus region of a Cas9 nickase, they were able to obtain single base conversion (C- > T, C- > A or C- > G) in specific genomic locations with relatively high efficiency and a reduced occurrence of indel mutations. Moreover, using a catalytically dead Cas9 (able to bind, but not to cut the DNA) instead of the Cas9 nickase, they were able to reduce the amount indels further, although with a lower base conversion efficiency (Zhang et al., 2017).

Conclusions

Once efficient, precise, and robust methods for knockout, and more importantly, site-specific knock-in of exogenous DNA are developed, the zebrafish animal model could emerge as the preferred vertebrate system for many functional genomic studies. Several features make zebrafish particularly attractive in this regard. First, external fertilization and nearly transparent early larvae allow for direct observation of phenotypes or pathologies in a live animal. Second, the small size and relatively simple husbandry allow for larger-scale approaches that would be much more challenging in mammalian disease models, such as rodents. Finally, zebrafish represent an opportunity to reduce the use of rodent models when possible, an effort supported by most regulatory bodies that govern animal use. Zebrafish models represents a valid and economical alternative to reproduce mutations found in human patients (personalized medicine and gene therapy), to model lethal disease caused by dominant mutations, and finally, to study the effects of specific amino acid alterations in protein sequence/structure in vivo, in order to develop new and better drug treatments.

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CHAPTER

50

Zebrafish as a Platform for Genetic Screening

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Introduction and History

> The mutant and its phenotype, caused by the lack of a single gene product, is a cleaner experiment than any transplantation, constriction or centrifugation could ever be. ***Christiane Nüsslein-Volhard (2012)***

What is a genetic screen? Broadly defined, a genetic screen is a research technique aimed at uncovering the characteristics of an organism, its phenotype, which arises from the DNA sequences contained within that organism, its genotype. By observing phenotypes in organisms which differ when compared with "normal" or wild-type organisms, and then determining what DNA change caused the new phenotype, the function of the mutated DNA can be deduced. Most often, these changes to the DNA occur in genes. Thus, a genetic screen is a way to learn the function of genes. For example, if a mutant does not develop eyes, then one can infer that the mutated gene is required for eye formation.

There are two major categories of genetic screens, known as forward and reverse. In a classical forward screen, the researcher starts with a phenotype that differs from the wild-type condition and then determines the gene that was changed. In contrast, in a reverse screen, the researcher starts with genes of interest and engineers the DNA changes to determine the phenotype that, if any, arises when the candidate genes are mutated. In zebrafish, reverse genetic approaches are enabled by recent advances in genome editing like CRISPR/Cas9, TALEN, zinc finger nucleases, and TILLING (Doyon et al., 2008; Huang et al., 2011; Hwang et al., 2013; Meng, Noyes, Zhu, Lawson, & Wolfe, 2008; Moens, Donn, Wolf-Saxon, & Ma, 2008; Rissone, Ledin, & Burgess, 2018). Moreover, transient methods like chemical treatments and gene knockdown are sometimes grouped into the reverse genetic category, although these nonheritable methods do not address how DNA relates to phenotype, and therefore, by definition, they are not genetic. While reverse genetic approaches are useful for understanding gene function, they are well-described in this volume and elsewhere (Lawson, 2016; Rissone et al., 2018). Here, I only consider further unbiased forward genetic screens, which remain a valuable and relevant technique even in the current era of powerful genome editing technologies.

Why are zebrafish useful for screens? The similarities between humans and zebrafish may not be immediately apparent. But in fact, zebrafish development, anatomy, and genomes are all strikingly similar to other vertebrates including humans. Like all vertebrates, zebrafish develop from a single cell to a complex, integrated, multicellular animal and the cellular and molecular mechanisms of development that build these complex animals are largely similar, or conserved, across all vertebrates. During development, vertebrate organisms pass through several developmental stages and landmarks, which are shared with other vertebrates. One of these developmental stages, in particular, the pharyngula stage, is the so-called *phylotypic* period proposed to be the developmental stage when vertebrate morphology is most conserved between species (Duboule, 1994; Irie & Kuratani, 2011; Von Baer 1828). The outcome of development is the different anatomical features, which are also largely shared among fish and mammals. It is more unusual to find a derived character that is not shared across vertebrates than the vast number of anatomical features that are shared (Hyman, 1942). The "recipe book" guiding the development of the single cell to complex morphology is encoded in the full complement of DNA within the organism, its genome. The similarities between zebrafish and human genomes are evidenced by the discovery that approximately 70% of human genes have at least one clear zebrafish matching gene, or ortholog (Howe et al., 2013).

There are also species-specific advantages for genetic screens that zebrafish enjoy compared to other animal

model systems. Zebrafish, originally a popular aquarium pet, are very easy to keep because they are resistant to variable water quality, temperature, salinity, and pH. Zebrafish are straightforward to breed, and it is common to obtain hundreds of offspring per breeding pair each week, an asset for genetic studies. Zebrafish are translucent when young, so developing animals can be placed under the microscope to directly image tissues, cells, and molecules. Furthermore, they are externally fertilized so that all developmental stages are readily observed. These valuable characteristics allow screening for interesting changes in phenotype when fish genomes are mutated.

How did zebrafish become a premier platform for genetic screening? The ability to start with an interesting phenotype and discover the causative genotype in zebrafish was made possible by pioneering scientists. In the 1960s, George Streisinger was using viruses that infect bacteria to address questions about gene function but wished to develop a vertebrate model system capable of mutational analysis of genes (Grunwald & Eisen, 2002). To this end, he began working to develop genetic methods in the zebrafish culminating with his generation of homozygous clones for the first time in a vertebrate (Streisinger, Walker, Dower, Knauber, & Singer, 1981). The now-famous, spontaneously occurring mutant "golden," which alters pigmentation in the zebrafish was instrumental in this study and demonstrated that zebrafish could be used for mutational analyses like the forward screens.

To understand the phenotype of a mutant, one must first understand the phenotype of the wild type. While Streisinger was developing genetic techniques, his colleague Charles Kimmel developed techniques for studying zebrafish development. Capitalizing on the advantages of the zebrafish, Kimmel used microscopy to observe live animals as they develop wild-type phenotypes. First developing techniques for tracking the development of a single neuron in live animals (Kimmel, 1972; Kimmel et al., 1978, 1981), followed by cell lineage analyses (Kimmel, 1989; Kimmel, Warga, & Schilling, 1990; Kimmel and Warga, 1987, 1988), and then a comprehensive description of embryonic development (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). With the tools for genetic and developmental analyses in place, the first genetic screens were carried out, uncovering induced mutations that altered the development of varied tissues (Felsenfeld, Walker, Westerfield, Kimmel, & Streisinger, 1990; Grunwald, Kimmel, Westerfield, Walker, & Streisinger, 1988; Walker & Streisinger, 1983). Meanwhile, large-scale genetic screens were revealing fantastic insight into the development of the Drosophila embryo (Nüsslein-Volhard & Wieschaus, 1980), motivating a similar big-screen approach in the burgeoning vertebrate model (Mullins, Hammerschmidt, Haffter, & Nüsslein-Volhard, 1994), thrusting the once humble aquarium pet into the limelight as a premier organism for genetic screens.

What are the basic steps of a genetic screen? A genetic screen can be distilled into six steps which I will describe in detail in the remaining sections of this chapter. The simplified outline of a forward genetic screen is (1) mutagenesis, (2) recovery, (3) phenotyping, (4) genotyping, (5) validation, and (6) characterization.

Mutagenesis

The goal of mutagenesis is to generate a permanent change in genomic DNA. There are several different mutagenesis options, and I will highlight the strengths and weaknesses of some commonly used mutagens below. Some variables to consider when choosing a mutagen are the type of lesion, the hazards to the researcher, and the target number of DNA lesions per genome. The number of lesions per genome is particularly important to consider because too few will result in an inefficient recovery of mutants, while too many will cause general sickness and increased chances of complex, difficult to recover mutations.

N-ethyl-*N*-nitrosourea (ENU) is the most commonly used mutagen for the forward screens in zebrafish. This potent chemical mutagen has a high mutation rate and is known to primarily induce point mutations on a single strand (Kile & Hilton, 2005) (Fig. 50.1). Therefore, single genes are destroyed in ENU-induced mutant

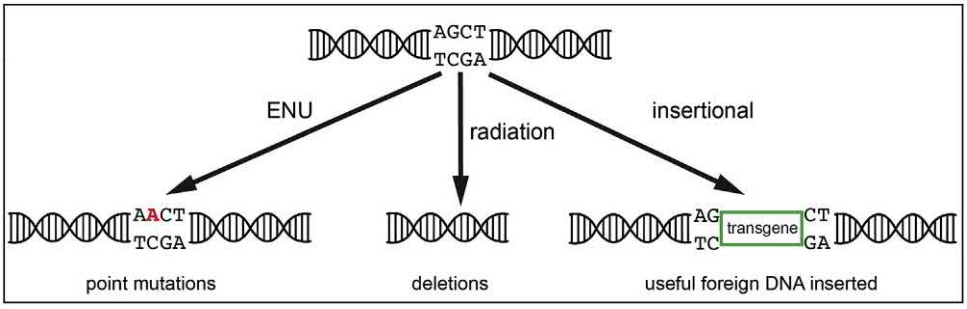

FIGURE 50.1 **Zebrafish mutagenesis.** Chemical (ENU), radiation, and insertional mutagenesis are commonly used methods of inducing genomic DNA changes, or mutations. Each mutagen is expected to induce different types of DNA changes as indicated.

lines, increasing the likelihood that a single candidate gene segregates with a phenotype. The primary negative to ENU mutagenesis is its toxicity; adult fish are known to jump out of tanks containing ENU to avoid exposure. Adding anesthetic to the mutagenesis solution helps to keep fish calm. At some doses, ENU is lethal to adult fish, and therefore, the concentrations must be kept low. However, the toxicity to adult fish can be overcome by repeated low-dose exposures, increasing mutagenesis while maintaining survival. Some of the dangers to humans from using ENU can be overcome by inactivating the mutagen with high pH and sodium thiosulfate (Westerfield, 1993).

A second useful mutagen is an ionizing radiation which induces double-stranded DNA breaks. Double-stranded breaks promote many different types of DNA changes, such as small or large deletions, as well as chromosome rearrangements. These various changes can create useful versions of genes, known as alleles. For example, a deletion can completely remove an entire gene or several entire genes for that matter. If an entire gene is deleted, it is known as a null allele, or a version, in which, the gene is completely dead. Null alleles can lead to a complete understanding of a gene compared to a point mutation in which residual function can remain, known as a hypomorph. A complete description of different classes of alleles is presented in the characterization section below. The primary negative of irradiation as a mutagen is that, often, only a few lethal mutants are isolated; perhaps, because they are not transmitted through the germ line effectively (Mullins et al., 1994; Nusslein-Volhard & Dahm, 2002).

Insertional mutagenesis is an effective method of introducing DNA changes into the zebrafish genome. Importantly, integrating foreign DNA sequences to disrupt genes fosters the rapid isolation of the DNA that was changed because such sequences act as "molecular flag" which can be easily located in the genome to determine the gene that was interrupted by the integrated foreign DNA. Moreover, experimentally useful sequences can be inserted, such as fluorescent tags to label cells or gene products or *loxP* sites which allow the removal of the causative DNA change (Clark et al., 2011). These sorts of reversion experiments address temporal questions about when a gene is required during development (Talbot et al., 2016). The downside of insertional mutagenesis is that there may be some sequence specificity, such that, the hotspots for integration may have increased insertion rates as opposed to uniform mutagenesis across the genome. In addition, the rate of insertional mutagenesis is lower than ENU mutagenesis.

A less-efficient way of obtaining mutants is by relying on spontaneous mutation. Indeed, the first described zebrafish mutant, *golden*, was a spontaneous mutation propagated in the pet industry as a favorite of fish hobbyists. Other spontaneous mutants from the pet trade include the *leopard* mutant, as well as *longfin* that has spotted and longfin phenotypes, respectively. While an inefficient way to generate mutants, interesting spontaneous mutant phenotypes can yield insight into the biology in zebrafish, just as in mouse, where the many "fancy mice" strains have been kept for centuries because of their appealing phenotype to hobbyists. Spontaneous mutations continue to occur in laboratory strains, and through careful inbreeding and husbandry techniques (Brooks & Nichols, 2017), these "fancy fish" mutants can easily be isolated when they appear.

The final method that I wish to discuss does not concern a specific mutagen, but rather the background to which mutagenesis is applied. Secondary screens are a method in which the mutagen is applied to a zebrafish line already harboring a known mutation, further altering the phenotype. Some second mutations will enhance, while some will suppress the original mutant phenotype, revealing information about gene logic and circuitry. Successful secondary screens in invertebrate models demonstrate their utility in gleaning information about genetic pathways. But, thus far, there have been few secondary screens in zebrafish. This is likely because most of the characterized zebrafish mutants are lethal as homozygotes and display no phenotypes as heterozygotes. One way to circumvent this restriction is to mutagenize a homozygous viable mutant, or a heterozygote that is sensitive to further mutation as done for a *chordin* mutant (Kramer et al., 2002). A second clever approach to perform a secondary screen is to generate a stable transgenic rescue line allowing the uncovering of the suppressors of an otherwise lethal mutant (Bai et al., 2010).

Recovery

After mutagenesis, the fish are screened to recover those with genetic mutations producing altered phenotypes. There are several different breeding strategies with advantages and disadvantages. Often, recovery strategy depends on the type of mutagenesis and what kinds of phenotypes the researcher is searching for. All recovery methods capitalize on the ability to readily breed zebrafish and propagate their genomes transgenerationally, either through natural crosses or laboratory manipulations.

The traditional method for recovering mutant families is through inbreeding. In the familial inbreeding method, the first step is to cross mutagenized animals, which are mosaic for mutations in their germline, to wild-type animals, a process referred to as outcrossing. These mosaic adults are the parental generation or P_0. The offspring of the P_0 will produce nonmosaic carriers

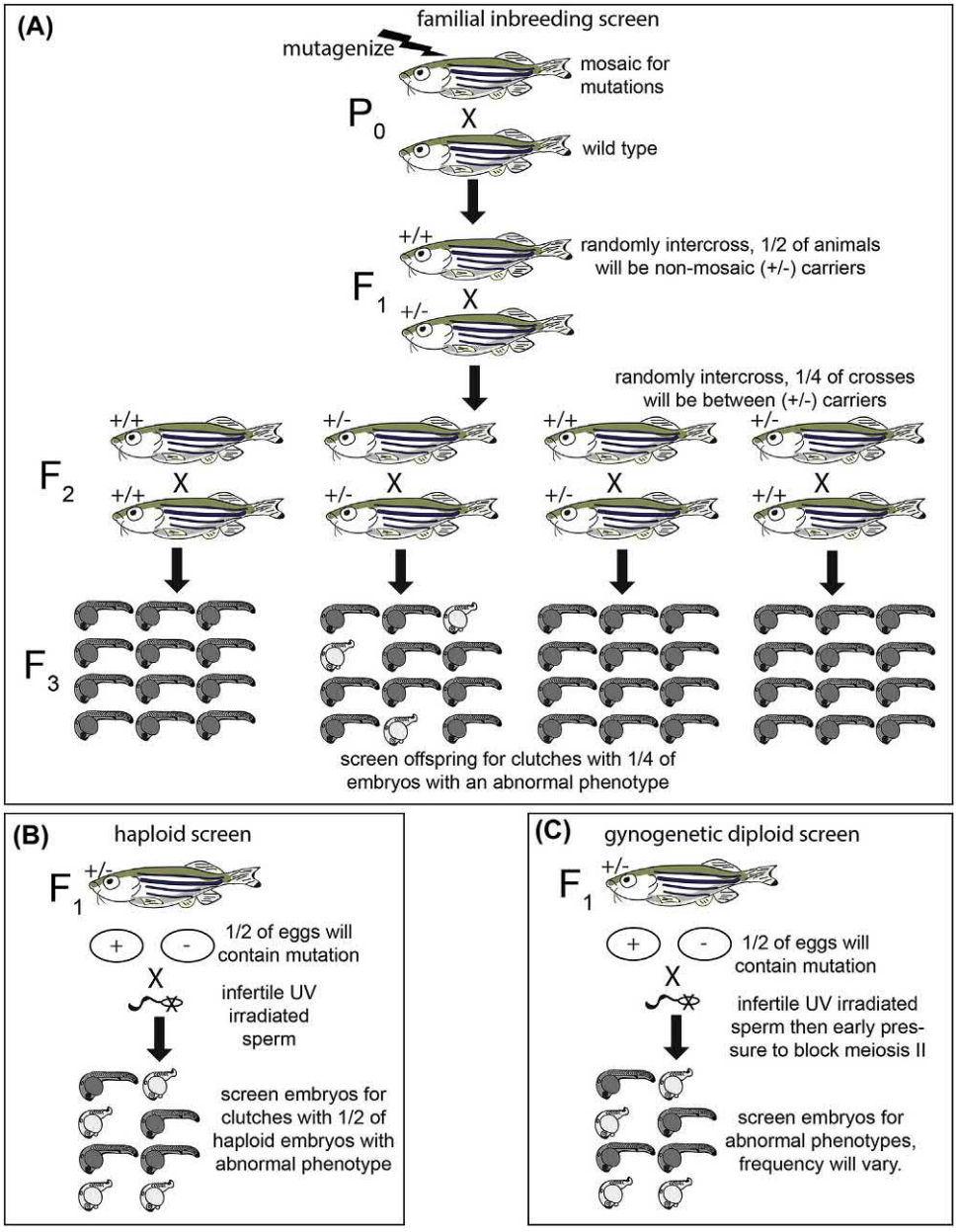

FIGURE 50.2 **Mutant recovery.** Careful breeding strategies are used to recover zebrafish mutants in a genetic screen. (A) Familial inbreeding requires more time and space than other methods because several generations of breeding are required, but it is not technically demanding and can identify phenotypes that manifest at all stages. (B) Haploid screening requires fewer generations, but it is only useful for screening early developmental stages and can be confounded by phenotypes that develop due to hemizygosity rather than a genetic lesion. (C) Gynogenetic diploid screening requires less time and space than familial inbreeding but is technically demanding.

of the mutation and are called the *first filial generation* or F_1. Randomly intercrossing animals from the F_1 produces the F_2 generation, which is then intercrossed again and their offspring, the F_3 generation, are screened for phenotypes. One-quarter of the F_2 intercrosses will be between animals heterozygous for the same newly created mutation, and one-quarter of the F_3 offspring from these crosses will be homozygous for the new mutations (Fig. 50.2A) (Haffter et al., 1996; Nusslein-Volhard & Dahm, 2002).

The family inbreeding method described above is time-consuming because of the requirement for several generations. Moreover, familial inbreeding requires substantial housing, heavily taxing facility space. Therefore, methods were developed to allow researchers to skip generations and screen families more rapidly. The simplest method to fast-forward a screen is to analyze haploid progeny. In this method, eggs from F1 females are fertilized with "dead" sperm that has had its genome destroyed by UV irradiation. The pseudo-fertilized egg

will undergo somewhat normal development for several days, and half of these eggs will carry the new mutation and can thus present mutant phenotypes (Fig. 50.2B) (Westerfield, 1993). Haploid animals do not develop completely normally, so haploid offspring to be screened must be thoroughly scrutinized to determine if the phenotype is due to a mutation or the nonspecific problems that come from developing with only one copy of the genome instead of the usual two copies. Moreover, because the embryos will not develop past a few days, any phenotypes that arise beyond the early larval stage cannot be screened in haploid animals. To circumvent some of the problems associated with analyzing haploid individuals, the pseudo-fertilized eggs can be treated with either hydrostatic pressure or heat shock to block the second meiotic division, or the first mitotic division respectively (Fig. 50.2C), making them diploid because they now carry two copies of the genome; however, both copies are identical and come from the mother. These "gynogenetic diploids," as well as haploid offspring are called *parthenogenetic*, meaning that they derive from an unfertilized egg from a single female parent. While parthenogenetic screens save time and space, they are technically more challenging.

Sometimes a special screening strategy is required, dictated by the type of phenotype that is hunted. For example, screening for maternal-effect mutants, that is, phenotypes in offspring that result from a mutation in the mother requires an extra generation because the phenotype manifests in the offspring of the homozygous mutant animals. That is, the F_3 homozygotes must be grown to adulthood to screen their F_4 offspring for phenotypes (Dosch et al., 2004; Wagner, Dosch, Mintzer, Wiemelt, & Mullins, 2004).

Phenotyping

Determining how the mutant phenotype differs from the wild type is a critical aspect of a genetic screen. A logical way to categorize phenotypes is by the developmental stage at which they manifest. Major lifestyle transitions like the onset of feeding and reproduction are rational ways to divide the zebrafish lifecycle and useful for dividing and categorizing mutant phenotypes based on when they appear (Fig. 50.3). By far, the largest class of characterized mutants are those that develop their phenotype during the embryonic and larval stage, appearing before the onset of feeding. Some mutants develop normally past the feeding stage, but manifest phenotypes in the late larva, juvenile or adult; these include many pigmentations and fin phenotypes and those that alter the larval to juvenile metamorphosis. The third class of mutants does not have obvious phenotypes, but are sterile because of defects in their germline (Fig. 50.3).

Finding a clean mutant phenotype requires an effective way to visualize and document the change in appearance. There are many ways of observing and phenotyping zebrafish mutants. The simplest method is observing the live, unstained organism with the naked eye for obvious phenotypes like those in the fancy fish trade (Fig. 50.3). Live unstained organisms can also be observed with microscopy-based methods, which reveal much more detail allowing a more comprehensive assessment of any mutant phenotypes. It is worth noting that the developing embryos are simply beautiful under the microscope, and phenotypes can be striking. Some phenotypes cannot be observed without first staining the animal. There are various methods for labeling cells with histological stains revealing anatomical features and tissues that cannot otherwise be seen. Molecular changes can also be observed when searching for phenotypes. For example, changes in gene expression patterns can be visualized with the RNA in situ hybridization technique, and proteins can be labeled with antibodies for visual and biochemical analysis. The advent of transgenes allows a dynamic look at phenotypes where traits like cell behavior and temporal changes in gene expression through development can be recorded from live animals. Sensory mutants are unresponsive to stimulation like touch and sound, and behavioral tests can assay cognitive ability.

Careful phenotyping is important, and sometimes very subtle phenotypes can be found that will later turn out to be very interesting. Subtle phenotypes can later become more striking through selective breeding to make the phenotype more severe, or by recovering another more severe allele (Brooks & Nichols, 2017; Miller et al., 2007a). To harness the power of a genetic screen in order to determine gene function, the researcher must first start with a good clean phenotype. A robust phenotype is the biggest advantage of a forward screen, and great care should be dedicated to phenotyping.

Genotyping

Genotyping is the determination of what DNA alteration is responsible for the altered phenotype. Often when screens are focused on specific tissues, many mutants will have similar phenotypes. The first step to genotyping these mutants is to determine if any of the mutants are disrupted in the same gene. A simple "complementation test" achieves this. Two mutants are said to complement each other if their hybrid offspring do not have the phenotype that is present within each

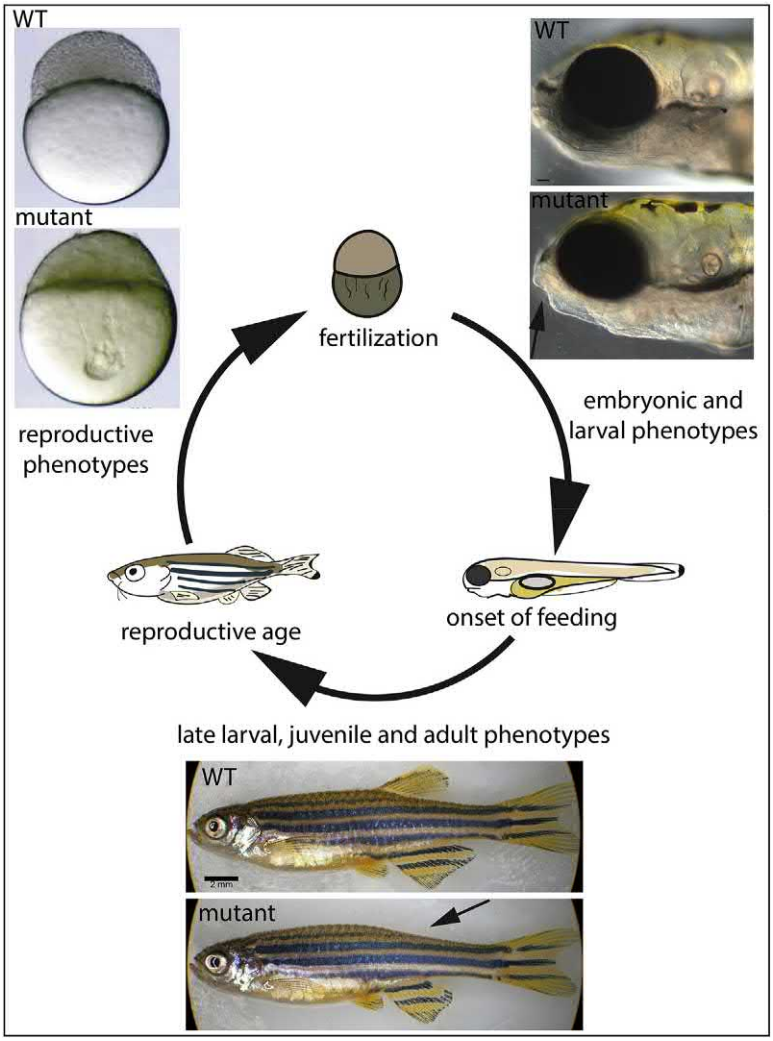

FIGURE 50.3 **Phenotyping.** Genetic screens can identify phenotypes that manifest at different life stages. Wild-type phenotypes and examples of mutant phenotypes observable in live unstained animals are illustrated. *Reproductive phenotypes images reproduced from Dosch, R., et al. (2004). Maternal control of vertebrate development before the midblastula transition: Mutants from the zebrafish I.* Developmental Cell 6, *771–780 and embryonic and larval phenotypes images from Nichols, J. T., Pan, L., Moens, C. B., Kimmel, C. B. (2013). barx1 represses joints and promotes cartilage in the craniofacial skeleton.* Development *140, 2765–2775 doi:10.1242/dev.090639.*

mutant line. Therefore, simply outcrossing mutant lines to each other and observing the mutant phenotype informs the researcher that the same gene is disrupted in the two lines. This is known as failure to complement and allows the researcher to place mutants into complementation groups that likely, all are mutated in the same gene (Fig. 50.4). While, generally, very useful, this rule is not infallible as there can be nonallelic failure to complement when two different mutagenized genes can present a phenotype as "trans heterozygotes" and technically fail to complement. However, these cases are rare.

To determine the precise DNA change in mutants, researchers take advantage of the fact that the DNA that the fish pass to the offspring are different than their own because their germ cells "mix-up" their genomes when they divide during meiosis. When this mix-up, called *recombination*, occurs, any given two parts of the genome are more or less likely to stick together depending on how close together they are on the DNA strands. If they stick together, they are said to be linked. Genes that are very close together are rarely broken up and are said to be tightly linked. Because we know the location of many genomic landmarks, linkage analyses can give rough information about where in the genome the mutation is located. That is, by looking for genomic landmarks that are inherited in the next generation alongside the mutation, the researcher can narrow down, or map, the location of the mutation. Once a region or interval of the genome that contains the mutation is known, candidates in the area can be sequenced and, hopefully, a lesion can be discovered that

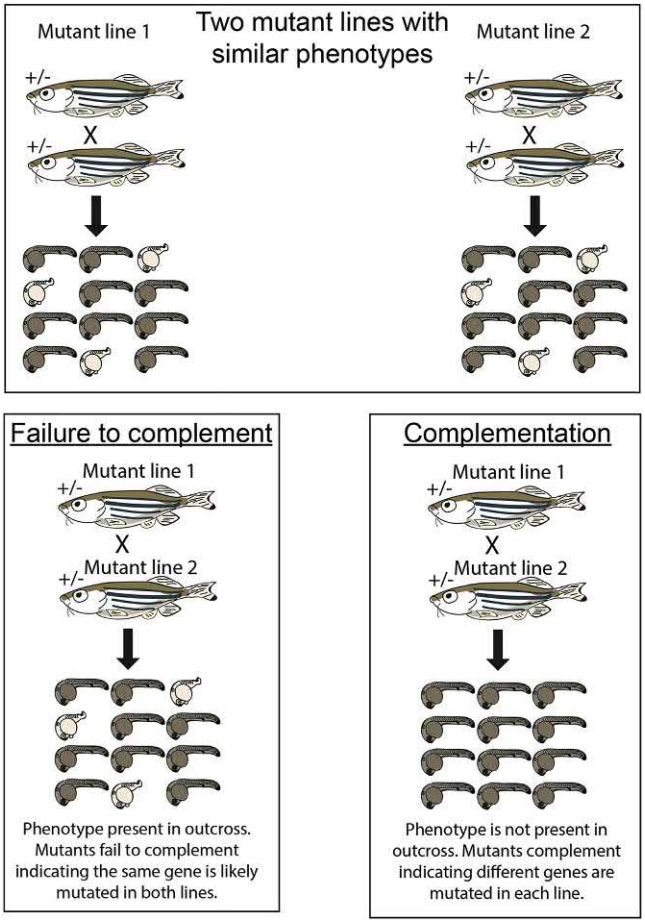

FIGURE 50.4 Complementation. A complementation test is an easy way to determine if two mutant lines with similar phenotypes harbor mutations in the same gene. If crossing two mutant lines to each other yield phenotypically mutant offspring, the mutants "fail to complement" and are likely caused by mutations in the same gene. If crossing the two mutant lines yields only wild-type offspring, the mutants "complement" each other, suggesting different genes are mutated in each line.

segregates with the mutant phenotype. Sophisticated methods of tracking genomic landmarks continue to be developed including sequencing all the genes in an animal or even the entire genome (Bowen, Henke, Siegfried, Warman, & Harris, 2012; Miller et al., 2007b, 2013; Obholzer et al., 2012; Stickney et al., 2002).

Validation

Once a candidate mutation has isolated the causality of the mutation needs to be demonstrated. There are several methods of validation that are often used in concert. Multiple noncomplementing alleles with mutations in the same gene are perhaps the most convincing evidence the mutations are causative, but complementation is only useful for homozygous recessive alleles. A common feature of many mutants is loss of the mRNA that the candidate gene encodes because mutated gene products that are not translated into protein properly are targeted for destruction through a cell-safety mechanism to remove mutated gene products called *nonsense-mediated decay*. If a candidate mutated gene has lower levels of mRNA in mutants, this can be evidence that the mutation is causative. Similarly, if the protein product of the candidate gene is somehow changed, either quantity or size or localization, further evidence has been obtained. However, this protein work requires a way to detect the protein, like an antibody, which unfortunately is not available for many gene products. Another convincing validation is to rescue the mutation by adding back either wild-type DNA sequences or mRNA from the suspected mutated gene. Finally, injecting an antisense morpholino (Stainier et al., 2017) which blocks either translation or splicing of mRNA gene products can phenocopy, or produce the same phenotype as the mutant, bolstering the case that a genetic lesion is causative. It is worth mentioning that morpholino phenotypes in isolation, that is, without an accompanying mutant, must be carefully considered as the field has come to realize that morpholino induced phenotypes often do not match those of the genetic mutant and can be misleading (Stainier et al., 2017). Nevertheless, they can provide supplementary evidence that a mutation is causative when mutant and morpholino phenotypes match. Researchers, to convincingly validate that a mutation is causative, should aim to satisfy several of the above validation methods in order to build a complete case that can be trusted.

Characterization

The specific version of the gene that is mutated is called the allele. Alleles are assigned a letter code to designate the institution that generated the genomic feature, followed by a unique identifying number. For example, alleles generated the University of Colorado are denoted with co followed by a series of digits that are only assigned to a singular specific DNA sequence. For more nomenclature information, see The Zebrafish Information Network (ZFIN) nomenclature page: https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines. The traditional way to characterize mutant alleles is by the rules of Muller's Morphs (Muller, 1932). According to this system, mutants come in four types or morphs. (1) Amorphs that are a complete loss of function or null. (2) Hypomorphs are a partial or weak loss of function. (3) Hypermorphs increase normal function and (3) Antimorphs oppose normal gene activity. Antimorphs are sometimes called *dominant negatives*, but antimorphs can also be recessive

(Nichols et al., 2016; Sijacic, Wang, & Liu, 2011). There are established rules for interpreting what type of morph a mutant is, but they often rely on comparing multiple alleles within a single complementation group, which is not always possible.

Up until this point, we have focused on mutations that occur within genes, coding mutations. But vast swaths of the genome that do not code for mRNA or protein are also important, and it is likely many unidentified mutants are in noncoding regions that act as regulatory regions. There are few noncoding mutants described in zebrafish (Schauerte et al., 1998; Sepich, Wegner, O'Shea, & Westerfield, 1998) because it can be difficult to assign noncoding genomic changes to a particular gene. As we learn more about gene regulation and noncoding DNA, it is likely more mutants in noncoding DNA will materialize in the zebrafish literature.

Concluding Remarks

One outstanding question regarding zebrafish genetic screens is, are zebrafish screens saturated? A screen is said to be saturated if all the genes involved in a biological process are found. If no new mutations come from the genetic screen, only different alleles of the same genes, the screen is approaching saturation. Interestingly, with the rapid generation of CRISPR/Cas9 based reverse genetic mutants, we are learning that many single-gene mutants have no overt phenotype but when multiple family members are mutagenized a phenotype becomes apparent (Askary et al., 2017; Barske et al., 2016). These results suggest that even as screens approach saturation, we will still not know all the genes that are involved in a process because of genetic redundancy, or multiple genes doing the same job. These results motivate designing secondary screens in CRISPR/Cas9 reverse genetic backgrounds lacking overt phenotypes. This approach is predicted to uncover new complementation groups that might not be found by mutagenizing wild types.

Amazing advancements in genome sequencing technologies have provided the full "ingredients list" for the DNA recipe book that guides development in many organisms, including zebrafish. But much work remains, the list of ingredients is not enough, and the challenge now is to understand how the ingredients work in concert to build a complex organism. That is, we need to learn about gene function. Staring with a clean mutant phenotype and working to find what genetic change causes that phenotype remains one of the most powerful ways to determine gene function. Clearly, there is still an important place in zebrafish biomedical research for unbiased forward genetic screens.

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CHAPTER

51

Zebrafish as a Platform for Drug Screening

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Introduction

Since the first demonstrated use of live zebrafish for chemical screening in 96-well format in 2000 (Peterson, Link, Dowling, & Schreiber, 2000), zebrafish chemical screens have expanded significantly to encompass a wide range of fish models, targeted pathways and phenotypic readouts (Rennekamp & Peterson, 2015). In the context of chemical screening, the zebrafish offers unique advantages, including rapid development, high fecundity, and small size, and optical transparency at the larval stage. These attributes allow high-throughput screening with larvae in 96- or 384-well plate format, and imaging-based readouts (Rennekamp & Peterson, 2015). In this chapter, we review chemical screening in the zebrafish model organism; we begin with a statistical overview of zebrafish chemical screens conducted since 2000, followed by more detailed discussions that address specific screens based on their areas of focus. We conclude by highlighting a few screens with significant clinical implications and bring to attention some of the current limitations and novel approaches toward addressing these limitations for future studies.

Zebrafish Chemical Screening, a Statistical Overview

A survey of the existing literature between 2000 and June 2017 (Ncbi Resource Coordinators, 2017) uncovered 114 studies involving zebrafish chemical screening; while we highlight a number of these studies in this chapter, we do not claim this list to be exhaustive and apologize for any omissions made. Of the 114 studies identified, 56 focus on specific tissues to identify modulators of organogenesis or rescuers of tissue-specific pathologies (Fig. 51.1A and D) (Alvarez et al., 2009; Buckley et al., 2010; Chiu, Cunningham, Raible, Rubel, & Ou, 2008; Crawford et al., 2011; de Vrieze et al., 2015; Fleming, Sato, & Goldsmith, 2005; Hirose, Simon, & Ou, 2011; Huang, Lindgren, Wu, Liu, & Lin, 2012; Kitambi, McCulloch, Peterson, & Malicki, 2009; Lam et al., 2012; Lake, Tusheva, Graham, & Heuckeroth, 2013; Liang et al., 2015; Oppedal & Goldsmith, 2010; Papakyriakou et al., 2014; Sun, Dong, Khodabakhsh, Chatterjee, & Guo, 2012; Tran et al., 2007; Wang et al., 2010) (Asimaki et al., 2014; Choi et al., 2013; Coffin, Williamson, Mamiya, Raible, & Rubel, 2013; Gallardo et al., 2015; Kannan & Vincent, 2012; Leet et al., 2014; Liu et al., 2014; Milan, Peterson, Ruskin, Peterson, & MacRae, 2003; Namdaran, Reinhart, Owens, Raible, & Rubel, 2012; Ni et al., 2011; North et al., 2007; Ou et al., 2009; Owens et al., 2008; Peal et al., 2011; Shimizu et al., 2015; Tang, Xie, & Feng, 2015; Thomas et al., 2015; Vlasits, Simon, Raible, Rubel, & Owens, 2012; Yozzo, Isales, Raftery, & Volz, 2013; Yang et al., 2015), (Arulmozhivarman et al., 2016; Astin et al., 2014; Cao et al., 2009; Colanesi et al., 2012; de Groh et al., 2010; Garnaas et al., 2012; Hultman, Scott, & Johnson, 2008; Kawahara et al., 2011, 2014; Ko et al., 2016; Shafizadeh, Peterson, & Lin, 2004; Tamplin et al., 2015; Waugh et al., 2014; Westhoff et al., 2013; White et al., 2011; Yeh et al., 2009; Yeh & Munson, 2010; Yin, Evason, Maher, & Stainier, 2012; Zhen et al., 2013). Twelve studies use zebrafish in metabolic screens (Fig. 51.1A,B). The emergence of behaviors, such as eye movement, light response, and food seeking within the first week of development has enabled high-throughput behavioral screening in zebrafish larvae (Kalueff et al., 2013), and 10 such studies were identified by our search (Fig. 51.1B). Several screens have also been designed to target the inflammatory response (Hall et al., 2014; Liu et al., 2013; Robertson et al., 2014; Wang et al., 2014; Wittmann et al., 2015; Ye et al., 2017), toxin metabolism (Dimri et al., 2015; Jin et al., 2013; Legler et al., 2011; Nath et al., 2013; North et al., 2010; Padilla et al., 2012), and a diverse set of signaling pathways (Fig. 51.1A and C) (Gebruers et al., 2013; Hao et al., 2013; Le et al., 2013; Molina et al., 2009; Molina, Watkins, & Tsang, 2007;

The Zebrafish in Biomedical Research
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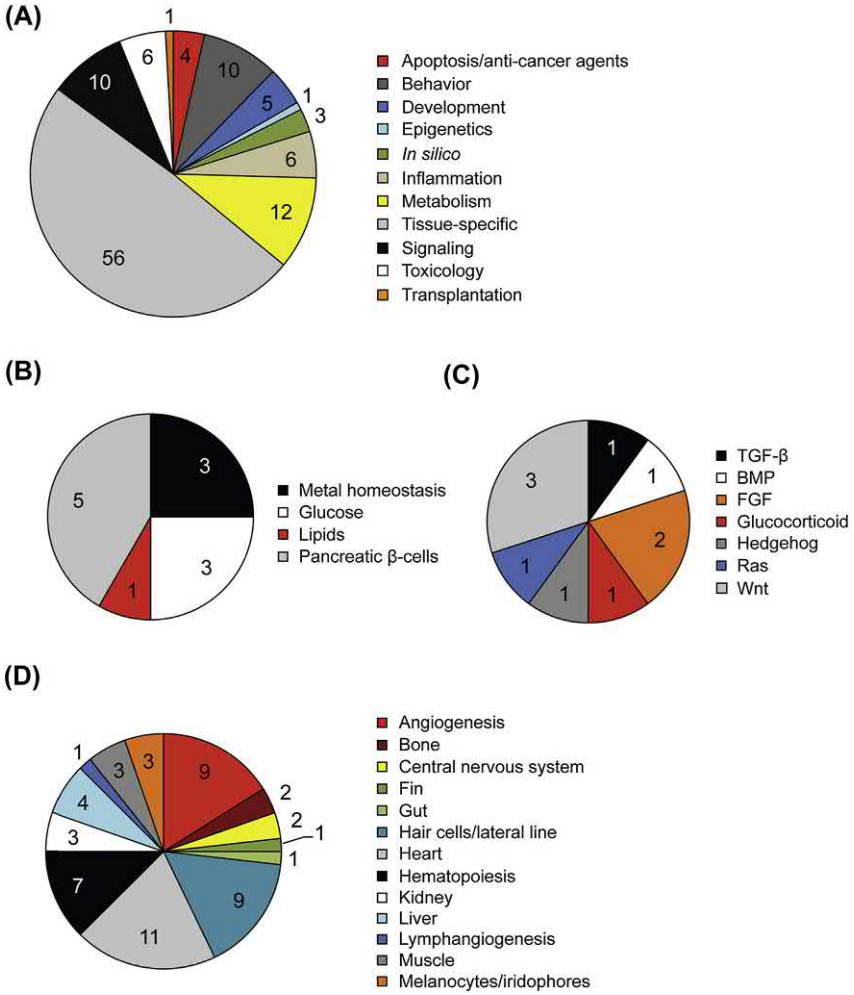


FIGURE 51.1 17 years of zebrafish chemical screening. (A) Zebrafish chemical screens by category, 2000–2017. (B) Metabolism-based zebrafish chemical screens. (C) Zebrafish chemical screens focusing on signaling pathways. (D) Tissue-specific zebrafish chemical screens.

Robertson et al., 2014; Torregroza, Evans, & Das, 2009; Weger, Weger, Nusser, Brenner-Weiss, & Dickmeis, 2012; Williams et al., 2015; Yu et al, 2008a, 2008b).

Fifty five studies rely on wildtype zebrafish (Astin et al., 2014; Bouwmeester et al., 2016; Chiu et al., 2008; Coffin et al., 2013; Colanesi et al., 2012; Challal et al., 2014; Das, McCartin, Liu, Peterson, & Evans, 2010; de Groh et al., 2010; Fleming et al., 2005; Garnaas et al., 2012; Hirose et al., 2011; Kannan & Vincent, 2012; Kokel et al., 2010; Laggner et al., 2012; Lake et al., 2013; Li, Huang, Huang, Du, & Huang, 2012; Long et al., 2014; Lu et al., 2013; Maximino et al., 2014; Mendelsohn et al., 2006; Milan et al., 2003; Morash et al., 2011; Nath et al., 2015; North et al., 2007; Oppedal & Goldsmith, 2010; Ou et al., 2009; Owens et al., 2008; Peterson et al., 2000; Rahn et al., 2014; Rennekamp et al., 2016; Rihel et al., 2010; Sandoval et al., 2013; Sun et al., 2012; Tamplin et al., 2015; Tang et al., 2015; Thomas et al., 2015; Thorsteinson et al., 2009; Vlasits et al., 2012; Wang et al., 2014; White et al., 2011; Zhen et al., 2013), 50 use transgenic lines (Alvarez et al., 2009; Andersson et al., 2012; Arulmozhivarman et al., 2016; Asimaki et al., 2014; Buckley et al., 2010; Choi et al., 2013; Crawford et al., 2011; de Vrieze et al., 2015; Gallardo et al., 2015; Gut et al., 2013; Gutierrez et al., 2014; Hall et al., 2014; Huang et al., 2012; Jimenez et al., 2016; Kitambi et al., 2009; Ko et al., 2016; Lam et al., 2012; Le et al., 2013; Leet et al., 2014; Li et al., 2015; Li et al., 2016; Li, Page-McCaw, & Chen, 2016; Liang et al., 2015; Liu et al., 2013; Liu et al., 2014; Molina et al., 2007; Molina et al., 2009; Namdaran et al., 2012; Ni et al., 2011; North et al., 2010; Papakyriakou et al., 2014; Raftery, Isales, Yozzo, & Volz, 2014; Robertson et al., 2014; Ridges et al., 2012; Rovira et al., 2011; Shafizadeh et al., 2004; Tran et al., 2007; Tsuji et al., 2014; Wang et al., 2010; Wang et al., 2014; Wang et al., 2015; Weger et al., 2012; Westhoff et al., 2013; Wittmann et al., 2015; Ye et al., 2017; Yeh & Munson, 2010; Yeh et al., 2009; Yin et al., 2012; Yozzo et al., 2013), and nine (Baraban, Dinday,

& Hortopan, 2013; Cao et al., 2009; Hultman et al., 2008; Kawahara et al., 2011; Kawahara et al., 2014; Paik, de Jong, Pugach, Opara, & Zon, 2010; Peal et al., 2011; Shimizu et al., 2015; Waugh et al., 2014) use mutant models (Fig. 51.2A), with the majority of studies focusing on larvae before eight days-post-fertilization (dpf), and four studies on adult zebrafish (Fig. 51.2B). (de Vrieze et al., 2015; Li et al., 2015; Maximino et al., 2014; Oppedal & Goldsmith, 2010). The majority of transgenic zebrafish encode fluorescent proteins (with GFP and its variants being the most common) for cell type-specific visualization: nine out of the nine angiogenesis screens use *fli1*- or *flik1*-driven reporter lines that fluorescently label the entire vasculature (Alvarez et al., 2009; Crawford et al., 2011; Huang et al., 2012; Kitambi et al., 2009; Lam et al., 2012; Liang et al., 2015; Papakyriakou et al., 2014; Tran et al., 2007; Wang et al., 2010); and five out of the six inflammation screens use neutrophil-specific reporter lines with *lyz*- or *mpx*-driven fluorescence (Hall et al., 2014; Liu et al., 2013; Robertson et al., 2014; Ye et al., 2017; Wang et al., 2014). Three studies rely on luminescence (luciferase)-based reporters (Asimaki et al., 2014; de Vrieze et al., 2015; Weger et al., 2012). Five studies use zebrafish carrying human oncogenes or additional human mutations of interest (Asimaki et al., 2014; Gutierrez et al., 2014; Le et al., 2013; Yeh & Munson, 2010; Yeh et al., 2009), and two studies use nitroreductase-expressing systems for tissue-specific ablation (Andersson et al., 2012; Ko et al., 2016), with or without additional fluorescent or luminescent reporters to facilitate tissue visualization.

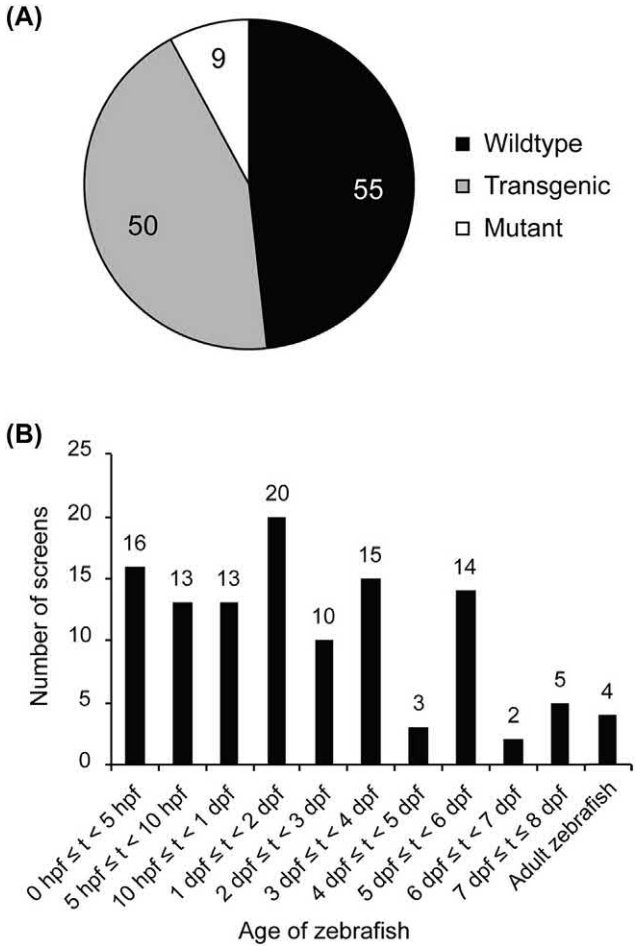
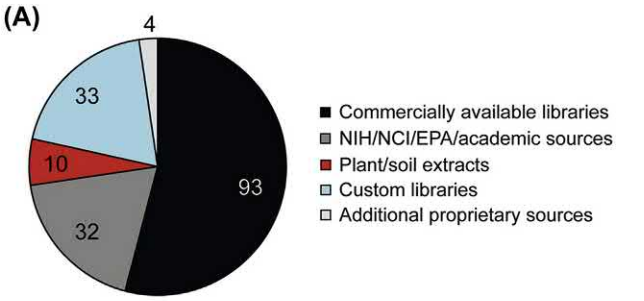


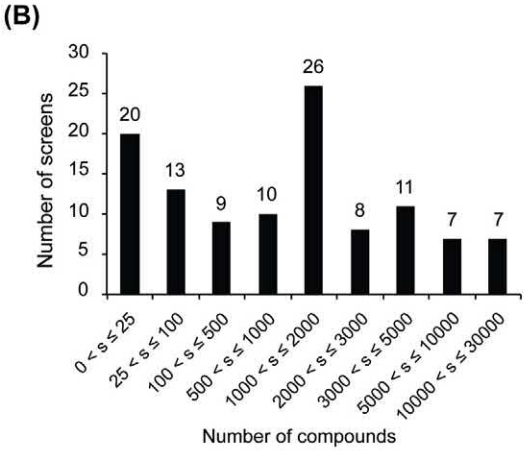
FIGURE 51.2 Zebrafish in chemical screening. (A) Types of zebrafish models for chemical screening, 2000–2017. (B) Age distribution of zebrafish screened. Studies with unclear or undesignated fish age were excluded; a few studies included screens in multiple age groups. t = age of zebrafish.

All nine mutant zebrafish lines used in screening were identified from forward genetic screens (Baraban et al., 2013; Cao et al., 2009; Hultman et al., 2008; Kawahara et al., 2011; Kawahara et al., 2014; Paik et al., 2010; Peal et al., 2011; Shimizu et al., 2015; Waugh et al., 2014). While forward genetics has contributed significantly toward the availability of zebrafish mutants for chemical screening, recent advancements in genome editing technologies, such as TALENS (Hwang, Peterson, & Yeh, 2014; Zu et al., 2013) and CRISPR-Cas9 (Gagnon et al., 2014; Liu et al., 2017) in zebrafish point toward a likely rise in targeted model generation, and pave the way for future chemical screens in new models.

Approximately half of the 114 screens use commercially available libraries, while another 20% rely on academic and government sources (Fig. 51.3A). Approximately 6% of screens are performed with extracts from plants, fungi or soil rather than purified compounds (Fig. 51.3A). 1000–2000 is the most widely used compound range, with the largest screens falling within the 10,000–30,000 range (Fig. 51.3B). A number of studies were conducted with a relatively small number of compounds (<25), and we have included these in our data as they often serve as proof-of-concept studies toward the execution of larger screens (Fig. 51.3B).

The top 10 most frequently used screening libraries are listed in Fig. 51.3C, and include both commercial sources and collections from the NIH and EPA. The top three most commonly used libraries contain FDA-approved drugs, natural products, and additional pharmacologically relevant compounds, providing a wide range of structures for chemical screening (Rennekamp & Peterson, 2015). More category-based libraries, such as the Enzo SCREEN-WELL Nuclear Receptor Ligands library, could be selected for screens targeting specific pathways. It is also worth noting that many screening libraries have been formatted to include control wells and be compatible with robotic liquid handling systems, thus allowing direct transfer of contents into a working plate for screening.





(C)

Library name	Number of screens
Prestwick Chemical Library (Prestwick Chemical)®	16
The Spectrum Collection (MicroSource Discovery Systems)	15
LOPAC®1280 (Millipore Sigma)	11
SCREEN-WELL® ICCB Known Bioactives library (Enzo)	9
NINDS Custom Collection 2	8
DIVERSet® Libraries (ChemBridge)	8
SCREEN-WELL® FDA Approved Drug Library (Enzo)	6
NIH Clinical Collection (1 and/or 2)	4
EPA ToxCast™ Phase-I Library	3
SCREEN-WELL® Nuclear Receptor ligand library (Enzo)	3

FIGURE 51.3 Chemical screening libraries. (A) Compound libraries by category, 2000–2017. (B) Distribution of number of compounds tested in screens; a number of studies employed multiple libraries, studies with ambiguous or undesignated compound source were excluded. s = number of compounds used in screen. (C) Top 10 most frequently used compound libraries.

Zebrafish Chemical Screens by Category

Zebrafish chemical screens have evolved over the past 17 years to encompass a wide range of biological processes (Fig. 51.1A). (Rennekamp & Peterson, 2015) The vast majority of these studies are phenotype-driven rather than target-driven, where hit compounds are identified based on downstream biological responses in the live, vertebrate zebrafish; this approach confers several advantages, such as the ability to identify hits even in the absence of known target or mechanism of action, and the inclusion of multiple targets that could boost the efficiency of drug discovery (Rennekamp & Peterson, 2015). While the lack of target information could prove detrimental toward compound optimization for therapeutic development, correlation of compound-induced phenotypes with those of established mutant lines may aid in elucidation of the mechanism of action (Mendelsohn et al., 2006; Sandoval et al., 2013). Additional biochemical and analytical approaches, such as affinity-based pull-down, crosslinking, and proteomics could also shed light on the pathways perturbed by the compound of interest (Rennekamp & Peterson, 2015).

We highlight the examples of zebrafish chemical screens in the following sections.

Metabolic Screens

Implementation of zebrafish in metabolic studies is aided by conservation of key nodes of metabolic regulation across zebrafish and mammals (Keatinge et al., 2015; Peal et al., 2011; Seth, Stemple, & Barroso, 2013). While the poikilothermic nature and still undiscovered existence of brown adipose tissue in zebrafish limit the study of thermogenesis in this organism, the preservation of key aspects of appetite regulation, lipid storage and insulin-sensitive tissues, has contributed to a wealth of studies in these areas (Schlegel & Gut, 2015; Seth et al., 2013). The zebrafish pancreas, compartmentalized into endocrine and exocrine portions, is one of the most extensively studied organs in the context of metabolism, which is also reflected in the focus of the majority of zebrafish metabolic screens on pancreatic β-cells (Fig. 51.1B) (Seth et al., 2013; Schlegel & Gut, 2015).

Zebrafish metabolic screens employ a noteworthy set of fluorescent reporters. The Tg(*tp1*:hmgb1-mCherry); Tg(*pax6b*:GFP) line was used in one of the earliest zebrafish β-cell screens (Rovira et al., 2011). This doubly transgenic system, consisting of mCherry-labeled Notch-responsive cells and GFP-labeled endocrine cells, enabled screening for inducers of secondary islet formation while also monitoring for potential adverse effects on Notch signaling (Rovira et al., 2011). This approach identified six candidate compounds, with mechanisms of action, including inhibition of GTP production and retinoic acid biosynthesis (Rovira et al., 2011). Another labeling technology implemented in zebrafish screens is the fluorescent ubiquitination-based cell cycle indicator (FUCCI), which uses dual fluorescent probes of oscillating cell cycle regulatory proteins to track phase-specific proliferation (Zielke & Edgar, 2015). Zebrafish larvae expressing mAG-zGeminin (fluorescent $S/G_2/M$ phase marker) under the *insulin* promoter has been used to screen for enhancers of β-cell proliferation (Tsuji et al., 2014). Importantly, it is also possible to incorporate additional dietary manipulations into transgenic larvae

prior to chemical screening; Li et al. established an overnutrition model in β-cell-specific transgenic zebrafish by feeding the larvae chicken egg yolk to induce compensatory β-cell proliferation; screening in this model identified FGF1 signaling as a key component of the overnutrition-induced β-cell proliferation response (Li et al., 2016).

Additional transgenic models for metabolic screening include the nitroreductase system (Andersson et al., 2012), and a transgenic reporter for gluconeogenesis (Gut et al., 2013). In the Tg(*ins*:CFP-NTR); Tg(*ins*:Kaede) line, *insulin* promoter drives expression of nitroreductase and Kaede fluorescent protein, allowing β-cell-specific ablation by metronidazole, and screening for enhancers of β-cell regeneration via increased Kaede fluorescence (Andersson et al., 2012). This approach identified the small molecule NECA as a promoter of β-cell regeneration through the adenosine receptor A2aa, an effect that was replicated in mice (Andersson et al., 2012). The transgenic zebrafish Tg(*pck1*:Luc2) expresses luciferase under the promoter of the fasting-inducible gluconeogenesis gene *pck1*; chemical screening in this model identified two compounds that raised *pck1* expression while lowering glucose, one of which improved the metabolic parameters in diet-induced obese mice (Gut et al., 2013).

As zebrafish chemical screens become fine-tuned to address increasingly complex biological questions, a similar development has taken place in high-throughput technologies for the examination of downstream phenotypic outputs. As an example, a 2016 screen conducted by Wang et al. to identify modulators of β-cell mass automated the entire screening process through automated reporter quantification in vivo (ARQiv), which included a microplate reader setup for high-throughput fluorescence quantification in live larvae, real-time data analysis, and robotic liquid handling and sorting systems for larva and compound transfer (Wang et al., 2015). Additional bioenergetic profiling methods, such as colorimetric detection of acid secretion from individual larva (Makky, Duvnjak, Pramanik, Ramchandran, & Mayer, 2008), and measurement of larval oxygen consumption rate via flux analyzer (Stackley, Beeson, Rahn, & Chan, 2011; Kumar et al., 2016), have been achieved for either 96- or additional multiwell formats. Fluorescence-based glucose detection kit has been used in a zebrafish chemical screen for modulators of glucose level (Nath et al., 2015), and the rise in omics-based technologies, such as high-throughput metabolomics supports a widening in the repertoire of measurable endpoints to include metabolic profiles in addition to imaging-based readouts (Li et al., 2016; Wang et al., 2017).

Efforts have also been directed toward optimization of fluorescent chemical probes to track metabolite uptake to complement the rich set of transgenic zebrafish models for metabolic screening. Two of the probes optimized for zebrafish larvae are the BOPIDY-labeled C5 fatty acid analog PED-6 (Clifton et al., 2010) and the fluorescent glucose analog 2-NBDG (Lee et al., 2013). Incubation with compounds followed by PED-6 addition in a chemical screen identified inhibitors of intestinal lipid absorption with potential pharmacological applications in cardiovascular disease (Clifton et al., 2010). 2-NBDG is taken up by zebrafish larvae in a time-dependent manner as visualized by increased fluorescence in the GLUT receptor-rich eye (Lee et al., 2013); this model was screened against selected compounds and plant-based fractions, leading to discovery of a triazine-based compound as a potential insulin-mimetic (Lee et al., 2013).

Additional zebrafish metabolic screens have focused on metal homeostasis (Li et al., 2016; Mendelsohn et al., 2006; Sandoval et al., 2013). Iron homeostasis in vertebrates is regulated by the hepcidin/ferroportin axis, defects in which cause iron deficiency or overload (Li et al., 2016). A recent zebrafish screen identified three steroids that lowered ferroportin level by increasing hepcidin biosynthesis, an effect that was recapitulated in mice and humans (Li et al., 2016). A screen for modulators of copper homeostasis identified the copper chelator neocuproine, which caused progressive appearance of phenotypes from pigmentation loss to wavy notochord and enlarged brain ventricle with increasing treatment time or dosage, supporting a role for copper metabolism in notochord development; a forward genetic screen identified the mutant *calamity* that phenocopied these copper deficiency-induced defects (Mendelsohn et al., 2006). Importantly, a separate chemical screen conducted several years later revealed that treatment of zebrafish larvae with kalihinol F, a diterpenoid originally isolated from a marine sponge, resulted in *calamity*-like phenotypes, and led to the discovery of kalihinol F's mechanism of action as a copper chelator (Sandoval et al., 2013). These studies demonstrate the ability to identify novel biological pathways and mechanisms of action for tested compounds through a combination of chemical and forward genetic screens.

Behavioral Screens

Zebrafish larvae have proven to be highly amenable to behavioral screening due to small size and early emergence of screenable behaviors (Kalueff et al., 2013). Within the first week of development, zebrafish larvae display a range of environment-triggered behaviors, such as eye tracking, threat responses, and food seeking (Kalueff et al., 2013). Zebrafish between 30 and 42 hpf demonstrate a reproducible pattern of mobile and immobile behaviors in response to pulses of light, a phenomenon termed the photomotor response (PMR)

(Kokel & Peterson, 2011). While the purpose of the PRM is undefined, the reproducibility, detectability, and scalability associated with this behavior have resulted in its inclusion in several screens (Kokel & Peterson, 2011). A chemical screen in seven dpf larvae was conducted by Rennekamp et al. to identify modifiers of freezing behavior in response to a strobe light, yielding three candidate classes of compounds that shifted freezing to escape (Rennekamp et al., 2016). In vitro binding and CRISPR-Cas9 knockout studies identified σ_1 receptor binding as the mechanism of action for the finazine class of compounds, and impaired freezing was also detected in a finazine-treated mouse fear model (Rennekamp et al., 2016). These findings implicate novel pathways underlying the decision between passive and active threat responses and suggests potential therapeutic targets for psychiatric disorders, in which, these responses are dysregulated (Rennekamp et al., 2016).

While the study of behavior in zebrafish may still be considered as an emerging field, past and recent findings of the ability of zebrafish to develop addiction (Bossé & Peterson, 2017), respond to psychotropic drugs, and navigate behavioral assays support conservation of key aspects of neurocircuitry between zebrafish and mammalian models (Kalueff, Stewart, & Gerlai, 2014). Treatment of zebrafish larvae with the common convulsant and $GABA_A$ antagonist pentylenetetrazol (PTZ) leads to behavioral and biochemical changes consistent with rodent seizure models (Baraban, Taylor, Castro, & Baier, 2005). Chemical screens in the zebrafish PTZ model have yielded several compounds, including vitamin K_3 derivatives (Rahn et al., 2014), and plant and marine natural products (Challal et al., 2014; Long et al., 2014) as potential anticonvulsants. A zebrafish model of epilepsy was discovered in a chemical mutagenesis screen (Schoonheim, Arrenberg, Del Bene, & Baier, 2010), and carries a mutation in the sodium-gated ion channel $Na_v1.1$ (*scn1Lab)* (Baraban et al., 2013). Small molecule screening in *scn1Lab* zebrafish identified clemizole as a seizure suppressant (Baraban et al., 2013), and paved the way for testing of the compound lorcaserin in a clinical trial (Griffin et al., 2017).

Given the multidimensionality of zebrafish behaviors in response to characterized and uncharacterized compounds, behavior barcoding has emerged as a method for behavior categorization and target prediction (Rennekamp & Peterson, 2015). By monitoring larval behaviors in a chemical screen, and performing hierarchical clustering of the recorded behavioral barcode corresponding to each tested compound, it was shown that compounds with similar biological targets often fall into the same phenocluster, thus making it possible to predict targets for uncharacterized compounds clustering with those with known modes of action (Kokel et al., 2010; Rihel et al., 2010). This approach was adapted by Rihel et al. (2010) and Kokel et al. (2010) toward sleep/wake and PMR behavioral data in response to psychotropic compounds. Hierarchical clustering yielded phenoclusters of compounds with similar known targets and identified previously unknown protein functions and novel small molecule modulators of known pathways (Kokel et al., 2010; Rihel et al., 2010). Clustering analysis has since been adapted toward additional screens with various behavioral outputs and compound types (Maximino et al., 2014; Wang et al., 2014). In a significant expansion of previous behavioral readouts, Bruni et al. tested 10 acoustic and/or visual stimulus-based behaviors against 14 antipsychotic, antidepressant or anxiolytic drugs, with data clustering demonstrating class-specific effects (Bruni et al., 2016). The behavioral signature from the antipsychotic haloperidol was further screened against 24,760 compounds, identifying a number of hits with haloperidol-like phenotypic profiles and in vitro receptor binding properties; the compound finazine reduced locomotion in a psychostimulant-induced schizophrenia mouse model (Bruni et al., 2016). This last study is worth noting for its demonstration of the resolution of complex zebrafish behavior barcodes in distinguishing different psychotropic drugs and recalling compounds with potentially shared mechanisms of action; this approach could prove useful toward the identification of novel psychotropics as therapeutics.

Selected Tissue-Specific Screens

56 out of the 114 zebrafish chemical screens focus on specific tissues and rely heavily on fluorescence or luminescence-based reporters for visualization following compound treatment. We highlight screens on heart, lateral line, and tissue-specific disease models in the following sections.

Heart

The zebrafish heart is a two-chambered organ consisting of a single atrium and ventricle (Asnani & Peterson, 2014). Electrophysiological studies demonstrate close resemblance in the shape of zebrafish ventricle action potentials to those of humans, with a conserved plateau phase that is not recapitulated in mice (Nemtsas, Wettwer, Christ, Weidinger, & Ravens, 2010; Vornanen & Hassinen, 2016). Compounds that are known to induce repolarization defects in humans also consistently cause bradycardia in zebrafish (Milan et al., 2003). In the context of chemical screening, the zebrafish heart confers several advantages, including small size, fairly rapid development, and optical transparency that allows high-throughput imaging and quantification of cardiac parameters (Asnani & Peterson, 2014; Rennekamp & Peterson, 2015). Zebrafish larvae are able to oxygenate

through diffusion alone during early development, and adult zebrafish heart regenerates following partial ventricle amputation (Zhao et al., 2014); these unique qualities allow regeneration studies and modeling of severe cardiomyopathy phenotypes that would otherwise be embryonically lethal (Asnani & Peterson, 2014).

Cardiovascular screens in the zebrafish employ a number of disease models (Asimaki et al., 2014; Peal et al., 2011; Shimizu et al., 2015). The zebrafish Kcnh2 mutant (*breakdance*) faithfully reproduces key aspects of human genetic long QT syndrome, a life-threatening disorder of prolonged cardiac repolarization caused predominantly by failed membrane trafficking of KCHN2 ion channel proteins (Milan et al., 2003; Peal et al., 2011). A small-molecule screen in this model identified flurandrenolide, a steroid that suppressed prolonged cardiac repolarization by acting through the glucocorticoid receptor, a mechanism distinct from trafficking rescue of defective ion channels (Peal et al., 2011). A zebrafish model of arrhythmogenic cardiomyopathy (ACM) bearing a known human mutation demonstrated progressive cardiomyopathy mirroring human pathologies (Asimaki et al., 2014). Combination of ACM zebrafish with the cardiomyocyte stress reporter line Tg(*nppb*: luciferase) allowed chemical screening for suppressors of ACM phenotypes and identified a compound that normalized ACM-associated action potential defects in mammalian and zebrafish myocytes (Asimaki et al., 2014). A zebrafish model for cardiac fibrillation, *tremblor*, exhibited uncoordinated heart contractions due to a defect in calcium extrusion (Shimizu et al., 2015); a chemical screening of *tremblor* larvae identified the small molecule efsevin, which bind to the mitochondrial channel protein VDAC2 and increase mitochondrial calcium uptake (Shimizu et al., 2015).

As mentioned previously, zebrafish have the remarkable capability of regenerating a number of tissues following injury, including portions of fins, heart, and brain (Gemberling et al., 2013); partial heart ventricle resection in adult zebrafish activates signaling cascades that drive formation of new cardiac wall typically by 30 days postinjury (Gemberling et al., 2013). While the non-high-throughput nature of heart surgery and size of adult zebrafish present challenges for chemical screening in adult models of heart injury, heart-specific transgenic larvae have been used in screens for modulators of cardiomyocyte proliferation with the potential to alter pathways involved in heart regeneration (Choi et al., 2013). Choi et al. used FUCCI to distinguish populations of proliferating and nonproliferating cardiomyocytes in developing larvae (Choi et al., 2013); combination of a larval chemical screen and adult heart-injury studies revealed involvement of hedgehog, insulin-like growth factor and transforming growth factor β signaling in cardiomyocyte proliferation postinjury (Choi et al., 2013). In a separate study, cardiomyocyte-specific transgenic larvae (Tg(*cmlc2*: EGFP)) were screened to identify the small molecules cardionogen-1, -2 and -3, which caused an enlarged heart phenotype via expansion of cardiac progenitor cells (Ni et al., 2011).

In the clinical setting, doxorubicin is a potent chemotherapeutic with a broad application but restricted use due to acute and/or delayed cardiotoxicity (Liu et al., 2014). Chemical screening in a zebrafish model of doxorubicin-induced cardiotoxicity identified the small molecules visnagin and diphenylurea, which rescued doxorubicin-induced cardiotoxicity in zebrafish and mice; visnagin bound to mitochondrial malate dehydrogenase (MDH2), and several MDH2 inhibitors exhibited similar cardioprotective effects, supporting visnagin and modulators of MDH2 activity as potential options for treating doxorubicin-induced cardiotoxicty in patients (Liu et al., 2014).

For carrying out further cardiac function-focused screens, there exists a need for imaging technologies with adequate throughput and speed for monitoring cardiac parameters in real time; this has been addressed in several studies that employ automated imaging systems to quantify body length, circulation, pericardial area, heart rate and/or intersegmental vessel area in live, fluorescent larvae (Asnani & Peterson, 2014; Liu et al., 2014; Lin, Chang, Lai, & Liau, 2014). Notably, Lin et al. used confocal laser-scanning microscopy to generate time-lapse images of live zebrafish heart at different depths, from which, a pseudodynamic 3D reconstruction of the cardiac cycle could be achieved (Lin et al., 2014). Availability of disease models and continued improvements in live imaging are likely to pave the way for additional heart-specific screens (Asnani & Peterson, 2014).

Lateral Line

The zebrafish lateral line is a sensory system on the surface of the fish that allows detection of changes in water flow and contributes to predator/prey sensing, shoaling, and mating behaviors (Chitnis, Nogare, & Matsuda, 2012; ZFIN). The lateral line is comprised of neuromasts, clusters of mechanosensory hair cells anatomically similar to those found in the human inner ear (Owens et al., 2008; ZFIN). Given the implication of hair cell loss in deafness and balance disorders, and challenge of accessing the inner ear in mammals (Ou, Santos, Raible, Simon, & Rubel, 2010), several zebrafish chemical screens have been designed with a focus on ototoxicity (Chiu et al., 2008; Coffin et al., 2013; Hirose et al., 2011; Namdaran et al., 2012; Ou et al., 2009; Ou et al., 2010; Ou, Simon, Rubel, & Raible, 2012; Ou et al., 2012; Owens et al., 2008; Thomas et al., 2015; Vlasits et al., 2012); an additional screen identifies inhibitors

of posterior lateral line primordium migration during development (Gallardo et al., 2015).

Ototoxicity-based screens fall into two categories: identification of toxic agents (Chiu et al., 2008; Coffin et al., 2013; Hirose et al., 2011) and identification of modulators of hair cell response to known ototoxins (Namdaran et al., 2012; Ou et al., 2009; Owens et al., 2008; Thomas et al., 2015; Vlasits et al., 2012). The vital DNA dye YO-PRO-1 is often used to fluorescently label hair cell nuclei (Coffin et al., 2010). A high-throughput ototoxicity screen was conducted in 2008 by Chiu et al., using wildtype larvae and 1040 compounds from NINDS Custom Collection 2 (Chiu et al., 2008). This screen identified seven known and 14 previously unknown ototoxins; two of the unknown compounds were further examined to show ototoxic effects in mouse utricle explant cultures (Chiu et al., 2008). Another ototoxicity screen was performed with 88 anticancer compounds from the NCI Approved Oncology Drugs Set, identifying four out of five known, four out of seven suspected, and five potentially novel ototoxins (Hirose et al., 2011). Additional ototoxicity-based screens identified modulators of hair cell response to known ototoxins, such as the aminoglycosides neomycin, gentamicin, and kanamycin, and the anticancer drug, cisplatin (Namdaran et al., 2012; Ou et al., 2009; Owens et al., 2008; Thomas et al., 2015; Vlasits et al., 2012). While some hit compounds reduce hair cell death by blocking ototoxin uptake (Thomas et al., 2015; Vlasits et al., 2012), others function via additional mechanisms, such as stimulating hair cell precursor division (Namdaran et al., 2012).

One screen focused on the posterior lateral line primordium (pLLP), a cluster of around a 100 cells, a large portion of which migrates posteriorly under the skin and periodically deposits neuromasts along the trunk of the developing larva (Chitnis et al., 2012). In a chemical screen with the pLLP reporter line Tg(*cldnb*:EGFP) (Haas & Gilmour, 2006), Gallardo et al. identified 165 inhibitors of pLLP migration (Gallardo et al., 2015). Three of the hit compounds were Src inhibitors, and CRISPR-Cas9 targeting of *src* or its downstream target *tks5* inhibited or delayed pLLP migration in injected larvae (Gallardo et al., 2015). The Src inhibitor SU6656 also exhibited antimetastatic activity in a mouse tumor model (Gallardo et al., 2015). These findings taken together, suggest that high-throughput screening in zebrafish, complemented by genome-editing technology and orthogonal secondary assays, could facilitate identification of compounds with high therapeutic potential.

Tissue-Specific Disease Models

Several screens were conducted with focus on specific diseases. Duchenne muscular dystrophy (DMD) is an X-linked neuromuscular disorder due to loss-of-function mutations in the protein dystrophin; patients experience progressive muscle weakening and often early death from cardiac and/or respiratory failure (Waugh et al., 2014). A dystrophin-null zebrafish DMD model, *sapje*, was identified from the large-scale 1996 Tuebingen mutagenesis screen by its reduced muscle birefringence (Guyon et al., 2007; Granato et al., 1996), while a similar model, *sapje-like*, was generated by early-pressure screen (Guyon et al., 2009); both models lack dystrophin expression and exhibit muscle degeneration within a few days of development (Guyon et al., 2007; Guyon et al., 2009).

In a chemical screen in *sapje* and *sapje-like* larvae with birefringence as readout, Kawahara et al. identified seven compounds that decreased the proportion of affected fish without altering dystrophin expression (Kawahara et al., 2011). In particular, prolonged treatment with the phosphodiesterase (PDE) inhibitor aminophylline normalized muscle structure as observed at 30 dpf (Kawahara et al., 2011). Protein kinase A (PKA) activity was upregulated in treated fish, suggesting that the cAMP-dependent PKA axis is likely involved in a compound activity (Kawahara et al., 2011). Several additional candidates were identified in subsequent screens, including modulators of heme oxygenase signaling (Kawahara et al., 2014) and the serotonin pathway (Waugh et al., 2014); the PDE5 inhibitor sildenafil was efficacious in a mouse DMD model (Adamo et al., 2010).

Two zebrafish models for polycystic kidney disease (PKD), $pkd2^{hi4166}$ and $ift172^{hi2211}$, were identified from mutagenesis screens. Known association between PKD and body curvature/left-right symmetry defects in zebrafish larvae enabled screening of PKD modulators with body curvature and laterality as readout (Cao et al., 2009; Sun et al., 2004). Chemical screening with $pkd2^{hi4166}$ and $ift172^{hi2211}$ embryos identified trichostatin A, a pan-HDAC inhibitor that rescued body curvature defects in $pkd2^{hi4166}$ larvae; excessive body curvature was also suppressed by *hdac1* knockdown or the class I-specific HDAC inhibitor valproic acid (VPA), and VPA slowed disease progression in a mouse PKD model (Cao et al., 2009).

One study focused on Hirschsprung's disease, a common birth defect characterized by failure of nerve cells to colonize the distal bowel (aganglionosis), leading to growth failure, intestinal blockage and life-threatening infections (Hirschsprung Disease; Lake et al., 2013). Lake et al. identified nine inhibitors of enteric nervous system development in a chemical screen with wildtype zebrafish larvae (Lake et al., 2013). Among these compounds, the immunosuppression drug mycophenolate caused aganglionosis in zebrafish and mice via GTP depletion from proliferating enteric neural crest-derived cells (Lake et al., 2013). Given partial penetrance and overall unclear etiology associated with this disease, the authors' findings hint at additional factors, such as

medication and perturbed nucleotide homeostasis as potential contributors toward disease phenotype (Lake et al., 2013).

Clinical Implications

Several screens have led to additional investigations toward therapeutic development. Dorsomorphin is a bone morphogenic protein (BMP) signaling inhibitor identified in a zebrafish chemical screen, where it triggered dorsalization of the developing embryo (Yu et al, 2008a, 2008b). Follow-up studies revealed dorsomorphin inhibited type I BMP receptors, leading to reduced transcription of BMP-responsive genes, such as the iron transport regulator hepcidin (Yu et al, 2008a, 2008b). Dysregulated BMP signaling is implicated in a number of diseases, including anemia of chronic inflammation (AI) and fibrodysplasia ossificans progressiva (FOP), in which progressive ossification of muscle and connective tissues leads to restrained movement and early mortality (Rennekamp & Peterson, 2015). A dorsomorphin derivative alleviated disease symptoms in mouse models of AI (Steinbicker et al., 2011) and FOP (Yu et al, 2008a, 2008b), and derivatives of dorsomorphin are being investigated as potential FOP therapeutics under NIH's Therapeutics for Rare and Neglected Diseases (TRND) program (Bone Morphogenetic Protein).

A screen in wildtype zebrafish larvae for modulators of the hematopoietic stem cell (HSC) pool identified a role for prostaglandin agonists and antagonists in increasing and decreasing HSC number, respectively (North et al., 2007). PGE_2 synthesis was required for HSC formation, and pre-ex vivo treatment of donor mouse whole bone marrow with a long-acting PGE_2 derivative significantly increased HSC repopulation frequency in recipient mice (North et al., 2007). Results from this study have contributed to the testing of ProHema (Fate Therapeutics) in human patients to assess its effect on neutrophil engraftment from blood cord hematopoietic cell transplantation (HCT) (Rennekamp & Peterson, 2015).

In addition to these findings, the ability to model oncogene-driven leukemia (Yeh et al., 2009) and perform HCT (Li et al., 2015) in zebrafish provides valuable tools in the study of transplantation biology and hematologic malignancies.

More recently, chemical screening in a zebrafish epilepsy model was directly translated into a clinical trial (Griffin et al., 2017). Mutations in *SCN1A* are the primary monogenic cause of Dravet syndrome, pediatric epilepsy with impaired development and severe seizures (Baraban et al., 2013; Griffin et al., 2017). Chemical screening in *scn1Lab* zebrafish identified the antihistamine clemizole as a seizure suppressant (Baraban et al., 2013). Clemizole exhibited a binding affinity for serotonin receptors, and several additional modulators of serotonin signaling also suppressed seizure events in zebrafish (Griffin et al., 2017). These findings led to a clinical trial with the FDA-approved serotonin agonist lorcaserin in five Dravet syndrome patients, with initial results demonstrating a reduction in seizure severity and/or frequency (Griffin et al., 2017).

Chemical screens in zebrafish models of doxorubicin cardiotoxicity (Liu et al., 2014) and long QT syndrome (Peal et al., 2011) have also yielded candidate compounds with promising therapeutic potential. Structural activity relationship studies are currently underway for visnagin. Dexamethasone, an approved drug structurally similar to the long QT rescue candidate flurandrenolide, is under investigation for its potential effects on long QT syndrome (Rennekamp & Peterson, 2015). While testing and approval of new compounds require significant time (Lo B), drug repositioning, the process of screening, either experimentally or computationally, previously approved drugs to identify additional therapeutic targets, could improve speed along the drug discovery pipeline given availability of data, such as mechanism of action, bioavailability, and toxicity (Ashburn & Thor, 2004; Brown & Patel, 2016; Lotfi Shahreza, Ghadiri, Mousavi, Varshosaz, & Green, 2017).

Limitations and Potential Solutions

While small larval size, high fecundity, and optic transparency of zebrafish confer distinct advantages in the realm of chemical screening, there also exist limitations unique to this model organism (Rennekamp & Peterson, 2015). Approximately 70% of human genes have at least one ortholog in zebrafish, with over 3100 genes having at least two zebrafish orthologs, a likely consequence of teleost-specific genome duplication (Howe et al., 2013; Vornanen & Hassinen, 2016). While this overlap supports conservation of the associated biological pathways, duplicates arising from genomic duplication could undergo loss and/or change of a function over time, thus creating additional downstream variability (Howe et al., 2013). The human and zebrafish genomes also contain a few thousand genes with no mutual orthologs, thereby placing some restrictions over a model generation; notable examples include the absence of identifiable zebrafish orthologs for human *IL6* and *BRCA1* (Howe et al., 2013).

Evolutionary divergence of the teleost lineage from other vertebrates has also resulted in significant physiological differences. As an aquatic organism, the zebrafish does not possess lungs, and therefore, would not be suitable for modeling pulmonary diseases. The poikilothermic nature of zebrafish hinders the study of thermogenesis, and the existence of brown adipose tissue in this

organism remains unexplored (Seth et al., 2013). Additional differences from mammals include location of hematopoietic tissue, and organization of pancreatic cells and hepatocytes (Menke, Spitsbergen, Wolterbeek, & Woutersen, 2011), though similarities in the types and functions of these cells have enabled studies of metabolic disorders and hematopoiesis (Rennekamp & Peterson, 2015; Seth et al., 2013).

In addition to differences in biology, chemical screening in zebrafish faces its unique technical challenges. The aquatic nature of zebrafish necessitates compound addition into an aqueous buffer, thus limiting testing of compounds with high hydrophobicity; when possible, synthesis of water-soluble analogs could improve solubility, though at the risk of potentially altering compound activity (Dang et a., 2016). In place of water or aqueous buffer, organic solvents, such as dimethylsulfoxide and alcohols, could be used as carrier solvents, though only low concentrations (typically averaging at around 1% volume/volume) are tolerable by live larvae (Maes et al., 2012). When available, partition coefficients (logP) could be used as a guide in the selection of compounds with a greater chance of solubility in the zebrafish environment (Savjani, Gajjar, & Savjani, 2012).

Another issue is compound uptake, which could vary depending on the developmental stage due to differences in the maturity of the gills, mouth, and/or skin (Dang et al., 2016). Zebrafish larvae hatch sporadically, typically on the third day of development, with most larvae hatched by the end of the fourth day (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995); this difference in hatching rate even within the same clutch could introduce inconsistencies in compound absorption, as the chorion may in some cases be a physical barrier to compound entry (Zhang, Qin, Zhang, & Hu, 2015). While manual and mechanical chorion removal is time-intensive, chemical methods, such as pronase have routinely been used on large numbers of embryos without lowering survival; an automated approach, combining pronase treatment with automated mechanical agitation followed by dechorionated larva placement into well plates, has also been developed (Mandrell et al., 2012).

As has been encountered in most mammalian models, increased size and opacity of adult zebrafish hinders high-throughput screening (Rennekamp & Peterson, 2015). While rapid development of zebrafish enables the study of many key aspects of behavior, metabolism, and organogenesis at the larval stage (Kimmel et al., 1995; Rennekamp & Peterson, 2015), it is difficult to integrate a high-throughput screening platform into aging, adult tissue regeneration, and some behavioral assays (Rennekamp & Peterson, 2015). Despite the unfeasibility of a 96-well format, it still remains possible to screen adult fish using smaller, custom compound collections, as was demonstrated in a study by Maximino et al., in which a panel of anxiolytic and anxiogenic drugs was screened in adult zebrafish to identify modifiers of anxiety-like behavior (Maximino et al., 2014). Generation of the highly optically transparent *Casper* zebrafish, which lack melanocytes and iridophores in all stages of life, has enabled imaging-based readout in adult fish (Li et al., 2015; Rennekamp & Peterson, 2015; White et al., 2008). As more complex behavioral assays, such as opioid self-administration (Bossé & Peterson, 2017) and maze learning (Roberts, Bill, & Glanzman, 2013) become optimized for adult zebrafish, it is likely that behavior-based chemical screens will also expand alongside these novel setups. The availability of automated, motion-tracking instruments and software, such as ZebraBox (ViewPoint Behavior Technology) and ActualTrack (Actual Analytics) have also facilitated behavior monitoring in both multiwell and single-fish formats.

Given the frequent use of fluorescent zebrafish reporter lines and imaging-based readouts, it is crucial for some screens that larval orientation remains consistent across all wells (Rennekamp & Peterson, 2015). Following swim bladder inflation around five dpf (Kimmel et al., 1995), zebrafish larvae typically take on a dorsal-ventral orientation when viewed from top, while larvae prior to swim bladder inflation tend to orient more laterally if undisturbed; differences in timeframe of swim bladder inflation, coupled with emergence of movement-related behaviors within the first few days of development, can lead to inconsistent larval orientation, with use of anesthesia not a guarantee for consistent orientation in all larvae.

Two major approaches toward improving larval positioning include modifications of the screening plate and design of automated systems (Rennekamp & Peterson, 2015). Rovira et al., in their screen for enhancers of β-cell differentiation, designed a 96-well SideView plate in which prisms adjacent to wells refract excitation and emission light, allowing consistent lateral visualization of fluorescent larvae (Rovira et al., 2011). A second approach by Wittbrodt et al. used a desktop 3D printer to create orientation tools that could be used as molds for generating cavities in agarose-filled multiwells, enabling positioning and imaging of larvae in either dorsal-ventral or lateral view in the resulting plate (Wittbrodt, Liebel, & Gehrig, 2014). An automated, capillary-based larva orientation and imaging system (VAST BioImager Platform) has been designed by Union Biometrica based on technology from the Yanik research group (Chang, Pardo-Martin, Allalou, Wahlby, & Yanik, 2012; Pardo-Martin et al., 2010; Pardo-Martin et al., 2013; Union Biometrica); a single larva is loaded and positioned in a capillary until reaching the desired view, the resulting instrument parameters can then be

rapidly replicated in each additional larva until all larvae are imaged (Union Biometrica). As previously mentioned, Wang et al., in their screen for modulators of β-cell mass, fully automated the screening process from larva transfer to data analysis, relying on the COPAS-XL system (Union Biometrica) for dispensing of larvae into individual wells of a 96-well plate (Wang et al., 2015). While the manual transfer of larva and compound has traditionally been one of the major bottlenecks of zebrafish chemical screens, it is likely solvable through advancements in automation.

While whole-organism phenotypic screens are more adept than cell-based approaches at capturing system-level responses and more suited toward discovery of novel drug targets than target-based methods, their limitation lies in the challenge of target identification (Hart, 2005). As the availability of target information is crucial for compound optimization, a number of target discovery methodologies have been explored. Two major approaches are direct methods, such as affinity chromatography and microarrays that examine compound-target interactions, and indirect methods that compare compound-induced biological changes against those associated with known compounds or chemical/genetic modulations (Hart, 2005). Affinity chromatography identified an interaction between visnagin and mitochondrial malate dehydrogenase (Liu et al., 2014), while comparison of kalihinol F-induced larval phenotypes against those of the established *calamity* model revealed kalihinol F's role as a copper chelator (Sandoval et al., 2013); however, it is worth noting that both of these approaches also carry limitations, such as challenge of maintaining compound activity in the former, and model availability in the latter (Hart, 2005). Ultimately, the choice of target identification methods will depend on compound structure, model organism, and availability of additional reference data.

Conclusion

In this book chapter, we provide an overview of zebrafish chemical screening over the past 17 years, highlighting key examples in metabolism, behavior, and organogenesis. Our literature search (Ncbi Resource Coordinators, 2017) yielded 114 examples of zebrafish chemical screens, approximately half of which were tissue-specific, focusing on modulators of organogenesis or rescuers of tissue-specific pathologies. The next largest categories were metabolism, signaling, and behavior, with additional screens covering a wide range of topics, such as inflammation, toxicology, and epigenetics. 55 studies use wildtype zebrafish, 50 use transgenic lines and nine use genetic mutants, with the majority of studies focusing on larvae before eight dpf, and four studies using adult zebrafish. The top three most frequently selected compound libraries were Prestwick Chemical Library, Spectrum Collection and Sigma LOPAC^{1280}.

While the optical transparency, high fecundity, rapid development and small size of the larval zebrafish confer clear advantages in the realm of high-throughput chemical screening, we also note limitations, including imperfectly conserved biology, challenge of target identification, difficulty of high-throughput screening in adult fish, compound insolubility, undefined absorption profile, and inconsistent larval orientation (Rennekamp & Peterson, 2015). However, continued improvements in automation suggest that some of the aforementioned limitations are likely to be solvable issues in future screens.

We also bring to attention chemical screens with significant clinical impact. While the rise in number of zebrafish screens in recent years supports a corresponding increase in the number of compounds with therapeutic potential, the fairly large span (10 to 15 years) (Lo B) between hit identification and drug approval suggests that additional time is needed before one could fully evaluate the impact of zebrafish screening in the realm of approved drugs and make comparisons with other drug discovery approaches.

As zebrafish chemical screening looks toward the future, we conclude the current chapter by proposing the following as potential rising trends in this evolving field:

(1) Optimization of more complex behavioral assays in adult zebrafish is likely to promote development of behavior-based chemical screens capable of accommodating these novel setups as well as older fish.
(2) Improved efficiency and availability of genome-editing technologies have contributed to an increase in targeted disease model generation in zebrafish, which could translate into a rise in disease-focused screening in these models.
(3) Advancements in high-throughput quantification of biological parameters (i.e., metabolomics) could broaden the range of phenotypic readouts.
(4) Improved automation in all aspects of the zebrafish screening platform is likely to significantly reduce experiment time and lead to an overall increase in the number of future screens.

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The Zebrafish in Biomedical Research

Biology, Husbandry, Diseases, and Research Applications

EDITED BY:
Samuel C. Cartner, Judith S. Eisen, Susan C. Farmer, Karen J. Guillemin, Michael L. Kent, and George E. Sanders

The Zebrafish in Biomedical Research: Biology, Husbandry, Diseases, and Research Applications is a comprehensive work that fulfills a critical need for a thorough compilation of information on this species. The text provides significant updates for working vivarium professionals maintaining zebrafish colonies, veterinarians responsible for their care and well-being, zoologists and ethologists studying the species, and investigators using the species to gain critical insights into human physiology and disease. As the zebrafish has become an important model organism for the study of vertebrate development and disease, organ function, behavior, toxicology, cancer, and drug discovery, this book presents an important resource for future research.

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