

Sex Control in Aquaculture

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Volume I

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WILEY Blackwell

This edition first published 2019
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Registered Office(s)

John Wiley & Sons, Inc., 111 River Street, Hoboken, NJ 07030, USA

John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK

Editorial Office

The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK

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Cataloging-in-Publication Data

Names: Wang, Han-Ping, 1958– editor. | Piferrer, Francesc, 1960– editor. | Chen, Songlin, 1960 – editor.

Title: Sex control in aquaculture / edited by Han-Ping Wang, Francesc Piferrer, and Song-Lin Chen.

Description: Hoboken, NJ : John Wiley & Sons, 2018. | Includes bibliographical references and index. |

Identifiers: LCCN 2018007574 (print) | LCCN 2018014556 (ebook) | ISBN 9781119127284 (pdf) |

ISBN 9781119127277 (epub) | ISBN 9781119127260 (cloth)

Subjects: LCSH: Aquaculture—Management. | Brood stock assessment.

Classification: LCC SH155.5 (ebook) | LCC SH155.5 .S49 2018 (print) | DDC 639.8—dc23

LC record available at <https://lcn.loc.gov/2018007574>

Cover Concept: Han-Ping Wang

Cover Design: Wiley

Cover Image: © Pgiam/Getty Images; © Ann and Steve Toon/Alamy Stock Photo; © Wolfgang Pölzer/Alamy Stock Photo; © Brandon Broderick/Alamy Stock Photo; © doidam10/Getty Images; © Kenneth Chamberlain for front cover; © Amaury Herpin, Chantal Cauty, Catherine Labbé for front cover

Set in 10/12pt Warnock by SPi Global, Pondicherry, India

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To 97-year old Zhennan Wang and 90-year-old Dusheng Peng, Hong Yao, Alan and Eileen Wang – superior parents, lovely wife, and fast-growing male and female offspring that one of the editors of the book is lucky to have.

To Arianna and Núria Piferrer, the most precious all-female progeny that one of the editors of this book has ever produced.

To Youmei Li, Shengqin Xia and Ying Chen, the most important three ladies – mother, wife, and daughter of one of the editors of this book.

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Preface

This book was motivated by an increasing, strong need for the control of sex ratios and monosex production knowledge and technology by the rapid growing global aquaculture industry. Currently, aquaculture – the fastest growing food-producing sector – contributes about 50% of the world's food fish, based on the Food and Agriculture Organization (FAO) latest reports. Sex control in aquaculture serves different purposes.

First and foremost, a wide spectrum of aquacultured species show sexual dimorphism in growth and ultimate size, whereby one sex grows faster than the other or attains a larger size. Thus, there are important benefits in rearing only the fastest-growing sex or monosex production. Second, in some species, precocious maturation and uncontrolled reproduction need to be prevented. Third, some negative impacts of reproduction on product quality or disease resistance need to be prevented in some species. Fourth, in sex-changing hermaphrodites, sex ratio control can benefit broodstock management. Finally, there are some species where the gonads or gametes of females have special economic value, e.g., caviar.

Therefore, sex control for the production of monosex or sterile stocks is extremely important for aquaculture professionals and industries to improve production or to increase revenue, reduce energy consumption for reproduction, and eliminate a series of problems caused by mixed-sex rearing or sexual maturation. Incidentally, the same principles used for sex control in aquaculture can be used in population control to eliminate

undesired invasive species – an aspect that is also dealt with in this book.

The two volumes of “*Sex Control in Aquaculture*” together is composed of 11 parts and a total of 41 chapters, which have been written by leading experts in the field. Volume I consists of Parts I to V (Chapters 1–19), while the remaining Parts VI to XI (Chapters 20–41) make up Volume II.

With eight chapters, Part I is concerned with the theoretical and practical basis of sex determination/differentiation and sex control in aquaculture. These chapters provide the concepts and rationale for sex control in aquaculture, and present our current knowledge on basic aspects of the genetic, endocrine, and environmental mechanisms for sex determination and sex differentiation, including epigenetic regulation. Readers will find a detailed, most up-to-date description of the underlying mechanisms responsible for the establishment of the sexes and, hence, the sex ratios. Several chapters also provide information on chromosome set manipulation techniques, hybridization and new gene knockout, and the application of these different approaches to aquaculture. There is also a chapter on the application of sex ratio manipulation for population control (e.g., for the management of invasive species).

Parts II to XI, or Chapters 9 to 41, contain detailed protocols and key summarizing information for the sex control practice of 35 major aquaculture species or groups with sexual size dimorphism, monosex, or polyploidy culture advantages. These major

aquaculture species include Nile tilapia, blue tilapia, Mozambique tilapia, black-chin tilapia, salmonids, European sea bass, bluegill, largemouth bass, crappies, yellow perch, Eurasian perch, channel catfish, yellow catfish, southern catfish, half-smooth tongue sole, turbot, southern flounder, summer flounder, Japanese flounder, Atlantic halibut, Pacific halibut, spotted halibut, sturgeon, shrimp, prawn, Atlantic cod, malabar grouper, honeycomb grouper, large yellow croaker, rice field eel, the Japanese eel, the European eel, the American eel, and common carp.

All chapters are arranged in the same structure and format for easier reading and the extraction of useful information, but each chapter has its own unique story. Therefore, the two volumes of the book can be read cover to cover, or you can pick any chapter, depending on your interests. However, we suggest that all readers start with Chapters 1 through 8 (Part I), in order to get a comprehensive background before moving to a particular species or group of species.

In summary, the use of sex control in aquaculture is becoming one of the most important topics for both aquaculture research and the aquaculture production industry. This book synthesizes relevant and recent information on sexual development principles and sex control practice, and emphasizes

their applications for use in the aquaculture industry. It bridges the gap between theory and practice in sex control of farmed species, including new developments and methodologies used in sex determination, differentiation, monosex, and polyploidy production for aquaculture.

Thus, the book will appeal to a large audience: Scientists working directly in aquaculture research or food production will find relevant information on the principle and practical aspects of sex control in aquaculture; and scientists working with basic aspects of fish/shrimp biology, reproductive endocrinology, genetics, and evolutionary biology will find abundant information regarding sex in related species. Likewise, biologists working in the farming industry, hatchery management, fisheries, as well as related administrators, will benefit from clear and practical information on how to apply sex control in aquatic animals. Finally, young researchers and graduate students will learn about a field – the establishment of sex in fish/crustaceans and its control – with both basic and applied connotations.

May, 2018

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Acknowledgements

We thank Sarah Swanson at The Ohio State University for her efforts in chapter coordination, format review, and editing assistance. Thanks also go to Joy Bauman, Jordan Maxwell, and Bradford Sherman at The Ohio State University for their English editing. We thank Amaury Herpin, Chantal Cauty, Catherine

Labbé, and Ken Chamberlain for providing photos for the front cover.

We thank all the anonymous reviewers for their peer-review of the book chapters and constructive comments for improvement of the book quality.

Part I

Theoretical and Practical Bases of Sex Control in Aquaculture

1

Sex Control in Aquaculture: Concept to Practice

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1.1 Introduction

With over 30,000 recognized species, fish constitute the largest and most diverse taxa of vertebrates [1, 2] and display all kinds of reproductive strategies and sex determining (SD) mechanisms. These include genotypic sex determination (GSD), environmental sex determination (ESD), hermaphroditism, parthenogenesis, gynogenesis, and hybridogenesis [3, 4], as shown in Table 1.1.

Of the 709 species with a recorded sexual system [5], SD mechanisms have only been extensively investigated in limited numbers – for example: tilapia (mainly of the genus *Oreochromis*); European sea bass (*Dicentrarchus labrax*); medaka (*Oryzias latipes*); tiger Pufferfish (*Takifugu rubripes*); rainbow trout (*Oncorhynchus mykiss*); pejerrey (*Odontesthes bonariensis*); Atlantic silverside (*Menidia menidia*); zebrafish (*Danio rerio*); Japanese flounder (*Paralichthys olivaceus*); and yellow catfish (*Pelteobagrus fulvidraco*). However, regardless of reproductive strategy, the sex ratio is determined by a sex determining mechanism, can be influenced during the process of sex differentiation, and is the vital demographic parameter that determines/influences population structure, reproductive potential, and economic value for a given species.

Besides the diversity, phenotypic sex of fish is characterized by plasticity/lability, changeability, and complexity. In short, sex determination in fish is much more complex than we ever thought, and having clear pictures of the related terminologies (Boxes 1.1 and 1.2) will help us understand the complexity of sex determination in fish and sex control in aquaculture.

The study of sex determination and sex differentiation in fish is important both from academic and practical aspects. Thus, research on the SD mechanism in a given species, and production of its monosex population, supplement each other. The diversity of sex determining mechanisms in fish offer extraordinarily unique material for broadening our understanding of the evolution of the mechanisms and the force that drive the formation and maintenance of sexes. The conserved, yet diverse, pathways involved in sex differentiation of fish [4, 5] allow researchers to even develop “medical models” (e.g., zebrafish, medaka [6]), and explore alternative regulatory mechanisms related to sexual dysfunction of vertebrates, including humans.

The more practical reason for studying sex determination and sex differentiation in fish is to obtain potential benefits of monosex production, with higher growth rate, superior

Table 1.1 Summary of sex determination in fish.

Sex determination or reproductive mode	Inheritance of sex	Sex-determining mode or reproductive strategy	Time of sex determination	Female genotype(s)	Male genotype(s)	Morphology of sex chromosome	Example species	Reference
Genotypic sex determination	Sex chromosomal systems	Mono-factorial system	At the point of fertilization and shortly later	XX	XY	Homomorphic	Tilapias	[51]
						Heteromorphic	Rainbow trout	[184]
				XX	XO	Heteromorphic	<i>Triacanthus brevirostris</i>	[185]
				ZW	ZZ	Homomorphic	<i>Tilapia mariae</i>	[41]
						Heteromorphic	<i>Leporinus sp.</i>	[186]
				ZW	ZO	Heteromorphic	<i>Colisa lalius</i>	[187]
				X ₁ X ₁ X ₂ X ₂	X ₁ X ₂ Y	Heteromorphic	<i>Lutjanus quinquelineatus</i>	[188]
				XX	XY ₁ Y ₂	Heteromorphic	<i>Hoplias malabaricus</i>	[189]
				XX	X ₁ X ₂ Y	Heteromorphic	<i>Hoplias malabaricus</i>	[190]
				Z W ₁ W ₂	ZZ	Heteromorphic	<i>Apareiodon affinis</i>	[191]
Environment-dependent sex determination	Minor sex factors	Multifactorial system	Thermo-sensitive period	XX, XW, WY	XY, YY	Homomorphic	Platyfish	[192]
		Poly-factorial system		N/A	N/A	N/A	zebrafish	[193]
		Temperature-dependent SD		N/A	N/A	N/A	Pejerrey	[122]
		pH-dependent SD		N/A	N/A	N/A	<i>Apistogramma sp.</i>	[194]
Hermaphrodite	Genetic factors	Proterandrous~	N/A	N/A	N/A	N/A	<i>Sparus aurata</i>	[195]
		Protogynous~	N/A	N/A	N/A	N/A	<i>Coris julis</i>	[196]
		Simultaneous~	N/A	N/A	N/A	N/A	<i>Serranus subligarius</i>	[197]
Unisexuality	Genetic factors	Gynogenesis	At the point of fertilization and	XX	XY	Homomorphic	<i>Carassius auratus gibelio</i>	[198]
		Hybridogenesis	shortly later	Not known	Not known	Not known	<i>Poeciliopsis</i>	[199]
		Parthenogenesis		Not known	Not known	Not known	<i>Poecilia formosa</i>	[200]

Note: sex determining mode assigned in the table only represents specific geographic population, not the species as a whole.
SD, sex determination; N/A, not applicable.
Table adapted from [64].

Box 1.1 Glossary of reproductive strategies, sex determining mechanisms, sex differentiation, and sex control

- **Gonochorists:** individual organisms that contain only male or female sex organs throughout their lifetime.
- **Hermaphrodites:** individual organisms that contain both male and female sex organs.
- **Sequential hermaphroditism:** individual organisms that change sex at some point during their life.
- **Unisexuality:** a mode of reproduction whereby offspring are formed exclusively from maternal or paternal genetic information.
- **Sex determination:** the genetic or environmental process that establishes the sex of an organism.
- **Sex differentiation:** the process by which an undifferentiated gonad is transformed into an ovary or a testis. Specifically, it is the realization of the phenotypic sex.
- **Genotypic sex determination:** an individual's sex is established by its genotype.
- **Environmental-dependent sex determination:** sex is triggered by environmental cues, such as ambient temperature or pH during a sensitive period, usually in larval states.
- **Temperature-dependent sex determination (TSD):** sex is determined by ambient temperature rather than genotype in early stages of development. TSD is the most popular type of ESD, which has received the most extensive attention.
- **Genotypic sex determination plus temperature effects (GSD + TE):** sex ratio is determined by genotype while affected by temperature.
- **Polygenic sex determination (PSD):** sex is dependent on the combined effects of multiple pro-female and pro-male factors (e.g., it is determined by multiple, independently segregating sex “switch” loci or alleles).
- **Sex control:** to change an individual or population's sex ratio through one of several possible approaches, such as direct modification through sex-reversal by hormone administration or gene knockout, or by indirect methods such as chromosome manipulation, hybridization, or a combination of several.
- **Neomale:** a genotypic female that develops into a phenotypic male (e.g., XX males in yellow perch (XX / XY)).
- **Neofemale:** a genotypic male that develops into a phenotypic female.

flesh quality, and so on. (Table 1.2). Studies on sex differentiation with relevance to aquaculture have been conducted in more than 100 fish species [4, 7–11] over 40 years since the publication of Yamamoto's [12] review on sex differentiation in fish. Monosex production has been achieved in several commercially important fish, including tilapia, turbot (*Scophthalmus maximus*), European sea bass salmonids, yellow catfish, Eurasian perch (*Perca fluviatilis*), yellow perch (*Perca flavescens*), bluegill (*Lepomis macrochirus*), etc. [7, 13–17; Chapter 17 of this book and Chapters 20–21 in Volume 2].

Importantly, the advancements of molecular biology and biotechnologies – especially the molecular marker technologies and next

generation sequencing – accelerate, deepen, and embolden the studies in this field. In this chapter, we provide a brief summary of concept and practices of sex control in fish with XY or ZW SD systems.

1.2 Establishment of Phenotypic Sex - “Promoter” to “Modulator”

The establishment of gender can be triggered by the action of a major SD gene, several sex-associated loci (poly-factorial sex determination), an environmental factor (Table 1.1), or a combination of these in gonochoristic fish. Once the orientation of a sex is initiated,

Table 1.2 Potential benefits of monosex production.

Potential benefit	Apply to Female (F)/Male (M)	Example	Reference
Higher growth rate	F	Rainbow trout	[201]
	M	Tilapia	[7]
Higher value of specific organ (e.g. ovary)	F	Sturgeon	[202]
		Mud crab	[203]
Greater uniformity of harvest size	F, M	Mud crab	[203]
Higher reproductive value	F	Swamp eel	[204]
Eliminating/reducing energy transfer into:			
gonad production	F	Atlantic salmon	
courtship/colonization related behavior	M	Bluegill	
production of uneconomic recruits	F, M	Tilapia	
Reducing aggressive interactions/cannibalism	F, M	Red mud crab	[207]
Superior flesh quality/taste	F	Rainbow trout	[201]
Ornamental value	M	Ornamental fish	
Controlling invasive species	F, M	Not studied yet	

Box 1.2 Confusing terminologies

Several terms, such as sex determination and sex differentiation, as well as the differences between GSD and ESD (especially TSD), are very important and need to be clearly defined.

Sex determination and sex differentiation

Sex determination and sex differentiation are often misused, because the distinction between the two terms is difficult, since the criteria of sex differentiation (morphological/histological, cellular, molecular) are frequently used to state whether the phenotypic sex has been determined [4]. Sex determination indicates how and when the genotypic or environmental sex is determined, while sex differentiation describes the realization process of phenotypic male or female.

Sex determination usually happens prior to, or at the same time as sex differentiation, and influences sex differentiation in a sex-specific manner. Both sex determination and sex differentiation are usually case- and species-specific. Sex determination happens at the point of fertilization, or shortly thereafter, for fish with GSD, while it happens later, usually at the larvae stage, for fish with TSD. Sex differentiation occurs either shortly after fertilization during the embryonic stage for a few fish, or at the larval stage for most others. For some fish species, gonadal differentiation is much later – for

example, in European sea bass (*Dicentrarchus Labrax*), grass carp (*Ctenopharyngodon idella*), black carp (*Mylopharyngodon piceus*), paddlefish, and sturgeons, it occurs from months to years post-hatching. Meanwhile, the criteria to infer the onset of sex differentiation are changing with the development of molecular biology. Furthermore, clarification of several terms, such as sex determination systems/modes, master sex determining genes [18, 3, 5], labile/sensitive period of sex differentiation, and molecular players involved in sex differentiation will help readers to understand the difference.

GSD and TSD

TSD, which has been extensively investigated in the past four decades as the most common form of ESD, is frequently misused to indicate the effects of rearing temperature on sex differentiation [5, 19], which is actually GSD+TE (genotypic sex determination plus temperature effects). TSD, as one of the sex determination mechanisms, is widely considered to be parallel to GSD. There is a continuous transition between GSD and TSD, both at the population level of a given species and at species level among different fish. Furthermore, they are considered the extreme ends of the transition (Figure 1.1, and also refer to [20] and Chapter 4).

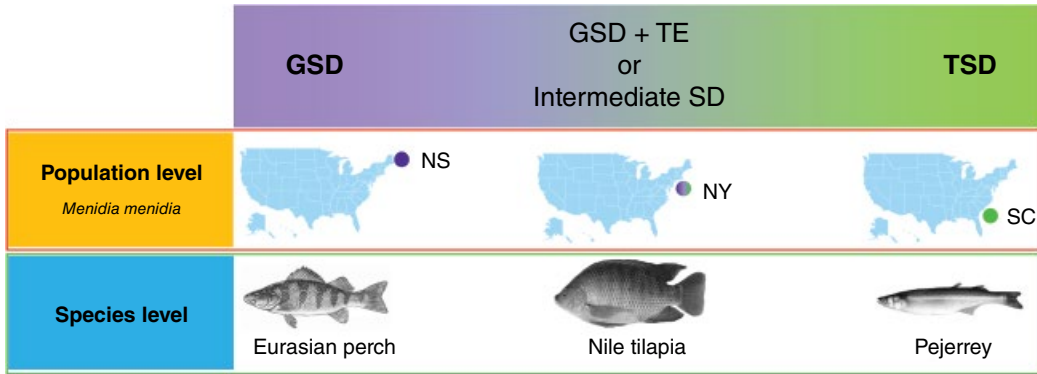


Figure 1.1 Sex determining mechanisms – relationship and examples.

GSD, genotypic sex determination; TSD, temperature-dependent sex determination; GSD + TE, GSD plus temperature effects. NS, Nova Scotia; NY, New York; SC, South Carolina.

The data in population level are adopted from [63]. The data in species level refer to [16, 119, 122].

The corresponding fish assigned to each sex determining mode represent the status of some populations, not the species as a whole.

related molecular players will be activated/suppressed thereafter, and display a sex-specific expression pattern and interact with one another, leading to the formation of ovary or testis. In the past two decades, several breakthrough advancements have been achieved in the studies of sex determination.

1.2.1 Sex Determining Factors – the Promoter

1.2.1.1 Known Master Sex Determining Genes

Five master SD genes, *dmy/dmrt1Y* in medaka *Oryzias latipes* [21, 22] and in *Orzias curvinotus* [23], *amhy* in Patagonian pejerrey *Odontesthes hatchery* [24], *gsdf^Y* in *Oryzias luzonensis* [25], *amhr²* in fugu (tiger pufferfish) *Takifugu rubripes* and other two *Takifugu* species [26], and *sdY* in rainbow trout *Oncorhynchus mykiss* and many salmonids [27, 28], have been identified from 2002 to 2012. Also, three outstanding candidate master sex determining genes, *dmrt1* in half-smooth tongue sole (*Cynoglossus semilaevis*), *amhy* in Nile tilapia [29, 30] and cobaltcap silverside *Hypoatherina tsurugae* [31], and *Sox3^Y* in *Oryzias dancena* [29], have been discovered recently [33]. Of these genes, five of them (*dmy*, *amhy*, *gsdf^Y*, *sdY*, *Sox3^Y*) reside on the Y chromosome, while *amhr²* is located both on

the X and Y chromosomes. Functional copies of *dmrt1* were only found on Z chromosomes, with a heavily corrupted, pseudogenized copy found on the W chromosome.

Interestingly, the master sex determining gene in fugu, *amhr²*, is expressed in both differentiating testis and ovary and before the onset of morphological differentiation of the gonads [26]. This finding suggests that the master SD gene needs not to be expressed in a sex-specific manner, like the mammalian *Sry* or the other five master SD genes that reside on the Y chromosome, probably because the sex-specific pathway can be generated by the male-specific isoform. Furthermore, the fugu SD locus shows no sign of recombination suppression between the X and Y chromosome [26], indicating that the sex chromosomes in fugu have not been differentiated.

Morphologically distinct sex chromosomes are present in only about 10 percent of the approximately 1700 species of fishes that have been characterized cytogenetically [4], suggesting that sex chromosomes have not been differentiated in most fish species. Therefore, the SD genes residing in a recombining region may be more common than previously thought. It is worth mentioning, however, a morphologically indistinguishable sex chromosome does not infer recombination of X and Y chromosomes. Association mapping,

applying next-generation sequencing, will be a powerful approach to unveil the SD genes/loci for fish with an undifferentiated sex chromosome.

Recently, *dmrt1* has been suggested to be a strong candidate for the SD gene in the half-smooth tongue sole, according to its association with sex and its pseudogenization in the W chromosome [33], although functional demonstration has not been reported to date. A functional copy of this gene was only detected on the Z chromosome, contrary to the other four SD genes residing on the Y chromosome, as we mentioned above. In addition, male expression of 763 Z-linked genes in whole-body transcriptomes was, on average, 1.32 times higher than female expression [33], indicating incomplete gene dosage compensation.

Meanwhile, this finding suggests that this SD gene may act through a threshold manner, like the SD gene in medaka [21] and sex differentiation-related genes in fish. Contrary to the XX/XY sex determination system, where the Y chromosome determines sex, it is the Z chromosome that determines sex in the tongue sole. DNA methylation of Z-linked male-determining gene was found to be involved in sex determination and inheritance of sex reversal [33, 34], suggesting that epigenetic changes can be linked to sex determination in vertebrates.

Among the 16 master SD genes identified so far in vertebrates and insects [3, 30, 32, 35], the direct downstream targets of SD genes have been only found for the mammalian SD gene, *Sry* [36, 37], and for *amhy* in Nile tilapia [30]. To our knowledge, *dmy* in medaka has been found to acquire a feedback downregulation of its expression; specifically, it is an indirect target itself [38], although it has been characterized for more than 10 years [21, 22]. Two independent studies, using either knock-down or injection of antisense morpholinos of *dmy*, have proved that the SD gene *dmy* (*dmrt1bY*) in medaka negatively regulates the proliferation of primordial germ cells via repressing the expression of *dmy* [39, 40]. However, little is

known about the molecular mechanisms by which the *dmy* expression-supporting cells interact with germ cells.

It is surprising to see that the pace of identifying new SD genes in the past six years (2012–2017) is moving so quickly, mainly because of fast-developing biotechnologies such as sequencing, mapping, transgenesis and knockout technologies. Especially, it is strongly suggested that SD genes have evolved unexpectedly fast. The SD gene *amhy* in one strain of Nile tilapia [29] has been found not to be the SD gene in another strain [30], while the tandem duplicate located immediately downstream of it, also denoted as *amhy*, which is residing on sex determining linkage LG23 of Nile tilapia, is essential for male sex determination in that strain. SD genes or SD mechanism diverged in closely related species [18, 21, 22, 25], and even in different populations of the same species [29, 30, 41–44]. In addition, the transition from GSD to TSD can be rapid, in just one generation in the Australian bearded dragon (*Pogona vitticeps*) [45], further suggesting the fast evolution of SD mechanisms and SD genes.

The difficulties of clarifying the SD pathway at this point involves understanding a complex hierarchy of genes, which can amount to hundreds of sex-specific expression patterns, a nearly impossible task. Although gene pathway analysis has yielded advances in mammals, it has not been generally used in aquaculture species, except in a few fish [29, 30, 46–49]. Gene set enrichment analysis (GSEA) has been employed to provide clues as to which gene pathways may be switched on or off via specific editing [50]. Several pathway analysis programs, such as Pathway Studio (<http://www.elsevier.com/solutions/pathway-studio>) and MetaCore™ (<http://lsresearch.thomsonreuters.com/pages/solutions/1/metacore>), are able to find out upregulated or downregulated genes and put them into the gene pathways in which these genes are involved, and then determine the gene pathways that are modulating under the specific condition of the editing. Promisingly,

incorporation of genome or transcriptome resources, genome editing technologies (e.g. knockout/knockdown, overexpression), GSEA, and pathway analysis programs will be able to clarify the molecular pathways involved in sex determination in the near future.

1.2.1.2 Sex Loci

Apart from the seven master SD genes, as we mentioned above, SD loci, on which master SD genes may reside, have been found in a many fishes (Table 1.3). Of these, more than

one locus associated with sex determination, either residing on the same or different linkage groups (LG)/chromosomes, has been detected in several fish species. These include loci on LG1 and LG3 in *Oreochromis aureus* and *O. mossambicus* [51], LG1 and LG23 or LG8 in Nile tilapia [41, 43], chromosome 5 and 16 in zebrafish [52], three LGs in Tasmanian Atlantic salmon *Salmo salar* [53], LG5 and LG7 in several cichlid fish [54], and two loci on LG21 in gilthead sea bream *Sparus aurata* L. [55]. It should be noted that

Table 1.3 Sex determining loci in fish.

Species	SD loci	SD system	Main technique employed	Reference
<i>Oreochromis karongae</i>	LG3	ZW/ZZ	BAC Sequencing	[41]
<i>Oreochromis tanganyicae</i>	LG3	ZW/ZZ	BAC Sequencing	[41]
<i>Tilapia mariae</i>	LG3	ZW/ZZ	BAC Sequencing	[41]
<i>Tilapia zillii</i>	LG1	XX/XY	BAC Sequencing	[41]
<i>Oreochromis mossambicus</i> Mozambique tilapia	LG1, LG3	XYZW complex	BAC Sequencing	[41]
<i>Oreochromis niloticus</i> Nile tilapia	LG8 LG1, LG23	XX/XY	Bulked segregant analysis	[43] [41]
<i>Oreochromis aureus</i> Blue tilapia	LG1, LG3	XYZW complex	Bulked segregant analysis	[41, 56]
<i>Hippoglossus hippoglossus</i> Atlantic halibut	LG13	XX/XY	RAD sequencing	[158]
<i>Danio rerio</i> Zebrafish	Chr3, Chr4 Chr5, Chr16	Poly-factorial Poly-factorial	RAD sequencing SNP genotyping	[152] [52]
<i>Scophthalmus maximus</i> Turbot	LG5	ZW/ZZ	QTL	[208]
<i>Gasterosteus aculeatus</i> Threespine stickleback	LG19	XX/XY	BAC Sequencing	[209]
<i>Pungitius pungitius</i> Ninespine Stickleback	LG12	XX/XY	BAC Sequencing	[210]
<i>Gasterosteus wheatlandi</i> Blackspotted stickleback	LG12, LG19	X ₁ X ₁ X ₂ X ₂ /X ₁ X ₂ Y	Genotyping	[59]
<i>Metriaclima</i> sp.	LG7	XX/XY	QTL	[54]
<i>Metriaclima</i> sp.	LG5	ZW/ZZ	QTL	[54]
<i>Metriaclima pyrrsonotus</i>	LG5, LG7	XYZW complex	QTL	[54]
<i>Salmo salar</i> Atlantic salmon	Chr2, Chr3, Chr6	XX/XY	SNP genotyping	[53]

Note: LG, linkage group; Chr, chromosome; BAC, Bacterial artificial chromosome; RAD, Restriction site associated DNA; SNP, Single-nucleotide polymorphism; QTL, Quantitative Trait Locus.

more than one SD locus on different LGs in a given species could be the same or distinct, and one SD locus could be segregated into several LGs.

Intriguingly, several studies have shown that SD genomic regions are non-homologous in closely related species, or distinct in different populations of one species. In tilapias (family Cichlidae, order Perciformes; genera *Oreochromis*, *Sarotherodon* and *Tilapia*), both male and female heterogametic sex determination systems (XY and ZW) have been characterized, and three LGs have been determined as sex-linked chromosomes [41, 51, 56–58]. Three sex determination systems – XY, ZW, and X_1X_2Y – have been discovered in several stickleback species (Gasterosteidae) [59]. Sex determining loci have been mapped to different genome regions in North American and European derived Atlantic salmon [53].

The evidence leads us to speculate that the evolution of sex determining mechanisms plays an important role in speciation. Actually, several studies indicate that transitions in the mode of sex determination have occurred in closely related species [51, 60]. Furthermore, the fact that phenotypes (e.g., tail color, body color pattern) have been mapped into the same LGs with SD loci [54, 61, 62], strengthens the idea that sex determining mechanisms have contributed to the radiation of fish.

Detection of quantitative trait loci (QTL) involved in sex determination has initiated investigations on the evolution of molecular pathways of sex determination, and provides useful information for further studies. The synthesis of high-resolution genetic maps and feasible deep sequencing, detailed analysis of content, and order of genes and other genetic elements in SD loci, as well as functional analysis of genes involved and complex hierarchy network of sex determination will be next steps in further understanding the mechanism of sex determination.

It is worthy of note that a recent study provides evidence that the B chromosomes, which were believed to be selfish genetic elements with little effect on phenotype, and

lacking functional genes, have a functional effect on female sex determination in Lake Victoria cichlid fishes [63]. Sex determining mechanisms may be more complex than previously thought; if this is the case in general, then investigations in this field will be more interesting, although much research is yet to be done.

1.2.1.3 Environmental Promoter

Several environmental factors, including temperature, pH, photoperiod, and salinity are assumed to determine or affect sex during sensitive periods of early development [4, 64]. Temperature has been the factor investigated in most detail in fish, and the effects of temperature on sex ratio have been observed in more than 60 species [4, 19, 64, 65]. The definition and exact criteria of TSD has been debated for several years, mainly focusing on how, or whether, it is necessary to distinguish TSD and GSD + TE (GSD plus temperature effects) [5, 19, 65–67, and see Chapter 4 of this book]. We advocate that the TSD should be clearly distinguished from GSD + TE because TSD has been extensively accepted as a sex determining mechanism that parallels GSD (Figure 1.1), and the fact that the sex determining mechanism should be relevant to ecology and adaptive significance [66, 68].

Meanwhile, we also propose that any significant effect of an environmental factor on sex ratio deserves to be studied in both field and laboratory, for several reasons. First, the influence of pollutants (e.g., endocrine disrupting chemicals, ocean acidification) and global warming on population development through changing sex ratio need to be addressed generally. Second, comparative analysis of molecular players and downstream pathways of the sex determining cascade between TSD and GSD + TE will provide important information on the plasticity of sex differentiation and evolution of sexual selection.

As we proposed (Figure 1.1), GSD + TE, the transition status or intermediate sex determining mechanism between GSD and TSD, may be important for the dynamics and

stability of fish populations when experiencing dramatic climate change. Both empirical and experimental studies suggest that the transition between sex determining modes have occurred many times in fish, reptiles, amphibians, and so on [69–73], and thermo-sensitivity in sex determination has been assumed to be the key factor in those transitions [73]. Finally, yet importantly, in practical aspects, pros and cons of the effects of environmental factors on sex ratio should be evaluated, in order to take full advantage in monosex production.

The immediate target of temperature in TSD has not been characterized. Three pathways are proposed here to speculate how temperature transduces sex determining signals into target organs and determines the orientation of the sex.

First, temperature may transduce the signals via altering methylation patterns of sex-related loci/genes. Sexually dimorphic DNA methylation patterning of sex differentiation-related genes and factors (e.g., *cyp19a*, *sox9*, estrogen receptor, and candidate SD gene *dmrt1*) have been observed in several fish and reptile species [33, 34, 74–79]. Furthermore, DNA methylation of gonadal aromatase *cyp19a1a* promoter has been found to be involved in temperature-dependent sex differentiation in the European sea bass [76]. In American alligator (*Alligator mississippiensis*), a reptile with TSD, differential incubation temperature leads to dimorphic DNA methylation patterning of *cyp19a1a* and *sox9*. Temperature-dependent DNA methylation of *cyp19a1a* promoter has also been detected in another reptile with TSD [75]. These results indicate that ambient temperatures cause differential methylation patterns/levels of sex-specific genes/factors, which lead the temperature-specific expression of these genes/factors, consequently bringing about the formation of ovary or testis.

Second, temperature may transduce sex determining signals through immediately altering the expression of sex-specific

genes/factors. Temperature has extensive modulatory effects on every stage of development [80]. Effects of rearing temperatures on sex differentiation-related genes (e.g., *dmrt1*, *amh*, *sox9*, *cyp19a1a*, and *foxl2*) have been observed in several fishes and reptiles with TSD or GSD+TE [5], indicating the involvement of these genes in temperature-dependent sex differentiation.

Finally, temperature may determine sex through the endocrine system. As early as 1985, it was found that exposure to cortisol and cortisone inhibited ovarian growth, and increased the proportion of males in rainbow trout larvae [81]. In recent years, several studies have reported that exposure to high temperature elevated cortisol levels and led to the masculinization of fish species with TSD and GSD+TE. In 2010, Hayashi *et al.* [82] reported that, in medaka, exposure to a high temperature (33°C) induced masculinization of XX females by elevating the cortisol level which, in turn, suppressed germ cell proliferation and expression of *fshr* mRNA. Thus, cortisol can cause female-to-male sex reversal in this species.

In Pejerrey, a fish species with TSD, individuals treated with cortisol presented elevated levels of 11-ketotestosterone (11-KT) and testosterone and typical molecular signatures of masculinization, including upregulation of *amh* expression and downregulation of *cyp19a1a* expression [83]. Moreover, in the same species, it has been observed that, during high-temperature-induced masculinization, cortisol promotes the production of 11-KT by modulating the expression of *hsd11b2*.

Cortisol also produces a dose-dependent sex reversal from females to males in the southern flounder (*Paralichthys lethostigma*), where exposure to high (28°C) and low (18°C) temperatures produce a preponderance of males, while an intermediate temperature (23°C) favors a 1 : 1 sex ratio [84]. In addition, in the Japanese flounder, exposure to cortisol causes masculinization by directly suppressing the expression of

cyp19a1a mRNA due to disrupting cAMP-mediated activation [85]. These results provide evidence on the relationships between temperature conditions and the responses of the organism, and allow us to conceptualize the endocrine-stress axis in terms of gonadal fate under temperature effects. They suggest that cortisol may be the “lost” link between temperature and the sex determining mechanism in species with TSD as well as GSD+TE and may, as a stress indicator, be involved in the adaptive modification of sex ratio in a spatially and temporally variable environment during the evolution of such species.

1.2.2 Molecular Players in Sex Differentiation – the Modulator

Sex differentiation involves a complex module of genes with germ cells and gonadal somatic cells. Little information of the molecular

cascade involved in sex differentiation is available, even though the expression profile of pertinent genes (e.g., testicular differentiation genes *dmrt1*, *amh* (also known as *mis*), and *sox9* and ovarian differentiation genes *foxl2* and *cyp19a1a*) have been well characterized in a large number of fish species. These fishes include economically important species (e.g., tilapia, rainbow trout) and model species (e.g., medaka, zebrafish) [5]. According to available reports, we have constructed a model to describe the general molecular pathway involved in sex differentiation, regardless of the genetic sexual background of the individual (Figure 1.2), and hope that it helps readers better visualize the cascade of sex differentiation.

Herein, we summarize four general characteristics of the molecular pathways involved in sex differentiation. These have been derived from extensive comparative analyses of expression profiles in a large number of

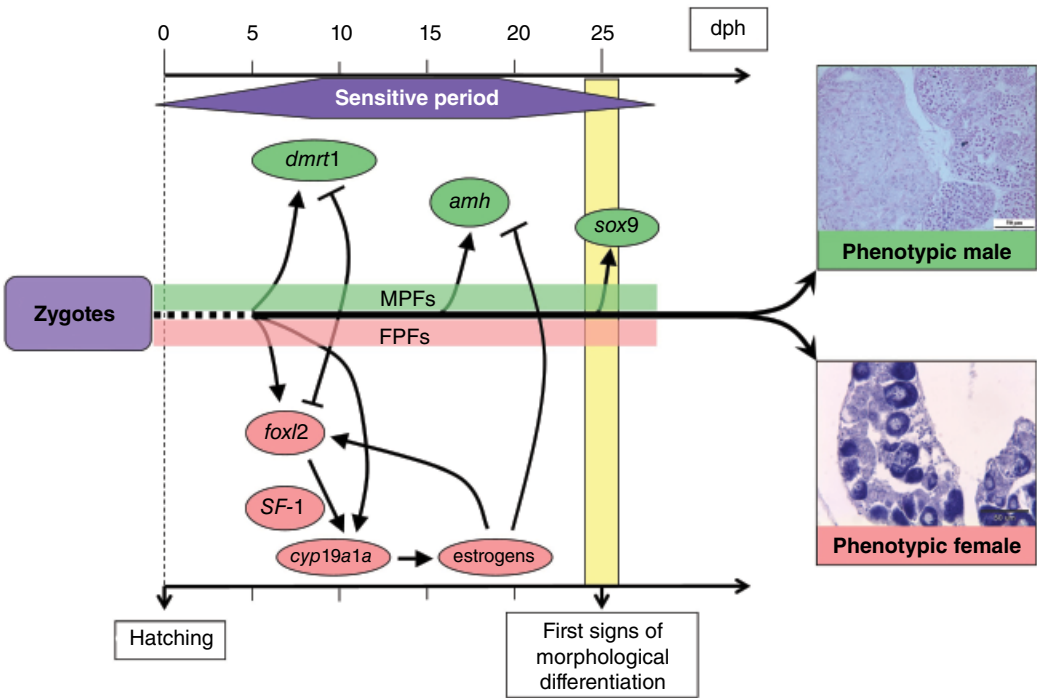


Figure 1.2 Molecular pathways involved in sex differentiation, taking Nile tilapia as an example. MPFs, male producing factors; FPFs, female producing factors; the factors could be environmental factors such as temperature, exogenous hormones, etc. dph, days post-hatching. The full names of these genes can be found in the main text. The data presented are a compilation from Shen and Wang [5].

fish species, based on available reports. The summary will facilitate researchers to compare their results with others, and better understand sex differentiation in a wide range of taxa. The four general characteristics of the molecular pathways involved in sex differentiation are explained below:

1.2.2.1 Conserved Genes Yet Diverse Expression Profiles

It seems that the genes involved in sex differentiation are quite conserved in a wide range of taxa, from fish, reptiles, and chicken, to mammals, including humans. For example, the discovery of SD genes *dmy*, *dmrt1* and *DM-W* in medaka, tongue sole and *Xenopus*, respectively [21, 22, 33, 86, 87], makes the *dmrt1* gene more interesting and important, even though an abundance of reports indicate *dmrt1* plays a decisive role in testicular differentiation [5, 88–90]. In *dmrt1*-deficient testes (through the introduction of transcription activator-like effector nucleases, TALENs) of tilapia, significant testicular regression, including deformed efferent duct, degenerated spermatogonia, or even a complete loss of germ cells, have been observed [88].

A mice model with *dmrt1*-deficient germ cells suggests that *dmrt1* regulates tubule morphology, spermatogenesis, and sperm function [91]. A series of studies in the red-eared slider turtle, a reptile with TSD, place *dmrt1* at the top upstream of the testicular differentiation cascade. These results suggest that, irrespective of sex determining modes (GSD, GSD + TE, or TSD), *dmrt1* is involved as a key factor in testicular formation and function. Besides *dmrt1*, testicular differentiation genes *amh* and *sox9*, and ovarian differentiation genes *foxl2* and *cyp19a1a*, have been suggested to be involved in sex differentiation across a wide range of animals [5, 90, 92–98].

Expression patterns are considerably diverse, yet the genes involved in sex differentiation are relatively conservative. For example, *sox9* expression in the developing XY gonad is activated by the SD gene *sry*, and

it upregulates the expression of *amh* thereafter and plays a decisive role in testicular formation and function of mammals [37, 99] while, in fish, reptiles and chicken, sexually dimorphic *sox9* expression was observed later than sexually dimorphic *amh* expression [90, 96, 100–103]; (also see Figure 1.2), suggesting a divergent relationship between genes involved in sex differentiation.

In tilapia and trout (*O. mykiss*), *dmrt1* is expressed in males prior to sex differentiation, but not in females [93, 104, 105], which indicates that in these fish species, *dmrt1* is involved in testis formation and differentiation. However, in other fish species, like medaka, pejerrey (*Odontesthes bonariensis*), and European sea bass (*Dicentrarchus labrax*), sexually dimorphic expression of *dmrt1* in males and females was reported [5], which indicates that, in these cases, *dmrt1* participates in testis and ovarian development.

1.2.2.2 Paralogues Play Different Roles

The ray-finned fish (*Actinopterygii*) have two paralogous copies for many genes (e.g., *dmrt1a* and *dmrt1b*, *cyp19a1a* and *cyp19a1b*, *sox9a* and *sox9b*), due to fish-specific genome duplication dated between 335 and 404 million years ago [106]. With the increasing availability of whole-genome sequences, the comparative analysis of genes and genomes will reveal the evolution and phenotypic diversification of the third round (and fourth round in some fish species such as common carp, *Cyprinus carpio*) of genome duplication [106–109]. Some duplicated genes have evolved new functions, while others have disappeared [108].

For example, the gene that encodes aromatase is a duplicated gene in all investigated teleost fish [110–113], except in eels, which belong to the ancient group of Elopomorpha [114]. The gene duplication gave rise to two different genes (isoforms), namely *cyp19a1a* and *cyp19a1b*, in most teleost fish. The *cyp19a1a* gene is also known as "gonadal aromatase" or "ovarian aromatase" (also referred to as *p450aromA*, *cyp19a* or *cyp19a1*), since it is mainly

expressed in the differentiating and adult gonads of teleost fish. The *cyp19a1b* gene is called the “neural aromatase” or “brain aromatase” (also referred as *P450aromB*, *cyp19b* or *cyp19a2*), since it is highly expressed in the brain of both male and female teleost species [115], but no sexually dimorphic brain expression during gonad sex differentiation has been demonstrated. *Sox9a* and *sox9b* are likely to play different roles in fish. In medaka, *sox9a* was not expressed in the somatic cells during gonadal differentiation, while *sox9* was found to be involved in germ cell maintenance, but does not directly regulate testis determination [116].

1.2.2.3 Antagonistic Roles of Testicular Differentiation Genes and Ovarian Differentiation Genes

Phenotypic sex is referred to as the result of the balance of two camps of antagonistic/competitive signaling pathways and transcription networks. A complex, dynamic molecular network underlies this process, as approximately half of the genome is being transcribed during sex differentiation, and many genes and factors are expressed in a sexually dimorphic manner [117]. In mammals, antagonistic action to reach threshold levels between *wnt4* and *fgf9/sox9* may tip the balance between female and male development [117]. In Nile tilapia, *dmrt1* may be the top upstream gene in testicular differentiation, while *foxl2* plays a decisive role in ovarian differentiation. These two genes have been found to play antagonistic roles in sex differentiation via regulating *cyp19a1a* expression and estrogen production, being demonstrated through a knockout technology called TALENs [88]. In a similar fashion, it has been suggested that two antagonists to the *Wnt* cascade, dickkopf-1 (*dkk1*) and dapper-1 (*dact1*), may play important roles in sex differentiation and gonadal development in sturgeon [118]. Therefore, sexual fate is actually determined by activating the testis or ovarian pathway and suppressing the alternative pathway.

1.2.2.4 Temperature Sensitivity is Limited and Heritable

The temperature effect on offspring sex ratio is not overwhelming when we see it in a wide range of fish species, although extreme temperatures can induce all-male or all-female populations in some fish species, including all-male populations in tilapia [119, 120], and all-male and all-female populations in Pejerrey, *Odontesthes bonariensis* [121, 122]. This is probably due to a “protection mechanism” which can avoid extinction because of so-called Trojan sex genes and/or an extremely unbalanced operational sex ratio [123–131]. This is absolutely distinctive to some reptiles in which TSD is universal and monosex induction by incubation temperature is common [132]. The temperature during early development of a given fish species is relatively stable, even though fish live in changing environmental conditions throughout their life [19]. This may partially explain the difference of temperature sensitivities between fish and reptiles, since reptile eggs are exposed to more variable temperature conditions during the period of sex differentiation (see Chapter 4 of this book for more details).

It is interesting, but reasonable, that temperature sensitivity is found to be heritable and can be selected for as a quantitative trait, both in Nile tilapia and rainbow trout [133–136]. These results reinforce the notion that GSD+TE may be a relatively stable status during the evolution of sex determination, and more common in fish. The promising findings – specifically, the heritability of temperature sensitivity – have already served in selective breeding programs, increasing the proportion of desired sex as quantitative trait, such as growth performance in several species, through a consumer- and environment-friendly approach (see Chapter 4 of this book).

1.3 Sex control Practice in Aquaculture

Generally, sex control includes many aspects, including producing sex-skewed/monosex populations through induction

of sex reversal, chromosome manipulation (gynogenesis and androgenesis, polyploidy induction), hybridization, selection, or a combination of these. Here, we focus only on large-scale monosex production, which could continuously provide a sufficient supply of monosex seeds for commercialization. Large-scale monosex production in fish usually requires the researchers to acquire basic information on the sex determining mechanism, and also meet two conditions: first, that the sex can be reversed; and second, that the phenotypically sex-reversed fish are fertile. Therefore, because of these constraints, large-scale monosex production has not been accomplished in many fish species.

1.3.1 Large-Scale Monosex Production

Many benefits can be generated in monosex production for aquaculture (Table 1.2). The most frequent consideration is the advantage of one sex growing faster and/or reaching a larger size than the other; this size disparity may be aggravated under aquaculture conditions [137]. In addition to growth differential, there are several additional reasons for monosex culture, including greater uniformity of harvest size, reducing the energy cost of gonad development, and aggressive interaction.

Large-scale monosex production of gonochoristic fish involves four major procedures: induction of sex reversal, identification of sex-reversed individuals, population expansion of sex-reversed individuals, and monosex production (Figure 1.3).

Nowadays, with the increase of aquaculture industries, growth-improved lines are available in many commercially important species. Therefore, monosex production and genetic improvement need to be combined in order to maximize benefits. There are hundreds of fish species for which monosex production could be advantageous, but sex-linked markers (SLMs) have been identified in very few fishes. Therefore, so far, progeny testing is the only way to distinguish sex-reversed individuals in most species when SLMs are not available.

Here, we propose an approach that could reduce the period of monosex production (Figure 1.3). We describe a program of inducing sex reversal in a F_2 generation, while the F_1 generation is being progeny tested, and prior to knowing the genotype of the F_1 individuals. These apply to all-male or all-female production protocols, regardless of sex determining mode (XY or ZW, Figure 1.3). The induction of sex reversal could also be conducted from the majority of the F_3 generation, when F_2 generation is being progeny tested, to enlarge the population of sex-reversed superfemales (YY females) or sex-reversed supermales (WW males). This proposed approach requires only one additional generation, compared to the approach with available SLMs, regardless of whether all-male or all-female stocks are the goal, or the SD system (XY or ZW, Figure 1.3) of the species; however, more labor and facilities are needed.

There have been several excellent reviews about sex control in fish recently [14, 15, 138] for some selected species. Here we describe the entire process using four basic procedures through some schematic diagrams (Figure 1.3) and forecast some cutting-edge technologies that can be applied in large-scale monosex production.

1.3.1.1 Sex Reversal

So far, 27 sex-reversal chemicals, including steroids, steroid enzyme inhibitors, and steroid receptor antagonists, have been applied for feminization or masculinization in more than a hundred different fish species, in order to produce a monosex population directly or indirectly. Besides the 22 steroids summarized by Baroiller *et al.* [139], one steroidal aromatase inhibitor, Exemestane, and three steroidal inhibitors, including Fadrozole, Letrozole, Anastrozole, and one steroid receptor antagonist, Tamoxifen, have been shown to be effective in sex reversal, suggesting that any interference in the steroid signaling pathway could result in sex reversal.

Five factors need to be considered before chemical treatment: method of administration, chemical; concentration; starting time; and

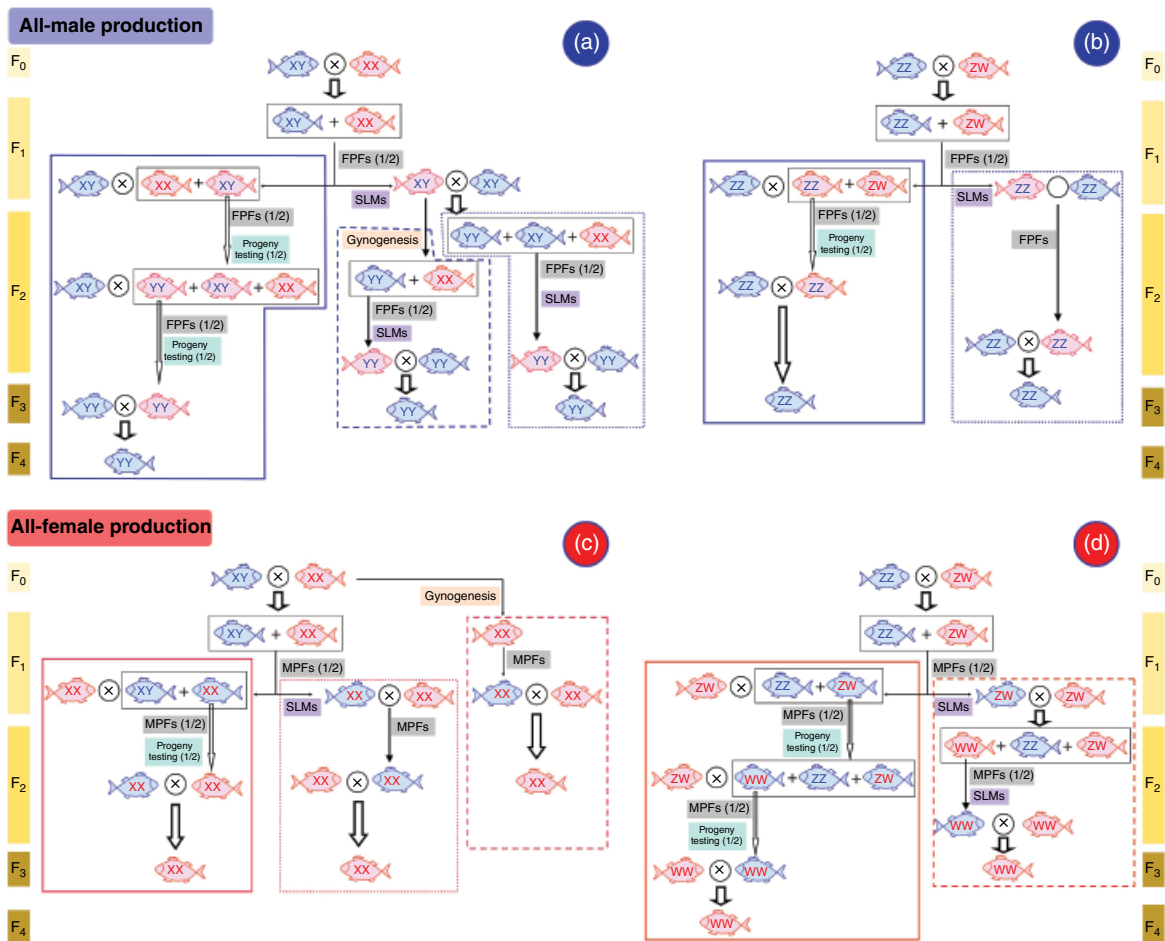


Figure 1.3 Workflow of large-scale monosex production in fish with a XY or ZW sex determination system.

MPFs, male producing factors; FPFs, female producing factors (the factors could be environmental factors such as temperature, exogenous hormones, etc.); SLMs, sex-linked markers.

A: all-male production in fish with XY sex determination.

B: all-male production in fish with ZW sex determination.

C: all-female production in fish with XY sex determination.

D: all-female production in fish with ZW sex determination.

F₀, F₁, F₂, ..., represent parental generation, 1st generation, 2nd generation, etc.

duration of treatment. Immersion and dietary treatments are appropriate for commercial practice. Concentration, starting time, and duration of treatment are dependent on the species and the age/size of sex differentiation. A histological study must be conducted to identify the size and age of gonadal differentiation, otherwise treatment is only “shooting in the dark”, depending on empirical results.

The age of sexual maturity can be used as a rough proportional estimate as to the pattern of gonadal differentiation – later-maturing species, such as Chinese carps and Acipenseriforms, take months to years, whereas common carp, tilapias and so on, differentiate earlier and at a small size. Administration through feed is the most widely used method for sex reversal, while immersion is more suitable for those species in which the most sensitive period occurs prior to the initiation of external feeding, or if formula feeds are not accepted by larvae (e.g., the live fish-eating carnivore *Siniperca sp.*), or with other specialized dietary habits, such as filter feeding.

The use of live feed (e.g., artemia) or fish (frozen or live) as a vehicle for steroids has been investigated in some fish species [9], and is considered a promising alternative to immersion treatments. Fabricated or more sophisticated means of controlled release implants are applicable for species with peculiar feeding habits and those whose gonads differentiate at a larger size, such as silver carp (*Hypophthalmichthys molitrix*), grass carp, paddlefish, and sturgeon.

Speaking of the usage of chemicals, the appropriate timing and duration of treatment can allow successful sex reversal and, meanwhile, reduce chemical usage. As illustrated in Figure 1.2, the most sensitive period frequently locates prior to the first signs of morphological gonad differentiation. Therefore, chemical administration should be started before the first signs of morphological gonad differentiation, and continued until after a short period when sex is differentiated. Interestingly, experiments also found that a very brief immersion

treatment of androgens for several hours in Nile tilapia larvae produced 100% males [140, 141], suggesting that we do still have room to minimize the usage of chemicals via optimizing protocols.

In addition, recent work has found that differentiated ovaries were transdifferentiated by longer treatment of the aromatase inhibitor Fadrozole (after 25 days post-hatching, Figure 1.2), and 100% of ovaries were induced to become functional testes [142]. This new finding provides a promising approach for those species where training to a formula diet is not fully successful (e.g., low survival) in larval stages when sex is differentiating.

Sex reversal through regulating the rearing conditions might be considered a more ecologically friendly method for large-scale monosex production. However, the thoroughness or completeness in terms of actual single-sex populations should be demonstrated on a commercial scale. As displayed in Figure 4.2 of Chapter 4 in this book, more males are produced when larvae are subjected to several stress conditions, including high temperature, hypoxia, bright background color, acid pH, higher social interactions (e.g., high density), and low food availability. High temperature, acidic water, or bright tank color can produce mostly (or close) males in several species, including tilapia, Japanese flounder, southern flounder, swordtail (*Xiphophorus helleri*), and black-belly limia (*Poecilia melanogaster*) [65]. Therefore, the effects of environmental conditions on sex ratio in fish species in which monosex is strongly desired need to be addressed extensively, in order to produce monosex population via environment- and consumer-friendly approach.

In addition to these traditional approaches for sex reversal, progress in gene editing technology in recent years provides a promising alternative to eliminate hormone usage in large-scale monosex production. Moreover, it has been observed that the ovaries of sex-reversed (estrogen-induced) YY females were damaged, and did not generate normal eggs in some fish species [143];

dosages which are too high can have a sterilizing effect, and the efficacy of estrogen treatments is not as predictable as that of androgen treatment. It is expected that fertile YY females can be produced by using gene editing techniques, although this has yet to be demonstrated. Loss-of-function mutants of several genes involved in the pathway in sex determination, sex differentiation, or steroidogenesis (e.g. *dax1*, *cyp19a1a*, and *bmp15* in zebrafish, *dmy*, *foxl3*, and *R-spondin1* in medaka, *foxl2*, *sf-1*, *amhy*, and *amhr2* in Nile tilapia) lead to masculinization or feminization, and the sex-reversed individuals are fertile [21, 30, 143–146].

We would strongly recommend that alternative protocols should be established for large-scale monosex production, so as to minimize or eliminate chemical treatment. Currently, the synthetic hormone 17 α -methyltestosterone (MT) has been used for direct masculinization for several decades in some aquaculture species (e.g., Nile tilapia) and many ornamental fishes [140, 147–151]. In the United States, because the drug is strictly controlled, use of MT in food fishes must be done under a government-managed INAD (Investigational New Animal Drug), but use for masculinizing ornamental fish is less

regulated. The use of MT for this purpose is clearly only for esthetics, and is not necessary. We urge that this type of steroid application should be well-controlled worldwide.

1.3.1.2 Identification of Sex-Reversed Individuals

Sex-reversed fish have reproductive morphology largely unaltered by the treatment, with few exceptions (see following section). For several decades in monosex production, progeny testing has been used to identify the genotype of sex-reversed individuals. Figure 1.4 displays the schematic diagram, which is also a means of identifying the sex determining mode in a given species. Progeny testing is the most challenging work in the whole process of large-scale monosex production:

- 1) it takes from a few months to more than a year before the sex of the sex-reversed progeny can be identified;
- 2) physiological and morphological characteristics of sex-reversed fish in reproductive systems are usually different from regular same-sex fish, and it is frequently observed that they have difficulties in spawning (e.g., bluegill, tilapia, yellow perch, European perch);

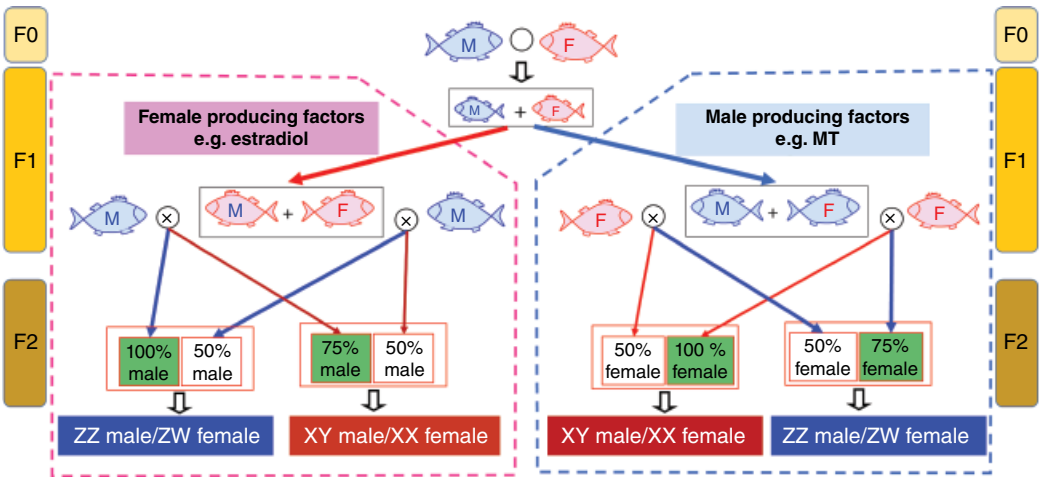


Figure 1.4 Progeny testing – identification of sex determining mode and sex ratio. M, male; F, female; MT, 17 α -methyltestosterone. F0, F1, F2, represent parental generation, 1st generation and 2nd generation, respectively.

- 3) more facilities and labor are required largely because of the requirement to maintain strict group identity. Therefore, development of molecular markers is particularly important so as to shorten the whole process.

SLMs are useful for identification of sex-reversed individuals from hormone-induced monosex population. SLMs have been identified in more than 20 fish species, and have been applied in monosex production and related research [15]. Many techniques, including AFLP (amplified fragment length polymorphism), SNP (Single Nucleotide Polymorphisms), RAPD (random amplified polymorphic DNA), SSR (simple sequence repeats), QTL (quantitative trait locus), and genomic sequencing have been employed for identification of SLMs. The identification of sex-linked markers depends on the frequency of the genome cuts by the restriction enzyme, the divergence level (or the size of the non-recombining portion of the Y or W chromosome) between sex chromosomes (X and Y, or Z and W), and the complexity of the sex determining mechanism (e.g., polygenic sex determination).

In recent years, restriction site-associated DNA (RAD) sequencing has been used to identify SLMs in several species [152–159]. There are some advantages of identifying SLMs via RAD-seq, compared with using microsatellites or AFLP. First, the sequence data created by RAD-seq allow for rapid generation of PCR primers and subsequent validation of SLMs. Second, if any of the restriction enzyme fails to identify a sex-linked marker, switching to another enzyme that cuts more frequently in the genome can be an option. Last but not least, the data obtained via RAD-seq, combined with genomic resources in the future, will be helpful for characterizing SLMs [155] and sex chromosomes. RAD-seq has the potential to rapidly screen large numbers of fish species to identify SLMs, and subsequently use them in monosex production. In addition, it will accelerate the identification of sex

determining mechanisms, facilitate comparative analysis of sex chromosome evolution across a wide range of animals, and spark a rapid turnover of sex determining mechanisms in closely related species.

Some subtle distinctions in morphology or physiology between sex-reversed and normal gonads have been observed. Yellow perch, in which females grow faster and reach larger sizes than males, display a XX/XY sex determining mode. Normal females have a single ovary, while males have paired testes. However, sex-reversed males (XX males) have a single testis, and this characteristic has been useful in all-female perch production (See Chapter 20). This altered feature has been found in Eurasian perch as well [16, 17, 160, 161].

We found that the testes in sex-reversed individuals (XX testes) could be readily differentiated from the testes in normal or untreated fish (XY testes), because of their irregular morphological characteristics: unsmooth surface and cyst-like structure (Figure 1.5). However, we have to dissect the fish to identify a reversed individual. Ultrasound examination was tested to distinguish possible differences between these different types of testes, but it failed. Nevertheless, the unique gonads in perch have already accelerated all-female production in these two perch species. Appearance differences between reversed gonads and normal gonads may also exist in many other species. The discovery of these differences will accelerate large-scale monosex production when SLMs have not been identified, or do not exist.

1.3.1.3 Population Expansion of Sex-Reversed Fish

As mentioned earlier, population expansion of sex-reversed fish can start when the genotype of individuals undergoing a progeny test is unknown. Theoretically, in a treated mixed sex group, about 50% of the individuals will be sex-reversed. Taking all-male production in an XX/XY system (Figure 1.3A) as an example, the progeny of individual A in the F_1 generation can be divided into two

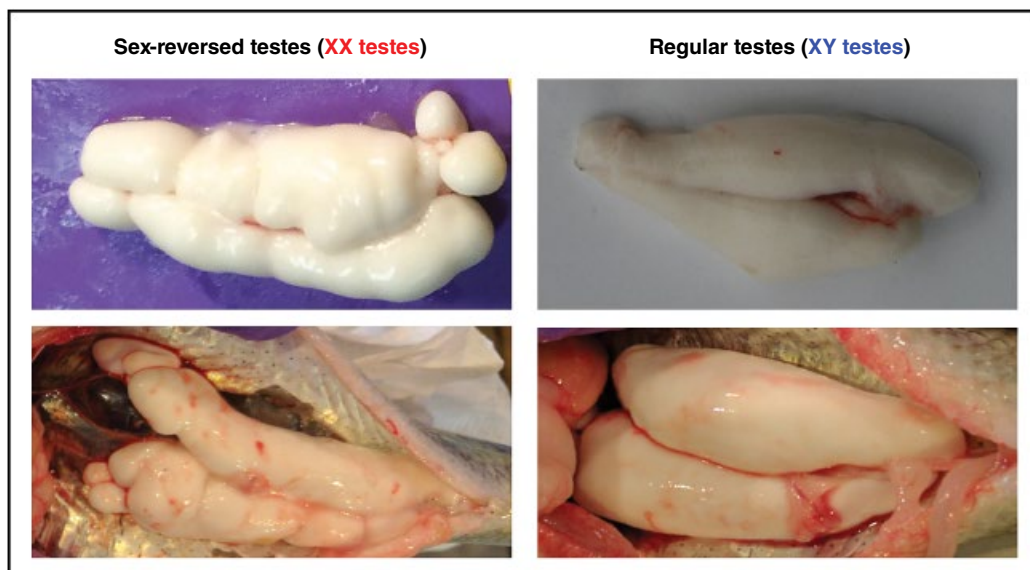


Figure 1.5 Morphological difference between the testes of neomales (XX-males) and regular males (XY-males) of yellow perch. (See inserts for the color representation of this figure.)

Sex-reversed testes (left) are characterized by rough surface, cyst-like structures, indivisible single part, which have never been observed in regular testes.

batches. One batch is treated with female-producing factors (FPFs, e.g. 17β -estradiol), and another batch is raised until the sex ratio can be identified. Once the sex ratio of individual A is identified, the sex-reversed batch (progeny of XY female) in the F_2 generation either can be recruited for the following production, or discarded (progeny of XX female). Similarly, most progeny from an individual in the F_2 generation can be treated with FPFs. In this way, there will be YY neofemales for all-male production when they reach sexual maturation. The proposed approach can shorten the entire process of large-scale monosex production by several months, or even several years, depending on how long it takes before sex can be identified in the given species.

Chromosome manipulation (gynogenesis or androgenesis) is a suboptimal alternative for population expansion of sex-reversed fish. Taking all-male production in an XX/XY system (Figure 1.3A) as an example again, gynogenesis can be applied for eggs produced by XY neofemales, so as to produce

YY supermales and YY neofemales [13]. Application of gynogenesis in this case can save facilities and labor, but cannot actually accelerate large-scale monosex production when compared to the abovementioned protocol. However, gynogenesis for all-female production in an XX/XY system, and androgenesis for all-male production in a ZW/ZZ system, could reduce the process by one generation (or one spawning cycle). From a practical point of view, variation in survival, induction rate, growth, and fertility of chromosome-manipulated fish is the most important impediment for its application in sex control. Importantly, damage and mutations induced by irradiation, pressure or temperature shock, or chemical treatment and their negative influence on growth and performance of following generations, cannot be neglected [162, 163].

1.3.1.4 Integration of Monosex Production with Genetic Selection

The final step of large-scale monosex production is relatively simple, such as mating

YY neomales with XX females (for commercial production), or with YY neofemales (for sustainable production), or ZZ neofemales with ZZ males. From a developer's point of view, they would like to maintain higher economic benefits through selling XY all-male or ZW all-female populations, rather than YY or WW individuals (Figures 1.3A and 1.3D). For all-male production in species with a ZW/ZZ system (Figure 1.3B), or all-female production in species with an XX/XY system, they may want to sell all-male ZZ fry or all-female XX fry until sex differentiation is completed.

Currently, all-female eggs for rainbow trout and coho salmon are commercially available. The availability of all-female eggs (XX) will allow new developers to catch up within one generation, through masculinizing the XX fry and mating them with regular XX females when they reach maturity. While rearing a monosex population can have many benefits (Table 1.2), we strongly suggest integrating genetic selection into monosex production, starting at the very beginning.

1.4 Sex Control Practices in Fisheries

Non-native fishes have resulted in problems in many parts of the world; they create both an economic burden and a threat to the environment [164, 165]. Asian carps (grass carp, silver carp, bighead carp, and black carp) in North America are problematic [166], and it seems that it is difficult to eradicate them in natural water systems at this stage. The practice of producing triploid sterile grass carp in the United States is a developed industry that has been operating for sex control in fisheries for decades (see Chapter 41). Several theoretical proposals for population control by using the Trojan sex gene approach suggest some potential. Herein, we summarize the potential application of the Trojan sex genes.

The fertility of sex-reversed fish, YY supermales, and WW superfemales have been demonstrated for several fishes. There

appears to be no serious difference between the sperm of sex-reversed and wild-type males; systematic review and meta-analysis of the literature that compares sperm characteristics of these two types of males indicates that sex-reversed individuals may be comparable to normal individuals in reproduction if they enter natural water [167]. None of the sperm traits, including total motility, progressive motility, curvilinear velocity, straight-line velocity, average path velocity and linearity, significantly differ between XX neomales, XY regular males, and YY supermales in tilapia [168]. Thus, individuals with these atypical genotypic-phenotypic combinations can spawn and produce viable offspring.

This viability issue is the primary basis on which the Trojan sex genes can be employed to control invasive species. However, developing these unique individual fish in sufficient numbers for release is the real challenge. Since the beginning of this century, several theoretical works predicted that a certain amount of introduction of XY females or YY genotype fish via environmental sex reversal in natural water, with intentional or unintentional release, could cause extreme male-biased sex ratios, and lead to the eventual eradication of a given population [69, 119, 121, 122, 125–127, 165, 166, 170]. However, no experimental verification has been reported.

Medaka and Nile tilapia might be good model species for demonstration of this theory. They have a short reproductive cycle, well-developed husbandry and handling technologies, a known sex determining gene, strong adaptability to limited living space, and should serve as an excellent species to test the consequence of introduction of XY female or YY female genotypes into natural water.

However, the consequence of releasing these fish into natural water is unpredictable, similar to the stated impacts of introducing non-native species into natural ecosystems. Furthermore, both studies have shown that new sex determining mechanisms (e.g., TSD)

can be evolved rapidly [66, 69, 175], and transition between GSD and TSD can occur rapidly and readily [45, 68, 175, 176], suggesting that the target population may evolve a new sex determining mechanism and live and multiply. On the other hand, the consequence of releasing an atypical phenotype is species-specific, and depends on many parameters [126]. Therefore, theoretical assumption, or the experimental verification in model species, may still be a suboptimal proxy for a given species.

1.5 Future Perspectives

1.5.1 Population Level-Based Identification of Sex Determining Mechanism

Fish are well adapted to their environments, and have evolved condition-specific characteristics, including sex determining mechanisms. Distinct sex determining modes in the same fish species have been reported, including Atlantic silverside *Menidia menidia* [66], Nile tilapia [119, 135, 177], zebrafish [178], rainbow trout [134], European sea bass [179], and bluegill [180–182]. A recent study suggests that domestic strains of zebrafish had lost their natural sex-determination system, but may have evolved different sex determining mechanisms during two decades in laboratory culture [178]. These studies suggest that the identification of the sex determination mechanism in a given fish species, based solely on laboratory studies, may not necessarily represent the situation of the wild population.

1.5.2 Targets of Sex Determining Factors and Molecular Network Involved in Sex Differentiation

Immediate targets of sex determining genes or temperature have not been identified, although several genes involved in sex differentiation have been found to be sensitive to temperature conditions, including *foxl2*, *cyp19a1a*, *dmrt1*, *amh*, *sox9*, both in species

with GSD + TE and TSD [5, 89, 90]. Male-producing temperatures increased the *cyp19a* promoter methylation levels, resulting in suppression of the aromatase gene and temperature-induced masculinization [75–77]. This suggests that DNA methylation could act as a key mediator integrating temperature into a molecular trigger that determines sex in thermosensitive species.

Sex is considered as a threshold phenotype, based on a sex differentiation network as soon as sex determining factors have taken action [183]. Interacting gene networks involve a complex interplay between different signals, and contribute to differentiate sexually undifferentiated gonads into ovaries or testes. The genome editing tools will be useful to uncover the complex networks involved in sex differentiation. Especially, knockdown/knockout/overexpression of *cyp19a* gene in species displaying different sex determining modes will shed light on the networks, since it codes the key enzyme for the synthesis of estrogens. This is the connecting link between the upstream gene *foxl2* and estrogens (Figure 1.2), and may link the environmental factors and sex ratios.

1.5.3 Environmental- and Consumer-Friendly Monosex Production

There is currently no alternative to the use of hormones for large-scale monosex production (Figure 1.3). The residues and metabolite of hormones can enter into the ecosystem in various ways, even though the market fish have not been directly exposed to hormones. In order to minimize the use of hormones, researchers need to make best use of environmental (e.g., temperature, background color, and pH) sex differentiation, as observed in many fish species, and could select sensitive lines or use temperature-induced sex-reversed individuals in monosex production (Figure 1.3; and see Chapter 4). In addition, progress in gene editing technology in recent years provides a promising alternative to eliminate hormone usage in large-scale monosex production, and should be extensively evaluated.

On the other hand, attention should be paid to the management of a sex-reversed population containing perhaps atypical genotypes, considering the genetic risk of releasing these populations [171].

1.6 Conclusions

Establishment of phenotypic sex is triggered by SD factor(s), modulated by complex molecular networks, and influenced by environmental conditions, steroid hormones, and endocrine-disrupting chemicals. Sex determination in fish is much more complex than we ever thought. NGS techniques and genome editing technologies can help find SD genes, molecular players involved in sex differentiation, and SLMs, which can be beneficial for our understanding of the diverse sex determination in fish and can play a key role in speeding up large-scale monosex production.

Large-scale monosex production can be achieved in the fourth generation when no SLMs are available, irrespective of whether it is

an XY or ZW SD system. There is a good potential for producing large-scale breeding systems for females in much less time if gynogenesis and sex reversal of XX-female are combined. Sex control achievements, such as atypical genotypes YY-neomales and WW-neofemales, can serve as biological tools to control invasive species. However, the risk should be comprehensively assessed in a species-specific manner. Researchers should take full advantage of environmental sex differentiation and gene editing technologies, in order to produce monosex populations with an environmental- and consumer-friendly approach.

Acknowledgments

This work was supported by the U.S. National Oceanic and Atmospheric Administration (NOAA) and Ohio Sea Grant. Salaries and research support were provided by state and federal funds appropriated to The Ohio State University, Ohio Agricultural Research and Development Center.

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2

Sex Determination and Differentiation in Fish: Genetic, Genomic, and Endocrine Aspects

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2.1 Introduction – Sex Determination in Fish: from Sex Control Applications in Cultured Fish Species to Basic Science Interests

In stark contrast to traditional ovine, bovine, or porcine animal production, most of the farmed fish species, due to the recent expansion of aquaculture, are only slightly domesticated [1]. While genetic improvement, as it is in other, more mature forms of animal production, could be also considered as a conventional contributing factor in developing efficient fish farming, it has only recently been applied to just a handful of species (e.g., common carp, *Cyprinus carpio* [2]; Atlantic salmon, *Salmo salar* [3, 4]; channel catfish, *Ictalurus punctatus* [5]; rainbow trout, *Oncorhynchus mykiss* [6]; European sea bass, *Dicentrarchus labrax* [7, 8]; turbot, *Scophthalmus maximus* [9] and Nile tilapia, *Oreochromis niloticus* [10]; see [11] for review).

While the number of fish species suitable for aquaculture is steadily increasing worldwide, nearly all farmed aquatic species are still very similar to their wild relatives [12]. As a matter of fact, most fish did not, and still do not, benefit from a deep basic

understanding of their biology, often precluding the development of a rational and sustainable aquaculture. To this end, efforts should converge toward an improvement of production efficiency (growth, disease resistance, fertility) and adaptation to new contexts (fluctuating environment, pollution, density).

Into that direction, control of sex is an important issue of modern aquaculture, as it allows the mass production of either all-female or all-male populations of fish, which are often economically more valuable to breed than normal mixed-sex populations. In salmonid species, for instance, all-female populations are often preferred, because males have the propensity to mature precociously, resulting in reduced growth rates, lower food conversion efficiency, lesser flesh quality and a high sensitivity to pathological problems. In other fish species, sex control can either:

- i) facilitate broodstock management – for instance by optimizing the ratio between males and females in hermaphrodite species;
- ii) prevent uncontrolled reproductions that favor energy investment into the gonad instead of body growth, as in the tilapias; or

- iii) allow the production of a sex-specific product, like the caviar in female sturgeons.

A better knowledge on fish sex determination and sex differentiation mechanisms is then a prerequisite toward a more rational and efficient control. This is especially needed for fish, as the number of farmed fish species is important, and diversification is still a current challenge in many countries.

As sex determination systems, genetic sex determinants, and even their downstream regulations, are not well conserved, the transfer of a sex-control technique from one species to another is often problematic. Even considering species in which biotechnologies are available that allow the mass production of all-male or all-female populations, a better knowledge of the sex determination and sex differentiation would still allow a potential improvement of the current biotechnologies toward more sustainability (see terminologies in Boxes 2.1 and 2.2 of Chapter 1).

Sex can be determined by different mechanisms. First, signals can be purely genetically driven (**genetic sex determination**, GSD), where the presence or absence of genetic factors decides whether the undifferentiated gonad anlage will become a testis or an ovary. Second, the main signals can come from the environment (**environmental sex determination**, ESD), and these signals could be temperature, water quality (pH, oxygen), or other “external” signals including social factors (dominance), size, or age. Importantly, these different mechanisms do not follow any evolutionary pattern, indicating that they evolved repeatedly and independently. Such a diversity of SD mechanisms is especially obvious in fish, with closely related species relaying on different GSD or ESD systems. However, despite huge efforts in the last decades, our knowledge of the diversity of sex determination and the evolution of master genes controlling genetic sex determination remains limited in fish.

2.2 From Genetic Sex Determination to Environmental Sex Determination and the Other Way Round

Although sexual dimorphism is probably the most penetrant feature of animal physiology, morphology, and behavior—at the same time—it is incredibly plastic. Despite the *quasi*-universality of that phenomenon itself, the different mechanisms of how sex is determined are very diverse among various organismic groups. Hence, across metazoans, such underlying molecular pathways tend to evolve recurrently and independently, and adjust and adapt during the course of evolution. As a result, initial molecular trigger(s) or regulation of the gene regulatory network(s) leading to sex determination and gonadal development and differentiation can be significantly different among closely related groups, despite sharing undistinguishable morphological, histological or cell biological gonadal features. The evolutionary triggers favoring/allowing such a high molecular degree of plasticity of an otherwise disarmingly common developmental program are unknown.

In birds and mammals, the same genetic sex determination systems are shared within all species (XX/XY in mammals and ZZ/ZW in birds), with only puzzling exceptions [13, 14]. In contrast, similar to the situation in amphibians and reptiles, the diversity of sex determination systems is especially obvious in fish where, within groups of closely related species, a wide spectrum of different systems can be found (Table 2.1; and see also Figure 2.1 and [15] for review). Interestingly, among each type, a multitude of mechanisms of how to spark either male or female gonadal development have been described. Practically, combinations – at various degrees – of the different systems (GSD and ESD) are also frequently observed (see [16–18] for reviews).

In fish species with GSD, all possible variations on the theme have been found. Such variations extend from “classical” male or

Table 2.1 Master sex-determining genes in vertebrates, evolution and diversity in fish.

Master Sex-Determining Gene	Organism	Sex-Determination System	Sex-Determining Gene Ancestor	Establishment	Ancestor Gene Function
<i>SRY</i>	Therian mammals	XY	SOX3	Allelic Diversification	Transcription factor. Expressed in developing gonads. Required for formation of the hypothalamo-pituitary axis and neuronal differentiation
<i>DMRT1</i>	Birds	ZW	<i>Dmrt1</i>	Allelic Diversification	Transcription factor. Expressed in the developing gonads. Major role during male sex determination, differentiation and maintenance.
<i>DM-W</i>	African clawed frog (<i>Xenopus laevis</i>)	ZW	<i>dmrt1</i>	Gene Duplication	Transcription factor. Expressed in the developing gonads. Major role during male sex determination, differentiation and maintenance.
<i>dmrt1bY</i>	Medaka (<i>Oryzias latipes</i>)	XY	<i>dmrt1</i>	Gene Duplication	Transcription factor. Expressed in the developing gonads. Major role during male sex determination, differentiation and maintenance.
<i>gsdf-Y</i>	Luzon rice fish (<i>Oryzias luzonensis</i>)	XY	<i>gsdf</i>	Allelic diversification	TGF-β-related factor. Important role during fish gonadal development.
<i>sox3-Y</i>	Indian rice fish (<i>Oryzias dancena</i>)	XY	<i>sox3</i>	Allelic Diversification	Transcription factor. Weakly expressed in developing gonads.
<i>sdY</i>	Rainbow trout (<i>Oncorhynchus mykiss</i>)	XY	<i>irf9</i>	Gene Duplication	Interferon response factor. No known gonadal function.
<i>amhbY</i>	European pike (<i>Esox lucius</i>)	XY	<i>amh</i>	Gene Duplication	Anti-Muellerian hormone. Expressed in the developing gonads. Important role during male gonadal differentiation.
<i>amhY</i>	Pejerrey (<i>Odontesthes hatcheri</i>)	XY	<i>amh</i>	Gene Duplication	Anti-Muellerian hormone. Expressed in the developing gonads. Important role during male gonadal differentiation.
<i>amhr2-Y</i>	Torafugu (<i>Takifugu rubripes</i>)	XY	<i>amhr2</i>	Allelic Diversification	Type II receptor for anti-Muellerian hormone. Expressed in the developing gonads. Important role during male gonadal differentiation.
<i>dmrt1</i>	Chinese tongue sole (<i>Cynoglossus semilaevis</i>)	ZW	<i>dmrt1</i>	Allelic Diversification	Transcription factor. Expressed in the developing gonads. Major role during male sex determination, differentiation and maintenance.
<i>gsdf-Y</i>	Sablefish (<i>Anoplopoma fimbria</i>)	XY	<i>gsdf</i>	Allelic Diversification	TGF-β-related factor. Important role during fish gonadal development.
<i>gdf6-Y</i>	Killyfish (<i>Nothobranchius furzeri</i>)	XY	<i>gdf6</i>	Allelic Diversification	TGF-β-related factor. Vertebral segmentation; cell differentiation. No known gonadal function.

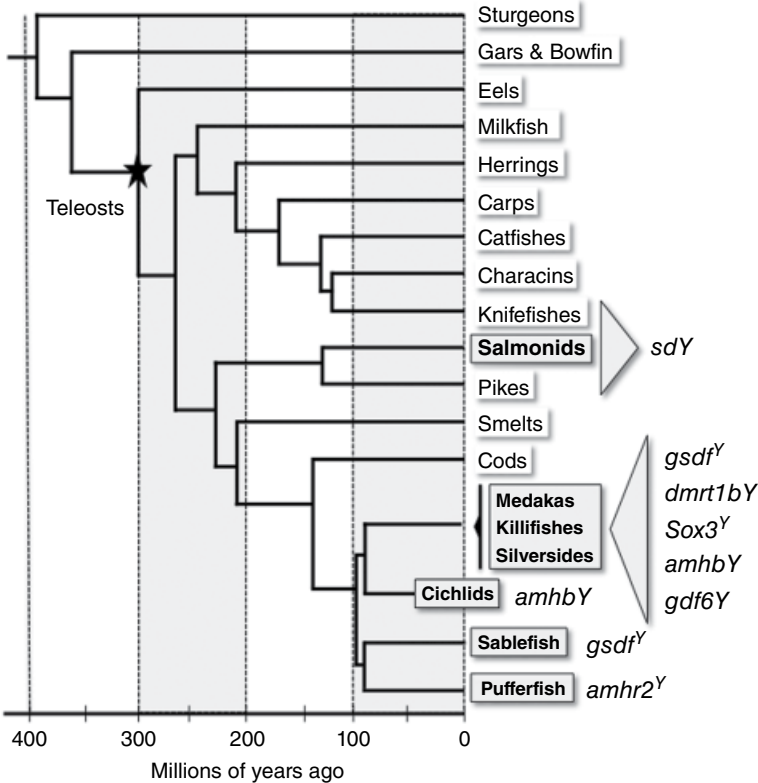


Figure 2.1 Diversity and evolution of master sex-determining genes in fish. Fish are particularly well suited for study of the evolution of sex determination and sex differentiation genes. Representing over half of the approximately 60,000 vertebrate species, they also display the greatest variety of master sex-determining genes and mechanisms. Interestingly, these different mechanisms do not follow any evolutionary pattern, indicating that they likely evolved repeatedly and independently. Species or phyla for which a master sex-determining gene has been revealed are indicated in bold. The star represents the position of the teleost-specific whole-genome duplication. See Table 2.1 for more details of each type of sex-determining gene.

female heterogamety to polygenic, multiple sex chromosomes (different Y- or X- chromosomes) or autosomal modifiers enhancing/antagonizing the sex-determining gene on gonosomes [17, 19–21] (Figure 2.1). Within teleosts, for instance, in the poeciliid fish (which include guppies, mollies, platyfish and swordtails), there are reports on temperature-dependent SD and various forms of GSD, ranging from polyfactorial SD to female and male heterogamety, multiple sex chromosomes and autosomal modifiers ([22, 23]; and see Chapters 1 and 4). Even within the same species, several SD mechanisms can occur [21]. The coexistence

of two or more of these systems has also been reported within the same genus – for instance, the XY/XX and ZW/ZZ genotypes that are found in different *Oreochromis* [24], rice fishes [25] or sticklebacks [26], or even within the same species, as in some platyfish populations, or some cichlids [21]. Interestingly, such a high diversity regarding the systems of sex determination is also observed when both GSD and ESD coexist and modulate each other (see Chapters 1, 4 and 17). Hence, it has been shown that in the “Atlantic silverside (*Menidia menidia*), a fish with temperature-dependent sex determination, populations at different latitudes

compensate for differences in thermal environment and seasonality by adjusting the response of sex ratio to temperature, and by altering the level of environmental as opposed to genetic control” [27].

Similarly, laboratory strains of zebrafish (*Danio rerio*) exhibit a polyfactorial system involving at least four different chromosomes (i.e., Chapters 3, 4, 5, and 6) [28], as a result of the loss of the tip of chromosome 4, harboring the primary sex-determining gene, during the process of domestication. Hence, they have become more susceptible to environmental modulators (gamma rays [29], hypoxia [30], high density [31] or temperatures [32, 33], altered thermocycles or poor nutrition [34, 35]) for sex determination. One step further, this is reminiscent of the situation observed in the Japanese medaka, which has an even stronger genetic sex determination system, but is still sensitive to environmental conditions (temperature [36, 37]) (Figure 2.1).

2.2.1 Genetic Sex Determination: “Usual Suspects, Newcomers, and Usurpers”

Following is a short review of the different actors or pathways that have been demonstrated to be strong genetic triggers (sex-determining genes) for sexual development in fish. The most classical ones, referred below as “usual suspects,” are those that have been pre-empted from known actors of the sex differentiation regulatory gene network. Most of the currently known fish sex-determining genes actually fall into this “usual suspect” category, with *dmrt1bY* in the Japanese medaka (Table 2.1) being the archetype and first described one [38, 39], but newcomers have also been recently described in the “usual suspect” category (see [40] for review). Right now, a single exception (or “usurper”) to that rule has been found with the recent discovery of the conserved sex-determining gene of salmonids [41]. Whether these “usurpers” are exceptions to a “usual suspects” rule, or just more difficult to characterize, remains to be solved.

Recent “omics” approaches may help to get a deeper and more precise evolutionary view of sex-determining genes in fish [42].

2.2.1.1 *Dmrt1*, the First Described “Usual Suspect” Sex-Determining Gene

Among the highest evolutionarily conserved and widespread factors unequivocally involved in sex determination, differentiation, or maintenance of genetic cascades in the whole animal kingdom are the *dmrt1* transcription factors. Being able to substitute for each other across species, the *dmrt1* factors are the inevitable key players of the sexual gene regulatory networks regulating various aspects of sexual dimorphism. Hence, male-restricted, or at least strong male-biased, expression of *dmrt1* has been reported in numerous fish, including the Japanese medaka (*Oryzias latipes*), rainbow trout, Atlantic cod (*Gadus morhua*), Nile tilapia, African catfish (*Clarias gariepinus*), rare minnow (*Gobiocypris rarus*), European pike (*Esox lucius*), olive flounder (*Paralichthys olivaceus*), lake and shovelnose sturgeons (*Acipenser fulvescens* and *Scaphirhynchus platyrhynchus*, respectively), and southern catfish (*Silurus meridionalis*) (see [43] for review).

Remarkably, in annual breeders gonochoric fish species, such as the southern catfish [44], the African catfish [45], or the rainbow trout [46], that alternate gonadal resting and recrudescence, rise in *Dmrt1* expression correlates with preparatory, pre-spawning, and spermatogenesis cycles, while decreased expression is observed during spawning or spermiation.

Hermaphrodite fish species (protandrous or protogynous) found a way to best exploit the high plasticity occurring during gonadal development and maintenance. Interestingly, in such species, the dynamic of expression of *Dmrt1* has been shown to constantly parallel either:

- i) testicular development in protogynous species (gilthead seabream, *Sparus auratus* [47], black porgy, *Acanthopagrus schlegelii* [48]); or

- ii) testicular regression in protandrous species (rice field eel, *Monopterus albus* [49], grouper, *Epinephelus coioides* [50], wrasse, *Halichoeres tenuispinis* [51]).

Notably, in *Odontesthes bonariensis*, a pejerrey species for which sex is determined by temperature, *dmrt1* expression again correlates with the rearing temperature, being up- or downregulated at male- or female-producing temperatures, respectively [52].

Being at the functional interface between sex determination and differentiation, *Dmrt1* is also one of the genes belonging to the sex-determining network that made it – repeatedly and independently – up to the top, most frequently in birds [53], African clawed frog, *Xenopus laevis* [54], and several fish species, including the Japanese medaka, [38, 39], the Malabar rice fish, *Oryzias curvirostris* [55], and the half-smooth tongue sole, *Cynoglossus semilaevis* [56] (see Table 2.1). In the Japanese medaka, *Dmrt1* took the leadership of the sex-determining cascade after a duplication event.

Remaining the only functional gene located onto the sex-specific region of the Y-chromosome, *Dmrt1BY* (also named *DMY*) has been demonstrated to be not only necessary, but also sufficient for triggering testicular induction and development [38, 39]. Reminiscent of the chicken's (*Gallus gallus*) case [53], in the half-smooth tongue sole, *dmrt1* locates on the Z-chromosome, while it is absent on the W [56] and, thus, its expression suggests it is acting as a dosage-dependent master male trigger.

In contrast, and while also lying on the W-chromosome in the African clawed frog, *Dmrt1* (as in the Japanese medaka) has been duplicated [56]. Truncated and lacking its dimerization domain, the African clawed frog *Dmrt1* has been proposed to act as a dominant negative protein, suppressing male development in ZW animals [56] (see Figure 2.2 for summary).

With respect to their molecular functions, *Dmrt1* proteins act as transcription factors

and recognize evolutionary conserved DNA target motifs [57, 58]. In that direction, it has been shown that *Dmrt1* transactivates various testicular genes while, on the other hand, downregulates ovarian genes ([59]; see Figure 2.2). Further on, targeted deletion of either *dmrt1* in mice [60, 61], or the autosomal *dmrt1a* in the Japanese medaka [62], have revealed a major role during gonadal maintenance, but not during primary sex determination.

Interestingly, in the Japanese medaka, a fish species for which *Dmrt1* has been recruited as a master sex-determining gene (see Table 2.1 and Figure 2.1), it has been shown that *dmrt1bY* is able to regulate germ cell proliferation shortly before the sex determination stage [63]. Thus, it is now emerging that *dmrt1*, depending on the cellular context, is able to tune the gene regulatory networks, either controlling early sexual differentiation or, later on, regulating and maintaining sexual identity [40, 59]. Figure 2.3 shows how different fish species and other organisms having different sex determination systems make use of the *dmrt1* factor as male sex-determining triggers.

2.2.1.2 From Mammalian *Sry* Back to *Sox3* in Fish

Sry is a conserved male master sex-determining gene in all therian mammals, with only a few exceptions to the rule – for example, in two species of rodents (the pygmy mouse, *Mus minutoides*, and the mole vole, *Ellobius*) that lack *Sry* as the master sex-determining gene [14, 64]. Interestingly, comparative molecular, cytogenetic, and now functional studies suggest that *Sry* probably arose after *Sox3* transcriptional rewiring and neo-functionalization (see [40] for review). *Sox* family proteins are transcription factors, displaying an evolutionarily conserved DNA binding domain (the high mobility group or HMG box), flanked by weakly preserved N- and C- terminal transactivating domains. In line with a potential ancestral role during sex determination/

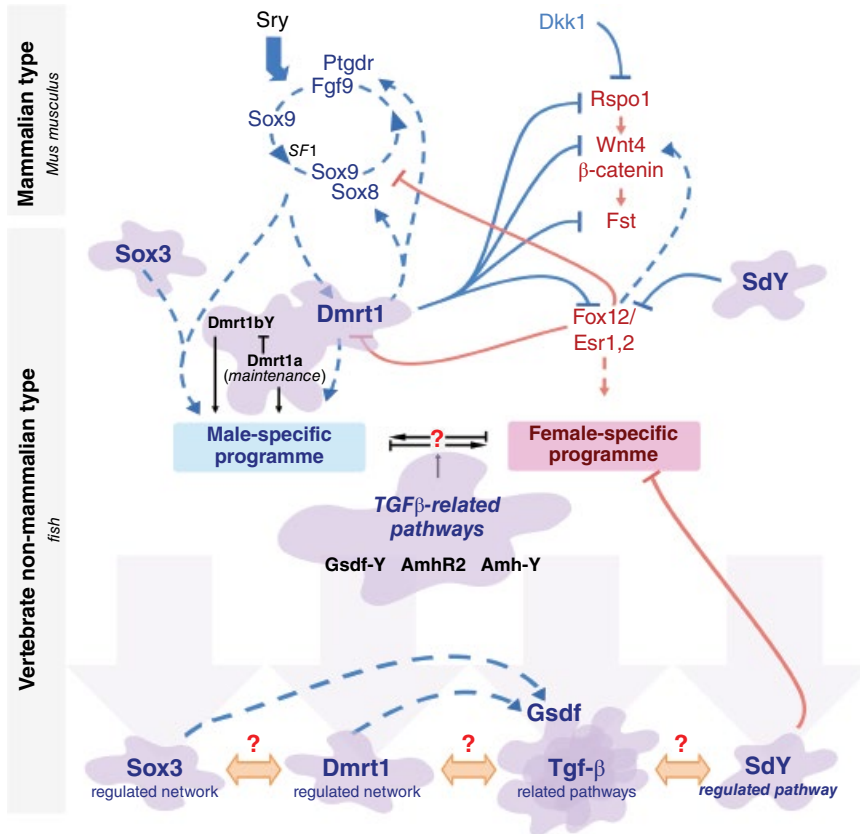


Figure 2.2 In fish, diverse genetic triggers modulate the vertebrate canonical gonadal sex-induction gene regulatory network.

An unstable equilibrium between the conflicting male and female genetic pathways underlies the regulation of the somatic gonadal development toward either testis or ovary. Central to this balance is the mutual cross-inhibition between *Dmrt1* and *Foxl2*, two evolutionary conserved key factors of the male and female differentiation pathways, respectively. Fostering one or the other genetic pathway will momentarily regulate the somatic gonadal fate, also suppressing the counterpart genetic pathway. Master sex-determining factors (tache-shaped highlighted) are able to initiate such tipping of the balance. Positive regulations are represented with dashed lines, while solid lines represent negative regulations. Tache-shaped highlighted factors represent genes that have been recruited as master sex-determining factors in fish (*Sox3*, *SdY*, *Dmrt1*, *Dmrt1bY*, *GsdF-Y*, *AmhR2*, *Amh-Y*).

differentiation, the expression of *Sox3* has been constantly reported in the gonads of mice, chicken [65], frog [66], and fish [67].

It has recently been shown that *Sox3* is not only the evolutionary precursor of *Sry* in therian mammals, but has also been selected as the master sex-determining gene of the Indian rice fish, *Oryzias dancena*, on the Y-chromosome [67]. Indeed, positional cloning revealed that, due to the presence of a Y-specific *cis*-regulatory DNA sequence

nested within the sex-determining locus, expression the Y-chromosomal copy of *Sox3* was upregulated during male gonadal development [67] (Figure 2.4).

Further on, loss and gain of functions, resulting in either XX male or XY female sex reversions, respectively, has confirmed the primary role of *Sox3* as the master male sex-determining gene in the Indian rice fish [67]. Curiously, the sex-determining locus of the Indian rice fish – encompassing the

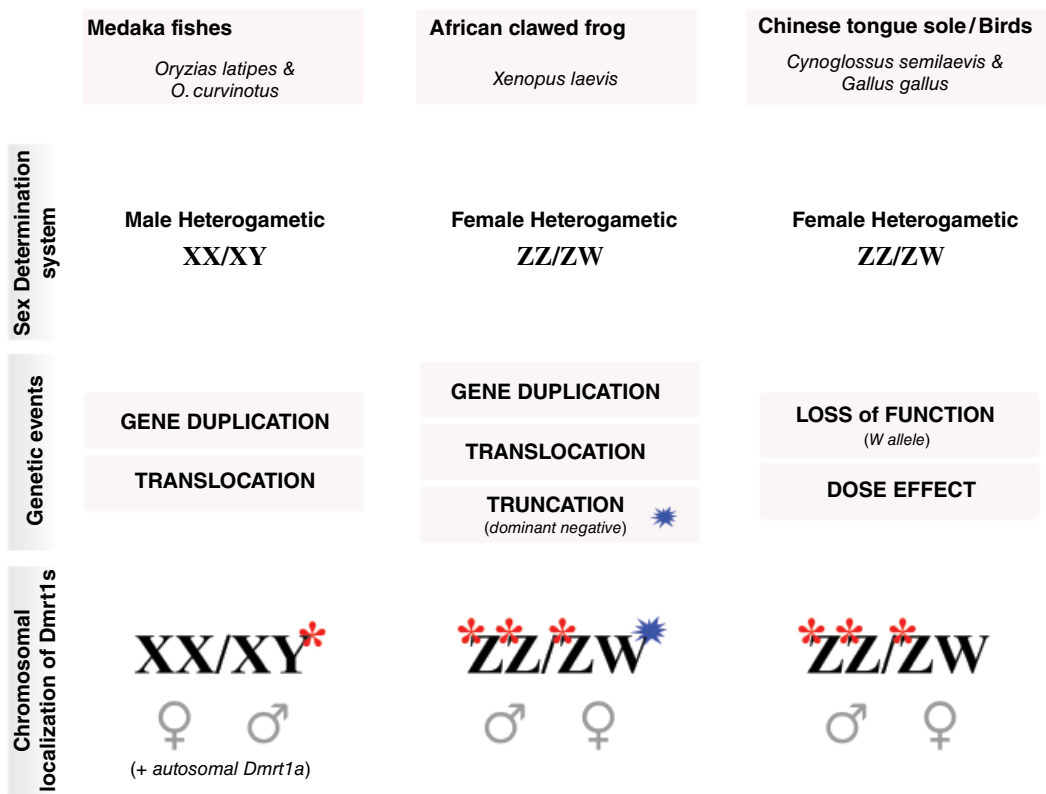


Figure 2.3 The different ways of creating a master sex-determining gene from *Dmrt1* factors.

From worms, frogs, and up to fish or mammals, the *Dmrt1* factors have been shown to be key players of the male gonadal gene regulatory network (see [43] and [40] for reviews, and also Figure 2.2). Interestingly, in a few organisms, these *Dmrt1* factors managed to hijack on top of the sex-determining cascade, recurrently and independently (see also Table 2.1) regardless of the type of GSD systems (male or female heterogametic). Given that *Dmrt1* factors are already involved into the process of male gonadal induction, the first steps toward the acquisition of a master sex-determining function deal with (i) transcriptional control and (ii) alteration of function. Ultimately, such transcriptional control or functional alteration aims at favoring either the male or the female gene regulatory networks (see also Figure 2.2). In the case of medakas (*Oryzias latipes* and *O. curvinotus*, male heterogametic system), the *Dmrt1* gene first underwent gene duplication. This new *Dmrt1* copy then translocated into another chromosome (proto Y-chromosome), which became the Y-chromosome [158, 159]. After transcriptional rewiring, conferring an early gonadal expression pattern and laying on the new Y-chromosome, the duplicated *Dmrt1bY* gene became the primary determinant of the Japanese medaka male sexual development.

In contrast to medakas, the frog has a female heterogametic system (ZZ/ZW). Similarly to medakas, the frog *Dmrt1* gene underwent gene duplication and translocation. Additionally, a truncation of the *Dmrt1* gene occurred, creating a dominant negative version (DM-W). Lying on the W-chromosome, the DM-W protein antagonizes the action of the only other Z-chromosome-located *Dmrt1* gene, leading to female gonadal development. ZZ individuals, having two copies of the *Dmrt1* gene (and no DM-W), develop as males [54, 160].

The Chinese half-smoothed tongue sole (as in birds) also has a female heterogametic system (ZZ/ZW). There, a single loss of function event of the *Dmrt1* gene occurred (W allele). Hence, by means of dose effect, ZW individuals have only one copy of the *Dmrt1* gene, while a ZZ individual harbors two copies, leading to either female or male gonadal development, respectively [56].

Gray or red stars indicate functional *dmrt1* genes; Black or blue stars indicate dominant-negative version of the *dmrt1* gene.

Sox3 gene – when introduced into a closely related species – namely, the Japanese medaka – was unable to trigger male gonadal development [67]. This suggests that acquisition of *Sox3* function as the male trigger of the Indian rice fish went along with the co-evolution of the downstream gonadal gene regulatory network (Figure 2.4).

In line with a potential key conserved role of *Sox*-related genes during gonadal determination and, although it has not yet been shown to be recruited on the very top of the cascade, the *Sox9b* gene is a recurrent and evolutionary conserved gene upregulated in the male gonads of numerous species, in which its role is essential for testis determination (see Figure 2.2). Particularly in the Japanese medaka, it has been shown that although not directly regulating testis determination, *Sox9* genes are nevertheless regulating cellular association, and are required only for proper germ cell maintenance (survival and proper proliferation) in the gonads of both sexes [68].

2.2.1.3 A Conserved Central Role for Gonadal TGF- β Signaling Molecules

From nematodes to mammals, cell signaling mediated by the TGF- β superfamily of active polypeptides has attracted attention, because of its ability to regulate diverse cellular functions controlling embryo development and, more generally, tissue homeostasis [69]. Based on sequence similarities, TGF- β molecules can be subdivided into:

- i) TGF- β *sensu stricto*;
- ii) bone morphogenetic proteins (BMPs); and
- iii) activins [69].

Despite the diversity and physiological importance of responses that this family elicits, an astonishingly simple system is the core of this signaling pathway. TGF- β superfamily members transmit signals through heteromeric complexes consisting of type I and type II serine/threonine kinase receptors. Type II receptors are constitutively active kinases

capable of binding ligands alone, while type I receptors only bind ligands in cooperation with type II receptors. Ligand binding induces the formation of a heterotetramer complex, in which two type II receptors transphosphorylate two type I receptors. Next, activated type I receptors phosphorylate specific receptor substrates (Smads), organized in multi-subunit complexes that move into the nucleus to regulate transcription of target genes (see [69, 70] for review).

Amh and *Amhr2*

The anti-Müllerian hormone (*Amh*) is a growth factor belonging to the TGF- β superfamily. In mammals, *Amh* plays a major role, accounting for the degradation of the Müllerian duct forming part of the female reproductive tract during male embryo development. It is nevertheless not essential for mouse testis development, although apparently playing a major role in testis formation in non-mammalian species. Fish do not have any Müllerian duct, but do have an *Amh* homolog, which has been shown for the first time in the Japanese medaka [71]. Interestingly, in *hotei* mutants of this species, disrupted for *Amh* signaling, germline stem cells overproliferate, resulting in male-to-female sex reversal [72].

Although being a recurrent subordinate actor of the sex gene-regulatory network, *Amh* signaling has, nevertheless, regularly made it to the top in different fish species, including the Patagonian pejerrey (*Odontesthes hatcheri*), the torafugu (*Takifugu rubripes*), the European pike, and possibly the lingcod (*Ophiodon elongatus*) (Table 2.1). For instance, after a duplication event and laying on the Y-chromosome, *AmhY* became the male sex-determining gene of the Patagonian pejerrey, a freshwater fish species [73].

In the genome of the torafugu, two versions of the receptor for *Amh* are present, and only differ by one amino acid (H384D) [74]. Being located on the X-chromosome and conserved in other pufferfish species, the H384 allele encodes for a hypo-active

Japanese medaka

Oryzias latipes

Dmrt1

DM domain TAD 3'UTR

Expression in adult somatic gonadal cells (Sertoli)

1-Duplication

3'UTR (plus insertion: proto Y-chromosome)

2-Transcriptional Rewiring

Insertion of transposable elements

Izanagi 3'UTR

Early expression in the somatic gonad

3- Neo-functionalization/Specialization

Co-evolution of the downstream gene regulatory network

Dmrt1bY 3'UTR

* Point mutations

Early expression in the somatic gonad and master sex-determining function

Indian rice fish

Oryzias dancena

Sox3

TAD HMG-Box TAD

Expression in neural tube, brain, eyes and gills. Low in somatic cell of the forming gonad (XY)

1-Transcriptional Rewiring...

...and evolution of the downstream gene regulatory network

Sox3^Y

* Cis-regulatory element

Expression up-regulated in the developing gonad

Rainbow trout

Oncorhynchus mykiss

Irf9

DNA bd IAD

Interferon response factor No known gonadal function

1-Duplication

2-Transcriptional Rewiring...

3- Neo-functionalization/ Specialization (truncation)

NO NEED FOR co-evolution of the downstream gene regulatory network

SdY

Interferon response factor

No known gonadal function

GENE DUPLICATION

ALLELIC DIVERSIFICATION

GENE DUPLICATION

: DM domain (DNA binding)

: Transactivating domains (TAD)

: HMG-Box

: Transactivating domains (TAD)

: DNA binding domain

: IAD = protein-protein interaction domain

Figure 2.4 Scenarios possibly explaining the mechanisms accounting for the emergence of new master sex-determining genes in the Japanese medaka, the Indian rice fish and the rainbow trout.

Although not always *a priori* predestined to practice a direct function during sex determination (i.e., *Sox3* or *IRF9*), it seems that common mechanisms of evolution are repeatedly utilized for the emergence of new master sex-determining genes.

The rise of *Dmrt1* as a master sex-determining gene in the Japanese medaka was somehow facilitated, since *Dmrt1* factors are already intrinsically involved during sex determination, differentiation or maintenance of genetic cascades in the whole animal kingdom. Nevertheless, in the Japanese medaka, the *Dmrt1* gene, together with neighboring genes (including *Dmrt2* and *Dmrt3*), first underwent gene duplication. This whole duplicated segment was then inserted into another chromosome, creating a proto Y-chromosome. Due to a lack of recombination between the proto Y- and X- chromosomes, part of the proto Y segment degenerated and transposable elements and repetitive sequences accumulated, resulting into a transcriptional rewiring of the *Dmrt1bY* gene, together with the acquisition of an early gonadal expression pattern compatible with a master sex-determining function. Further on, likely due to point mutations, neo-functionalization/specialization occurred, together with co-evolution of the downstream gene regulatory network.

In the Indian rice fish, given that the *Sox3* gene is not *a priori* involved or expressed during neither gonadal induction nor development, the first step toward the acquisition of a sex-determining function went along with transcriptional rewiring in order to acquire a specific expression pattern compatible with sex determination (point mutation within a cis-regulatory element). Surprisingly, in the Indian rice fish, this step alone was sufficient for conferring *Sox3* its master sex-determining function and was not accompanied by any other functional specialization. Astonishingly, *SdY*, the master sex-determining gene of the rainbow trout, unexpectedly evolved from an immune-related gene (*IRF9*), and therefore is not related to any known gene in the sex determination pathways. It could be traced back that *SdY* arose after first duplication and second truncation events of the *Irf9* autosomal gene. Again, the duplication event was accompanied by a transcriptional rewiring, conferring a pattern of expression compatible with sex determination. Interestingly, in a second step, a truncation event totally re-specialized *SdY*. The functional significance of this neo-functionalization is still under investigation. Hence, *SdY* is indeed the first example of a totally *de novo* evolved gene functionally capable of taking the leadership of the gonadal gene regulatory network in salmonids.

version of the type-II *Amh* receptor (*AmhR2*) [74]. As in the *hotei* Japanese medaka mutant [72], lower *Amh* signaling from the receptor results in the feminization of the gonad. Thus it seems that, alone, modulation of quantitative thresholds of *Amh* signaling could be master triggers for gonadal development in fish.

In zebrafish, the fine genetic basis of sex determination is unknown. Recent data on wild zebrafish populations suggests that sex determination in zebrafish might rely on discrete genetic mechanisms influenced by environmental cues [75]. Interestingly, all juvenile zebrafish display an ovarian phase in which the gonads are only filled with meiotic oocytes. In some juveniles, oocytes survive and the individual becomes a female but, in others, oocytes die from about 19–27 days post-fertilization (dpf), and the fish becomes a male. Being expressed during the gonadal differentiation period, the *Amh* ligand has been shown to be implicated in zebrafish testis formation, notably controlling primordial germ cell number [76, 77].

Gsdf

Phylogenetically closely related to *Amh* ligands, the gonadal soma-derived factor (*Gsdf*) is another growth factor belonging to the TGF- β superfamily. Only present in fish genomes [78, 79], its precise biochemical characterization has not been carried out. Being – in all fish species examined so far – exclusively expressed during the early phase of testis differentiation, it is anticipated to have a major role for male gonadal development. In the Japanese medaka, *Gsdf* is first detected six dpf when the male gonadal primordium is forming, and later on localizes in the Sertoli cells of the adult testis [80]. In terms of functions, *Gsdf* has been shown to be implicated with primordial germ cell and spermatogonia proliferation [81], and is suspected to act as a male sex initiator in the Japanese medaka [80].

Besides its expected and further demonstrated autosomal contribution to the fish gonadal downstream regulatory network [80], *Gsdf* has also been recruited as a master sex-determining gene in two fish species: the

Luzon rice fish (*Oryzias luzonensis*) [82], a sister species to the Japanese medaka (taking over the *Dmrt1bY* former master sex-determining gene) and, most likely, in the sablefish (genus *Anoplopoma* genus) [83]. It may also be the case in some rockfishes [84]. Although functional data is scarce, *Gsdf* is involved in primordial germ cell and spermatogonia proliferation in rainbow trout [81]. Additionally, in the three spot wrasse, *Halichoeres trimaculatus*, *Gsdf* also promotes spermatogonia proliferation and spermatogenesis, and has been proposed to act during sex change from female to male in this species [85].

BMP/Gdf

In the mouse embryo, independent *BMP* signals are necessary for proper PGC induction; the primary induction of PGCs at the posterior proximal epiblast is driven by *BMP4* [86], whereas the number of PGCs is guided by *BMP2*, *BMP4*, and *BMP8b* in synergistic action [87]. Hence, although not apparently involved during the triggering events of sex determination, the *BMP* signaling pathway is rather implicated during either mammalian germ cell specification or gametogenesis [88, 89]. Notably, only *Gdf9* and *BMP15* are important players in mammals [90] and fish [91], but only during the late phase of ovarian development.

Intriguingly, although *Gdf6* was not originally found to be implicated in gonadal development, recently it has been found that this gene was recruited as the master sex-determining gene, through allelic diversification in the killifish (*Nothobranchius furzeri*) [92]. In this fish, *Gdf6Y* (lying on the Y-chromosome) differs from its X-linked counterpart by having 15 and three amino acids exchanges and deletions, respectively [92]. This probably impacts upon protein interaction, notably regarding to its receptor, or during the phase of dimerization [92]. Likely due to a truncated 3'UTR, the expression of the *Gdf6Y* allele is also rewired, resulting in a high and early male-specific gonadal expression [92].

Taken together it appears that different components of the TGF- β signaling pathway (*Amh*, *AmhR2*, *Gsdf* or *Gdf6*, for instance),

Box 2.1 Fish TGF- β in need of answers

TGF- β is implied in fish sex determination. An emerging concept is that the response of a given cell to extrinsic signals relies not only on the effect of a single pathway, but more on the integration of multiple signals from a plethora of cross talking pathways [1]. But behind facts a number of issues remain to be addressed. For instance:

- 1) How is signaling specificity and crosstalk of the different gonadal TGF- β signal transducing factors (Amh/AmhR2/Gsdf/Gdf6) achieved?
- 2) How does integration of these TGF- β signaling pathway(s) with the canonical sex determination/gonadal gene regulatory network occur? (see Figure 2.2).
- 3) How is the sex-determining function of these TGF- β pathway/signaling physiologically achieved during sex determination and maintenance?

are not only regularly acting downstream in the sex determination network, but can become the master sex-determining genes recurrently and independently (Table 2.1 and [40]). In that respect, and due to the imperative need to integrate multiple signals from a plethora of cross talking pathways, a number of issues remain to be addressed regarding to TGF- β signaling in relation to gonadal commitment (Box 2.1).

2.2.1.4 *sdY* – the “Usurper” Salmonid Sex-Determining Gene

While most master sex-determining genes described and discussed above are always independently popping up again and again at the top of the genetic cascades in different fish species, as well as the whole animal kingdom, the recent report on the discovery of a new master sex-determining gene conserved in most salmonids has deeply changed our evolutionary concepts about the turnover of master sex-determining genes. Indeed, *sdY*, an immune-related gene, evolved into the master sex-determining gene in rainbow trout [41, 93].

Located at the sex-determining locus of the rainbow trout, *sdY* (standing for **s**exual **d**imorphic on the **Y**-chromosome) is, astonishingly, not related to any known gene in the sex determination pathways but, rather, with an immune-related gene (*irf9*) [41]. It could be traced back that *sdY* arose after (i) duplication and (ii) truncation events of the *irf9* autosomal gene [41] (Figure 2.4). Out of 15 salmonid species examined, 13 displayed complete sex-linkage together with the presence of *sdY* [41] and, interestingly, the Y-chromosomes are not syntenic with SD loci located onto different chromosomes. Hence, *sdY* is, indeed, the first example of a totally *de novo* evolved gene functionally capable of hijacking the leadership of the gonadal gene regulatory network (see Figures 2.2 and 2.4).

Because early maturation proceeds differently between male and female salmonids, and is also associated with challenging issues regarding to modern aquaculture (growth rates, food conversion efficiency, flesh quality, high sensitivity to pathological problems or broodstock management), females are often preferred for production. To this end, and regardless of its chromosomal location, *sdY* represents a major advance and useful tool for sexing (most) salmonids [93].

2.2.2 A Glimpse into Environmental Sex Determination in Fish

Environmental factors have been reported to affect sex ratio in many different fish species, ranging from basal teleost lineages, such as in eels (Elopomorpha), to a number of Percomorphas' species (see the following reviews [16, 17] and Chapter 4 for more details). Temperature has, indeed, been the most studied of these environmental factors, with the first precise description of an effect of temperature on sex-ratio described in the early 1980s in the Atlantic silverside [27, 94]. Since then, temperature sex determination (TSD) has been described as being quite widespread in fish. However, in most of these so-called TSD fish species, temperature may

be acting more on the sex differentiation process by bypassing a known GSD, a process that may be more accurately named thermal-effect (TE) on GSD (GSD + TE) [95]. Interestingly, there is a recurring theme in these TSD or GSD + TE species, as high temperature is nearly always linked with masculinization effects [95], with only very few exceptions (see [96] for review on the Southern flounder, *Paralichthys lethostigma*).

Among other environmental factors affecting sex-ratios, pH has been described in a few cichlid species belonging to the *Apistogramma* [97] and *Pelvicachromis* [98] genera. In these species, a low pH tends to produce more males, and a high pH more females. Social control is a well-known trigger of sex-inversion in many hermaphrodite species [99, 100], but it has also been found to influence sex-ratio in at least one gonochoristic species, namely, the Midas cichlid, *Amphilophus citrinellum*, in which larger animals within a group will become males, and smaller individual will become females [101].

Density in eels [102] and in the paradise fish, *Macropodus opercularis* [103], and growth rate in the European sea bass [104] have also been described to affect sex-ratios, even if these effects could be related together, and potentially also mediated, through social control, as in the Midas cichlid. Hypoxia (dissolved oxygen level in water below 2.8 mg O₂ l⁻¹) is also described as a masculinizing environmental factor, at least in the Japanese medaka [105] and zebrafish [30]. Even if questionable from a physiological point of view, as these very low water oxygen concentrations are likely to induce lethal or sub-lethal effects, it is, however, noteworthy that hypoxia masculinization is associated with a strong deregulation of the sex steroid synthesis pathway [30], or of genes involved in sex determination and differentiation [105, 106].

Finally, an influence of the background tank color has been found in the Southern flounder, a species with a known TSD, in which extreme low and high temperatures promote masculinization [107]. Blue tank backgrounds

induced higher masculinization, compared with black and grey tanks. This effect could be triggered by an increasing stress, with higher cortisol values in animals maintained in blue tanks, compared with other colors [107].

Stress is also a common theme of most, or potentially all, these environmental effects, including background color effects, social interactions, density, hypoxia, extreme pHs, or temperatures, which can all be related to stress (reviewed in [108]). This stress implication in ESD in fish has been actually well demonstrated, mostly in some temperature-sensitive species, in which cortisol has been found to be correlated with high temperature masculinization (pejerrey [109], Japanese medaka [110], Japanese flounder [111]), or rearing density (zebrafish [112]), but also in relation with the blue background tank color (Southern flounder [107]).

2.2.3 When GSD and TSD Blend

While it appears that the phenotypic expression of sex might be better seen as a threshold trait for which very plastic and modular networks of interactions are – at different degrees – influenced by a variety of “masters” or “minor” triggers, another piece of evidence going in that direction comes with the coexistence of GSD and ESD. Indeed, examples show that, in many cases, what was thought to be a strict genetic hierarchy can be simply shunted, or at least influenced, by other triggers or associations of minor triggers (genetic again, environmental or epigenetic).

For instance, in fish species thought to have clear genetic sex determination, temperature has nevertheless been revealed to be a substantial modulator of the sex ratio (see [17] for review). In the Japanese medaka, although a strong genetic sex determination system was described decades ago, naturally occurring male-biased sex ratios have been reported during hot summers in Japan [113]. More recently, complete female-to-male sex reversal after high temperature treatment has been observed [114] and potentially

linked to an upregulation of the *Dmrt1* gene expression [36] or elevation of cortisol levels [37].

In tilapias, while sex is under the control of dominant genetic factors on sex chromosomes (XY/XX or ZW/ZZ), discrete genetic factors or temperature influence sex ratios [17, 24]. In the European sea bass, a fish species with polygenic sex determination modulated by temperature [115], it was shown that methylation of the promoter of the aromatase gene (*cyp19a1a*) – regulating its expression level – was positively correlated with water temperature, accounting for masculinization [116, Chapter 3].

Further on, transcriptomic analyses of early forming gonads of larvae exposed to elevated temperatures revealed a parallel increase in genes involved in stress response, cholesterol transport, epigenetic regulation, or testis differentiation, together with a repression ovarian differentiation related genes [117]. Interestingly, the weak polyfactorial GSD of laboratory strains of zebrafish is easily overwhelmed by various environmental cues, such as high temperatures [118] or density [119], hypoxia [30], or even growth rates [31], which all tend to have masculinizing effects.

Conversely, in *Odontesthes bonariensis*, a species of pejerrey known to have strong temperature dependent sex determination, screening for the Y-specific copy of *Amh*, namely *AmhY* (the otherwise master sex-determining gene of *O. hatcheri*, a sister species) revealed high but incomplete linkage, together with phenotypic sex in wild population [120]. This, indeed, suggests the “coexistence of genotypic and temperature-dependent sex determination in pejerrey *Odontesthes bonariensis*” [120].

Taken together, these data suggest that mainly temperature, but also other environmental cues, influence sex ratios at different physiological or genetic levels. Hence, while variations of temperatures, related to higher stress and elevation of cortisol levels, could act on primordial germ cell proliferation modulation through FSH receptor

upregulation [37], it is also suggested that cortisol could act on the metabolism of androgen synthesis [108]. Ultimately, such a scenario would result in “physiological sex reversion/modulation” only, (ultimately controlling germ cell number) and not support any real inflection of the genetic sex determination networks.

On the other hand, there are data that, indeed, support an environmental-triggered inflection of the primary sex-determining genetic network. In the Japanese medaka, for instance, modulation of temperature not only translates into higher levels of cortisol [37], but is also associated with the transcriptional rewiring of the autosomal *Dmrt1* gene expression, being then earlier expressed, like its duplicated *Dmrt1bY* co-ortholog and master sex-determining gene would be [36]. In the same direction, temperature-induced differential methylation of genes belonging to the gonadal gene regulatory network [116] would also support the idea that environmental cues might also act in a similar manner to primary genetic sex triggers.

2.3 Sex Differentiation as a Threshold Phenotype Relying on Fine Regulations of a Plastic Gene Regulatory Network

While many recent studies dealing with sex determination/differentiation in fish have been focused on the quest for new master sex-determining genes, the genetic architecture of such a complex phenotypic trait as sex cannot be simply limited or restricted to the action of unique and totipotent master sex-determining triggers. Then, what happens when “masters change?” The classical view of sexual development suggests that not much would change downstream, since “slaves remain” [121]. Potentially a new master would pop up at the top from a postulated conserved downstream gene regulatory network, possibly slightly adjusting

(bottom-up theory [122, 123]). However, more and more data, notably gathered within teleost fish species, indicate that the phenotypic expression of sex is a rather plastic trait, relying on a complex and unstable equilibrium of a constantly adjusting network of regulatory interactions.

In addition, and challenging our initial view of sex-determining pathways evolution and gonadal maintenance, it has been documented that primary specified gonadal identity has to be asserted actively and maintained lifelong by adjusting down the opposing sex-determining program(s) (see [124] for review and Figure 2.2). For instance, in two medaka species, *Oryzias latipes* and *Oryzias curvinotus*, which share the same master sex-determining gene (*Dmrt1bY*), interspecific hybridization results in XY sex reversal [125] and sterility [126]. This points out that:

- i) an autosomal locus controls sex reversal in the interspecific hybrids [127]; and
- ii) the downstream gene regulatory network has most likely evolved quickly in these two closely related species.

In the same vein, *Sox3*, the master sex-determining gene of the Indian rice fish, after BAC clone transfection, is not potent enough for triggering male gonadal development in genetic females of *O. latipes*, although the two species are closely related [67]. On a larger evolutionary scale, between mammals and medaka fishes, it has been reported that, although some transcription factors (like *Sox9*, *Dmrt1*, *Foxl2*) or signaling pathway transducers (like Hedgehog, R-spondin1 pathway, together with Wnt or Follistatin), are key players of the gonadal gene regulatory network and are preserved across phyla, their specific regulation and function and interplay can be drastically different [128]. Altogether, this supports the idea that the acquisition of a new master sex-determining function goes along with the concomitant adjustments of the downstream gonadal gene regulatory network (see also Figure 2.4).

2.3.1 The Classical Actors of the Fish Sex Differentiation Cascade

Although the “canonical” primary actors of the early sex determination process – such as the *Dmrt1* and *Sox* molecules or *Tgf-β* (*Amh*, *Gsdf*, *Gdf*) signaling pathways – have been shown to manage regularly to be recruited as master sex-determining genes, surprisingly, other evolutionary conserved and recurrent components (*R-spondin1/Wnt4/β-catenin*, *Foxl2*) are nevertheless classical actors of the downstream gonadal gene regulatory network (see also Figure 2.2).

2.3.1.1 The *R-spondin1/wnt4/β-Catenin* Pathway

Belonging to a family of secreted growth factors, *R-spondin1* (*Rspo1*) is a central female-determining factor. Operating through the canonical *Wnt* signaling pathway [129], *Rspo1* proteins activate the β -catenin pathway and also upregulate Follistatin (*Fst*) via *Wnt4* [130] (see also Figure 2.2). In mammals, it is known that *R-spondin1/Wnt4/β-catenin* and *Fst* are all acting within the same pathway, in order to promote ovarian development on the one hand, while repressing the formation of the testis cord on the other hand [131].

2.3.1.2 The Winged Helix/Forkhead Transcription Factors and *Foxl2*

Fox proteins display an evolutionary highly conserved DNA binding domain referred to as the Forkhead Box (*Fox*) (see [132] for review). Interestingly, various members of this family are implicated during either sexual development or gonadal regulation (*Foxc1* and *Foxl2*), or more generally controlling the ovarian function (*FoxO* genes, for instance [133]) or spermatogenesis (*Foxj2*, *Foxp3* and *Foxo1*) ([134–136] and [132] for review).

More specifically, one fraction of *Fox* proteins, the *Foxl2* factors, displays an interesting evolutionary conserved pattern of expression, being mainly present in the somatic cells of the female gonad [137]. Further on, *Foxl2* has been shown to be the key player of the female gonadal gene

regulatory network, notably via its mutual antagonizing relationship, together with *Dmrt1* (see Figure 2.2). Suppressing each other's expression, *Foxl2* and *Dmrt1* maintain either female or male gonadal fate, respectively [61, 138], also avoiding transdifferentiation (Figure 2.2). Additionally, in mammals, cooperation of *Foxl2* together with *Wnt4* has been reported during ovarian development [139]. Finally, it is interesting to note that the expression profile of *Foxl2* highly correlates with that of the *aromatase* (*Cyp19*), suggesting an additional role during endocrine regulation of fish sex differentiation via estrogen synthesis, through direct regulation of the aromatase promoter by *Foxl2* [140].

2.3.2 Endocrine Regulation of Fish Sex Differentiation

2.3.2.1 Are Steroids Natural Inducers of Gonadal Sex Differentiation in Fish?

Since Yamamoto's experiments in the early 1950s [141, 142], steroids have been found to be effective molecules able to induce phenotypic sex-inversion in fish (reviewed in [143]). However, the question of their implication as natural inducers of gonadal differentiation has been debated, with controversies on the respective roles of androgens and estrogens that were initially defined, respectively, after Yamamoto, as "androinducers" (male-inducers) and "gynoiducers" (female-inducers) (for more details on steroid implication on fish sex differentiation, see the following review [144]). Most experiments supporting this initial assumption were actually based on the ability of these steroids to induce an effective masculinization or feminization following experiments with steroid treatments in many fish species [145].

Additional experiments, using sex-steroid assays, steroid metabolism and, more recently, steroid-related gene expression and transcriptomic studies, revealed that male and female fish differentiating gonads exhibit contrasted abilities in the synthesis of sex-steroids [146]. Ovarian differentiation is

generally characterized by early estrogen production, and testicular differentiation by an absence of estrogen synthesis and the synthesis of specific male androgens – namely, 11-oxygenated androgens. These results supported a theoretical model close to Yamamoto's model, in which the balance between estrogens and androgens (11-oxygenated androgens), instead of their absence/presence, would determine the fate of gonadal sex differentiation. However, by preventing estrogen synthesis with enzymatic inhibitors specifically blocking aromatase (*Cyp19a1a*) enzyme activity, complete phenotypic masculinization of genetically female populations were first demonstrated in Chinook salmon [146].

Since then, masculinizing effects of aromatase inhibitors (AI) have been described in many different fish species, suggesting that the mere absence of estrogens is sufficient for male sex differentiation, which would not require a stimulatory action of androgens but, rather, a lack of estrogen to proceed. This has led to an estrogens-centric model, in which estrogens would be required for female sex differentiation, while their absence would be required for male sex differentiation [147]. This pivotal role of estrogens is also supported by results showing that steroid synthesis is a rather late event during fish testicular differentiation, in contrast to the differentiating ovary, which has been shown to express many genes involved in steroid synthesis, but much earlier during the development of the gonads [148, 149].

Questions on the physiological roles of androgens, and whether they are really implicated in early testicular differentiation, remain. However, they may be seen as a late requirement for maintenance of the male phenotype, by being an additional lock, preventing estrogen production and, thus, maintaining the male sex by inhibiting the expression of the aromatase gene. This question of sex phenotype maintenance is now increasingly prevalent, despite an initial dogma stipulating that fish sex phenotype was only sensitive to exogenous treatments around the sex differentiation period. More

recent experiments have demonstrated that inhibition of estrogen synthesis in adult females can lead to partial or even fully functional masculinization [150–153]. This demonstrates that, in fish, like probably most vertebrates, ovarian maintenance needs a continuous estrogen synthesis. It also further supports the idea that estrogens are pivotal hormones for gonadal sex differentiation in fish.

Apart from the sex steroids (i.e., mainly estrogens and androgens), a relatively large body of literature has been recently published on the implication of cortisol as a natural trigger of temperature- or stress-induced masculinization [37, 107, 109–111]. This effect of cortisol could either be mediated by the conversion of cortisol into 11-oxygenated androgens, potentially more physiologically active on testicular differentiation, [154], or by a direct effect on the downregulation of the *cyp19a1a* gene [109, 111]. It should be also mentioned that temperature can also induce masculinization via epigenetic inhibition of the same target gene, *cyp19a1a* [116].

2.3.2.2 How Steroid Treatments Impact Gonadal Sex Differentiation in Fish

Independent of their action as potential natural inducers of gonadal differentiation, steroids are still widely used in fish aquaculture to produce sex-reversed animals such as neomales in salmonids and other species that are phenotypic males, although genetically females (XX or ZW males). The mechanisms of action of these steroids, when applied as masculinizing or feminizing treatments, are then of special interest for eventually developing better strategies for controlling sex. Results obtained in rainbow trout show that these treatments do not induce global expression profiles comparable to those observed during the natural differentiation of the gonad, and that androgens produce a strong deregulation of the normal early testicular physiology [155–157].

However, within these massive gonadal deregulations, there is a conserved action, as all these treatments have a direct and fast inhibitory effect on the expression of genes

involved in steroid synthesis, including the downregulation by androgens of *cyp19a1a*, the gene encoding the Aromatase enzyme. This inhibition of estrogens synthesis following androgen treatments may be actually the unique physiological requirement needed for masculinization, in line with the idea that estrogens are pivotal hormones for gonadal sex differentiation in fish. Interestingly enough, in that context, is the fact that masculinizing treatments with AI are much less disruptive on their overall impact on gene expression profiles [157]. This, again, supports the hypothesis that inhibition of estrogen synthesis could be the single physiological mechanism needed for testicular differentiation, and opens new avenues for using more physiological exogenous AI treatments for a better sex control in aquaculture.

2.4 Mechanisms for the Emergence of New Master Sex-Determining Genes and Gene Regulatory Networks

Because the amazing diversity of sex triggers in fishes emphasizes the many options possible at the sex determination stage (and possibly beyond) to switch and supervise over the destiny of the gonad, fishes on the whole are an attractive system for studying the evolution of sex-determining genes and regulatory networks in relation to the emergence or turnover of master sex-determining genes.

It is now clear that the phenotypic expression of sex translates from either genetic triggers, environmental triggers, endocrine triggers, or a blend of all. The main emerging idea is that sex determination gene regulatory cascades should no longer be seen as simply hierarchical but, rather, as a regulatory network or, even more, as connections of interdependent regulatory networks (Figure 2.5). Hence, the above-described *Dmrt1*, *Sox*, *TGF- β* or *R-spondin1/Wnt4/ β -catenin*, and *Fst* gene regulatory networks, although looking to be acting in parallel are, at some points, indeed intimately linked together

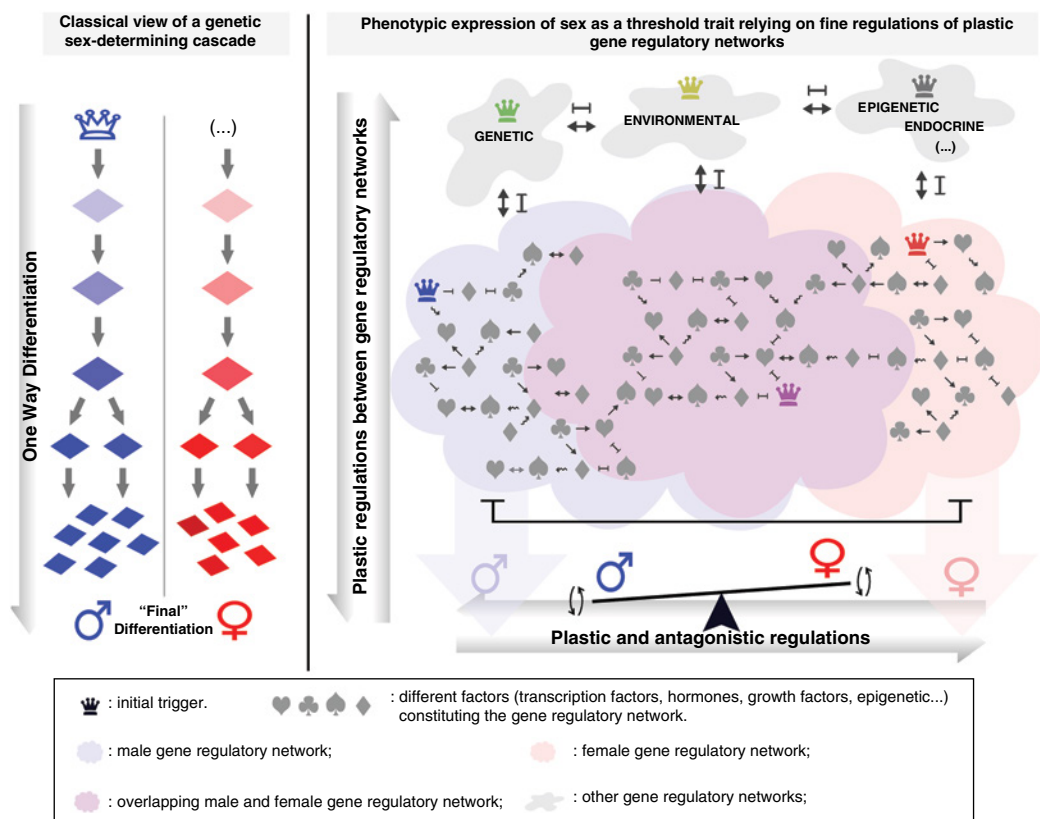


Figure 2.5 Sex differentiation as a threshold phenotype relying on fine regulations of plastic gene regulatory networks.

It is obvious that the phenotypic expression of sex cannot be seen any longer as a simple one-way top-down differentiation process under the action of a unique and totipotent master sex-determining trigger (left). Sex determination should, rather, be seen through the prism of a developmental perspective as the emanation of either genetic, environmental, endocrine triggers, or a blend of all, acting among interconnected gene regulatory networks (right).

(Figure 2.2). It also seems that the sex determination case should now be treated more like a developmental perspective, rather than a simple one-way, top-down differentiation process (neither genetically nor physiologically). Indeed, the process of gonadal differentiation and maintenance is highly plastic lifelong, with formed gonads able to transdifferentiate after what was thought to be “final” differentiation (Figure 2.5).

Up to now, the emergence of master sex-determining genes was seen through the prism that sex determination was a hierarchized cascade. This led to the view that master sex-determining genes were necessarily up-recruited from the pre-existing sex

cascade (duplication, transcriptional rewiring and subfunctionalization), and then added to the top. Further on, but still as a variation on the theme, one could say that such up-recruitment is not necessary: The gene can stay at its place in the cascade and just become more powerful.

But these views are rather mechanistic. Indeed, the underlying mechanisms are always the same: gene duplication, transcriptional rewiring, neo-/sub-functionalization (coding change, truncation ...), and specialization. The translation of these mechanisms into physiology opens many more options for evolution (presence or

Box 2.2 Outlook and future prospects

Sex determination is probably one of the most basal, ubiquitous, and ancestral developmental processes. Nevertheless, the fact that, even between closely related species or organisms, it is so variable and plastic, provides many fascinating questions:

- 1) Have hermaphroditic (fish) species found a way to control/utilize the observed lifelong plasticity of the gonad, or is such plasticity just remnant of an evolutionary ancient mechanism?
- 2) How stable are SD mechanisms in evolutionary terms? What is the evolutionary meaning of the high variability of sex determination mechanisms?
- 3) What is the evolutionary dynamic driving such variability? Is it purely stochastic, de-convergent in order to promote speciation?
- 4) Are different SD mechanisms triggering the same, related, or totally specific molecular pathways during the process of the fate differentiation of the gonad anlage?
- 5) Are *SdY* and *Sox3* factors indeed components of the sex determination gene regulatory network that have been neglected so far, or true usurpers?
- 6) Why do the same usual suspects always pop up at the top of the genetic cascades leading to sex determination? Why do others appear to never make it (*Foxl2*, for instance)?
- 7) Do the three main genetic cascades (*Dmrt1*, *Sox* and *TGF-β*) crosstalk or converge at one point, in order to regulate the same physiological output (germ cell number, specification of the somatic gonad)?

absence does not make it all). Hence, mechanistically speaking, it might be much “easier” to find a gene already known for being able to influence gene regulatory network(s), although others, essentially any other gene, could do the same function. *Sdy* in salmonids does not play any physiological role on its own but, rather, provokes a slight bend into the gonadal gene regulatory network. This is enough for doing the job. Indeed, for such

bending, maybe some signaling pathway components are better at doing it – for instance, as seen with the emergence of the *TGF-β* signaling pathway. The existence of such intricate and plastic regulatory networks has drastically changed our traditional perception of a standard linear developmental process for initiating and developing either a male or a female gonad and now opens up fascinating questions for future research (Box 2.2).

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3

Epigenetics of Sex Determination and Differentiation in Fish

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3.1 Introduction

With over 33,000 species (according to *Fishbase*), fish constitute the largest and most diverse group of vertebrates. This diversity includes size, shape, morphology, behavior, physiology, life-history, habitat, distribution, etc. [1]. In terms of reproductive strategies, fish exhibit not only gonochorism (separate sexes), but also hermaphroditism and unisexuality [2]. Fish are also diverse regarding sex determination, the process by which genetic and/or environmental factors establish the sex of an individual.

Thus, in contrast to birds and mammals, which have genetic sex determination (GSD), fish exhibit several types of sex-determining mechanisms. These include species also with GSD, where sex is determined by the action of a “master” sex-determining gene [3, 4] (see Chapters 1 and 2 of this book), species with polygenic sex determination (PSD), where the sex of an individual depends on the combined effects of multiple pro-female and pro-male factors (Chapter 14), and species with environmental sex determination (ESD), where sex is determined by the magnitude of an environmental cue during early sensitive periods [2].

The most common form of ESD in fish is temperature-dependent sex determination (TSD), in which temperature during what is

called the thermosensitive period (TSP) determines the sex of individuals [5, 6] (see Chapter 4). Currently, rather than being considered as two mutually exclusive types, GSD and ESD are regarded as two ends of a continuum [7] sharing the same molecular players [8]. Thus, even in species with GSD, there can be some environmental influences on sex determination if the magnitude of the environmental cue is above a certain threshold. This may not normally occur in nature under normal conditions, but it can occur in laboratory conditions, or in nature in specific places, or during specific events. Thus, species with GSD, where sex is genetically canalized, may end up being strongly influenced by the environment if a given population encounters naturally or man-made exceptional conditions.

In this regard, some natural populations of Nile tilapia, *Oerochromis niloticus*, from lakes Volta and Koka in Africa, which have a GSD system based on a predominant male heterogametic factor, with additional influences of polymorphism at this locus and/or action of minor factors, exhibit natural sex-reversal [9]. Conversely, even in species with TSD, there is a genetic substrate that explains different reaction norms in response to the environmental factor in question. This has been documented also in fish species such as the Atlantic silverside, *Menidia menidia*,

where populations exhibit GSD and/or TSD, depending on adaptation to a given geographic range with specific environmental conditions [10]. Finally, in PSD species such as the European sea bass, *Dicentrarchus labrax*, there can be an approximately equal contribution of the genotype (G) and the environment (E) with $G \times E$ interactions seen in the reaction norms [11].

In the same way that pluripotent stem cells can differentiate in a variety of cell types, seen from a developmental point of view, sex differentiation (SD) is the process by which an undifferentiated gonad develops into either a testis or an ovary [12]. Before that process, both somatic (precursor of supporting cells and interstitial cells) and germ cells (gonia) are bipotential. During the early stages of SD, gonia differentiate into spermatogonia in future males and oogonia in future females. Likewise, supporting cells will differentiate into Sertoli cells in testis or granulosa cells in ovaries. Thus, starting with the same genome, each type of cell will acquire a specific identity and give rise, through mitotic divisions, to daughter cells of the same identity. In cell biology, it is well known that the same genotype is capable of giving rise to various phenotypes. The process by which cells that share the same genome differentiate, acquire and maintain their identity, and thus a cell-specific phenotype, is in the realm of epigenetics.

3.2 Definition of Epigenetics

The term epigenetics literally means “above genetics,” and was coined by British developmental biologist Conrad Waddington in the 1940s, to describe the “branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being” [13]. It is neither the purpose of this chapter to review the context of scientific affairs in which the concept of epigenetics emerged, nor to describe the more common or different definitions of the term (see [14]). Here, epigenetics is defined

as the branch of science concerned with the study of alterations (marks) of the DNA and chromatin, but not of the underlying DNA sequence, that result in mitotically or meiotically heritable changes in gene expression (see also Box 3.1). However, unlike genetic changes, epigenetic marks may be reversible.

Epigenetic regulation of gene expression is present in bacteria, plants, fungi, and animals. However, not all types of epigenetic regulatory mechanisms exist in all types of organisms (see next section below). Some familiar epigenetically regulated phenomena include mating type silencing in yeast, temperature-dependent vernalization in plants, gametic imprinting, position-effect variegation, and X-chromosome inactivation in mammals [15, 16]. Epigenetics allows organisms to integrate internal (differentiation signals, metabolites, etc.) or external (nutrients, temperature, biotic stress, etc.) environmental information on top of genomic information, to produce a particular phenotype [17]. Therefore, there is a strong component of phenotypic plasticity that is dependent on epigenetics [18].

3.3 Epigenetic Regulatory Mechanisms

Epigenetic regulation of gene expression is accomplished by three major and distinct mechanisms that, in practice, act in coordination. These mechanisms include DNA methylation, histone modifications, and non-coding RNAs, and they can act together to orchestrate gene expression [19].

3.3.1 DNA Methylation

DNA methylation is a chemical modification of the DNA chain itself, whereby the 5' carbon atom of cytosine is replaced (“methylated”) by a methyl (CH_3) group, becoming 5'-methylcytosine (^5mC). In vertebrates, DNA methylation only occurs in a CpG context (i.e., a cytosine followed by a guanine and linked by a phosphate bond).

Box 3.1 Glossary of terms used in the text

Epigenetics: The branch of science concerned with the study of alterations (marks) of the DNA and chromatin, but not of the underlying DNA sequence, that result in mitotically or meiotically heritable changes in gene expression.

Epigenetic trap: Any epigenetic change that arises in response to unique environmental cues that produce maladaptive phenotypes, with no increase in phenotypic variance, and that can have negative consequences for fitness in nature, but be of advantage for farming.

Essential epigenetic marks (EEMs): The number of measurable and identifiable epigenetic marks, such as DMCs, DMRs, or a given set of histone modifications in specific loci that are strictly necessary, albeit perhaps not sufficient, to bring about a specific, measurable phenotype.

Conserved model of epigenetic regulation of sexual development in fish: A model based on

the assumption that there are “pro-male” and “pro-female” genes, and aimed at understanding the relationship between gene silencing states and gene expression levels during sex differentiation in gonochoristic species, or sex change in hermaphroditic species. The model predicts that a given set of epigenetic and gene expression patterns are more associated with a particular gonadal phenotype than the means by which this phenotype is obtained.

Reproductive programming: The selection of broodstock based not only on genetics, but also taking into account a specified set of epigenetic characteristics, to produce offspring with certain desired reproductive characteristics.

Sex determination: The process by which genetic and/or environmental factors establish the sex of an individual.

Sex differentiation: The process by which an undifferentiated gonad develops into either a testis or an ovary.

Enzymes called DNA methyltransferases (DNMTs) are in charge of catalyzing this reaction.

There are several DNMTs, classified according to the status of the DNA they target. DNMT1 methylates the unmethylated opposing pair of a hemimethylated site. DNMT1 is called maintenance DNMT, because is responsible for copying the existing methylation profile during cell division and, thus, participates in the transmission of epigenetic marks and contributes to the epigenetic inheritance mechanism. On the other hand, DNMT3 methylates previously unmethylated CpGs, and is thus responsible for the *de novo* DNA methylation [20]. In fish, because of the ancestral genome duplications, there can be several isoforms of each type of DNMT [21].

In the genome, CpGs are usually methylated and evenly distributed, except in regions where there is an elevated content of CpGs. These regions are called CpG islands (CGIs), and they are normally associated with

promoter or regulatory regions. Changes in methylation levels in these CGIs are associated with gene expression regulation. Across tissues, within the same tissue with age, or in other sorts of comparisons, a difference in the methylation of a given CpG loci is referred to as differentially methylated cytosine (DMC), and many of them as DMCs. Likewise, and with a stronger association with gene expression changes, genomic regions with different DNA methylation are called differentially methylated regions (DMRs). Also, certain loci can influence DNA methylation, and these are called methylation quantitative trait loci (meQTLs). They can influence methylation across extended genomic regions, and may underlie direct single nucleotide polymorphism (SNP) associations or gene-environment interactions [22].

3.3.2 Histone Modifications

Histones are the proteins that form the nucleosome, the basic unit of DNA packaging

in eukaryotes. Each nucleosome is formed by a histone octamer comprising the core histones H2A, H2B, H3, and H4, with two copies of each [23]. Histone variants and biochemical modifications of the histone amino acid residues conform a series of modifications associated to euchromatin or heterochromatin states and, thus, to gene transcription activation and silencing, respectively [24].

For example, trimethylation of lysine 4 (H3K4me3) is a histone H3 modification that is usually associated with transcriptional activity. There is evidence that H3K4me3 can also attract and activate DNMTs. On the other hand, methylation of lysine 9 (H3K9me) is usually associated with transcriptional repression. The biochemical modifications of histones are carried out by “epigenetic writers” such as histone acetyltransferases (HATs), decoded by “epigenetic readers” such as polycomb proteins, and wiped out by “epigenetic erasers” such as histone deacetylases (HDACs), which reverse the epigenetic marks made by HATs. Several enzymes involved in chromatin conformational changes, including DNMTs, HATs, and HDACs, are sensitive to environmental variations and metabolic cues, and can act together [25]. These enzymes, therefore, act as sensors through which the environment can alter gene expression [26].

3.3.3 Non-Coding RNAs

Non-coding RNAs (ncRNAs) are RNA molecules that are transcribed, but not translated into proteins, and have been implicated in some of the most studied complex epigenetic phenomena, including transposon silencing, X-chromosome inactivation, and dosage compensation [27, 28]. ncRNAs are classified according to their nucleotide (nt) length, structure, and function. The best characterized ncRNAs, in terms of epigenetic regulation, are microRNAs (miRNAs; 19–25 nt) and long-ncRNAs (lncRNAs; > 200 nt).

miRNAs are involved in the fine-tuning of translational regulation by repression or degradation of specific mRNAs. ncRNAs regu-

late gene transcription by the recruitment of epigenetic silencing complexes to loci in the genome recognized by those complexes [29]. Identifying tissue-specific ncRNAs is an essential first step toward understanding the biological functions of these molecules, which include the regulation of sexual fate determination.

On the other hand, lncRNAs, such as *roX* and *XIST*, have been implicated in dosage compensation in *Drosophila melanogaster* and *Mus musculus*, respectively. Dosage compensation is a phenomenon present in animals with GSD, in which one of the two sex chromosomes, or a part of it becomes, in terms of gene expression, either inactivated (mammals) or boosted (birds) by an epigenetic chromatin modification, in order to compensate for the disparity arising from having a different number of a given sex chromosome (one or two X in a XX/XY system and one or two Z in a ZZ/ZW system) [30]. In the Senegalese sole, *Solea senegalensis*, miRNAs may play a role in temperature-induced phenotypic plasticity of growth in teleosts [31].

Considered globally, epigenetic modifications determine the phenotype by allowing differential access of the transcriptional machinery to the DNA by altering the chromatin structure. Thus, specific, discernible DMCs and DMRs can be used as epigenetic marks, as an additional, very important type of genomic information, aside from pure genetic variation. There are high expectations for the application of epigenetic marks in livestock [32].

3.4 Transgenerational Effects

As seen above, *dnmt1* is able to replicate the methylation patterns during cell division. This mitotic type of inheritance allows each type of cells to maintain not only their identity across generations, but also to carry-on specifically acquired epigenetic marks in response to intrinsic (e.g., age, metabolites) or extrinsic (e.g.,

temperature) influences. The other form of inheritance involves the germ cells, which can also pass to the next generation epigenetic changes that occurred in the parents. If the generation that is exposed to a certain environmental stimulus is called the F_0 generation, effects observed in the F_1 generation are called multigenerational, and effects observed in the F_2 and beyond are called transgenerational.

Thus, in fish, “true” epigenetic effects are only those observed in the F_2 and beyond, because effects observed in the F_1 may be direct effects of the stimulus through the exposed F_0 , rather than inherited ones. This applies to oviparous fish, the great majority of species. In ovoviviparous species, and in the few that are viviparous (some sharks), transgenerational effects, to be called so, should be recognizable, like in mammals, in the F_3 generation and beyond. Transgenerational epigenetic inheritance is thus a form of non-Mendelian inheritance, for which a tremendous importance for population acclimation to new environments is just nowadays being recognized [33, 34], as well as for evolutionary rescue [35]. Furthermore, some epimutations can induce genetic changes in subsequent generations [36], suggesting a mechanism by which environmental information can be eventually and permanently integrated into the genome.

From a mechanistic point of view, research in parental imprinting in fish gametes is still very scarce, compared with that carried out in mammals. Nevertheless, some advances have been made. For example, in zebrafish, *Danio rerio*, it has been determined that the sperm – but not the oocyte – DNA methylome is inherited in early embryos [37]. Recently, the epigenetic mechanisms acting in fish germ cells and embryos was reviewed by Labbé *et al.* [38], calling attention to the fact that the erasure-establishment of the epigenetic marks during gametogenesis in fish is still not well resolved. Further, in fish, the germline is programmed very early during embryo development, compared with mammals.

Transgenerational changes in gene expression in response to an environmental stimulus can recapitulate developmental changes in the same species [39], a situation that confers a certain advantage when searching for transgenerationally affected loci.

3.5 Epigenetics and sex – General Considerations

Some general considerations seem appropriate before case studies concerning epigenetics and sex in explicit species are discussed in the following sections.

3.5.1 What Species can be More Fruitful to Study?

In the introduction, it was mentioned that, during sex determination and differentiation, somatic and germ cells that start with the same genome end up giving rise to two mutually exclusive, male or female, somatic and germ cell types, respectively. Thus, it can be argued that all cases of sex determination and differentiation involve the concurrence of the epigenetic regulatory mechanisms to bring about each sex phenotype. While this is true, here I want to draw attention not only to strict GSD species but, particularly, to species where their sexual fate depends mainly or partially on the integration of an environmental cue. This mostly includes PSD and ESD species.

Likewise, hermaphroditic species, in which also the environment determines when to change sex, are another clear example of epigenetic regulation of sexual identity. Thus, organisms in which the same genome is able to produce two distinct sexual phenotypes in response to environmental cues are among the clearest examples of phenotypic plasticity, and a good place to study epigenetic mechanisms influenced by the environment.

Similarly, it has been suggested that epigenetic mechanisms could play a crucial role in the evolutionary persistence of unisexual complexes, such as *Chrosomus eos-neogaeus*

(Cyprinidae), since genetically identical organisms could rely on phenotypic plasticity to face environmental variation [40]. Also, epigenetics probably plays an important role in the stabilization of genomes in induced polyploids. This is an area of research that has not received the attention it deserves. One of the few exception include the study of Covelo-Soto *et al.* [41] on global analysis of DNA methylation between triploid and diploid brown trout, *Salmo trutta*.

3.5.2 What is the Best Developmental Period to Target?

One aspect worth considering is the observation that organisms are not equally responsive to environmental perturbations throughout their lifetime. In terms of epigenetic modifications, the early stages are undoubtedly the most sensitive ones. This is illustrated, for example, in zebrafish, where treatment of embryos (26–56 hours post-fertilization) with androgen results in changes in global DNA methylation when examined as adults. However, the same treatment administered between 21–28 days post-fertilization did not have any appreciable effect [42]. Thus, pertaining to epigenetics and sex determination-differentiation, the best period to target is from fertilization until the completion of the differentiation of the gonads in gonochoristic species.

3.5.3 Are there Organs Other than the Gonads that should be Considered?

It is also worth mentioning that in some fish species, one sex, typically the males, can come in two distinct phenotypes that have distinct social behavior (polyethism). This is the case of the African cichlid fish, *Astatotilapia (Haplochromis) burtoni*, in which 10–20% of males have large testis, bright coloration, and are reproductively active, exhibiting a dominant role. In contrast, the remaining males have smaller testes, dull coloration, are non-dominant, and do not reproduce. The interesting thing about these fishes is that the two male phenotypes are interchangeable: fish can

switch from one to another, in a matter of about two weeks, depending on the presence of other males. Thus, a dominant male will become non-dominant in the presence of an even larger dominant male [43]. This polyethism is rooted in gene expression changes in the brain [44]. It has been argued that epigenetics must play a very relevant role in the stabilization of alternative brain stages and the repertoire of behavioral outcomes [45].

3.5.4 Links with Ecotoxicology

Substances present in the aquatic environment that are considered endocrine-disrupting chemicals (EDCs) can cause a variety of changes in fish, including alterations in the reproductive performance of affected individuals, as well as exposed populations. The incorporation of epigenetics in ecotoxicological research has been regarded as fundamental [46], because the effects of exposure to EDCs during early development can epigenetically persist until adulthood, with deleterious consequences. In zebrafish, exposure to 17 α -ethynylestradiol—a potent synthetic estrogen and an active compound of the contraceptive pill, the rest of which are detectable in urban effluents—caused DNA methylation changes in the promoter of *vitellogenin1* (*vtg1*) in the liver and brain, potentially affecting reproductive capacity [47]. Exposure to EDCs can affect sex differentiation-related genes, with consequences on sex ratios. However, this is outside the scope of this chapter and, thus, it will not be discussed further.

3.5.5 Does the Study of Epigenetics of Sex Determination-Differentiation have an Added Comparative Value?

Finally, for comparison, the effects of environmental factors mediated by epigenetic mechanisms observed in fish, such as the ones to be described in the next sections, have also been observed in reptiles with TSD. These include the red-eared slider turtles,

Chrysemis picta, regarding *cyp19a1* [48] and the alligator, *Alligator mississippiensis*, regarding *cyp19a1* and *sox9* [49], suggesting that the underlying mechanisms of epigenetic gene regulation are conserved. Thus, insights made in one temperature-sensitive species may be of value for other species, even if they are from a different vertebrate class. The relevance of epigenetics for sex determination across a wide phyletic window, from plants through mammals, has been reviewed elsewhere [50].

3.6 Epigenetics and Sex in Gonochoristic Species – Case Studies

3.6.1 European Sea Bass

The European sea bass, *Dicentrarchus labrax*, is a gonochoristic species with polygenic sex determination [11], and its sex is determined by both genetic and temperature influences (see Chapter 14). In this species, for the first time in any animal, a link has been demonstrated between environmental temperature during early development and *cyp19a1a* expression, through an epigenetic mechanism involving *cyp19a1a* promoter DNA methylation in the gonads [51].

Bisulfite sequencing was carried out for aromatase (*cyp19a1a*) and β -actin as a housekeeping control gene. Results showed that males had higher levels of DNA methylation in the *cyp19a1a* promoter than females (≈ 80 vs. $\approx 40\%$). First, this observation is in agreement with the constitutive lower expression levels of *cyp19a1a* in males, compared to females. Interestingly, exposure to elevated temperature during the critical thermosensitive period increased *cyp19a1a* DNA methylation levels in both sexes. However, the increase in males was not significant, probably due to levels at control temperature already being constitutively high whereas, contrastingly, in females the increase was significant.

Furthermore, a weak but statistically significant inverse relationship was found

between DNA methylation and *cyp19a1a* expression levels in females. Temperature or sex did not affect *cyp19a1a* promoter methylation levels in the brain, which were very high in both sexes, regardless of temperature, corroborating the observation that, in fish, *cyp19a1a* is not expressed in the brain. Also, β -actin exhibited very low levels of DNA methylation, in agreement with the constitutive expression of this housekeeping gene.

In vitro studies confirmed that DNA methylation of the European sea bass *cyp19a1a* promoter prevented its transcriptional activation by *foxl2* and *sf-1*. It was concluded that temperature-induced hypermethylation of the *cyp19a1a* promoter prevents the transcriptional activation of this gene, reducing aromatase levels and, thus, estrogen production. This was believed to result in a fraction of the fish that, under a lower temperature regime, would develop as females, actually developing as males when exposed to elevated temperature [51]. These masculinized females are called neomales [52].

These observations fit well with the fact that, in vertebrates, *cyp19a1a* is the main enzyme responsible for the androgen-to-estrogen ratio. However, being such a powerful environmental cue, it is possible that temperature can also affect other genes, including genes related to sexual differentiation. Another interesting aspect to consider is that the genetic makeup can influence the epigenome and, in turn, how it responds to environmental influences.

The contribution of epigenetic regulatory mechanisms in European sea bass sex determination was further investigated. In this species, fish exposed to elevated temperature when larvae, and sampled at the time of sex differentiation when juveniles, were found to have upregulated the expression levels of genes related to epigenetic regulatory mechanisms: *dicer1*, a helicase needed to produce an active small RNA component that represses gene expression; *jarid2a*, a DNA binding protein that acts as a transcriptional repressor; *pcgf2*, which contains a RING finger motif and forms

protein-protein interactions to maintain transcriptional repression; and *hdac11*, a histone deacetylase [53]. It was noted that, although further studies are clearly needed, these genes are involved, in different ways, in transcriptional repression functions, which here may be connected with the long-lasting effects of early heat exposure.

As mentioned in Chapter 14, which deals with European sea bass sex determination, it has been noticed recently that highly biased sex ratios are observed in farms, even in broods that have been raised under a non-masculinizing thermal regime. The underlying reason for this is not known, but one possibility would be the epigenetic inheritance of masculinization of broodstock that were exposed to elevated temperature some years ago, when they were in the larval stages.

3.6.2 Half-Smooth Tongue Sole

The half-smooth tongue sole, *Cynoglossus semilaevis*, has a ZW/ZZ (female/male) system of sex determination, where male-specific expression of the Z-linked *dmrt1* gene is associated with testicular differentiation [21]. As in the European sea bass, exposure to elevated temperature during early development induces some genotypic females to develop as neomales. In a landmark study, Shao *et al.* [54] used whole genome bisulfite sequencing (WGBS) to interrogate gonadal DNA methylation patterns across the whole genome of males, females, and neomales (called pseudomales in that study). *Dmrt1* was hypomethylated in males and pseudomales, but hypermethylated in females, indicating that males and pseudomales not only are equivalent in terms of gonadal morphology, but also in the epigenetic regulation of this important gene.

However, other genes involved in testicular and ovarian differentiation, including *cyp19a1a*, did not show the expected inverse relationship between DNA methylation and gene expression levels. Furthermore, it was found that some neomales can spontaneously generate more neomales, even in the

absence of elevated temperature, suggesting a transgenerational epigenetic inheritance of sex reversal in this species [54]. The consequences of this are twofold. The first arises because these changes are, in many instances, maladaptive [55], constituting a sort of “epigenetic trap” [35, 56] that can have negative consequences for population fitness. On the other hand, fish farming could take profit from these epigenetic traps since, for example, one can immediately think of the advantage represented by having epigenetically-produced neomales that could be incorporated in a monosex production system.

3.6.3 Olive Flounder

The olive flounder, *Paralichthys olivaceus*, is a gonochoristic species with a XX/XY female/male sex determination system, with sexual growth dimorphism in favor of females [57]. The quantitative expression, cellular distributions, and methylation patterns of *cyp19a1a* and *dmrt1* have been investigated in this species [58]. Following the accepted pattern in fish, results showed that while *dmrt1* expression was ≈ 70 times higher in the testis than in the ovary, in contrast, *cyp19a1a* expression was ≈ 40 times higher in the ovary than in the testis. The *dmrt1* promoter CpGs were completely unmethylated in the testis but, in the ovary, methylation was close to 60%. In contrast, *cyp19a1a* promoter methylation in the testis was close to 100% while this figure dropped to about 75% in the ovary. These observations show that, in this species, *dmrt1* and *cyp19a1a* are sex-related genes with sexual dimorphic expression and CpG methylation. However, how such opposing methylation patterns are generated and regulated is at present still unknown.

Building from these results, Si *et al.* [59] found a strong relationship between *cyp19a1a* and its transcriptional activator *foxl2* DNA methylation and gene expression levels during ovarian differentiation of the olive flounder. These two genes are known to

exhibit parallel expression patterns, and this study shows that this link is also found in terms of epigenetic silencing-activation. Together, these findings show that methylation of the *dmrt1*, *cyp19a1a*, and *foxl2* genes are important for the sexual differentiation of olive flounder. This pattern likely applies to many fish species.

The candidate gene approach is not exempt of risks. Genes that are transcribed under a variety of different conditions, and that have a CGI immediately upstream of the transcription start site, are characterized by having a low level of methylation in this area. Thus, they may not experience appreciable changes in DNA methylation, something that does not make them ideal candidates without *a priori* knowledge of differential methylation [60].

On the other hand, a single CpG may make the difference. In this regard, Ding *et al.* [61] found that, in the coding region of the olive flounder *cyp17-II*—a gene involved in growth, gonad differentiation and development, as well as other reproductive traits of fish—there were three CpG-rich regions. Three SNPs were identified and located in exons 4 and 6. One of these added a new methylation site to the *cyp17-II* coding region, and this was always methylated, with concomitant lower *cyp17-II* expression and lower testosterone levels [61]. These findings illustrate the phenotypic difference that a single DMC can make. Incidentally, this was a gene-targeted study, but a similar finding would likely be passed unnoticed in a study targeting other genes. Thus, if possible, it is important to target a representative part of the whole genome.

3.6.4 Nile Tilapia

The Nile tilapia is a gonochoristic teleost with a XX/XY sex determination system, with sexual growth dimorphism in favor of males. Many factors may contribute to this growth superiority of males but, incidentally, Zhong *et al.* [62] found that DNA methylation

of pituitary growth hormone is linked to this sex-related growth superiority.

Chen *et al.* [21] used qPCR results to show that the expression level of fibroblast growth factor 16 (*fgf16*), *sialidase-3-like*, *fgf20*, *cyp19a1a*, *estrogen receptor*, and *gonadotropin receptor II precursor* were negatively correlated to their methylation levels in the ovary and testis, as assessed by MeDIP-seq. Sun *et al.* [63] also used MeDIP-seq to determine the genome-wide DNA methylation patterns in the ovary and testis of Nile tilapia. Results showed that, while gene bodies exhibited high levels of DNA methylation, the promoter regions had low levels. Again, *cyp19a1a* DNA methylation and gene expression were inversely correlated. The DNA methylation level in females was higher than that in males for various chromosomes.

It is interesting to note that, the half-smooth tongue sole average global methylation levels were about 10% higher in testes with respect to the ovaries, except for the W chromosome [54] while, in contrast, the Nile tilapia females had higher global methylation levels than the males in various chromosomes [63]. The underlying reason for this global interspecific difference is not known.

3.7 Epigenetics and Sex in Hermaphrodite Species – Case Studies

Only about 5% of the more than 33,000 species of teleost fish are hermaphrodites. Among these, the majority are sequential hermaphrodites, implying that they go through sex change, while the rest are simultaneous hermaphrodites, which do not change sex. Within sequential hermaphrodites, most are protogynous (female-to-male sex reversal), and the rest are protandrous (male-to-female sex reversal) [64]. In most hermaphrodites, external stimuli dictate the sexual phase of the gonads so that, in sequential hermaphrodites, while one sex becomes functional, the other sex becomes inactive.

Hermaphrodites use the same genetic toolkit that gonochoristic species use to organize their gonads and to accomplish protogynous or protandrous sex differentiation and, in sequential hermaphrodites, also sex change.

Transcriptomic studies, for example as in the protogynous bluehead wrasse, *Thalassoma bifasciatum*, showed much larger sex-related differences in the gonads, compared with the brain [65], with most genes involved in sex differentiation in other species exhibiting an “orthodox” behavior. This means that, for example, genes related with ovarian differentiation and maintenance, such as *cyp19a1a*, become downregulated during protogynous sex change, while, in contrast, genes related with testicular development, such as *dmrt1*, become upregulated. Among this framework, there can be exceptions and, thus, some genes related to ovarian differentiation, such as *rspo1* and *wnt4b*, can exhibit unanticipated expression patterns, as observed in the sharp-snout seabream, *Diplodus puntazzo* [66]. Thus, observations, in terms of epigenetic changes during male and female sex differentiation in gonochoristic species, are expected to be also found in hermaphroditic species during protogynous and protandrous sex change, respectively. The examples below involve species of protogynous, protandrous, and simultaneous hermaphrodites, and constitute the first reports on epigenetics of sex change in hermaphrodite fish. They seem to confirm the framework stated above.

3.7.1 Ricefield Eel

The ricefield eel, *Monopterus albus*, is a protogynous monandric hermaphrodite fish in which the smaller and younger individuals are females, which develop and mature as such, while the larger individuals are functional males that reach this condition only after sex reversal. Thus, no primary males are known in this species. Individuals of an intermediate age have ovotestes.

In order to investigate the contribution of epigenetic regulation of gene expression, Zhang *et al.* [67] conducted a series of

experiments focusing on *cyp19a1a*, given its importance for estrogen production, which is essential for accomplishing protogynous sex change. Similar to what occurs in other species, the ricefield eel *cyp19a1a* promoter can be stimulated via cAMP, through its cAMP response element (CRE). Analysis of the *cyp19a1a* promoter showed that it was hypomethylated in the ovary, and hypermethylated in the ovotestis and testis. In accordance with this observation, the methylation levels of CpG sites around CRE in the distal region (region II), and around steroidogenic factor 1/adrenal 4 binding protein sites and TATA box in the proximal region (region I), were inversely correlated with *cyp19a1a* expression during female-to-male natural sex change. In addition, chromatin immunoprecipitation (ChIP) assays showed that H3K9 in regions I and II of the *cyp19a1a* promoter were deacetylated and trimethylated in the testis, in accordance with the lower *cyp19a1a* expression levels in this organ [67].

These authors also found that treatment with a DNMT inhibitor, 5-aza-2-deoxycytidine (5-aza-dC), reversed the natural sex change of ricefield eels. 5-aza-dC does not target a specific gene but, rather, has a genome-wide effect and, thus, the participation of other genes cannot be ruled out. Nevertheless, taken together, these results indicate that DNA methylation and histone deacetylation and methylation may inhibit the gonadotropin stimulation of *cyp19a1a* in males, and that this is part of an important underlying mechanism of sex change in hermaphroditic species.

3.7.2 Black Porgy

The protandrous black porgy, *Acanthopagrus schlegelii*, is a species that has gonads with testes and ovaries topographically separated by connective tissue, as in all hermaphroditic members of the Sparidae family. They are males during their first two reproductive cycles, and then they start changing to females. Treatment of juveniles

with exogenous estrogen is able to induce a transient ovary that reverts to testis upon steroid withdrawal. On the other hand if, during the second year, the testicular part of the gonad is removed, the animals precociously turn into females. This species, therefore, provides yet another excellent model to study the epigenetic control of the process of sex change in fish.

Focusing also on *cyp19a1a*, Wu *et al.* [68] found that, in the testis, *cyp19a1a* is hypermethylated, and that the first signs of protandrous sex change were decreased methylation levels and increased numbers of hypomethylated clones of the *cyp19a1a* promoter. Then the ovarian follicle cells exhibited low levels (0%–20%) of *cyp19a1a* promoter region methylation. In addition, Wu *et al.* found low *cyp19a1a* DNA methylation levels in the gonad (ovary), in which the testicular part had previously been removed one month after surgery. Furthermore, treatment with estradiol-17 β maintained low levels of *cyp19a1a* promoter methylation. These results were interpreted as that in the digonic gonad of the black porgy, the testis portion, which is the first one to mature, controls the epigenetic changes of at least the *cyp19a1a* promoter methylation in the ovary [68].

3.7.3 Barramundi

The barramundi, *Lates calcarifer*, is a large protandrous hermaphrodite that undergoes male-to-female sex change when it attains 3–5 years of age. It has great aquaculture potential, but the time needed to reach sex change implies that producers need to maintain fish as males before they can be bred as females. Domingos *et al.* [69] investigated the methylation levels in the promoter and first exon of six sex-related genes. *Dmrt1* and *nr5a1* methylation levels were lower in testis than in ovaries, *foxl2* and *sox8* had low (<10%) methylation levels in both sexes, and *cyp19a1a* and *amh* methylation levels were higher in testis than in ovaries [69].

3.7.4 Mangrove Killifish

The mangrove killifish, *Kryptolebias marmoratus*, is a simultaneous hermaphrodite that is capable of self-fertilization (selfing), to favor reproductive success when finding a mate is difficult. However, since selfing renders populations more vulnerable to environmental change by reducing genetic variability, a mixed-breeding strategy, relying on the alternation between selfing and outcrossing depending on context, may allow species to balance these needs, but requires a system for regulating sexual identity precisely. This species, therefore, constitutes another excellent model to investigate sex determination and mating strategy associated with environmentally-induced epigenetic modifications [55].

In this regard, Ellison *et al.* [70] exposed selfing animals to different temperatures, and encountered a significant interaction between temperature, methylation patterns of genes associated with sex differentiation, and sexual identity (male or hermaphrodite). These genes represent candidates for the temperature-mediated regulation of sexual identity, and the researchers concluded that epigenetics provides a mechanism by which environmental change may influence selfing rates [70].

3.8 The “Conserved Model of Epigenetic Regulation of Sexual Development in Fish”

The examples discussed so far in the sections above indicate that epigenetic regulation of gene expression is involved in the sexual development of gonochoristic fish with different types of sex-determining mechanisms, as well as in driving the process of sex change in different types of hermaphrodites. Based on what we know so far, it is possible to build a model that relates epigenetic states of gene expression regulation and actual expression levels for pro-male and pro-female genes (see Figure 3.1 and Figure 3.1 legend for details).

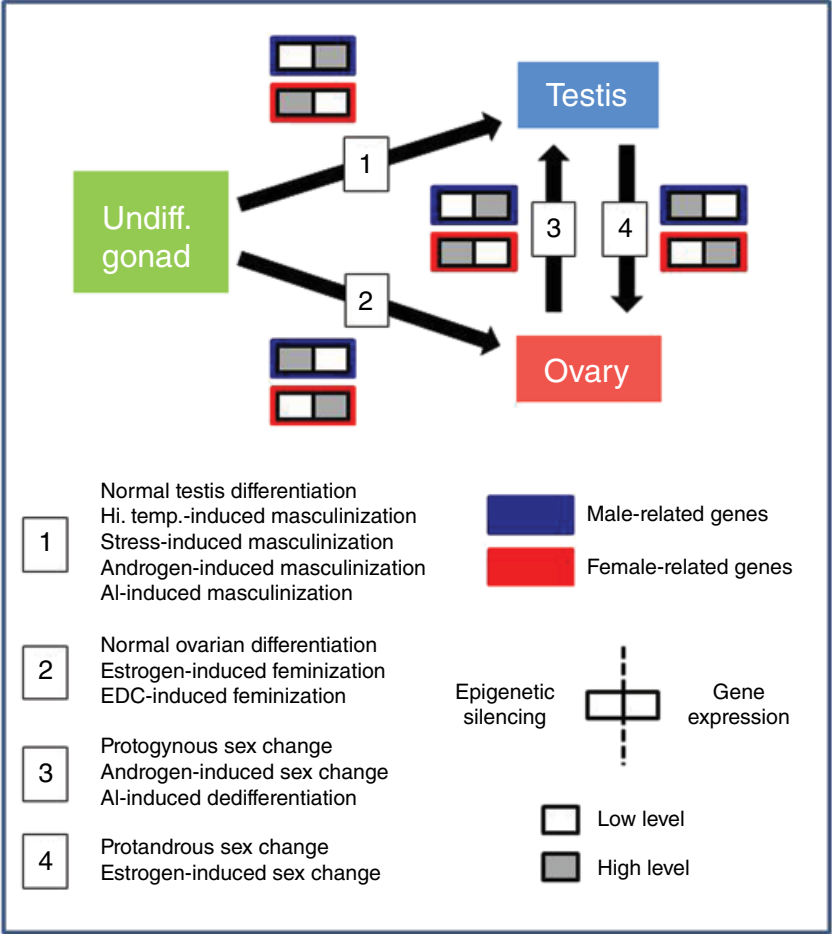


Figure 3.1 Proposed model for the epigenetic regulation of sexual development in fish. This model deals with the relationship between gene-silencing features, such as DNA methylation, H3K9me enrichment, etc., and gene expression levels during either sex differentiation in gonochoristic species or sex change in hermaphroditic species. In each type of development (arrows 1–4), pro-male (Black or blue outline) and pro-female (Gray or red outline) genes refer to genes that are exclusively or preferentially expressed in one sex with respect to the other. In each box, the left side refers to epigenetic silencing, and the right side to gene expression. White and grey squares indicate lower and higher levels, respectively, of epigenetic silencing and gene expression. Arrows between the three different gonadal phenotypes (undifferentiated [Undiff.] gonad, testis and ovary) indicate some of the possible different means to reach this phenotype, outlined by the 1–4 boxed numbers. There might be other means. This model is based on the assumption that there are “pro-male” and “pro-female” genes, and on the canonical inverse relationship between (promoter) gene silencing and gene expression levels. Importantly, it also postulates that, in terms of the epigenetic and gene expression patterns involved, the final gonadal phenotype is more relevant than the means to achieve it. Abbreviations: AI, aromatase inhibitor; EDC, endocrine disrupting chemical. Hi. Temp., high temperature.

This model for the epigenetic regulation of sexual development in fish deals with the relationship between gene-silencing states and gene expression levels during sex differentiation in gonochoristic species, or sex change in hermaphroditic species.

We can call this model the *Conserved model of epigenetic regulation of sexual development in fish*, because the underlying mechanisms are postulated to be conserved across species and reproductive strategies. The model is based on the assumption that there

are “pro-male” and “pro-female” genes, and on the canonical inverse relationship between gene silencing and gene expression levels. Importantly, it also postulates that a given set of epigenetic and gene expression patterns is more associated with a particular gonadal phenotype (e.g., males) than the means by which this phenotype is obtained, be it either the result of a natural process (e.g., male sex differentiation) or of human intervention (e.g., androgen-induced masculinization).

So far, then, we have seen what we know takes place in terms of epigenetics in a natural context. In the next sections, we will explore how we can take advantage of what we know on epigenetics in order to control sex.

3.9 Epigenetics and Sex Control in Fish

It should be clearly stated right from the beginning that, nowadays, there is no such thing as epigenetics and sex control in fish. Thus, what follows below are some insights based on our current understanding of epigenetics and of sex control in fish, with the purpose of stimulating research. To frame the issue, let us first recapitulate that sex control – or, more specifically, sex ratio control – in fish has two major applications: in aquaculture, to favor the most desirable sex [52, 71], and in population control, either to boost endangered populations [72], or to eliminate invasive species [73]. The rest of this section is devoted to sex control in finfish aquaculture.

In many species of farmed fish, one sex – usually females – grows more than the other sex. This has implications for production. In order to achieve monosex stocks, several approaches are available, namely:

- hormonal treatment by direct or indirect (no hormone exposure in the fish destined for human consumption) methods [4, 52, 71];
- through the combination of triploidy and indirect feminization, or by gynogenesis [74]; or
- through hybridization and selection [71].

Each one of the above-mentioned methods has its own set of pros and cons.

In a context in which breeding programs are gaining importance for an increasing number of farmed fish species, epigenetic programming could find its place [75, 76]. Here, I define epigenetic programming as the actions undertaken to exploit the effect of the early environment on the establishment of a series of long-lasting epigenetic marks that will, later in life, confer an advantage to face a certain environment. For example, in nutritional programming, larvae or juveniles may be exposed for a period of time to a diet of defined characteristics – for example, with substituted animal protein for plant proteins – in order to condition these animals when adults to have better growth and survival rates than non-conditioned counterparts [77].

In the same way, one could envisage “reproductive programming,” in which broodstock would be selected – based not only in genetics but also taking into account a specified set of epigenetic characteristics – to produce offspring with certain desired reproductive characteristics (for example, with a sex ratio biased toward the sex with superior growth rates, delayed maturation or better food conversion efficiency). Another possibility would be to select broodstock with a given epigenetic makeup that would pass to the offspring the capacity of not succumbing to masculinization, even if reared at elevated temperatures to promote initial fast growth, or at high stocking density to optimize space. Thus, in a sort of epigenetic programming applied to fish farming, the identification of broodstock fish with a particular methylation profile holds promise, because these animals could pass specific DNA methylation marks to their offspring.

Preliminary results of research carried out at the Institute of Marine Sciences in Barcelona along these last lines hold promise. However, looking at the other side of the coin, the knowledge on epigenetics can no longer be ignored, if not to improve things, then at least to avoid going along the wrong

path. Thus, an unfavorable programming going unnoticed could lead to unwanted sex ratios, higher susceptibility to temperature, etc. In summary, the above two types of epigenetic programming have been witnessed – one intragenerational (the example of nutritional programming), and the other that could be transmitted to the next generation in a sex-specific manner (reproductive programming). However, one cannot ignore the two faces of Janus looming over epigenetic programming.

Many questions, then, remain in order to have a clear picture of the patterns of gene expression programming during early development, throughout life and down to the next generation. Attention has been recently called to the fact that, although a specific epigenetic modification may be present or not, thus resembling a digital on-off state, in fact, epigenetic modifications should be regarded not as discrete (yes/no), but as graded [78], because a trait can change over time in its intensity depending, for example, on the number of cells affected.

Further, this gradation brings the additional concept of “epigenetic wash-in” and “epigenetic wash-out,” referring to those epigenetic changes that, either intragenerationally or intergenerationally, increase or decrease, respectively, in a non-linear fashion over time [78]. Thus, for a given type of sequence:

- Is it equally affected in every individual in response to a particular environmental stimulus?
- Are different tissues responding differently?
- Taking into account that is not the same whether somatic tissues or germ cells are affected, do the phenotypic consequences depend on the type of sequence or tissue affected?
- Are there specific genetic makeups that confer different susceptibility/resistance to environmentally induced epigenetic alterations and epigenetic inheritance?

These are just some of the questions still to be answered.

3.10 Open Questions and Future Perspectives

Despite recent advances and interesting insights, our knowledge of the role of epigenetics in sex determination and differentiation in fish is still rudimentary. The pioneering work of Navarro-Martín *et al.* [51] with the European sea bass showed that, by virtue of being the sole steroidogenic enzyme responsible for the balance between androgens and estrogens, and given that estrogens are needed for ovarian differentiation in all non-mammalian vertebrates [79], *cyp19a1a* was strategically placed to be among the first target genes to be identified as being under epigenetic regulation during sexual development. However, the whole genome study of Shao *et al.* [54] with the half-smooth tongue sole clearly showed that differences between sexes, in terms of DNA methylation, can occur genome-wide, and that temperature can affect genes other than *cyp19a1a*.

So, an open question is: what effect does temperature really have in the gonads in terms of setting meaningful epigenetic modifications? Here, once again, the challenge is to disentangle causal from consequential changes in DNA methylation patterns. Further, the challenge also consists of being able to identify, from all sorts of epigenetic alterations (i.e., a sort of “background epigenetic noise”) taking place during the process SD in gonochoristic species or during sex change in hermaphroditic ones, the essential epigenetic marks (EEMs) that actually are responsible. Here I define EEMs as the number – one, two, several – of measurable and identifiable epigenetic marks, such as DMCs, DMRs, or a given set of histone modifications in specific loci, that are strictly necessary, albeit perhaps not sufficient, to bring about a specific, measurable phenotype.

To illustrate this, hypermethylation of the promoter of, for example, *cyp19a1*, *foxl2*, and concomitant hypomethylation of the promoter of *dmrt1* and *cyp11b* could perhaps be expected to be required for proper male

development in normal conditions or after heat exposure in a gonochoristic species – or, also, for maintaining the male phenotype in a sex-changing hermaphrodite.

From a more practical point of view, another challenge would be to use these EEMs. For example, a defined set of meQTLs could be used as EEMs to aid in selection, in order to identify in those broodstock fish with a certain epigenetic profile that is suitable to withstand a masculinization environment due to elevated density or temperature, two masculinizing factors, the underlying mechanisms of which start to be known [80, 81].

The application of the concepts of epigenetics, epigenetic research methods, and epigenetic programming to help us to understand sexual development in fish and, eventually, to aid in fish sex control, is still in its infancy. Thus, much research is needed in the years to come. The *conserved model of epigenetic regulation of sexual development*

in fish and the concept of EEMs outlined above are just two examples of tools we can develop, to foster and better focus research efforts. In the upcoming years, many more examples and questions will undoubtedly arise, as progress in this exciting field continues to be made.

Acknowledgments

Research at the FP lab was supported by Government of Spain grants ref. AGL2013-41047-R and AGL2016-78710-R and Government of Catalonia grant ref. 2009SGR1050. Thanks are due to Silvia Joly for her help with the references; to Drs. Carlos Guerrero-Bosagna, Laia Ribas, and Dafni Anastasiadi, as well as to Susanna Pla and Alejandro Valdivieso, for helpful comments on the manuscript.

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4

Environmental Sex Determination and Sex Differentiation in Teleosts – How Sex Is Established

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4.1 Introduction

Fish are a fascinating clade of animals that contains more than 30,000 recognized species [1, 2]. This great biological diversity also contains the most diverse reproductive strategies, compared with other groups [3], including gonochorism (genotypic sex determination (GSD); environmental-dependent sex determination (ESD)), hermaphroditism (protandrous, protogynous, both-way and simultaneous hermaphroditism), and unisexuality (e.g., gynogenesis, androgenesis) (see terminologies in Chapter 1).

Intriguingly, as investigation progresses, it seems that sex determination is much more complex than we ever thought, not only within a single species, but in a phylogenetic context as well [3, 6–15]. Sex could be determined by a single master sex-determining gene [3, 8, 16–20], by multiple sex-associated loci [9, 21–26], by environmental factors (e.g., temperature, density, social interactions) [3–5, 27–30], or by a combination of genetic and environmental factors [7, 8, 16, 31–33] (Box 4.1). Sex differentiation, the process by which an undifferentiated gonad is transformed into an ovary or a testis, involves a bewildering network of multi-level, multi-gene, multi-hormone, multi-target interactions on undifferentiated and differentiating gonads. Meanwhile, sex differentiation can

be influenced by abiotic and biotic environmental factors, including temperature, exogenous hormones, endocrine-disrupting chemicals, pH, background color, hypoxia, social interactions (e.g., density), and food availability in a wide range of fish species [5, 34–39]. Therefore, in some instances, phenotypic sex may not coincide with the genotypic sex.

ESD, a concept raised 40 years ago [40] as an alternative sex-determining mechanism to GSD in fish and reptiles, however, has been misused over and over again [16, 27, 41], due to the confusion between sex determination and sex differentiation, and the unresolved mechanism involved in ESD. Temperature-dependent sex determination (TSD), one type of ESD, has received the most extensive attention. TSD, as other ESDs, has been misused partly because, in many cases, it is difficult to distinguish it from GSD plus temperature effects (GSD + TE).

Different terms, including “temperature effects on sex ratio”, “temperature effects on sex differentiation”, “temperature influences on sex determination”, “thermolabile sex determination”, “temperature-dependent sex ratios”, “temperature-dependent sex differentiation”, “temperature induced sex reversal”, and so on, have been used to explicitly or implicitly indicate TSD. Even though there are unambiguous definitions and

Box 4.1 How sex is determined in ESD

- The direct target(s) of environmental factors involved in ESD in fish have not been characterized.
- The assumption is that the environmental master switch (e.g., thermo-sensitive, pH-sensitive) gene(s) activate or specify responses (testis- or ovary-determining pathway) during the plastic time window.
- Two pathways are proposed to deduce how sex is determined in ESD:
 - epigenetics is assumed to be the key mechanism for sex determination in ESD.
 - sex is determined via the interactions of hormones, genes, and cells, which are modulated by cortisol, the main stress hormone in fish.

criteria to distinguish GSD, GSD + TE, and TSD [27, 41], how sexes are determined by temperature remains unknown. Sex ratio variation could be a result of environmental factors, as we mentioned, differential embryo or larvae mortality, or parental influence. In addition, studies that indicate TSD may represent only the status of a particular population, not the entire species [6–8, 21, 42].

Many researchers have pointed out that the genetic difference between sexes in TSD is little [27, 41, 43], but what *is* the “little” has not been proposed? Ecology and adaptive significance, which are considered as important components of TSD [14, 40, 41, 43], have only been verified in one species to date [43]. All of these lead to the misuse of ESD/TSD.

Sex control is to produce a monosex population for aquaculture or research purposes, using the sex-determining mechanism knowledge in a given species. In turn, monosex production can facilitate the pace of uncovering the underlying sex-determining mechanisms. In a commercial environment, sex control may involve many aspects, including hormone- or environment-induced sex reversion, chromosome manipulation, hybridization, searching sex-linked markers,

genetic selection, and identification of sex-reversed individuals, in order to produce large numbers of monosex fry continuously. In view of the effects of temperature on sex differentiation in a large number of species [4, 16, 27, 32, 36, 41], temperature treatment, a chemical-free and, thus, pollution-free approach, is considered as a promising environment- and consumer-friendly method in monosex production. Actually, temperature sensitivity is demonstrated to be inheritable, and can be accumulated through selection [45–48]. Furthermore, other environmental factors, such as rearing background color and density, should be considered in aquaculture activities, since they can potentially influence the sex ratios [28, 30, 49].

In this chapter, we review the current knowledge on how to distinguish TSD from GSD + TE, how sex is determined in ESD, and molecular networks involved in sex differentiation. We propose that environmental factors, which could be considered as moderate stress factors, transduce signals via the stress response pathways, with cortisol as the key mediator on related genes, hormones, and cells in all types of ESD. We also discuss the pros and cons of ESD in aquaculture and fisheries.

4.2 Distinguishing TSD from GSD + TE

It is interesting, while not surprising, to find there are so many similarities between TSD and GSD + TE in terms of pathways in sex differentiation, if we see the transition of sex-determining mechanisms as a continuous event (Figure 4.1), even though they are essentially different mechanisms. Empirical studies suggest that the transition between sex-determining modes have occurred several times in fish, reptiles, amphibians, and so on [15, 50–53], and thermosensitivity in sex determination has been assumed to be the key factor in those transitions [53]. It has been demonstrated that transition from GSD to TSD can be achieved in the first generation

in a reptile, the Australian bearded dragon (*Pogona vitticeps*) [54]. Recently, it has been found that TSD and GSD + TE can coexist in the same population in Atlantic silverside and pejerrey [7, 8], indicating that the sex-determining mechanism may respond and transform quickly, especially in species that experience changing environments. These findings also prove that identification and confirmation of sex-determining mechanisms will be much more complex and time-consuming than ever.

Nevertheless, there are recognized criteria to distinguish ESD from GSD (Figure 4.1). In the present review, we consider that ESD is stress-induced sex determination and, specifically, we consider that all environmental conditions that are beyond the range of optimum conditions (e.g., higher/lower temperature, acidic/alkaline pH) are stress factors, as we will discuss later. Four criteria are proposed here to distinguish ESD from GSD, based on previous reports [27, 41]. The essential difference between ESD and GSD (or GSD + TE) is how sex is determined. Therefore, confirming the existence of sex chromosomes (heteromorphic or homomorphic) or sex-determining genes will rule out ESD immediately (Figure 4.1).

First, it has been estimated that about 10% of species have cytogenetically distinct sex chromosomes [5, 55]. Other than the classical cytogenetic method, phenotypic and molecular markers have also been applied in identification of sex chromosomes [5]. Seven sex-determining genes, *dmy/dmrt1Y* in medaka [56, 57], *amhy* in pejerrey and tilapia [18, 19, 58], *gsd^h* in *Oryzias luzonensis* [59], *amhr2* in fugu (tiger pufferfish) *Takifugu rubripes* and another two *Takifugu* species [60], *sdY* in rainbow trout and many salmonids [61, 62], *Sox3^Y* in *Oryzias dancena* [20], and *dmrt1* in half-smooth tongue sole (*Cynoglossus semilaevis*) [17], have been identified in fish to date.

Second, if sex ratio does change when exposed to a wide range of environmental conditions, including abiotic and biotic factors (e.g., temperature, pH, dissolved oxygen,

food availability, habitat background color, and social interactions), ESD will be ruled out (Figure 4.1).

Third, sexually differential fertilization or mortality should be taken into account and, if they are the reason for the variation of sex ratio, then the given species should be classified as GSD + TE rather than ESD.

Fourth, if variation of sex ratio is caused by extreme environmental conditions (e.g., high temperature), specifically, beyond the range of environmental conditions during development (including the sensitive period of sex differentiation), the given species is classified as GSD + TE.

The latter two, especially GSD + TE, have not been given enough attention. As the end of the continuous event (Figure 4.1), ESD should be considered as a counterpart of GSD [14, 27, 43]. It has been proposed that ESD is preferred when the environment experienced by offspring influences the fitness (e.g., fecundity, size) of the two sexes differently [7, 14, 40].

We expect that “no populations display pure ESD or GSD” [7] in more species, especially widely distributed fish species. It is also worth mentioning that, though sex is predetermined at fertilization by an individual's genotype for GSD, sex-determining genes are expressed later (Figure 4.2a) [56, 57]. Further, it is possible that expression of sex-determining genes could be affected by environmental factors, consequently influencing sex differentiation.

4.3 How Sex is Determined in ESD

The direct target(s) of environmental factors involved in ESD in fish have not yet been characterized. It is speculated that the environmental master switch (e.g., thermo-sensitive, pH-sensitive), which transduces the physical, chemical or biological signal into molecular, hormonal, and cellular responses, and drives the undifferentiated gonads to follow the male or female pathway, are the gene(s) that activate or specify responses (the

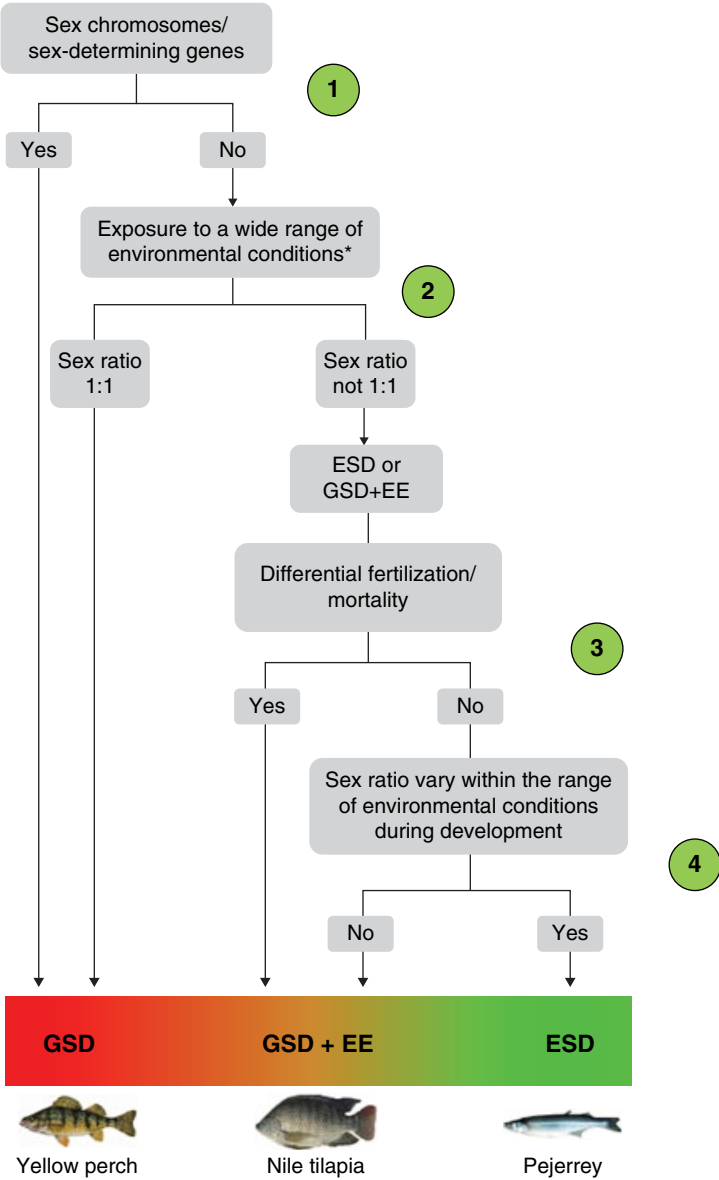


Figure 4.1 Criteria for distinguishing temperature-dependent sex determination (TSD) from genotypic sex determination (GSD) and GSD plus environmental effects (EE). The example for each sex-determining mechanism only represents geographic population(s) of this species, rather than the whole species. The data presented are a compilation from [27, 41]. Numbers indicate the criteria. Environmental conditions include temperature, dissolved oxygen, density, pH, food availability, social interactions, background color, etc.

testis- or ovary-determining pathway) during this plastic time window. Candidates for this role would be genes that are expressed prior to, or exactly at, the onset of the sensitive period, rather than genes that are differen-

tially expressed after this period [26], or genes that are differentially expressed as a result of testicular or ovarian development. Key information on how sex is determined in ESD is summarized in Box 4.1.

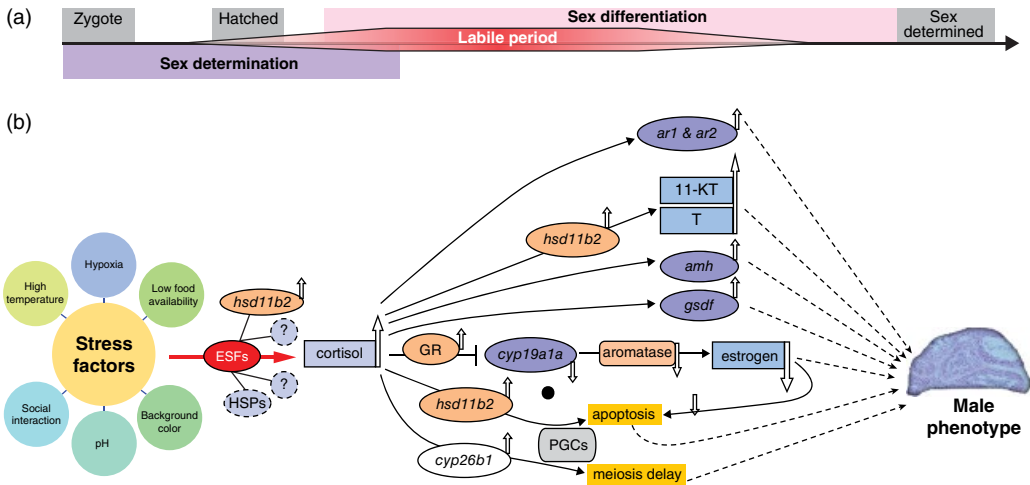


Figure 4.2 Timeline of sex determination and sex differentiation (a), and the pathway of stress-induced testis differentiation (b).

ESFs, environmental-sensitive factors; HSPs, heat shock protein; GR, glucocorticoid receptor; PGCs, primordial germ cells; 11-KT, 11-ketotestosterone; T, testosterone; *hsd11b2*, 11 β -hydroxysteroid dehydrogenase gene; *cyp26b1*, cytochrome P450 family 26 subfamily B member 1, codes for cytochrome P450 enzyme that metabolizes retinoic acid; *cyp19a1a*, cytochrome P450, family 19, subfamily A, polypeptide 1a, codes for ovary type aromatase; *ar*, androgen receptor gene; *amh*, anti-Müllerian hormone gene (also known as *mis*, Müllerian-inhibiting substance gene); *gsdf*, gonadal soma-derived growth factor gene.

Data refers to [49, 97–102, 104, 147, 213, 214].

Two such potential “master switch” genes, the *sf-1* and *wt-1* that are involved in the formation of a bipotential gonad, have been proposed, based on their early significant differential expression before the onset of the labile period in reptiles with TSD [63–66]. A large amount of molecular players located downstream of the pathway in fish with TSD have been studied, and are found to be conserved [16]. Expression of some of these genes, including *dmrt1*, *amh*, and *sox9* for the testis-determining pathway, and *foxl2*, *cyp19a1a*, and *sf-1* for the ovary-determining pathway, have been found to be sexually different and thermo-sensitive [16], indicating their involvement in temperature-dependent sex differentiation in both TSD and GSD + TE.

Since there is very little genetic difference between sexes in ESD [27, 41, 43], we propose two pathways here to deduce how sex is determined in ESD and, specifically, how environmental signals are transduced into target organs and decide the fate of sex.

4.3.1 Epigenetics

Epigenetics is an exciting area of biology that is currently proceeding at an amazing pace. Epigenetics is the study of changes in gene function that cannot be explained by changes in the DNA sequence [67]. Three mechanisms of epigenetics – DNA methylation, modification of histones and histone variants, and the presence of non-coding RNAs – are all found to be involved in sex determination/sex differentiation in fish, reptiles, and mammals [68–72].

Sexually dimorphic DNA methylation patterning of sex differentiation-related genes and factors (e.g., *cyp19a*, *foxl2*, *sox9*, *dmrt1*, *sf-1*), and estrogen receptors, have been observed in several fish and reptile species [17, 73–80]. DNA methylation of gonadal aromatase (*cyp19a1a*) promoter has been found to be involved in temperature-dependent sex differentiation in European sea bass [75], a species with strong temperature effect on sex ratio (GSD + TE) [81]. High temperature treatment during the thermo-sensitive

period, which is well before morphological sex differentiation, increased DNA methylation of gonadal *cyp19a1a* (but not brain type *cyp19a1b*) promoter and decreased *cyp19a1a* mRNA expression via blocking *sf-1* and *foxl2* stimulated *cyp19a1a* expression, consequently causing masculinization [75]. This is the direct evidence that methylation is involved in temperature-induced masculinization, though how temperature transduces the thermal signal into a molecular trigger remains unknown.

The *in vitro* findings in this study also indicate that the regulation by temperature is endogenous within cells in the bipotential gonad, as demonstrated in the red-eared slider turtle (*Trachemys scripta*, TSD) [82], specifically, temperature functions directly on undifferentiated gonads, and does not require other embryonic tissues to be expressed in a normal pattern.

Elevated methylation of *cyp19a1a* promoter and decreased expression at male-producing temperature, relative to female-producing temperature, and conversely at the *sox9* locus, from embryonic gonads in American alligator (*Alligator mississippiensis*, TSD) have been reported recently [76]. Similar results regarding *cyp19a1a* were also observed in another TSD species, the red-eared slider turtle [74]. These results indicate that male-producing temperatures cause methylation of ovary-determining genes and/or demethylation of testis-determining genes, and female-producing temperatures cause methylation of testis-determining genes and/or demethylation of ovary-determining genes, leading to the development of testes and ovaries, respectively, in TSD species.

It is worth mentioning for future studies that we expect that epigenetic mechanisms will be involved in many aspects and components of sex differentiation and sex determination. Epigenetic regulation of the action of steroid and thyroid hormones [83, 84] should also be taken into account. In mice, a histone demethylase, *Jmjd1a*, directly and positively controls the mammalian Y chromosome-bound sex-determining gene *Sry*, and male-to-female sex reversal has been observed in

mice lacking the H3K9 demethylase *Jmjd1a* [72], indicating the involvement of epigenetics in sex determination. Therefore, epigenetics is also assumed to be the key mechanism for sex determination in ESD, since there is little genetic difference between two sexes.

4.3.2 Hormone-Gene-Cell Interactions

As early as 1985, it was reported that cortisol and cortisone administration inhibited ovary differentiation and increased male ratio in rainbow trout larvae [85]. In recent years, several studies have found that high temperature treatments elevated cortisol levels and resulted in the masculinization of fish species with TSD and GSD + TE. Hormones are considered as the primary links between environmental conditions and physiological activities, because environmental information must first be transduced into a physiological signal to affect sex ratio [86].

Cortisol is the major glucocorticoid produced by the interrenal cells, and is used as a key indicator of stress since its production is increased by stress factors such as rapid temperature changes, hypoxia, handling, and acid water in fish [87]. Cortisol regulates a diverse array of systems, including metabolism, ion regulation, growth, and reproduction [88]. We speculated earlier that cortisol may be the “lost” link between temperature and the sex-determining mechanism in species with TSD [16]. Here, we extend the speculation to ESD species. Specifically, we consider that mild fluctuation of environmental conditions or environmental conditions (e.g., high temperature, hypoxia, low food availability, non-neutral pH, bright background color, social interactions) beyond the range of optima are stress factors. These stress factors could induce masculinization. Thus, for the first time, we propose that for ESD species, environmental factors transduce signals via the stress response pathway. Sex is then determined via the interactions of hormones, genes, and cells, which are modulated by cortisol, the main stress hormone in fish (Figure 4.2b).

4.3.2.1 Females May be the Default Sex and Males are Induced in ESD

There is evidence to indicate that female may be the default sex in ESD. Ospina-Álvarez and Piferrer analyzed field and laboratory data for 59 fish species in which sex ratio can be influenced by temperature to some extent, and found that TSD only exhibits one single-sex ratio response pattern to temperature – namely, increasing temperature results in a male-biased sex ratio [27], though TSD have been grouped according to three patterns of sex ratio response to temperature [5, 34, 36, 43, 89].

In most fish studied, germ cells in putative ovaries outnumber those in putative testes, and mitosis and meiosis of germ cells occur earlier in ovaries than in testes [5, 90]. In some other special cases, such as in zebrafish and *Barbus tetrazona*, all gonads initially develop as ovaries. In about half of the individuals, immature oocytes undergo degeneration, and then testis differentiation takes place [5, 91, 92]. Further studies found that fish without germ cells, or with degenerated germ cells induced by high temperature, develop as phenotypic males [93–95]. In addition, in Atlantic silverside displaying TSD, low temperature during larval development produces more females, because of the significantly long growing season and enhanced relative fitness of females [7]. Interestingly, researchers proved that the ovarian phenotype is an active process throughout life [96]. All of this evidence suggests that female is the default sex, and male is induced.

4.3.2.2 Effects of Different Types of Stress Factors in Sex Determination and Differentiation

Various potential stressors, namely high temperature, hypoxia, bright background color, acidic pH, and social interactions (e.g., high density) have been found to be involved in masculinization so far (see Table 4.1, Figure 4.2b).

As mentioned earlier, it has been suggested that TSD species only exhibit one single-sex ratio response pattern to temperature: high temperature produces more males [27].

Masculinization induced by cortisol treatment during the critical period has been reported in several fish species with TSD or GSD, including rainbow trout [85], pejerrey [97, 98], Japanese flounder [99, 100], medaka [101, 102], southern flounder (*Paralichthys lethostigma*) [49], or even in hermaphrodite species, such as the wrasse (*Halichoeres trimaculatus*) [103].

Hypoxia has been found to cause male-dominated populations in zebrafish, with the involvement of various genes controlling the synthesis of steroid hormones, *3β-hsd*, *cyp11a*, *cyp19a*, and *cyp19b* [104], possibly by acting directly on steroidogenic gene expression via HIF-1-induced leptin expression [105]. Hypoxia also impairs primordial germ cell migration, which is found to be important for ovarian development in zebrafish embryos [12, 106, 107, 107, 108].

Interestingly, rearing with a relatively bright background color (blue, compared with black and grey) also leads to a significant male-biased sex ratio in the southern flounder, with the involvement of higher cortisol level during the time of sex determination [49]. Acidic pH has also been found to be related to male-biased sex ratios in several cichlids and poeciliids [109–111]. Density, one of social interactions, is vital for sex determination in Anguilliformes, with low densities being associated with a high percentage of females and high densities induce a male-biased sex ratio [28, 30, 112–117].

The involvement of each of the mentioned environmental factors that can be considered as stress factors, and male-biased sex ratios induced by cortisol treatments in both ESD and GSD species, suggest that stress-induced testicular differentiation may be a common mechanism in sex differentiation.

4.3.2.3 Links Between Stress Factors and Male Phenotype: Cortisol as a Mediator

The influences of cortisol on gonadal differentiation, and the involvement of cortisol in high temperature-induced masculinization, have begun to receive attention in recent years. Even though the direct responsor(s) of

Table 4.1 Environmental stress factors – induced masculinization or testis differentiation and involved hormones and genes.

Species	SD mode	Stress factors/ treatment	Cortisol response	Sex steroids	Steroidogenesis and sex differentiation related genes	other signatures	Reference
<i>Odontesthes bonariensis</i> pejerrey	TSD or GSD + TE	high temperature treatment	↑	↑ 11-KT	↑ <i>hsd11b2</i> , ↑ <i>gr1</i> , ↑ <i>ar1</i> , ↑ <i>ar2</i>	male-skewed population	[97]
		cortisol administration	↑	↑ 11-KT	↑ <i>hsd11b2</i> , ↑ <i>ar2</i>	male-skewed population	
		high temperature treatment	↑	↑ 11-KT, ↑ T	↑ <i>amh</i> , ↓ <i>cyp19a1a</i> , ↑ <i>dmrt1</i>	germ cell apoptosis	[98, 125–127, 215, 216]
		cortisol administration	↑	↑ 11-KT, ↑ T	↑ <i>amh</i> , ↓ <i>cyp19a1a</i>	germ cell apoptosis	[98]
<i>Paralichthys olivaceus</i> Japanese flounder	XX/XY GSD + TE	cortisol administration	↑	N.S.	↓ <i>cyp19a1a</i> , ↓ <i>foxl2</i>	male-skewed population	[99]
<i>Oryzias latipes</i> medaka	XX/XY GSD + TE	high temperature treatment	↑	N.S.	↑ <i>cyp26b1</i> , ↓ <i>cyp19a1a</i> , ↓ <i>foxl2</i>	delayed meiotic initiation of germ cells	[99, 100]
		high temperature treatment	↑	N.S.	↓ <i>fshr</i> ; ↓ <i>cyp19a1a</i> , ↑ <i>gsdf</i>	inhibited proliferation of germ cells	[101]
		cortisol administration	↑	N.S.	↓ <i>fshr</i> ; ↓ <i>cyp19a1a</i> , ↑ <i>gsdf</i>	inhibited proliferation of germ cells	
		Metyrapone - an inhibitor of cortisol synthesis	↓	N.S.	↑ <i>fshr</i>	inhibited high temperature-induced masculinization	
		E ₂ + cortisol / E ₂ + high temperature	↓	N.S.	↑ <i>cyp19a1a</i> , ↓ <i>gsdf</i>	completely rescued cortisol- and high temperature-induced masculinization	[102]
<i>Paralichthys lethostigma</i> southern flounder	GSD + EE	brighter background color	↑	N.S.	N.S.	male-skewed population	[49]
		cortisol administration	↑	N.S.	N.S.	dose-dependent masculinization	
<i>Anguilla</i> eels	unknown	high density	↑	N.S.	N.S.	male-skewed population	[28, 30]
		cortisol administration	↑	↑ 11-KT	N.S.	male-skewed population	
		adding shelters (de-stress)	N.S.	N.S.	N.S.	female-skewed population	[30]
<i>Danio rerio</i> zebrafish	GSD + EE	hypoxia	N.S.	↑ T/E2	↑ 3β- <i>hsd</i> , ↑ <i>cyp11a</i> , ↑ <i>cyp19a1a</i> , ↑ <i>cyp19a1b</i>	male-skewed population	[104]
		high density	↑	N.S.	N.S.	male-skewed population	[217]
<i>Pseudocrenilabrus</i> <i>multicolor victoriae</i>	GSD + EE	hypoxia	N.S.	↑ T/E2	N.S.	germ cells apoptosis	[214]
<i>Apistogramma</i> sp.	GSD + EE	acidic pH	N.S.	N.S.	N.S.	male-skewed population	[218]

stress factors have not been identified, some of the pathways involved in stress-induced testicular differentiation, in which cortisol was a mediator, have been characterized (Figure 4.2b). The production of glucocorticoids by the interrenal gland is mainly regulated by the adrenocorticotrophic hormone, whose production is modulated by the hypothalamic peptide corticotrophin-releasing hormone [88, 118–120].

Cortisol, the main glucocorticoid in teleosts, and the main stress hormone in vertebrates, plays an important role in the regulation of the adaptive intermediary metabolism, ionic regulation, and immune function [88, 121, 122]. The action of cortisol involves its passage through the plasma membrane and binding to cytoplasmic receptors thereafter. The hormone receptor complex is then transported to the nucleus, and functions as a ligand-dependent transcription factor on the transactivation or repression of glucocorticoid responsive genes, through binding to glucocorticoid response elements (GRE) within the promoter of the target gene [123].

It has been suggested that cortisol plays important roles in stress-induced testicular differentiation through different mechanisms of action, including promotion of 11-KT synthesis via upregulation of *hsd11b2* (gene that codes for 11 β -HSD), inhibition of aromatase, and/or hepatic catabolism of cortisol, regulation of the androgen/estrogen ratio via downregulation of *cyp19a1a*, promotion of germ cell apoptosis or delay meiosis initiation of germ cells, and/or regulation of sex differentiation related genes (e.g. *amh*, *gsdf*, *ars* (androgen receptors)) (Figure 4.2b). Thus, the existence of interactions between the hypothalamic-pituitary-adrenal/interrenal gland (HPA) axis, the hypothalamic-pituitary-gonadal (HPG) axis, and the hypothalamic-pituitary-thyroid (HPT) axis in stress-induced testis differentiation has also been suggested [124].

In pejerrey, a TSD species in which high or low temperature could produce a monosex population (29°C, 100% males; 17°C, 100%

females) [8], cortisol and high temperature-treated groups displayed typical molecular signature of masculinization (e.g., *cyp19a1a* downregulation and *amh* upregulation), higher incidence of gonadal apoptosis, which is found to be important in gonadal sex differentiation, and a higher proportion of males [98, 125–127]. Inhibition of female-type proliferation of germ cells, or delayed meiotic initiation of germ cells in cortisol- and high temperature-treated animals during sexual differentiation has also been observed in medaka, pufferfish, and Japanese flounder [93, 99, 101], suggesting the involvement of the regulation of germ cell numbers in ESD (Figure 4.2b).

Germ cell activities are important in mediating the direction of gonadal development, and degeneration/inhibition of germ cells are related to masculinization in many species. It is interesting to find that germ cell apoptosis-induced sex reversal from females to males has been reported in some species, including zebrafish, medaka, pufferfish, Nile tilapia, and carp (*Carassius gibelio*) [93, 94, 107, 128–130], while not in others (e.g., loach (*Misgurnus anguillicaudatus*) and goldfish (*Carassius auratus*)) [131, 132], indicating the diversity in terms of the involvement of germ cells in sex differentiation across species. Evidence showed that sexual differentiation of germ cells is controlled by the somatic micro-environment, rather than being cell autonomous [133]. Sperm-egg (male or female) decision and mitosis-meiosis decision of germ cells are found to be two independent events, and sex decision precedes mitosis-meiosis decision; germ cells display distinct sexuality prior to meiosis, in rainbow trout [134]. This evidence suggests the sexuality of germ cells is labile to environmental conditions, and inhibition of germ cell proliferation is important for testis development.

In Japanese flounder, it is suggested that high temperature or cortisol treatment induces masculinization by delaying meiotic initiation of germ cells, through upregulation of *cyp26b1* expression [100] (Figure 4.2b). *Cyp26b1*, which codes retinoid-degrading

enzyme, can regulate retinoic acid signaling during meiotic initiation of germ cells, and determine whether or not germ cells enter meiosis, in mice, chicks, and amphibians [135–138], indicating the conversed role of these genes on meiotic initiation of germ cells. Evidence also shows that *cyp26b1* may be regulated by *cyp19a1a* [138], indicating the interaction of male- and female-specific gene expression on germ cell activities (Figure 4.2b). Taken together, this evidence suggests that germ cells are one of the downstream responders of environmental conditions, and determine the direction of gonadal development.

Male-producing temperature promotes production of cortisol, 11-ketotestosterone (11-KT), and testosterone (T), compared with female-producing temperature in pejerrey [97, 98]. Interestingly, cortisol administration at an intermediate, mixed sex-producing temperature (24°C) also causes increases in 11-KT, T, and in the proportion of males in this species. The increase of 11-KT by cortisol administration could be explained through three different mechanisms of action, including upregulation of *hsd11b2* expression, downregulation of *cyp19a1a*, and/or through the hepatic catabolism of cortisol.

Cortisol administration and high temperature treatment both increase *hsd11b2* expression at the critical period of sex determination in pejerrey. 11 β -hydroxysteroid dehydrogenase (11 β -HSD), which is coded by *hsd11b2*, is one of the two enzymes (the other one is 11 β -hydroxylase, CYP11B) that are shared in the synthesis of 11-oxygenated androgens and cortisol. Differential expression of *cyp11b* has only been observed at later stages of morphological gonad differentiation [29], excluding the involvement of this gene in the increase of 11-KT induced by stress factors at the critical period of sex determination. The *in situ* hybridization demonstrated that the expression of *hsd11b2* is restricted to somatic gonadal cells (Leydig cells) [97, 139]. The evidence indicates that *hsd11b2* may play an important role in ESD, though whether its upregulation is directly induced

by high temperature (or stress factors), or by a higher level of stress-induced cortisol, remains unclear.

Downregulation of *cyp19a1* expression has been observed in stress-induced masculinization or cortisol-related testis differentiation in several species [97–101]. Importantly, it has been demonstrated that cortisol can directly suppress *cyp19a1* expression via glucocorticoid receptor (GR) in Japanese flounder [99]. Co-localization of CYP19A1 and GR was detected in the somatic cells of XX gonads in this species. GR bound directly or indirectly to the cAMP-responsive element within the *cyp19a1* promoter in gonads at male-producing temperature, but not in female-producing temperature [99]. These results strongly suggest that downregulation of *cyp19a1* and subsequent downregulation of aromatase are important for stress-induced testis differentiation in ESD.

Several gonadal differentiation-related genes are involved in stress-induced testis differentiation in fish with ESD or GSD + EE, including *dmrt1*, *amh*, *sox9*, *ars*, *gsdf*, *foxl2*, and *cyp19a1* [16, 29, 97]. However, none of these genes is a direct target of environmental stress factors. Because of the importance of steroid hormones (estrogens and androgens) in sex differentiation in a wide range of species, genes that are related to synthesis or regulation of steroid hormones, or their receptors that could be regulated by stress factors, could be the sex-determining factor(s) of ESD.

According to the current reports, there are two candidate molecular players, *hsd11b2* and heat shock proteins (HSPs), that may respond to stress factors and determine the direction of gonadal development. The *hsd11b2* gene, as we mentioned earlier, which is involved in metabolism of both 11-oxygenated androgens and glucocorticoids, responds to stress factors at the critical period of sex determination. However, further studies need to address whether its upregulation is modulated directly by stress factors, or regulated by stress-induced cortisol.

The HSPs were originally identified as the proteins whose expression is induced by heat and other stress factors [140–142]. Therefore, HSPs are interesting candidates to play important roles in stress-induced testis differentiation. The HSP gene families consist of stress-inducible genes and constitutively expressed genes. Inducible genes maintain low expression levels under non-stress conditions, while their expression boosts rapidly under different stress factors. All of the steroid receptors in higher vertebrates, except ER β – including ARs, other ERs, GR, mineralocorticoid, and progesterone receptors – are associated with HSP90 in the absence of their cognate ligands. *Hsp27* can suppress estrogen response element-mediated transcription by competing with the ER [143, 144].

In a TSD species, American alligator, *hsp27* expression was dramatically elevated in testicular tissue, compared with ovarian tissue. Sexual dimorphism in mRNA expression of gonadal *hsp70a* and adrenal *hsp90* were also observed in this species [145]. The involvement of *hsp27* and *hsp70a* in the E₂ signal [145] suggests that HSPs may play important roles in stress-induced testis differentiation. Therefore, we speculate that the small genetic difference between two sexes in ESD [27, 41, 43] is environmentally sensitive (e.g., thermo-sensitive) to factors related to cortisol or steroidal hormones metabolism (e.g., genes [97], protein, or even just intein [146]). These environmental-sensitive factors (ESFs) are species-specific as various sex-determining genes observed in different species.

It has also been proposed that testicular development is the result of hormonal interactions with the involvement of three axes, hypothalamic-pituitary-thyroid (HPT), HPG, and HPA [124]. The presence of 11-KT at very early stages of testis development [147], and high levels of 11-KT in stress conditions during the critical period of sex determination, also demonstrate that androgens play very important roles in stress-induced testis differentiation and normal testis differentiation in both ESD and GSD + EE species.

It is worth mentioning that proper levels of cortisol, rather than high levels, promote testis differentiation or masculinization [49, 97–99, 101]. This fact indicates that, as we mentioned, mild stress factors promote testis differentiation or cause masculinization.

4.4 Temperature-Dependent Sex Differentiation

The trigger(s) of the ovary- or testis-differentiating pathway are essentially different between ESD and GSD (or GSD + EE). However, the molecular plays involved in sex differentiation are conserved across taxa, with different sex-determining mechanisms [16]. Hence, sex determination systems in fish could be considered as one evolutionarily conserved network, regulated by species-specific upstream triggers. Even though different types of ESD have been proposed in a large amount of fish species, only TSD in one species – the Atlantic silverside – has been demonstrated to have an adaptive significance [7, 43].

The effects of temperature and exogenous hormones on sex differentiation have received the most attention, with a considerable amount of reports. In recent years, researchers have made important advancements and have benefited from the accessibility of next-generation sequencing. Therefore, in this part, we mainly focus on molecular players, their interactions, and pathways involved in temperature-dependent and hormone-induced sex differentiation. We also address the future studies needed, on the basis of our previous review [16].

4.4.1 Independent Genes to Interactions, Networks, and Comparative Analysis

Many genes, catalytic enzymes, and receptors have been indicated to be involved in temperature- or hormone-induced sex differentiation, through evaluation of expression or activities among different conditions. Increasing evidence strongly suggests that the realization of phenotypic sex is the

consequence of the interactions of a large set of genes, factors, hormones, and the feedback and response of primordial germ cells, rather than a simple cascade event. As the development of experimental molecular biology and sequencing technologies mushrooms, research works have been shifting from quantitative analysis of single or multiple genes' expression, to comparative analysis, functional analysis, interaction or pathway analysis.

Taking the *foxl2* gene as an example, its expression has been detected prior to morphological gonadal differentiation in all species studied, except in medaka, probably

because of different criteria for gonadal differentiation (Table 4.2). Sexual dimorphic expression of *foxl2* during sex differentiation has also been observed in all species investigated except American Alligator, *Alligator mississippiensis*, including species with either TSD or GSD (Table 4.2). Its expression also generally displays a parabolic trend, with a climax at the critical point of sex differentiation. With regard to temperature-dependent sex differentiation, its expression displays a thermo-sensitive pattern, with female-producing (promoting) temperature increasing, and male-producing temperature decreasing its expression (Table 4.2).

Table 4.2 *Foxl2* expression profile.

Species	Express before MGD	Sexual dimorphic expression during SDi	"n-shaped expression pattern	Thermo-sensitive	Population used	Positively correlated with <i>cyp19a</i>	Reference
Nile tilapia	√	√	×	NS	XX female XY male	√	[219]
	√	√	NS	NS	XX female XY male XY female XX male	√	[154]
Japanese flounder	√	√	NS	√	XX female	√	[155]
Medaka	×	√	NS	NS	Mixed sexes [#]	NS	[220]
Air breathing catfish	√	√	√	NS	Mixed sexes	√	[221]
Rainbow trout	√	√	NS	NS	XX female XY male	√	[222]
	√	√	√	NS	XX female XY male	×	[223]
Willow minnow	√	NS	NS	NS	Mixed sexes	NS	[224]
Zebrafish	√	√	√	NS	Mixed sexes [§]	NS	[225]
<i>Oryzias luzonensis</i>	√	√	NS	NS	Mixed sexes [§]	NS	[226]
Pacific oyster	NS	NS	√	√	Mixed sexes	NS	[227]
American alligators	√	×	NS	×	Mixed sexes [‡]	NS	[228]
Snapping turtle	√	√	√	√	Mixed sexes [‡]	NS	[82]
Red-eared slider turtle	√	√	NS	√	Mixed sexes [‡]	NS	[229]
	√	√	NS	√	Mixed sexes [‡]	NS	[230]

MGD, morphological gonadal differentiation; SDi, sex differentiation; √, yes; ×, no; NS, not studied.[#], genetic sex could be identified by a PCR-based strategy.[§], sex could be distinguished by a molecular marker.[‡], mono-sex was produced at female/male producing temperature.

In vertebrates, *foxl2*-knockout mice have a total absence of secondary follicles and oocyte atresia, and XX mouse gonads without *foxl2* develop into males [148–150]. Goats with the function of *foxl2* disrupted have a downregulated expression of aromatase, compared with control animals [151, 152]. In human beings, mutations in the *foxl2* gene lead to the loss of the ability to suppress the induction of *cyp17* mediated by SF-1 [153]. All the functional analysis strongly suggests that *foxl2* plays a vital role in ovarian differentiation and maintenance of ovary functions.

Further studies in fish species found that *foxl2* can directly activate the transcription of *cyp19a1a* and, meanwhile, interact with *sf-1* to promote the *sf-1* mediated *cyp19a1a* expression in species with different SD mechanisms [154, 155]. All the evidence places the *foxl2* gene at the very top of sex differentiation in teleosts [16]. Studies in several fish species revealed that treatment of exogenous estrogens upregulate the expression of *foxl2* while exposure to aromatase inhibitor or exogenous androgens suppress it [156–159], and suggest that expression of *foxl2* is regulated by downstream hormones through a feedback regulation. There is evidence that the strongest unregulated gene in the ovary upon the deletion of *foxl2* is *dmrt1* [96], and that *foxl2* and *dmrt1* display opposing effects on the regulation of estrogen production [160], strongly suggesting that *foxl2* may suppress *dmrt1* transcription directly. Studies in mice demonstrate that *dmrt1* directly represses expression of *foxl2* and other ovary-specific signaling, such as *esr1*, *esr2*, *wnt4*, *rspo1*, in both fetus and adults [161]. Antagonistic roles of *foxl2* and *dmrt1* have also been confirmed in fish [162].

RNA-seq analysis of both sexes is able to discover a large amount of unidentified genes, and identify differentially expressed genes (DEGs), specifically expressed genes (SEGs), and enhanced/repressed genes during development. In the recent decade, especially in the past four years, RNA-seq analyses of both male and female reproductive organs have been reported in more than 10 aquatic

animals (Table 4.3), and a lot more are ongoing. These reports and datasets provide a large amount of genomic resource for future studies. In particular, comparative analysis of datasets from fish with different sex-determining mechanisms will shed light on the evolution of sex-determining mechanisms, sexual selection, and maintenance of sexual phenotypes.

Generally, RNA-seq analyses of testes and ovaries in different development stages (e.g., sexually undifferentiated gonads, sexually differentiating gonads, sexually differentiated gonads, maturing and mature gonads), result in a large number of DEGs and SEGs (see references in Table 4.3). However, it must be mentioned that those DEGs and SEGs from the gonads of juveniles and adults, namely differentiated gonads, are not necessarily involved in sex determination and sex differentiation, as claimed by several researchers. These genes are good candidates involved in gonad development, gonad maintenance, reproductive activities, secondary sex characteristics, alternative reproductive tactics, and so on. Only those DEGs and SEGs obtained from sexually undifferentiated and differentiating gonads are excellent candidates that are involved in sex determination and sex differentiation [163, 164]. Nevertheless, these are comparatively challenging tasks for the following three reasons:

- a) Undifferentiated gonads are extremely tiny in most species, which hamper obtaining enough RNA samples unless sample pooling is adopted.
- b) Contamination with surrounding tissue is almost inevitable when dissecting undifferentiated gonads.
- c) Giving the first two issues could be solved, genetic males and females should not be mixed for the sample pool. In this case, researchers need to produce both all-male and all-female populations for a given species unless sex-linked markers have been developed. However, this could only be achieved in a limited number of species.

Table 4.3 RNA-seq analysis of ovary and testis regarding sex differentiation and sex maintenance.

Species	SD mode	Genotype	Sampling points	Organ(s)	Analysis	Literature
Zebrafish <i>Danio rerio</i>	PSD	unknown*	Sex differentiated and adult	gonad, brain	DEGs	[231]
Nile tilapia <i>Oreochromis niloticus</i>	GSD + EE	XX/XY	sex undifferentiated, differentiating, differentiated, and adult	gonad	DEGs, SEGs	[163]
Cichlids	GSD + EE	unknown	mature adult	gonad, brain	DEGs, SEGs	[232]
Channel catfish <i>Ictalurus punctatus</i>	GSD + EE	XX/XY	Sex differentiated and adult	gonad	DEGs	[233, 234]
Tuna <i>Thunnus maccoyii</i>	unknown	unknown	Maturing adult	gonad	DEGs, SEGs	[235]
Olive flounder <i>Paralichthys olivaceus</i>	GSD + EE	XX/XY	Sex differentiated and adult	gonad	DEGs, SEGs	[236]
Yellow catfish <i>Pelteobagrus fulvidraco</i>	GSD + EE	XX/XY	Sex differentiated and adult	gonad	DEGs, SEGs	[237]
Pacific white shrimp <i>Litopenaeus vannamei</i>	GSD	ZW/ZZ	mature adult	gonad	DEGs, SEGs	[238]
Mosquitofish <i>Gambusia affinis</i>	GSD	ZW/ZZ	Sex differentiated	gonad	DEGs, SEGs	[239]
Turbot <i>Scophthalmus maximus</i>	GSD + EE	ZW/ZZ	sex undifferentiated, differentiating, differentiated	gonad	DEGs, SEGs	[164]
Rock bream <i>Oplegnathus fasciatus</i>	GSD	X ₁ X ₁ X ₂ X ₂ / X ₁ X ₂ Y	mature adult	gonad	DEGs	[240]
Japanese scallop <i>Patinopecten yessoensis</i>	unknown	unknown	mature adult	gonad	DEGs	[241]
European sea bass <i>Dicentrarchus labrax</i>	PSD	unknown*	Sex differentiating and differentiated	gonad	DEGs	[165]
American alligator <i>Alligator mississippiensis</i>	TSD	N.A.	sex undifferentiated, differentiating	gonad	DEGs	[188]

Note: PSD, polygenic sex determination; GSD, genotypic sex determination; EE, environmental effects; TSD, temperature-dependent sex determination; N.A., not applicable; DEGs, differentially expressed genes; SEGs, specifically expressed genes.

*refer to [12] and [242] for sex determination in zebrafish and European sea bass, respectively.

This work is challenging yet feasible. Three hundred gonadal samples for each sex from all-male (XY) and female (XX) larvae, at five days post-hatching, were successfully pooled in Nile tilapia [161]. Differentiating gonads were also dissected without any contamination of surrounding tissue in the European sea bass [165].

Comparatively analyzing these reports, we found that more genes were expressed in testes than in ovaries, more DEGs and SEGs were found in testes, and more upregulated genes were observed in testes at all developmental stages, regardless of sex determination mode (GSD, TSD, or polygenic sex determination, PSD) or genotypes (XX/XY, ZW/ZZ, or

$X_1X_1X_2X_2/X_1X_2Y$) (Table 4.3). These results reinforce our abovementioned hypothesis that female is the default sex, and male is the induced one that requires activation of a set of molecular players.

Why are there more genes and more enriched genes expressed in males? “Dosage” of a chromosome or a gene refers to its copy numbers in the entire genome. Dosage compensation is a mechanism to harmonize the expression of X- or Z-linked genes between sex chromosomes and autosomes. In mammals which display XX and XY genotypes, dosage compensation for X-linked gene products between male and female individuals is realized by silencing one of the two X chromosomes in female cells [166]. Another form of dosage compensation balances expression of X-linked and autosomal genes by promoting the transcripts of the active X in the male genotype XY [167]. These extraordinary regulatory processes derive from the evolution of the sex chromosomes [167], and are thought to play important roles in sex differentiation and maintenance of sexual phenotypes. This explains why more upregulated genes were found in testes, throughout all the development stages.

By analogy with the XY system, one might expect that the ZW system will also be profiled by the upregulation of genes on single Z-chromosome individuals, namely females. However, evidence has demonstrated an increased expression of some Z-linked genes in males (ZZ), compared with females [168–171]. In birds and silkworms, the ratio of Z-linked gene expression between male and female range from 1 to 2 [172–174], indicating distinctive sex difference in gene expression when compared with mice and humans [173, 175]. Similar results were also reported in a fish species with ZW sex chromosome.

Male expression in whole-body (without gonad) transcriptomes is 1.32 times higher than female expression in half-smooth tongue sole *Cynoglossus semilaevis* [17]. Generally higher gene expression in testes, deduced from Table 4.3, which is consistent

with previous reports in both XY and ZW sex chromosome systems, indicates that upregulation of sex chromosome genes in testes may be ubiquitous, though distinct mechanisms are involved. Furthermore, as complete dosage compensation is observed in humans [173, 176], the level of dosage compensation may also reflect the evolution status of sex chromosomes. It will be very interesting to find out whether there is a certain degree of dosage compensation in reptiles and fish displaying TSD, in which it is thought that there is minuscule genomic difference between males and females. It will also be exciting to see the changes of sex-reversed individuals (XY with female phenotype or ZW with male phenotype) regarding dosage compensation, compared with normal individuals.

4.4.2 Yin and Yang in Sex Differentiation

The origin and maintenance of sex are everlasting questions for evolutionary biologists. Yin-yang is a concept in Chinese philosophy describing how seemingly two opposite or antagonistic forces may be interdependent, interconnected, and complementary, and how they may be derived from each other [177]. As displayed in Figure 4.3, Yin represents black (in the symbol) and feminine, while Yang represents white and masculine. As the symbol illustrates, each side has its core as an element of the other; an increase in one brings a corresponding decrease in the other [178]. The Yin-yang philosophy may not represent and match each one of the cases in the natural world, but it gives us some general ideas of how things are operating and connecting to each other.

Ovary or testis originate from the unique part of an organism, the primordial germ cells, which display bipotential features, and are determined by essential differences in the genome, or environmental conditions, or both in gonochoristic species. As yin and yang, recent studies strongly suggest that male-specific genes and female-specific genes

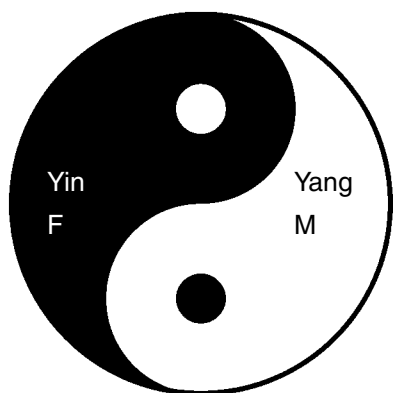


Figure 4.3 Yin-yang philosophy in sex differentiation.

As the symbol illustrates, each side has its core as an element of the other; an increase in one brings a corresponding decrease in the other. Sex differentiation is a battle for primacy, and the commitment to ovary or testis is the result of success of one sex-specific camp of molecular players.

F, female; M, male.

play antagonistic roles in sex differentiation and maintenance of sexual phenotypes [96, 161, 162].

Research in mice demonstrated that female-specific players *foxl2* and *esr1* work cooperatively to repress expression of the male-specific players *sox9*, which is the direct target of the sex-determining gene *sry* in mammals [96]. Another study in mice discovered that *dmrt1*, a sequence-specific transcriptional regulator capable of regulating transcription of target genes, represses the network of female-specific genes, including *foxl2*, *esr1*, *esr2*, *wnt4*, and *rspo1*, while it may upregulate other male-specific genes, such as *sox9* and *fgf9* [161].

In a teleost fish, Nile tilapia, *foxl2* and *dmrt1* play antagonistic roles in sex differentiation. AMH, known as **Anti-Müllerian Hormone** or **Müllerian-Inhibitory Substance** (MIS), is responsible for the suppression of Müllerian ducts during male fetal development in mammals, birds, and reptiles [179–181], and is associated with early sex differentiation and later gonadal development in higher vertebrate species [182]. Studies have also shown that AMH functions to suppress primordial

follicle transition and assembly and, therefore, maintains primordial follicles in their arrested state [183–187].

Amhy and *amhr2* are found to be sex-determining genes in some fish species, and loss of function will cause male-to-female sex reversal, while overexpression will lead to female-to-male expression [18, 19, 58, 60]. Further, *amhy* knockdown results in upregulation of the expression of female-specific genes (*foxl2* and *cyp19a1a*), and promotes ovarian development. Intriguingly, RNA-seq analysis in American alligator (with TSD) found significant upregulation of *amh* under both **male-** and **female-producing temperatures** (MPT, FPT), while the degree of upregulation was dramatically greater at MPT, during the critical time of sex determination [188].

Recent work in Nile tilapia found that female ovaries could be reversed into functional testes by treatment of an aromatase inhibitor, even starting at the age of 90 days post-hatching (dph). This is far later than the time of molecular sex differentiation (5 dph) and morphological sex differentiation (23–26 dph) in this species, through transdifferentiation of germ cells and somatic cells driven by the repression of female-specific genes and activation of male-specific genes [156].

Therefore, sex differentiation actually is a battle for primacy, and commitment of primordial germ cells to an ovary or testis is the result of success in one sex-specific camp of molecular players, through upregulating of its own camp and/or repressing the opposite camp. As estrogens and androgens both play important roles in males and females, some sex-specific molecular players also function importantly in another sex regarding sex differentiation and sex maintenance, just as the yin and yang illustrated, but not black or white. Stress-induced masculinization, as mentioned earlier, (or hormone-induced sex reversal) needs to break the initial status of network and/or lead to apoptosis/degeneration of developed gonad, and establish an adequate environment for primordial germ cells, to bring about the development of the opposite sex.

4.5 ESD in Aquaculture and Fisheries

Environmental conditions (e.g., temperature, pH, and background color), could determine sex and/or influence sex differentiation in a large number of fish. Several studies have demonstrated that temperature sensitivity of sex ratio, specifically the variation extent of sex ratio responding to different temperature, could be selected as a quantitative trait [46–48, 189–192]. Further, the allelic variant in the *amh* gene is closely related to phenotypic sex in Nile tilapia [192], indicating that marker-assisted selection for *amh* variant could be applied to select temperature-sensitive families, so as to produce a high proportion male or all-male population.

High temperature, acidic water, or bright tank color can produce 100% (or close) males in several species, such as tilapia, Japanese flounder, Southern flounder, swordtail (*Xiphophorus helleri*), and blackbelly limia (*Poecilia melanogaster*) [32]. Therefore, the effects of friendly environmental conditions on sex ratio in other species, in which one sex is strongly desired, need to be addressed extensively in order to produce a monosex population via an environmental- and consumer-friendly approach.

On the other hand, at this stage, large-scale monosex production is limited in a few species. Effects of environmental factors on sex ratios in many species are not wanted. For example, females grow faster and reach a larger size than males in several flounders (genus *Paralichthys*) [193]. However, high rearing temperature-induced masculinization in real-world production has been reported in many flounders [193–195]. In Southern flounder, blue tanks produced 95% males, compared with 50% males reared in black and gray tanks [49].

As we mentioned, some potential environmental stress factors, such as high or low temperature, bright tank color, high density, handling, hypoxia, or acidic pH, may produce a high proportion of unwanted sex in commercial hatchery production or research facilities. In this consideration, extensive research about

the effects of various stress factors on sex differentiation need to be addressed, and these should be taken into consideration in commercial production and research activities.

Stock enhancement through hatchery-produced populations could lead to the extermination of the wild population, depending on the sex reversal percentage in the hatchery, the relative reproductive success of hatchery fish in the wild, and the source of hatchery broodstock used (wild-born or hatchery-born) [196]. Although it is generally considered that these changes in living conditions are not sufficiently drastic to bring about the extinction of wild populations, their negative effects on effective population size, population growth, and biological diversity cannot be neglected [196–206].

Stock enhancement in rainbow trout for recreational fisheries has raised general concerns about the consequences of releasing a considerable number of hatchery-born fish into the ecosystem. Early maturity of the male rainbow trout, compared with the female, is a major bottleneck in production of such commercially important fish, due to inferior meat quality after maturation, and selection experiments to increase the proportion of females by applying a temperature treatment are ongoing [46].

Magerhans *et al.* have reported the production of female- or male-biased progenies under high-temperature treatment (18°C) versus control temperature (12°C) in different populations of rainbow trout [45]. Thus, environmentally sex-reversed females (i.e., XY or ZZ females, XX or ZW male) could also be induced intentionally or unintentionally in farm hatcheries for this fish species. For example, all-female eggs of rainbow trout are available for sale at a commercial scale.

The introduction of hatchery-born fish into natural waters of other commercially important fish with TSD or GSD + TE, such as carps, Nile tilapia, sockeye salmon (*Oncorhynchus nerka*), chinook salmon (*Oncorhynchus tshawytscha*), Japanese eel, yellow catfish, pufferfish, turbot (*Scophthalmus maximus*), European sea bass

and flounders [32, 193, 207–210], should also be evaluated for their consequences on population sex ratios and stability. Moreover, strong evidence suggests the presence of naturally sex-reversed individuals due to change in temperature in grayling (*Thymallus thymallus*, Salmonidae) from a wild lake [211], in two natural populations of Nile tilapia [212], in zebrafish [6], pejerrey [8], and half-smooth tongue sole [17]. These findings stress the importance of extensive investigation of environmental sex reversal (either artificially or naturally induced), especially for those commercially important

species with considerable amounts of hatchery stockings.

Acknowledgments

This work was supported by the National Institute of Food and Agriculture (NIFA) and North Central Region Aquaculture Center (NCRAC), U.S. Department of Agriculture. Salaries and research support were provided by state and federal funds appropriated to The Ohio State University, Ohio Agricultural Research and Development Center.

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5

Gene Knockout and Its Principle and Application in Sex Control of Fish Species

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5.1 Introduction

Controlling the sex ratio is essential in fish farming. A balanced sex ratio is usually good for broodstock management, since it enables the development of appropriate breeding schemes. However, in some species, the production of monosex populations is desirable because of the existence of sexual dimorphism, primarily in growth or time to sexual maturation. Additionally, dimorphic color or shape can render one sex more valuable. Thus, knowledge of the genetic architecture of sex determination (SD) can allow for control of sex ratios and for the implementation of breeding programs [1].

The gonads are unique among vertebrate organs, in that they have two normal options for development. In most vertebrates, embryos have a bipotential gonad that can develop into an ovary or a testis. The course of differentiation selected by a gonad determines the future sexual development of the organism. Therefore, the most upstream event in sex determination of individuals is the sex determination of the gonads, and the sex-determining gene decides the direction of development of a bipotential gonad [2]. In fish species, all kinds of sex-determining systems observed in other vertebrate classes have been observed, including male

heterogametic (XX/XY), female heterogametic (ZZ/ZW), temperature-dependent systems, as well as natural hermaphroditism, either simultaneous or sequential [3]. Knowledge about the sex-determining mechanisms is limited for most fish species.

In the case of animals where sex is determined by genetic factors, the molecular processes that lead to the formation of either testis or ovary are evolutionarily labile [4–6]. For example, while sex determination in most mammals is triggered by the testis-determining gene, *SRY*, this role is played by *dmy/dmrt1bY* and *DMRT1* in medaka (*Oryzias latipes*) and chicken, respectively [7–9]. In addition, sex determination in frog (*Xenopus laevis*) is regulated by the ovary-determining W-linked gene, *DM-W*, which is thought to inhibit the function of the Z-linked male-determining gene, *DMRT1* [10]. The identification of these master sex-determining genes in the past two decades has provided valuable insights into our understanding of the mechanisms of sex determination and how they have evolved.

Teleost fishes represent about half of all extant vertebrates, and show a wide variety of sex determination mechanisms. Their sex can be determined by genetic factors, environmental factors, or both [11–13]. The genetic sex determination includes

monofactorial systems involving a single master SD gene, such as *dmy* in medaka, and polyfactorial systems involving several genes on multiple chromosomes [13–17]. Recently, four novel SD genes (or strong candidates) in fishes were reported – *amhy* in the Patagonian pejerrey (*Odontesthes hatcheri*) [18], *amhr2* in fugu (*Takifugu rubripes*) [19], *gsdf* in *Oryzias luzonensis* (a relative of medaka) [20], and *sdY* in rainbow trout (*Oncorhynchus mykiss*) [21]. Knowledge of the sex-determining mechanisms in fish will allow for more control over sex ratios in species of aquacultural importance.

Once the sex determination genes are identified, gene knockout as a genetic technique could be used to obtain mutant fish without the function of these genes, which may have a sex-reversed phenotype. For example, knockout of a female-determining gene may result in an all-male population, whereas knockout of a male-determining gene may result in an all-female population. In this chapter, we will review the techniques for gene knockout in fish species, as well as sex control through gene knockout in model fish species and other economically important species.

5.2 Approaches for Gene Knockout

Currently, the most efficient methods for producing gene knockouts in both model and non-model organisms utilize programmable, sequence-specific DNA nucleases, which allow the precise production of a DNA double-stranded break (DSB) at the genomic locus to be modified. Nuclease-induced DSBs can be repaired by one of two pathways that operate in nearly all cell types and organisms: nonhomologous end-joining (NHEJ); and homology-directed repair (HDR) [22]. NHEJ can lead to the efficient introduction of insertion/deletion (indel) mutations of various lengths, which can disrupt the translational reading frame of a coding sequence

or the binding sites of transacting factors in promoters or enhancers. HDR-mediated repair can be used to introduce specific point mutations, or to insert desired sequences through recombination of the target locus with exogenously supplied DNA “donor templates.” With targeted nuclease-induced DSBs, the frequencies of these alterations are typically greater than 1% and, in some cases, can be 100% (i.e., bi-allelic knockout). At these rates, desired mutations can be identified using simple screening techniques.

During the past decades, the approaches for producing targeted gene knockouts have been developed and greatly improved. Genome-editing tools based on site-specific DNA nucleases, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeat (CRISPR)-associated (Cas9) effector proteins, have been developed to facilitate site-specific genomic modifications. These approaches have been applied to many research fields in many species, including fish species.

5.2.1 ZFNs

Zinc finger nucleases (ZFNs) are a class of engineered DNA-binding proteins that facilitate targeted editing of the genome by creating double-strand breaks in DNA at user-specified locations. ZFN represents a chimeric fusion protein, consisting of a zinc finger protein (ZFP) and the cleavage domain from the FokI endonuclease [23]. The DNA binding specificity is defined by the ZFP, which can be engineered to recognize a variety of the target DNA sequences (Figure 5.1) [24, 25]. The important part in designing ZFN is the ZFP optimization for the target recognition. The use of ZFN will generate the breaks, which are then repaired by non-homologous end joining, resulting in small insertions and deletions.

ZFNs have been shown to effectively stimulate NHEJ-mediated repair of targeted DSBs and, thus, generate gene-specific mutations. ZFN-mediated gene targeting has been

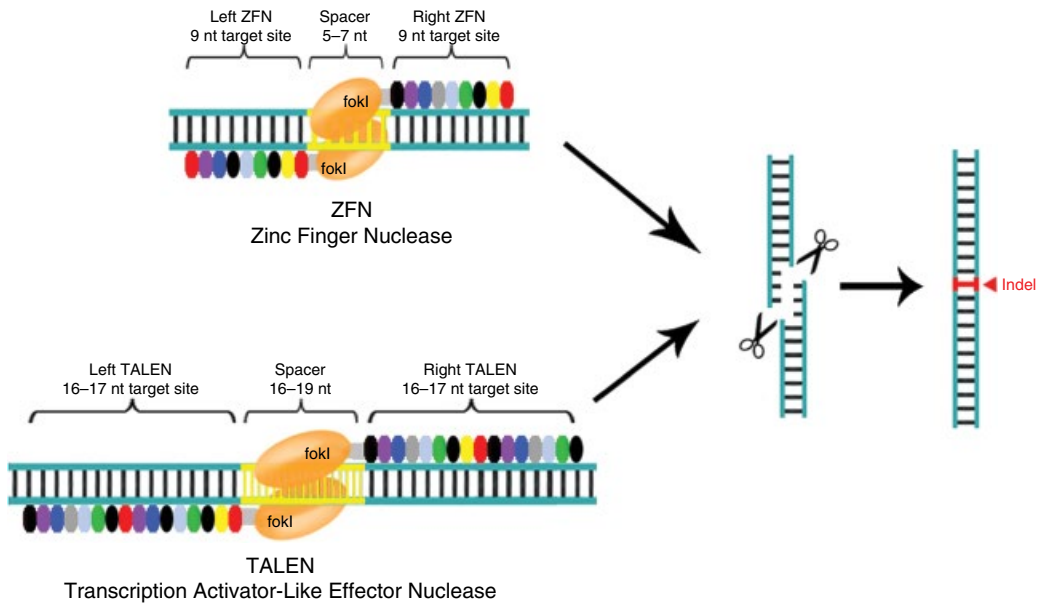


Figure 5.1 Genome engineering using ZFNs and TALENs (revised from [26]).

ZFNs utilize DNA-binding domains that recognize 3 bp sequences and are joined together to create arrays that can target specific DNA sequences. TALENs bind DNA using TAL effector repeat domains, derived from *Xanthomonas*, that recognize individual nucleotides. These TALE repeats are ligated together to create binding arrays that recognize extended DNA sequences. Each ZFN or TALEN binds to a half-site with dimeric FokI nuclease domains, cleaving the DNA within the intervening spacer region. The mechanism responsible for inducing DNA mutations is identical using either methodology, where nuclease-induced, double-stranded DNA breaks are repaired by error-prone non-homologous end joining (NHEJ), resulting in the creation of insertion or deletion mutations (indels).

used successfully in *Xenopus* [27], *Drosophila* [28–30], *Caenorhabditis elegans* [31], rats [32], and also zebrafish [24, 25, 33]. Targeted gene inactivation via ZFN technology in zebrafish has also demonstrated that successfully generated mutant zebrafish were able to pass on their mutagenized genes to the next generation by both groups [24, 25]. In zebrafish, ZFNs have been successfully used to mutate several genes with known loss-of-function phenotypes (e.g., *no tail*, *golden* and *kdrl*) and, in all cases, the expected phenotypes were obtained.

Also, ZFNs have been successfully used in the aquaculturally important species, rainbow trout [21, 34] and yellow catfish (*Pelteobagrus fulvidraco*) [35]. In rainbow trout, ZFNs were used to test the necessity of the candidate male sex-determining gene, *sdY*: targeted inactivation of *sdY* in males

caused ovarian differentiation, resulting male-to-female sex reversal [20, 33].

Even though ZFNs have been used for targeted genome editing in various organisms, two major limitations prevent their wider applications. ZF domains have limited modularity, due to the context-dependent DNA-binding effects, making it difficult for ZFNs to target all desired DNA sequence [36]. Moreover, lack of specificity of some ZF domains can generate off-target cleavage, leading to undesired mutations and chromosomal aberrations [37, 38].

5.2.2 TALENs

Transcription activator-like effector nucleases are restriction enzymes that can be engineered to cut specific sequences of DNA. TALENs bind to DNA through a highly

conserved 33–35 amino acid transcription activator-like (TAL) effector repeat domain which was originally isolated from plant pathogenic bacteria from the genus *Xanthomonas*. Each TAL effector repeat domain is comprised of 33–35 amino acids, and binds to a single bp of DNA. The nucleotide specificity is determined by just two variable amino acids within each repeat, known as the repeat variable di-residues (RVDs) [39–41] (e.g., the repeat containing the NI RVD sequence recognizes adenine, whereas HD recognizes cytosine, NG recognizes thymine, and NN recognizes primarily guanine) [39, 40].

Importantly, TAL effector repeats can be readily assembled into extended arrays that can bind to DNA sequences as long as 30 nucleotides. As with zinc fingers, TAL effector repeats can be fused to the FokI nuclease domain to create TALENs capable of cleaving DNA as a dimer of two sequence-specific modules, thus allowing for greater specificity. As noted for ZFNs, DSBs induced by TALENs can be repaired by NHEJ, thus producing indel mutations at a high frequency (Figure 5.1) [43]. A main advantage over ZFNs is that TALENs can be easily and rapidly constructed to target almost any DNA sequence, due to the simple protein-DNA code and their modular nature. In addition, TALENs exhibit significantly reduced off-target effects and cytotoxicities, compared with ZFNs, making them an efficient genome-editing tool [26, 42, 43]. TALENs have been widely applied to modify endogenous genes in a variety of organisms.

As for fish species, TALENs had been used for targeted genome editing of many genes in zebrafish, such as *cyp19a1a* [45, 46], *dmrt1* [47], and *bmp15* [45]; in medaka for *dmy* [48] and *dj-1* genes [49]; and in Nile tilapia (*Oreochromis niloticus*), an important species for worldwide aquaculture, for *dmrt1*, *foxl2*, *cyp19a1a*, *gsdf*, *igf3*, and *nrob1b* genes [50].

5.2.3 CRISPR/Cas9

Early methods for targeting DSB-inducing nucleases to specific genomic sites relied on

protein-based systems with customizable DNA-binding specificities, such as ZFNs and TALENs. These platforms made possible important advances, but each has its own set of associated advantages and disadvantages (Table 5.1) [22]. More recently, a platform based on a bacterial CRISPR-associated protein 9 nuclease from *Streptococcus pyogenes* (hereafter referred to as Cas9) has been developed. This is unique and flexible, owing to its dependence on RNA as the moiety that targets the nuclease to a desired DNA sequence via Watson-Crick base-pairing. To create gene disruptions (Figure 5.2), a single guide RNA (sgRNA) is generated to direct the Cas9 nuclease to a specific genomic location. Cas9-induced double strand breaks are repaired via the NHEJ DNA repair pathway, thus resulting in indels.

Compared to ZFNs and TALENs, the easy programmability of the DNA-binding domain via sgRNAs is the most advantageous feature of the CRISPR/Cas9 system, making it the most amenable approach for high-throughput mutagenesis projects in most organisms. Moreover, there are increasing numbers of organism-specific tools that aid in the design of gene-specific sgRNAs sequences. For example, for zebrafish, there are web-based sgRNA design programs that minimize possible off-target effects. These include CRISPR MultiTargeter, CRISPRdirect, CCTop, CHOPCHOP, sgRNAcas9, CRISPRscan, and CRISPOR [51–57]. Overall, these tools notably increase the ease of the CRISPR/Cas9-editing system in zebrafish. The typical advantages of CRISPR/Cas9, compared to ZFNs and TALENs, are summarized in Box 5.1.

A number of successful studies have exploited the CRISPR/Cas9-mutated zebrafish to test the causal role of specific genetic perturbations in a genotype-to-phenotype approach [58–61]. For example, Perles *et al.* [62] employed the CRISPR/Cas9-mutated zebrafish to investigate the effect of *mmp21* knock-out. Moreover, the precision of CRISPR/Cas9 editing has been used to test candidate genes while cloning phenotype-causing mutations isolated in

Table 5.1 Comparison of ZFN, TALEN, and CRISPR/Cas9 techniques.

Tool name	ZFN	TALEN	CRISPR/Cas9
Molecular target	DNA	DNA	DNA
Result of targeting	Irreversible knockout	Irreversible knockout	Irreversible knockout
Target sequence	Every 140–400 bp	Every 1–3 bp	N20-PAM sequence (NGG; N = A, C, G or T))
Recognition module	Zinc finger domain	TALE	sgRNA
Transmission efficiency	Low	Variable	High
Ease of generating target specificity	Difficult: substantial cloning and protein engineering required	Moderate: substantial cloning steps required	Easy; simple oligo synthesis and cloning steps
Off-target activity	Moderate	Low	Low
Ease of multi-plexing	Low	Moderate	High, proper for reverse genetic screening
Transcriptional and epigenetic control	DNA-binding ZF domains can be fused to new functional domains	DNA-binding domains can be fused to new functional domains	Enzymatically inactivated Cas9 can be fused to new functional domains
Ease of generating large-scale libraries	Low: complex protein engineering required for each gene	Moderate: technically challenging cloning steps	High: simple oligo synthesis and cloning required
Costs	High	Moderate	Low

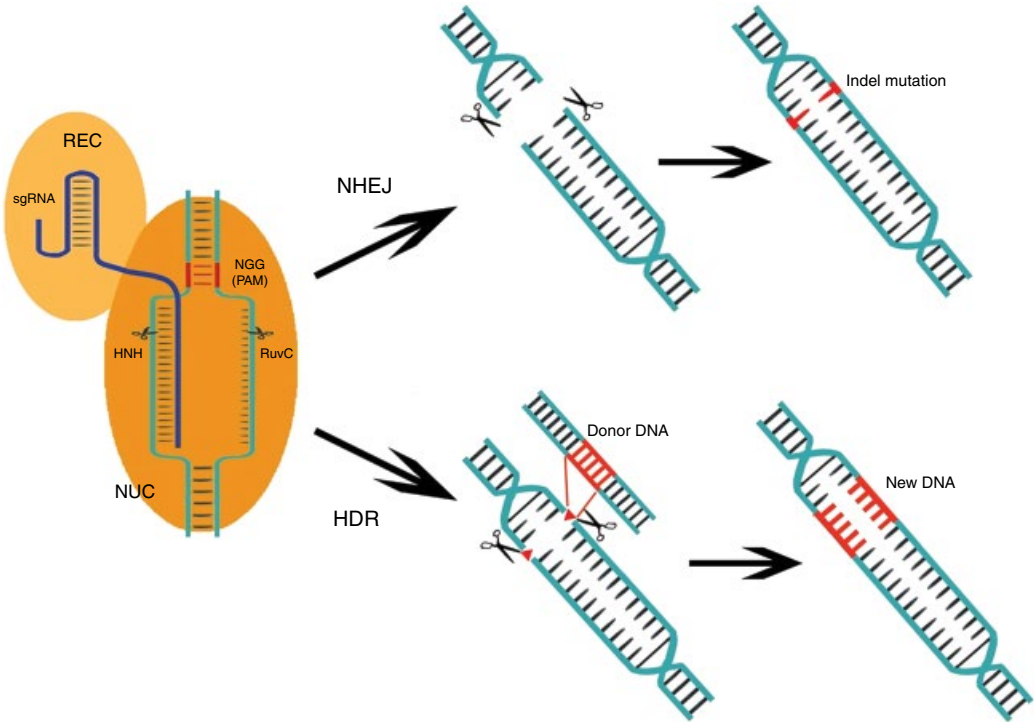


Figure 5.2 The principle of CRISPR/Cas9-mediated gene disruption.

A single guide RNA (sgRNA), consisting of a crRNA sequence that is specific to the DNA target, and a tracrRNA sequence that interacts with the Cas9 protein, binds to a recombinant form of Cas9 protein that has DNA endonuclease activity. The resulting complex will cause target-specific, double-stranded DNA cleavage. The cleavage site will be repaired by the non-homologous end-joining (NHEJ) DNA repair pathway, an error-prone process that may result in insertions/deletions (indels) that may disrupt gene function.

Box 5.1 The CRISPR/Cas system offers several advantages over the ZNF and TALEN mutagenesis strategies:

- 1) *Target design simplicity.* Because the target specificity relies on ribonucleotide complex formation and not protein/DNA recognition, gRNAs can be designed readily and cheaply to target nearly any sequence in the genome specifically.
- 2) *Efficiency.* The system is super-efficient. Modifications can be introduced by directly injecting RNAs encoding the Cas protein and gRNA into embryos or cells. This eliminates the long and laborious processes of transfecting and selecting cells that are required to create targeted mutant, using classical homologous recombination techniques.
- 3) *Multiplexed mutations.* Mutations can be introduced in multiple genes at the same time by injecting them with multiple gRNAs.

traditional forward genetic screens. For example, Reischauer *et al.* [63] systematically genome-edited each candidate genes in the *cloche* mutant-containing region, and successfully identified the *cloche* gene, which is involved in haemato-vascular development.

These are typical cases showing that genomic functional studies could be easily conducted with the combination of the CRISPR/Cas9 system and zebrafish. Besides zebrafish, CRISPR/Cas9 technique has been also used in some other aquaculture species, such as in common carp (*Cyprinus carpio*) for *sp7* and *myostatin* genes knockout [64], and in Tilapia for *nanos2*, *nanos3*, *dmrt1*, and *foxl2* gene knockout [65].

The above three approaches, including ZFNs, TALENs, and CRISPR/Cas9, all result in loss-of-function (LOF) mutations based on targeting genes. Besides these, there are also some traditional techniques which could result in LOF with different targeting level [66], such as targeting the genome by using mutagenic high-energy

particles (X-rays and gamma rays), chemical mutagens (ethyl methane sulfonate (EMS) and N-ethyl-N-nitrosourea (ENU)), and transposons elements (insertions or deletions), targeting RNA using RNAi against gene target (degradation of mRNA) or morpholinos (block translation or splicing), targeting proteins using degron-mediated proteolysis (via peptide or GFP). These approaches have also been used in gene function analysis in fish during the past two decades. All three technologies – ZNFs, TALENs, and CRISPR/Cas9 – offer researchers alternative methods to develop mutant animals and human disease models, faster than traditional gene-targeting methods. However, there are also some limitations and complications, which are summarized in Box 5.2.

5.3 Sex Control in Zebrafish

5.3.1 Sex Determination Mechanism of Zebrafish

In the last 30 years, the zebrafish has become a widely used model organism for research on vertebrate development and disease [67]. Through a powerful combination of genetics and experimental embryology, significant inroads have been made into the regulation of embryonic axis formation, organogenesis, and the development of neural networks. Research with this model has also expanded into other areas, including the genetic regulation of aging, regeneration, and animal behavior. Zebrafish are an attractive model organism, because of the ease with which they can be maintained, their small size and low cost, the ability to obtain hundreds of embryos on a daily basis, and the accessibility, translucency, and rapid development.

Notwithstanding numerous efforts, the primary mechanisms that determine zebrafish sex still remain controversial. Domesticated zebrafish do not have a sex chromosome-based system of sex determination [68–71].

Box 5.2 Limitations and complications for ZNFs, TALENs, and CRISPR/Cas9

- 1) *Off-site effects.* Mutation introduced at non-specific loci with similar, but not identical, homology to the target sites are one of the most important complications of these technologies. These can be difficult to identify, and require scanning the genome for mutations at sites with sequence similarity to the gRNA target sequence.
- 2) *Mosaicism.* Animals with a mutant allele in only some of their cells can be produced, because the nucleases may not necessarily cut the DNA at the one cell stage of embryonic development.
- 3) *Multiple alleles.* Healing of the nuclease cleavage site by non-homologous end-joining can produce cohorts of animals with different mutations from the same targeting constructs, requiring genome sequencing to verify the nature and position of the specific mutation. The production of animals with mosaics of multiple mutations is also possible, and breeding may be required to segregate and isolate animals that carry single mutations. The production of animals with multiple variants also creates phenotyping bottlenecks.

Instead, several groups have shown that domesticated zebrafish use a polygenic system that is, so far, ill-defined [69]. Consistent with this, selection experiments with specific crosses can yield predictable sex ratios, often with a bias toward males. Regardless, there is also evidence that sex differentiation in zebrafish can be influenced by environmental factors. For example, multiple factors, such as nutrition, hypoxia, temperature, and rearing density, have been shown to influence sex ratios [72–74]. Although influenced by environment, zebrafish do not have a typical environmental sex determination system like some reptiles, where temperature acts as the definitive determinant [12].

Most results from cytogenetic analyses suggest that the zebrafish does not possess heterogametic sex chromosomes, where the inheritance of a particular chromosome would be the predominant determiner of sex [17]. A recent genome-wide association study, using RAD-tags to identify sex-linked SNPs, concluded that wild zebrafish have a ZW-ZZ sex determination mechanism, where females are ZW [75]. The sex-linked SNPs are found clustered at the tip of the right arm of chromosome 4. Intriguingly, the laboratory strains of zebrafish (AB and TU) seem to lack, or have greatly weakened, sex determinants on this locus of chromosome 4. Since these

strains can make males and females despite the apparent lack of full function of the natural sex determinants, it suggests the existence of an alternative sex-determining mechanism. This could be due to unmasking of “weak sex determinants” of the polygenic system, and/or unmasking of the latent and pre-existing environmental sex determination mechanisms [75].

The molecular mechanisms of sex differentiation (the developmental pathways of gonadal differentiation) in zebrafish are better understood. Zebrafish are a gonochoristic species, but exhibit juvenile or false hermaphroditism, where the early bipotential gonad first forms an immature ovary during the juvenile stage, before transforming into an adult ovary in females or a testis in males [76, 77]. Various studies have analyzed the expression patterns of genes during gonad development of zebrafish (Table 5.2) [78–81], and loss-of-function studies have tested the role of many of these genes in ovary and testis development (Table 5.3).

5.3.2 Genes Required for Male Development

Dmrt1 is the most conserved and characterized downstream component of sex determination, and has been shown to be involved

in male sex determination and differentiation in a wide variety of phylogenetically divergent groups like corals, annelids, arthropods, and vertebrates, from fishes to mammals [82].

To investigate the role of *Dmrt1* in zebrafish sex determination and gonad development, Webster *et al.* [47] isolated mutations disrupting this gene from ENU mutagenized fish, as well as targeted mutations using

TALEN-mediated mutagenesis. Their results showed that the majority of *dmrt1* mutant fish developed as fertile females, suggesting a complete male-to-female sex reversal in mutant animals that would have otherwise developed as males. A small percentage of mutant animals became males, but were sterile and displayed testicular dysgenesis. Therefore, zebrafish *Dmrt1* functions in male sex determination or differentiation and testis development.

It was also found that *Dmrt1* is necessary for normal transcriptional regulation of the *amh* (anti-Müllerian hormone) and *foxl2* (forkhead box L2) genes, which are thought to be important for male or female sexual development, respectively. Thus, in zebrafish, *Dmrt1* likely promotes male development by transcriptionally regulating male and female genes, as it does in mammals [83].

5.3.3 Genes Required for Female Development

The process of ovarian differentiation is believed to involve gonadal aromatase, which is encoded by the *cyp19a1* gene in all vertebrate species studied. The zebrafish

Table 5.2 The expression patterns of genes during gonad development of zebrafish.

Protein	Male	Female
Sox9a	Testis (Sertoli cells)	Juvenile ovary
Sox9b	–	Ovary and oocytes
Dmrt1	High in testis, germ cells	Low in ovary, germ cells
Amh	Testis (Sertoli cells)	Adult ovary
Cyp19a1a	–	Ovary (follicular cells)
Foxl2		Ovary

Table 5.3 Gene function studies together with sex control in zebrafish.

Genes	Approaches for loss of function mutations	Results	References
<i>dmrt1</i>	ENU and TALEN	Male-to-female sex reversal	[47]
<i>cyp19a1a</i>	TALEN and CRISPR/Cas9	Female-to-male sex reversal	[45, 46]
<i>bmp15</i>	TALEN	Female-to-male sex reversal	[45]
<i>dnd</i>	Morpholinos	Sterile fish	[84]
<i>cxcr4</i>	ENU-induced mutation project	Female-to-male sex reversal	[85, 87]
<i>sdf-1</i>	Morpholinos	Female-to-male sex reversal	[87]
<i>nanos3</i>	ENU-induced mutation library	females developed completely sterile; males developed as normal	[85, 88]
<i>vasa</i>	ENU-induced mutation project	Sterile males	[92]
<i>ziwi</i>	ENU-induced mutation project	Sterile males	[95]
<i>zili</i>	ENU-induced mutation project	Sterile males	[96]
<i>mlh1</i>	ENU-induced mutation project	Sterile males; fertile females with high rates of dysmorphology and mortality	[99]

genome contains two ohnologs of mammalian aromatase, called *cyp19a1a* and *cyp19a1b*, which are mainly expressed in the ovary and brain, respectively. Using TALEN- and CRISPR/Cas9-mediated mutagenesis, loss-of-function mutations for both genes have been produced, and it was found that all homozygous *cyp19a1a* mutants were fertile males as adults [45, 46]. By contrast, the sex ratios of *cyp19a1b* mutants were indistinguishable from their wild-type siblings, indicating that *cyp19a1b* does not play a major role, if any, in zebrafish sex determination [45]. These results also demonstrated that aromatase does not seem to influence the formation of juvenile ovary with oocyte-like germ cells. However, it starts to function afterwards, by promoting oocyte growth and maintaining their femaleness, which is essential for resisting germ cell apoptosis and further development into the true ovary.

In vertebrates, bidirectional paracrine signaling between the oocyte and the surrounding granulosa cells is critical for the proper development and function of the follicle. Oocytes receive signals and nutrients from follicle cells throughout their development, while the oocyte, in turn, signals to surrounding follicle cells to regulate their function. Two of these oocyte-produced signals that regulate granulosa cell development are growth differentiation factor 9 (Gdf9) and bone morphogenetic protein 15 (Bmp15), which are closely related members of the TGF- β superfamily of signaling molecules, and are expressed primarily by the oocyte in mice and zebrafish.

In order to test whether the oocyte-expressed signaling molecules Gdf9 and Bmp15 have any role in female sex determination or maintenance of female sex differentiation in zebrafish, Dranow *et al.* [45] generated targeted mutations for *gdf9* and *bmp15* using the TALEN approach. They found that *bmp15* mutant females initially have normal development but, during the juvenile stage, oocytes are degraded after they arrest at early stages and the premeiotic germ cells switch to a spermatogenic

program, as the gonad transforms to a fully functional testis. Consequently, all *bmp15* mutant adults are fertile males.

5.3.4 Genes Required for General Fertility

Aquaculture is progressively becoming more prevalent and vital to resolve the current and projected shortages in aquatic food availability. While the shift in reliance from fishery harvests to artificially propagated aquatic species continues, the increase in aquaculture activities poses a great threat to our ecosystem and environment. Non-native, selectively bred and, eventually, genetically modified farmed fish might escape from aquaculture containments, and propagate and/or interbreed with wild stock, subsequently changing the genetic composition of populations or causing species extinction. The use of reproductively sterile farmed fish will be the most effective strategy for genetic containment, particularly in large scale operations, thereby achieving environmentally responsible aquaculture practices [84].

The fetal gonad is composed of a mixture of somatic cell lineages and germ cells. The fate of the gonad, male or female, is determined by a population of somatic cells that differentiate into Sertoli or granulosa cells, and direct testis or ovary development. It is well established in zebrafish that germ cells are not required for the establishment or maintenance of Sertoli cells or testis cords in the male gonad. By contrast, in the absence of germ cells, granulosa cells do not form. Therefore, mutations that affect germ cell survival lead to an all-males sterile phenotype [85].

Zebrafish dead end (*dnd*) mRNA is specifically expressed in primordial germ cells (PGCs) and is required for PGC migration and survival [86]. Wong and Zohar [84] discovered that a molecular transporter, comprised of a dendrimeric oligoguanidine with a triazine core, can effectively traverse the morpholino oligomer (MO) across the chorion and into early embryos. Vivo-conjugated MO against zebrafish dead end

(dnd-MO-Vivo) effectively disrupted PGC development, leading to the elimination of germ cells and resulting in the development of reproductive sterile male adults.

In many migratory cells, chemoattractants are sensed by G-protein-coupled receptors that signal through phosphatidylinositol-3-OH kinase (PI(3)K) to recruit pleckstrin homology (PH) domain-containing proteins to the leading edge. In zebrafish, Knaut *et al.* [85] demonstrated that a zebrafish homolog of the G-protein-coupled chemokine receptor *cxcr4* is required specifically in germ cells for their chemotaxis. In *cxcr4* mutants from the ENU-induced mutation project, germ cells are able to activate the migratory program, but fail to undergo directed migration toward their target tissue, resulting in randomly dispersed germ cells. The resulting germ cell-deficient zebrafish are all males as adults.

Moreover, studies from Doitsidou *et al.* [87] also showed that chemokine stromal-cell-derived-factor (Sdf)-1a are key molecules directing the PGCs toward their intermediate and final targets. Knocking down Sdf1a or its receptor Cxcr4 through MO technology results in severe defects in PGC migration.

In both vertebrates and invertebrates, *nanos*-related genes, which encode RNA-binding zinc finger proteins, have been shown to play essential and conserved roles during germ cell formation [88, 89]. The study of Köprunner *et al.* [88] indicated *nanos3* (previously named *nanos1*) is necessary for PGC survival through using MO technology. Draper *et al.* [89] identified a mutation in *nanos3* from an ENU-induced mutation library. Their results showed that female *nanos3* mutants contain oocytes, but fail to maintain oocyte production, which indicates that *nanos3* is required for maintaining oocyte production in adult females. All *nanos3* mutant males developed normally, while females were sterile by six months of age. Interestingly, once *nanos3* mutant females become agametic, they sex-revert to a male phenotype [90].

Vasa is a universal marker of the germ line in animals, yet mutations disrupting *Vasa* cause sexually dimorphic infertility, with impaired development of the ovary in some animals and the testis in others [91]. Identification of the Vasa homolog in zebrafish allowed for the first description of zebrafish primordial germ cell development [92]. Vasa is expressed in mitotic and early meiotic germ cells throughout life. Hartung *et al.* [92] characterized a loss-of-function mutation disrupting zebrafish *vasa* from the ENU-induced mutation project [93]. Their results showed that maternally provided *vasa* is stable through the first 10 days of zebrafish development and, thus, could fulfill any roles for Vasa during germ-line specification, migration, and survival in the larval gonad. Although Vasa is not required to form the juvenile gonad, *vasa* mutants develop exclusively as sterile males: Germ cells fail to progress beyond the pachytene stage of meiosis, and the eventual loss of the germ-line stem cells causes all *vasa*-mutant fish to form an immature testis. Thus, zebrafish Vasa appears to be required for female and male meiosis, differentiation, and maintenance of germ-line stem cells.

Piwi proteins specify an animal-specific subclass of the Argonaute family that, in vertebrates, is specifically expressed in germ cells. In the zebrafish genome, two clear Piwi homologs can be identified, called Ziwi and Zili. Loss-of-function *ziwi* and *zili* mutants, isolated from the ENU-induced mutation project, result in activation of transposable elements [94–96]. *ziwi* mutant germ cells undergo apoptosis, and loss of *zili* results in a failure of germ cells to differentiate into mature oocytes or sperm. Thus all *ziwi* and *zili* mutants develop as sterile males.

Besides disrupting germ cells' development, disrupting of meiosis also displays fertility problems in mammals. MLH1 has been demonstrated to have functions during meiosis in mice [97, 98]. In a *mlh1* knockout line of zebrafish from the ENU-induced mutation project, Feitsma *et al.* [99] found that male *mlh1* mutants are sterile and display an arrest in spermatogenesis

at metaphase I, resulting in increased testis weight, due to accumulation of prophase I spermatocytes. In contrast, females are fully fertile, but their progeny shows high rates of dysmorphology and mortality within the first days of development, presumably due to chromosomal aneuploidy.

5.4 Sex control in Medaka

The medaka has also been established as a model organism that is particularly useful in the analysis of gonadogenesis. The medaka has an XX–XY male heterogametic sex determination system, like mammals. *Dmrt1* and *Sox9b* are preferentially expressed in the male gonads, whereas *Foxl2* and *Cyp19a1* are expressed in the female gonads [100–102].

The key sex determinant in medaka was identified as the DM-domain-related gene on the Y chromosome, namely *dmy* or *dmrt1by* [8, 103]. *dmy* is considered to have arisen via gene duplication of *dmrt1*. In the medaka, both genes are expressed in Sertoli cells, but with distinct temporal expression patterns: *dmy* expression starts just before the sex-determining period, whereas *dmrt1* expression first occurs during the testicular differentiation period.

Two naturally occurring mutations establish *Dmy*'s critical role in male development [8]. The first heritable mutant, a single insertion in exon 3 and the subsequent truncation of *Dmy*, resulted in all XY female offspring. Similarly, the second *dmy* mutant showed reduced *dmy* expression, with a high proportion of XY female offspring [2]. During normal development, *dmy* is expressed only in somatic cells of XY gonads. These findings strongly suggest that the sex-specific *dmy* is required for testicular development, and is a prime candidate for the medaka sex-determining gene.

Luo *et al.* [48] assembled improved TALENs targeting the *dmy* gene, and generated XY(*dmy*-) mutants to investigate gonadal dysgenesis in medaka. *dmy*-TALENs resulted in indel mutations at the targeted loci. XY(*dmy*-)

mutants developed into females, laid eggs, and stably passed the Y(*dmy*-) chromosome to next generation. In a *dmrt1* mutant line, which was found by screening an ENU-induced mutation library, XY mutants also developed into normal females and laid eggs. Histological analyses of this mutant revealed that the XY mutant gonads first developed into the normal testis type, which then sex-reverted into a functional ovary. The mutant phenotype could be rescued by transgenesis of the *dmrt1* genomic region. These results show that *dmrt1* is essential to maintain testis differentiation after a *dmy*-triggered male differentiation pathway [104].

Similar to zebrafish, the medaka *cxcr4* ortholog had been reported to be involved in the migration of primordial germ cells during gastrulation, as this process is severely impaired by inhibiting *cxcr4* using morpholino oligos [105]. *cxcr4* morphants are germ cell-deficient and showed female-to-male sex reversal [106].

A recent analysis identified *foxl3* as a gene that determines the sexual fate decision of germ cells in medaka. *foxl3* acts in female germline stem cells to repress commitment to male fate (spermatogenesis) [107]. Nishimura *et al.* [107] generated TALEN-induced mutants of *foxl3*, and their results showed that the adult XX *foxl3* mutants developed functional sperm in the expanded germinal epithelium of a histologically functional ovary.

In one medaka species, *O. luzonensis*, Myosho *et al.* [20] demonstrated that *gsdfy* (gonadal soma derived growth factor on the Y chromosome) is the master sex-determining gene in this species. Overexpression of *gsdfy* in XX animals using a genomic clone containing the *gsdfy* locus resulted in all-male development in both the F1 and F2 progeny, whereas all XX fish without the transgene developed as females. In another medaka-related fish, *O. dancena*, Takehana *et al.* [108] demonstrated that *sox3* is the male-determining factor on the Y chromosome. They generated transgenic *O. dancena* by introducing a Y chromosomal BAC clone,

which contained a part of the mapped region and the adjacent *sox3* and *p2ry4* genes, and induced sex-reversed XX males. Knocking out of the *sox3* gene was produced by using ZFN technology. The results showed that all heterozygous XY fish having the mutant alleles on the Y chromosome had ovary-type gonads at 20 dph (days post hatching), and developed as fertile females.

5.5 Sex control in Economic Fish Species

In addition to zebrafish and medaka, genome editing techniques have recently been widely applied in several economic fish species for sex control (Table 5.4), such as Nile tilapia [50, 65, 109], Chinese half-smooth tongue sole (*Cynoglossus semilaevis*) [110], rainbow trout [22], Atlantic salmon (*Salmo salar*) [111], and Patagonian pejerrey [18].

Numerous studies have investigated the mechanisms of sex determination in Nile tilapia, motivated in part by commercial interest, because males have a higher growth rate than females. Tilapia are gonochoristic teleosts, in which sex is largely genetically determined [112], although environmental factors can also play a role [113]. A XX/XY sex determination system has been described for Nile tilapia [114, 115].

Through using TALENs, somatic mutations for target genes related to sex differentiation including *dmrt1*, *foxl2*, *cyp19a1a*, *gsdf*, *igf3*, and *nrob1b* were generated [50]. In *dmrt1*-deficient testes, phenotypes of significant testicular regression, including deformed efferent ducts, degenerated spermatogonia, or even a complete loss of germ cells, and proliferation of steroidogenic cells, were observed. In contrast, deficiency of *foxl2* in XX fish induced varying degrees of oocyte degeneration and significantly decreased aromatase gene expression and serum estradiol-17 β levels. Some *foxl2*-deficient fish even exhibited complete sex reversal, with high expression of *dmrt1* and *cyp11b2*.

Disruption of *cyp19a1a* in XX fish led to partial sex reversal, with increased expression of the male-specific genes *dmrt1* and *cyp11b2*. Through using CRISPR/Cas9 approach, mutations for *nanos2*, *nanos3*, *dmrt1*, and *foxl2* genes were induced [65]. In agreement with the gonadal phenotype of *dmrt1* and *foxl2* deficiency induced by TALENs [50], *foxl2* mutations induced by Cas9/gRNA lead to downregulation of aromatase expression and sex reversal, and *dmrt1* deficiency resulted in upregulation of aromatase expression in the testis. Recently, studies from Li *et al.* [109] showed that *amhy*, a Y-specific duplicate of the

Table 5.4 Sex control using loss-of-function approaches in economic species.

Species	Genes	Approaches for loss-of-function mutations	Results	References
Nile tilapia <i>Oreochromis niloticus</i>	<i>cyp19a1a</i>	TALEN	Partial female-to-male sex reversal	[50]
	<i>nanos2</i>	CRISPR/Cas9	Female-to-male sex reversal	[65]
Chinese half-smooth tongue sole <i>Cynoglossus semilaevis</i>	<i>dmrt1</i>	TALEN	<i>Dmrt1</i> -deficient fish showed ovary-like testis and disrupted spermatogenesis	[110]
Rainbow trout <i>Oncorhynchus mykiss</i>	<i>sdY</i>	ZFNs	Male-to-female sex reversal	[21, 34]
Atlantic salmon <i>Salmo salar</i>	<i>dnd</i>	CRISPR-Cas9	Sterile fish	[111]

anti-Müllerian hormone (*amh*) gene, is the likely male sex determination gene in Nile tilapia.

The Chinese half-smooth tongue sole is a very important cultured marine flatfish with a ZW sex chromosome system. Male and female tongue sole are considerably different in size and growth rate, with mature females being twice as large in length and six times greater in weight than their male counterparts [116]. Thus, understanding the underpinning of sexual dimorphism and sex determination in this species is essential for developing methods to boost its productivity to meet the aquaculture market demands. Genome sequencing suggested that the Z-linked *dmrt1* is a putative male determination gene.

Cui *et al.* [110] induced efficient *dmrt1* mutations through TALEN technology. Their results showed that ZZ *dmrt1* mutant fish developed ovary-like testes with disrupted spermatogenesis. The female-related genes *foxl2* and *cyp19a1a* were significantly increased in the gonad of the ZZ *dmrt1* mutant. Conversely, the male-related genes *sox9a* and *amh* were significantly decreased. Importantly, the *dmrt1*-deficient ZZ fish grew much faster than the ZZ male controls.

Salmonids are generally considered to have a robust genetic sex determination system, with a simple male heterogamety (XX/XY) [117]. However, many studies have also reported that their sex differentiation can be modulated by temperature [118]. In rainbow trout, females exhibit higher growth rates than males and achieve larger sizes. In addition, males mature before reaching marketable size. Therefore, there is great interest from the fish farmers to produce all-female rainbow trout stocks.

Yano *et al.* [21] identified one master sex-determining gene for this species, named *sdY* for sexually dimorphic on the Y chromosome. Mutations of *sdY* induced a clear ovarian phenotype, indicating a male-to-female sex reversal [34]. In Atlantic salmon, the CRISPR-Cas9 system was used to simultaneously mutant *dead end* (*dnd*), a factor required for

germ cell survival in vertebrates, and the pigmentation gene *albino* (*alb*). Induced mutations for the tracer (*alb*) and the target (*dnd*) genes were highly correlated, and produced germ cell-less fish lacking pigmentation [111]. These results indicate that sterility in Atlantic salmon, which would allow for genetic containment, could be achieved by *dnd* gene knockout.

5.6 Implications for Aquaculture

It is imperative that highly efficient aquaculture practices are developed that enhance production, so that the growing demand for seafood can be met while, at the same time, reducing the need to harvest wild fishery stocks, which are in decline. However, the expansion of aquaculture operations also creates a great risk to our ecosystems and environment. Effective and practical fish sterilization technologies are crucial to resolve current and predicted threats posed by escapees from fish farms.

Manipulating chromosome set normality by triploidization or interspecies hybridization is the most common method used to produce infertile fish [119]. However, some hybrids and triploids were found to be fertile and/or sub-fertile [120–123]. In the last half decade, a transgenic approach has been developed to produce sterile fish through disrupting PGC migration. For example, disruption of the *Sdf1a* signaling pathway can prevent normal PGC migration in the fish embryo [85, 87]. Therefore, an inducible over-expression of *sdf1a* in the zebrafish embryo has been designed to disrupt the formation of the *Sdf1a* gradient that guides PGC migration and to saturate the *Sdf1a* receptor, *Cxcr4b*, on PGCs, which prevents PGCs from responding to the endogenous *Sdf1a* signal. As a result, PGCs mis-migrate to an ectopic region without reaching the developing gonads and, consequently, the treated fish develop into sterile individuals with severely under-developed gonads that lack germ cells [124].

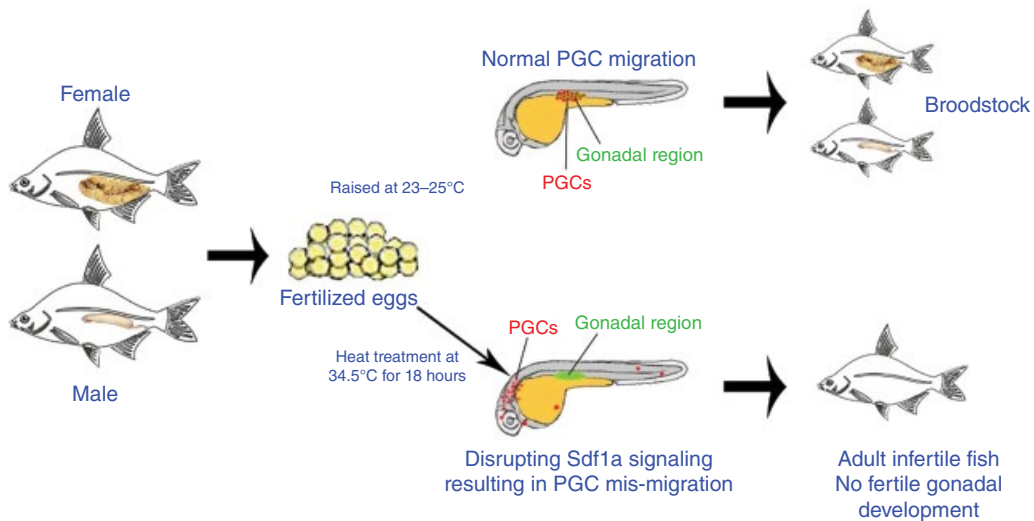


Figure 5.3 A flow chart for production of fertile broodstock and sterile fish using the disruption of PGC migration.

This technology uses a heat shock promoter (*hsp70*) and a *nanos3* 3' UTR to drive *sdf1a* expression [125]. Upon heat induction, over-expression of *sdf1a* disrupts the *sdf1a* signaling that guides PGC migration. The heat-treated transgenic fish eventually develop into sterile fish. When transgenic embryos are grown at lower temperatures, they become fertile broodstock.

This technology uses a heat shock promoter (*hsp70*) and a *nanos3* 3' UTR to drive *sdf1a* expression (Figure 5.3, cited from Wong and Zohar [125]). Upon heat induction, over-expression of *Sdf1a* disrupts the *Sdf1a* signaling that guides PGC migration. The heat-treated transgenic fish eventually develop into sterile fish. When transgenic embryos are grown at lower temperatures, they become fertile broodstock. Therefore, gene knock-out approach could be used as the first step to identify the genes' specific functions in PGCs for aquaculture species, then transgenic approach can be used to produce sterile fish.

For some fish species, sex control could be successfully accomplished simply through direct hormone induction, technology combining hormone induction and artificial interspecific hybridization, as well as gynogenesis and androgenesis. In addition, current gene knockout techniques show real promise to genetically modify organisms. Several all-male or all-female populations have been generated through gene knockout technologies in the laboratory level, as reviewed above. Hopefully, its practical application in aquaculture-relevant species will be realized in the near future.

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6

Chromosome Manipulation Techniques and Applications to Aquaculture

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6.1 Introduction

Chromosome (set) manipulation is defined as a system of techniques to alter the number and combination of homo- and hetero-specific genome(s) or chromosome set(s). It was initially investigated at the beginning of the 20th century from the viewpoint of basic biology, mainly in amphibians. From the 1950s to 1970s, several pioneer studies in fish species were conducted to investigate the effect of ploidy elevation, as well as uniparental development on animals by scientists in the former Soviet Union [1–4], the United Kingdom [5–7], the United States [8, 9], Norway [10], Hungary [11, 12], Japan [13], and other countries.

In the 1980s, aquaculture-oriented studies began to improve performances of farmed strains by chromosome manipulation techniques in various species of finfish and aquatic invertebrates, and became widely prevalent [14–16]. In Japan, research programs of “*Regional Biotechnology*” were allotted to several prefectural governments, to promote the application of chromosome manipulation in aquatic farmed species that were commercially important to each prefecture [17].

In the last three decades, rationales underlying the treatment to achieve ploidy

elevation and induction of all-female and all-male inheritance (i.e., gynogenesis and androgenesis, respectively) have been well understood. Optimum treatment conditions have been subsequently determined in each target species, followed by precise evaluations of aquaculture performances, such as survival, growth, maturation, disease-resistance, and other traits in resultant polyploid and gyno- and androgenetic progenies, as reviewed by [16–26].

Considering the research history of chromosome manipulations, these techniques seem to be classic ones. However, Arai [17] remarked that chromosome manipulation should not be viewed as an obsolete technology to be discarded in future breeding of aquatic organisms, because further advancement could be expected if it was used in conjunction with other genetic technology, including hypervariable DNA markers, transgenics, and germ cell technology. Recently, this prediction has come true in several aspects.

Linkage maps were constructed in the progeny of chromosomally manipulated or clonal strains [27, 28]:

- 1) Genome annotation was facilitated by using homozygous strains [29].
- 2) Inbred lines with transgenic genotypes were established by induced androgenesis [30].

- 3) Induced sterile triploids were used as host of xeno- or allo-genesis, i.e., surrogate production [31].

In addition, biological containment tools were used to minimize the risk of escapees of transgenic and other genetically manipulated organisms into the wild populations [32].

In this chapter, we review not only well-known principles and methods of chromosome manipulation, but also unsolved technical problems. Triploids and gynogenetic diploids can be now easily induced in many commercially important aquatic species, by the inhibition of second polar body releases with hydrostatic pressure, or temperature shocks after fertilization with normal and genetically inert sperm, respectively. However, acceptable yields have never been achieved in most cases of the production of tetraploids from diploid zygotes and in the induction of doubled haploids (DHs) from gynogenetic or androgenetic haploids. Thus, we focus the discussion on the mechanisms underlying whole genome duplication and effects of

chromosome doublings on survival from various viewpoints.

Next, we review techniques and results of androgenesis. Then, we summarize recent innovations, such as cold shock induced androgenesis without irradiation of eggs, dispermic androgenesis, and the usage of hybrid gametes to overcome nucleo-cytoplasmic incompatibility. We then discuss the use of distant hybridization as an alternative method of chromosome manipulation in aquaculture, because it often causes unreduced gametogenesis and atypical reproduction. Finally, the last section summarizes the sex determination system inferred from sex ratios observed in the chromosomally manipulated fishes.

Technical terms in this chapter and explanations of their meanings appear in Box. 6.1.

6.2 Induced Triploidy

6.2.1 Induction of Triploid Fish and Shellfish

Nowadays, principles for the production of triploid fishes are well known. In fish,

Box 6.1 Glossary of terms

- **Androgenesis:** A form of asexual reproduction in which embryos develop without any genetic contribution of egg nucleus and, consequently, progeny have paternally derived genotypes
- **Diploid:** Cells or individuals with two sets of homologous chromosomes, one set from mother (egg) and another set from father (sperm), and thus is shown by the symbol $2n$. A state of diploid condition of cells or individuals is referred to as *diploidy*.
- **Doubled haploid:** Cells or individuals with completely homozygous diploid genotypes, comprising two identical sets of haploid chromosomes.
- **Gynogenesis:** A form of asexual reproduction in which development occurs without any genetic contribution of the father, but it requires sperm to trigger development.
- **Haploid:** Cells or individuals with a single set of chromosomes, and thus is shown by the symbol n . A state of haploid condition of cells or individuals is referred to as *haploidy*.
- **Hybrid:** Progeny with chromosome sets from maternal and paternal species (strains) after fertilization between two different species (strains).
- **Polyploid:** Cells or individuals with extra set(s) of chromosomes are collectively referred to as polyploid. A state of polyploid condition of cells or individuals is referred to as *polyploidy*. Polyploid cells or individuals are described, according to the number of chromosome sets that they have, as: *triploid* (3 sets, $3n$); *tetraploid* (4 sets, $4n$); *pentaploid* (5 sets, $5n$); *hexaploid* (6 sets, $6n$); *heptaploid* (7 sets, $7n$); *octaploid* (8 sets, $8n$); *nanoploid* (9 sets, $9n$); *decaploid* (10 sets, $10n$) and so on. *Autopolyploid* (ex. *autotriploid*) includes homospecific homologous chromosome sets, while *allopolyploid* (ex. *allotriploid*) includes at least one set of heterospecific non-homologous chromosomes.

mature oocytes are ovulated at the metaphase of meiosis II (MII) after the intrusion of sperm and, thus, the second polar body will be released just after fertilization [14–24]. Thus, meiosis is completed by the release of the second polar body after the intrusion of sperm through micropyle of an egg of finfish species. Inhibition of this process results in triploid ($3n$) zygotes, comprising two sets of chromosomes ($2n$) from female ($1n$ egg pronucleus + $1n$ second polar body nucleus) and one set ($1n$) from male (sperm) pronucleus.

To induce triploidy by inhibiting the second polar body release, physical treatments such as cold shock, heat shock, and hydrostatic pressure shock have been frequently used in teleosts. Treatment conditions must be optimized in each target species, because physiological conditions related to the successful manipulation were species-specific (see more details in Chapter 41 of Volume 2). When triploids are produced in a same species with homospecific genomes, we call such triploids *autotriploids*. When we produce triploids with heterospecific genomes in interspecific hybrids, we call such triploid hybrids *allotriploids*.

In shellfish, chemical treatments using cytocharasin B, 6-dimethylaminopurine and caffeine are generally preferred for induction of triploidy, but physical treatments (hydrostatic pressure, cold, and heat shocks) have been also utilized in some species [24, 25, 33]. Sometimes, double treatment with both chemical and physical shocks gave better results in abalone [33]. There are generally two timings to produce triploids in shellfish species, because mature eggs first accept sperm for fertilization and then release the first and the second polar body (i.e., completion of meiosis I (MI) and II (MII), respectively). However, inhibition of the MI is no longer considered a good method to induce triploid shellfish, because different types of aneuploidies appear in high percentages, due to the frequent formation of tripolar spindles and separated bipolar spindles by the blocking of MI [25].

Successful induction of triploid animals has recently been verified by measuring the DNA content of somatic cells by flow-cytometry in most cases. However, the cytogenetic method (chromosome counting and karyotyping) is still active for precise determination of polyploidy and/or aneuploidy. In early phases of triploid studies, measuring cell or cellular nuclear sizes and counting Ag-NOR (nucleolar organizing regions by silver staining) per cell were common methods [24].

6.2.2 Performance of Triploid Fish and Shellfish

The performance of triploids relative to survival, growth, maturation, meat quality, disease resistance, and other traits has been studied in many aquatic species, from the viewpoint of aquaculture applications. In commercially important traits, better growth and increase of edible parts have been observed in shellfish species, although the results in teleost triploids have been variable. Some exhibited better growth under certain conditions, but others gave undesirable results. In allotriploids, recovery of viability in hybrids has been frequently observed: Some interspecific salmonid hybrids die before hatching, but induced triploidy in such hybrids often results in viable progeny that grow until adult stages [34–40].

Several allotriploid salmonid hybrids have been used in aquaculture to vitalize the local economy in Japan [17, 41]. Different results among artificially produced triploid fish and shellfish species were compiled and summarized, and then compared to discuss merits and demerits of induced triploids in several reviews [18, 19, 24–26]. Generally, outperformance of triploid shellfish has been explained by the integrated effects of cell size hypothesis (gigantism), increased heterozygosity, and energy reallocation from maturation to somatic growth, due to sterility [25].

6.2.3 Reversion of Triploids to Diploids – Newly Recognized Problem in Shellfish

A newly recognized problem in triploid aquaculture is *reversion* from triploid to diploid status, via diploid-triploid mosaicism and aneuploidies, which was noticed in *Crassostrea* oyster species [25]. Such a reverting by chromosome elimination in somatic cells will lose their aquaculture advantages, due to the recovery of reproductive capacity [42, 43]. The reversion phenomenon has not been found in other triploid and polyploid animals used in aquaculture so far. Reversion of ploidy will provide serious biological and practical problems. Thus, the reversion should be investigated in as many cases of farmed triploid animals as possible.

6.3 Induced Gynogenesis

6.3.1 Induction of Gynogenetic Haploids by Using Irradiated Sperm

Artificial gynogenesis can be induced by activation of eggs with genetically inert sperm, prepared by gamma-, X-ray, or UV irradiation prior to fertilization [17, 23]. Generally, UV irradiation is widely used to inactivate sperm genetically, because this method does not require any special facilities or equipment to keep safe, except for a relatively cheap, commercially available germicidal lamp. Sperm is diluted by physiological saline or natural/artificial seminal fluid, which ensures maintaining fertility, and then should be kept in a thin layer during the irradiation, due to the low penetrating power of UV. Successfully induced gynogenesis will result in haploid embryos, which are not able to develop beyond the stages of hatching and/or soon after the initiation of feeding, in most cases, due to the expression of abnormalities, referred as so-called haploid syndrome.

6.3.2 Induction of Gynogenetic Diploids by Inhibition of Meiosis

Diploidy must be recovered in gynogenetically developing haploid embryos for further uses by the retention of the nucleus of the second polar body, by means of the inhibition of its release. The mechanism of chromosome duplication is essentially the same as the induction of triploids – namely, inhibition of the release of the second polar body in teleosts and inhibition of the release of the first or second polar body in most shellfish [24]. As in triploids, either temperature (cold- or heat-) shock and hydrostatic pressure shock are normally applied to inhibit polar body release in teleosts. However, induced gynogenesis is still in the stages of academic research in shellfish, and transfer of technology from the laboratory to the aquaculture farm has not yet been achieved [25].

This type of gynogenetic diploid progeny is called meiotic gynogenetic diploids (or diploid gynogens, or diploid gynogenotes, or diploid gynogenetics), but some researchers use the term meiogynes or polar-body gynogens [17, 26]. The most important point is that such meiotic gynogenetic diploids are not completely homozygous, due to the influences of recombination (crossing-over) between gene locus and centromere during meiosis [44], as shown in Figure 6.1.

In induced triploid and meiotic gynogenetic diploid fish ($2n$ gynogens), chromosomes in eggs are half-tetrad, in which tetrad means bivalent (i.e., two meiotic products [bivalents] from a single meiosis). All half-tetrads of a diploid heterozygous female will be homozygous if they are non-recombinants. An odd number of recombination (crossing-over) will produce recombinant heterozygous progeny. The proportion of heterozygous progeny is a measure of the frequency of recombination (y). G-C map distance (cM) can be estimated as $y/2 \times 100$. Generally, in teleosts, due to strong interference [44], only one chiasma is formed and, thus, recombination provides heterozygous genotypes.

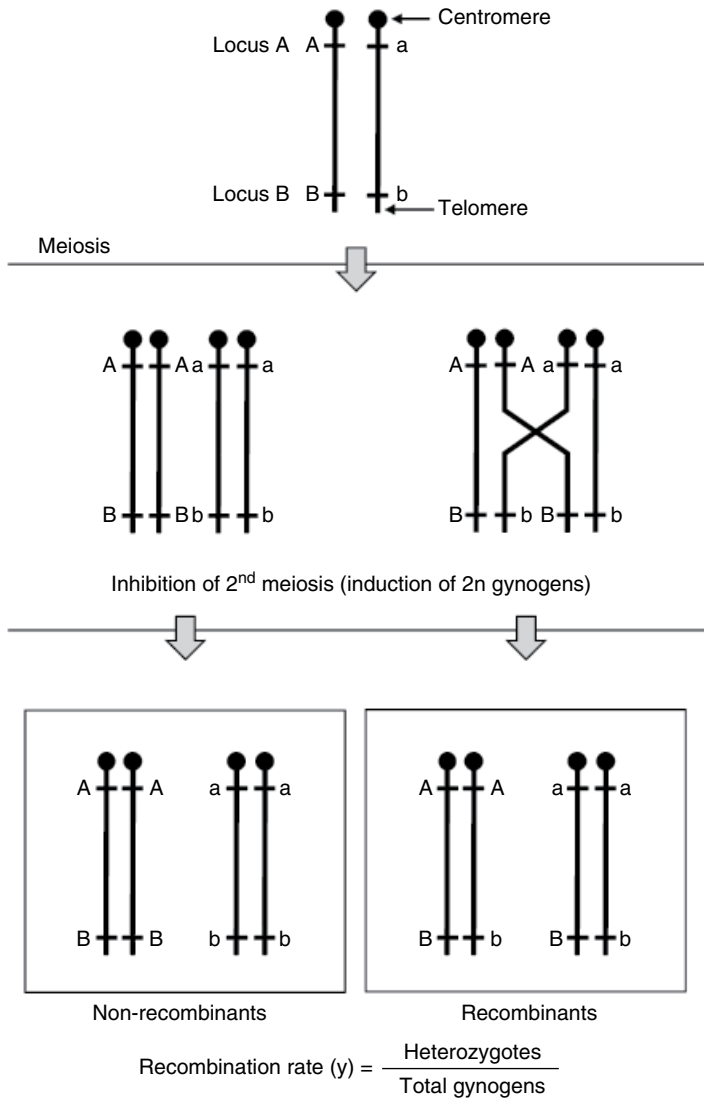


Figure 6.1 Non-recombinants and recombinants in meiotic gynogenetic diploids (2n gynogens) induced by the inhibition of second polar body release, from a female heterozygous at centromeric A and telomeric B loci.

Based on this rationale, gene-centromere mapping has been done to accommodate microsatellite DNA loci by the method of half-tetrad analyses using triploid and gynogenetic diploid progeny in zebrafish, *Danio rerio* [45], pink salmon, *Oncorhynchus gorbuscha* [46], dojo loach, *Misgurnus anguillicaudatus* [47], eel, *Anguilla japonica* [48], barfin flounder, *Verasper moseri* [49], and others. The production of meiotic gynogenetic diploids is much easier than gynogenetic doubled haploids

(DHs), as we will see in Section 6.5. However, it is possible to produce genetically similar lines by two, three, or more cycles of meiotic gynogenesis, because the distal and proximal loci on homologous chromosomes would be fixed in the heterozygous and homozygous conditions, respectively. This suggests resultant genetic fixation of same heterozygous and homozygous genotypes in the second, third, and later generations of gynogenetic progenies [17].

There are many reports of sexual dimorphisms on commercially important traits such as growth, external appearance, taste, and so on in meiotic gynogenetic diploids [15, 16, 22, 26]. Females exhibit better growth than males in some aquaculture species, while males exhibit much more attractive color patterns in several aquarium fishes. Such differences between sexes readily led to the necessity to develop sex control techniques in aquaculture species. Sex manipulation using gynogenetic techniques will be reviewed later, in Section 6.9.

6.4 Induced Tetraploidy

6.4.1 Induction of Tetraploid Fish

If we obtain fertile tetraploid fish, they are expected to ovulate tetraploid oocytes comprising diploid ($2n$) egg nucleus and diploid ($2n$) polar body nucleus in females, and to ejaculate diploid ($2n$) sperm in males. Diploid gametes (eggs and sperm) are especially important, because they provide various applications to manipulate ploidy status. For example, tetraploid lines can be developed by fertilization of diploid eggs with diploid sperm, and mass production of sterile triploids can be easily realized by fertilization of diploid eggs with normal haploid sperm.

Viable gynogenetic diploids can be induced by activation of diploid eggs with UV-irradiated sperm: no chromosomal duplication is required when diploid eggs are available. Moreover, a combination of diploid gametes and chromosome manipulation may realize production of higher polyploid animals. Pentaploids can be induced by fertilization of diploid eggs with haploid sperm, followed by the inhibition of the second polar body release (i.e., $2n$ egg nucleus + $2n$ polar body nucleus + $1n$ sperm nucleus), while hexaploids can be induced by fertilization of diploid eggs with diploid sperm, followed by the inhibition of the second polar body (i.e., $2n$ egg nucleus + $2n$ polar body nucleus + $2n$ sperm nucleus) [17].

As mentioned above, tetraploids are considered an important source of fertile diploid gametes and, thus, a large number of trials have been done to produce mature tetraploid animals. Tetraploids have been believed to be induced by endomitosis (chromosome duplication without cytokinesis) of whole genome through the inhibition of the mitotic cell cycle (i.e., suppression of the early cleavage). However, in such a manipulation, it is very difficult to obtain acceptable numbers of viable and fertile tetraploid adults. In almost all cases, tetraploid individuals were verified in treated groups, but most progenies died in embryonic, larval, and fingerling stages, and very few individuals survived to adult stage. In induced tetraploids, extremely low survival rate is the most serious obstacle. Only a very few successful examples have been reported in teleosts such as rainbow trout, *Oncorhynchus mykiss* [50, 51], blunt nose bream, *Megalobrama amblycephala* [52], and mud loach, *Misgurnus mizolepis* [53, 54].

There have been many attempts to determine optimum conditions for tetraploid induction, including temperature of heat or cold shock, strength of hydrostatic pressure, concentration of chemicals, duration of treatment, and timing to start and stop treatment [24]. Optimization of induction is a matter of trial and error, with re-iterations not considering female to female individual variation. Further, within a population of gametes, not all are synchronously correlated, but optimization produces the greatest number of viable progeny with the desired chromosome number. Double treatment was also tried to have better results: heat shock 40.5°C for two minutes at 28 minutes after fertilization, immediately followed by cold shock 1.5°C for 45 minutes in mud loach [55].

To improve success rates of tetraploidization, timing of the treatment has been intensively optimized by using a relative embryological age, showing the duration of one mitotic cycle, such as the first cleavage interval (FCI) and tau zero (τ_0) [56, 57].

In salmonid, embryos likely developed from non-optimum conditions, and various kinds of aneuploids and mosaics occurred [58, 59]. A small number of tetraploid fry that successfully hatched frequently exhibited abnormal morphology, including body flexure, edema, congestion of blood, poor vascular system, and other conditions that readily gave rise to lethal physiological disorders [58, 59].

6.4.2 Induction of Tetraploid Shellfish

In contrast to tetraploid finfish, the induction of tetraploidy in oysters has been successful by inhibiting the first meiosis of fertile eggs of mature triploids [60]. Triploid oysters produced from crosses between tetraploids and diploids are widely used in aquaculture [25]. However, there are some problems in induction of tetraploid oysters, such as very low reproductive ability of triploids (few mature triploids and very small numbers of normal eggs) and very low percentage (normally less than 0.1%) of induced tetraploid spats [24]. Production of tetraploids by using eggs of induced triploids and inhibition of MI can be applicable to other shellfish species, but practical use has been limited to oyster farming, so far [24]. Other approaches to produce tetraploid shellfish by inhibiting both MI and MII have been challenged, but industrial application has not been realized [61].

6.4.3 Cellular Mechanisms Responsible for Whole Genome Doubling

Chromosome doubling has been considered to be realized by suppression of cell division, due to the disorganization of spindles by physical treatment [62, 63]. Zhang and Onozato [64] found that hydrostatic pressure or heat shocks did not suppress the first cleavage because of regeneration of the bipolar spindle, but was achieved as the second cleavage was suppressed due to a monopolar spindle formation in rainbow trout. Thus, actual chromosome doubling

should occur in the second cleavage, so the two-cell stage should be expanded longer after the treatment on the optimal timing. On this process, behavior of the centriole is also very important; daughter centriole in one centrosome that normally contains two centrioles (mother and daughter) was depolymerized, but the first cleavage occurred by regenerated bipolar spindle. After this process, chromosome doubling occurred.

According to Zhang and Onozato [64], *“the centrosome with two centrioles entered a daughter cell and resulted in the normal mitosis. The centrosome with single centriole (the mother centriole) entered the daughter cell, although a new daughter centriole budded from mother, they together formed only one centrosome resulting in the monopolar division, leading to the tetraploidization of this cell.”* Similar results have also been observed in the process of chromosome duplication of the gynogenetically developing haploid embryos after the cold shocks, heat shocks, or hydrostatic pressure shocks targeted at the first cleavage in flounder [65, 66] and zebrafish [67]. Co-existence of a monopolar spindle in one blastomere, and a bipolar spindle in the other, may lead to diploid-tetraploid mosaic individuals [64].

6.4.4 Performance of Tetraploids

Even in the most successful case of viable and reproducible tetraploid rainbow trout, low growth rate (approximately 40% of control diploids) was recorded in about 2.5-year-old tetraploid rainbow trout [50]. Such a reduced growth may have been related to the deleterious effect of higher ploidy status itself, because hexaploid dojo loach, induced using diploid gametes of natural tetraploids, showed a retarded growth when compared with tetraploids [68]. When compared between diploid and tetraploid clonal dojo loach, both of which were genetically identical, tetraploids were significantly smaller than diploids [69].

In successfully induced tetraploid fishes, problems occurred not only in survival and growth, but also in reproductive capacity. Out of 48 artificially induced tetraploid mud loach males, 36 males had normal gonads, but 26 out of 36 males generated unexpected haploid sperm, and only three produced fertile diploid sperm [54]. Similar results were also obtained in tetraploid and diploid-tetraploid mosaic males of phylogenetically close dojo loach; these individuals matured at the age of one year, but they produced haploid sperm [70]. In contrast, induced tetraploid rainbow trout males, which matured in two or three years, were reported to produce triploid progeny after the fertilization of eggs of control diploid females, suggesting production of diploid sperm by tetraploid males [50].

Similarly, tetraploid females were reported to produce fertile diploid eggs, based on the results of artificial gynogenesis and fertilization by normal sperm of diploid males [51]. They also succeeded in the production of tetraploids, pentaploids, and hexaploids by the combination of diploid gametes and chromosome manipulation techniques [51]. In artificial tetraploid oyster, maturation at one year, normal sex ratio (1 female : 1 male), and production of fertile diploid gametes were reported [71].

6.4.5 Mosaics Including Tetraploid Cells

After the induced tetraploidization, very few survivors actually appeared as mentioned above. In the adult stage of amago salmon, *O. masou ishikawae*, pure tetraploid individuals disappeared in the treated group, and a few diploid-tetraploid mosaics remained [72, 73]. Diploid cells might have acted as a prophylactic to help survival of the individual, by reducing the undesirable effects of tetraploid cells, considering the deleterious effects of tetraploid cells themselves. Germ-line mosaicism is important, because one female with diploid somatic cells gave both diploid and triploid progeny after fertilization with normal haploid sperm. This female was concluded to be a diploid-tetraploid mosaic

individual, including only diploid red blood cells, but had both diploid and tetraploid germ cells in its ovary, and then generated both haploid and diploid fertile eggs [72]. Thus, a fertile diploid-tetraploid germ line mosaic can be used as a source of valuable diploid gametes for further breeding practices instead of pure tetraploid lines.

6.5 Gynogenetic Doubled Haploids (DHs)

6.5.1 Induction of Gynogenetic DHs

Gynogenetic DHs can be produced by whole-genome duplication of gynogenetically developing haploid embryos, induced by activation of eggs with genetically inert sperm. Genetic inactivation is easily achieved by irradiation of sperm, as described in Section 6.3.1. However, whole-genome duplication is achieved by the artificial induction of endomitosis at the initial stages of mitotic cleavage with temperature or hydrostatic pressure shocks. If the shock is applied at around pro-metaphase of the first cleavage, the treatment gives rise to duplication of a haploid set of homologous chromosomes (i.e., complete homozygosity of gynogenetic diploids). Thus, such gynogenetic DHs are also called mitotic gynogenetic diploids, mitotic gynogens, mitogynes, late shock gynogenotes, etc.

Optimum treatment conditions have been determined to produce gynogenetic DHs in more than 20 species, and were well summarized in the review paper [23]. Survival rates of resultant gynogenetic DHs were generally very low, but yields were much better than those of artificially induced tetraploids, even though the same treatments were applied to zygotes [59]. There is no conclusive explanation why DHs exhibit better survival than tetraploid progeny, which die in the embryonic and larval stages. Severe mortality of tetraploid is unlikely to be a side-effect of the chromosome doubling treatment, but is the result of the elevation of ploidy status itself [59].

Cytological studies revealed that the mitotic process of gynogenetic haploids was delayed, compared with control diploids, and such a time lag was presumably related to the presence of dense chromatin body derived from irradiated spermatozoon [59]. The stage of late pro-metaphase was the optimum timing for the treatment to obtain gynogenetic salmonid DHs [59]. However, as in the case of tetraploidization, the treatment did not suppress the first cleavage, but the second cleavage. This was because a bipolar spindle was regenerated to undergo the first cleavage after the treatment, but a monopolar spindle formation led to the arrest of mitosis, as well as chromosome duplication by interference of centrosome duplication [64–67].

6.5.2 Complete Homozygosity of Gynogenetic DHs

Gynogenetic DHs have been successfully induced in about 20 fish species [23] since the first success in zebrafish [74]. Gynogenetic DHs have been frequently reported to be contaminated by the spontaneous occurrence of meiotic gynogenetic diploids, which may appear by natural inhibition of the second polar body release [75]. Consequently, such meiotic gynogenetic diploids always exhibit much better survival rates than gynogenetic DHs, due to their higher heterozygosity than completely homozygous DHs (i.e., low influence of unmasking of deleterious recessive genes or genetic loads). These facts strongly suggest the importance of genetic screening of homozygous DHs. Microsatellite loci located on telomeric regions of chromosomes are especially useful markers to screen and verify completely homozygous DH individuals, because these loci are likely heterozygous in meiotic gynogenetic diploids, due to the locus-centromere recombination [47, 49, 75] (see Section 6.3.2 and Figure 6.1).

6.5.3 Performance of Gynogenetic DHs

Very low survival of gynogenetic DHs has been explained by the side-effect of

heat/pressure shocks, as well as inbreeding depression [23]. All the gynogenetic DHs that survived to the adult stages no longer had deleterious genes related to survival capacity, because all such genes should be eliminated by death during the embryonic, larval, and juvenile stages. However, the effects of inbreeding still remain for fertility-linked traits. In Nile tilapia *Oreochromis niloticus*, 10 of 77 (13%) gynogenetic DH females produced viable eggs [76]. In marine fish, only one of 13 (7.7%) gynogenetic DH red sea bream *Pagrus major* was reported to produce gametes [77]. Similar reductions in fertility have been observed in gynogenetic DHs of common carp *Cyprinus carpio*, tiger barb *Puntius tetrazona*, and rainbow trout [23].

Once gynogenetic DHs are produced, each DH individual can be used as a source of isogenic gametes. Because DH individuals are completely homozygous at any locus of chromosomes (coefficient of inbreeding $F=1$), they are thus equivalent to an inbred line that will be established by sib-mating more than 20 generations (estimated $F=0.99$).

6.6 Induced Androgenesis

6.6.1 Induction of Androgenetic Haploids by Using Irradiated Eggs

To induce androgenetic development, the egg nucleus must be exposed to gamma irradiation or X-rays, as both have strong penetrating power, but special facilities or equipment are necessary for safe practice [23]. In fish with relatively small eggs, such as common carp and dojo loach, UV irradiation has been used after detailed optimization of irradiating conditions [78, 79]. In any case, viability of the eggs should be maintained during irradiation. Several media have been tested: water [80]; synthetic ovarian fluid [78]; ovarian fluid [81]; Ringer solution [82]; seminal plasma [79]; and Hank's solution [83]. Genetic inactivation of egg nucleus of large size eggs from salmonids and sturgeons has not been successfully performed so far using UV irradiation.

In lower doses of irradiation, small chromosome fragments and other unusual structures have frequently been observed, and certain traits were expressed from these fragments [84]. UV irradiation of eggs also gave chromosome fragments, even in optimal conditions [79, 85], but their behavior and function are unknown. Irradiation should damage the egg nucleus, as well as mitochondrial DNA or cytoplasmic mRNA stored in oocytes. Until now, however, no evidence of serious damage to mitochondrial DNA has been reported. Mitochondrial DNA is presumably protected by its double membrane and large amount of yolk, and a large number of copies of mitochondrial DNA may prevent the damage due to the irradiation [23].

6.6.2 Induction of Androgenetic Doubled Haploids

Inducing the method of androgenetic DHs is essentially the same as gynogenetic DHs, as described in Section 6.5.1. In common carp DHs, there is no difference in survival results between gynogenetic DHs and androgenetic DHs. Successful yields (feeding fry) of androgenetic DHs were generally low: for example, 4.8 (inbred sperm) to 6.2% (outbred sperm) in rainbow trout, 5.6% in amago salmon, 19.8% in common carp, and 19% in mud loach [23].

These androgenetic DHs are considered a predictable genetic resource of isogenic gametes for cloning, because completely homozygous DHs are genetically equivalent to the inbred line. The other use of androgenesis is restoration of endangered genotypes from cryopreserved sperm. Sex manipulation using androgenetic DHs will be reviewed later, in Section 6.9.

6.6.3 Androgenesis by Diploid Sperm and Dispermic Fertilization

As mentioned in Section 6.4, it is very difficult to induce mature and fertile tetraploids by chromosome manipulation. However, once

pure tetraploid individuals are successfully produced, diploid gametes can be expected for further ploidy manipulation. When diploid sperm inseminated gamma irradiated eggs of rainbow trout, viable androgenetic diploid progeny appeared [86]. In dojo loach, viable androgenetic diploid progeny were produced using diploid sperm of natural tetraploid males and of neo-tetraploid males [83, 87, 88]. These neo-tetraploid males were produced by inhibition of the second polar body release after cross-breeding between wild-type diploid female and natural tetraploid male [88]. Androgenesis using diploid sperm gave better survival than DHs, suggesting that chromosome-duplicating treatments should mainly cause low survival of androgenetic DHs. Better survival of androgenetic progeny using diploid sperm also suggests a deleterious effect of homozygosity to survival and growth.

Production of viable androgenetic diploid progeny by dispermic androgenesis has been challenged in salmonids and sturgeons. Araki *et al.* [89] produced androgenetic diploid rainbow trout by fertilization of irradiated eggs with fused (diploid) sperm. A similar approach was done by using fused sperm of the endangered Biwa salmon, *O. masou* subsp. [90]. For fusion of spermatozoa, the authors used polyethylene glycol (PEG). Much better survival rates were expected in dispermic androgenetic diploids, because they were not complete homozygotes, but heterozygotes, similar to fertilization within a sibling if spermatozoa of single male were used. Thus, influences of unmasked deleterious recessive genes were lower than DHs. However, survival rates of androgenetic diploid progeny were not drastically improved when compared with the results in androgenetic DHs. Thus, further technical elaboration is required on this approach.

Dispermic androgenesis in sturgeons is essentially different from the abovementioned experimental sperm fusion by chemical treatment. The developed method is closely linked to biological characteristics specific to sturgeon eggs, which have several

micropyles to assure physiological polyspermy (spermatozoa intrude via several micropyles, but only one spermatozoon contributes to the zygote). The method in sturgeon comprises fertilization of X-ray irradiated eggs with concentrated normal sperm ensuring polyspermy, followed by heat shock, which facilitates the fusion of male pronuclei [91–93]. Using this dispermic androgenesis, viable androgenetic progeny were produced in sturgeons. More details on this topic will be given in Volume II, Chapter 34 (Polyploidization and hybridization in sturgeon).

6.6.4 Cold Shock-Induced Androgenesis

Androgenetic haploid development induced without egg irradiation was developed in dojo loach by Morishima *et al.* [94]. They found that cold shock treatment (0°C or 3°C for 60 minutes) of dojo loach eggs immediately after fertilization successfully induced androgenetic development. Cytological observation strongly suggested that both the egg nucleus and the second polar body nucleus were eliminated, and the remaining paternally derived sperm nucleus initiated haploid development. All-male inheritance of these androgenetic progeny was verified by the phenotype of male-specific recessive color gene and microsatellite genotyping. However, cold shock (3°C, for 30 minutes) induced androgenetic progeny exhibited abnormality due to haploidy. Next, viable androgenetic diploid progeny were artificially produced by cold shocking just after fertilization (within 10 seconds) with diploid sperm of a neo-tetraploid male [95].

Using the cold shock-induced technique, androgenetic DHs were induced in dojo loach [96]. Cold shock (about 3°C for 30 min) was applied within 10s just after fertilization, then the eggs were incubated at about 20°C for 35 min, then heat shocked about 42°C for 2 min beginning 65 min after fertilization [96]. The yield of putative DHs was about 10% relative to the initial number of eggs.

All-male genotypes were genetically confirmed by the expression of recessive orange body color of sire and complete homozygosity was verified by 28 microsatellite loci, which covered 27 linkage groups [97].

To show the effectiveness of cold shock androgenesis, Hou *et al.* [98] reported the production of androgenetic haploid progeny in a typical model animal, zebrafish, in which about 7°C was the optimal temperature, and cold shock androgenesis occurred in almost the same frequencies between 20-minute and 60-minute durations. Histological observation indicated that the same mechanisms eliminated all maternally derived nuclei (egg nucleus and second polar body nucleus) [98].

Corley-Smith *et al.* [99] reported the production of DHs at 1.3–2.1% yield rates by heat shock after irradiation androgenesis. Hou *et al.* [98] also induced DHs at about a 1% yield rate, according to their heat shock condition after cold shock androgenesis. All-male inheritance and homozygosity of resultant DHs were genetically verified by 30 microsatellite loci covering all 25 linkage groups [98]. Finally, a clonal line was established by the second cycle of androgenetic diploid production, using both cold shock (initiation of androgenesis) and heat shock (whole genome duplication), using sperm of an androgenetic DH. Genetic identity among progeny of a clonal line was verified by completely identical AFLP (amplified fragment length polymorphisms) fingerprinting [98].

The success of androgenetic induction without irradiation of eggs was confirmed in at least two species – dojo loach and zebrafish. Recently, this method was further extended to marine fish such as Japanese flounder, *Paralichthys olivaceus* [100]. The next challenge is to induce androgenetic development in species with large-sized eggs, such as salmonids and sturgeons. If androgenesis is induced in salmonids and sturgeons with the cold (temperature) shock method, we can perform the experiments to induce DHs and clones by routine procedure, without any irradiation facilities and equipment.

6.6.5 Nucleo-Cytoplasmic Hybrids by Androgenetic Techniques

Nucleo-cytoplasmic hybrids (“cybrids”), which consist of the nucleus of one species and the cytoplasm of a different species, are normally produced by nuclear transplantation [101]. Interspecific androgenesis can be an alternative method to induce nucleo-cytoplasmic hybrids. In teleosts, interspecific androgenesis, that is, fertilization of irradiated (genetically inert) eggs from one species with sperm from another species, results in an induction of interspecific nucleo-cytoplasmic hybrids, which provide an experimental system to investigate interactions between nucleus and cytoplasm in the course of embryonic development and cellular differentiations.

Fujimoto *et al.* [102] produced androgenetic nucleo-cytoplasmic hybrids comprising goldfish *Carassius auratus* haploid nucleus and dojo loach cytoplasm, and found that these nucleo-cytoplasmic hybrids arrested at the late blastula stage of embryonic development, and never entered into the gastrula stage. However, in these nucleo-cytoplasmic hybrids, *gooseoid* (*gsc*) and *no tail* (*ntl*) genes were expressed normally before the gastrula stage, as in diploid. Thus, *gsc* and *ntl* expressions in the nucleo-cytoplasmic hybrids indicate that the goldfish haploid genome is regulated by loach cytoplasm. The expression of these genes was not maintained, and all the signals disappeared finally.

When chimeras were produced by transplanting blastomeres of the nucleo-cytoplasmic hybrids into loach embryos, blastomeres were mixed with the cells of host loach embryos at the gastrula stage, but transplanted blastomeres finally formed clusters at the somitogenesis stage. In contrast, when blastomeres were transplanted to goldfish embryos, transplanted blastomeres aggregated. This suggested that embryonic cells from the nucleo-cytoplasmic hybrids that arrest before gastrula stage could survive beyond the somitogenesis stage, depending

on the cytoplasmic conditions of the host embryos. Thus, in an *in vivo* study, nucleo-cytoplasmic hybrid cell viability could be improved if these cells were grafted to embryos of the cytoplasmic species, suggesting that signaling with cytoplasmic species cells might reduce cellular incompatibility of nucleo-cytoplasmic hybrids.

As mentioned above, interspecific androgenetic haploid progeny provide excellent material for academic studies on basic developmental biology, but genetic studies including sex determination require the production of acceptable numbers of interspecific androgenetic diploid progeny. However, induction of viable androgenetic nucleo-cytoplasmic diploid hybrids has not been successful in fishes, except for a very few examples.

As an intergeneric androgenesis, Bercsenyi *et al.* [103] reported the production of viable goldfish from common carp irradiated eggs. The most surprising interfamilial androgenesis was a production of transgenic diploid mud loach from irradiated carp eggs; these progeny were used to establish an isogenic line with transgenic genotypes [30, 104]. In all other cases reported, viable androgenetic diploid progeny have not been induced in the interspecific and intergeneric combinations of species, which give rise to viable and fertile hybrid progeny [105, 106]. As a reason why interspecific androgenetic progeny could not survive, nucleo-cytoplasmic incompatibility is concluded.

To overcome such an incompatibility, the use of a hybrid was proposed for successful interspecific androgenesis. When mature eggs were obtained in a hybrid between species B female and species A male, sperm of species A and irradiated eggs of the hybrid were used. In this case, nucleus from species A is surrounded by cytoplasm of the hybrid B female × A male and, thus, half of the cytoplasmic substances are presumably synthesized, according to the nucleus from species A. Based on this idea, interspecific androgenesis was challenged [107]. When unreduced diploid eggs from hybrid crucian

carp, *Carassius auratus gibelio* × common carp were irradiated and then fertilized with carp sperm, followed by heat shock for doubling chromosomes, viable intergeneric androgenetic nucleo-cytoplasmic hybrid progeny were obtained, and their all-male inheritance was genetically verified by the expression of recessive color genes [108].

In salmonids, viable androgenetic nucleo-cytoplasmic hybrids have never been produced, even in species combinations that provide fertile hybrids [106]. Recently, however, a few viable androgenetic nucleo-cytoplasmic hybrids were successfully induced by activating irradiated eggs of mature hybrids between brook char (trout) *Salvelinus fontinalis* and Arctic char *S. alpinus* with sperm of brook trout, followed by hydrostatic pressure shock to double chromosomes to restore diploidy [109]. When eggs of pure char species were irradiated, and then activated with sperm of interspecific fertile hybrids, brook char × Arctic char, followed by pressure shock for chromosome doubling, androgenetic development of hybrid nucleus successfully initiated in eggs of pure parental species, and several nucleo-cytoplasmic hybrids survived [110].

Viable androgenetic nucleo-cytoplasmic hybrids were also obtained in sturgeons by dispermic fertilization, as mentioned in Section 6.6.3. An androgenetic nucleo-cytoplasmic hybrid between stellate sturgeon, *Acipenser stellatus* cytoplasm and beluga sturgeon, *Huso huso* nucleus was obtained and survived beyond the age of six years old [91]. Production of androgenetic nucleo-cytoplasmic hybrids between Siberian sturgeon, *A. baerii* egg cytoplasm and Russian sturgeon, *A. gueldenstaedtii* sperm nucleus was also reported by the same approach [93].

6.7 Clonal Lines Using Isogenic Gametes of DHs

As mentioned in Sections 6.4.1. and 6.5.1., the most difficult chromosome manipulation is a duplication of chromosomes in an early

somatic division (cleavage). Once gynogenetic and/or androgenetic DHs are successfully produced from haploid embryos, clonal lines can be induced by the second round of gynogenesis and/or androgenesis. Because each DH individual is completely homozygous at all loci, genetically identical gametes are produced. According to Komen and Thorgaard [23], clonal lines were established from the second cycle gynogenesis of eggs of gynogenetic DH females in zebrafish, medaka *Oryzias latipes*, common carp, Nile tilapia, amago salmon, rainbow trout, ayu *Plecoglossus altivelis*, Japanese flounder, and red sea bream (nine species). Since the nucleus of the second polar body and the egg are genetically identical in DHs, homozygous progeny can be induced from eggs of a DH by inhibiting the second polar body release just after the beginning of the second cycle gynogenesis.

In contrast, clonal lines established from androgenetic DHs were produced in carp, Nile tilapia, amago salmon, and rainbow trout (four species) [23]. Very recently, clonal lines were produced from androgenetic DHs of zebrafish and Japanese flounder [98, 100]. When androgenetic DH females are obtained, a clonal line can be produced by the second cycle of gynogenesis of eggs of an androgenetic DH female. When androgenetic DHs are all-male, second round androgenesis is necessary to establish clonal lines. After activation of genetically inert eggs of wild-type diploid female(s) with sperm of a DH, followed by chromosome duplication in cleavage stage, all of the androgenetic diploid progeny become members of a clonal line.

The abovementioned clones are all homozygous ($F=1$) and, thus, are equivalent to inbred lines after more than 20 generations of sib-mating ($F=0.99$). In contrast, cross-breeding between different DHs should provide a heterozygous, but isogenic line; all members of a clone are genetically identical to each other, but a large number of genetic loci are heterozygous. Such “heterozygous clones” (hetero-clone) are expected to exhibit better performance than “homozygous clones”

(homo-clone), due to the masking effect of deleterious genes, and have been produced in nine species [23]. When homozygous clones are all-female in the species with male heterogamety (female XX, male XY), some parts of members of a clone are sex-reversed by hormonal or environmental treatments. They are then used for the large-scale production of heterozygous clone(s), which are applicable for real aquaculture of fish with uniform quality in external appearance, meat texture, size, etc. [111].

Clonal fish are genetically identical and, thus, can be used for studies of quantitative traits, because common environmental variance within a clone and maternal effect are assumed to be zero. According to Taniguchi *et al.* [112], variances of quantitative traits were increased in DHs and reduced in clonal ayu strains. They estimated heritability of body size, morphometric, and meristic traits by applying the human twin model [113]. Applications of DHs and clones, selective breeding, and QTL mapping were well discussed by Komen and Thorgaard [23]. These topics will be explained in later chapters, specified for each important species.

6.8 Distant Hybridization and Chromosome Manipulation

When fertilization is conducted between different species, the resultant hybrids exhibit different performance, ranging from inviable in the early development, to fertile, as in pure species [114]. Thus, various results are expected in survival and reproductive capacity of hybrids. Although hybrids between closely related species generally tend to be viable and/or fertile, those between distant species may exhibit low viability and low fertility or absolute sterility, even though they can survive. Infrequently, interspecific hybrids show atypical formation of unreduced gametes, which likely

causes the appearance of polyploid and/or unisexual strains [115]. The use of such unreduced gametes is an alternative approach to realize polyploid breeding in fish, because it is very difficult to induce fertile tetraploid individuals by chromosome manipulation, as already discussed in Section 6.4.

6.8.1 Allotetraploid Hybrid Strain of Crucian Carp \times Common Carp

Scientists in the former Soviet Union and Israel found that hybrids between crucian carp and common carp produced diploid eggs [107, 108]. In this case, no males that produce diploid sperm appeared, and thus they maintained the strain by induced gynogenesis.

Scientists in China also observed similar phenomena in intergeneric hybrids between a red variety of crucian carp and common carp [116, 117]. F_1 and F_2 hybrids were diploid, with $2n=100$ chromosomes, but some males and females of F_2 produced diploid sperm and diploid eggs, respectively. Thus, allotetraploid F_3 hybrids with $4n=200$ were produced by cross-fertilization between diploid eggs and diploid sperm from F_2 hybrids. Then, F_4 hybrids stably generated diploid gametes, and consecutive generations F_4 – F_{18} hybrids, stably maintained the tetraploid situation over 20 years by bisexual reproduction. In the allotetraploid strain, 100 bivalents were observed in meiosis, and the system of premeiotic endomitosis (chromosome doubling without cytokinesis) was presumably involved in unreduced gametogenesis.

Using diploid gametes of this allotetraploid strain, triploid hybrids with three different genomes were produced by cross-breeding between allotetraploid and Japanese Gengorou buna, *Carassius auratus cuvieri* [118]. These hybrids are sterile from 1–6 years old and exhibit outperformance in disease resistance, growth, and meat quality. Using diploid gametes, gynogenetic lines have also been established [119, 120].

6.8.2 Allopolyploid Hybrid Strain of Crucian Carp × Blunt Snout Bream

Inter-subfamilial hybridization normally results in production of inviable progeny, but cross-fertilization between crucian carp red var. females (subfamily Cyprininae) and blunt snout bream males (subfamily Cultrinae) gave a high fertilization rate (>60%) and hatching rate (>50%) and, subsequently, large numbers of living progeny (5,000 to 100,000) [121]. In F_1 progeny, 23% progeny were allotriploid ($3n=124$), comprising two genomes of crucian carp ($2n=100$) and one genome of blunt snout bream ($2n=48$), and the other 77% were allotetraploid ($4n=148$), comprising two genomes of crucian carp and two genomes of blunt snout bream. Allotriploid hybrids were sterile, but allotetraploid hybrids were fertile. Female tetraploids produced 95% unreduced tetraploid eggs with large size (2.0 mm), as well as 5% meiotic diploid eggs with small size (1.7 mm). In contrast, male tetraploids showed low fertility and generated very low concentrated sperm. Cross-breeding between allotetraploid females and wild-type diploid blunt snout bream males produced allopentaploid progeny with $5n=172$.

6.8.3 Natural Nucleo-Cytoplasmic Hybrid Clonal Strain of Crucian Carp

In Japanese silver crucian carp, *Carassius langsdorfii*, gynogenetically reproducing triploids ($3n=156$) and tetraploids ($4n=206$) live together with gonochoristic bisexually reproducing diploids ($2n=100$) [122]. However, Chinese gibel crucian carp, *Carassius gibelio*, triploid ($3n=156-162$) had about 10% males and had a special reproductive mode, which is much more complicated than Japanese silver crucian carp. Chinese triploid crucian carp has:

- 1) bisexual reproduction in response to sperm from the same clone male;
- 2) typical gynogenesis in response to sperm from the males of another species; and

- 3) unusual hybrid-similar development mode in response to sperm from another different clone [123].

When eggs of clone D strain gibel carp were fertilized with sperm of clone A, only 9% of eggs survived. Among survivors, about 80% were clone D, 15% were genetically polymorphic, and the other 5% were clone A. In Clone A-like progeny, it was genetically verified that the nucleus was derived from clone A, while mitochondrial DNA was derived from clone D. Thus, they were supposed to be an androgenetically developed natural nucleo-cytoplasmic hybrid clone between clone A (nucleus) and D (cytoplasm) strains [124]. These nucleo-cytoplasmic hybrids have been utilized in real aquaculture, because they exhibit good aquaculture performances [124].

6.8.4 Applications of Atypical Reproduction of Artificial Hybrid and Hybrid-Origin Species

In certain hybridizations, unexpected polyploid and uniparental development have been reported in the progeny. Stanley [8] reported the appearance of viable polyploid hybrid, androgenetic and gynogenetic progeny in survivors from the cross-breeding between carp females ($2n=100$) and grass carp, *Ctenopharyngodon idella* males ($2n=48$). Marian and Krasznai [125] reported the occurrence of triploid progeny ($3n=72$) in hybrids between grass carp females ($2n=48$) and big head carp, *Hypophthalmichthys nobilis* males ($2n=48$); no diploid hybrids survived.

In salmonids, intergeneric hybridization between rainbow trout females and brook trout males resulted in the production of allotriploid hybrids [126, 127] while, in hybrids between coho salmon *O. kisutch* females and brook trout males, spontaneous gynogenetic diploids appeared [128]. All these examples have indicated that heterospecific fertilization should cause the occurrence of polyploidy and uniparental

development, but the mechanism responsible is poorly understood at present.

Distant hybridization often gives rise to the production of unreduced gametes (mostly eggs) in resultant hybrids. Hybrid brown trout, *Salmo trutta* × Atlantic salmon, *S. salar* were reported to generate gynogenetic diploid and triploid progeny when back-crossed to male Atlantic salmon [129]. This is an example of unreduced egg formation in salmonid hybrids, suggesting the possible use of such diploid gametes for further breeding programs in commercial aquaculture. Aquaculture of allotriploid salmonids has been conducted for vitalization of the local economy as described in Section 6.2.2.

Hybrid origin of unisexually reproducing fishes has been strongly suggested [115]. Clonal diploid lines of dojo loach have been considered to be a descendant of hybrids between genetically distinct groups and, thus, they formed isogenic diploid eggs that developed by spontaneous gynogenesis [115].

Unreduced oogenesis and spermatogenesis likely occurs, even in diploids and polyploids, where homologous chromosomes do not have counterparts for pairing. There are several ways for unreduced gametogenesis. Here, only two typical cases, *apomixis* and *premeiotic endomitosis* in triploids, are shown in Figure 6.2. Both routes also show subsequent gynogenetic development of triploids.

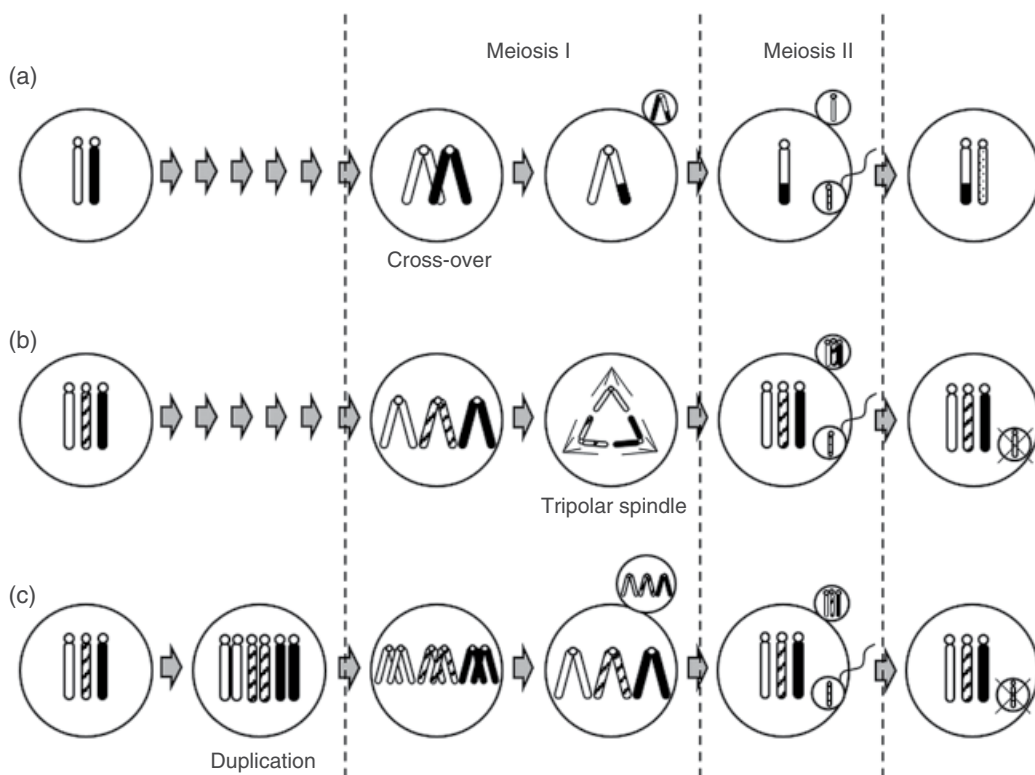


Figure 6.2 Oogenesis and meiosis in normal diploid (a), unreduced egg formation by apomixis (b), and that by premeiotic endomitosis (c) in triploid.

In apomixis, three homologous chromosomes form tripolar spindle, and replicated homologous chromosomes skip MI to enter MII. Consequently, unreduced triploid eggs identical to the mother are produced. In premeiotic endomitosis, each chromosome duplicates to form two sister chromosomes before entering into the meiosis, and these sister chromosomes behave like homologous chromosomes. Crossing over never generates genetic variation, due to the exchange of identical elements between sister chromosomes, duplicated from a single chromosome. Consequently, unreduced triploid eggs with the mother genotypes are produced.

6.9 Sex Determination Inferred from Results of Chromosome Manipulation

Morphologically distinct sex chromosomes have not been clarified in most fish species. Among sex chromosome systems described so far in teleosts, besides major male heterogamety (XX female-XY male) and female heterogamety (ZW female-ZZ male), various systems such as XX female-XO male, $X_1X_2X_1X_2$ female- X_1X_2Y male, XX female- $X_1Y_1Y_2$ male, ZO female-ZZ male, ZW_1W_2 female-ZZ male, and others have been reported, as reviewed by [22, 26]. Sex determination genes were recently reported in medaka (*dmy*) [130], puffer fish *Takifugu rubripes* (*amhr2*) [131], and rainbow trout (*sdY*) [132], but neither sex determination genes, nor sex linked markers, were detected in most other fish species.

Under such situations, gynogenesis and androgenesis will provide information on the

sex determination system of the target species as reviewed [9, 16, 17, 22, 23, 26, 133] (see Table 6.1). In species with the XX-XY system, both meiotic gynogenetic diploids and gynogenetic DHs are predicted to be all-female, because there is no contribution of Y-chromosome on resultant progeny. Androgenetic DHs are expected to bring XX females and YY supermales. If YY supermales are viable and fertile, they produce all-male progeny by crossing with normal females with XX chromosomes.

In species with the ZW-ZZ system, meiotic gynogenetic diploids have ZZ males and WW superfemales, together with ZW females, which appear based on recombination between Z and W chromosomes. If recombination is random, 67% females appear in progeny, while if recombination is 100%, all-female population appeared. Whereas in gynogenetic DHs, ZZ and WW are expected. If WW superfemales are viable and fertile,

Table 6.1 Expected sex ratios (female/male rates) in progeny of meiotic gynogenetic diploids, gynogenetic DHs, androgenetic DHs, triploids, and tetraploids under the assumption of sex determination system of male heterogamety (XX female: XY males) and female heterogamety (ZW females: ZZ males) in fishes.

	Male heterogametic sex determination system, XX female: XY male	Female heterogametic sex determination system, ZW female : ZZ male
Meiotic gynogenetic diploids	XX 100% females	ZZ 50–0% males WW* 50–0% superfemales ZW 0–100% females (ZW appears depending on recombination)
Gynogenetic DHs	XX 100% females	ZZ 50% males WW* 50% superfemales
Androgenetic DHs	XX 50% females YY* 50% supermales	ZZ 100% males
Triploids	XXX 50% females XXY [†] 50% males	ZZZ 50–0% males ZZW [‡] 50–0% females ZZW [‡] 0–100% females (ZZW appears depending on recombination)
Tetraploids	XXXX 50% females XXYY 50% males	ZZZZ 50% males ZZWW 50% females

*Superfemales and supermales are often inviable in certain species.

†If presence of Y chromosome determines male, XXY is male.

‡If presence of W chromosome determines female, ZZW and ZWW are females.

they produce all-female progeny by crossing with normal males with ZZ chromosomes.

By observing sex ratios in resultant progeny from gynogenesis and/or androgenesis, a sex determination system has been estimated, even in species in which the sex chromosome, sex-linked marker, and/or sex determination gene have not been clarified yet. Using such approaches, the XX-XY system has been estimated from all-female results of gynogenetic diploids in grass carp [134, 135], Atlantic halibut *Hippoglossus hippoglossus* [136], spotted halibut *Verasper variegatus* [137], Eurasian Perch *Perca fluviatilis* [138], Atlantic cod *Gadus morhua* [139], and others.

From sex ratios (65–80% females) in meiotic gynogenetic diploids, the ZW-ZZ system has been estimated in several sturgeon species (see Volume II, Chapter 34) and paddle fish *Polyodon spathula* [140]. The ZW-ZZ system is also estimated based on superfemale (WW) : male (ZZ) = 1 : 1 ratio of gynogenetic DHs in turbot *Scophthalmus maximus* [141]. The same system was also estimated in half-smooth tongue sole *Cynoglossus semilaevis* [142]. There are many arguments about the sex determination system in zebrafish, since the first report on gynogenetic DHs by Streisinger *et al.* [74]. Recently, our androgenetic DH zebrafish provided only males [98] as in [99], suggesting the basic involvement of the ZW-ZZ system.

Based on deviations from expected sex ratios in gynogenetic or androgenetic progeny, female-to-male sex reversal due to environmental (temperature) factors has been clarified in Japanese flounder [111] and red sea bream [77]. Overrule of temperature on the genetic sex determination system, as well as influence of minor sex determination genes, were also proven in a series of chromosome manipulation studies in tilapia (see review [22]). Recently, the minor sex determination by recessive genes related to female-to-male sex reversal was verified by Karayucel *et al.* [143]. In DH and clonal common carp, sex reversal mutation *mas-1* was discovered [144, 145].

Sex ratios in triploid, tetraploid, and higher polyploid fishes are considered much more

complicated [22]. In species with male heterogamety, both females (XXX) and males (XXY) appear in triploid progeny, if the presence of Y determines male. In tetraploids, XXXX females and XXYY males are expected, but the sex ratio in the next generation may shift to males, due to the occurrence of XXXX, XXXY, and XXYY.

Sex ratio biased to males (94.5%) was observed in the second generation of tetraploid male (XXYY) in rainbow trout [50]. In subsequent generations, tetraploid males will be XXXY, and they may produce gametes in a 1 female : 1 male sex ratio when bivalent pairing occurs. In bisexually reproducing natural tetraploid dojo loach, a sex ratio of about 1 female : 1 male was reported [115]. In species with female heterogamety, both ZZZ males and ZWW females are expected in triploid progeny, but ZZW females can occur in relation to the recombination frequencies between sex chromosomes [22, 146]. In tetraploid progeny, ZZZZ males and ZZWW females are expected, but no experimental confirmation has been done on these genotypes. At present, the mechanism responsible for stable sex ratio is poorly understood in tetraploid and polyploid animals [115].

6.10 Conclusion and Perspectives

In most finfish, triploidy and meiotic gynogenetic diploidy can be easily induced for experimental purposes, to estimate the sex determination system, as well as aquaculture practices (see Sections 6.2.1, 6.3.2, and Volume II, Chapter 41). Induced triploidy is presumably applicable for biological containment in cases where triploids are fully sterile. Meiotic gynogenetic diploids are also useful for sex control, as well as fixation of preference traits. Low survival rates of tetraploids and gynogenetic or androgenetic DHs are the most serious unsolved problem.

Recently, cellular mechanisms responsible for whole genome duplication have been clarified [64–67], and technical improvement for successful production of tetraploids

and DHs are expected. Both gynogenetic and androgenetic DHs are especially important as a source of isogenic gametes, which are required for cloning by the second cycle of gynogenesis or androgenesis.

Newly developed cold (temperature) shock androgenesis may open a new possibility of androgenesis without egg irradiation. This innovation may realize induced androgenesis, without special facilities and equipment

for gamma or X-ray irradiation, of relatively large eggs in fish species such as salmonids and sturgeons. Distant hybridization can be used as an alternative technique to obtain unreduced diploid gametes, and has been practiced as a method to develop aquaculture strains in China. All abovementioned chromosomally manipulated products will provide information to infer the sex determination system in the target fish species.

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7

Hybridization and Its Application in Aquaculture

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7.1 Introduction

With global population expansion, the demand for high quality protein, especially from aquatic sources, is rising dramatically. Increased aquaculture production is clearly needed to meet this demand. In the third millennium, because capture fishes are at capacity or showing precipitous declines due to over-fishing, habitat destruction, and increasing population, increase in capture fishes is not anticipated under the global conditions [1].

Development of better fish breeds that can contribute to increased fish production, while ensuring protection of biodiversity and the environment, is seen as one of the key solutions to meet future food demands of the growing world population [2, 3]. The advent of induced spawning techniques, such as hypophysation (the use of pituitary gland extract to induce ovulation), synthetic hormones, *in vitro* fertilization technologies, and increased knowledge of reproductive biology, has enabled aquaculturists to induce breeding and domesticate many fish species for aquaculture. As domestication of fish species increases, the possibility of increasing fish production through appropriate genetic

improvement methods also increases. Hybridization is considered as one of the simple, inexpensive, and potential tools of such enhancement programs in fishes; it is a useful method for combining the desirable traits of selected species.

The mating of two different species is a process called hybridization, with the offspring known as hybrids. Hybrids can have some characteristics of both parental species. A hybrid with selected or favored characteristics of each parent is one of the goals of animal husbandry. When a hybrid has characteristics superior to both parents, it is said to have hybrid vigor or positive heterosis, which, of course, is the ultimate breeding goal.

Hybridization occurs widely in fishes under natural conditions [4–6], and is observed in fish more commonly than in other vertebrate animal groups [7, 8]. Several factors have been suggested as contributing to the high incidence of natural hybridization among closely related fish species, including external fertilization, weak behavioral isolating mechanisms, unequal abundance of the two parental species, competition for limited spawning habitat, and decreasing habitat complexity [4, 7]. Hybrid zones are defined

as areas of narrow regions where genetically distinct populations or species meet, mate, and produce hybrids [9].

Intraspecific hybridization (cross-breeding) is a classical approach for the genetic improvement of livestock animals [10–21]. This method has also shown its potential in aquaculture. Increases by 55% and 22% in the growth rate of channel catfish and rainbow trout hybrids, respectively, were achieved using this technique [22, 23]. However, there was no increase in growth rates in Chum Salmon crossbreeds when compared with parental strains [24]. Cross-breeds of different strains of European catfish, *Silurus glanis*, exhibited higher adaptability under warm water conditions and mixed diet feeding regimes [25].

Gjerde and Refstie [26] investigated the heterosis effect between crosses of five Norwegian strains of Atlantic salmon. They did not find a significant heterosis effect for either growth rate or survival rate. Similarly Friars *et al.* [27] found no heterosis effect for growth rate of Atlantic salmon fry.

Interspecific hybrids have, thus, attracted attention because they can improve productivity through hybrid vigor, transfer desirable traits, or produce sterile animals [28–33]. Hybridization may also be used to combine other valuable traits, such as better growth and flesh quality, disease resistance, and increased environmental tolerances. In recent years, hybrids of major carps have been successfully produced in public and private hatcheries, and are available for farming, due to high resistance against unfavorable ecological conditions [34, 35].

Many molecular biologists and fish geneticists have realized that the use of inter-specific hybrids in global fisheries production is not well-reported nor examined properly. On the other hand, there have still been some controversies in global acceptance for using interspecific hybrid organisms that have been genetically modified (GMOs) [36–38]. Intraspecific hybrids are GMOs. They are sometimes created through natural means, but represent a combination of the genes of

two different “species.” This study focuses on the crossing among different genetically distinct species and rearing of hybrids, to understand the potentiality of hybrids in the world’s aquaculture production.

7.2 Inter-specific Hybrids and Their Applications in Aquaculture

Inter-specific hybridization has long been practiced in various species of fishes to increase growth rate, improve flesh quality, produce sterile animals, increase disease resistance and environmental tolerance, and to improve other quality traits to make fish more profitable (Table 7.1) [25].

The majority of the earlier works on hybridization was conducted for salmonid fishes, but these species did not usually produce hybrids of commercial importance [25]. For this reason, hybrids in these fishes do not draw the attention of fish culturists [39]. Due to the increased expansion of fish farming throughout the world, hybrids produced from inter-specific crosses play a substantial role for global aquaculture production. The increased use of artificial breeding and *in vitro* fertilization techniques, and increased knowledge of reproductive biology, encourage aquaculturists to produce hybrids in order to improve the quality traits over their pure parental siblings. Some of the important traits and performances that have been improved through hybridization among different species of fishes are evaluated below.

7.2.1 Improved Growth Performances

Increased growth rate is the most desirable trait for stock improvement in aquaculture. Growth increase may result from dominant variance [40], or from increasing the number of polymorphic loci in an individual. Increased heterozygosity has been implicated in improved growth in a variety of species, as

Table 7.1 Summarization of hybrid fishes in global aquaculture production and stock enhancements [25].

Hybrids	Characteristics, effects, and advantages	References
Cyprinid fishes		
Rohu × catla (<i>Labeo rohita</i> × <i>Catla catla</i>)	Hybrid is hardy and combines first growth of catla with desirable small head shape of rohu.	[34]
Catla × fringe-lipped peninsular carp (<i>C. catla</i> × <i>L. fimbriatus</i>)	Hybrid has desirable head and body shape, improved dressing percentage, and growth performances similar to those exhibited by catla	[34]
Silver carp × bighead carp (<i>Hypophthalmichthys molitrix</i> × <i>Aristichthys nobilis</i>)	Hybrids are fertile and exhibits positive heterosis in growth. Pure lines may have disappeared because of the fertility of hybrids. Food and feeding strategy is intermediate to parental species.	[46]
Grass carp × bighead carp (<i>Ctenopharyngodon idella</i> × <i>A. nobilis</i>)	Hybrids are generally sterile and functional triploids with higher growth rates.	[57]
Common carp × catla (<i>Cyprinus carpio</i> × <i>C. catla</i>) and common carp × mrigal (<i>C. carpio</i> × <i>Cirrhinus mrigala</i>)	Hybrids are usually functional triploids and sterile, having higher growth and survival in monoculture practices and with good seinability	[49]
Tilapia fishes		
Nile tilapia × blue tilapia (<i>Oreochromis niloticus</i> × <i>O. aureus</i>)	Hybrids of some strains yield all-male offspring with superior growth. Some hybrids are fertile with increased cold and salinity tolerance. Reciprocal cross gives 50% males and females.	[61], [65], [66]
Nile tilapia × long-finned tilapia (<i>O. niloticus</i> × <i>O. macrochir</i>)	Hybrid yields predominately male offspring, but strain of Nile tilapia is important for good fry production.	[61]
Nile tilapia × Wami tilapia (<i>O. niloticus</i> × <i>O. hornorum</i>)	Hybrid yields predominately male offspring with some strains producing red-skinned fish with salt tolerance.	[61]
Mozambique tilapia × Nile tilapia (<i>O. mossambicus</i> × <i>O. niloticus</i>)	Recognized as Taiwan red with higher salinity tolerance; progeny of these hybrids display a variety of different skin colors.	[76]
Mozambique tilapia × Wami tilapia (<i>O. mossambicus</i> × <i>O. hornorum</i>)	Hybrid yields predominately male offspring and are fertile. Certain strains produce Florida red tilapia with salinity tolerance and good growth.	[46], [59], [60], [61]
Salmon and Trout		
Atlantic salmon × brown trout (<i>Salmo salar</i> × <i>S. trutta</i>)	Triploid hybrid exhibits the higher growth and survival to a comparable level to Atlantic salmon, but offspring becomes sterile.	[81]
Brown trout × brook trout (<i>Salmo trutta</i> × <i>Salvelinus fontinalis</i>)	Hybrid known as tiger trout is sterile, with low early survival, but grows well in later stages.	[63]
Rainbow trout × char trout (<i>Oncorhynchus mykiss</i> × <i>Salvelinus</i> sp.)	Hybrid shows increased disease resistance to salmonid viruses.	[73]
Lake trout × brook trout (<i>Salvelinus namaycush</i> × <i>S. fontinalis</i>)	Hybrid commonly recognized as splake, and is fertile, fast growing, and tolerant of acid water.	[77]
Chum salmon × Chinook salmon (<i>O. keta</i> × <i>O. tshawytscha</i>)	Triploid hybrids have early seawater tolerances.	[82]
Hybridization among the Pacific salmon (<i>Oncorhynchus</i> spp.)	Majority of the diploid hybrids are not useful for aquaculture, but have potential for disease resistance, sterility, and early seawater tolerance when the diploid hybrids are made triploid. These are also useful for production of all-female using denatured sperm and rediploidized eggs.	[63], [73], [80], [82]

well as other desirable characteristics such as developmental compatibility [41], food conversion efficiency, and oxygen metabolism [42, 43].

A hybrid between white bass (*Morone chrysops*) and the striped bass (*M. saxatilis*), called sunshine bass, exhibits faster growth and has many more good culture characteristics than either of the parents under captive culture systems [44]. Crosses of the black crappie \times white crappie (*Pomoxis nigromaculatus* \times *P. annularis*), stocked in small ponds and impoundments [45]; silver carp \times bighead carp (*Hypophthalmichthys molitrix* \times *Aristichthys nobilis*) [46] in polyculture systems; and catfish hybrids between the African catfish (*Clarias gariepinus*) and the Vundu (*Heterosneustes longifilis* or *H. bisorsalis*) in intensive concrete tanks [47, 48], were reported to grow faster (positive heterosis) than conspecific parents.

Improved growth performances were also obtained from crosses of mrigal (*Cirrhinus mrigala*) and catla (*Catla catla*), and common carp (*Cyprinus carpio*) with rohu (*Labeo rohita*) in pond culture systems in India [49]. Intergeneric hybrids between catla (*Catla catla*) and fimbriatus (*Labeo fimbriatus*) were observed to combine desirable qualities, such as the small head of the fimbriatus and the deep body of the catla, and exhibited heterosis in terms of meat yield with higher flesh content than either of the parents [50].

Hybrids between tambaqui (*Clossoma macropomum*) and pacu (*Piaractus brachypoma*) in Brazil and Venezuela raceways and ponds grew faster than either parent [51]. Crosses of the green sunfish (*Lepomis cyanelus*) with bluegill (*L. macrochirus*) [52, 53], and crosses of the gilthead sea bream (*Sparus auratus*) with red sea bream (*Pagrus major*), also had positive heterosis in growth and other culture characteristics [54]. Several hybrids have been produced in the Mediterranean, with the cross between red sea bream and common dentex (*Dentex dentex*) being especially fast growing in cage culture management [55].

7.2.2 Production of Sterile Animals

Hybridization often results in offspring that are either sterile or have reduced reproductive capacity. Production of sterile animals may be advantageous to diminish unwanted reproduction, or to improve growth rate and avoid energy loss due to prolific breeding. Examination of species karyotype is a good general indication of whether or not hybridization will result in offspring that are sterile [25, 39]. Karyotypes describe the chromosome count from the nucleus in a eukaryotic cell of an organism, and what these chromosomes look like under a light microscope, where attention is usually paid to their length, the position of the centromeres, banding pattern, differences between the sex chromosomes, and any other physical characteristics [56].

Natural hybrids produced from the cross between grass carp (*Ctenopharyngodon idella*) and bighead carp (*Aristichthys nobilis*) are functionally triploids, generally sterile, but with a small proportion being diploid and fertile [57]. Hybrids between Indian major carps are generally fertile because of similar chromosome numbers ($2n=50$). Indian major carps crossed with Common Carp ($4n=102$) results in hybrids that are sterile because they are functionally triploid [34, 49]. However, crosses of some sturgeon species with different chromosome numbers, as well as most tilapia crosses, produce fertile offspring [58–61].

The cross between the black crappie (*Pomoxis nigromaculatus*) and white crappie (*P. annularis*) exhibits positive heterosis, and is often recommended for stocking in small impoundments, because of reduced fertility of the F_2 generation that would prevent overpopulation [45]. The sunshine bass is generally sterile but, apparently, an undetermined percentage of these hybrids are capable of reproduction, as evidenced by hybrid mating and backcrossing [62]. The red sea bream \times gilthead sea bream cross also produces sterile hybrids, and this may be an important quality in marine aquaculture due

to improved growth rate and good overall performance in cage culture [54]. The tiger trout, a hybrid between brown trout (*Salmo trutta*) and brook trout (*Salvelinus fontinalis*) is sterile, with poor early survival, but good growth rate, and therefore is useful for stocking areas where reproduction is very limited [63].

7.2.3 Manipulation of Sex Ratio

Production of monosex populations in fish is often preferable for aquaculture development. This preference may be due to growth differences between sexes (e.g., male tilapia grow faster than females, whereas female salmonids and sparids grow better than males). A specific sex chromosome (XX chromosomes for female and XY for male individuals) may produce a valuable product and monosex populations, and help reduce unwanted reproduction [39].

Hybridization between some species of tilapias, such as the Nile tilapia (*Oreochromis niloticus*) and the blue tilapia (*O. aureus*), results in the production of predominantly male offspring, and reduces unwanted reproduction in grow-out pond culture management [64]. This cross produces predominantly males, because of different sex-determining mechanisms in the two species, and the hybrid males have superior growth over pure parental species. Nile tilapia has the XX, XY system, with the male being heterogametic, whereas blue tilapia has the ZZ, ZW, with the heterogametic genotype being female [61, 65, 66].

Similarly, crosses between Nile tilapia (*O. niloticus*) and Wami tilapia (*O. honorum*), Nile tilapia and long-finned tilapia (*O. macrochir*), and Mozambique tilapia (*O. mossambicus*) and Wami tilapia produce hybrid offspring that are predominantly male, with excellent growth and production [61]. Hybridization between striped bass (*Morone saxatilis*) and yellow bass (*M. mississippiensis*) produces 100% females, with excellent survival and growth in culture systems [67].

7.2.4 Overall Improvement

The principal aim of hybridization is to combine desirable traits from different species to increase the overall production or marketability of a cultured species. The major hybrid catfish cultured in Thailand is a cross between African (*Clarias gariepinus*) and Thai (*C. macrocephalus*) catfish, which combines the fast growth rate of the African catfish with the desirable flesh characters of the Thai catfish [48]. The overall product is improved, and the flesh is still acceptable to Thai consumers, although it does not grow as fast as the pure African catfish.

The rohu × catla hybrid grows almost as fast as pure catla, but has the small head of the rohu and is, therefore, useful in Indian aquaculture [34]. Catla × fringed-lipped peninsula carp (*Labeo fimbriatus*) were reported to have small heads of the fringed-lipped peninsula carp, and deep body and nearly equal growth rate to the catla; the dressing percentage also improved in this hybrid [50]. The sunshine bass hybrid (white bass × striped bass) has a suite of advantageous traits, including good osmoregulation, high thermal tolerance, resistance to stress and disease, high survival in culture and modified water-bodies, and ability to utilize soy beans as a protein source [44, 55]. The overall growth performances of hybrids (*C. catla* × *L. rohita*) fed on wheat bran was consistently higher, followed by rice broken, and blood meal [35].

Among the cultivatable hybrids, red tilapia is more desirable than darker skinned tilapia in Cuba, Venezuela, Thailand, Europe, and the United States. Most red tilapia are descended from the Nile × blue tilapia cross [66], but red tilapia also result from the cross of Wami tilapia (*O. urolepis hornorum*) × Mozambique tilapia [60]. It has been reported that red tilapia from Nile tilapia × Mozambique tilapia, and Nile tilapia × Wami tilapia, are being farmed in central Thailand to Lao PDR for aquaculture purposes (Welcomme, personal communication). The latter cross is also salt-tolerant and used for coastal aquaculture in

parts of Southeast Asia [68]. Stability of the skin coloration is often a problem in successive generations, and studies have been undertaken to understand the genetic mechanisms of color inheritance [69, 70].

Hybrids between different species of North American catfish have been researched for more than 30 years. Among the interspecific catfish hybrids, crosses between channel catfish (*Ictalurus punctatus*) and blue catfish (*I. furcatus*) exhibit good culture characters of the channel catfish, with the ease of harvesting characteristics of the blue catfish, such as better angling and increased seinability [71]. Once breeding problems are worked out, these hybrids may be useful in culture, as they show heterosis for growth rate and are superior to channel catfish in low oxygen tolerance, disease resistance uniformity in body shape, angling vulnerability, seinability, and dress-out percentage [71].

The hybrid produced from the crosses between the muskellunge (*Esox masquinongy*) and the pike (*E. luscious*) is sterile and well-adapted to intensive culture systems. However, the hybrid has similar sport fish characteristics to the pure parental muskellunge, but higher protein requirements than both parental species [72].

7.2.5 Disease Resistance and Environmental Tolerances

Hybridization may be used to improve disease resistance by breeding a higher resistant species with a less resistant one. Dorson *et al.* [73] reported that crosses of coho salmon (*Oncorhynchus kisutch*) with other species, such as rainbow trout, had increased disease resistance to a variety of salmonid viruses, but other culture characteristics were poor. Viability was increased when hybridization was followed with triploidization, and Dorson *et al.* [73] stated that the rainbow trout (*O. mykiss*) × char (*Salvelinus* spp.) triploid hybrids had increased resistance to several pathogenic salmonid viruses and early sea water tolerance.

Hybrids may have increased environmental tolerances when one parental species has a wide range of tolerance (e.g., euryhaline species), a specific tolerance (cold tolerance species), or because of increased heterozygosity sometimes being associated with a broad niche [74, 75]. Mozambique tilapia and Wami tilapia can reproduce in saline waters, but the Nile tilapia has improved culture performance in many aquaculture systems. Hybridization between Mozambique and Nile tilapias yields a red tilapia with salinity tolerance [76]. Hybrids between Mozambique and Wami tilapia, called the Florida Red strains, have high growth rates and can reproduce in salinities of 19 ppt [59]. Crosses between Nile tilapia and blue tilapia also resulted in progeny with good salinity tolerances [61, 65]. Hybrids also may be used to exploit degraded aquatic environments.

Lakes affected by acid rain may not be suitable for native salmonids, but splake, a hybrid between lake trout (*Salvelinus namaycush*) and brook trout (*S. fontinalis*) can tolerate the reduced pH levels of 4.9–5.4 of the acid lakes of Ontario. Lake trout reproduce successfully only in waters with pH values above 5.5 [77]. The splake has also been shown to have higher survival and growth than both brook and lake trout in lakes with pH in the range of 5.5–7.2 [78].

7.2.6 Hybrid Polyploidization

Hybridization combined with chromosome manipulation may increase the viability and developmental stability of hybrid fishes during early life history stages [79]. Polyploid hybrid salmon appear to be better suited for a variety of culture situations than either polyploid or hybrid salmon are on their own. Although many diploid salmonid hybrids are not used for culture, triploidization of the hybrids may confer increased viability, growth, and survival [80].

Triploidization of Atlantic salmon (*Salmo salar*) × brown trout (*S. trutta*) hybrids increased survival and growth rate to a level comparable to Atlantic salmon [81].

General disease resistance was improved by triploidizing the cross between rainbow trout and char; rainbow trout and coho salmon triploid hybrids had increased resistance to infectious disease, but the latter hybrids grew more slowly [73]. Triploid Pacific salmon hybrids between chum salmon (*Oncorhynchus keta*) and Chinook salmon (*O. tshawytscha*) have earlier seawater acclimatization times [82].

7.2.7 Experimental Hybridization

Laboratory hybridization experiments have been utilized extensively to confirm the probable hybrid nature of certain individuals, by demonstrating that two taxa will interbreed when provided with the opportunity to do so, or that gametes from two taxa can be artificially cross-fertilized. Hybrids produced from appropriate cross-fertilization techniques among commercially important fish species have been tested for their growth performance, viability, and fertility. A hybrid recently produced experimentally between sheim (*Acanthopagrus latus*) and sobiaty (*Sparidentex hasta*) in Kuwait appears to have good growth, flesh quality, and is fertile (Khaled Al-Abdul-Elah Kuwait Institute of Scientific Research, personal communication).

Hybrids resulting from crossing several sunfish species have been used for the past three decades to improve farm pond fishing. The most desirable hybrids result from crossing the female green sunfish (*Lepomis cyanellus*) with males from one of three other species. These include the bluegill (*L. macrochirus*), the redear, or shellcracker (*L. microlophus*), and the warmouth, or goggleye (*L. gulosus*). The most commonly used hybrid in the southeast United States is the male bluegill (BG) \times female green sunfish (GS) cross. This BG \times GS hybrid has the most desirable set of characteristics, which means that the hybrids can outperform their parental species in one or more ways.

Rapid and superior growth is one way hybrid sunfish exhibit hybrid vigor. Experimental hybrids between dusky grouper (*E. marginatus*) and the white grouper (*E. aeneus*) were

evaluated, but all the hybrids died within 10 days post-hatching [83]. The camouflage grouper (*Epinephelus polyphkadion*) is more resistant to environmental stress and disease than the marbled grouper (*E. fuscoguttatus*). Experimental hybrids (marbled grouper \times camouflage grouper) exhibited faster growth performances and increased conversion efficiency [84]. A hybrid between the beluga (*Huso huso*) and Russian sturgeons (*Acipenser guldenstati*) was evaluated, and appeared to have a wide salinity tolerance to both fresh and seawater, as well as good growth rate [85]. These hybrids are now being considered for culture in Russia and Iran (Shilat, Iranian Fisheries Company, personal communication).

Two loach (*Misgurnus* spp.) are cultured both for food and for ceremonial purposes by Buddhists in Korea [86] – the mud loach (*M. mizolepis*) and the cyprinid loach (*M. anguillicaudatus*). The mud loach grows to a larger size, has a faster growth rate, and is more resistant to diseases, while the cyprinid loach has a more desirable body color. These two species of loach were hybridized to combine the fast growth and large size of the mud loach with the desirable body color of the cyprinid loach. Fertilization, hatching, survival, and karyology of the hybrids were very similar to the parents [87]. These hybrids are now being cultured commercially, and continued studies are planned to combine other desirable characteristics of the hybrids and their fertility.

Hybrids produced using the eggs of Asian catfish (*Clarias batrachus*) and African catfish (*C. gariepinus*) perform as well as either parental control during the alevin stage, and better in the fry and advanced fry stages, while the reciprocal hybrids are inferior in all performance traits. During the different experiments, this hybrid group showed the highest survival from post-larval stage to market size fish [88, 89]. Growth performance was always better than maternal control and, in some cases, better than or close to paternal control. Preliminary observations of organoleptic testing revealed that the hybrid showed superior taste performance,

compared with parental groups [88]. Further research is needed to examine other desirable traits of the hybrids and their sterility.

Hybridization between giant catfish (*Pangasiodon gigas*) and giant pangus (*Pangasius sanitwongsei*) are now being practiced in Thailand (Pongthana, National Aquaculture Genetics Research Institute, Thailand, personal communication). Both of these catfishes are extraordinarily large, reaching 3 m and 300 kg, with the giant catfish considered as an endangered species whose trade is restricted under the Convention on International Trade in Endangered Species of Wild Flora and Fauna. Hybrids between these two catfish species show good growth performance, and should be used to reduce pressure on the giant catfish, so as not to endanger it through excessive catch of brood fish from the wild, or through genetic introgression of the two parental species [25, 90].

Due to the wide geographical distribution of yellow bass (*Morone mississippiensis*), hybridization tests with striped bass, and comparisons with the sunshine bass have been conducted. The yellow bass hybrid exhibited 65% survival to harvest, compared with 45% for the sunshine bass, but poorer growth rate and condition factor when raised in tanks continuously supplied with pond water [67]. Further research has been undertaken to explore the possibility of combining other desirable traits in the above hybrid progeny.

7.2.8 Unplanned/Accidental Hybridization

Unplanned and accidental hybridization in hatchery stocks may cause a genetic deterioration in aquaculture production and open water fisheries. During the production of Indian major carp seeds, different species often are induced to spawn in a common spawning tank, thus providing the opportunity for unintentional hybridization [91]. Silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*Aristichthys nobilis*) are sometimes hybridized inadvertently, because of their similar appearance, and because of

the shortage of “the correct” species at spawning time, due to differences in maturation times between males and females. This hybridization often results in a fish that does not feed efficiently, as its gill rakers are intermediate in shape between those of the silver carp (which eats phytoplankton) and those of the bighead carp (which consumes zooplankton).

There is much anecdotal evidence of genetic deterioration of carp hatchery stocks in Bangladesh, through inbreeding, negative selection, and hybridization [92]. Stocks of exotic (i.e., non-indigenous) carps are particularly vulnerable to such degradation, given that the opportunities to go back to wild populations for brood stock replenishment are very limited. Furthermore, anecdotal evidence suggests that hybridization between silver carp and bighead carp is common, at least partly due to a shortage of mature bighead carp males toward the end of the breeding season. Reported aquaculture production of the silver carp in Bangladesh in 2001 was 130,000 tons, or 21.7% of freshwater aquaculture production [93], while there was no reported production figure for bighead carp. Bighead carp brood stock are present in many hatcheries so, presumably, aquaculture production of bighead carp is present, but not high enough to be reported separately.

Hybridization between silver carp has also been reported to occur fairly frequently in commercial aquaculture hatcheries in Bangladesh. The consequences of hybridization for brood stock purity have recently been investigated. Allelic variation at three microsatellite DNA loci isolated from silver carp routinely distinguished between silver carp and bighead carp. These markers were used in the analysis of samples collected from hatcheries in different regions of Bangladesh. Of 422 hatchery broodstock that were morphologically identified as silver carp, 8.3% had bighead allele(s) at one or more of the three microsatellite loci, while 23.3% of the 236 fish morphologically identified as bighead carp had silver carp allele(s) at one or

more loci. The results suggested that, while some of these fish might be F_1 hybrids, others had more complex genotypes, suggesting further generations of hybridization, or introgression between the species in hatcheries, with potentially damaging consequences for the integrity of these stocks and their performance in aquaculture [94].

Interspecific hybridization in some carp species has recently been reported in Bangladesh [93]. Either out of scientific interest, or shortage of adequate hatchery populations (i.e., brood stock), introgressed hybrids are being produced intentionally or unintentionally by private hatchery operators, and sold to hatchery and nursery owners. These hybrids are being ultimately stocked, knowingly or unknowingly, either in grow-out ponds, or in open water bodies like floodplains under the government's massive carp seed stocking program. There is widespread concern that mass stocking of such hybrids in the floodplains and other related open water might cause a serious genetic introgression problem, which could adversely affect aquaculture and inland open water fish production. There is every possibility of segregation of genes, with the result that some of the fish carrying the introgressed genes could not be easily distinguished from the pure species [92].

Hybrid introgression in major carp species is very likely to have negative consequences, as a result of loss of distinct feeding strategies of the pure species, which are the basis of successful polyculture systems [95]. If the introgressed hybrids reproduce in natural water bodies, or are used as broodstock in hatcheries, they will not be true breeders; therefore, collection of carp seed from the pure species/strains will be difficult.

Hybridization with wild fish is especially prevalent in tilapia ponds connected to natural water bodies that contain indigenous or feral tilapia populations. Such uncontrolled and unintentional hybridization could undermine the performance of cultured stocks, and make future use of the contaminated stocks as broodstock questionable. For example,

wild three-spotted tilapia (*Oreochromis andersoni*) invaded Nile tilapia ponds in Mozambique, and produced hybrid tilapia that was less marketable than pure Nile tilapia. Inadvertent hybridization at a Chinook salmon hatchery was suggested as the probable explanation for the appearance of Chinook \times Coho salmon hybrids in a California stream [96]. The level of unintentional or accidental hybridization has important considerations of aquatic biodiversity, and will influence risk assessment on the use of hybrid fishes in aquaculture.

7.3 Discussion

A number of hybridization studies in fishes have been reported [25, 55] but certainly not all of the hybrids are contributing to commercial aquaculture production. However, the contributions that hybrid fishes make to global aquaculture production are underestimated. Approximately 80% of Thai catfish production is from hybrids, and there is a growing concern that these hybrids may be impacting native catfish [90]. The tilapia hybrids in Israel are the main tilapia produced, but the 6,691 mt reported were not identified as hybrid [54]. Production of 4,257 mt of hybrid striped bass was reported from the United States, but production of no other fishes was reported, in spite of the fact that red tilapia and other tilapia hybrids are being produced and sold in Florida [25].

Accurate identification of hybrids is important, not only for sustainable aquaculture development, but also to allow for a better understanding of biodiversity and conservation issues. It would be unfortunate to experience widespread loss of pure species in aquaculture, as happened with tilapia, as a result of widespread loss of pure species and subsequent hybridization [97]. It would be a significant cause for concern if hybrid Thai catfish or hybrid Venezuelan characids poses more of a threat to local species than the pure species. The following points need to be addressed to overcome the above situations,

as well as to understand the role of hybrids in global aquaculture production [25, 39].

- 1) Good broodstock management needs to be promoted to avoid inbreeding and interbreeding problems.
- 2) Species and traits relevant to low-input systems need to be prioritized for genetic enhancement, through proper hybridization programs that better address food security issues.
- 3) Genetic stock improvement through inter-generic or inter-specific hybridization of cultured fish species should be initiated under well-designed breeding plans at research institutes and lead central hatcheries, under the guidance of fish breeding specialists/biologists.
- 4) Data on parental origins and stock identity should be recorded for each hybrid. When crosses are made, the female species should be listed first; random crosses in regards to sex of each parent should also be identified.
- 5) As much information as possible should be made available concerning the hybrid. Necessary information includes the stock and sex of each parental species, a comparative evaluation of the reciprocal crosses including a basic description of culture facility of environment, and an assessment of the fertility of the hybrids [25].
- 6) Consideration should be given to establishing a recognizable name for established hybrids and those that appear to have good potential for aquaculture and fisheries [25]. The bester and sunshine bass are examples of two accepted names of interspecific hybrids that signify specific hybrids. A number of researchers working on the hybridization of sparids in the Mediterranean have adopted an informal nomenclature, where the cross between the genera *Dentex* and *Pagrus* was regarded as “dentagrus,” while the reciprocal cross was named as “pantex” [55].
- 7) In order to maintain genetic integrity, proper care needs to be undertaken so that the hybrids should not be

intermingled and do backcross with their parental siblings [33, 98–100].

- 8) Many private hatchery operators hybridize fish without knowledge of breeding biology and genetics that may cause deterioration of hatchery populations. Therefore, governments should immediately ban the unplanned/intentional hybridization practices being carried out by the hatchery operators and fish seed multiplication farms.
- 9) Linkages should be established among the general public, organizations, scientists, industry, and governments, to address hybridization issues and to support the development of practical regulation and sound policy.
- 10) Dissemination of genetically improved aquatic organisms for aquaculture should only be carried out within the framework of adequate regulations and policy.

The management and conservation issues associated with hybridization and introgression in aquatic species are experiencing a renewed interest, based in part on scholarly treatments of the subject [101], and in part because of controversies and difficulties associated with legal mandates such as the Endangered Species Act. In the half century since Hubbs's [4] seminal synthesis on his work with interspecific hybrids, our view of hybridization has drifted away from doctrines that considered it a rare “mistake,” toward a more evolutionary perspective that considers it a more common and, occasionally, constructive process. We hope this information serves as a springboard toward more scientific endeavors to understand hybridization as an evolutionarily important phenomenon, and an important living resource management issue, rather than an idle curiosity in nature.

7.4 Conclusion

It should be concluded that hybridization is not only a preferred method of genetic improvement, but also a potential tool for

stock improvement through transmitting desirable traits to the inferior parents. Appropriate evaluation of hybridization depends solely on the genetic structure, crossing patterns, gamete compatibility, and gene flow patterns of the parental species. Practical knowledge on the genetic constitution of brood fishes, including the maintenance of true parental species and avoidance of inbreeding, inadvertent hybridization, or backcrossing, is very crucial before initiating

hybridization experiments. It cannot be ignored that some non-generic factors, such as weather conditions, culture systems, seasons, and stresses associated with selecting, collecting, handling, breeding, and rearing of broodstock and progeny, may influence hybridization success to a greater extent. Further studies are also required for large-scale production of fish hybrids that can be utilized for species conservation and commercial aquaculture.

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8

Population Consequences of Releasing Sex-Reversed Fish: Applications and Concerns

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8.1 Introduction

8.1.1 The Threats of Distorted Population Sex Ratios

Sexual reproduction creates strong frequency-dependent selection on the production of sons and daughters [1], which explains why we can usually expect about 1 : 1 sex ratios in undisturbed populations, at least at some early life-history stages. However, adult sex ratios often deviate significantly from 1 : 1 [2]. Among the factors that can bias sex ratios in one direction or another are sex-specific life histories and life-history association mortality rates [2], non-random harvest [3, 4], or sex-specific tolerance to anthropogenic stress.

Any deviations from equal sex ratios can be a threat to natural population, because they increase effects of stochasticity. For example, the last individuals of the dusky seaside sparrow (*Ammodramus maritimus nigrescens*) that were meant to be used in a breeding program turned out to be all male [5]. Deviations from equal sex ratios also reduce the genetically effective population size (N_e) relative to the census size (N_c) (Box 8.1), because $N_e = 4N_mN_f/(N_m + N_f)$, with N_m and N_f being the number of mature males and females, respectively [6]. Sex ratio biases, therefore, reduce the genetic diversity and,

hence, the evolutionary potential of a population, and may contribute to an extinction vortex, especially in small or declining populations [7].

The potentially damaging effects of shifted sex ratios may be more obvious in a male-biased population than in a female-biased one if the available number of eggs constrains population growth. One of the most spectacular examples of this is the case of the critically endangered Kakapo (*Strigops habroptilus*), which typically lay only one or two eggs per season. It appears that management measures unintentionally affected parental strategies and, thereby, caused an overproduction of sons [8]. The lack of daughters then further threatened the survival of the Kakapo as a species [8]. Fish, with their usually high reproductive potential (e.g., high number of eggs per female), may seem less susceptible to these kinds of threats. However, fish typically show high embryo, larval, and juvenile mortality, and male-biased shifts in sex ratios have been discussed as a possible cause of further declines of already protected populations [9].

It often seems possible to manage population sex ratios by manipulating ecological or social factors that affect sex-specific growth and survival, or that affect maternal life-history and, hence, family sex ratio [10]. Such measures could aim to support small and

Box 8.1 Terms used:

- N_c : census population size
- N_e : genetically effective population size, i.e. the size of an idealized model population that loses genetic diversity at the same rate as the study population [6].
- Environmental sex reversal: mismatch between genetic and phenotypic sex that is induced by environmental factors (e.g., extreme temperatures or micropollutants) during a sensitive period in life.
- “Trojan” genetic element: genetic factor that can change the demography of a population.

endangered populations, either by preventing distorted sex ratios, or by inducing small female-biased sex ratio distortions to increase N_e of the later generations, even if this means to first reduce N_e in the F1 and possibly the F2. The immediate negative effect of such an induced genetic bottleneck would have to be compensated by additional population growth, as a consequence of the manipulation [11, 12].

Distorted population sex ratios are not only an important topic in the management of threatened populations. Sometimes, intentionally distorting sex ratios may help us to control populations that have been identified as problematic for a given ecosystem; for example, an exotic and invasive species that has successfully established in a system. This is especially the case if manual or chemical eradication of such undesirable populations is not practical [13].

8.1.2 Sex Determination and Sex Differentiation Fish

Fishes show a great diversity of gonadal development and sex differentiation, including: gonochoristic species with individuals developing either testis or ovaries; simultaneous hermaphrodites; sequential hermaphrodites that mature as males or as females first and

may change sex later in life; and all-female species that reproduce gynogenetically [14, 15]. The mechanisms of sex determination are very diverse in fish, too [16], and sex differentiation is typically more labile, compared with birds and mammals [17, 18]. The diversity ranges from purely genetic sex determination, with males or with females as the heterogametic sex, to purely environmental sex determination [14, 15].

In fish and amphibia, this range can be seen as a continuum, with phenotypic sex as a threshold trait dependent on the interaction between genetic and environmental factors that may influence physiological processes during sex differentiation [19]. Importantly, in this context, sex determination then also includes environmental sex reversal (Box 8.1). The environmental factor that induces the sex change can be, for example: extreme water temperatures or temperature variation [20–23]; municipal wastewater effluents that contain endocrine disrupting chemicals [24, 25]; or exogenous hormones such as the synthetic 17 α -ethynylestradiol (EE₂), the natural 17 β -estradiol (E₂), or 11-ketotestosterone (KT) applied during a sensitive period in ontogeny to manage population sex ratios [26]. It even seems that, in many teleost fish, environmental sex reversal occurs regularly over evolutionary time, and has contributed to the maintenance of homomorphic sex chromosomes [27, 28].

8.2 Sex reversal and “Trojan” Genetic Elements















Genetic elements are called “Trojan” (Box 8.1) if they have the potential to change the demography of populations and even, potentially, drive them to extinction [29]. There are various types of genetically engineered organisms whose Trojan elements are used, or could potentially be used, in controlling problem populations – including, for example, sex-specific lethality constructs [30], or a genetically engineered aromatase inhibitor

gene (D) that, when introduced into a population, may lead D-gene carriers to phenotypically develop into males, regardless of their sex chromosome [31]. For many fish, arguably the most promising methods in this context are based on the “Trojan sex chromosome” idea originally suggested and modeled by Gutierrez and Teem [32, 33]. This idea is not based on a recombinant approach, but on sex reversal [34]. It therefore avoids the danger of gene constructs jumping to other species [35] and may, hence, be more likely to be accepted by the public and approved by local authorities [36, 37].

The basic idea of the Trojan sex chromosomes hypothesis is to change the frequencies of sex chromosomes in natural populations,

in order to influence population demography. This may be possible if the target species is gonochoristic, and has sex chromosomes that are not significantly decayed as a result of suppressed recombination between the sex chromosomes (i.e., not like in most mammals) [38]. Interestingly, sex chromosomes of fish and amphibians are, indeed, typically not significantly decayed [16]. Therefore, in fish and amphibians with an X-Y sex determination system (i.e., with normally XX females and XY males), individuals with a YY genotype are mostly viable.

YY genotypes can be produced by mating, for example, a feminized XY individual with a wild-type XY male (Figure 8.1). YY individuals would be males who, when

	X-Y sex determination			W-Z sex determination		
released type:	 YY	 YY	 XX	 WW	 WW	 ZZ
mating in the wild with:	 XX	 XY	 XX	 ZZ	 ZW	 ZZ
F1 males	100%*	100% (50%  YY)	0%	0%	0%	100%*
F1 females	0%	0%	100%*	100%*	100% (50%  WW)	0%
X-chromosomes in F1	50%	25%	100%			
Y-chromosomes in F1	50%	75%	0%			
W-chromosomes in F1				50%	75%	0%
Z-chromosomes in F1				50%	25%	100%

* only regular karyotypes

Figure 8.1 The expected consequences of the release of different types of Trojan chromosome carriers into natural populations with an X-Y or an W-Z sex determination system.

Trojan chromosome carriers are individuals with karyotypes that can result from sex reversal in the parental generation (grey symbols) and/or that have been sex-reversed themselves (black symbols). The expected frequency of males and females in the F1 generation are based on the assumption that all mating types are possible, and have the same effect on the viability of all types of offspring. The figure gives the expected frequencies of wild-type males and females, the frequencies of offspring males and females with unusual karyotypes, and the expected frequencies of X-, Y- W- and Z-chromosomes.

mated with a wild-type XX female, would only produce XY sons. Feminized YY individuals would, when mated with a wild-type XY male, produce 50% XY sons and 50% YY sons who, themselves, could only produce sons. Therefore, when YY males or YY females are released into the wild, the Y-chromosome would act as a Trojan element to reduce the frequency of females in the following populations, assuming that the Trojan chromosome carriers successfully reproduce and produce viable offspring. Analogous Trojan chromosome carriers can be constructed in a W-Z sex determination system, and the Trojan element can be used to create both male- or female-biased sex ratios in the following generation. While male-biased sex ratios would usually aim to control the growth of an undesired population [32], an induced female-biased sex ratio could potentially be used to boost population growth [39].

Figure 8.1 illustrates the expected demographic and genetic effects of the release of various types of Trojan chromosome carriers in a X-Y and a W-Z sex determination system, assuming that all mating types are possible, and that all types of offspring have the same viability. The Trojan chromosome carriers, with their unusual karyotypes, would either be offspring of sex-reversed individuals but not hormone-treated themselves, or they would be individuals that have been sex-reversed before release into the wild.

8.3 Trojan Chromosome Carriers Produced in Brood Stocks

Population management based on Trojan sex chromosomes is ideally based on brood stocks. If the release of Trojan sex chromosome carriers is meant to lead to male-biased population sex ratios in the following generation, the brood stock would ideally consist of YY males and YY females if males are normally the heterogametic sex, and of ZZ males and ZZ females if females are normally the heterogametic sex (Figure 8.1). If the release

of Trojan sex chromosome carriers is meant to lead to female-biased population sex ratios in the following generation, the brood stock would ideally consist of XX males and XX females, or of WW males and WW females, respectively (Figure 8.1).

The establishment of such brood stocks is greatly simplified if genetic sex markers are available. At the time that Gutierrez and Teem [32] suggested their idea, such genetic sex markers were not available for many fishes. Meanwhile, master sex-determining genes have been found in various fish [40, 41], including the *sdY* locus in rainbow trout (*Oncorhynchus mykiss*) [42], which proved to be highly conserved among many salmonids [43]. However, there are examples of within-species variation in sex determination in other taxa [44]. It may, therefore, often be necessary to verify a sex-linked marker for a given population. The latest developments in restriction-site associated DNA sequencing technology (RAD-seq) allow for cost-effective identification of sex-specific markers in fish with no reference genome [45, 46].

Recently, Schill *et al.* [47] successfully produced a brood stock of brook trout (*Salvelinus fontinalis*) that can now be used to produce and release Trojan chromosome carriers to possibly eradicate brook trout populations outside of their native range. The brook trout has a X-Y sex determination system, and is sensitive to steroids during the sensitive stage in sex differentiation (i.e. during early larval stages). Schill *et al.* [47] therefore produced a YY brood stock to produce untreated YY males for release into the wild (the first scenario in Figure 8.1).

The authors followed the three-step approach that was originally suggested by Gutierrez and Teem [32] (see also Figure 8.2):

- **Phase 1:** They fertilized eggs, incubated the embryos at standard hatchery conditions, and exposed half of the resulting swim-up fry to estrogens over a period of 60 days. For exposure, they used food pellets that had been sprayed with natural 17 β -estradiol to create an estradiol concentration of 20 mg/kg diet, following recommendations

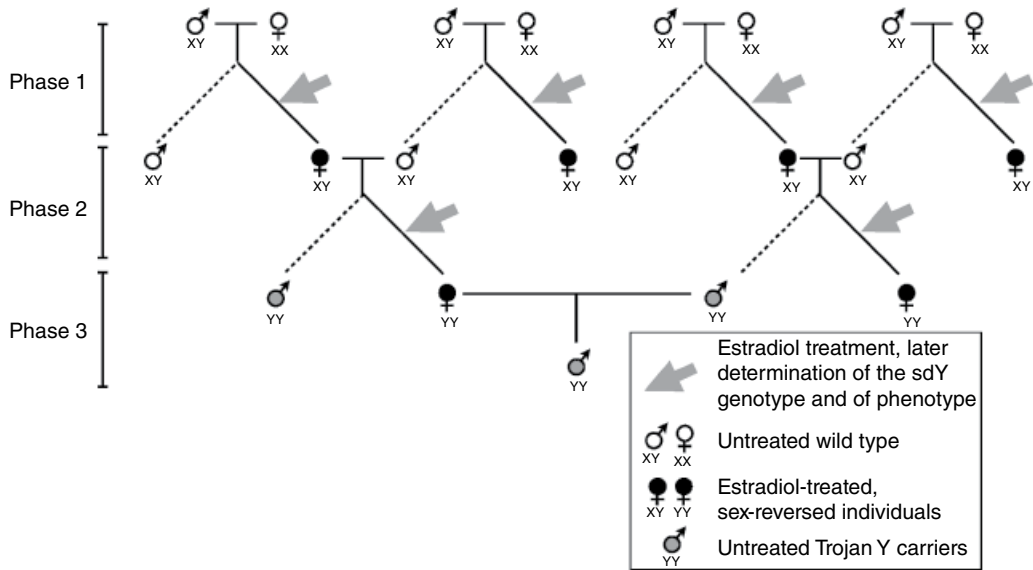


Figure 8.2 Production of outbred Trojan Y carriers that are not hormone treated.

In Phase 1, various sibgroups are produced. Some individuals per sibgroup are estradiol-treated to induce sex reversal. Sex reversal is verified via genetic screening (of the *sdY* genotype) and phenotypic sexing.

In Phase 2, sex-reversed XY females are crossed with XY males from other families. Some individuals per sibgroup are again estradiol-treated to induce sex reversal, and sex reversal is verified via genetic screening and phenotypic sexing.

In Phase 3, sex-reversed YY females are crossed with YY males from other families to produce YY males. The figure only shows combinations of genotypes and phenotypes that are essential for the brood-stock production of Trojan Y carriers.

from Johnstone *et al.* [48], except that they did not defatten the diet pellets prior to treatment. The fish were then raised to about fingerling size, when fin clips could be taken for genetic sex identification (based on the *sdY* genotype [43]). Hormone-treated and untreated XY individuals were then raised to maturity.

- **Phase 2:** Eggs of hormone-treated XY females were fertilized with sperm from untreated XY males, the embryos raised, the clutches split, and half of the swim-up fry again exposed to estradiol-treated food pellets, as in Phase 1. After genetic screening (again based on fin clips taken from fingerlings), YY females from the hormone-treated group and YY males from the untreated group were raised to maturity.
- **Phase 3:** Eggs of YY females were fertilized with sperm from YY males to produce and maintain a YY brood stock.

Analogous procedures are likely to work in many fish species. In aquaculture, monosex cultures are often economically advantageous – for example, because they avoid the problems of early maturation and uncontrolled reproduction [49]. There are, therefore, a number of species for which the large-scale production of monosex progeny has already been established, including the Nile tilapia (*Oreochromis niloticus*) [50] and the rainbow trout [51, 52], and many estrogenic substances have been tested on many different fish species in this context [49]. However, instead of producing a YY brood stock, masculinization of XX individuals is often used to eradicate Y chromosomes and, hence, produce a female monosex culture (the third scenario in Figure 8.1).

In the case of the brook trout, reaching Phase 3 (i.e., the production of YY offspring only), takes at least four years, because the

minimal generation time in this species is two years. Schill *et al.* [47] describe the investment in terms of manpower and financial costs as “modest,” despite the fact that they worked with rather large sample sizes and kept families separate until PIT-tagging the fish to avoid crossing full-sibs later. The total financial costs for the development of their YY brood stock that produced 5,000 YY males at the beginning of Phase 3, and 15,000 YY males two years later, were “... less than \$10,000” [47], including genetic testing (“ca \$5/fish”), feed, and labor. Manpower needs, apart from maintaining the fish stock, included only 2–3 days per spawning period at the end of each phase, and a day per generation for fin clipping and PIT tagging.

The sex-reversal protocol that Schill *et al.* [47] used proved very effective in the first phase, with 99.6% feminization of XY individuals. Feminization of YY individuals in Phase 2 was less successful, with 93.8% of the hormone-treated YY individuals showing intersex characteristics. In order to avoid self-fertilization during stripping, Schill *et al.* [47] had to open the body cavity and to remove ovulated eggs by hand. Hence, the production of YY females that could be released into the wild (Scenario 2 in Figure 8.1) seems not sufficiently established yet for brook trout. However, Schill *et al.* [47] discussed techniques that could be tried to potentially achieve better feminization rates of YY individuals, including immersion in estradiol-treated water around the time of hatching from eggs, which has been found before to lead to very high rates of sex reversal [53, 54]. Moreover, attempts to feminize YY individuals have been successful in several other species [55, 56].

8.4 Consequences of Releasing Sex-Reversed Fish

The demographic and genetic consequences of releasing Trojan chromosome carriers are not yet well understood. Figure 8.1 only lists the expected consequences of various types of

releases, under the assumption that there is no reduction in viability and reproductive capacity in any sex-reversed fish or any of the unusual karyotypes, compared with the wild types. This assumption is currently not well supported and, in fact, there are various indications that sex reversal and, especially, unusual karyotypes (the chromosomally aberrant YY and WW), reduce viability or reproductive potential [57]. As mentioned above, intersex characteristics are frequently observed, possibly as a result of non-complete sex reversal. However, among various taxa, masculinized fish generally show similar sperm characteristics to wild-type males [58], suggesting that sex-reversed female genotypes have reproductive success, comparable to genotypic males. Moreover, Schill *et al.* [47] found no reduction of fecundity of sex-reversed XY females, compared with XX females.

Theoretical analyses of the effects of environmental sex reversal and/or the release of sex-reversed individuals or of offspring of sex-reversed individuals, are either based on strong assumptions about viability and fertility, or they include treatment-induced effects on viability and fertility in their models as further factors [32, 59–62]. Laboratory-based estimates of these key variables are scarce and potentially misleading, if not confirmed by field studies. Therefore, data-based modeling is currently constrained, and may give only rough ideas about whether a certain stocking strategy could drive undesired populations toward extirpation, or support a declining population whose population growth is constrained by their number of females.

8.5 Public and Legal Acceptance of Releasing Sex-Reversed Fish

I currently do not know of any experiments that include the release of carriers of Trojan sex chromosomes into a natural population. However, public and legal acceptance of field trials based on Trojan sex chromosomes may not pose a major challenge. First, introducing

Trojan sex chromosomes into a population is a method that specifically targets the undesired population, with little risk of direct ecological collateral damage [63]. Second, the release of hormone-treated individuals can be avoided, if necessary, from a food safety standpoint – for example, if the targeted species has a X-Y sex determination system and male-biased sex ratios are the aim (Scenario 1 in Figure 8.1). Third, a management measure based on the release of certain types of fish is quickly reversible, and genetic long-term effects are unlikely. Last

but not least, the Trojan sex-chromosome approach to population management may often be the only realistic chance to eradicate a problem population, as long as manual or chemical measures are not practical.

Acknowledgements

I thank H.P. Wang and an anonymous reviewer for comments, and the Swiss National Science Foundation for financial support.

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Part II

Sex Determination and Control in Cichlidae

9

Sex Control in Tilapias

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9.1 Tilapia Species and their Aquaculture

Tilapias belong to the large and diverse cichlid family (order Perciformes) native to Africa and the Near and Middle East (the Levant). Due to their amazing adaptability, more than 70 tilapia species have colonized an impressive range of habitats: equatorial, tropical and subtropical rivers, deep lakes, alkaline, saline or acidic waters, marsh, hot springs or cold volcanic crater lakes, estuaries, and lagoons. These habitats are found within a very large distribution area, consisting of the Nilo-Sudanian, the Ethiopian Rift Valley, the Kivu, the north Tanganyika provinces, and the Northern part of the East African Rift Valley [1–4].

There is a debate on the systematics of tilapiine cichlids [5, 6], with the subdivision of the Tilapiini tribe based on several genera, including *Tilapia*, *Oreochromis*, and *Sarotherodon*. Nevertheless, the use of only these three genera is still predominant in literature [2], and relies upon several characteristics, among which the most critical are the reproductive and behavioral traits. *Tilapia* are substrate spawners, with a biparental nest-guarding care, whereas *Sarotherodon* and *Oreochromis* have, respectively, a paternal/biparental and a strict maternal mouth-brooding behavior.

Various subspecies (i.e., seven in the Nile tilapia) have also been identified by their divergent eco-morphological traits [2, 4] that could present differences, including their sex-determining system [7]. Considering this amazing plasticity, it is not surprising that tilapias became a major aquaculture group. Although some forms of tilapia farming have been related on pharaonic tomb friezes (2000 BC), the global development of its aquaculture began during the 1970s, involving a dozen species: *Oreochromis niloticus* (On), *O. mossambicus* (Om), *O. andersonii*, *O. aureus* (Oa), *Tilapia rendalli*, *O. macrochir* (Omc), *O. shiranus*, *O. spilurus*, *Sarotherodon melanotheron* (Sm), *O. tangericae*, *S. galilaeus*, and *O. hornorum* (Oh).

Tilapia farming success, particularly with the Nile tilapia, stems from being an “ideal aquaculture species,” with well suited traits, such as aptitude for domestication, good quality flesh and palatability, a good growth rate, and an efficient reproduction continuously throughout the year. They have a great plasticity to a wide range of culture conditions (e.g., density, pH, dissolved oxygen, temperature), and have high resistance to disease. In addition, they are mainly “herbivorous” (low-trophic level fish), but also have opportunistic feeding habits (primarily phytoplanktonophagous). Therefore, tilapia can

be produced on relatively low inputs [1, 3, 4] and, finally, important domestic and natural genetic resources exist.

They are a major source of animal protein for developing and emerging countries, but tilapias are now also consumed in various northern countries, such as the United States (300,000 tons) and the EU (10–15,000 tons). Driven by an increasing domestic and international demand, tilapias have become a traded prime white fish commodity worldwide, with one of the most significant production increases (quadrupled over the past decade) of all aquaculture fish species [8, 9].

However, the major drawback of tilapias is their early sexual maturity, occurring at 4–6 months under culture conditions, with a continuous reproduction under favorable conditions (temperature > 22°C; photoperiod ≥ 12 : 12). These characteristics, together with female mouth-brooding behavior, will often favor the males' better growth rates and, therefore, the use of all-male populations is often promoted for tilapia farming [3, 10, 11].

Since the 1970s, and during subsequent decades, some populations belonging to the 12 tilapia farmed species have been widely transferred outside of their natural ranges, and introduced into every continent (to more than 135 countries), but especially to Asia and South America, where most of the tilapias are now produced. The largest producing countries are China, Indonesia, Egypt, Brazil, Thailand, and the Philippines, but other countries, such as Israel, Ghana, Zimbabwe, and Costa Rica [12] also have significant tilapia production.

These introductions have impacted local aquatic biodiversity, due to escapees [13]. They have also affected the genetic diversity of the introduced stocks, due to the limited number of founding breeders, causing genetic bottlenecks, inbreeding (absence/inappropriate brood stock management), and/or selection. Inbreeding has been demonstrated to reveal deleterious or rare alleles, and lead to a genetic drift, with possible consequences on aquaculture traits, including survival rates and sex ratios [14].

The use of inter-specific hybrids in aquaculture and fisheries of some teleost groups can favor hybridizations or introgressions in wild or domestic populations [15]. Because tilapia hybrids are fertile, it is not surprising to find evidence of hybridizations/introgressions in wild or domestic populations, especially when introductions were done inside their native continent [13, 16, 17]. These may result in a decrease of the biodiversity within Africa [18].

Among the 12 species and related hybrids ($On \times Oa$; $On \times Om$ and some red strains, such as the red Florida) used for aquaculture, the Nile tilapia alone accounted for 70–73% (3.4 and 3.7 million tons) of the global tilapia production, reported to be 4.6 and 5.3 million tons in 2013 and 2014, respectively [12]. In fact, if one excludes some carp species that are mainly produced and consumed in China and, to a lesser extent, in some other Asian countries, the Nile tilapia is already the most important species for fish farming. It is sometimes named “the aquatic chicken,” and is assumed to eventually overtake carp as the most important farmed fish within the next decade [19].

Tilapia farms range from small-scale farms to commercial investments, with all types of extensive to intensive production systems (earthen ponds, cages, raceways, tanks, recirculating systems, aquaponics, etc.). In the last 30 years, the growing demand for Nile tilapia has often favored the development of several genetically improved strains. Some of them rely on a single strain (i.e., Chitralada-Thailand), some others upon the hybridization between different wild or/and domestic populations of Nile tilapia (e.g., GIFT-Genetically Improved Farmed Tilapia, GenoMar Supreme Tilapia, Abbassa-Egypt, Akosombo-Ghana) or, finally, between different tilapia species.

Three main species have been hybridized with *On* for two non-exclusive farming objectives – first as a way to combine key traits such as growth rate, cold or salinity tolerance, and color brought by each species for selective breeding programs and, second, for sex control purposes. The blue tilapia, *Oa*, has a good growth rate and presents a better cold and saline tolerance than *On*; this is why

$On \times Oa$ hybrids are extensively produced in China (420,000 tons in 2014; [12]) or have long been used in Israel. Similarly, the Mozambique tilapia, *Om*, can live in marine/saline and cold waters.

Moreover, reddish-orange mutants that naturally appear in domestic populations can be used to produce red tilapias. Because they usually have a higher market value than wild-type tilapia, these red lines have become very popular in various countries, such as China, Malaysia, and Thailand. Therefore, both classic (i.e., *Molobicus*: $Om \times On$) or red hybrids have been developed (Red Florida: $Om \times Oh \times On \times Oa$). Finally, all-male populations favoring the best male growth rate can be produced through the crossbreeding of *Oa* or *Oh* with *On* (see Section 9.6.2.2). Sex-determining systems may differ, depending upon the tilapia species and, probably, even between populations (see Section 9.3), because species or strains have been sometimes introduced within their native African continent. Therefore, the first step to control sex in tilapia is often (or should be) the characterization of its sex-determining (SDS) in the target species/populations, which then permits choosing the best approach for sex control.

The present chapter will, therefore, review the culture conditions that favor sex control, the major basis of sex determination/differentiation of the main tilapia species and hybrids that allow male monosex production. The current methods will be reviewed and compared from different point of views: easiness to apply; production cost and cost-in-use; management strategies; environmental impact; and brand image. Finally, other possible sustainable approaches, based on up-to-date data or technologies, will be proposed.

9.2 Is Sex Control Always Necessary for Tilapia Farming?

The amazing worldwide expansion of tilapia culture has been based on eliminating unwanted reproduction, especially in

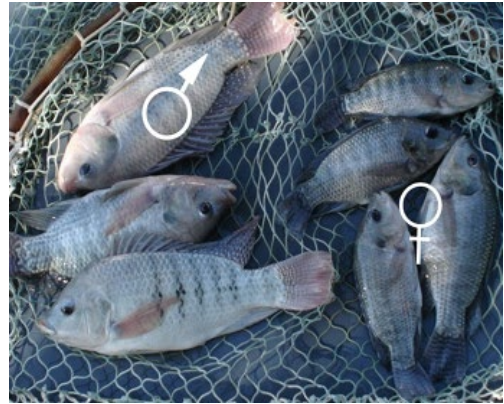


Figure 9.1 Nile tilapia males and females from the same batch showing the males' faster growth rate.

pond-based systems, through efficient sex control [11, 20]. However, as far as growth rate is concerned, male-monosex tilapia culture is not systematically more profitable than mixed-sex culture [21–24]. The growth dimorphism in favor of males, reported in both natural and different culture conditions of Nile tilapia (Figure 9.1), is due to several factors, starting with an important energy investment of females in multiple asynchronous spawning cycles, associated with successive fasting periods (10 days per cycle for mouth-brooding mothers) [25]. In addition, there are the inhibitory effects of endogenous estradiol on female growth rate [26].

Furthermore, males' better efficiency of food conversion (lower food conversion ratio) with higher metabolic capacity, as well as the effects of social interactions on food conversion efficiency [27, 28], strongly contribute to better growth rates in males [3, 29]. The relative magnitude of this dimorphism also depends upon some strain-related traits, such as the age of sexual maturity and, consequently, upon the age of the fish at harvest. Therefore, the advantages of sex control will depend upon the culture conditions (including the species/strains) that favor (or not) the appearance and magnitude of the sex-linked growth dimorphism [30].

In domestic strains of Nile tilapia considered to have a late sexual maturity (22–27 weeks), such as the Chitralada strain [23], mixed populations can perform equally well when compared to monosex populations, and might even be more profitable (when considering additional costs associated with the sex reversal treatment), at least in the presence of piscivorous fish species. After five months, net fish yields are similar but, whereas mixed systems produce large proportions of small and medium size individuals, monosex systems give large-sized fish [23].

When other strains are used under mixed culture conditions, different approaches have been proposed in order to control reproduction: cage culture; high densities; selective harvest; and polyculture with predators of tilapia eggs/fry (reviewed previously by Guerrero, Mair and Little [31, 32]). Among these, the use of predators is probably the most efficient. This approach is still used in several rural aquaculture systems, especially in Africa. Various predators, such as catfish species including *Clarias gariepinus*, *Heterobranchus isopterus*, and *Parachanna obscura*; latids such as *Lates niloticus*; cichlids such as *Hemichromis fasciatus* and *Cichlasoma urophthalmus*; and cyprinids such as *Tor putitora* (the Himalayan mahseer) have been efficiently used with mixed sex populations of tilapia [9, 31, 33, 34].

To oversimplify, the best farming system for tilapia will first depend upon the targeted commercial sizes, but also upon the availability and costs of the inputs [33]. Small-size tilapias are usually produced for household consumption and rural markets, whereas larger fish are needed for urban or international markets. Systems based upon stimulating pond productivity, stocking young fingerlings, and using predators to control fry recruitment, will be more efficient to produce large amounts of small- and medium-size individuals than male monosex populations [23]. Indeed, there is still a huge demand for fish of small and medium size (<200 g) in rural Africa and Asia [35].

However, this approach prevents recruitment, but not the reproduction and its negative consequences on female growth. Therefore, at least when homogeneous large-size tilapias are targeted using food with high protein content, all-male (or male monosex) populations will allow the farmers to benefit from the higher growth rate of the males, and also to avoid the negative effects of excessive recruitment (overcrowding results in food competition and subsequent stunting), as reported by Baroiller and D'Cotta, Beardmore *et al.*, and Baroiller and Jalabert [10, 11, 36].

Under pond-based farming systems, the reproductive efficiency (early maturation, continuous spawning with short sexual cycles, and mouth-brooding behavior) and the poor market value of small individuals (usually the females) can impact the profitability of Nile tilapia aquaculture when mixed sex populations are used [23, 37]. Avoiding these drawbacks associated with female reproduction and consequent recruitment, male monosex populations allow achievement of a better global growth rate, a more homogeneous size/weight at harvest [38], and limit escapees and associated negative impacts on wild populations [11]. Several approaches have been developed: manual sexing/sorting, associated (or not) with the use of predators; hybridization; genetic control; or hormonal sex reversal [10, 11, 39].

9.2.1 Survey on Sex Control Methods in Tilapia Aquaculture and Interest in a Sexing Kit

We performed a survey in order to obtain information on the sex controlling methods currently used in tilapia aquaculture. This was followed by interviews to evaluate the marketing possibilities for a precocious sexing procedure (Tome *et al.*, unpublished data). The survey was very informative, although we only received 44 responses, which might be due to internet-forum distributions, language problems (although the survey was sent in four languages), lack of interest, or perhaps unwillingness to question

the use of hormones to obtain males. Among the responses, 43.2% were marketable tilapia producers, 22.7% fry or juvenile producers, 25% researchers, 4.6% YY producers, and 2.3% were either technical consultants or investors.

Amongst the producers (three of them produced 30–100 million fry/year), 83% control the sex during production, while 17% do not (farming mixed-sex batches corresponding probably to small-medium farmers). Among the farmers controlling the sex of their tilapia populations, the majority (91.6%) use hormones, and their major criteria for this choice was its price, followed by its effi-

ciency (Figure 9.2). Only 21% control their sex ratio monthly, whereas 32% never do. Twenty-one percent of monosex fry producers never control their sex ratios, 25% do so systematically.

The use of YY males was not extensive (2.8%) in our survey, and comparable to the use of temperature or high densities (used in a Malawi and European Farms). The criticisms for not using YY male breeders were low fry quality and growth, and difficulty in obtaining and conserving high male proportions (>95–99% males). These reasons are why YY usage has not been a success in Thailand. We consider that these results are

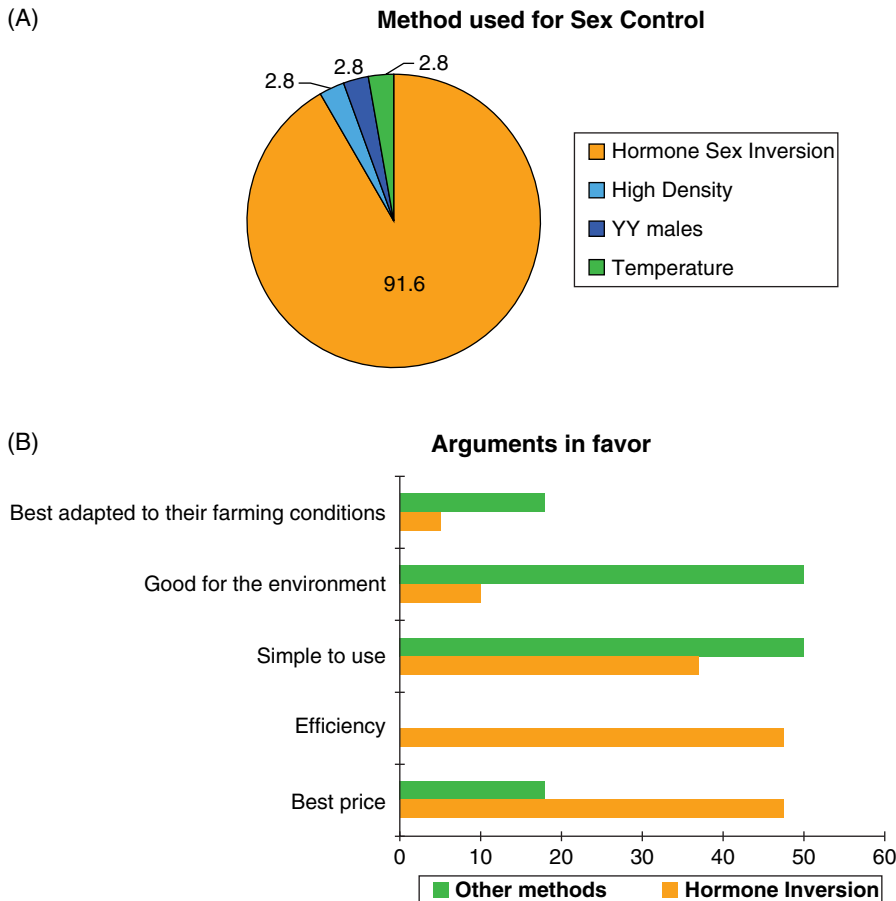


Figure 9.2 Survey of different tilapia actors on sex control.

A. Methods used by the 83% of producers who control tilapia sex, showing that 91.6% use hormonal sex reversal.

B. The two major arguments in favor of hormonal use are efficiency and price.

linked to the strain, where minor parental/environmental factors complicate the use of a genetic approach. Additional reproaches concerning YY males were their cost (41% of respondents) for small-to-medium producers who cannot generate YYs from their own strain, having to buy non-local strain fish that are not adapted to their country/production conditions (45%), and the complicated management required (35%). Nevertheless, 80% of the producers were interested in generating YY males from their own strain. A South African farmer was also interested in producing YY males for *O. mossambicus*, the only tilapia species allowed in that country.

An important argument in favor was that the use of YY males allowed producers to follow European rules that prohibit hormone use for commercial animals. In addition, participants answered favorably for the use of a precocious sexing kit, if the price were reduced and if it was simple. A precocious sexing procedure would permit comparisons, rapidly, easily and efficiently, of different local strains, and/or eliminate the minor factors through selection.

9.3 Genetic Sex Determination in the Four Most Important Tilapia Species

A considerable amount of research on sex determination has been focused on the Nile tilapia for decades, in view of the economic importance of producing males in this species. The most sustainable method for achieving this is genetically. However, sex determination in Nile tilapia has been “a long winding” process, and has proved to be much more complex than initially thought. Sex is multifactorial in this species, with a genetic sex determination, parental influences which we call minor genetic factors, and is also influenced by temperature [40] (Box 9.1). This implies that the genotype might be different from the phenotype, due to sex reversals that

Box 9.1 Sex in Nile Tilapia is under multifactorial control

Genetic sex determination: Females are XX and males XY. Two different sex-determining loci exist, varying depending on the strain: 1) a still unknown gene on LG1 found in a Manzala and Ghana strains; and 2) A Y-linked *amh* gene on LG23 that appears critical for maleness in a Japanese strain.

Temperature-influence on sex is inherited genetically: Female-to-male sex-reversed individuals can be induced with high temperatures in XX genotypes. Some individuals have no or low thermosensitivity. Selection of high thermosensitive individuals has been performed successfully in only three generations, giving >92% males after 10 days' treatment at 36 °C.

Minor genetic factors, each parent contributing, can affect the sex ratios with some breeders: This is the case of certain YY males that give high % of females. Likewise, both parents contribute to the thermosensitivity.

result in XX males or XY females that considerably complicate the search for the sex-determining locus [7].

The development of environmentally- and consumer-friendly approaches based on a non-hormonal sex control to produce all-males is still a major challenge for tilapia aquaculture, particularly in the context of sustainable aquaculture and conservation of water resources. To use these genetic and environmental approaches commercially requires knowledge of the sex-determining system, and the finding of the sex-determining locus or the factors under environmental influence.

The first necessary step was to characterize the sex-determining system of the different tilapia species. Sex chromosomes in tilapia species, as in most fish species, are homomorphic, meaning that they are not sufficiently distinct and, consequently, males or females cannot be identified directly by a simple karyotype analysis [41,

42]. The sex-determining system has been first identified in tilapia by indirect approaches, using progeny testing after sex reversal with hormonal treatment, as well as the use of gynogenesis and interspecies hybridizations.

Two types of sex-determining systems have been identified, co-existing in the same genus. The Nile tilapia, *Oreochromis niloticus* (*On*), and the Mozambique tilapia, *O. mossambicus* (*Om*), have a male heterogametic XX/XY system [43–45] whereas their sister species, the blue tilapia, *O. aureus* (*Oa*), has a female heterogametic ZZ/ZW system similar to birds [40, 45, 46]. Likewise, females are heterogametic (ZZ/ZW) in *O. hornorum* (*Oh*), *O. karongae*, and *O. tanganicae*, as well as in *Tilapia mariae* [45, 47–49]. Homogametic broodstocks giving monosex male progenies have been produced with YY males in *On* [36, 50], and ZZ neofemales in the blue tilapia, *Oa* [49, 51]. However, significant proportions of females may appear in these theoretically monosex male progenies [10, 45, 52]. Genetic analyses based on diallel patterns of individuals and repeated matings of YY males or XX males from *On*, ZZ females from *Oa*, or red tilapia hybrids, have all concluded on the existence of additional minor genetic factors (parental factors) that can modulate sex ratios [10, 40].

9.3.1 Genetic Sex Determination in Nile Tilapia, *O. niloticus*

Attempts have been made to identify the X and Y chromosomes in Nile tilapia *On* by analyzing the synaptonemal complexes of pachytene chromosome preparations [53, 54]. In XY males, no terminal pairing was observed for the largest chromosome bivalent [53, 55], whereas pairing was visualized in the terminal regions for homogametic XX and YY male tilapias [53]. Chromosome measurements and accumulation of heterochromatin led to the suggestion that this terminal region corresponded to the sex-determining region of the Y chromosome [56]. An accumulation of repeated elements of type SATA,

SINE and LINE, and of Rex type retrotransposons, have also been evidenced in this large pair [42, 45, 57]. Hence, this large pair has all the features of a sex chromosome that has started to be differentiated due to its large dimensions, recombination suppression, and high amount of repetitive sequences [45, 57].

Microdissection of the large bivalent has been performed to obtain chromosome probes developed by DOP-PCR [54], which hybridized differently between XX, XY and YY genotypes. We also microdissected the large chromosome and amplified it using GenomiPhi and GenomPlex, which were much more effective in obtaining larger non-repetitive DNA fragments of > 5 kb, and using them as a means to identify new genes on this chromosome or for species comparisons (D'Cotta, Ozouf *et al.*, unpublished data).

9.3.1.1 Genetic Mapping to Search for the Sex-Determining Locus

In order to find quantitative trait loci (QTL) for important aquaculture traits, including sex determination, genetic maps have been constructed progressively for *On*, with DNA polymorphic markers consisting of amplified fragment length polymorphism (AFLP) and, subsequently, with microsatellite markers and single nucleotide polymorphisms (SNP) [58, 59]. The sex-determining locus of XY was located on LG1 within the 10cM region of three markers: GM201, UNH995 and UNH104 [60]. The phenotypic sex could be predicted with 95% accuracy in two families. However, in a third family, sex could not be associated to LG1, suggesting that additional genetic and/or environmental factors were controlling sex in this family. Linkage to LG3 was shown for three Y-linked (OniY425, OniY382, OniY227) and one X-linked (OniX420) AFLP markers identified in homozygous XX and in YY individuals obtained from gynogenesis of XY females, which did not systematically identify males and females in all families [61].

The strong genetic segregation for a male Y locus located on LG1 was confirmed in five families of *On* belonging to the Manzala

strain (from Lake Manzala in Egypt), kept at the University of Stirling and ARO-Israel, and from Lake Volta (Ghana), kept by the University of Maryland [45]. No recombination suppression was observed for XY in LG1, and no sex-specific differences in rates were found around the 11 cM region of the sex determinant [45].

Another strategy to search for the tilapia sex-determining loci was mapping genes implicated in the sex-determining or sex-differentiating cascade of other vertebrates. This was a promising approach, considering that duplications of downstream genes have since been shown to have taken on the role of the Y master-determining gene in many fish species, such as in the medaka, *O. latipes*, with *dmy/dmrt1by* [62], and *amhy* in the pejerrey, *O. hatcheri* [63]. Out of the 11 genes mapped, the ovarian aromatase *cyp19a1a* gene was mapped to LG1, while two genes were mapped to LG23, the anti-Müllerian *amh* gene located 5 cM from marker UNH879, and within a QTL region for sex determination, and the *dmrta2* Doublesex mab3-related transcription factor 5 gene, positioned at 2 cM from UNH216 within a QTL region for sex-specific mortality [59].

Both the aromatase *cyp19a1a* and Wilms tumor *wt1b* genes were mapped to LG1 in *On*, and considered to be promising candidates for the sex-determining gene, since both are involved in the sex-determining/differentiating cascade of vertebrates [64]. *Cyp19a1a* encodes the enzyme that converts androgens into estrogens and, therefore, plays a critical role in regulating gonad sex differentiation—inducing ovarian differentiation if estrogens levels are high or, when low, testis development [65–67]. *Wt1* upregulates *Sry* transcription in mammals and of *dmy/dmrt1by* in medaka [68]. Males were heterozygotes (g.2124AG) for an SNP in the *cyp19a1a* promoter, whereas females were homozygotes (g.2124AA) [64]. Likewise, a SNP located in *wt1b* showed that males were heterozygotes (g.686CG), whereas females were (g.686CC) homozygotes [64]. Linkage

analyses located *cyp19a1a* far from the sex locus at 27.1 cM, while *wt1b* was only 2.5 cM [64]. *Wt1b* was nevertheless excluded at the time as a possible sex determinant, due to the two recombinant male individuals. *Wt1b* has since been re-established as a putative male determinant for Nile tilapia, using more powerful genomic tools [69].

9.3.1.2 Physical Location of the Sex-Linked LG1 and LG3

Several genomic resources were generated for *On*, before the whole genome sequencing, that have been important tools to fine-map the sex determinant and sex-differentiating genes [64]. The genetic map of Nile tilapia was linked to the physical map by the construction of Bacterial Artificial Chromosome (BACs) libraries, containing inserts of large tilapia genomic fragments (average size 145–194 kb) [70]. A physical map was generated with 35,245 fingerprinted BAC clones, resulting in ≈ 1.752 Gb (a $1.65\times$ coverage of the genome) [70], as well as a comparative physical map with the BAC end sequences [71], using numerous bioinformatics tools (GBrowse interface) accessible at <http://www.BouillaBase.org>, built by Thomas Kocher's group at the University of Maryland.

Screening of these BACs for microsatellite markers located on LG1 and LG3 permitted the physical location of the corresponding sex chromosomes, by fluorescence *in situ* hybridization (FISH) onto metaphase chromosome preparations. LG3 has been anchored to the large chromosome pair in both tilapias *On* and *Oa* by FISH, while LG1 has been anchored to a smaller chromosome pair in *On* [45]. Similarly, BAC clones for three Y-linked and one X-linked AFLP markers were mapped onto the large chromosome arm of the large bivalent (LG3) [61]. A radiation hybrid (RH) map was also constructed for *On*, allowing the mapping of 1,296 non-polymorphic markers (genes, BACs, microsatellites and SNPs) to 81 RH groups, covering 88% of the entire genome (937,310 kb) [72]. The RH map increased the density of markers

on the sex-linked groups, and allowed synteny detection.

9.3.1.3 Refinement of the Sex-Determining Region by Whole-Genome Sequencing

The whole-genome sequencing of *On*, together with that of four other cichlids from the large Eastern African lakes, was possible due to an International Cichlid Consortium, driven by Thomas Kocher, with Illumina (a Next Generation Sequencing NGS procedure) performed by the Broad Institute of MIT and Harvard [73]. The assembly of the whole genome sequences of the five cichlids was helped by the *On* RH map, and resulted in thousands of DNA markers. In order to simplify the computation assembly of the sequences, only the genome of a female homozygous XX clone [74] from the Manzala strain of Stirling University was sequenced and, thus, the Y chromosome is lacking. Currently, a new assembly of 44× coverage is being generated with PacBio reads, which provide long reads using the previous XX clonal line (Kocher, Penman *et al.*, unpublished data).

Families from crossings of the female homozygous XX clonal line with XY males were subsequently used for Restriction Associated DNA (RAD) sequencing, to identify additional SNPs and map the sex-determining region(s) [75]. They generated a linkage map consisting of 3,802 SNPs, performed a QTL analysis based on these SNPs, and found those showing the highest association to the phenotypic sex in two families, with females being homozygous and males heterozygous for two significant SNPs (Oni23063 and Oni28137). The major sex-determining region was found on LG1 in a 2 cM interval, which comprised ≈ 1.2 Mb of the genome (at 28–30 cM), showing 96% association to the phenotypic sex. The highest associations were shown at 14.95 Mb (LOD score 18.5).

The two sex-linked SNPs are located 400 kb apart in the *On* genome, containing within this region 10 genes that are putative sex-determining candidates [75]. Exceptions

were found when genotyping some progeny and broodstock males that were homozygous for the sex-linked SNPs. Progeny testing of some of these males gave biased female sex ratios, suggesting that they were XX males reversed by other genetic factors or/and environmental factors [75].

Using a family-based method with two crosses, and then pooling separately males and females, large amounts of functional SNPs were found within an 8.8 Mb region on LG1, which had sharply defined edges, indicating that it corresponded to an inverted region [69]. The researchers identified a cluster of SNPs with alleles that were significantly different between males and females within this region. These findings suggest that recombination, if it exists, is reduced between X and Y alleles. The inverted region comprises 257 RefSeq annotated genes and, taking into account the gene expression data of the gonad, gene models show female-biased enrichment (69.2%), while only 29.3% showed a male-bias. This study suggests that LG1 was a sex chromosome at a relatively early evolutionary stage, where the degradation had begun with low expression of Y-linked genes [69].

Based on functional SNPs, differential gene expression and involvement in sex-determining/differentiating pathways, there are eight candidate sex-determining genes in the inverted sex region: Transcription factor *SOX-6*; Ras-related protein *R-Ras2*; Suppression of tumorigenicity 5 protein; Ras association domain-containing protein 10; (ATPase Family Gene 3)-like protein 1; *Wt1b*; estrogen-related receptor gamma *ERRγ*; and Growth regulation by estrogen in breast cancer 1 (*GREB1*) [69].

9.3.1.4 Finding of a Y-Linked *amh* Gene Critical for Male Sex Determination in a Japanese Strain

The whole-genome sequencing allowed the refinement of the QTL for sex determination located on LG23, which was physically mapped onto scaffold 101. This sex region contains 51 genes, with the *amh* gene located

in the center [76]. *Amh* could well be the male determinant in tilapia, since it is involved in testis differentiation of vertebrates, repressing the development of the female Müllerian ducts. In teleosts that lack these ducts, *amh* is expressed early during testis differentiation [67, 77]. We have also detected an early *amh* male-specific expression in the Nile tilapia brain [78].

Amh and other members of the superfamily of *TGF-β* genes have taken on the role of sex determinants in several fish lineages [63, 79]. A Y-linked duplicated *amh* gene named *amhy* was identified in *On* in a Manzala Israeli strain by coupling sequencing and transcriptome microarray analyses [80]. This *amhy* form has a 233 bp deletion in exon VII that gives a truncated protein [80]. Another group working on a Japanese strain (which also originated from Egypt), detected sex-specific insertions and deletions in scaffold 101 near the *amh* gene, when comparing XX, XY, and YY genotypes [81]. They then screened an XY genomic library (fosmid clones), identifying X-specific and Y-specific clones that were thoroughly sequenced [82]. The analyses showed the presence of three *amh* genes, two *amh* genes located in tandem on the Y chromosome termed *amhy* and *amhΔY*, this last corresponding to the previously truncated gene [80] and an X-linked *amh*.

The *amhy* gene, when compared to its X-linked homologous, has lost 5608 bp in its promoter, and has a SNP (C/T) in exon II causing a change in amino acid Ser for Leu92. *AmhΔY* has numerous insertions and deletions, compared with the X-linked *amh*, but it is a 5 bp insertion in exon VI that causes a frameshift mutation, resulting in a premature stop codon, giving a truncated protein that lacks the *TGF-β* domain [82]. This domain is important for the binding of *amh* to its receptor *amhr2*, which might imply that *AmhΔY* is a degenerated gene [82].

Expressions of *amhy* and *amhΔY* were only detected in XY-differentiating gonads, starting at 9 dpf and then peaking at 34 dpf, with the *amh* antibody detecting both proteins in XY and YY testis extracts. The *amhy*

knockout by CRISPR/cas9 gave male to female sex reversals in F0-XY fish, with simultaneous elevated aromatase *cyp19a1a* expression, which yields high E_2 blood levels [82]. The F1 mutant of the *amhy* allele showed sex-reversal, while F1 mutants with the *amhΔY* allele did not. It is interesting to note that knockout of the *amhr2* gene gave 100% male-to-female sex reversal. Moreover, overexpression of the *amhy* gene in XX fish caused testis differentiation [82]. This study shows that the *amhy* gene is critical for maleness, and may likely be the sex determinant in the Japanese strain, although this is not the case in the Manzala [75] or Ghana strains [69]. Studying wild populations, we have also found some of these where the *amhy* and *amhΔY* genes are not associated to maleness, suggesting sex-linkage to another LG, most likely LG1 (Sissao *et al.*, unpublished data).

9.3.2 Genetic Sex Determination in the Blue Tilapia, *O. aureus*

In the blue tilapia *Oa*, the female is heterogametic ZW [40, 45, 46, 48]. A sexually growth dimorphism in favor of males also exists in this species. ZZ females have been produced (see section 9.6.2.3) which, in theory, should give 100% males when crossed with a normal ZZ male. However, in several cases, distortions from the expected sex ratio have been observed [46, 48], emphasizing that other factors, such as the environment, can also cause sex-reversals in this species [83].

The analyses of the synaptonemal complex showed an unpairing in the terminal region of the large chromosome, and a complete uniform unpairing of a smaller chromosome in all ZW females [84]. Sex-linked markers segregated for a dominant female W locus (male repressor) on LG3, located near markers GM354, UNH168, GM271, and UNH131 [85]. An additional association to sex with an epistatic interaction was also revealed, with a dominant locus for a Y haplotype located on LG1 [85]. These results were enhanced with more markers from LG1 and LG3, using six families, showing segregation differences depending on whether the strain was the

Manzala (Egypt) or from Lake Hula (Israel) [45]. The phenotypic sex was associated with markers for LG3 in the Israeli strain, with maternal inherited alleles concordant with a ZW heterogametic sex. Sex-specific rates of recombination were detected between markers UNH131 to GM354 [45]. In the Manzala families, however, the mechanism was more complex, with a strong association for markers of LG1, but also a weak association with markers for LG3.

A reconstruction of the four parental chromosome combinations on LG1 with the segregation distortions suggested that lethal alleles were associated strongly with the sex determiner on LG1 [45]. FISH using BACs showed that LG3 corresponded to the large bivalent chromosome, and that the genetic region of recombination suppression of ~ 80 cM comprised, in fact, more than 50 Mb [45]. Sex-specific recombination rates with the finding of double recombinants, suggested also that, along the W chromosome, there are several inverted regions [45] (Ozouf *et al.*, unpublished data).

9.3.3 Genetic Sex Determination in the Mozambique Tilapia, *O. mossambicus*

O. mossambicus (*Om*) and several of its hybrids have been farmed, due to their salinity tolerance and red coloring [86, 87]. Progeny testing was performed for a Florida red tilapia, a hybrid obtained from crossing an *Om* male and *O. urolepis hornorum* female, which was then crossed with *On* and *Oa* to improve the growth rate and resistance to low temperatures [46]. Male frequencies of 27%, 50%, and 70%, respectively, suggested a complex polygenic sex determination of the hybrid.

In *Om*, where the male has been considered to be XY, a synaptonemal complex study revealed the pairing along the whole large bivalent chromosome [84]. The first genetic mapping using an *Om* and *Oa* hybrid identified two QTLs linked to sex in LG23 [88, 89]. Segregation analyses of markers from LG1 and LG3 in families of *Om* from Natal (South Africa) showed association of both LG1 and

LG3 with the phenotypic sex, but were not able to define whether it corresponded to a male or female heterogametic sex [45]. Males might require the allele present in LG1, while females only that of LG3 [18].

An integrated genetic map of *Om* and red tilapia was constructed, with 401 markers composed of microsatellites, and expressed sequence tag (ESTs), mapping in males 351 markers that spanned 1104.3 cM, while the female map had 299 markers spanning 1051.3 cM [90]. *Om* males and hybrids of *Om* males showed only linkage to a supposedly XY locus, mapped onto LG1 between markers OMO086 and OMO287, and analyzed in five families consisting of 549 individuals [90]. In contrast, in red tilapia (hybrids of *Om* and *On*) males, the main sex-determining locus was located in LG22, mapped between GM047 and OMO049. In 30% of the individuals (58 females and eight males), there was no correlation of sex with either LG1 or LG22, indicating that other factors, such as the environment, also appear to be influencing sex in these individuals [90].

Recently, the zinc finger AN1-type domain 3 gene (termed *OsZFAND3*) was found to be expressed exclusively in testis and ovaries in a hybrid of an *Om* male crossed with a red female tilapia [91]. Transcripts were localized predominantly in spermatocytes and spermatids, indicating that the gene is implicated in male germ cell maturation. Three SNPs were identified in the gene, which were strongly associated to the phenotypic sex and mapped onto the sex-determining locus of LG1, suggesting that it could be playing a major role in sex determination or differentiation in this hybrid [91].

9.3.4 Genetic Sex Determination in the Black-Chin Tilapia, *Sarotherodon melanotheron*

In *Sarotherodon melanotheron* (*Sm*), sex determination has been investigated through hybridization with *On* and by progeny testing of sex-reversed *Sm* males (Baroiller *et al.*, unpublished data). Masculinizing treatments were applied via the feed, using

11 β -hydroxyandrostenedione at a dosage of 50 mg/kg food, starting from 10 days after fertilization, during a period of 30 days. Both types of hybridization (Female *On* \times Male *Sm* and Female *Sm* \times Male *On*) provided balanced sex ratios, suggesting a XX/XY type of sex determination in *Sm*. Progeny testing of sex-reversed *Sm* males revealed either balanced sex ratios (genetic males) or highly skewed sex ratios towards females. Such very high proportions of females are usually sired by XX males in *On* [10, 40].

From these unpublished data from our group, it can be concluded that *Sm* has a XX/XY sex-determining system. Furthermore *Sm* sex ratios are clearly modulated by some additional minor genetic factors, as already demonstrated in *On* or *Oa* [40, 48]. Finally, preliminary experimentations (data not shown) at high temperatures suggested that *Sm* fry are not tolerant to high temperatures. Therefore, we did not succeed in analyzing possible thermosensitivity in this species.

Female and male DNA pools were sequenced for *Sm* and then compared to female and male pools of *On*, and both were aligned to the Nile genome. Shared SNPs and quantifying regions rich in sex-linked SNPs were analyzed, together with copy number variants (CNV) [92]. A strong signal was found for LG1, which overlapped with the sex determining region of *On*, where it spans from 10.1 to 18.9 Mb, while in *Sm* it is broader, spanning from 10.1 to 28 Mb (10.1 to 18.9 Mb and from 21.7 to 23.6 Mb). In addition, a sex-patterned signal was also seen for LG22 of less importance, and none found for LG3.

The highest density of sex-patterned SNPs was found between 10.1 and 18.9 Mb in both species. Twenty-one SNPs showed similar X- and Y-alleles patterns between *On* and *Sm*, and 16 of these were found in the LG1 region between 10.1 and 18.9 Mb. One SNP mutation was 19 kb downstream of the sex-determining candidate *Wt1b* (position 14,895,959), and the other 5 kb downstream (position 11,400,015) of the Ras association domain containing protein 10, both previously identified [69]. This last

did not affect the binding site for any transcription factor. However, the SNP close to *Wt1b* affected the binding site of the transcription factor *Gata4*. This loss of *gata* binding might impact in females on the activation of *Wt1b* and, subsequently, of *amhr*, important for maleness [92].

Results from this study suggest that both *Sm* and *On* share the same sex-determining region on LG1 (a Y chromosome), which arose before these species diverged [92]. The sex region is not inverted in *Sm*, with mutations accumulated throughout the region whereas, in *On*, two blocks have been maintained in linkage disequilibrium (an antagonistic locus and the sex-determining locus?).

9.4 Thermosensitivity: a Hereditary Factor that Affects Gonad Differentiation

Part of the frequent distortions in Nile tilapia sex ratios from the expected 50 XX/50 XY might be due to environmental factors. Temperature has been shown to override the genetic sex determination in some progenies of *On* [40] (Box 9.2). Temperatures above 32°C applied during the sex differentiating period induce sex reversal of XX females into functional phenotypic males [93]. This temperature male-induction, termed thermosensitivity, was first evidenced in the Bouaké strain (a synthetic strain developed by crossing stocks from the Volta and Nile basins) [93] but, subsequently, was also observed in the Manzala strains [94–96]. It does not stem from domestication of *On*, since it has been shown to exist also in several wild populations in East and West Africa living in different temperature regimes [52]. However, the temperature-induced male proportions varied in the different populations depending upon the families, indicating different degrees of thermosensitivity [52].

Diallel crossings (5 males \times 5 females), followed by masculinizing temperature treatments, indicated that parental genetic factors from both dam and sire contribute

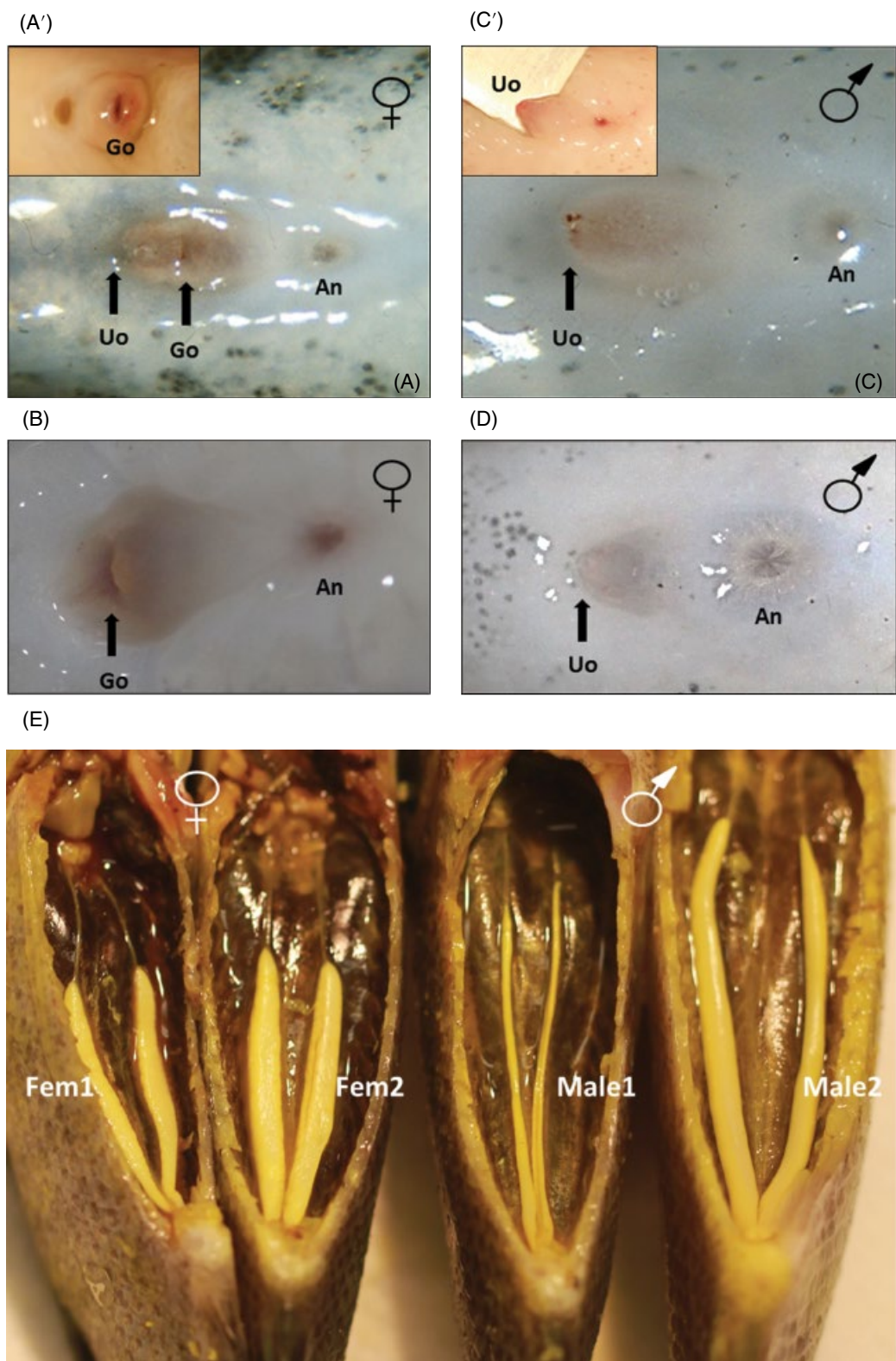


Figure 9.3 A, A', B: Females' urogenital papilla that are roundish with a horizontal genital orifice (Go), as well as the oviduct (Uo) and anus (An). (See inserts for the color representation of this figure.)

C, C', D: Males' urogenital papilla, oval in appearance, with only one oviduct and the anus.

E: Macroscopic differences between young ovaries (left) and testis (right) in shape (roundish/oval in ovaries), diameter (bigger in ovaries), and length (testis occupy the whole peritoneal cavity, whereas ovaries 2/3).

Box 9.2 Temperature masculinizing treatments

High temperatures of 35–36°C should be applied from 10 dpf (swim-up fry/first feeding stage), and must last at least 10 days. Thirty-day treatments do not cause any deformities but, when applied earlier (<10 dpf), they can induce some mortalities.

Heat two aquaria: one at 27–28°C (control group) and the other at 36°C. Divide the fish into two equal batches. To avoid temperature shock, place the future 36°C batch in a plastic water container/small aquaria, and let it float until the temperature reaches 36°C. Fish can then be liberated. Follow the temperature daily (try to $\pm 1^\circ\text{C}$). Well-adapted fish grow faster than controls.

After 10 days of treatment at 36°C, reduce temperature to 28°C, avoiding shock (as above). Raise the fish for ≈ 90 days, or when the sex can be distinguished. Try to avoid cannibalism/sex-specific mortality by keeping the density high.

to the thermosensitivity [10, 40, 52], with similar parental effects also shown in the Manzala strain by Tessema *et al.* [95]. Stable sex ratios are, nevertheless, obtained in repeated crossings of single pair matings for thermosensitivity.

Paradoxically high temperatures have also shown feminizing effects when the genotypes were YY and, possibly, in some XY males [94, 97]. In these studies, heat treatment of YY individuals resulted in 49.2 and 32% females. A selected low-thermosensitive line (see below) potentiated these feminizing effects of high temperatures in mixed sex batches [98]. These feminizing effects of high temperature require more research. It remains to be seen if these feminizing effects also exist in the wild.

Thermosensitivity was treated like a QTL by Wessels and Hörstgen-Schwark, selecting individuals from a Manzala strain that gave progenies with high male proportions in their sex ratios [96, 98]. In only three generations, they achieved progenies with

92.7% males, following 36°C treatments during 10 days [98]. Cumulative heritability of the high thermosensitive line was 0.69, while the low or non-thermosensitive line had a heritability of 0.86, with a male proportion of $\approx 54\%$ [96].

These researchers subsequently used six families of XX females, and compared these to 36°C sex-reversed males to map the temperature genetic factors using microsatellites [99]. Temperature phenotypic associations were found on LG1, LG3, and LG23. Further fine mapping revealed associations with allelic variants found within the *amh* gene, located on LG23 [100]. High proportions of temperature-induced males were associated to a missense SNP located in exon 6, which differed from the Y-linked SNP found for the *amhy* gene [81] or the SNP located for *amh Δy* by Eshel *et al.* [80]. However, there was no knowledge at that time [100] of the presence of the three *amh* genes.

Palaikostas *et al.* [75] studied several males where the sex could not be assigned correctly with the LG1 markers, finding that they corresponded to XX males. They mapped SNPs identified by ddRADseq in a highly thermosensitive XX family, which gave $\approx 66\%$ males when treated to 36°C, and found a QTL linked to thermosensitivity in LG20, a new LG not previously linked to sex [101]. Further resequencing and mapping studies need to be done on temperature-induced males, in order to find the loci linked to these phenotypic males.

Application of high temperatures at a large scale to control sex and produce monosex male progenies is only beginning. The University of Göttingen has developed a thermosensitive line/strain (“Tilapia Augusta”). A tilapia fish farm is using this procedure in Brazil with success, with the aim of commercializing fish under an ecological label (personal communication). Lastly, a large selection company has begun a selection program to obtain high thermosensitive Nile tilapias (personal communication).

Sex ratio analysis of genetic progenies, hormone or temperature treated fish is summarized in Box 9.3.

Box 9.3 Sex ratio analysis of genetic progenies, hormone or temperature treated fish

Male percentages are analyzed at three months of age and are necessary to validate the parental genotype (i.e., for YY male production, or to define sex reversal efficiencies of temperature or hormonal treatments). It is important to see, in cases of sex reversal treatments, whether the gonad shows complete sex reversal, or presents sterile portions.

Sexing using the genital papilla which, in females is rounded and has a horizontal opening and a second urinary orifice (Figure 9.3A/B), while it is oval in males, with only one orifice (Figure 9.3C/D). This procedure is used when fish are not sacrificed, keeping them for the F1.

Sexing the gonad macroscopically. This is done by sacrificing the fish; if mature, it is possible to see oocytes or sperm. Otherwise ovaries appear oval and pinkish, occupying 2/3 of

the peritoneal cavity (Figure 9.3E), while testes are narrower, more string-lined, and occupy most of the peritoneal cavity.

Sexing by gonadal squash, which is the most reliable procedure, implies sacrificing and dissecting the fish, removing the viscera and cutting a small portion of one gonad, and adding a bit of water and squashing the gonad between a slide and a cover slip to observe it under a microscope (10 and 40×). Higher contrast can be obtained using a vital staining (e.g., aceto-carmine) [102] if needed. Ovaries have large cells (oocytes) with a large central nucleus (Figure 9.4A). Testes appear as an accumulation of very small cells with no nucleus visible (Figure 9.4C); the testis lobular structure, observed by histology (Figure 9.4D), is more difficult to observe.

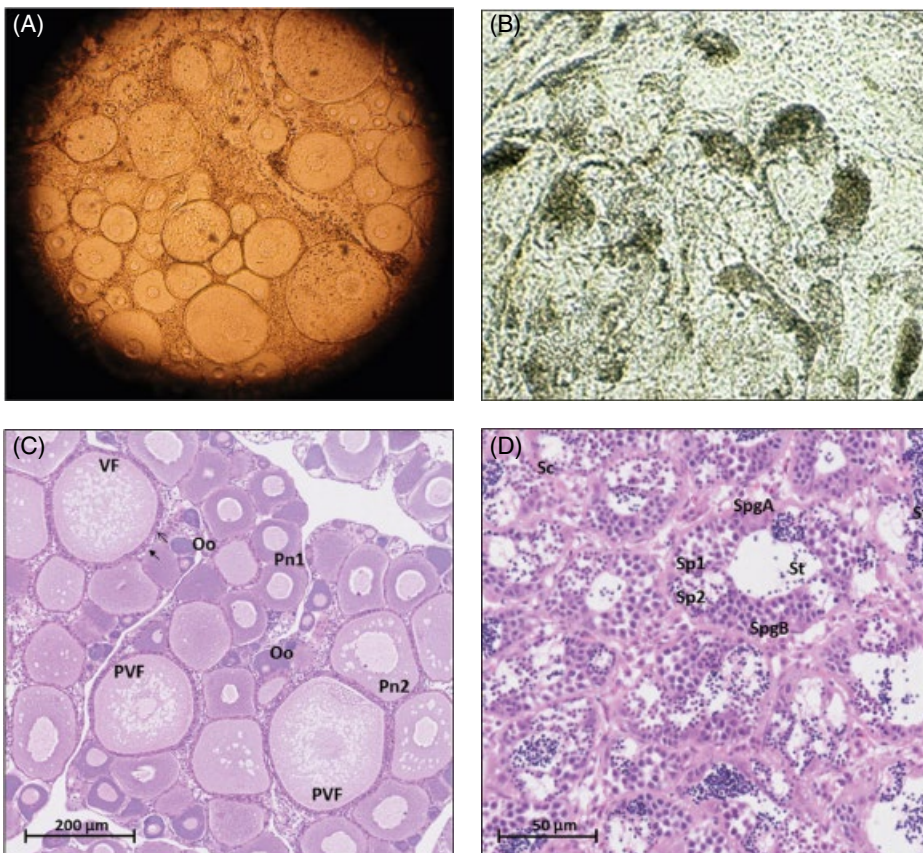


Figure 9.4 A. Ovary squash microscope appearance showing large oocytes and nucleus; B. Histology of a maturing ovary with primary oocytes (Oo), primary perinuclear oocytes (Pn1), secondary perinuclear oocytes (Pn2), previtellogenic oocytes (PVF), and vitellogenic oocytes (VF) with follicles in which the granulosa and theca cells can be distinguished (arrows); C. Testis squash microscope appearance with numerous small cells (spermatocytes); D. Histology of a testis with several tubules showing cysts of spermatocytes I (Sp1) and II (Sp2), spermatogonia A (SpgA), and slightly smaller spermatogonia B (SpgB) cells and spermatids (St). (See inserts for the color representation of this figure.)

9.5 Sex Differentiation in Nile Tilapia: Molecular Markers for Selection of the Phenotypic Sex

Nile tilapia is one of the most studied teleosts regarding sex-differentiating mechanisms, because all-female XX and all-male XY offspring can be produced, which allows gonad sampling and analyses throughout development, knowing the future fish sex. This is important, because gonads at these stages require pooling, since they are too small to be analyzed individually. Several tilapia studies have shown the important role of estrogens (and not androgens) on the gonad fate, regulated at the level of the aromatase enzyme or the *cyp19a1a* gene [65–67, 103, 104]. The decrease in aromatase levels leads to lower estrogen biosynthesis, inducing testis development in XX fish. Conversely, elevated estrogen levels induce ovarian differentiation in XY tilapia.

RNAseq data from XX females and XY males at different stages have confirmed the important role of estrogen biosynthesis in ovarian differentiation [105], together with knockdown studies [106]. It is particularly fascinating to know that gonad bipotentiality persists in tilapia juvenile females, since long treatments of fadrozole (an aromatase inhibitor) were able to reprogram the ovaries to develop as functional testes, and these fish showed male sexual behaviors [107, 108]. A regulator of the ovarian pathway is the transcription factor *Foxl2* (putative winged helix/forkhead transcription factor), and its deficiency by CRISPR/Cas9 causes decrease in the aromatase *cyp19a1a* expression and female-to-male sex reversal [106].

Testis differentiation is characterized at the molecular level basically by an early increase in the *dmrt1* gene expression at ≈ 8 –10 dpf [67, 77], a gene which, when mutated, causes increase in *cyp19a1a* expression in testis [106]. *Dmrt1* expression is followed by *gsdf* upregulation, a fish-specific gene postulated to repress estrogen levels in males [109]. *Dmrt1* also seems to be an activator of *gsdf*

shown by *in vitro* studies [109]. *Amh* was found to be differentially expressed in genetic XY males from ≈ 17 –19 days onwards [67, 77, 78]. However, transcriptome studies detected earlier high expressions at 9 dpf for the *amhy* gene [82], and also the *amhΔy* gene [80, 82], exclusively observed in testis.

Any of the genes briefly described above could be used as molecular markers of the phenotypic sex, particularly useful in selection for thermosensitivity. The reprogramming, or trans-differentiation, of the ovaries of tilapia XX females into testes is already observed after only three days of treatment at 36°C, with an upregulation of both *dmrt1* and *amh* [77]. At this time, there was no knowledge about the existence of three *amhs*, so it remains to be seen which of them is/are upregulated. Earlier activation of male testis-developing genes, such as *sox9* and 11 β -hydroxylase *11βhsd*, usually expressed later, was also apparent [7]. Following the activation of the initial testis-differentiating *dmrt1* and *amh* genes, we observed the repression of the ovarian pathway genes, such as *foxl2* and *cyp19a1a* [77], which we also saw in a high temperature-sensitive line developed at the University of Göttingen (Wessels *et al.*, unpublished data).

9.6 Current Approaches for Sex Control in Tilapias

9.6.1 Sex Reversal Through Hormonal Treatments

9.6.1.1 Critical Period, Hormones, Feed Preparation, Feeding, and Survival

Most Nile tilapia farmers use hormonal treatments to produce their male monosex populations, because it is a simple, highly efficient ($\geq 95\%$ males on a commercial scale, and even 99% under very strict and rigorous management), reliable, and cheap approach (around 1€ or 1.1\$ per 1,000 treated fry) [11, 110]. Based upon the available literature, but also based upon our own survey (Section 9.2.1), more than 91% of the traded tilapias have been produced using hormones.

Readers who are not familiar with the practical aspects of fry production and sex reversal are strongly recommended to read the excellent review published by Phelps and Popma, and the guide for efficient sex reversals under commercial conditions, published by Popma and Green [39, 111]; they will usefully complement our present review.

In order to consistently produce the highest percentage of males at a commercial level, treatment has to be applied from first feeding (this corresponds to the swim-up stage), which is around 10 days post-fertilization (dpf), or six days post-hatching (dph) at 27°C. Pooled fry have to be as homogeneous as possible in their age/size/weight. To produce and collect homogeneous fry for sex reversal treatment, three possible approaches have been proposed, [39, 111] depending on the facilities (water availability, ponds, tanks, nursery/incubation systems, etc.) and targets (number of fry, expected male proportion):

- a) daily “scooping/skimming” (fine mesh scoop net) along the edges of spawning ponds/large tanks;
- b) seining fry in a catch basin after draining the ponds/large tanks and removing breeders (every 2–3 weeks);
- c) egg/fry collection from each mouth-brooding female in spawning hapas/race-ways (every 5–10 days (see [49] for the fry production in race-ways).

Approach **a)** is time-consuming and adapted to small production; moreover, the pond has to be drained regularly (1–2 months), because escaped fry will become very efficient predators, and sorting is needed in order to eliminate larger fry (>13 mm) (Figure 9.5A). Approach **b)** needs to systematically drain the ponds/tanks, and has also to be associated with sorting. Among the three techniques, **c)**, which does involve a need for draining, is the most efficient, both in terms of fry production and homogeneity (uniform size); collected eggs and embryos are then artificially incubated (Zug jars or McDonald bottles) (Figure 9.5B), and swim-up fry leaving the incubators will be ready for an efficient sex reversal treatment.

Due to its relatively low price and availability and to its high masculinizing efficiency, 17 α -methyltestosterone (17 α -Methyl-4-androsten-17 β -ol-3-one=MT) is the most widely used androgen for sex reversal in tilapia farming [11]. However, various other androgens have been also tested for the production of male monosex populations in tilapia, most of them being synthetic androgens: 19-norethyltestosterone, fluoxymesterone, ethyltestosterone [112], mestrolone, trenbolone acetate, 17 α -ethynyltestosterone, dihydrotestosterone [113], and 17 α -methyl-dihydrotestosterone [114–116].

Following studies on the mechanisms of tilapia sex differentiation, some natural androgens involved in early testis differentiation, such as 11 β -hydroxyandrostenedione (11 β OHA4), have been very efficiently tested on both Nile and red tilapia [49, 117]. In both species, 11 β OHA4 presented a very similar masculinizing effect to MT when administered at similar dosage and duration (50 mg/kg during 28 days). A mean male percentage of 99.1% was produced at a commercial level for the red tilapia [49] and, in aquaria, 100% for the Nile tilapia [117]. Lower doses have proved also to be efficient, at least under controlled conditions (aquariums, recirculating systems, indoor facilities). For instance, a four-week treatment using MT or 11 β OHA4, at a dose of 20 mg/kg, generated 98–100% males in *On* under aquarium conditions [117]. Such natural androgens present the advantage of being eliminated more easily by the fry than artificial steroids such as MT. Conversely, however, because they are not yet used extensively, these natural androgens are still more expensive than MT.

To be optimal, hormonal treatments have to be applied for at least three weeks. However, in order to take into account possible differences between and within progenies, a four-week treatment is more reliable. Similarly, MT dosages will depend upon the farming conditions. If the fry are treated directly in the ponds, or in outdoor systems where plankton will develop, and with possible loss of part of the feed, higher dosages



Figure 9.5 Sex reversal treatments in tilapias.

A: fry are collected from ponds or from tanks/raceways.

B: they then need to be sorted by size, by passing them through specific mesh size (C), in order to eliminate fry >13 mm, which are no longer suitable for an efficient sex reversal treatment. For sex reversal to be efficient, they must be at 8–10 days (first feeding). Treatment will be performed in hapas placed in the ponds (shown in A).

High 36°C temperature treatments can be performed in aquaria (D) or in large tanks, but closed systems are preferable in order to control the temperature.

E: A farm worker giving hormone feed to fry in hapas can be exposed to the androgen daily.

(60 mg/kg) will often be suggested, whereas, in indoor systems, lower dosages (30–45 mg/kg) can have the same efficiency. On average, 50 mg/kg will be the best dosage.

The preparation of the treated feed for masculinizing treatments requires MT that has to be incorporated in the commercial feed (Box 9.4). For steroid manipulation, it is necessary to use gloves and, ideally, a lab mask. A 1 mg/mL stock solution can be prepared by weighing 50 mg of MT in a 50 mL volumetric flask. A volume of 50 mL of 95% ethanol is then added into the flask. In order to dissolve MT, close the flask using a glass stopper, and shake the solution thoroughly. This stock solution will be stocked in a refrigerator.

It is possible to avoid the weighing step by dissolving the whole content of MT in the appropriate volume of ethanol (i.e. 1 g of MT in 1 L of ethanol). Prepare the needed amount of feed: For treating 100 g of feed, preferentially use a Pyrex/glass crystallizing

dish (adsorption of steroids is higher on plastic than on glass), add the feed, and then 5 mL of the stock solution and 100 mL of 95% ethanol (this additional ethanol will allow the MT to perfectly spread into the whole feed), and mix it in the diet using a glass holder/stick. Ethanol is then air-dried overnight in a ventilated room, or under a hood. If needed, the dry feed can be crushed into powder by using a pestle and mortar, or by rolling a glass bottle over it. The dry treated feed has to be stored in a refrigerator until its use.

Usually, survival rates are not affected by the MT masculinizing treatments, whereas high dosages of EE (feminizing treatments to produce XY females) can induce some mortalities.

Sex reversal treatments can be done in various aquatic systems. Hapas (fine meshed net cages) were first used in Asia for sex reversal treatments, and are now classic structures notably for this purpose (larger hapas can also be used for fry production) in many other tilapia-producing countries (Figure 9.5C), because they are particularly appropriate for swim-up fry (protection against predators, easy to install and manage in ponds, and well adapted for fry collection at the end of the treatment). However, because of their fine mesh, and especially when high protein feed is used, which is the case for sex reversal treatments, hapas can be fouled quickly by plankton over-development, limiting water exchange. Therefore, they need to be cleaned regularly with a hard brush, and changed for a clean one after each sex reversal treatment. Other structures can be used efficiently indoors or outdoors for sex reversal treatment, such as tanks or raceways [49] (Figure 9.5D).

Box 9.4 Hormonal sex reversal treatments in Nile Tilapia

Dose: 50 mg/kg MT (M-7252 Sigma) for masculinizing treatments, or 175 mg/kg 17 α -Ethinylestradiol (EE) (E-4876 Sigma) for feminizing treatments (in order to produce XY females and further YY males).

Duration: 28–30 days.

Age/weight/length of the fry at the beginning of the treatment: 9–12 dpf or 5–8 dph/9–11 mg/10–13 mm.

Critical period: 9–21 dpf at 27 °C.

Feeding rates: 20 % of biomass per day for the first week, 18 % for the second, 16% for the third week, and 15 % for the fourth week.

Feed distribution: The ration is distributed to fry 4–6 times by day, 7 days a week.

Feed quality: 40–50% crude protein.

Feed particle size: 400–1000 μ m in diameter.

Density: 3000–10,000 fry/m², depending upon the structure (i.e., 3000 fry/m² in ponds, 10,000 fry/m² in tanks).

9.6.1.2 Impacts of Hormonal Treatments on Human Food Safety

Although MT is widely used in tilapia farming, its safety in human food, and possible impacts on the environment, are still under debate [37, 110, 118]. We will analyze these two aspects. Concerning human food safety, it is generally considered that the use of MT

for sex reversal in tilapia does not induce any risk to the consumers. These assumptions usually rely upon three arguments:

- 1) Treatments are applied *via* feeding at early stages of fry development (beginning at the 10 dpf stage), with low dosages (usually 50–60 µg/g of feed), and during short durations (3–4 weeks). At the beginning of the treatment, the average weight of the fry is 9–10 mg. At this age, the classic feeding rate is 20%, meaning that a fry will receive 2 mg of feed/day, containing 100 ng of MT (700 ng for the first week). For the second week, based on a feeding rate of 18%, the fry (average weight of 56 mg) will receive 10.1 mg of feed/day, containing 505 ng of MT (3535 ng for the second week). For the third week, the feeding rate will be 16%, so the fry (average weight of 200 mg) will receive 32 mg of feed/day, containing 1600 ng of MT (11,200 ng for the third week). Finally, for the final week, the average weight of the fry is 600 mg, and it will be fed at a feeding rate of 15%, thus receiving 90 mg of feed/day, containing 4500 ng of MT (31,500 ng for the last week). Therefore, the total amount of hormone administered to an individual fry is low (0.047 mg), especially compared to the dosages used in human medicine or livestock production.
- 2) The MT is rapidly degraded by the fry, and excreted partly as a free un-metabolized compound (MT), and partly as free or conjugated metabolites [119–121].
- 3) Following the treatment, there is a grow-out period for the fry of at least five additional months before they are harvested; during this period, the fish are fed with untreated feed.

Therefore, it is usually considered that MT is no longer present in the muscle when the adult fish will be commercialized (information about the persistence of its metabolites in the different tissues is scarce). At this point, it is important to recall some general principles of androgen/steroid metabolism. MT

(17 α -methylandroster-4-en-17 β -ol-3-one) is a synthetic 17 α -methylated derivative of testosterone. Its methyl group at the C17 α position gives it an oral bioavailability, but also prevents deactivation by sterically hindering oxidation of the 17 β -hydroxyl group [122]. In order to be eliminated through branchial, fecal, or urinary excreta, a lipophilic steroid has to be first metabolized into polar end-products, allowing water solubility, but part of the parent compound and residues can also be excreted as free metabolites. In vertebrates, the main biochemical reactions allowing their elimination are reductions, oxidations, hydroxylations, and conjugations (formation of hydrophilic products: sulfate and/or glucuronide derivatives). However, sulfated steroids can also be precursors for free hormone synthesis, through hydrolysis by a sulfatase (i.e., dehydroepiandrosterone sulfate, which can be used for estrogen biosynthesis in the fetoplacental unit of mammals).

This rapid reversibility between inactivated and biologically active compounds has to be kept in mind as far as MT degradation is concerned. If MT residues can be stored either in the tissues of treated (intentionally or not) individuals or/and in the sediments (see the next paragraph), their possible bioavailability and further biological effects has to be questioned.

Based upon current tilapia production (5.3 million tons), the estimated amounts of MT used today for sex control in this group of species range between 200 kg (100 kg for a projected tilapia production of 3 million tons in 2010, according to [123]), to more than 1 ton (A Fostier and JF Baroiller, unpublished data). In fish and tilapias, little information exists on MT metabolic pathways. Using 3H- or 14C-MT, Goudie *et al.*, Cravedi *et al.*, and Curtis *et al.* demonstrated that total radioactivity, respectively given through a typical feeding treatment in *Oa*, or through a single intragastric dose in the rainbow trout, or a single dietary dose in *On*, is rapidly eliminated from fry [119–121, 124]. The extensive metabolization of MT in rainbow trout was

studied, further showing that, although the parent compound was rapidly eliminated, some metabolites persisted in the tissues [125, 126].

In the liver, radioactivity is mainly associated to glucuronides whereas, in the muscles, it is mainly linked to free (unconjugated) metabolites. In the gall bladder, radioactivity (mainly associated to glucuronides) is 200–2,000 fold higher than in other tissues. Excreted radioactivity was primarily found through free metabolites (unconjugated) in the water, and through glucuronides in the feces [125, 126]. Through hydroxylation and/or reduction, methyl-dihydrotestosterone and methyl-androstane-diol metabolites are produced.

Further biotransformation resulted in metabolites that have been tentatively identified as 17 α -methyl-4-androsten-6 β , 17 β -ol-3-one, 17 α -methyl-4-androsten-7 ξ , 17 β -ol-3-one, and 17 α -methyl-5- ξ -androstan-3 ξ , 7 ξ -triol, 17 α -methyl-4-androsten-17 β -ol-3, 11-dione and 17 α -methyl-17 β -hydroxy-4,6-androstadiene-3-one [125]. Lack of references for such metabolites precludes their definitive identification. However, these results show the extent of the MT biotransformation, and the likely existence of several metabolic pathways for 17MT. Not much is known about these metabolic pathways, and even less about possible impacts of these metabolites on fish, their predators, and environment.

9.6.1.3 Effect of Sediment Environment on MT Accumulations, Bioavailability, and Ultimate Fate

There is little information in the literature regarding steroid accumulation, bioavailability, transport, and ultimate fate in the sediments of tilapia ponds and adjacent water bodies. Promising results, showing that some environmental conditions prevailing in the sediments can facilitate or impede MT accumulation/degradation, have been published during the last 5–10 years, and deserve to be presented below in this review.

Because of their lipophilicity with low water solubility, steroids, rather than dissolving in the water, can easily be adsorbed on soil or in sediment particles. Therefore, in aquatic systems, the soil plays a key role in the control of steroid bioavailability, transport, and ultimate fate [127]. Depending upon the soil/sediment type, particles have different size fractions (i.e., sand: 0.425–0.075 mm; silt: 0.045–0.002 mm; and clay: 0.6–2 μ m). Adsorption and desorption abilities of soil/sediment particles will strongly depend upon particle sizes, but also upon temperature, pH, ionic strength, soil/water ratio, and organic matter [127]. Hence, the adsorption and release of testosterone, onto or from all of the particles, will be respectively increased and decreased by low temperatures/pH or high organic matter.

Also, an increase of the soil/water ratio will decrease the androgen desorption from the particles. Adsorption mechanisms will depend upon the nature of the soil and the size fractions of their particles. Interactions between hormones and particles will mainly rely upon the electrostatic attraction for sand, and upon hydrogen bonding and functional groups for clay, whereas the interactions will depend upon both mechanisms for silt [127].

As suggested by Sangster *et al.*, association between sediment/soil and steroids can preserve the molecule, with transformation occurring in the aqueous phase, with the size of the sediment particles being a key parameter for preservation efficiency [128]. Fine particles in silty loam will not only preserve the steroids longer but also, because they can be easily suspended in the water column, they will also favor their transport and their contacts with aquatic organisms. The biological effects of sediment-protected steroids could rely either upon their release (desorption) in the aqueous phase and/or upon a direct effect of the complex steroid-sediment particle. However, these hypotheses have to be further confirmed.

Nevertheless, as far as the impacts of MT treatments on aquatic systems are considered, interactions between steroids and

sediment, rather than water alone, should be better characterized, because they will influence the subsequent bioavailability of the androgen and its residues. Because they can strongly influence androgen preservation, bioavailability, transport, and ultimate fate, a survey of the soil/water characteristics in the ponds and surrounding water systems could allow us to predict and, perhaps, to control the fate of MT and its residues.

Under aerobic conditions, various steroids, including testosterone (T), can be completely degraded by specific bacteria. For instance, T is mineralized to carbon dioxide and water by *Comamonas testosteroni* [129], but key reactions leading to this metabolic pathway do not occur under anoxic environments. Because oxygen is rapidly consumed in freshwater sediments/carbon-rich soils, Fahrbach *et al.*, have isolated a gammaproteobacterium *Steroidobacter denitrificans* strain FST that is able to use testosterone as a source of carbon and energy, and nitrate as an electron acceptor under denitrifying conditions [130]. Through several dehydrogenation and hydrogenation processes, transformation products are generated from T, which will be further degraded efficiently and rapidly by *S. denitrificans* [129]. However, these results have been obtained through bioassay tests and therefore, using T, they have to be confirmed with MT in more complex aquatic systems, where the abundance and activity of the *S. denitrificans* strain are unknown.

Indeed, three MT-degrading bacteria closely related to *Rhodococcus equi*, *Nocardioides aromaticivorans*, and *Nocardioides nitrophenolicus* have been isolated from the sediment/water of a treatment pond where Nile tilapia fry were usually masculinized [131]. Although the growth of these bacteria strains was inhibited by high MT concentrations (1.0–10 mg/L), they were able to degrade MT to products without androgenic potency [131]. These results suggest that bacteria conditioning (through pond/sediment management) could be a way to

naturally accelerate the total degradation of MT and its residues.

In addition, bioassay tests demonstrated that the electron acceptor conditions prevailing in the sediment (42.4% clay, 27.8% silt, and 29.8% sand) were important factors for biotransformation efficiency. Biotransformation of MT was rapid (half-life of 4–5 days) under aerobic, sulfate-reducing, and methanogenic conditions, whereas it was slow under iron(III)-reducing conditions, and extremely slow to absent under nitrate-reducing conditions. Moreover, androgenic activity was completely lost under aerobic and sulfate-reducing conditions, but still persistent after 45 days of incubation under methanogenic conditions [132].

Absence of biotransformation under nitrate-reducing conditions could be associated with the presence of a methyl group in MT. These results strongly suggest that MT (and its androgenic residues) accumulation depends upon the characteristics of the sediments and, especially, the prevailing electron acceptor conditions; iron(III)-reducing, nitrate-reducing and methanogenic conditions may favor the accumulation and persistence of androgenic activities associated to MT or its residues in masculinization ponds and surrounding water bodies [132].

Again, these results have to be confirmed using more complex aquatic systems. However, they suggest that efficient remediation of MT-contaminated sediments should be possible through pond/sediment management conducting to aerobic and sulfate reducing conditions.

9.6.1.4 Impacts of Hormonal Treatments on the Environment: MT Water Release

During sex reversal treatments, part of the feed will be lost. Some will fall at the bottom of the rearing structure (hapas in ponds, tanks, etc.), and the rest will be spread over the surface through the mesh (hapas). In both cases, this treated feed can then be eaten by wild juvenile and adult tilapias (and other fish species, but also mollusks, amphibians, etc.) aggregated around the cages and/or

downstream of the farms. Therefore, MT and its residues could be expected to be found in the surrounding water bodies.

Evidence that significant amounts of MT can be released into the pond environment during and after hormonal treatments has been previously reported using radioimmunoassay [133–135]. Such environmental contaminations raise many questions about the impacts of these residues on water quality, biodiversity, and so on. Therefore, other methods have been developed to better detect MT and some of its residues in the water, in tilapia and in some other aquatic species.

A high-performance liquid chromatographic approach using ultraviolet detection (245 nm) recently allowed identification and quantification of MT in fish muscle [136] in tilapia ponds (un-metabolized residue and MT from uneaten treated feed) and surrounding water bodies [137]. MT was detected in the surface water samples from a Thai tilapia farm at a concentration of 617.4 µg/L. Indeed, important differences can be observed between the MT concentrations in the water reported in the literature. We believe that these differences could reflect differences between the experimental conditions, especially regarding the sediment/water characteristics.

As reported in this review, a pond's sediment and water characteristics can strongly favor or impend MT accumulation. Moreover, depending upon the farms, the period of treatments can be limited to a few months, followed by a long period without any treatment or, conversely, be continuous during the optimum period for tilapia reproduction (8–9 months/year in many tropical countries). Such conditions probably do not result in the same MT concentrations in the water/sediment.

9.6.1.5 Possible Impacts of Hormonal Treatments on Biodiversity

American crocodile populations have been reported to have male-skewed sex ratios in some Costa Rican drainages, despite living under female-producing temperature regimes

[138–140]. The levels of accumulated MT in all the field collected eggs and in the plasma of wild crocodile hatchlings were similar to those that found in experimentally masculinized hatchlings, even when eggs were exposed to a female-producing temperature [139]. These results strongly suggest that the observed natural bias could result from the masculinizing effects of this synthetic androgen [139–141].

Based upon blood plasma (juveniles and adults) and egg yolk analysis, crocodiles of the Tempisque Basin and Tarcoles River seem to be exposed chronically to MT and/or retain it. The higher plasmatic concentrations of MT in hatchlings, compared with eggs (after 1/3 of incubation), juveniles, and adults, suggest either less frequent exposure and/or more rapid utilization, or storage of this exogenous androgen in growing or mature individuals.

A possible mechanism to explain chronic exposure to MT and subsequent effects on crocodile sex ratios in the Tempisque and surrounding basins in Costa Rica relies on tilapia as a possible biotransporter of the environmental androgen [140]. Since 2004, Costa Rica has become an important Nile tilapia producer, with 15,500 tons in 2014 and 25,000 tons in 2013 [12]. The Costa Rican production mainly relies upon intensive earthen-pond and cage systems. An important part of this production is exported mostly to the US markets. All-male tilapia populations in Costa Rica are produced using either MT or YY males. In all the systems relying upon earthen-pond or cages, it is quite impossible to prevent escapees altogether and, therefore, treated fry, juveniles, and adults escape from the numerous farms.

Moreover, the authors hypothesize that, in hapas installed in pond culture systems, excess treated feed can pass through the mesh and can accumulate on the bottom beneath the hapa, and/or be released into the environment, especially during water exchanges. In both cases, this feed escaping the farm can either contaminate the surrounding water bodies and/or be eaten by

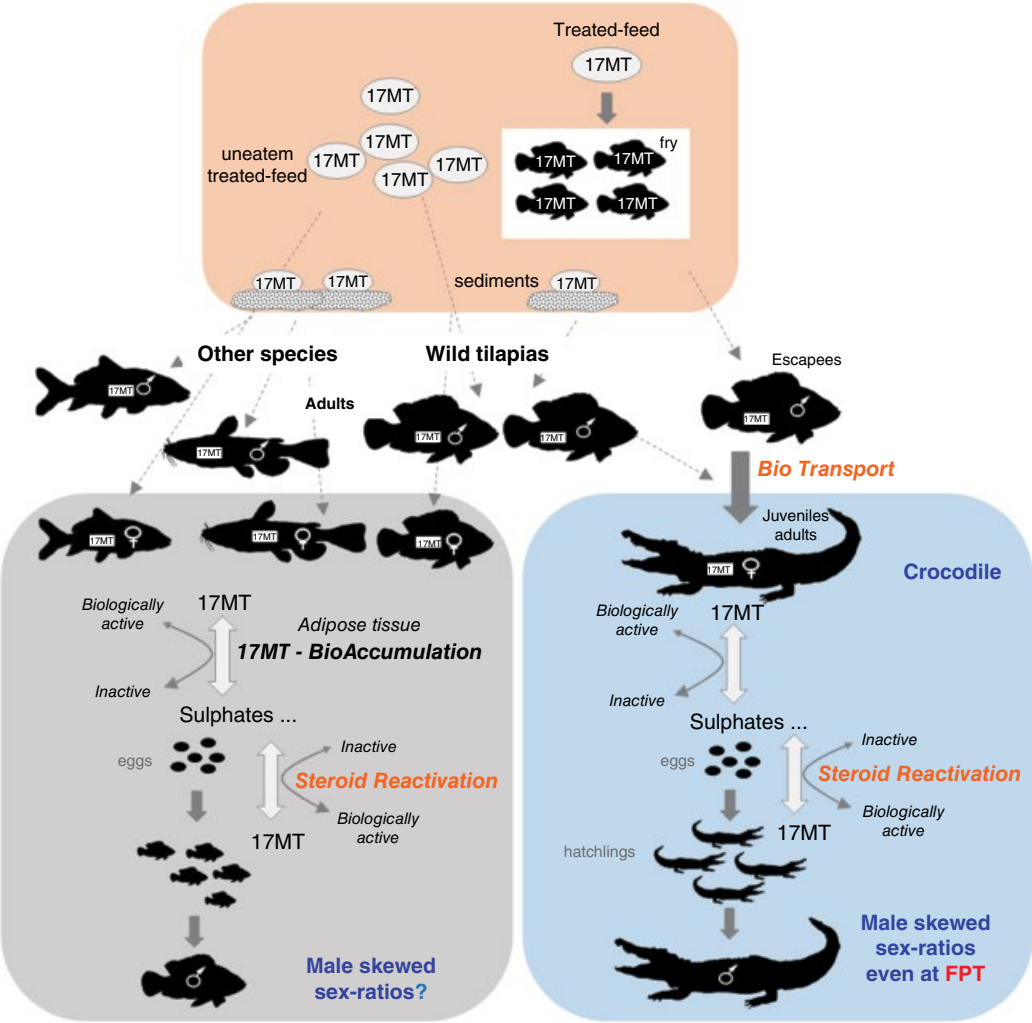


Figure 9.6 Possible impact scenario of MT bioaccumulation in adipose tissues of tilapia and other species (via treated food or water), to explain the male skewed sex ratios reported by Murray *et al.* [138–140] for the American Crocodile in Costa Rica, via the activation and inactivation of sulphate conjugates. Abbreviations: FPT, female-producing temperature.

wild tilapias (and other species) aggregated around the cages and/or downstream of the farms (Figure 9.6).

Because of its rapid degradation, MT is not supposed to accumulate, either in the environment (except perhaps in the sediments that have not been analyzed in these studies) or in the treated fish [119, 121, 124]. Hence, the study suggests the existence of a biotransporter [140]. The authors consider that tilapias could be the biotransporter; escaped

farmed tilapia, but also wild tilapias, aggregated around the cages and around the farms, could bio-accumulate MT from the lost treated feed in their adipose tissue (a classic site for bio-accumulation of exogenous steroids [142]). Following a single meal of a diet containing both unlabeled and labeled (3H- and 14C-) 17MT, adult tilapia can accumulate MT in various tissues (adipose tissue was not analyzed in this study) [124]. At four and 21 days after the single

meal, estimated concentrations of 13 µg/g and 67 ng/g, respectively, were still detected in the fish. This means that an adult of 300 g could contain between 3.9 mg and 20 µg of MT.

It is, then, easy to hypothesize that a crocodile (adults or juveniles eat tilapia) can, in turn, also accumulate MT in its adipose tissue (Figure 9.6).

During the four years needed to become an adult, a crocodile will eat 260 kg of feed (and tilapia is a usual prey for it). If a female crocodile has accumulated synthetic androgen in its adipose tissues, stored MT could be mobilized during oogenesis and deposit in the yolk, as already suggested [142, 143] (Figure 9.6). This would explain the high concentrations of MT found in the eggs of these crocodiles, and these doses have been demonstrated to be able to masculinize an embryo, even under feminizing temperatures [139, 141].

The higher concentration of MT found in hatchling plasma, compared to dose detected before in the eggs, cannot be explained by tilapia consumption (hatchlings are too small to eat tilapias). However, previous studies in reptiles [144, 145] have postulated that maternally derived steroid hormones can be conjugated as sulfates in the yolk, then transferred to embryo for later use during development. Because conjugation of steroids can occur very rapidly [146], storage of MT could be undetectable, but high concentrations could be expected in hatchlings when (and if) MT is reactivated during this critical period of development [139, 140].

Based on this scenario, MT could masculinize crocodile embryos in the Tempisque basins in Costa Rica through the consumption by their mothers of treated tilapias (both escapees and wild individuals that have eaten lost treated feed), and through the bioaccumulation (as sulfate conjugates) of MT in the adipose tissues of tilapias and crocodiles and its reactivation during the hatchling stage (Figure 9.6). If this hypothesis is confirmed, MT release from the

ponds to the environment could impact not only fish, but also any fish predator, such as piscivorous fish and birds [139, 140].

In conclusion about hormone impacts, further studies will have to analyze the metabolism of MT (especially conjugation), its possible persistence in sediments under specific conditions, its possible storage in adipose tissues of fish and associated predators, and the mechanisms of maternal transfers to yolk and embryos, as well as its bio-reactivation at some specific stages.

9.6.2 Genetic Approaches

9.6.2.1 Strain Management for an Efficient Genetic Sex Control

Within *On*, sex-linkage varies between LG1 and LG23, depending upon strains/populations. To what extent are these conflicting associations the result of numerous strain hybridizations, due to excessive inbreeding, to low number of founders, or stem from the use of few YY males? Our studies are showing that the complexity is not all due to processes of domestication, since we are seeing variations in wild populations (Sissao *et al.*, unpublished data). Nevertheless, it is necessary to continue and improve the characterization of tilapia species and wild populations, and to determine the broodstock purity of the species used to produce hybrid all-male crosses.

Several molecular techniques with different markers have been used in the past for this, which have then been used for the development, improvement, and management of farmed strains. Moderately polymorphic markers (allozymes and mtDNA) were first used to characterize tilapia species, hybrids, and subspecies [147–151]. Subsequently, RAPD and microsatellite markers have been used respectively to analyze species/subspecies and populations/pedigrees [4]. More recently, Cytochrome C oxidase subunit I (COI) and SNPs have allowed us to discriminate between 10 tilapia species [152, 153].

9.6.2.2 Production and Use of All-Male Hybrids in Tilapia

Following the first evidence that hybrid progenies between a male *Om* (XX/XY Sex Determination System) and a female *Oh* (ZZ/ZW SDS) were all males [154], similar results have then been obtained using *Omc* and *On* [155] or *Oa* and *On* [156]. These results, and studies of the reciprocal hybrids (i.e., a female *Omc* and a male *On*), have led to the hypothesis that the Z chromosome in tilapia is dominant over the X chromosome. This explains why XZ hybrids are males in tilapia.

Following these results, tilapia hybridization has been widely adopted in commercial or experimental hatcheries, using various tilapia species. This has been facilitated by the behavioral plasticity found in tilapias [157]. However, it has also led to an impressive wave of transfers of tilapia species/populations/strains to most of the countries where tilapia aquaculture was already implanted. Apart from the ecological impacts of hybrids, which will not be discussed in the present review, another negative consequence of these transfers has been their genetic impact on tilapia genomes (introgressions, loss of variability, etc.), and possible effects on the sex determination system(s) by mixing two complex systems.

Several drawbacks have, however, impaired the use of this approach for sex control:

- 1) Very few species' combinations can lead to true all-male hybrid populations (100% males) in tilapia. The main efficient combinations are the following: [158] *fOm* × *mOh*; *fOn* × *mOh*; *fOn* × *mOa*; *fOn* × *mOmc*; *fOn* × *mO. variabilis*; and *fO. spilurus niger* × *mOh* (with very possible misidentifications of the species in the 1960s). However, for each species, only a few strains/populations can be used in order to get true monosex populations. Unexpected proportions of females in the hybrid progenies are usually explained by the presence of some minor genetic factors in the genome of some breeders/populations/strains.

- 2) Misidentifications and subsequent mixing between hybrids and parental species, and difficulties in keeping the original parental stocks genetically pure (partially related to the tilapia's behavioral plasticity – see [157]), progressively lead to a decrease in the male proportion. This can be explained either by the presence of minor genetic factors in some breeders, and/or by unwanted mixing between hybrids and parental species.
- 3) Lower zootechnical performances of the hybrids compared to the pure parental species.
- 4) Difficulties in obtaining large number of hybrid progenies (despite its plasticity, differences in reproductive/parental care behaviors mainly explain this point).

Because of its better tolerance to low temperatures and salinity, *Oa* has been used in countries where these two parameters were limiting factors for tilapia farming, such as Israel and China. It has also been used for sex control purposes for decades, because its hybridization with *On* (female *On* × male *Oa*) led to all or nearly all-male populations [156]. However, several drawbacks have led to the progressive abandonment of this approach:

- misidentifications between hybrids and their parental species (hybrids have intermediate traits);
- broodstock management (strict conservation of two pure species' stocks is difficult);
- complexity of hybrid sex determination (variable sex ratios depending not only upon the parental strains/populations but also upon the individuals); and also
- tilapia genetic diversity/biodiversity concerns (not discussed in the present review).

Because of these difficulties, in the rare countries where hybrids were still used (mainly Israel and in some parts of China), farmers first associated MT treatments to ensure the production of nearly all-male population (95–99% males). Finally, hybridization is no longer used for sex control, with

farmers turning to *On* or *Oa* production with systematic hormonal treatment of the progenies. This is what occurred in Israel (G. Hulata, personal communication), where farmers have abandoned the use of the *On* × *Oa* hybrid. The majority of the 40 million tilapia fingerlings produced annually in Israel are sex-reversed fry from a local strain derived from the initial crossbreeding of these two species. There are some two million *On* produced (Chitralada strain), and five million all-male *Oa* (Nir David strain).

9.6.2.3 Genetic Selection: the YY Male or the ZZ Female Technologies

Up to now, the most sustainable method to produce large amounts of all-male monosex offspring has been the use of genetics using YY “supermales.” Future selection based on the male-determinant in Nile tilapia needs further research, in view of different loci (and chromosomes) implicated, dependent on the strain [69, 75, 82]. Genetic selection to eliminate minor factors can also be performed, and this has been done indirectly in some strains where all-female XX and all male-XY show no sex distortions, such as the Japanese strain.

The classic procedure to produce YY males, which are perfectly viable and functional, first involves the production of XY females by sex reversal treatment. Feminizing treatments require higher dosages of synthetic estrogens – EE usually used at a concentration of 150–200 mg/kg. The procedure used is similar to that of the masculinizing treatments but, usually, a limited number of individuals are concerned and, therefore, hapas in ponds or tanks will be used.

Progeny testing of feminized fish is then necessary. For this, several females generated by the feminizing treatment are removed from their growing structure, tagged and reproduced either semi-naturally (placed with a dominant male in an aquarium or a tank) or through artificial fertilization (based on the papilla development and nesting behavior). Females that can be either normal XX or functional sex reversed XY are selected

and stripped individually; eggs are fertilized *in vitro* with sperm that is collected by stripping untreated genetic XY males. After fertilization, eggs are incubated in a Zug or a McDonald bottle.

If the female is XY, the cross will give sex ratios of 3 males : 1 female, with 25% of the males being YY males or “supermales” [36, 50]. By crossing YY males with XY females followed by EE feminization of the progeny, we can generate a large amount of XY and YY females. Subsequently, crossing YY females with YY males allows the massive production of YY males [159] (Figure 9.7).

An accelerated procedure is through androgenesis, using the milt from an XY male with UV-inactivation of the eggs, followed by heat shock, so that the first mitosis is inhibited and duplication induced. Consequently, only the paternal genome contributes, with the offspring being either YY males or XX females [160, 161]. Higher survival rates can be obtained with a combination of hormonally sex-reversed XY females, and then performing diploid meiotic gynogenesis on the eggs. The sperm is UV-irradiated, followed by a heat shock, inducing the retention of the second polar body and duplication, so that the genome contribution is only from the female, thereby producing female XX or YY male individuals [47, 161].

Until recently, the procedure to produce YY males was long and tedious, taking about five years and requiring numerous infrastructures, due to an indirect procedure being necessary with several progeny testings, since it was not possible to distinguish XX, XY, and YY genotypes. Nowadays, some genetic and phenotypic markers exist (see Section 9.7) which allow an acceleration of the YY production. For instance, our phenotypic marker using the head *amh* expression (see Section 9.7.2) allows the precocious analyses of an offspring sex ratio, which indicates the maternal or paternal genotype and, thus, can accelerate the progeny testing.

Due to the absence of markers and sufficient infrastructures, a reduced number of

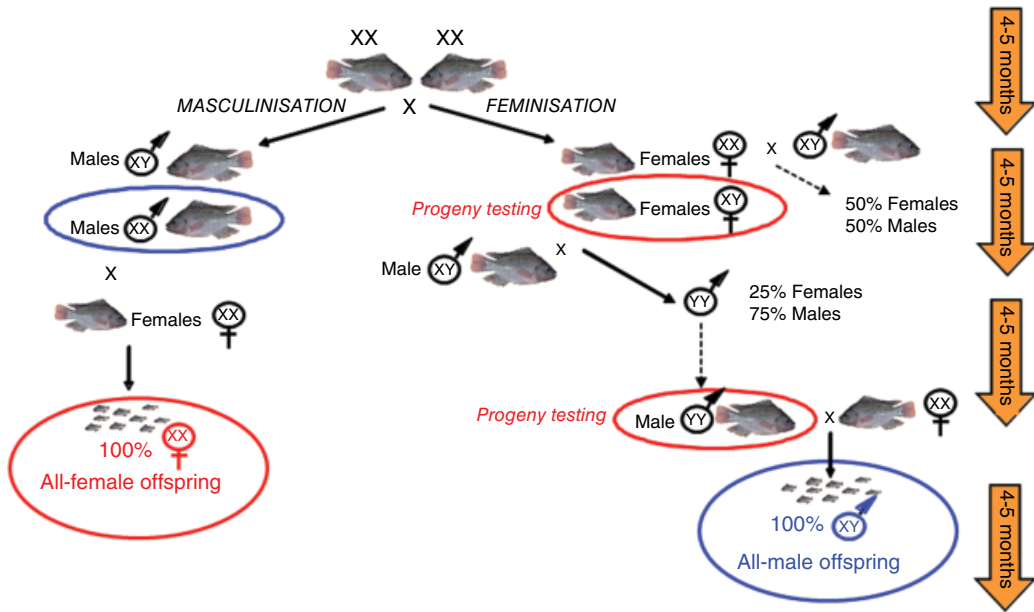


Figure 9.7 Steps needed to produce YY supermales in XX/XY tilapias, showing the different hormonal treatments and the progeny testings required, with the final crossings giving 100% XY males (monosex males), as well as the production process of monosex XX females.

YY breeders have usually been produced, causing genetic bottlenecks (generally associated to the loss of genetic variability). The other possibility is to buy YY males from one of the two companies that commercialize them worldwide. This implies that the farmer needs to buy, and then introduce in his country, one of the YY strains used by the companies. This is a serious problem if the strain is not already present in the country, causing genetic pollution of the local genetic resources used by the farmer; moreover, these strains are usually not well adapted to local conditions.

Comparative growth performances showed that progenies generated from YY males, such as the genetically male tilapias (GMT), had 58.9% higher growth rates than males from mixed sex batches, and were 31% larger than hormonally sex-reversed males in extensive ponds, but differences were not significant under intensive cultures [162, 163]. YY males have, nevertheless, a bad reputation among farmers (see Section 9.2.1), particularly due to their

progenies showing bad growth rates. This might be due to inbreeding, or to the fact of having some females in the batches, or to the use of strains that are not well adapted to local conditions. Despite their theoretically 100% XY males offspring, some YY males can give up to 30% females in certain cases, due to parental factors [10, 40, 159, 162]. It is, therefore, also necessary to select the YY males as well as the females that give progenies with >95% males and good growth rates, as well as producing YY males from local strains.

YY males present some biological differences, showing lower growth rates than genetic XY and XX males [28], as well as lower survival rates [159]. It is harder to feminize them, with EE treatments requiring much higher doses, such as 500 mg/kg feed, to produce 80–100% sex reversals, with earlier immersion studies being ineffective [163]. High temperatures also affect the sexual genotype differently (see Section 9.4), inducing up to 49% feminization of YY males [94, 97]. Finally, comparisons of the sperm

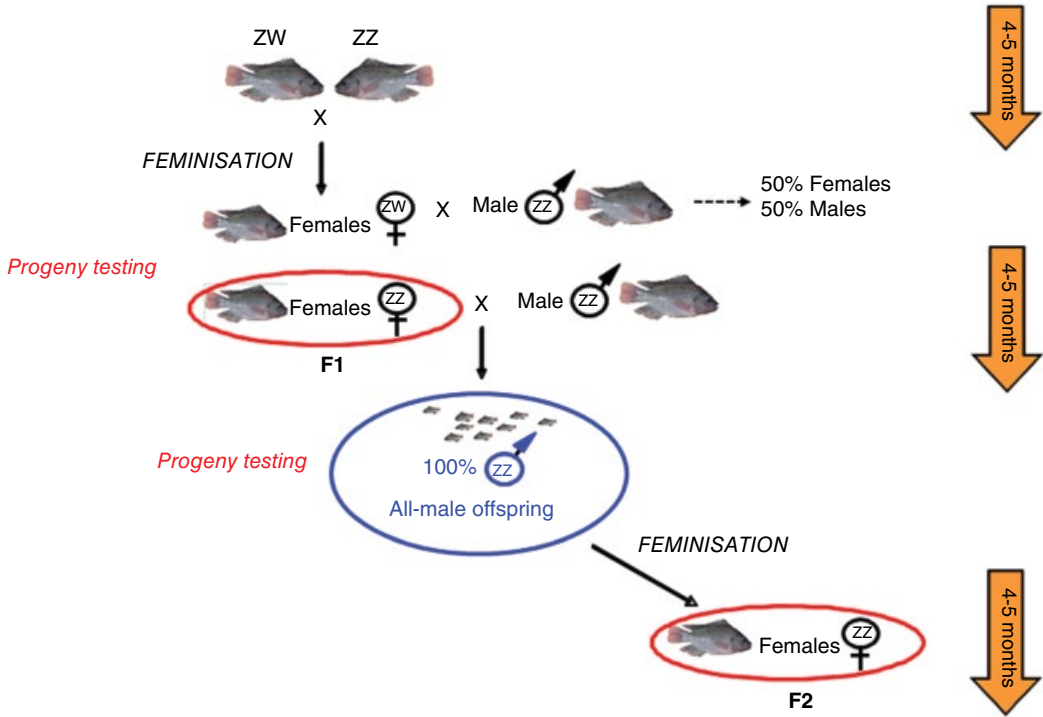


Figure 9.8 Steps needed to produce two generations of ZZ pseudofemales in *O. aureus*, showing the feminizing treatments and the progeny testing required in order to obtain 100% ZZ males (monosex males).

quality of YY males with XY and XX males showed no significant differences [164].

In the blue tilapia *Oa*, because of the sexual dimorphism in favor of males, the most interesting genotype is the ZZ pseudofemale. We generated ZZ females from Israeli and Egyptian Manzala strains [49]. Feminization was performed for 40 days, using 100–200 mg of EE/kg of food [165].

The F1 ZZ females could be identified by progeny testing when the sex ratio was significantly different from the expected (1 : 1) ratio. Only ZZ females that gave 100% males were used to create the successive generations (F2, F3, etc.) by feminizing them. This production was successfully applied at a small commercial scale in Reunion Island (France) (Figure 9.8). In the fifth generation, ZZ females generated 97–100% male progenies [164]. It is important to take into account that the fecundity of ZZ females is affected, since they spawned 35% less than normal ZW females [166].

9.7 Future Approaches for Sex Control in Tilapias

9.7.1 Precocious Identification of the Sexual Phenotype

Our search for sexually dimorphic markers led us to study the brain simultaneously with the gonad, during *On* sex differentiation. We observed sex differences in the activity of the brain aromatase enzyme, which was suppressed with temperature-masculinizing treatments [66]. We have subsequently found that the *amh* gene expression is sexually dimorphic in male brains between 10 and 15 dpf [78]. Our analyses revealed that *amh* expression levels at 14 dpf could be used to discriminate the sex ratio of a progeny precociously (Poonlapdech et al., unpublished data). We were able, for instance, to differentiate precociously males from females in offspring from the Manzala strain, or the Japanese strain (Fig 9A), as well as in wild

populations (i.e., Lake Kou from Burkina Faso [data not shown]), using *amh* expressions from individual heads (D'Cotta *et al.*, unpublished data).

Amh prediction of phenotypic males at 14 dpf was highly correlated to the proportions of males analyzed at 3–4 months in most families. Some exceptions have, nevertheless, been encountered, like in some GIFT families from Worldfish, where *amh* values were too low, which might have been due to early sampling or a somewhat lower growth rate at 14 dpf. Delay of a few dpf might be required for some strains/populations in evaluating *amh* expression peaks.

In temperature and hormonal treatments, the *amh* analyses are not done at 14 dpf, but have to be performed later (D'Cotta *et al.*, unpublished data). Precocious phenotypic sexing could considerably accelerate progeny testing in selection programs – for instance, selecting YY males that give 100% males, evaluating sex ratios rapidly by testing several YY males crossed with different dams. It also allows a more rapid analysis of the male proportions of thermosensitive breeders and, hence, could be useful for the selection of a thermosensitive line. A precocious phenotypic sexing could also be a means of guaranteeing that a hormonal treatment is being efficient.

9.7.2 Genotypic Sexing

Because sex in *On* is under the control of sex chromosomes, but also under the control of genetic parental factors and temperature, mismatches between the genotypic sex and phenotypic sex are common. Consequently, both phenotypic and genotypic sexing are required to follow natural sex-reversed tilapias, and those induced by treatments. Genotypic sexing of Nile tilapia is still not totally reliable, since the sex determinant appears to differ, depending on the strain or population [45, 69, 75, 82]. Nevertheless, we were able to accurately genotype the sex with the Y-linked *amhy* gene and the truncated *amhΔy* gene located on LG23 in the Japanese

strain [82] and in most known genotypes of our Tihange-Manzala strain, as well as in a semi-domesticated strain from lake Kou (Burkina Faso) (Figure 9.9B).

In this last strain, two XX males could be identified with the *amhΔy*-233 marker, and this was subsequently confirmed with progeny testing (Sissao *et al.*, unpublished data). These genotypes matched the phenotypic sexing done with *amh* expression at 14 dpf (shown for the Manzala in Figure 9.9A). We nevertheless found cases where the *amhΔy* gene was not systematically linked to the *amhy* marker (*amhDel5* in the promoter), such as in the wild populations of Ethiopia [7]. Additional analyses with other markers are needed to see whether sex is associated in these families/populations to LG1. Genotypic sexing can also be performed using SNPs from LG23, as well as those identified on LG1 that are strongly associated to sex [75, 101], with the KASP method, where different fluorescent primers are used for each allele.

9.7.3 Epigenetics of Sex

Epigenetic marks, together with genetic variability, are transmitted across generations, affecting the phenotype of a progeny. The environment might be changing tilapia sex through epigenetic modifications, affecting DNA without changing the nucleotides. Epigenetics modifications can involve chromatin folding, the way DNA is packaged around nucleosomes, can modify the histones, and can cause DNA methylation that will silence or activate gene expressions, as well as involving non-coding RNAs that regulate post-transcription, ultimately affecting the phenotype.

Higher DNA methylation levels of cytosines at CpG dinucleotides were found on the promoter of the aromatase *cyp19a1a* gene in gonads of temperature-treated European sea bass, *Dicentrarchus labrax*, which were correlated with lower *cyp19a1a* expression [167]. In this species, early temperature treatments >17°C are associated with higher male proportions.

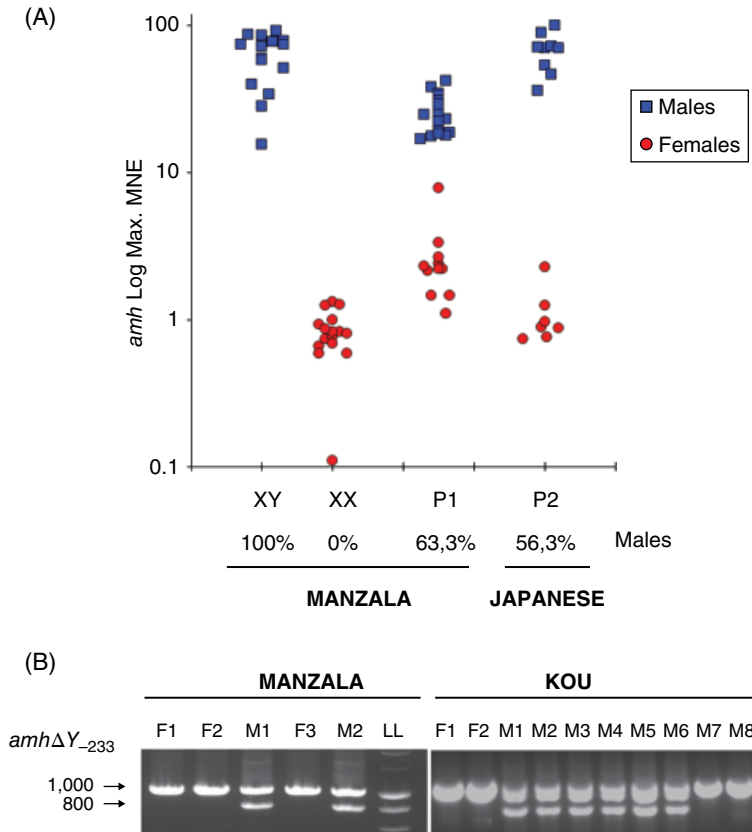


Figure 9.9 A: *Amh* expressions in Nile tilapia's heads at 14 dpf in three Manzala progenies treated at 27 °C: a monosex male XY, a monosex XX, and a mixed sex (P1), where the *amh* expressions identified 100%, 0% and 63.3% males, respectively. A mixed sex progeny (P2) from a Japanese strain where *amh* identified 53% males. All *amh* sex ratios correlated with the male sex ratio obtained by gonadal squash at four months of age. Values are represented as Log of Percent Maximum Mean Normalization Expression (MNE).

B: PCR genotyping of males (M) and females (F), using the *amh*ΔY 233 marker (Eshel *et al.*, 2014). The 800 bp band corresponds to the 233 bp deleted *amh*ΔY gene, associated to the Y chromosome (chr). The 1,000 bp band corresponds to two putative *amh* genes: the X-chr *amh*, and the sex determining Y-chr gene *amh*Y (Li *et al.*, 2015). Left gel corresponds to a Manzala mixed-sex progeny, with the absence of the *amh*ΔY band in XX females. Right gel corresponds to adult wild Kou fish, showing a mismatch of the sexual phenotype with the Y linked *amh*ΔY marker in M7 and M8 by the absence of the 800 bp band, suggesting they were XX males. (Sissao *et al.*, unpublished data).

Temperature treatments caused higher methylation levels in two CGs in both males and females, whereas methylation sex differences were observed in seven CGs, with the hypermethylation apparently blocking the activation sites of *Sf1* and *Foxl2* [167]. In the half-smooth tongue sole, 28 °C temperatures can override the ZZ/ZW genetic sex determination, inducing 73% masculinization of ZW females that had a demethylation of the *dmrt1* promoter [168].

Some epigenetic studies have been initiated in tilapia. A global methylation study was performed in *On*, revealing that females had higher levels of DNA methylation in several chromosomes, compared with males [169]. High temperature treatments resulted in higher methylation levels in induced males, with 1,100 differentially methylated regions (DMRs) found in gene bodies and promoters. Higher methylation levels were found, for instance, in the *dax1* (*nr0b1a*)

gene, involved in the aromatase/estrogen levels, while the male pathway gene *gsdf* had lower levels. A methylome analysis, together with RNAseq performed in the *On* × *Om* hybrid, revealed 4,757 sexually dimorphic DMRs in the skeletal muscle, with many located in LGs associated to sex determination [170]. High levels of hypermethylated DMRs were found in males, particularly on LG1, but they were also male-biased in LG23, whereas they were female-biased on LG7, LG16-21, LG18, and slightly on LG3.

The simultaneous analyses of RNA transcripts and microRNAs has recently been performed, comparing *On* XX and XY gonads at 9 dpf, and finding 635 miRNAs, of which 130 novel miRNAs had sex-biased expression [171]. These act on the gonads as single miRNA, targeting either a single or multiple 3' UTR position, or multiple miRNAs might be targeting various 3' UTR sites on the same gene. Nine miRNAs (among which are the miR-30 family members) were downregulated in females, and predicted to regulate *Cyp19a1a*, while seven miRNAs were downregulated in males and predicted to target *dmrt1*.

Since the epigenetic marks could be transgenerational, they should be better studied, so that they are also integrated in a genetic sex selection or thermosensitivity breeding program. Targeted epigenetics could also be a way to shape the sexual phenotype.

9.7.4 Genome Editing: CRISPR/Cas9 Technology

Exploring the function of a gene and performing genetic editing are important to define gene roles in the sex-determining or differentiating pathway. Several different genome-editing technologies have been created recently to change specific regions of a targeted gene. CRISPR/Cas9 (clustered regulatory interspaced short palindromic repeats) has supplanted TALEN (transcription activator-like effector nucleases) and zinc-finger nucleases (ZFNs), due to its low cost and extreme efficiency in directed mutations,

transmitted via the germline, that can be effective in just one generation [106]. Complete knockout can be achieved with low levels of mosaicism.

CRISPR/Cas9 was used as a means in *On* to validate the critical master role of the *amhy* gene for maleness and its receptor *amhr2* gene [82]. There are currently many ethical debates on whether a CRISPR individual is considered a genetic modified organism (GMO) or not, since no foreign DNA is introduced. Changes in phenotypes can be achieved and improved with CRISPR/Cas technology, so that it could be a means to generate all-male populations.

9.8 Conclusion and Perspectives

The group of tilapias (5.3 million tons) is of major importance for world aquaculture, both in southern and northern countries (>135 countries). Most of the tilapia farms rely upon male monosex populations that are mainly (92%) produced through hormonal treatment, using the synthetic androgen, MT. In use since the 1970s in most tropical countries, these treatments still raise controversial environmental and health issues and, consequently, have already been banned by several countries. As underlined by our review, considering that little is known about the catabolism of MT (conjugation pathways), and even less about the possible bioaccumulation and bioavailability of the androgen and/or its residues (especially glucuronide and sulfate conjugates) in complex aquatic systems, it is not surprising that these controversies persist.

Environmentally friendly products are becoming more and more popular for consumers, as well as the development of policies for protecting the citizens and the environment. Therefore, it is important to better characterize the fate of MT and its residues under various aquaculture conditions, in order to better evaluate the environmental and health issues associated to the hormonal treatments, to suggest measures

for mitigation or/and remediation, avoiding any possible bioaccumulation/bioavailability of residues in specific environment conditions, and also to propose more sustainable alternative methods to produce male monosex populations in tilapia farming.

Future directions should focus on the following priorities:

- **Better understand the fate of MT and its residues under various aquaculture conditions:**

- *Towards a possible remediation of MT sediment contamination through pond/sediment management*

As suggested in our review, MT degradation can be hastened or delayed by qualitative and quantitative traits of the pond sediment/soil. A better characterization of the sediment ecosystem should allow modulation of steroid-particle interactions and the development of strategies of bacteria “domestication,” in order to favor natural MT degradation through pond/sediment management (aerobic or sulfate-reducing conditions).

Additional methods can be used to remove or degrade MT and its residues from the water of treatment ponds or hatcheries, before their discharge into receiving waters/watersheds. Filtration on powder or granular activated carbon is effective for steroid adsorption (commonly used in sewage treatment plants), and could be easily installed at the pond/hatchery outlets. Although more difficult to be used under pond-based aquaculture systems, ozone treatments can efficiently degrade steroids, and could be used for treatments in recirculating systems (hatchery). Finally, aerated lagoons efficiently remove hormone contaminants from waters.

- *MT catabolism*

As reported in this review, various studies have demonstrated the rapid elimination of MT in fish, as well as in water (in soil, it will depend upon the sediment and water characteristics). However,

besides this parent compound, little is known about MT catabolism in fish. Approximately 10 free metabolites have been tentatively, but not definitively (lack of references), identified in rainbow trout [126]. However, in vertebrates, glucuronidation and sulfonation of hydroxyl groups are important pathways of steroid catabolism [172]. Among the few glucuronide metabolite residues identified in fish, OHMT-glu (a glucuronide of a hydroxylated MT) may persist much longer than MT, at least in the bile in tilapia [173], suggesting that further studies have to be done on other MT glucuronides. Moreover, other classic metabolites in vertebrates, like sulfate conjugates, have not been analyzed in fish.

- *Bioaccumulation and Biotransporters*

Although MT is supposed to be rapidly eliminated from treated fish and water, recent detection of important MT concentrations in crocodile eggs, hatchlings, and adults around some Costa Rican tilapia farms [140] raises many questions. The main hypothesis of these authors relies upon the possible role of biotransporter that wild tilapias could play through a possible MT storage in its adipose tissues (sulfate conjugate). While free MT elimination from aqueous matrices has been well described, little is known about its persistence in a hydrophobic environment, such as the adipose tissue [141]. As tilapia is a common prey for crocodiles, these reptiles will consequently bioaccumulate MT (brought by the biotransporter fish) in their adipose tissues. In female crocodiles, conjugated MT could be mobilized during oogenesis, and deposit in future eggs. Sulfate conjugates can be easily reactivated as an active steroid, and used by crocodile hatchlings. Similarly, we believe that conjugated MT, potentially stored in the adipose tissues of wild female tilapias, could also be mobilized during oogenesis, deposited in the future eggs, and perhaps influence their sex differentiation,

because early treatments by steroids have been demonstrated to sex-reverse part of the fry [163].

Therefore, it is important to verify that tilapia could accumulate MT in its adipose tissue, and to analyze its persistence, as well as the transfer mechanisms of maternal derived steroids to yolk and embryos and the reversibility between sulfate conjugates and active steroids, especially during the critical periods of sex differentiation.

- **Recommendations for best practices**

Skin contact with MT has to be avoided (use gloves and face masks). At least when indoor hatcheries are used for hormonal treatment, but also when the water of treatment ponds is discharged toward receiving waters/watersheds, treat the water effluents. Provided that strict/rigorous management is applied, a dose of 50 mg MT/kg feed is sufficient for an efficient sex reversal treatment (99–100%). Do not increase unnecessarily the amount of MT that will be used and released into the environment. Farm workers distributing the MT feed should wear gloves and protective boots/clothing if they enter into the ponds (Figure 9.5E) to collect the fry when treatment is completed, or when they clean the bottom of the treatment ponds.

- **Develop alternative methods to produce male monosex populations in tilapia farming**

The development of genetic or environmental approaches has been hampered by the complex sex determination of the Nile tilapia (interactions between major genetic factors, parental effects, and temperature influences). The development of various genomic resources, including the whole genome sequence of the Nile tilapia [73],

has strongly contributed to better characterize tilapia sex determination, and to identify sex-specific markers, as well as QTL associated with thermosensitivity. Powerful tools have been also developed for the characterization of tilapia species and wild populations. Altogether, these tools and knowledge should allow to optimize the development of specific strains for sustainable genetic (YY males), or environmental (temperature) treatments to control sex that will progressively replace the present hormonal sex-reversal technique.

Both approaches rely upon selective breeding programs that have to be developed at a regional level, and using local genetic resources, rather than transferring commercial strains that are not already present in the countries. Concerning the YY male approach, YY males can be sold to the farmers; conversely, temperature treatments have to be centralized in accredited and dedicated structures that will ensure rigorous and reliable treatments; markers of thermosensitivity will allow checking of the thermosensitivity, whereas markers of phenotypic sex will allow prediction of future sex ratios. Temperature-treated populations will then be sold to the farmers.

Acknowledgments

The authors are greatly appreciative, and would like to thank the editors of this book, and most particularly Francesc Piferrer, for inviting them to write this chapter. Part of our tilapia work was supported by two projects funded by the Agence Nationale de la Recherche (ANR), SEXTIL-ANR-11-EMMA-033 and CLIMSEX-ANR-15-CE02-0012.

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10

Quantitative Genetics of Sexual Dimorphism in Tilapia and Its Application to Aquaculture

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10.1 Introduction

Females and males of all living organisms differ in many biological characteristics, ranging from fitness, health, and diseases to behavior, physiology, morphology, weight, and growth [1]. This is primarily a result from natural or sexual selection pressurizing differently on males and females, due to their contrasting roles in reproduction or competition for resource utilization [2, 3]. In both terrestrial and aquatic animal species, considerable attempts have been made to understand the sexual dimorphism in body size because, for instance in tilapia, males grow faster and have a greater fillet yield than females [4] and, therefore, reach a marketable size at an earlier age [5]. As a consequence, farmers that only use males can produce more fish crops per year, reduce production costs per unit of culture and, hence, increase economic returns.

Quantitative studies have examined the genetic architecture of sexual size dimorphism (SSD) for growth-related traits such as body weight or daily weight gain. Understanding the genetic basis of sexual dimorphism in aquaculture species could provide information to enable the design of cost-effective selective breeding programs. If the expressions of body traits in both sexes are determined to a large extent by different

genes, female and male expressions should be treated as genetically different traits. When the genotype by sex ($G \times S$) interaction is significant, separate selective breeding programs may be needed for females and males.

To date, multivariate analysis of genetic parameters across species shows that there is no sex-specific (co)-variance for growth related traits, and that these characteristics may be under similar genetic control. This was indicated by the high, and close to unity, genetic correlations between the trait expressions in females and males such as in laboratory model organisms [6], fish [7], and/or farmed animals [8].

To gain a better understanding with regard to quantitative genetic basis of SSD in Cichlid, tilapia was chosen as a model species to study here. The main aim of this chapter is to:

- i) evaluate sexual size dimorphism among major tilapia species;
- ii) examine variation in SSD between populations within a strain/species;
- iii) study heritability for body weight in female and male;
- iv) assess genotype by sex interaction;
- v) measure genetic changes in body weight of female and male to selection for high growth; and
- vi) investigate genetic architecture of SSD in diverse culture environments.

In addition, the importance of SSD for other traits of commercial importance is also discussed.

10.2 Variation Between Species

Figure 10.1 presents percentage difference in body weight between females and males of important tilapia species. Among the species studied, Nile (*Oreochromis niloticus*, L.), black (*O mossambicus*), and red (*Oreochromis spp*) tilapias are the three candidates being widely cultured in aquaculture systems, and contributing about 71% of the global tilapia production (4,207,900 metric tons, with an estimated value of \$6,923 million in 2012) [9].

The differences in sexual size dimorphism (SSD) between the major tilapia species varies remarkably, being as low as 15.3% in GIFT (Genetically Improved Farmed Tilapia), and as high as 47.8% in the Egyptian

blue tilapia strain [10]. The large magnitude of the SSD difference between species is likely due to a range of factors involved, such as history of population, past selection, and management. Further, the tilapia populations used in this study were tested in different locations/countries at different times, and under diverse growing systems.

To enable a relative comparison of commercial tilapia strains, Thoa *et al.* (2016) conducted a series of systematic experiments to evaluate three genetic lines (salinity tolerant, GIFT-derived strain, and red tilapia) under two culture environments (fresh, 0ppt, and brackish water, 15ppt). The experimental animals were synchronously produced and tested simultaneously under the same rearing conditions and management practices.

In addition to the between-strain and between-environment differences in the magnitude of SSD, male Nile tilapia had 12.5–26.8% greater body weight than that of females. Also, there were significant

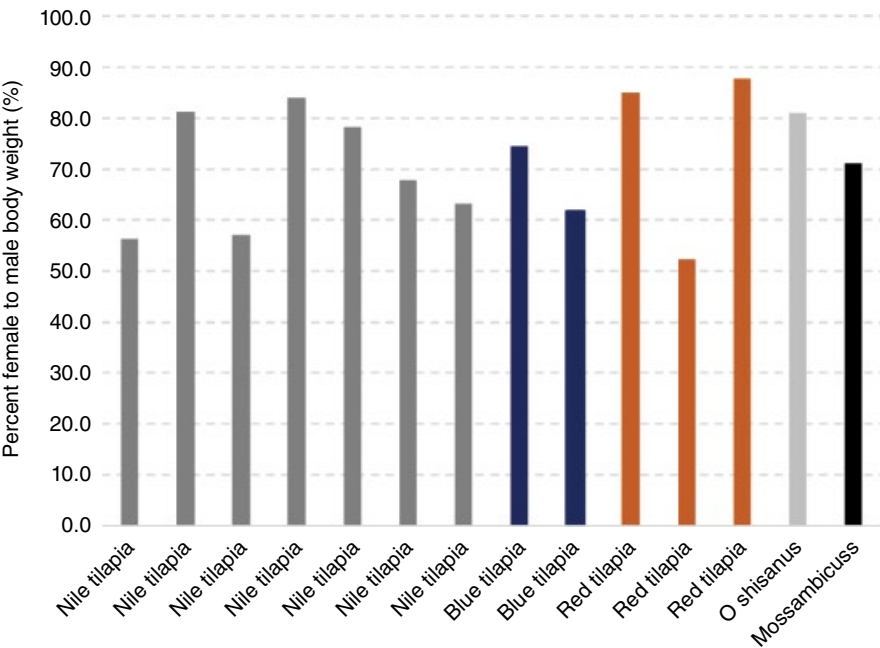


Figure 10.1 Between-species differences in sexual size dimorphism, expressed as percentage female to male body weight for Nile tilapia, *O. niloticus* [11, 19, 23, 29, 31, 35, 36], Blue tilapia, *O. aureus* [37], red tilapia, *O. spp.* [38, 39], Indigenous tilapia, *O. shiranus* [10], and *O. mossambicus* [40]

differences in maturity between the two environments, ranging from 9.7% in freshwater to 15.8% in brackish water. Between-sex differences were also found for fillet weight in both fresh and brackish water, and varied between 12.1% and 19.5%. To date, no relevant literatures on the sexual difference in maturity and fillet weight between diverse culture systems are available to compare with the results of this study.

Collectively, the findings of Thoa *et al.* (2016) indicated that the sexual differences in many biological characteristics varied with strains and the environments to which they were subjected – although many other factors, such as between-sex differences in behavior/social interaction and/or osmoregulation, may have been involved.

10.3 Differences Among Populations Within a Species

In addition to the between-species difference in SSD, variation among populations within a strain/species was also investigated. Here, the GIFT strain of Nile tilapia (*O. niloticus*) was chosen to illustrate sexual size differences within a strain. Due to the superior characteristics of the GIFT fish, they have been disseminated to major tilapia-producing countries, such as in Bangladesh, China, Philippines, Thailand,

or Vietnam. In these countries, the breeding nucleus of the GIFT strain has been maintained, and selection has been ongoing to continue to improve production characteristics. Routine data collection for important traits, such as body weight, was made during the course of the selection programs, to enable a rigorous statistical analysis. The SDD variation, calculated as percentage of female to male body weight, ranges from 69–84% for the GIFT strain being reared in different countries and environments (Table 10.1). In different terms, the SSD variation between populations of the GIFT strain was between 16 and 31%.

10.4 Heritability for Growth-Related Traits in Females and Males

To explore the additive heritable genetic variation in sexual dimorphism, trait expressions in females and males were treated as if they were different traits, and statistical analyses were carried out separately in females and males, in order to examine whether there were differences in heritability between sexes (see Box 10.1 for a detailed method of heritability estimation). Across populations, the heritabilities for body weight were not significantly different between females and males. One exception is

Table 10.1 Variation in sexual size dimorphism among populations of the Genetically Improved Farmed Tilapia strain *Oreochromis niloticus*.

Strain/pop	Reference	Offspring	Sire	Dam	Gen	Env	(F/M) %
China	Zaijie <i>et al.</i> (unpublished)	22,975	439	502	5	FWP	78.3
Bangladesh	Kohinoor <i>et al.</i>	20,895	505	514	9	FWP	76.4
Malaysia	Hamzah <i>et al.</i> (2014)	33,812	598	853	9	FWP	84.0
Vietnam	Thoa <i>et al.</i> (2016)	36,145	564	754	8	SWP	78.2
Brazil	Olliveria <i>et al.</i> (2016)	8,725	184	255	5	FWC	67.9

Zaijie *et al.* (unpublished), Kohinoor *et al.* (unpublished).

Gen = number of generations, Env = environment, FWP = freshwater pond, SWP = saline water (15–20 ppt) pond, and FWC = freshwater cages

the study of Rutten *et al.* [11], who reported greater heritability for females than males (0.60 vs. 0.26, respectively). The estimate in females can be considered as an outlier, due to the small sample size and shallow pedigree in comparison with other studies presented in Table 10.1.

In addition to separate estimates of heritability in females and males, the heritabilities for body traits were also jointly analyzed for the two sexes. In these analyses, the across-sex estimates of heritability were generally slightly greater than those in the single trait analyses in all studies (Table 10.2). However, there were no statistically significant differences in heritabilities when the expressions in both sexes were treated as a single trait or when they are analyzed separately. In addition to the additive genetic variances, the maternal and common environmental effects for growth related traits were almost identical in females and males (Table 10.2). In either single or combined analyses, the heritabilities

were moderate to high, ranging from 0.14–0.36 (Table 10.2) and significantly different from zero ($P < 0.05$ –0.001).

The similarity in the estimates of heritability and the amount of additive genetic (or phenotypic) variance for all traits in the two sexes (Table 10.2) indicates that female and male expressions of body traits will respond to selection in the same way. As a corollary, it also indicates that there are no differences in the sensitivity to the environment between females and males. These results support the hypothesis that there is no sex-specific response to selection. The conditional additive genetic variance ($V_{A(y|x)}$) for body trait in sex y that is conditioned upon the genetic variance of the same trait in the other sex x and their genetic covariance [12] was small relative to the actual values, and almost identical between females and males. The amount of the additive genetic variance in one sex that was independent of the other sex was trivial and, thus, the potential of a trait to respond

Table 10.2 Heritability ($h^2 \pm S.E.$) and maternal and common environmental effects ($c^2 \pm S.E.$) for body weight in female and male tilapia.

Reference	Species	Sex	Offspring	Sire	Dam	Mean	h^2	c^2
Rutten <i>et al.</i> (2005)	Tilapia	Female	1,024	51	69	418.7	0.60 ± 0.18	0.02 ± 0.06
		Male	1,459	51	69	744.0	0.26 ± 0.14	0.10 ± 0.06
		Both	2,483	51	69	609.8	0.26 ± 0.14	0.09 ± 0.06
Nguyen <i>et al.</i> (2007)	Tilapia	Female	6,582	232	340	168.3	0.36 ± 0.05	0.18 ± 0.02
		Male	5,726	232	340	206.8	0.33 ± 0.05	0.20 ± 0.02
		Both	12,308	232	340	188.9	0.35 ± 0.05	0.18 ± 0.02
Bentsen <i>et al.</i> (2012)	Tilapia	Female	24,909	461	815	154.0	0.10 ± 0.02	0.14 ± 0.02
		Male	18,157	461	815	270.0	0.10 ± 0.02	0.17 ± 0.02
		Both	43,066	461	815	212.0	0.10 ± 0.02	0.15 ± 0.02
Oliveira <i>et al.</i> (2016)	Tilapia	Female	4,496	188	255	642.4	0.60 ± 0.05	0.05 ± 0.05
		Male	4,207	188	255	418.8	0.53 ± 0.05	0.06 ± 0.05
		Both	8,725	188	255	534.3	0.49 ± 0.05	0.07 ± 0.02
Kause <i>et al.</i> (2003)*	Rainbow trout	Female	12,862	340	552	1,071.0	0.23 ± 0.02	0.02 ± 0.01
		Male	15,023	340	552	1,144.4	0.25 ± 0.03	0.03 ± 0.01
		Both	27,885	340	552	1,107.7	0.23 ± 0.03	0.03 ± 0.01

*included as a reference to compare with tilapia

in one sex independently of the other is very low. Our results suggest that sex-specific response or evolution has not occurred for body traits in selection programs for high growth. This conclusion can be predicted based on the genetic correlations for the expressions of body traits between sexes:

$$V_{A(y|x)} = V_{A(y)} - \frac{(Cov_{A(xy)})^2}{V_{A(x)}}$$

where $V_{A(y)}$ and $V_{A(x)}$ denotes the additive genetic variance in sexes y and x , respectively, and $Cov_{A(xy)}$ is the additive genetic covariance between the sexes.

Box 10.1 Method to estimate heritability in females and males

Examining heritability for homologous trait expressions in female and male provides useful information with regard to possible genotype by sex ($G \times S$) interaction in relation to scaling effect. The scaling effect is a result of heterogeneities in the additive genetic variance between the two genders. Note that, with the scaling $G \times S$ effect, there is no change in the ranking of individuals between females and males.

In complex pedigreed populations, heritability (the observed variations that are due to genetics) for trait expressions in females and males is often estimated using Restricted Maximum Likelihood Method (REML) in a univariate mixed model [13]. In a matrix notation, the mixed model is written as:

$$\mathbf{y} = \mathbf{X}\mathbf{b} + \mathbf{Z}\mathbf{a} + \mathbf{W}\mathbf{c} + \mathbf{e} \quad (1)$$

where \mathbf{y} is the vector of observations for trait expressions in females and males; \mathbf{b} is the vector of all possible systematic fixed effects such as generation, testing environments, and age of the animals.

Vector \mathbf{a} is the random animal additive genetic effects $\approx (0, \mathbf{A}\sigma_a^2)$, where \mathbf{A} is the additive genetic (numerator) relationship matrix among the animals, \mathbf{c} is the vector of dam effects (or maternal effects) $\approx (0, \mathbf{I}\sigma_c^2)$, and \mathbf{e} is the vector of residual effects $\approx (0, \mathbf{I}\sigma_e^2)$. The dam component (σ_D^2) is most likely a combination of maternal and common environmental effects (thus, $\sigma_D^2 = \sigma_{M+CE}^2$, referred to as σ_c^2), caused by the separate rearing of full-sib families until individuals reached a suitable size for physical tagging.

\mathbf{X} , \mathbf{Z} and \mathbf{W} are incidence matrices, relating observations to fixed effects, additive genetic

effect of the individual animal and common full-sib effect included in the model, respectively. Under model (1), $\text{var}(\mathbf{a}) = \mathbf{G} = \mathbf{A}\sigma_a^2$. The remaining effects are assumed to be distributed as $\text{var}(\mathbf{e}) = \mathbf{R} = \mathbf{I}\sigma_e^2$, $\text{var}(\mathbf{c}) = \mathbf{W} = \mathbf{I}\sigma_c^2$, where \mathbf{I} is an identity matrix. The expectations of all random effects are zero, $\text{cov}(\mathbf{a}, \mathbf{e}) = 0$ and $\text{cov}(\mathbf{a}, \mathbf{c}) = 0$ and, thus, $\text{var}(\mathbf{y}) = \mathbf{Z}\mathbf{G}\mathbf{Z}'\sigma_a^2 + \mathbf{W}\mathbf{I}\sigma_c^2 + \mathbf{W}'\mathbf{R}\mathbf{W}$.

The mixed model equation for the best linear unbiased estimator (BLUE) of estimable functions of \mathbf{b} and the best linear unbiased prediction of \mathbf{a} and \mathbf{c} are:

$$\begin{bmatrix} \hat{\mathbf{b}} \\ \hat{\mathbf{a}} \\ \hat{\mathbf{c}} \end{bmatrix} = \begin{bmatrix} \mathbf{X}'\mathbf{X} & \mathbf{X}'\mathbf{Z} & \mathbf{X}'\mathbf{W} \\ \mathbf{Z}'\mathbf{X} & \mathbf{Z}'\mathbf{Z} + \mathbf{A}^{-1}\alpha_1 & \mathbf{Z}'\mathbf{W} \\ \mathbf{W}'\mathbf{X} & \mathbf{W}'\mathbf{Z} & \mathbf{W}'\mathbf{W} + \mathbf{I}\alpha_2 \end{bmatrix}^{-1} \begin{bmatrix} \mathbf{X}'\mathbf{y} \\ \mathbf{Z}'\mathbf{y} \\ \mathbf{W}'\mathbf{y} \end{bmatrix} \quad (2)$$

where $\alpha_1 = \sigma_e^2 / \sigma_a^2$ and $\alpha_2 = \sigma_e^2 / \sigma_c^2$.

The variance components obtained from model (2) are used to derive heritability for trait expressions in females and males, as:

$$h^2 = \frac{\hat{\sigma}_a^2}{\hat{\sigma}_a^2 + \hat{\sigma}_c^2 + \hat{\sigma}_e^2}, \text{ and the maternal effect}$$

$$\text{as } c^2 = \frac{\hat{\sigma}_c^2}{\hat{\sigma}_a^2 + \hat{\sigma}_c^2 + \hat{\sigma}_e^2}, \text{ where } \sigma_a^2 \text{ is the additive}$$

genetic variance, the maternal variance (σ_c^2) and the residual variance (σ_e^2).

The REML and mixed model approach have been implemented in several software packages, such as *ASReml* version 4.0 [14]. *ASReml* provides flexibility to specify different co-variance structures or different fixed and random effects for each sex, to avoid any possible bias associated with the heritability estimates.

10.5 Genetic Correlations Between Sexes

In addition to the scaling effect (i.e., the differences in heritability between female and male – Section 10.4 and Box 10.1), the $G \times S$ interaction that is due to re-ranking effect is critical in both terrestrial and aquatic animal species. One way of approaching the study of the re-ranking $G \times S$ interaction effect is by treating the expressions of a trait in each sex as if they were different characters. Then, the estimates of genetic correlations between performances in different sexes can be used as a measure of the $G \times S$ interaction.

The genetic correlations for homologous traits between the two sexes are often estimated using a multi-trait analysis approach (Box 10.2). With this approach, the estimates were achieved through genetic relationships in the pedigree. However, there were no environmental covariances between the homologous traits, as phenotypical measurements of body traits were made on different animals. The multi-trait statistical model was basically the same as those used to estimate heritability (equation [1] in Box 10.1).

Figure 10.1 shows the genetic correlations of body traits between the trait expressions in both sexes. The genetic correlations were very high (0.91 to 0.96), and were not significantly different from unity ($z = -0.04$ to -0.08 , $P > 0.97$). A similar trend was also observed for the maternal and common

environmental correlations. The near-unity genetic correlations suggest that the expressions of body traits in females and males are controlled by a similar set of genes. Our results, together with the published information, are consistent with the between-sex genetic correlation estimates in other aquaculture species, such as common carp [15] or banana prawn [16].

These genetic correlation estimates for homologous traits between the two sexes also suggest that there is no genotype by sex interaction for body traits in fish. Hence, female and male expressions of body traits in tilapia can be safely treated as the same trait in practical breeding programs. However, when the traits exhibit heterogeneous variances between the sexes, appropriate transformation, such as using linear regression of phenotypic standard deviation (or variance) to a mean value, a multiplicative (anti-log) mixed model or a log-linear model [17] should be applied to account for possible bias in genetic evaluation systems.

Further, the close-to-one genetic correlations for the expressions of body traits between sexes impose a constraint on selection for sexual dimorphism. The response to selection for sexual dimorphism (R_{SD}) is defined as the difference of male and female response [18]:

$$R_{SD} = \frac{1}{2} \left[h_M^2 \sigma_{PM} i_M - h_F^2 \sigma_{PF} i_F + h_M h_F r_G (\sigma_{PM} i_F - \sigma_{PF} i_M) \right] \quad (3)$$

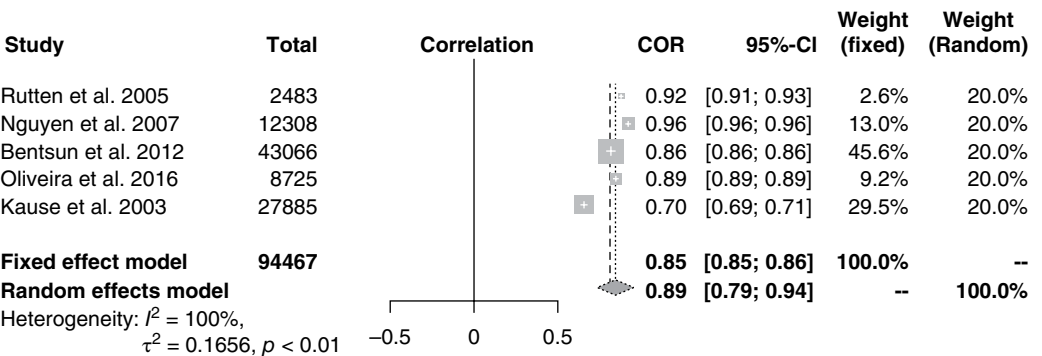


Figure 10.2 Forest plot of genetic correlations for body weight between sexes.

Box 10.2 Estimation of genetic correlations for the same trait between sexes

The large variation in performance of genotypes between sexes may cause possible genotype by sex interaction ($G \times S$). The $G \times S$ interaction is due to scaling and re-ranking effects. Scaling effect results in heterogeneous variance in traits recorded in females and males (see Box 10.1). Re-ranking effects impact selection decision, because animals are ranked differently between the two sexes. To examine the re-ranking $G \times S$ interaction effect, a multivariate mixed model approach was applied to estimate genetic correlations between performances in females and males, and the between-sex genetic correlations can be used as a measure of the $G \times S$ interaction [20]. The $G \times S$ effect may not be significant when the genetic correlation estimate between homologous traits is greater than 0.8. By contrast, the smaller than 0.80 genetic correlations between the two sexes indicate that the $G \times S$ could be biologically important. A multi-trait (bivariate) model was used to obtain (co)variance components for traits recorded in females and males, as follows:

$$\begin{bmatrix} y_f \\ y_m \end{bmatrix} = \begin{bmatrix} X_f & 0 \\ 0 & X_m \end{bmatrix} \begin{bmatrix} b_f \\ b_m \end{bmatrix} + \begin{bmatrix} Z_f & 0 \\ 0 & Z_m \end{bmatrix} \begin{bmatrix} a_f \\ a_m \end{bmatrix} + \begin{bmatrix} Z_f & 0 \\ 0 & Z_m \end{bmatrix} \begin{bmatrix} c_f \\ c_m \end{bmatrix} + \begin{bmatrix} e_f \\ e_m \end{bmatrix}$$

The subscript letters f and m stand for females and males, respectively. In these bivariate models, additive genetic effects (a) were assumed $\approx N(\mathbf{0}, \mathbf{G} \otimes \mathbf{A})$, the effect common to full-sib (c) $\approx N(\mathbf{0}, \mathbf{C} \otimes \mathbf{I})$, and the residuals (e) $\approx N(\mathbf{0}, \mathbf{R} \otimes \mathbf{I})$, where \otimes is the Kronecker direct product. As traits were recorded on individuals of different sexes, there was no environmental covariance between traits and, therefore, \mathbf{R} was assumed to be a diagonal matrix. The assumed co-variance structure is:

$$\text{Var} \begin{bmatrix} \mathbf{a} \\ \mathbf{c} \\ \mathbf{e} \end{bmatrix} = \begin{bmatrix} \mathbf{G} \otimes \mathbf{A} & 0 & 0 \\ 0 & \mathbf{W} \otimes \mathbf{I} & 0 \\ 0 & 0 & \mathbf{I} \sigma_e^2 \end{bmatrix}$$

The genetic correlations between trait expressions in females and males (r_{MF}) are calculated as:

$$r_{MF} = \frac{\sigma_{MF}}{\sqrt{\sigma_M^2} \sqrt{\sigma_F^2}}$$

where σ_{MF} is the estimated additive genetic or phenotypic covariance between the two sexes, and σ_M^2 and σ_F^2 are the additive genetic or phenotypic variances of traits in males and females, respectively.

where subscripts M and F refer to male and female parameters and h^2 represents heritability, while h is the square root of heritability, σ_P is the phenotypic standard deviation, i is the selection intensity, and r_G is the genetic correlation between the trait expression in the two sexes.

By using genetic co-variance components estimated in the GIFT strain [19], our theoretical calculation (Equation 10.3) showed that the predicted responses to selection for sexual dimorphism for all traits were close to zero, assuming that the same selection intensity was applied in both sexes ($i_M = i_F = 1$). Sensitivity analyses to more extreme differences in the selection intensity of females

(10%) and males (1%) also gave a small R_{SD} (-0.006 to $0.128 \sigma_P$ across traits). It can be concluded that there is very limited prospect for selection to reduce sexual dimorphism in tilapia, or in fish in general.

10.6 Can Sexual Size Dimorphism be Altered by Selection for High Growth?

Estimated breeding values (EBVs) for body traits in females and males can be used to measure genetic changes in sexual size dimorphism due to selection for high growth

(see Box 10.3). Here, I present the results from three independently large datasets:

- 1) an eight-generation pedigreed population of Nile tilapia, selected for high growth under moderately saline water (referred to here as salinity tolerance line);
- 2) a long-term 10-generation selection for increased harvest body weight in the GIFT strain in Malaysia; and
- 3) a genetic improvement program for Nile tilapia in Bangladesh from 2015 to 2016 (Kohinoor *et al.*, unpublished).

In brief, the salinity tolerance line originated from a selection program over eight generations (2007–2014) for increased harvest body weight in moderately saline water (15–20 ppt). There was a total of 36,145 animals, with individual body traits records collected over eight generations from 2007–2014. These were the offspring of 564 sires and 754 dams. Within- and between-family selection was practiced, based on estimated breeding values for body weight. About 4.43% of females and 3.48% of males were selected to become parents in each generation [21]. For the GIFT strain, a detailed description with regard to breeding and

selection procedures is given in earlier publications [22–26]. With regard to the breeding program for Nile tilapia at Bangladesh Fishery Research Institute (BFRI), a total of 20,895 fish were performance tested between 2005–2015. These were the offspring of 505 sires and 514 dams (Kohinoor *et al.*, unpublished).

Across the three populations, genetic gain, measured as EBVs in either actual unit of measurements or genetic standard deviation unit, did not differ between females and males (Table 10.3). The similar magnitude of genetic gain estimated in both sexes across the methods used, obtained from univariate or bivariate analyses, is consistent with the prediction made on the basis of high (nearly unity) genetic correlations for body weight between females and males. The non-significant differences in the sexual weight dimorphism traits observed from the selection program for high growth in the three populations of Nile tilapia studied here indicate that reduction in sexual dimorphism for body weight did not occur in selective breeding programs for this species. Collectively, our results also suggest that conducting separate genetic improvement for females and

Table 10.3 Genetic changes in female and male body weight to selection for high growth in tilapia.

Reference	Species	Sex	n	Genetic changes per generation		
				Actual unit	Genetic SD	%
Thoa <i>et al.</i> (2016)	Nile tilapia	Female	11,179	9.48	0.265	3.7
		Male	16,487	10.25	0.287	4.0
		Both	36,145	9.97	0.279	3.9
Hamzah <i>et al.</i> (2014)	Nile tilapia	Female	18,869	6.85	0.281	3.2
		Male	15,414	6.67	0.274	3.1
		Both	33,812	6.91	0.283	3.2
Kohinoor <i>et al.</i> *	Nile tilapia	Female	9,481	8.27	0.623	4.5
		Male	9,414	8.26	0.622	4.5
		Both	18,911	8.29	0.625	4.5

*unpublished. Genetic gain was estimated by regression of individual EBV on birth year (generation), and expressed in actual units (g), genetic standard deviation (SD) unit (i.e., EBV in actual unit/genetic standard deviation of body weight) and percentage of the population mean.

Box 10.3 Measure of genetic changes in females and males

Genetic changes in female and male body weight were estimated using mixed model methodology (as described in Box 10.2) that relies on the presence of genetic connectedness between generations. This is a realistic option in the context of commercial production where a control group is often not maintained in parallel with the selection line due to shortage of resources. Estimated breeding values obtained from the mixed model approach are expected with minimum bias, because the model can account for all possible systematic fixed effects, such as spawning years, culture environments, age of the animals, and the random effects of the additive genetic and common full-sibs. The genetic gain, based on EBVs, can be expressed for each generation, or be cumulative over all generations and/or showing a genetic trend achieved in the population.

- i) Response for each generation: The gain was calculated as the difference in estimated breeding values between successive generations (G): $\text{Gain} = G_n - G_{n-1}$
- ii) Cumulative genetic response: Cumulative genetic response (in percentage) over

generations was calculated using the following formula: $P_c = \prod_{i=1}^n (1 + p_i) - 1$, where P_c is the total genetic response (%); p_i is the genetic response (%) for the i^{th} generation; and i is the generation ($i = 1, 2, \dots, n$). The formula used here accounts for the fact that, as generations progress, there is change if there is genetic gain [27, 28]. Hence, the percentage in each generation is calculated relative to a different mean. Average genetic response (% per generation) was calculated as: $P_a = P_c / n$, where P_c is the cumulative genetic response over n generations.

- iii) Genetic trend: Genetic trend is estimated by linear regression analysis of individual EBV on year of birth or generation [29].

Across the three methods, the genetic changes in sexual size dimorphism can be expressed in the actual unit of measurements (i.e., gram for body weight), genetic standard deviation unit (the ratio of the EBV in actual unit over the square root of the additive genetic variance), or percentage of the population mean.

males in tilapia is not justified, provided that resources are limited in many developing countries.

10.7 Do Genetic Parameters for Sexual Dimorphism Differ Between Culture Environments?

To answer this question, we conducted a systematic study using the salinity tolerance line, selected over eight generations [21, 30, 31]. Offspring of the 2012 generation were also tested in both saline (15–20 ppt) and freshwater. Statistical and genetic analyses were carried out separately for each sex in saline and freshwater environments. Table 10.4 presents

heritability for body weight by sex and testing environments used. There were no significant differences in the heritabilities estimated for females and males between the two environments (saline vs. freshwater). Within each environment, the difference in the heritability estimates between females and males was also not significant.

Further, we estimated the genetic correlations for body trait expressions in females and males for saline and freshwater. In both testing environments, the genetic correlation estimates were high and close to unity (Table 10.5), and there was no statistical difference in the genetic correlation estimate between the two environments.

Both the estimates of heritability and genetic correlations for the trait expressions

Table 10.4 Heritability ($h^2 \pm S.E.$) and common environmental effects (c^2) for body weight of females and males cultured in diverse environments.

Traits	Sex	Brackish water		Freshwater		Both environments	
		h^2	c^2	h^2	c^2	h^2	c^2
Weight	Female	0.44 ± 0.17	0.01 ± 0.05	0.31 ± 0.05	0.22 ± 0.02	0.32 ± 0.04	0.22 ± 0.02
	Male	0.02 ± 0.11	0.18 ± 0.06	0.34 ± 0.04	0.23 ± 0.02	0.34 ± 0.05	0.22 ± 0.02
Maturity	Female	0.12 ± 0.08	0.03 ± 0.03	0.13 ± 0.09	0.02 ± 0.04	0.11 ± 0.06	0.02 ± 0.03
	Male	0.06 ± 0.09	0.12 ± 0.05	0.01 ± 0.03	0.14 ± 0.04	0.13 ± 0.07	0.12 ± 0.04
Survival	Female	0.31 ± 0.04	0.55 ± 0.02	0.12 ± 0.38	0.66 ± 0.19	0.54 ± 0.05	0.14 ± 0.02
	Male	0.28 ± 0.43	0.71 ± 0.21	0.27 ± 0.39	0.71 ± 0.19	0.30 ± 0.49	0.70 ± 0.24

Table 10.5 Across-sex genetic (r_g) and common environmental correlations (r_c) in each testing environment and in both environments.

Traits	Correlation	Brackish water	Freshwater	Both
Weight	r_g	0.97 ± 0.04	0.99 ± 0.23	0.99 ± 0.02
	r_c	0.87 ± 0.02	0.21 ± 0.58	0.92 ± 0.02
SSD	r_g	0.94 ± 0.11	0.93 ± 0.06	0.99 ± 0.01
	r_c	0.26 ± 1.18	0.96 ± 0.02	0.88 ± 0.02
Maturity	r_g	0.86 ± 0.35	0.91 ± 2.91	0.99 ± 0.77
	r_c	0.99 ± 0.42	0.99 ± 0.60	0.99 ± 0.37

in females and males indicate that genetic architect of sexual dimorphism did not change with the two culture environments used in this study. However, note that the genetic line used here has undergone seven generations of selection under a sub-optimal condition (i.e., moderate salinity of 15–20ppt), and their progeny were performance-tested in a conducive culture systems (i.e., freshwater pond). Based on the preposition of Falconer [32], selection under less favorable conditions may produce genotypes that can perform well across production systems.

In a previous study conducted to evaluate the G×E effect for a range of traits, Thoa *et al.* [31] found that the across-environment genetic correlations were high (close to one) for homologous traits, and suggested that the preposition of Falconer was applicable to the

present selected line (i.e., the salinity tolerance line can perform in freshwater systems). To gain a good understanding of genetic change in sexual dimorphism with culture environments, further studies are needed, such as in genetic lines selected in a favorable environment in the nucleus, and their offspring should be performance-tested in hash or low-input farming systems.

10.8 Sexual Dimorphism in Other Traits of Economic Importance

Body weight has been the primary determinant of income and expense at the farm level, and has significant impacts on economic return of aquaculture enterprises; therefore, a majority

of studies reported in the literature have focused on this trait (weight or growth-related characteristics). From the perspectives of commercial aquaculture enterprises, together with the primary economic returns, factors known as yield and production performance, fitness and functional traits (survival, deformity, disease resistance), flesh quality, and many other traits, are also of commercial importance, and they should be considered in future breeding programs for aquatic animal species [33]. To date, there is no published information regarding genetic aspects of sexual dimorphism for these new traits of economic importance. Future research in this area would aid our understanding in developing cost-effective methods, so as to reduce sexual differences in fish and other aquaculture species.

10.9 Concluding Remarks and Suggestions

Quantitative genetic studies show that body weight of females and males is under the control of a similar or same set of genes, and they are genetically dependent. Thus, there is little prospect to reduce sexual size dimorphism through genetic selection. Realized genetic response in female and male body weight from long-term selection programs for high growth has been similar between the

two sexes, and reaffirms our theoretical predictions, based on genetic co-variance components, that there is limited scope to change sexual size dimorphism. To date, genetic basis of sexual dimorphism in other traits of economic importance, such as diseases, fitness, and physiological characteristics, as well as flesh quality attributes or eating characteristics, are not known. Further research that could help our understanding of genetic architecture of SSD in fish may include:

- heritability of functional, fitness, and quality traits in females and males;
- genetic architecture of sexual dimorphism in a range of culture environments or farming systems;
- alternative measures of SSD, such as sexual dimorphism indexes, as proposed in human and model species [34];
- alternative selection strategies to reduce sexual size dimorphism in aquaculture species; and
- new “omic” tools to dissect molecular basis of sexual dimorphism in tilapia and important aquaculture species.

Acknowledgements

The author has received funds from the Collaborative Research Network (CRN) and University of the Sunshine Coast.

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Part III

Sex Determination and Control in Salmonidae

11

Sex Determination and Sex Control in Salmonidae

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11.1 Salmonids Family

Salmonids (Salmonidae family) belong to a basal teleost Protacanthopterygii sub-order (mostly pikes and salmons) group. The following phylogenetic classification has been proposed: Osteichthyes, Actinopterygii, Actinopteri, Neopterygii, Teleostei, Osteoglossoccephalai, Clupeocephala, Euteleosteomorpha, Protacanthopterygii, Salmoniformes, and Salmonidae [1, 2]. The family comprises three sub-families, with 11 genera and about 66 species, but the biological diversity in this family should be greater than what is recognized from current taxonomy [3].

The three sub-families include Coregoninae (whitefish and ciscoes, round whitefishes, beloribitsa), Thymallinae (graylings, one monogeneric group), and Salmoninae (huchen, lenok, trout, char and salmon) (see Box 11.1). Some interspecific hybrids can also be obtained [4]. The oldest known fossil ancestor, *Eosalmo driftwoodensis*, has been found in middle Eocene lacustrine rocks of North America [5, 6]. It has been dated to 50 million years old, but the family origin has been estimated to be 59 million years old [6], dating from the Paleocene geologic period, when the continents drifted farther apart, heading toward their modern positions. All extant salmonids are characterized by an additional specific whole genome duplication,

compared with most of the other teleosts [7], and this duplication dates from around 90–100 million years ago [8, 9]. Since then, major genome rearrangements can be suspected, considering the great disparity in the number of chromosomes within the family [10, 11].

The native distribution of salmonids is restricted to the Northern Hemisphere [3]. However, many salmonid species have been introduced in the temperate waters of the southern hemisphere during the 19th and mainly 20th centuries, raising some serious concerns about natural biodiversity [12].

Life history traits can differ a lot between salmonid species, such as the age of first reproduction, the frequency of reproduction in a lifetime, body size and the fecundity/body size ratio, sexual dimorphism, and parental care [13], although they all spawn in freshwater sites. Many species are anadromous, but a diversity of migratory patterns may co-exist within populations [14]. Juveniles can be sedentary in a river or a lake, migrate from a river to a lake, or migrate from freshwater to the sea. Their anadromy is often linked with a homing behavior, consisting of reproductive adults returning to the place where they were born [15].

The most-well-studied species belong to the *Oncorhynchus* or *Salmo* genus from the salmoninae sub-family (Box 11.1). This is

Box 11.1 Common and scientific names of salmonid species mentioned in the text

Subfamily Coregoninae:

Beloribitsa: *Stenodus leucichthys*
European whitefish: *Coregonus lavaretus*
Lake whitefish: *Coregonus clupeaformis*
Sardine cisco: *Coregonus sardinella*
Vendace: *Coregonus albula*

Subfamily Thymallinae:

Grayling: *Thymallus thymallus*

Subfamily Salmoninae:

Amago salmon: *Oncorhynchus rhodurus*
Arctic charr: *Salvelinus alpinus*
Atlantic salmon: *Salmo salar*
Brook trout: *Salvelinus fontinalis*
Brown trout: *Salmo trutta*

Chinook salmon: *Oncorhynchus tshawytscha*
Chum salmon: *Oncorhynchus keta*
Coho salmon: *Oncorhynchus kisutch*
Dolly Varden trout: *Salvelinus malma malma*
Huchen: *Hucho hucho*
Japanese huchen: *Parahucho perryi*
Lake trout: *Salvelinus namaycush*
Lenoks: Genus *Brachymystax*
Masu salmon: *Oncorhynchus masou*
Pink salmon: *Oncorhynchus gorbuscha*
Rainbow trout: *Oncorhynchus mykiss*
Sockeye salmon: *Oncorhynchus nerka*
Yellowstone cutthroat trout: *Oncorhynchus clarki bouvieri*

especially true for the rainbow trout, which has been considered as an equivalent of a freshwater lab rat model [16]. Like the vast majority of fish, salmonids use external fertilization. Salmoninae species show a gymnovarian type of ovaries (i.e. the ovigerous lamellae open in the coelomic cavity, where mature oocytes are directly released at ovulation [17]). Under low temperature conditions, they can remain unlaidd for a few days, or artificially stripped through the genital papilla [18]. These eggs are demersal, relatively large in size (about 2–6.5 mm), mechanically resistant, and they develop during several weeks (about 200–500 degree-days to reach hatching) at low temperature (usually, about 5–9°C) [19]. All these traits are propitious for experimental treatments during the egg and larvae development, and for their biotechnical applications.

11.2 Salmonid Aquaculture

The oldest known parietal picture of fish is a carved life-sized salmon dated 25,000 years ago on the roof of a cave called “TAbri du Poisson” in southwest France [20]. Salmonids are likely to have been a valuable human food resource for a very long time, including our

prehistoric ancestors from Eurasia [21–23] and North America [24]. During the Middle Ages, European monks reared brown trout (*Salmo trutta*). In the late modern period, salmonid hatcheries were first established in Europe during the second part of the 19th century, in order to enhance fisheries.

However, aquaculture contributed significantly to the food fish supply only during the second half of the 20th century. Trout farming grew slowly until the development of pelleted feeds in the 1950s, while salmon farming began in the 1960s in Norway and Scotland. Later on, public salmon enhancement programs were followed by a significant development of private pen-raised salmon in the 1980s [25]. The level of domestication of several salmonids can now be regarded as high, and this domestication has already impacted significantly on certain life history traits, such as adult body size, egg size, and time spent in the sea [26]. Farmed populations often have a reduced genetic diversity relative to their wild ancestors, and inadvertent genetic changes may arise in these populations [27].

According to the Food and Agriculture Organization (FAO) estimation, the world production of aquaculture was about 7,000 tons for salmonids in 1950, and was mainly

represented by rainbow trout produced in Europe [28]. This world production was 216 times higher at the end of the 20th century, and was mainly represented by Atlantic salmon, which has more than doubled over the last 15 years. In 2015, about 3.4 million tons of salmonids were produced (70% Atlantic salmon, 22% rainbow trout). This was more than three times the quantity of wild captured salmonids and 6.5% of global farmed fish. The two main aquaculture producers were Norway (40% of the production) and Chile (24%).

Today, huge international companies have an integrated value chain with the production and processing. Due to such a high and concentrated production, special attention has to be given to the potentially negative side effects of some breeding and farming techniques, particularly the issues and challenges faced at the level of human health and protection of the environment and animal welfare [27, 29].

Various production systems are used in salmonid aquaculture, including a sea phase for the anadromous species, freshwater open-pen system, land-based freshwater recirculating systems, marine conventional net-pen system, marine floating bag systems, and land-based saltwater flow-through systems. Theoretically, closed-containment systems can reduce proximate environmental impacts, including fish escapees, which will be discussed below [30].

11.3 Why Control the Sex of Salmonids?

The comprehensive review by E. Donaldson and G. Hunter, published in 1982, was probably the first one on this topic [31]. At that time, salmonids aquaculture was at an early stage of development, with a world production of about 200,000 tons (FAO, [28]), and environmental issues were already being discussed [32]. This debate should seriously be considered when developing new fish farming techniques.

Reasons for controlling sex in fish can be diverse, and here we will just focus on those pertaining to salmonids. Both male and female monosex populations of salmonids can be very useful for research purposes, since this makes it possible to work on all-male or all-female populations before any signs of sexual differentiation are visible [33, 34]. However, the farming industry is mainly interested in female monosex cultures, and this demand increased with the development of this economical sector, for various reasons.

At the beginning of salmonid aquaculture, the fish farmer and the consumer were satisfied with portion-sized rainbow trout or pan-sized coho salmon (*Oncorhynchus kitsuch*) – that is to say, about 0.20–0.40 Kg of body weight. At this time and for this size, the number of mature fish was low. Larger salmonids were obtained from fisheries. Then, fish farmers aimed at two goals: first, to improve growth performance; and second, to compete in the marketplace of large salmonids. They were encouraged by the food industry, which wanted to market steaks, filets, smoked slices, and ready meals – products that require processing of large fishes. Starting in the 1970s, family (in Norway, [35]) or individual (in France, [36]) selective breeding programs have been efficiently applied for selecting fast growing salmonid fishes.

Large fish often means mature fish. Furthermore, there is a positive genetic correlation between weight and maturity [37]. Early male maturation in fresh water [38] or after one winter in sea for Atlantic salmon ('grilsing') [39], occurs sometimes at a high frequency in salmonid farming. Unfortunately, the maturation process in salmonids reduces growth rate, causes a deterioration of flesh quality, and increases mortality [40], with an increase of susceptibility to diseases like fungal infections [41].

As a first step, the use of all-female stocks of fish could solve this problem, because females usually mature one year later than males [42]. This strategy can be still applied for 1–2 years of culture. For instance, rearing the all-female diploid Kamloops strain

(Canada) rainbow trout at 12–14°C in a freshwater recirculating system, Davidson *et al.* (2014) got 4.5 kg fish after 22 months at a time when the Feed Efficiency Ratio (kg feed/kg biomass gain) was 1.2 and the gonadosomatic index (GSI = percentage of gonad mass/total body mass) was around 3% [43]. However, this strategy can be inadequate to produce larger fish and, in addition, precocious maturity may happen in females [39]. Thus, methods have been proposed to delay puberty by selective breeding or photoperiod control, but there are some biological or technical limitations [44].

The other option is to prevent gonadal development. This is possible by producing triploid fish that are sterile (see Chapter 13). Triploid females may be sterile for several reasons: meiosis failure; deficiency of steroid hormones; and abortion of oocytes and inhibition of further differentiation of oögonia [45]. In this type of sterility, the ovaries do not develop and the GSI remains very low [46]. In contrast, testes are able to develop in triploid males, even if there is no production of fertile spermatozoa. Indeed, mitosis in germ cells is not affected, and meiosis failure occurs only at the end of spermatogenesis. Recently, a more sophisticated method has been experimentally developed to produce sterile Atlantic salmon and rainbow trout. In the last case, the *dead end* (*dnd*) gene has been knocked down, inducing germ cell deficiency [47, 48].

The other advantage of producing sterile fish is to limit the negative impact of escapees [49, 50]. Sterility is a solution to the problem of introgressive hybridizations between cultured and native populations that occur, to a greater or lesser extent, according to the affected native populations and sites [51]. However, competition and predation can be a problem, and the dissemination of salmonids in non-native ecosystems has also raised serious concerns – for instance, for the southern cool-temperate galaxioid fishes [52].

It can be also beneficial for large hatcheries to control the sex ratio of their brood stock by reducing the number of males in favor of

females, even using cryopreserved sperm [53]. Finally, alternative high-value added products to sturgeons' caviar are growing in international markets [54], so producers of salmonids' roe ("red caviar") may be also interested in obtaining all-female populations.

Finally, sex control could be interesting for purposes other than fish farming. It has been suggested that a Trojan Y chromosome could be used, in theory, to cause extinction of an introduced exotic species by shifting the sex ratio of the population in favor of males [55]. This sex-skewing approach has recently been chosen in order to eradicate exotic brook trout (*Salvelinus fontinalis*) populations [56]. Today, a large number of YY brook trout males have been produced to be released in the wild to test this hypothesis *in situ*, but doubts as to the effectiveness of such an approach have been expressed [57].

11.4 Genetic Sex Determination in Salmonids

11.4.1 Sex Chromosomes

Sex determination refers to the primary mechanism leading to the expression of the phenotypic sex, and is mostly triggered by the genome (genotypic sex determination) or by the environment (environmental sex determination [58, 59]). Salmonids are gonochoristic fishes, with a genotypic sex determination (GSD) system classically described as being male heterogametic (XX/XY) [31, 60, 61]. They do not show temperature-dependent sex determination (TSD), although some temperature effects (GSD + TE) have been reported in a limited number of cases [61]. Finally, intersexuality has been rarely macroscopically detected in individuals from wild populations, mostly in *Oncorhynchus* species [62, 63] (see Box 11.2).

Most of the information on sex chromosomes in salmonids came from studies on species of the subfamily Salmoninae. Heteromorphic sex chromosomes have only been identified in a few species, including

rainbow trout, sockeye salmon (*Oncorhynchus nerka*), lake trout (*Salvelinus namaycush*), brook trout, and suspected in the sardine cisco (*Coregonus sardinella*) and vendace (*Coregonus albula*) [10, 60, 64]. However, homomorphic X-like sex chromosomes can be observed in males of some rainbow trout strains obtained after selective breeding programs. The Y chromosome can exist in different morphological forms: a shorter form, unlike the X chromosome; and a longer form, like the X chromosome [65]. It was suggested that the shorter form of the Y chromosome lost the totality or most of its short arm, consisting of 5S rRNA genes [65].

Sex chromosomes have also been identified in other species, using a combination of chromosome mapping and fluorescence *in situ* hybridization, with probes containing sex-linked markers in the Chinook (*Oncorhynchus tshawytscha*), chum (*Oncorhynchus keta*) and pink (*Oncorhynchus gorbuscha*) salmon [66], Yellowstone cutthroat trout (*Oncorhynchus clarki bouvieri*) [67], and brown trout [68]. Also, sex chromosome polymorphisms may occur in various strains of rainbow trout [65]. Indeed, sex chromosomes in salmonids have been extensively studied, to understand how they merged after the initial tetraploidization, and how they evolved with speciation [69].

In this regard, using chromosome staining, it has been suggested that the addition of heterochromatin to the X could be the first step in the inhibition of crossing over between the X and Y chromosomes in lake trout [70]. Complex systems may also occur, as in the sockeye salmon, which shows a X_1X_2Y system. In this species, males have 57 chromosomes, while females have 58 chromosomes, but both sexes have 104 chromosome arms [71]. There are two pairs of acrocentric chromosomes (X_1 and X_2) in females, and one copy each of X_1 and X_2 are fused into a single metacentric chromosome in males [72]. The Y chromosome and an autosome fused to form a metacentric chromosome [71, 73]. Complex systems have also been detected in two species belonging to the

Coregoninae sub-family, sardine cisco [74] and vendace [75]. A XY_1Y_2 system has been suggested for the sardine cisco. Finally, it has been also considered that X and Y may be in an early stage of differentiation in salmonids [76].

11.4.2 Gynogenesis, Androgenesis and Sex Inversion

Gynogenesis, which can occur naturally by fertilization of eggs with heterologous sperm [77], has been experimentally obtained in many species of salmoninae, and can be easily induced with sperm inactivated by radiation or chemical treatments. Reconstitution of diploidy is obtained either by retention of the second polar body, or by suppression of the first mitotic division, using temperature or pressure shocks [78]. All diploid gynogenetic salmonids are females, and this supports the theory that females are the homogametic sex (XX). Also, spontaneous triploids, probably resulting from the fertilization of an unreduced oocyte, have been found in domesticated hatchery stocks of rainbow trout. Their karyotypes showed that XXY triploids were males, suggesting again that the Y chromosome is male-determining in this species [79].

Androgenic viable rainbow trout males have been obtained after egg irradiation before fertilization [80], or by transplantation of spermatogonia into female recipients [81]. They produced all-male offspring when mated with standard females, supporting the assumption that males are the heterogametic sex, bearing the Y sex chromosome, and that androgenesis produces YY males (sometimes called supermales). It should be stressed that, even if salmonid YY males are viable, indicating the Y chromosome is not deficient of vital genetic functions present on the X chromosome [82], these fish can show chromosomal aberrations, probably caused by the incomplete maternal nuclear DNA inactivation [83].

The conclusion that salmonid male is the heterogametic sex was also suggested by the

analysis of the sex ratio of progenies obtained from crosses between sex-reversed individuals. Genotypic females can be made to develop into phenotypic males by precocious androgen treatments, while genotypic males can be made to develop into phenotypic females by precocious estrogen treatments, as detailed below. The sex ratio of the offspring of such steroid-treated populations also agrees with the assumption of a XX/XY system of sex determination [84, 85]. Thus, when an androgen treatment is applied to a group of fertilized eggs or larvae from a population that if left untreated would show an equilibrated sex ratio, around 50% of the resulting males are, in fact, genetically XX females (usually called neomales), and will produce all-female offspring when mated with standard females.

Likewise, when an estrogen treatment is applied, about 50% of phenotypic females are genetically XY (usually called neofemales). These neofemales will produce about 75% males in their offspring when mated with standard males, including 25% YY supermales that can produce all-male offspring if mated with standard females. Such YY supermales have also been obtained in rainbow trout by self-fertilization of estrogen-induced hermaphrodites [86]. Incidentally, the viability of YY individuals raises environmental concerns, since environmental disturbances that would induce the appearance of sex-inverted males (XY females) could result in the eventual emergence of YY supermales and, thus, reduce the number of genetic females in a wild population with each successive generation [87].

11.4.3 Genetic Sex Markers and the Sex-Determining Gene

Davidson *et al.* (2009) reviewed the extensive searches undertaken to find male-specific genetic markers in Salmonids [60]. Linkage analyses with the phenotypic sex-determining locus (SEX) have been performed with allozymes or genetic markers. Genetic maps that include SEX have been constructed for

rainbow trout, brown trout, Atlantic salmon, Arctic charr (*Salvelinus alpinus*), coho salmon, and Chinook salmon. The SEX locus was located on different sex chromosomes, and also at different positions, in the sex chromosome of four different species. Indeed, genetic maps located the SEX locus in the telomeric region for three species (Arctic charr, Atlantic salmon, and brown trout), but in the centromeric region for rainbow trout [88]. In addition, a set of sex-specific markers linked to the SEX locus in one species was not spatially correlated to the same set of sex specific markers in the SEX locus in other species.

Furthermore, multiple sex-determining gene loci have even been found within the same species for Arctic char [89] and Tasmanian Atlantic salmon [90]. In fact, SEX mapped to a different locus in the Tasmanian (North American-derived) Atlantic salmon from that reported in the Scottish (European) Atlantic salmon [90]. It has been suggested that either a short chromosome arm containing SEX could be translocated, or a smaller region containing a single sex-determining gene could be transposed into different chromosomal contexts in different species or sub-species. Another hypothesis would be that all these species do not share the same sex-determining gene. This might explain why the identified sex-linked genetic markers were not fully reliable between species, or even between families of the same species.

A number of such markers have been identified in Chinook salmon [91], masu salmon (*Oncorhynchus masou*) [92], rainbow trout [93], brown trout, Atlantic salmon [68], brook trout, and Arctic charr [94]. Because of their usually low error rates, they could be useful for estimating a population sex-ratio or in detecting sex inversion. For instance, the Y-chromosome specific marker OmyY1 [76, 94], has been used for understanding the difference in sex ratio between hatchery and anadromous wild steelhead trout [95].

Recently, a novel master sex-determining gene has been characterized in rainbow trout. This gene, named *sdY* (*sexually dimorphic on*

the *Y chromosome*), has been identified as a *Y* chromosome-specific gene that encodes for a protein that displays similarity to the C-terminal domain of interferon regulatory factor 9 [96]. The targeted inactivation of *sdY* in males, using zinc-finger nuclease, induces ovarian differentiation, and the over-expression of *sdY* in females, using additive transgenesis, results in testicular differentiation [96, 97].

This master sex-determining gene is conserved in salmonids belonging to Salmoninae (10 species belonging to five different genders: Atlantic salmon, brown trout, Arctic charr, Dolly Varden trout, *Salvelinus malma malma*, masu salmon, rainbow trout, Chinook salmon, sockeye salmon, huchen, *Hucho hucho*, and Japanese huchen, *Parrahucho perryi*) and Thymallinae (1 species: grayling, *Thymallus thymallus*) subfamilies [98]. In the Coregoninae subfamily, *sdY* has been detected in both male and female genomes in European whitefish (*Coregonus lavaretus*) and lake whitefish (*Coregonus clupeaformis*), probably due to the existence of multiple sex chromosomes in some Coregoninae species, such as the vendace and sardine cisco, which have a possible XY_1Y_2 system [74, 75].

Interestingly, a significant number of mosaic intersex gonads have been found during the ontogenetic gonadal differentiation process in a population of European whitefish inhabiting a Swiss lake [99]. However, *sdY* is still male-specific in some other Coregoninae species belonging to another gender, the beloribitsa (*Stenodus leucichthys*) [98]. Besides, a recent study indicates that *sdY* is also tightly linked with the male phenotype in the sockeye salmon [100], which shows a X_1X_2Y system of sex determination [71].

The presence of *sdY* only in males of many salmonid species strongly suggests that this gene is a conserved sex-determining gene in these species. A recent analysis of the *sdY* locus (800 kb) in rainbow trout suggested the presence of transposons, ribosomal DNA, repetitive elements, and a few single copy genes, such as *CREB-regulated transcription activator* and *cAMP responsive element*

binding [76]. Moreover, a deeper comparative analysis of the sex-determining region shared by three salmonids (rainbow trout, Chinook salmon, and Atlantic salmon) revealed that only 4.1 kb of this *sdY* locus is conserved within species, suggesting that it contains the minimal region needed to trigger masculinization. This region also contains potential elements necessary for transposition, such as *transposase* and *RNA-directed DNA polymerase* [101]. These studies revealed that this single master sex-determining gene (*sdY*) is present and may transpose between the different chromosomes, thus behaving as a jumping-sex gene [101, 102].

11.5 Effect of Environmental Factors on Sex Differentiation

Regardless of the robust XX/XY GSD system reported in salmonids, thermal effects on GSD have been observed in some species, including sockeye salmon [103, 104] and rainbow trout [105–107]. In some populations of rainbow trout, masculinization in response to temperature was shown to be heritable [108]. Furthermore, spontaneous masculinization of XX females has been found in homozygous lines of rainbow trout [109, 110]. In this latter study, the analysis of the male phenotype transmission in a three-generations pedigree supported the hypothesis that a recessive mutation in one putative minor sex determination factor (named *mal*), together with other sex modifier loci, was responsible for the masculinization of XX individuals [110–112].

Further observations suggested that this maleness could be a consequence of the early disturbance of ovarian differentiation and that this disturbance would be amplified by exposure to high temperature (18°C, for trout) during the first stages of development [112]. Indeed, this might be caused by an effect of temperature on the sex differentiation cascade, rather than by a direct effect on the upstream steps of sex determination.

Interestingly, in a search for quantitative trait loci (QTLs) in rainbow trout, a Y-linked marker has been associated with the upper thermal tolerance phenotype, estimated by the duration of the ability of individual fish to maintain equilibrium when temperature was increased from 10°C to 25.7°C [113].

Biased sex ratios have been reported in several wild populations of salmonids, such as grayling [114], and phenotypic sex inversion has been suspected to play a role in Chinook salmon [87]. In this species, 12% of fish bearing *sdY* were found among about one hundred wild phenotypic females [115]. However, these sex ratio distortions are not necessarily a direct effect of temperature, and other factors like pollution, certain kinds of pathogens, or sex-specific predation [116] could also be involved. In regard to pollutants, there is evidence that some endocrine-disrupting compounds can disturb the salmonid sex differentiation process [117, 118].

11.6 Gonad Sex Differentiation in Salmonids

Sexual differentiation refers to the developmental consequence of sex determination, and is the process by which an undifferentiated gonad develops either into a testis or ovary [58]. The earliest molecular events of this process occur during the early embryogenesis, before any histological signs of differentiation. The speed of embryo development is temperature-dependent [119] (Figure 11.1; Box 11.2).

11.6.1 Histological Differentiation

With regard to histological criteria, it is generally acknowledged that salmonids belong to the “differentiated” type of gonochoristic species, meaning that sex differentiation proceeds from the undifferentiated gonad directly to an ovary or a testis, without

Box 11.2 Sex determination and differentiation in salmonids

Genetic sex determination: Salmonids are gonochoristic fishes that exhibit a genotypic sex determination (GSD) system with male heterogamety (XX/XY). Heteromorphic sex chromosomes have only been identified in a few species, but sex chromosomes have also been identified using sex-markers. Some temperature effects (GSD+TE) have been reported in a limited number of cases. A master sex-determining gene was first characterized in the rainbow trout. This gene, named *sdY* (*sexually dimorphic on the Y chromosome*), is Y chromosome-specific, and encodes a protein that displays similarity to the C-terminal domain of *interferon regulatory factor 9*. It has been shown to be conserved in species mostly belonging to the Salmoninae and Thymallinae subfamilies.

Gonad sex differentiation: Salmonids belong to the “differentiated” type of gonochoristic species, meaning that sex differentiation proceeds from the undifferentiated gonad,

directly to an ovary or a testis, without any intermediate phase. Salmonid eggs are relatively large in size (about 2–6.5 mm), mechanically resistant, and develop during several weeks (about 200–500 degree-days to reach hatching) at low temperature (usually, about 5–9°C). In rainbow trout, the first histological feature of the gonad sex differentiation occurs at around 75 days post-fertilization (dpf) at 10–12°C, when the first germ cell meiosis are observed. However, the first sign of *sdY* expression in the male gonad is detected at 32 dpf in somatic cells. Sex dimorphic expression of several genes has been found since that age, including genes involved in steroid biosynthesis. In particular, the early emergence of a positive loop of regulation between gonadal aromatase (*cyp19a1a*), and an estrogen-induced expression of *forkhead box protein L2* (*foxl2*), supports the hypothesis of a key role of estrogens in inducing and maintaining ovarian differentiation in salmonids.

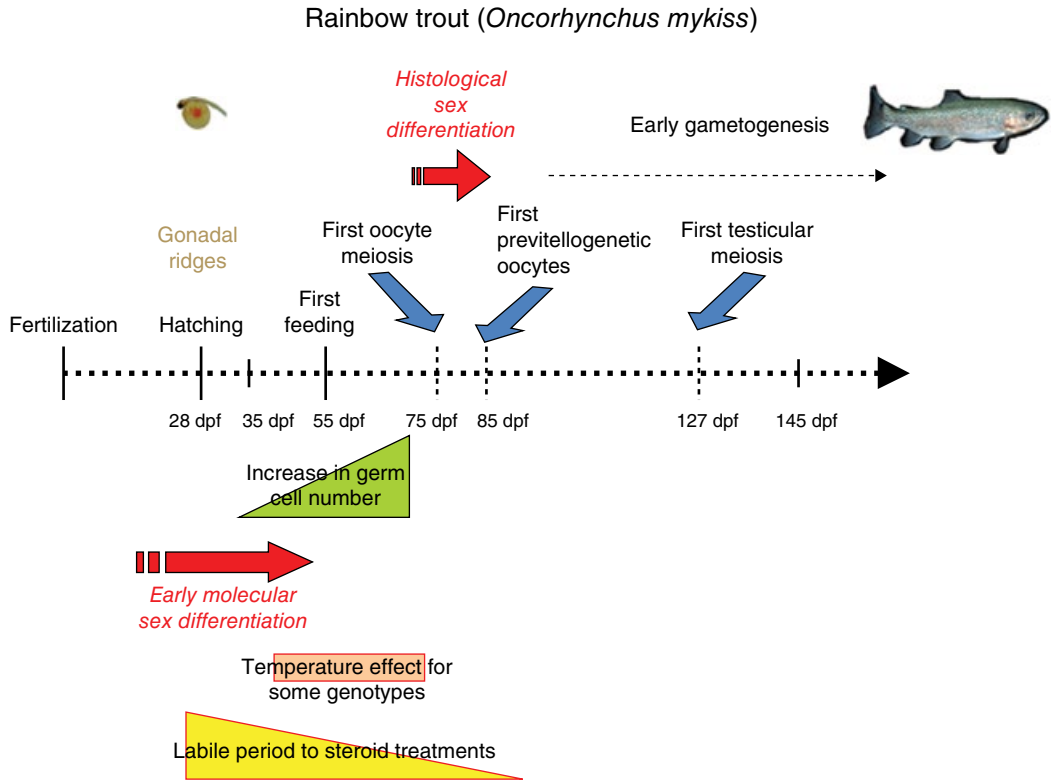


Figure 11.1 Kinetics of gonad sex differentiation in rainbow trout, where eggs are incubated at 10°C and then larvae reared at 11–12°C (dpf: days post-fertilization).

any intermediate phase [120]. This was questioned by Mršić in 1923 [121], with her first histological observation of rainbow trout gonad differentiation but, later on, Padoa (1939, [122]) for rainbow trout and Robertson (1953, [123]) for chum salmon, concluded the occurrence of a “differentiated” type of gonad development. Furthermore, as for other teleosts species, the somatic component of salmonid gonads has one single embryonic origin, and derives only from cells of the peritoneal wall [124, 125].

There is no single and simple answer to the questions of whether and how germ cells drive the sexualization of somatic tissues in teleost gonads. According to the species, germ cells depletion may result, or not, into masculinization of the gonad, regardless of its genetic sex [126]. However, *sdY* expression at 32 days post-fertilization (dpf) in the rainbow trout gonad was localized in somatic

cells [96]. Furthermore, in a recent study, germ cells have been ablated in Atlantic salmon gonad after *dead end (dnd)* knockout performed with the CrispR-Cas9 technology [47]. Sex differentiation of the somatic tissues was maintained in male and female germ cell-free fish, although the ovarian tissue appeared thin and unorganized. In the ovary of triploid rainbow trout, in which oocytes are not developing, scattered patches of spermatogenic-like cells can be occasionally detected [127]. Proper interactions between germ cells and ovarian tissue seem, therefore, necessary for normal ovarian development.

The first sign of genital ridges development in rainbow trout occurs only after the appearance of the germ cells just below the mesonephric duct [128]. An increase in the number of pre-meiotic germ cells starts after hatching, at around 35 dpf [129, 130].

The first histological sign of gonad sex differentiation takes place at around 75–80 dpf at 10–12°C (Figure 11.1), when first germ cell meiosis, an early female specific characteristic in embryonic gonads, is observed [129, 131]. Before this stage, the gonadal ridges appear as thin structures of the coelomic epithelium where germ cells proliferate [129]. However, the higher number of germ cells, which was observed in female gonads at a precocious stage of development [129], has not been confirmed after germ cell labeling using *vasa*-green fluorescent protein transgenic rainbow trout [130].

11.6.2 Molecular Differentiation

In a species bearing a sex-determining gene, the first sign of differentiation should be its expression. Until now in rainbow trout, the earliest expression of *sdY* in male gonads was detected at 32 dpf at 10°C, using whole-mount *in situ* hybridization [96]. In Atlantic salmon, *sdY* expression was first detected at 58 dpf at 8°C, using qPCR [132]. It has first been shown in trout that the sexually differential expression of several other genes occurs in gonads at the free-swimming stage (55 dpf, [133]). In subsequent studies, with more accurate and early sampling protocols, the onset of this period has been located around hatching in gonads (embryos of 35 dpf reared at 10°C, [134]), or before in whole embryos (of 15 dpf reared at 11°C, [135]).

Sex differentiation in salmonids has received a lot of attention with regards to the implication of sex steroids, following the early 1950s pioneering studies of Yamamoto on the Japanese medaka, *Oryzias latipes* (reviewed in [136]). Numerous sex steroid treatments were tested and found, in most cases, to be efficient in triggering phenotypic sex inversion (see section 11.7 below), with only a few unexplained cases of paradoxical inversions (reviewed in [137]).

However, the most informative treatments were probably those carried out with specific inhibitors of steroid synthesis. Among these, those inhibiting the action of the enzyme

that catalyzes the conversion of androgens to estrogens (i.e., aromatase [*Cyp19a1a*]) have provided key information on the mechanisms of gonad differentiation in salmonids. These experiments were first carried out in Chinook salmon, in which a single two-hour immersion treatment with a nonsteroidal aromatase inhibitor applied early in development (three days post-hatching) led to sex inversion of genetic females into phenotypic functional males [138]. These results were further confirmed in other salmonids, including rainbow trout [33] and Atlantic salmon [139], and provide a clear demonstration of the implication of sex steroids in gonad differentiation in salmonids, highlighting the pivotal role of estrogens in that process [140].

Other studies also support this implication of steroids and, especially, estrogens in salmonids. Thus, steroid-producing cells were ultrastructurally observed on the periphery of clusters containing meiotic oocytes at the onset of ovarian differentiation in amago salmon, *Oncorhynchus rhodurus* [141]. Several steroidogenic activities were detected at 50 dpf in rainbow trout after *in vitro* incubations of homogenized gonads with tritiated steroids precursors [142]. Furthermore, it has been shown in the same species, by using radioimmunoassay, that gonads are able to release androstenedione *in vitro* at 55 dpf [143]. At about the same age, aromatase expression was specifically detected by semi-quantitative RT-PCR in female gonad [33].

More recently, and using quantitative RT-PCR, this female-specific over-expression of aromatase (*cyp19a1a*) was shown earlier (i.e., at 32 dpf), while the expression of the gene coding for the enzyme giving rise to the male specific 11-oxygenated-androgens, namely, 11 β -hydroxylase (*cytochrome P450, family 11, subfamily b, polypeptide 2; cyp11b2*), was only found from 45 dpf [134]. In addition, other genes involved in steroid production regulation have been shown to be differentially expressed during early gonadal differentiation in rainbow trout, including the *anti-Mullerian hormone* (*amh*, [134]) and the *forkhead box*

protein L2 (foxl2) gene [134, 144], which is a key player in ovarian differentiation and oogenesis in vertebrates [145].

Interestingly, *foxl2* is known to be a positive regulator of *cyp19a1a*, along with *nr5a1* (*nuclear receptor subfamily 5 group A member 1* or *Steroid Factor 1*, or *Sf1*) in tilapia [146], and *foxl2* expression in rainbow trout is both tightly correlated with *cyp19a1a* [134] and is strongly and quickly upregulated by estrogens [144, 147]. These results strongly suggest the existence of a positive loop of regulation between *cyp19a1a* under the control of an estrogen-induced expression of *foxl2* [140]. Such a regulation would explain the important sex-dimorphic expression of these two genes during gonadal differentiation [134, 144], and further supports the key role of estrogens in inducing and maintaining ovarian differentiation in salmonids.

Apart from these studies on the potential physiological implication of steroids in the normal process of gonadal differentiation in salmonids, a few gene expression experiments were carried out to investigate the effects of steroid treatments on gonadal differentiation in rainbow trout. These experiments showed that both masculinization with androgens, and feminization with estrogens, induce important alterations in the gonadal transcriptomes of the treated animals, compared with untreated control males and females [147–149]. Interestingly, genes involved in sex-steroid synthesis, including *cyp19a1a*, were strongly inhibited by these treatments. This inhibition of *cyp19a1a* by androgen treatment could be seen as a causal trigger of female-to-male sex-inversion, as this prevents any conversion of androgens to estrogens, thus promoting maleness, as also seen with anti-aromatase treatments.

In the case of feminization treatments with estrogens, *cyp19a1a* is also downregulated only during the application of the treatment, but its expression is restored once it is finished. Comparisons of masculinizing treatments, using either androgens or anti-aromatase, also show that blocking the estrogen synthesis leads to gonadal expression profiles very

similar to those observed in untreated control males [150]. This also supports a physiological implication of estrogen synthesis in the control of ovarian differentiation, which would require estrogens to proceed, and also of testicular differentiation, which would proceed in the absence of estrogens. Male differentiation in salmonids would, then, simply require a down-regulation of *cyp19a1a*.

11.7 Methods of Sex Control

There is a great interest in the fish farming industry for controlling fish sex and, thus, several reviews have been published in the last three decades about sex control in fish, including salmonids [31, 120, 151–157].

11.7.1 Selective Sorting Based on Secondary Sexual Characters

The simplest way to select females from a population would rely on the identification of an early visual marker for gender dimorphism. However, clear sexual dimorphism or secondary sexual characters can be only detected in maturing or adult salmonids [158]. Sexual size dimorphism has been observed only in juvenile immature masu salmon [159]. Indeed, several non-lethal sexing methods based on phenotypic traits have been developed by field biologists for evaluating the reproductive potential of the wild populations. These include morphometry, external palpation, immunological techniques for vitellogenin or sex steroid detection, endoscopy, and ultrasonography [160]. However, these methods are time-consuming, and only work at a late stage of fish development; thus, they are rarely applied for fish farming purposes.

11.7.2 Direct Feminization

Direct feminization can be obtained by treatment with estrogen [155]. However, these treatments can induce larvae mortalities [161]. Chronic exposure by immersion with a

low level ($1 \mu\text{g l}^{-1}$) of estradiol-17 β (E_2) during the eyed embryo stage in rainbow trout can induce a decrease in the expression of growth-related genes, and high rates of mortality and deformities [162]. Estrogens can be added in the food. By instance, feminization has been obtained in rainbow trout by feeding larvae with 17 α -ethynylestradiol (EE_2 , 20 mg kg^{-1} food) during two months from first feeding. In that case, EE_2 treatment upregulated the expression of several genes involved in early ovarian differentiation, while it repressed genes involved in androgen synthesis and some Sertoli cell markers. However, some molecular markers of testicular differentiation were not downregulated [147].

Feminization can be obtained with earlier and shorter exposure to estrogen. All-female Chinook salmon populations were obtained with E_2 or EE_2 after a single immersion treatment at $400 \mu\text{g l}^{-1}$ three days after median hatch. EE_2 was more efficient, and needed only a single immersion for two hours, versus eight hours compared to E_2 [161]. This EE_2 property may be due to a lower degradation because of the impossibility of its hydroxylation at C-16.

The metabolic clearance rate of E_2 is relatively high, ranging between $18.7 \text{ ml of blood h}^{-1} \text{ kg}^{-1}$ body weight and $40.9 \text{ ml h}^{-1} \text{ kg}^{-1}$ in adult trout, according to the sexual stage [163]. The half-life of E_2 in whole tissues of a yearling trout fed with an E_2 -enriched diet was less than 12 hours [164]. The half-life of E_2 in eggs, newly hatched larvae and first feeding fry of coho salmon, measured after immersion for 96 hours in a water bath containing $400 \mu\text{g } E_2 \text{ L}^{-1}$, was 27.8, 27.5 and 4.4 hours, respectively [165].

Regarding these short half-lives, and the fact that treatments were performed long before the fish were marketed, the risk for the consumer to be exposed to estrogens has been considered to be negligible [155]. Besides this, the amount of E_2 released in the aquatic environment with such treatments was compared with other sources of contamination, such as the use of oral contraceptive, to minimize the potential environmental

impact of direct feminization of fish. However, we should also consider that metabolites toxicity is not always well known, and that was an important part of the debate in using steroids for improving cattle growth [166]. There is also a health risk for workers manipulating steroids and steroid-enriched diets if no special precautions are taken.

Regarding the environment, a global vision can be restrictive. We should be attentive to the particular ecosystem in which residual steroids could be released. In fact, it should be stressed that estrogens are now well known as active endocrine disruptors, with potential deleterious effects on both human health [167] and indigenous fauna [168]. Thus, sewage-treatment work (STW) effluents can be estrogenic to fish [169], and can contain E_2 and EE_2 at low, but efficient, doses [170]. Surface water can be also contaminated by these hormones [171]. Further, intersexuality was induced in male rainbow trout when they were exposed chronically to 10 ng l^{-1} of EE_2 for 76 days after the onset of first feeding [172]. Compared to E_2 , EE_2 was much more resistant to biodegradation [173]. In conclusion, there are, indeed, some facts that motivate a strong consumer rejection of steroid-treated food [155]. Thus, the widespread use of steroid hormones could irreversibly damage the image of fish as food, which usually receives a good perception on its nutritional value in comparison to other animal protein sources [174].

11.7.3 Gynogenesis and Diploidization

Gynogenesis is a pseudogamous parthenogenesis (i.e., a sperm-dependent parthenogenesis), which can occur naturally in some non-salmonid fish species [175]. Oocytes arrested at metaphase of the second meiotic division are induced to complete their second meiotic division (with the second polar body emission), and to start an early embryonic development after activation by spermatozoa, but without any spermatozoa genome contribution and without syngamy (fusion of pronuclei).

That could artificially be obtained in salmonids using sperm in which the genome is inactivated. This inactivation is usually performed by sperm irradiation. Gamma-ray irradiation needs a radioactive source not always available for the fish farmers, and not easy to handle. Ultraviolet (UV) irradiation gave better results when compared to gamma-irradiation, or even to chemical treatment of sperm [176, 177]. Indeed, UV-irradiation is the most frequently used method for sperm inactivation. Intensity and duration of irradiation need to be properly fixed, taking into account the pseudo ‘Hertwig effect,’ because of major differences with the ‘Hertwig effect’ observed with gamma-rays, which is a dose-dependent decrease in embryos’ survival rate at low doses of irradiation, but a better survival rate at higher doses [178, 179]. That could be due to the continued existence of residual paternal fragments of chromosomes at low doses of irradiation, leading to aneuploidy. Thus, this possibility deserves to be carefully explored [180]. Besides, it should be stressed that wide variations in the most efficient dose may occur between males and, thus, it is advised, for practical purposes, to use a pool of sperm from several males [178].

The gynogenetic embryo that bears one X sex chromosome is haploid, and dies within a few days after hatching [109, 181, 182]. This is also true for the androgenetic haploid embryos, whether they bear the X or the Y chromosome [180]. Diploidization allows getting viable diploid XX female embryos, and can be obtained by retention of the second polar body or suppression of the first embryonic mitosis [182]. These processes are usually called meiotic gynogenesis and mitotic gynogenesis, respectively. After pioneering studies performed on non-salmonid fish [183, 184] (based on methods first developed with amphibians [185]), diploidization has been induced in salmonids using heat shocks [182, 186, 187], or pressure shocks [78, 188] after fertilization with inactivated sperm. With that purpose, three parameters need to be fixed – namely, temperature or

pressure level, duration, and time of application. The success of all these techniques also relies on a proper brood stock and gamete management [189–191].

Meiotic gynogenesis produces homozygous individuals, except for regions where crossing over took place between maternal and paternal chromosomes of the donor oocytes and, thus, a high degree of residual heterozygosity can still be maintained [192]. Mitotic gynogenesis results in the production of individuals carrying only a duplicated set of chromosomes, and they are fully homozygous doubled-haploid fish [193]. Getting more or less homozygous fish can be very interesting for producing isogenic lines [194, 195] but these all-female populations often show low performance for production traits [109, 196]. However, these populations can be used to produce neomales, and the developed methods can be used to produce sterile triploid females.

11.7.4 Production and Use of Neomales

The easiest way to produce all-female salmonid populations is to get XX males, since salmonids have GSD with male heterogamety (see Box 11.3). The possibility of fully inverting the phenotypic sex from female to male in fish, using androgen treatments, has been known for more than half a century [197]. Regarding salmonids, partial or complete ovary reversal was first obtained in rainbow trout after treatment with the synthetic androgen 17 α -methyltestosterone (MT) [198–200], and all-female populations were obtained by crossing these neomales (XX males) with normal females [84] (Figure 11.2). A large number of studies have been performed on various salmoninae species (belonging to the genus *Oncorhynchus*, *Salmo*, or *Salvelinus*), and some comprehensive reviews have listed the species and treatments used [31, 59, 201]. Interestingly, a recent systematic review and meta-analyses of the literature did not show significant differences between sperm traits of masculinized fish and of wild-type males [202].

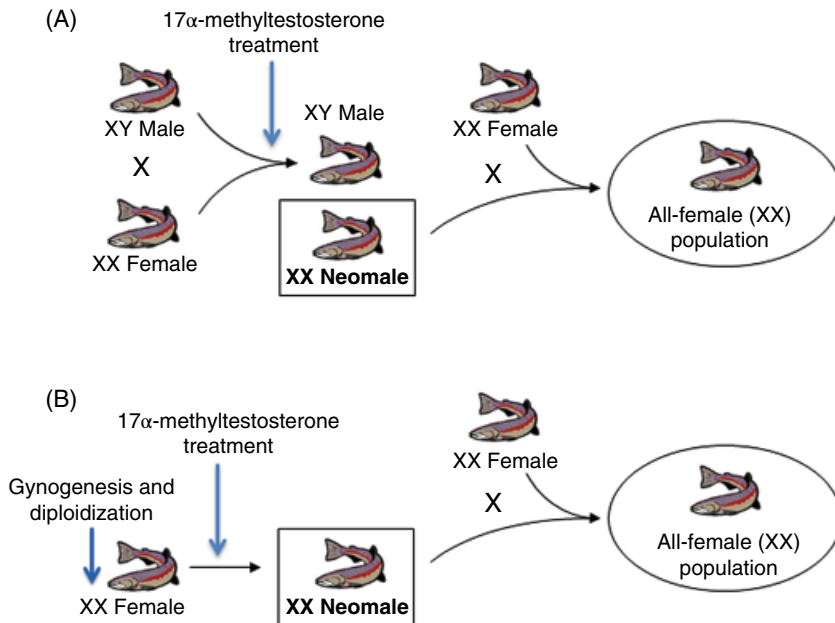


Figure 11.2 Production of all-female populations in salmonids using XX neomales. XX neomales are obtained through masculinization of genetic females (XX) after treatment with the synthetic androgen 17 α -methyltestosterone (MT). The androgen-treated fry can be issued from: (A) a normal population containing genetic XX females and XY males (in that case, XX neomales have to be screened from XY males); (B) A gynogenetic all-female population. In that case, all males are XX neomales.

Treatments should start at least before the histological sex differentiation of the gonad, and are usually performed by adding androgen into the food delivered to larvae. In terms of molecular regulation of sex differentiation, androgen treatments at this relatively late stage of development could induce testicular transdifferentiation, with gene expression patterns quite different from what is observed during natural testicular differentiation, as shown in rainbow trout [148].

It has been proposed that the crucial step of this masculinization process is the de-differentiation of the granulosa cells [149]. Moreover, a marked asymmetry in the response of the trout gonads to the treatment (the right ovary being more refractory) can be observed [203, 204]. Asymmetry in gonadal development was also observed in response to the disturbance caused by the *mal* mutation (see Section 11.5) but, in this case, the right gonad is more sensitive to the mutation-induced masculinization [63]. Additionally,

administration of an aromatase inhibitor to rainbow trout resulted in a much more specific testicular gene expression pattern than that observed following androgen-induced masculinization [150].

In fact, androgen treatment starts after the early occurrence of sex-specific steroid synthesis when administered by food. Gonadal aromatase (*cyp19a1a*) has a pivotal role in the ovarian differentiation [140], and its regulators are expressed before yolk resorption and first feeding, and even before hatching in rainbow trout [134, 205] and Atlantic salmon [206]. The expression of *cyp11b2*, which is involved in the synthesis of 11-oxygenated-androgens, increases after hatching, but before first feeding in trout [134]. Altogether, the previous results are a good reason to look for an early androgen treatment by immersion of eggs or young larvae [207–209]. These early stages also give the opportunity of using yolk as an endogenous reservoir of the incorporated lipophilic steroids [165].

The synthetic androgen MT has been the most widely used hormone, and has proved more efficient than natural androgens. Its oral bioavailability from food is determined to be about 70% in juvenile rainbow trout [210]. Its mechanism of action is complex and could be different according to the period of administration and the dosage, since it can produce paradoxical feminizing effects [211]. However, MT does not bind to the rainbow trout native liver estrogen receptor [212]. Its estrogenic effect could be due to its aromatization into 17 α -methyl estradiol (ME) which, indeed, can bind to the liver estradiol receptor in the fathead minnow (*Pimephales promelas*), with a lower affinity than E₂ [213]. Nevertheless, MT shows a high affinity for coho salmon and rainbow trout androgen receptor, even higher than fish natural androgens [214, 215]. However, its effect on aromatase and E₂ synthesis is unclear.

MT was shown to be a competitive inhibitor of aromatase activity in an *in vitro* human model [216]. MT inhibits *cyp19a1a* expression, but stimulates the expression of the brain isoform (*cyp19a1b*) after treatment of zebrafish (*Danio rerio*) larvae during the whole histological gonadal differentiation period [217]. In fact, ME could stimulate the brain aromatase, which shows an estrogen response element in its promoter [218, 219]. Interestingly, in rainbow trout, both *cyp19a1b* expression and *cyp19a1b* enzymatic activity were significantly higher in male brains than in female brains during the early period of morphological gonad differentiation [220].

All these results raise at least two questions relative to MT treatments. First, what could be the effects of the actual relative amounts of estrogens versus androgens, according to the dose of MT and duration of treatment, taking into account that MT could be metabolized into ME? Second, what could be the consequences of MT effect at the brain level, considering brain sexualization [221] and possible implication of the brain for a full and physiological gonadal differentiation [222]?

Failure of masculinization by androgen treatments may be due to several reasons. Timing and duration of androgen administration should properly be adapted to each species. For instance, immersions beginning one week before hatching, combined with an oral treatment, were necessary to efficiently masculinize female brook trout [223]. The actual androgen concentration in the food used for treatments should fit the expected value. Thus, MT concentration may decrease with storage time when the food is kept at room temperature [224, 225]. This decline is limited when the food is frozen at -20°C. Furthermore, androgen should be uniformly mixed with feed [224]. Finally, larvae health problems, or a suboptimal water temperature for the species, can reduce the food intake and, thus, the delivered quantity of androgen.

Another important issue is discrimination of neomales from standard males. This is mainly critical when a normal mixed population is used for masculinization, since all males should be XX males when female gynogenetic offspring is used, unless stocks are accidentally mixed in the fish farm (Figure 11.2). Of course, the way to unequivocally identify a neomale is to check that it gives an all-female progeny, but this is time-consuming and costly. Thus, treatments are sometimes fixed to get some easily identifiable phenotypes, such as hermaphrodites or males without functional *ductus deferens* [203, 226]. In that case, spermatozoa must be collected directly from the testis by sacrificing the fish and squeezing the testicular lobes [227]. Testicular sperm does not undergo the last steps of maturation which take place in spermatid ducts [190, 228], and it needs an exogenous maturation with an artificial medium mimicking seminal plasma before activation [229]. This sperm could also be cryopreserved for future use [230]. Finally, the use of genetic sex markers has also been proposed to identify neomales [231, 232].

Ingestion of MT residue in treated fish may be potentially hazardous to human consumers [210], but salmonid neomales are not

Box 11.3 Sex control in salmonids

Gynogenesis, followed by diploidization, can be used to produce all-female populations in salmonids, but these all-female populations show a high level of homozygosity. An alternative classical sex control method uses XX neomales, which give all-female populations after crossing with wild-type females. These XX neomales are produced by masculinization of females with an androgen, MT being the most frequently used. Androgen treatments are generally administered directly in the food from the first feeding of larvae, but dosage and timing should be adjusted according to the species. As an example, 3 mg MT kg⁻¹ of food given during 2–3 months gives satisfactory results in rainbow trout.

Even if these treatments are generally efficient in salmonids, they need precautions in their application with, for instance, proper storage of the treated food, and a satisfactory feeding rate of healthy larvae. Caution should be taken when preparing and handling these treatments. The risk of workers' contamination with steroids should be reduced by using personal protective equipment (gloves and face mask). The environmental impacts of these treatments should also be taken into account. Treatment methods using eggs and/or larvae bath immersion are also efficient in some salmonid species, and would be worth being considered, as these health and environment risks can be controlled more easily.

intended to be used for human consumption. They have a poor organoleptic quality, because they are killed after maturation. Environmental risk requires more attention [233], knowing that MT has already been detected in the aquatic environment, where it can act as an endocrine disruptor [234], even at low doses [235].

11.8 Conclusions and Future Perspectives

Salmonid fish farming has been substantially extended during recent decades. This development is largely based on the farming of large fish that need a delay or a suppression of sexual maturity. In some cases, the delay of puberty using photoperiod control can be good enough to suppress pre-harvest sexual maturation [236]. However, the most efficient strategies have been to produce all-female diploid populations showing a later onset of puberty than males, or to produce all-female triploid populations, which do not develop gonads. In some rare cases, hybridization between salmonids species can also give sterile fish [4], but their actual potentiality for fish farming has been poorly investigated.

Crossing wild-type females with sex-inverted females (XX neomales) is the most commonly used method today. Sex inversion is usually obtained by early treatment with androgens, and the most used chemical is MT. However, the various biological actions of this aromatizable androgen are not fully known. It needs to be cautiously manipulated in order to exclude any impact on fish farmer health, and it can also raise some environmental concerns. Thus, in several countries MT is an unapproved drug but, specific exemptions are usually delivered by competent authorities.

Other types of treatments, like the use of aromatase inhibitors, which give a more physiological testicular differentiation and a functional testis, could be more biologically appropriate than MT treatments. However, the same kind of reservations could be issued as for MT, with regards to human health risks and environmental impacts. These risks could at least be reduced if accurate labile periods, during which treatments are most effective, are identified for each species, together with the lowest efficient doses of the chemical [237]. Deciphering the molecular cascade of gene activation and repression during early differentiation should help for such a purpose.

Such a situation seeks to look for another approach to produce all-female populations. The recent finding that temperature can influence the sex ratio of some salmonid populations has led to consideration of other strategies. For instance, Magerhans and Höstgen-Schwark [105] have suggested either selection of families that show a high percentage of females in their sex ratios after temperature treatment, or the use of neomales derived from temperature treatments of gynogenetic offspring. However, achieving the production of all-female populations could be difficult to reach within the frame of such a genetic selection program, especially if selection is also directed to other traits of interest. The use of neomales derived from temperature treatments of gynogenetic offspring may be then more realistic from a practical point of view, because just a few males will be enough to fertilize tens of thousands of eggs.

Finally, gene modification has been proposed to feminize or sterilize growing fish. A recent strategy, called “sterile feral technology,” combines a stage-specific promoter with a disrupter of a critical development gene, under the regulation of a repressible element [238]. This disruption of gonadal development can be restored when needed, to produce brood stock. Germ cell

elimination can also be obtained by introducing a transgene that can be induced to kill germ cells, or to prevent their migration to the developing gonad [239].

Recently, a simple bath-immersion of eggs has been developed in zebrafish for the administration of an antisense morpholino oligonucleotide able to block *dnd* action, which is necessary for a proper germ cell migration [240]. All these technologies are still at the laboratory stage, but some of them are currently being assessed in salmonids for a potential transfer to the production sector [239]. Their transfer into standard practices needs several feasibility and assessment studies regarding their use in salmonids, repeatability, health and environmental security, cost for a large-scale application, and market acceptability.

Acknowledgments

We owe special thanks to Dr. Francesc Piferrer and an anonymous reviewer for their useful and relevant advices. Maryse Corvaisier has helped to collect the relevant literature. Sylvain Bertho was supported by an INRA/Region Bretagne Ph.D. fellowship followed by the “DAAD STIBET Doktoranden” Program.

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12

Development and Application of Sex-Linked Markers in Salmonidae

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12.1 Introduction

Most salmonid fish have an XY sex determination system, usually with no morphologically differentiated putative sex chromosomes [1] (see Box 12.1). Sockeye salmon (*Oncorhynchus nerka*) is an exception, with an X_1X_2Y sex determination system, in which females have one more chromosome ($2n=58$) than males ($2n=57$) [1–3]. Accurate sexing of salmonids provides many commercial benefits, motivating research to identify sex-linked markers for aquacultured fish. Sexual maturity affects growth, and increases male aggressive and competitive behaviors. Maturing fish may also stop feeding, show decreased vitality due to skin infections or other diseases, and produce lower quality meat (including fillets with altered color or flavor).

Due to the many maturity-related changes relevant to commercial salmonid production, aquaculturists seek to limit pre-harvest sexual maturation, producing sterile males and females by inducing triploidy (see Chapter 13), or monosex specimens, using gynogenesis or androgenesis (see Chapter 13). Given that the XY system is common to most salmonids, the research has focused on finding male-specific sex-linked molecular markers. Markers present in the male (putatively in the Y chromosome, called Y-linked markers) and absent in

females (or the X chromosome) have been detected using various molecular techniques that have evolved from the 1980s to the present day.

In the 1970s and 1980s, allozymes (biochemical markers) were used extensively to assess genetic variation in natural populations and were the first sex-linked markers identified in salmonids. Given their historical importance, we will dedicate a few lines to allozymes, keeping in mind that the polymorphisms underlying these biochemical markers have a genetic basis in the coding sequence of the enzyme. These polymorphisms are expressed in the phenotype, and may have adaptive implications. In rainbow trout (*Oncorhynchus mykiss*), the allozymic loci *bGLUA-2** (formerly *HEX-2*) and *sSOD-1** show linkage with the Y chromosome [14–16] and loci *Ldh-1**, *Aat-5**, and *Gpi-3** in the *Salvelinus* species [17]. Application of these markers for salmonid sexing has been very limited.

The development of polymerase chain reaction (PCR), molecular cloning, and automated Sanger sequencing, have made it possible to perform amplifications from small quantities of genetic material. As a result, small DNA segments are sufficient for performing genetic analyses, determining nucleotide sequences, and comparing

Box 12.1 Sex determination systems in salmonids

Sex determination systems are diverse among vertebrates. Genetic and environmental factors guide the process of determining whether the primordial gonad in the embryo becomes an ovary or testicle. When the gonads begin to function, the respective male or female sexual phenotype emerges.

Fish exemplify the diversity of sex determination systems. Various species have XX/XY, ZZ/ZW, or multiple chromosome systems and, in some species, sex is determined, or strongly influenced, by the environment [4]. Salmonids have separate sexes, and the sex determination is under genetic control. Experimental sex reversal experiments have confirmed that the male is the heterogametic sex. Crossing an XY female (sex-reversed male) with a normal male (XY) yields a 3 : 1 proportion of phenotypic

males and females, and crossing an XX male (sex-reversed female) with a normal female (XX) produces 100% phenotypic female progeny [5–7].

In some salmonids, such as rainbow trout (*Oncorhynchus mykiss*) and various *Salvelinus* species, chromosomal sex (XX/XY) is distinguishable by morphology [8], while other salmonids do not exhibit marked sex-linked morphology [1]. In the latter case, sex chromosomes have been identified using chromosome-banding techniques, such as fluorescence *in situ* hybridization (FISH), involving probes that carry sex-linked markers. Linkage studies and comparative analyses among species have characterized most of the sex chromosomes in this group of fishes [9–13].

findings with results from public databases to identify homologous sequences. Since the 1990s, these techniques have been used to develop PCR-based markers, such as RAPDs (random amplified polymorphic DNA [18, 19]), AFLPs (amplified fragment length polymorphisms [20]), SCARs (sequence-characterized amplified regions [21]), and microsatellites [22], to amplify partial sequences of genes and pseudogenes, and to evaluate associations between these markers and phenotypic sex.

Development of next-generation sequencing methods in the 2000s permitted massive sequencing of RNA from specific tissues (a technology called RNA sequencing). This technology was used to compare the genes transcribed in male and female gonadal tissues, shedding light on a potential salmonid master determining sex gene. This section will review the development of male-specific markers, through the 2012 discovery of the *sdY* gene and their applications, to 2017. The most relevant markers are described below, but various markers developed as an academic exercise with no practical utility are not listed. Only a few markers have been

applied massively to salmonid sexing and, to our knowledge, even these markers are not used routinely in commercial fish farming. Probably, when all these technologies become more cost-effective than echography, they will be routinely used by the industry – but now this is not the case.

12.2 Development of Sex-Linked Markers in Salmonids

Biological samples are required to evaluate genomic DNA for the presence of any of the markers discussed in this chapter. In alevins, the entire adipose fin is often removed. Because the fin may be difficult to cut in adult fish, a small sample called a fin clip is often used instead. This technique requires removing a small piece of dorsal fin – no more than 0.5 cm². Samples can be dried and then stored in paper or in a tube with 95–100% ethanol until DNA extraction. There are many protocols for extracting DNA, including commercial kits (available from many biotech suppliers worldwide),

rapid protocols using Chelex resin [23], and elaborated protocols using phenol and chloroform [24]. Regardless of the protocol, high-quality DNA is necessary for genotyping any molecular marker.

12.2.1 *OtY1/OtY8*

One of the first male-specific salmonid markers identified was the Y-chromosomal DNA probe *OtY1* in Chinook salmon (*Oncorhynchus tshawytscha*), by Devlin *et al.* [25]. This probe was initially developed using the subtractive hybridization method, to produce an enriched fraction of male-specific sequences for cloning. Eighteen clones were subjected to southern blotting, using a radioactive probe. A single 250 bp probe hybridized with an 8 kb fragment in all 30 males, but none of the 29 females were analyzed [25]. Segregation analysis of one family showed *OtY1* was inherited by male progeny from the sire. Because the blotting method was time-consuming and difficult to apply in commercial aquaculture, a rapid PCR-based test for *OtY1* was developed, producing a male-specific 209 bp amplicon [26].

The *OtY1* marker was explored in other salmonids, but found to be male-specific in the Chinook only. In rainbow trout, *OtY1* was not Y-linked, nor did it map in the linkage group bearing the sex-determining locus [27, 28]. Furthermore, the above studies detected no recombination between the *OtY1* marker and the sex-determining locus [25, 29]. Females positive for *OtY1* have been detected in some wild and hatchery populations (ranging from 4–84% of the female population), indicating a possible recombination event; however, this pattern may be attributable to environmental sex reversion mediated by temperature or estrogen pollution [30, 31].

In a subsequent analysis, the 8 kb fragment detected with the *OtY1* probe was cloned and subjected to southern blotting and PCR analyses, to characterize the genomic organization of the new marker, *OtY8*. As with *OtY1*, this clone was found to be Y-linked,

segregating from the male parent to male progeny [32]. Studies in eight other *Oncorhynchus* species (*O. keta*, *O. nerka*, *O. gorbuscha*, *O. kisutch*, *O. mykiss*, *O. masou*, and *O. clarki*) and Atlantic salmon revealed that *OtY8* is Y-linked only in Chinook salmon [28, 32].

12.2.2 *GH-Ψ/GH-2* Genes

Growth hormones (GH) play an important role in fish growth. Because the growth rate of captive fish has been (and still is) a primary target in fish breeding, there are ongoing efforts to clone, sequence, and characterize the genes associated with this process in salmonids [33, 34]. Salmonids have two expressed growth hormone genes (*gh1* and *gh2*), one of which has been identified as a sex-linked marker in Pacific salmon [35]. For example, in coho (*Oncorhynchus kisutch*) and Chinook salmon, two alleles (*a* and *b*) were identified in intron C of the *gh2* gene. These alleles differ in size (434 and 455 bp, respectively) and *HinfI* enzyme restriction sites [36]. In both species, segregation analyses have shown that allele *b* is male-specific and located in the Y-chromosome, while allele *a* is located in the X-chromosome. Therefore, all males are heterozygous for this allele (genotype *ab*), and females are homozygous for the *a* allele. This type of segregation is absent in rainbow trout, in which the *gh2* gene does not show a sex-linked pattern [36].

In addition to the sex-linked polymorphism in the *gh2* gene, a non-functional Y-linked growth hormone pseudogene (*ghΨ*) has been described in five Pacific salmon species: Chinook, coho, masu (*O. masou*), chum (*O. keta*), and pink salmon (*O. gorbuscha*) [29, 33, 35, 37]. In all male Chinook and coho salmon, a 290 bp fragment from *ghΨ* is amplified by PCR primers *GH5/6*, designed for intron E [33, 34]. In chum and pink salmon, the Y-linked specific fragments are amplified by primers *GH28/GH30*, designed for intron C, resulting in 160 bp and 175 bp amplicons [29]. In masu salmon, the male-specific fragment is 280 bp.

The inheritance pattern indicates some degree of recombination between Y and X chromosomes, and 97.5% and 24.3% of the male fragment is present in phenotypic males and females, respectively [35, 38]. It is likely that some recombination also occurs in Chinook salmon [29], as the estimated distance between *ghY* and the sex-determining gene is approximately 10 centimorgan (cM) in this species. However, no study to date has detected a recombination event with the sex-determining locus.

12.2.3 *OmyP9*

In rainbow trout, the first male-specific marker was identified by Iturra *et al.* [39] with bulked segregant analysis (BSA) and RAPD (random amplified polymorphic DNA) screening. These researchers used pooled samples from 12 males and 12 females from the Mount Lassen strain. An RAPD assay with 900 primers identified two sex-associated RAPD fragments (650 and 390 bp), amplified by the primers OP-A11 and OP-P9, respectively. The 390 bp fragment amplified by RAPD primer OP-P9 was present in all 12 males, and absent in all 12 females. When this polymorphism was tested in the Scottish strain, it amplified in all males, but also in 38% of females. The 650 bp fragment amplified by RAPD primer OP-A11 always amplified in a percentage of males, but never in females. Finally, only the fragment amplified by primer OP-P9 was converted to a SCAR (sequence-characterized amplified region) marker, designated *OmyP9*, enlarging the RAPD fragment to 899 bp [40].

A more detailed analysis of *OmyP9* identified three size polymorphisms (899, 894, and 840 bp) and one restriction polymorphism when digested with the *RsaI* enzyme. Combinations of size and restriction polymorphisms produced three *OmyP9* variants: variant A (894 bp, with two *RsaI* restriction sites), which generated three fragments (441, 114, and 339 bp); variant B (899 bp, with one *RsaI* site), which generated two fragments (555 and 344 bp); and variant C (840 bp, with

one *RsaI* restriction site), which generated two restriction fragments (501 and 339 bp). Segregation analyses, in 93 males and 93 females from six different strains of rainbow trout, showed that males are never homozygous for the C variant. However, none of the three variants are strictly associated with male or female phenotypes, indicating that *OmyP9* is not a fully Y-linked locus, and that some recombination between X and Y chromosomes can occur in the region bearing this marker.

In crosses with known parental genotypes, determining the progeny's sex is straightforward. For example, in 10 experimental crosses, the male parent always passed his variant A to male progeny and never to female progeny [40]. A similar pattern was observed by Lopez and Araneda [41] in crosses used to evaluate the performance of *OmyP9* in identifying the sex of rainbow trout.

12.2.4 *Omy-163*

This marker was also developed in rainbow trout to identify the Y-chromosome, using amplified fragment length polymorphism (AFLP) screening in pooled samples obtained from crosses between outbred females and F₁ males, derived from crosses between XX individuals from the Oregon State University female clonal line, with YY individuals from four different male clonal lines (SW, Swanson; ARL, Arlee; CW, Clearwater; and HC, Hot Creek) [42]. AFLP screening was performed with 486 primer combinations and three pairs of restriction enzymes (*EcoRI/MseI*, *PstI/MseI* and *BamHI/MseI*), resulting in 4,374 polymorphic fragments. Fifteen sex-linked AFLP markers were converted to SCAR markers, but only the *Omy-163* marker produced distinctive male vs. female fragment patterns in the trout – that is, a sex-linked amplification pattern [41, 43].

Omy-163 has been tested for genotyping in several strains of rainbow trout, but has not always shown a Y-chromosome

association [43]. In cases where a Y-linked pattern was identified, some recombination between the putative *sex*-determining locus and the SCAR was observed. For example, in the global analysis performed by Felip *et al.* [42], 29 of 380 males were negative for the male pattern, and nine of 396 females were positive for the male pattern. In Lopez and Araneda [41], 16 of 47 males were negative for the male pattern, and 8 of 84 females were positive for the male pattern. Linkage studies show that *Omy-163* is located near the *SEX* locus, separated by a distance ranging from 0.0 to 42.2 cM (average 7.2 cM), making recombination plausible [42, 43].

12.2.5 *OtY2/OtY3/OmyY1*

OtY2-WSU is another marker with a Y-linked inheritance pattern, developed for Chinook salmon and later detected in coho, chum, and sockeye salmon [44]. *OtY2*-WSU shows autosomal inheritance in rainbow trout. A small number of coho ($n=48$) and chum ($n=30$) salmon were also screened; in sockeye salmon, the segregation pattern detected in 119 samples was not fully Y-linked, as 12 phenotypic males were negative and three phenotypic females were positive for the marker. *OtY2*-WSU was detected using AFLP screening for sex-specific fragments in pools of androgenetic diploid Chinook salmon (males and females). It is thought that these androgenetic individuals typically carry two copies of the paternal X-chromosome (in females) or Y-chromosome (in males), facilitating the identification of Y-specific markers [44]. *OtY2*-WSU genotyping was performed using trio PCR, with two pairs of male-specific primers and a primer for the glyceraldehyde-3-phosphate dehydrogenase gene (*gapdh*) as an internal control [44].

OtY2-WSU was the basis for developing two other Y-linked molecular markers, one for Chinook salmon (*OtY3*) and the other for rainbow trout (*OmyY1*) [45]. Both markers were studied using PCR screening in 12.5 kb and 21 kb genomic regions flanking *OtY2*-WSU in

Chinook salmon and rainbow trout, respectively. Approximately 10 kb of the sequences were found to be similar between the species. Extensive characterization of these genomic regions indicated that, in Chinook salmon, this region contains an inactive retrotransposon and a minisatellite. These were used to develop a PCR assay to amplify the fully Y-linked marker *OtY3*, which shows two male-specific alleles (725 and 500 bp) [45].

In rainbow trout, the marker contains a region that shows sequence homology with 18S ribosomal RNA and internal transcribed spacer 1 (ITS), the major histocompatibility complex (MHC) class IB intronic region, a LINE-1 type reverse transcriptase, and the *OmyY1* Y-linked marker (in the genomic region homologous with Chinook salmon). However, the retrotransposable element detected in Chinook salmon is absent in rainbow trout. The Y-specific marker *OmyY1* amplifies a 792 bp fragment at a high frequency in males (96.5%) and a low frequency in females (3.7%). This finding may indicate either some degree of recombination with the sex-determining region (note that some evidence of mobile elements has been provided for this region) or, as has been argued for other Y-linked markers, may be attributable to environmental sex reversion of some individuals [45].

Several single-nucleotide polymorphism (SNPs) have been identified in a 1,058 bp region, including the *OmyY1* Y-specific marker in various male lineages [45]. This male-specific region is not believed to undergo recombination. A Y-haplotype phylogeographic analysis of 333 male rainbow trout obtained from 57 locations in western North America and Russia was recently performed, but no information regarding the inconsistencies between phenotypic sex and *OmyY1* was reported [46].

12.2.6 Microsatellite Markers

With the development of salmonid genetic maps that include phenotypic sex, a number of microsatellite markers have been mapped

near the putative sex-determining locus (*SEX*) in a named sex- or Y chromosome-linked group. The first comparative analysis of the *SEX* locus was performed for Arctic char, brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*), and rainbow trout, indicating that the microsatellites linked to the *SEX* locus are different in every species [47].

The first microsatellite map for rainbow trout identified the locus *OmyFGT19TUF*, located 1.15 cM from the putatively sex-determining locus in males [48]. Advances in rainbow trout genetic maps have confirmed this finding. Other microsatellites detected in this sex-linked group (RT-1) and used to assign sex in rainbow trout include *Ots517NWFSC*, *OMM1026*, and *OMM1372* [27, 42, 43, 47, 49–52]. Finally, the RT-1 linkage group was identified as the sex chromosome (OmySex) in later genetic maps for this species [9].

In Atlantic salmon (*Salmo salar*), the first sex-linked microsatellite reported was *Ssa202DU*, followed by other markers in the linkage group AS1 [47, 53]. This finding was confirmed when the physical map was integrated with the genetic map, anchoring the *SEX* locus between *Ssa202DU* and a large heterochromatin region [55] in the Ssa02 chromosome. Interestingly, the *SEX* locus in this species has also been mapped in two other chromosomes, Ssa06 and Ssa03, depending on mapping families [56].

There are obstacles to using microsatellite loci for sexing salmonids. For one, microsatellite loci are not the sex-determining loci. For another, some degree of recombination between the microsatellites and the *SEX* locus is always possible. For example, in Tasmanian Atlantic salmon, the prediction of a phenotypic male, based on a Y-specific haplotype for seven microsatellites inherited from grandsire to sire, fails about 11.4% of the time, probably due to recombination among these markers and the *SEX* locus [56]. Another drawback of microsatellites is that it is necessary to know the paternal and maternal haplotypes to genotype the progeny.

12.2.7 *sdY* Gene

2012 marked the discovery of the *sdY* gene (sexually dimorphic on the Y chromosome), the master sex-determining gene in rainbow trout by Yano *et al.* [57]. This gene was discovered by comparing the gonadal transcriptomics of true males and females at the onset of molecular sexual differentiation. The presence of *sdY* was evaluated in 425 trout, and all 218 males were positive for the gene, while all 207 females were negative [57]. *sdY* encodes for a putative protein of 192 amino acids, has four exons, and shares homology with the rainbow trout sex-specific marker *OmyY1* [45] and interferon regulatory factor 9 (*Irf9*). The rainbow trout linkage map containing *sdY* confirmed full linkage with the *SEX* locus in the chromosome OmySex (RT-01 linkage group).

After this revolutionary discovery, screening for the *sdY* gene was performed in other salmonid species, yielding generally similar results to those found in rainbow trout. Species evaluated included graylings (*Thymallus thymallus*), masu salmon, Chinook salmon, Dolly Varden trout (*Salvelinus malma malma*), Arctic charr, brook trout, lake char (*Salvelinus namaycush*), Atlantic salmon, brown trout (*S. trutta*), huchen (*Hucho hucho*), and sakhalin taimen (*Parahucho perryi*) [58]. In all of these species, *sdY* is present in males and absent in females, with few deviations from this pattern.

However, another study carried out in Asian populations from five species of *Oncorhynchus* genus showed high rate of incongruences between presence/absence of *sdY* and phenotypic sex: Chinook salmon (41.2%), chum salmon (18%), sockeye salmon (44%), and masu salmon (31%). Only pink salmon presented a 4% on incongruences [59]. These high rates of females positive to *sdY*, and males negative to *sdY*, indicate a possible instability of this sex-determining locus in Pacific salmon [59].

More extensive screening for *sdY* has been performed in cultivated Atlantic and wild Chinook salmon. In Chinook salmon, *sdY* is likely the sex-determining gene, but some discrepancies have been found

between phenotypic sex and the presence of *sdY*. For example, Yano *et al.* [58] found one female positive for *sdY* among 41 females tested from a wild Alaskan population (USA). Cavileer *et al.* [60] found 13 phenotypic females positive for *sdY* among 107 females tested. In this latter work, four *sdY* coding regions were examined in the *sdY* positive females. Seven females were negative for the *sdY* promoter region and exon 1, but the other six seemed to have the complete coding region, despite a female phenotype. The most probable explanation for females bearing the whole *sdY* gene is that expression was somehow disabled, possibly due to environmental factors (temperature or estrogen contamination), during early development [60].

In Tasmanian Atlantic salmon, there is strong evidence for association among regions bearing the *sdY* gene and phenotypic sex, but there are also some discrepancies [56]. For example, six individuals, evaluated using two sets of *sdY*-specific primers (exon 2 and exon 4), were positive for this gene but phenotypically female, and two phenotypic males were also negative for *sdY* [56].

Similarly, our laboratory tested for the *sdY* gene in Atlantic salmon (mowi strain) breeders from the Huililco aquaculture reproduction program in southern Chile (Figure 12.1). Two phenotypic females were found to be positive for *sdY* among 45 females, and one phenotypic male

was negative for *sdY* among 45 males. Our laboratory used a set of primers published by Yano *et al.* [58] for exon 2 (*sdY*-E2S1: CCCAGCACTGTTTCTTGTCTC and *sdY*-E2AS2: CTGTTGAAGAGCATCAGGGTC). Interestingly, in Tasmanian Atlantic salmon, *sdY* was found in three different chromosomes, depending on the male lineage of the family. For example, in 58.6% of the 58 families analyzed, this gene was in chromosome Ssa02, but mapped to chromosomes Ssa06 and Ssa03 in 37.9% and 3.5% of families, respectively [56]. Therefore, in this species, the *sdY*-bearing chromosome region and *SEX* locus can suffer recombination with other chromosomes.

Current evidence supports a strong consensus that the *sdY* gene is likely the master sex-determining gene in rainbow trout, Chinook salmon, and Atlantic salmon, and probably other salmonid species. The inconsistencies between female phenotypic sex and the presence of the complete *sdY* gene (excluding genotyping or phenotype assignment error) in Chinook and Atlantic salmon may be attributable to temperature-dependent sex reversal [56], contamination with estrogens during early development [60], or an as yet undiscovered factor that must interact with *sdY* gene to produce sex differentiation.

Due to its high rate of success in identifying phenotypic sex, several tests have been developed using the *sdY* gene. For example, a

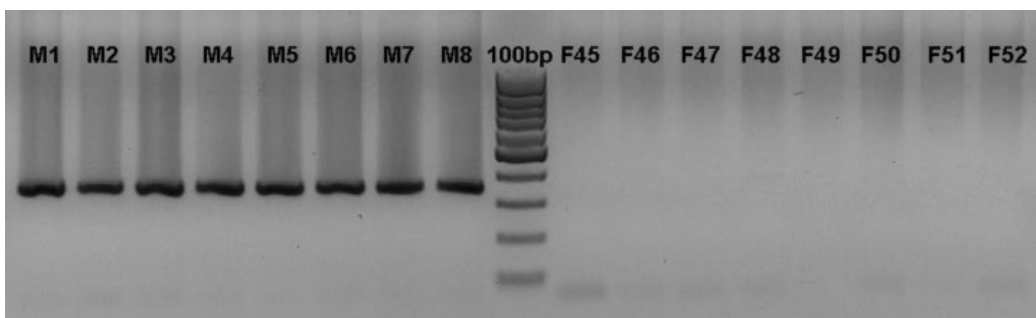


Figure 12.1 Agarose gel electrophoresis, showing the PCR amplification of *sdY* gene (exon 2) in eight males (M1 to M8) and eight females (F45 to F52) from Atlantic salmon. Males shown an amplicon of ≈ 350 bp, which is absent in females.

rapid test, based on high resolution melting analysis (HRM), simultaneously discriminates the sex and species of Atlantic salmon, brown trout, and their hybrids [61], using the two primer pairs published for co-amplification of *sdY* and 18S ribosomal RNA by Yano *et al.* [57]. The test has not been applied in many samples to date. However, it is an interesting, cost-effective, and quick method for sexing, as well as for species and hybrid identification, with potential applications in conservation biology and the food industry.

In the genus *Salmo*, a second assay, based on the amplification of a small section of 200bp of the *sdY* gene, was developed to be multiplexed with microsatellite markers [62]. The method was tested on 65 marine trout (*Salmo trutta*), with a mismatch of 3.2% [62]. Unfortunately, the authors did not provide raw data for a quantitative evaluation of their results using diagnostic tests.

A third quick method for sexing Atlantic salmon with *sdY* gene uses a TaqMan assay, based in the amplification of a fragment of 93bp from the 4th exon of the gene [63]. This method was tested on 2,583 individuals, detecting only one female among the 1,257 salmons positive to *sdY* (false positive rate = 0.08%), however the false negative rate (males negative to *sdY*) was not evaluated [64].

12.3 Evaluation of Sex Marker Applications in Salmonids

As described above, many sex-linked markers have been identified in salmonids, but only a few have been used extensively. To evaluate potential applicability to salmonid sexing, the approach described by Lopez and Araneda [41] is used here to estimate diagnostic statistics for each molecular assay: sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), likelihood ratio of a positive test result (LR+), accuracy (ACC), and diagnostic odds ratio (DOR). A basic description of all of these diagnostic tests can be found in Glas *et al.* [64]. Successful

Table 12.1 Contingency table for sex phenotyping and classification using a molecular assay.

		Genotype (Molecular Assay)		
		Positive (Male)	Negative (Female)	Total
Phenotype	Male	TP	FN	PM
	Female	FP	TN	PF
	Total	GM	GF	

performance was defined as correct identification of the male fish (XY individual), given that all of the molecular assays tested detect Y-chromosome gene or markers. In this type of analysis, individuals are classified in a 2 × 2 contingency table (Table 12.1), as follows:

TP, FP, FN, and TN denote the number of true positive, false positive, false negative, and true negative results, respectively. PM and PF are phenotypic males and females, respectively, identified through direct observation of gamete emission or gonads, and GM and GF are genotypic males and females, respectively, identified through genotyping with the molecular assay (Table 12.2).

The computational formulae for the tests are as follows:

Sensitivity (true positive rate) is the proportion of true (phenotypic) males correctly identified by the molecular assay.

Sensitivity = (P(PM ∩ GM) / P(PM)) = (TP / (TP + FN))

Specificity (true negative rate) is the proportion of true females correctly identified by the assay.

Specificity = (P(PF ∩ GF) / P(PF)) = (TN / (TN + FP))

To evaluate the probability that these molecular assays provide the correct gender identification, positive predictive value (PPV, i.e., the proportion of males with positive test results correctly sexed as male) and negative

Table 12.2 Performance of various molecular assays developed for salmonid sexing.

Marker positive fish										
Gen/Marker	Assay	Male	Female	Sensitivity	Specificity	PPV	NPV	LR+	DOR	ACC
Atlantic salmon:										
<i>sdY</i> ¹	PCR	542/555	4/384	0.9766	0.9896	0.9894	0.9769	93.75	3961	0.9819
<i>sdY</i> ²	PCR	64/65	2/65	0.9846	0.9692	0.9697	0.9844	32.00	2016	0.9769
Chinook salmon:										
<i>sdY</i> ³	TaqMan®	45/45	13/157	1.0000	0.9172	0.9235	1.0000	12.08	974 [†]	0.9356
<i>OtYI</i> ⁴	PCR	396/396	88/530	1.0000	0.8340	0.8576	1.0000	6.02	3965 [†]	0.9050
<i>GH-Ψ</i> ⁵	PCR	91/91	0/89	1.0000	1.0000	1.0000	1.0000	179.02 [†]	32757 [†]	1.0000
<i>OtY3</i> ⁶	PCR	143/143	0/127	1.0000	1.0000	1.0000	1.0000	255.11 [†]	73185 [†]	1.0000
Rainbow trout:										
<i>sdY</i> ⁷	PCR	218/218	0/207	1.0000	1.0000	1.0000	1.0000	415.05 [†]	181355 [†]	1.0000
<i>Omy-163</i> ⁸	PCR	386/427	21/480	0.9040	0.9563	0.9538	0.9088	20.66	206	0.9313
<i>OmyP9</i> ⁹	PCR	35/47	12/84	0.7447	0.8571	0.8390	0.7705	5.21	18	0.8168
<i>OtY2-WSL</i> ¹⁰	trio-PCR	94/94	0/104	1.0000	1.0000	1.0000	1.0000	208.89 [†]	39501 [†]	1.0000
<i>OmyYI</i> ⁶	PCR	139/144	5/134	0.9653	0.9627	0.9628	0.9652	25.87	717	0.9640
Brown trout:										
<i>sdY</i> ⁷	PCR	73/73	76/76	1.0000	1.0000	1.0000	1.0000	152.96 [†]	22491 [†]	1.0000

(Continued)

Table 12.2 (Continued)

Marker positive fish										
Gen/Marker	Assay	Male	Female	Sensitivity	Specificity	PPV	NPV	LR+	DOR	ACC
Coho salmon:										
<i>GH-2</i> ¹¹	PCR	41/41	0/47	1.0000	1.0000	1.0000	1.0000	94.86 [†]	7885 [†]	1.0000
Masu salmon:										
<i>GH-Ψ</i> ¹²	PCR	63/70	2/61	0.9000	0.9672	0.9649	0.9063	27.45	266	0.9313
Sockeye salmon:										
<i>OtY2-WSL</i> ¹⁰	Trio PCR*	49/61	3/58	0.8033	0.9483	0.9395	0.8282	15.53	75	0.8739

¹ Eisbrenner *et al.* [56].
² Combined data from Yano *et al.* [58] and Araneda (unpublished).
³ Cavileer *et al.* [60].
⁴ Combined data from Devlin *et al.* [25, 29], Nagler *et al.* [30] and Williamson and May [31].
⁵ Combined data from Du *et al.* [33] and Devlin *et al.* [29].
⁶ Brunelli *et al.* [45].
⁷ Yano *et al.* [57].
⁸ Combined data from Felip *et al.* [42] and López and Araneda [41].
⁹ López and Araneda [41].
¹⁰ Brunelli and Thorgaard [44].
¹¹ Forbes *et al.* [36].
¹² Zhang *et al.* [35] and Yamamoto and Kitanishi [38].
[†]Estimated adding 0.5 to all counts due to LR+, and DOR are undefined if the 2 × 2 contingency table contains zeroes.

predictive value (*NPV*, i.e., the proportion of females with negative results correctly sexed as female) were estimated with the equation from Altman and Bland [65]. In the next two equations, *Prevalence* was assumed to be 0.5, as this is the expected proportion of males in a normal population [41].

$$PPV = \frac{Sensitivity \cdot Prevalence}{Sensitivity \cdot Prevalence + (1 - Specificity) \cdot (1 - Prevalence)}$$

$$NPV = \frac{Sensitivity \cdot (1 - Prevalence)}{(1 - Sensitivity) \cdot Prevalence + Specificity \cdot (1 - Prevalence)}$$

The likelihood ratio of a positive test result (*LR+*) was estimated to evaluate the usefulness of molecular assays in identification of males. This statistic is the ratio of a positive “male” test result among phenotypic males to the same positive result among phenotypic females. Larger values of *LR+* indicate better performance.

$$LR+ = \frac{Sensitivity}{(1 - Specificity)}$$

Accuracy (*ACC*), that is, the proportion of correctly-identified subjects, was estimated as follows:

$$ACC = \frac{(TP + TN)}{(TP + TN + FP + FN)}$$

Finally, the diagnostic odds ratio (*DOR*) of a test is the ratio of the odds of a positive result among phenotypic males relative to the odds a positive result among phenotypic females.

$$DOR = \frac{\left(\frac{TP}{FP}\right)}{\left(\frac{FN}{TN}\right)} = \frac{\left(\frac{Sensitivity}{(1 - Sensitivity)}\right)}{\left(\frac{1 - Specificity}{(Specificity)}\right)} = \frac{\left(\frac{PPV}{1 - PPV}\right)}{\left(\frac{1 - NPV}{NPV}\right)}$$

Higher values of *DOR* indicate better discriminatory test performance, and values close to 1 indicate that the genetic test does not discriminate between the sexes. The *DOR* is highest when sensitivity and specificity are close to 1.0 [64].

The genotypic and phenotypic sex data published for each assay in each salmonid species were used for these estimations.

The only restriction was that the analyzed samples must include at least more than 40 individuals per sex (Table 12.2).

In general, nearly all of the markers developed for sexing salmonids showed high sensitivity and specificity for detecting a true male individual, with a *DOR* value above one (Table 12.2). The performance of various assays developed for different species shows that, in general, markers developed for the *sdY* gene performed better than other markers when enough data were available for analysis.

For Atlantic salmon, the assay developed by Eisbrenner *et al.* [56] showed the best performance. In Chinook salmon, an assay based on the *OtY3* marker [45] showed the best performance among four markers evaluated. In rainbow trout, a comparison of five different markers indicated that the best sexing test was based on the *sdY* gene developed by Yano *et al.* [57]. For brown trout, coho, masu, and sockeye salmon, only one marker was evaluated in each species, based on the *sdY* gene [58], *gh2* gene [36], *ghΨ* [35], and *OtY2-WSU* [44], respectively.

On the other hand, Podlesnykh *et al.* [59] have shown congruence in genotyping between the *sdY* gene and other Y-linked molecular markers in some Pacific salmon. For example, in Chinook salmon and sockeye salmon, sexing performance was similar, with *sdY* and with *OtY2-WSU* marker. Similarly, in masu salmon, sexing performance was also similar between *sdY* and *ghΨ* marker. These findings indicate that it is possible to use *sdY* instead of other Y-linked molecular markers in these species. However, considering the small samples used by species (29–50), these results should be considered preliminary.

It is highly probable that the application of the primer sets developed by Yano *et al.* [58], Eysturskarð *et al.* [63], or Quéméré *et al.* [62] in more individuals of other salmonid species would reveal that *sdY*-based tests show the best performance for salmonid sexing if *sdY* is truly the sex-determining master gene for all salmonids. However, molecular assay for salmonid sexing must be more cost effective, faster, and validated with international standards such ISO 17025, before they will be extended to the industry.

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13

Polyploidy Production in Salmonidae

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13.1 Introduction

Polyploidy is the special situation where one or more additional chromosome sets, beyond the normal condition (usually diploid), are found within all the nuclei of an individual organism. Fishes are among the members of several vertebrate classes that tolerate polyploidy [1]. Polyploidy has been observed at low levels within natural populations of fishes [2–5], but methods have been developed for some time now to induce polyploidy intentionally [6, 7].

Induced polyploidy has a long history in fishes of the family Salmonidae, chiefly because of an interest in farming these fishes, and the fact that they have genetic sex determination and external fertilization. The *in vitro* methods for collecting, handling gametes, and conducting fertilization in salmonids have long been established, and these features have aided polyploidy method development. Early experiments to induce polyploidy in salmonids used low temperature [8, 9] or chemical [10–12] treatments of the newly fertilized eggs, with limited success. The numbers of polyploid fish produced were low, methods worked inconsistently, and genetic mosaics sometimes occurred.

The first method in salmonids that became widely utilized was that of Chourrout [13],

using a hyperthermic treatment on rainbow trout (*Oncorhynchus mykiss*) eggs immediately after fertilization to produce triploids (containing one additional chromosome set beyond the diploid condition). Onozato [14] followed this with another method – the application of hydrostatic pressure to newly fertilized eggs – to produce triploid rainbow trout. Tetraploid (containing two additional chromosome sets beyond the diploid condition) rainbow trout were first induced experimentally by chemical [15] or hydrostatic pressure [16] treatments of eggs before the first mitotic division of the zygote. Subsequently, both triploid and/or tetraploid salmonids have been produced in other *Oncorhynchus* [16] and *Salmo* [18–20] genera, and the genus *Salvelinus* [21, 22].

The initial interest in the production of polyploid salmonids occurred for the aquacultural purposes of enhancing growth and controlling reproductive development (i.e., sterility). Secondly, there has been research using polyploidy to understand the biological consequences of chromosomal modification [23] and chromosome dosage effects [24] in salmonids. The following sections provide details on induced triploidy and tetraploidy production in salmonids.

13.2 Triploid Production

Two mechanisms have emerged for the production of triploids in salmonids – methods (i.e., chemical, high temperature, and hydrostatic pressure) that interfere with and prevent the release of the second polar body during metaphase II of meiosis (Figure 13.1), or the use of fused sperm for fertilization [25]. In practice, there have been varying degrees of efficacy and acceptance of these methods for producing triploid salmonids for aquaculture.

The chemicals cytochalasin B [10, 11], colchicine [12], and nitrous oxide [26] have all been used to treat salmonid eggs immediately after fertilization, to produce triploid fish (Box 13.1). Chemical treatments appeared on the scene early, but were not widely adopted, and their use is non-existent at the present time. This is due to other, more effective

triploid induction methods being available, that do not require the use of a chemical.

Early experimentation with temperature and salmonid triploidy used a low temperature shock during early development, but a high temperature shock proved more reproducible and effective [13, 27]. Delivery of a high temperature shock to eggs shortly after fertilization induced high levels of triploidy, and became popular in aquaculture because of the lack of a need for specialized equipment [29–31] (Box 13.1). In practice, this method does not routinely yield 100% triploid production, because it is difficult to apply the precise temperature treatment uniformly to all eggs [32]. Therefore, there are limitations for the use of this triploid method, if there is a stringent requirement for all-sterile fish.

The delivery of a hydrostatic pressure treatment to newly fertilized salmonid eggs is an effective method for the production of triploids (Box 13.1). It has been widely adopted, because it is more reliable than a high temperature shock at producing 100% triploid salmonids [32–34]. This is due to the fact that it is much easier to uniformly apply a hydrostatic pressure treatment to all eggs within a pressure chamber. The only drawback to this method is the initial investment for a specialized piece of equipment, in the form of the pressure chamber needed to treat the eggs.

Finally, dispermic fertilization in rainbow trout has been reported for triploid production whereby two sperm are first fused and then used to fertilize an egg [25]. This method has not seen use in salmonid aquaculture, because of the difficulty of reproducibly fusing the sperm, and the low numbers of triploid fish produced.

Triploid production for salmonid aquaculture has received much attention, because these fish are sterile. It has been demonstrated in several species that the development of the gonads is impaired in triploids [35–37]. Typically, germ cells in the ovaries of female triploids do not enter meiosis, and these individuals do not display secondary sexual characteristics. In triploid males, the testes do develop, but they usually lack a

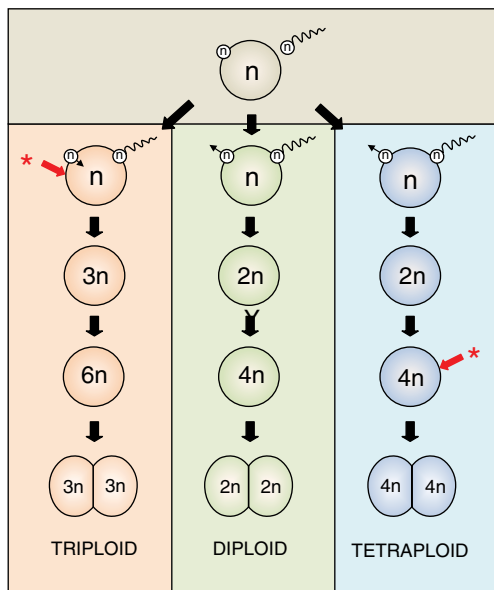


Figure 13.1 Schematic diagram to produce triploid, diploid, or tetraploid salmonids. This scheme shows a haploid (n) sperm fertilizing the female egg. A polar body is shown either being expelled or retained, depending on the developmental timing of the treatment applied (*). The treatment (*) to produce triploids or tetraploids could be either chemical, an elevated temperature, or hydrostatic pressure.

Box 13.1 Methods for inducing triploidy in salmonids**1) Chemical**

Cytochalasin B (10 mg/ml dissolved in 0.1% dimethylsulfoxide) is added to fertilized Atlantic salmon* eggs 45–70 hour degrees (the incubation temperature \times hours post fertilization) after fertilization, or rainbow trout eggs 35–50 hour degrees after fertilization at 8°C [10]. Colchicine (0.01% solution) is added just before the first cleavage (105 hour degrees) to fertilized brook trout eggs at 10°C [12]. For nitrous oxide, newly fertilized rainbow trout eggs are treated with elevated pressure (11 atm) and pure nitrous oxide gas for a period of 30 minutes at 9°C [26]. In all cases, eggs are then transferred to clean

water after the treatment period to complete the incubation period.

2) Temperature

Rainbow trout eggs held at 10°C approximately 10 minutes after fertilization were exposed to a heat shock of 34–37°C for one minute before moving them back to 10°C [27].

3) Hydrostatic pressure

Rainbow trout eggs were treated with 650–700 kg/cm² of hydrostatic pressure for 6–7 minutes, starting 5–15 minutes after fertilization. Water temperature was 10°C [14].

*use of other salmonid species would require empirical testing to determine optimal conditions

sperm duct, preventing the release of mature gametes. In the few instances where sperm is released, the semen is dilute [37], and high levels of sperm aneuploidy have been reported [38]. For these reasons, male triploid salmonids are considered sterile, although they do develop secondary sex characteristics, because the testes develop to the point that the necessary sex steroids are synthesized and released into the blood.

The perceived advantage of triploidy for salmonid aquaculture rests with the female, because most of the industry is geared toward a female-only culture. Given that triploid females do not develop ovaries, the energy that would normally be used for reproductive development could, theoretically, be used for enhanced somatic growth. This would, ideally, result in a greater meat yield more quickly. Unfortunately, this has not been achieved. Considerable research has failed to broadly establish that triploids grow better than diploid fish of the same species [39–41]. In some instances, salmonid interspecific or intergeneric triploid hybrids have demonstrated traits (e.g., survival, growth, seawater tolerance, and disease resistance) that may be useful for aquaculture [21, 43–48]. However, these hybrids have not seen significant use by the fish farming community.

Currently, the most important use of triploid salmonids, since they are sterile, is in freshwater stocking programs, where these fish can be released into waters without the possibility of them breeding and establishing a population, or hybridizing with related species [49]. This has been done to increase angling opportunities for salmonids both within and outside their native ranges [50]. The release of triploid salmonids into the environment does hinge on the method employed for the production of the triploid fish because, in most instances, it is desirable to release 100% triploids. The method of choice would be hydrostatic pressure treatment. An alternative would be the use of interspecific or intergeneric triploid crosses, to obviate any problem encountered with single-species triploids that are not 100% sterile [52].

An emerging use of triploidy, because the fish will be sterile, involves the production of transgenic fish. The AquAdvantageTM Atlantic salmon, a transgenic that has an added Chinook salmon growth hormone gene, can be marketed in the United States if it is triploid [51]. The concern is that transgenic fish might escape into the environment and breed with wild fish. Utilizing triploidy will dramatically reduce the probability of this

occurring, in the event of an accidental release into the environment.

13.3 Tetraploid Production

The desire for tetraploid salmonids emerged when the potential value of having triploids was realized. By crossing a tetraploid fish with a diploid mate, 100% triploids would result [53]. The hope was that this method would circumvent problems encountered with chemical or physical (e.g., temperature) methods of triploid induction. Tetraploid salmonids were first produced in rainbow trout, with either a chemical treatment [15] or high temperature shock [27] of the developing embryo in advance of the first mitotic division (Box 13.2). By suppressing this initial mitotic division, the chromosome number was doubled, resulting in a tetraploid individual (Figure 13.1).

Hydrostatic pressure treatment was also shown to be effective for making tetraploids in the rainbow trout [54, 55], brown trout (*Salmo trutta*) [56], and both intraspecies and interspecies salmonid hybrids [57] (Box 13.2). Much effort has been devoted to producing tetraploid salmonids, but instances of poor embryonic survival, abnormal development, and/or mosaicism have been observed [10, 59, 60]. The rainbow trout is the only species in which

tetraploids have been somewhat successful for aquaculture purposes [61–63]. It is not obvious that there is a particular advantage with rainbow trout – it may just be that this species was more readily available to the research community conducting this work. Therefore, with the appropriate investment, it should be possible to develop tetraploids with other salmonid species, which could be used to produce triploids on a production level scale.

13.4 Conclusion

The induction of polyploidy in salmonids for aquaculture improvement has a long history. Both triploid and tetraploid salmonids have been produced. The application of triploidy is much more widespread, because several methods, yielding high levels of efficacy (e.g., high temperature and hydrostatic pressure), have emerged. The desire for tetraploid salmonids stemmed from the resulting opportunity to make triploids, by crossing a tetraploid mate with a diploid mate. Unfortunately, the hoped-for advantages of improved growth performance and/or enhanced disease resistance of having a sterile (triploid) salmonid for the purpose of fish farming have not been widely realized. However, triploid fish are produced by the

Box 13.2 Methods for inducing tetraploidy in salmonids	
1) Chemical Cytochalasin B (10mg/ml dissolved in 0.1% dimethylsulfoxide) is added to fertilized rainbow trout* eggs, starting at 30–40 hour degrees (the incubation temperature×hours post fertilization) until the four-cell stage, at 10°C [15]. Eggs are then transferred to clean water to complete the incubation period.	exposed to a heat shock of 34–37°C for one minute before moving them back to 10°C [27].
2) Temperature Rainbow trout eggs held at 10°C approximately five hours after fertilization are	3) Hydrostatic pressure Rainbow Trout eggs are treated beginning at 62–65% of the first cleavage interval (i.e., proportion of eggs attaining first cleavage) for eight minutes in a pressure chamber at 633 kg/cm ² [55].
*use of other salmonid species would require empirical testing to determine optimal conditions	

aquaculture industry for stocking purposes. Because they are sterile, they can be added to the environment without the concern that they will establish self-supporting populations or hybridize with related species.

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Dedication

This chapter is dedicated to Professor Gary Thorgaard, one of the pioneers of induced polyploidy research in salmonids.

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Part IV

Sex Determination and Control in Moronidae

14

Genetic and Environmental Components of Sex Determination in the European Sea Bass

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14.1 Introduction to European Sea Bass Ecology and Reproductive Biology

The European sea bass (*Dicentrarchus labrax* L.) is a teleost fish of the order Perciformes (perch-like fishes) that belongs to the family Moronidae (temperate basses). Its closest relative is *Dicentrarchus punctatus*. The striped bass, *Morone saxatilis*, is also a member of the same family. However, there are other many “basses” that, although belonging to the same order, are placed in different families, such as the largemouth bass, *Micropterus salmoides* (F. Centrarchidae), the Asian sea bass, *Lates calcarifer* (F. Lateolabracidae), and black sea bass, *Centropristis striata* (F. Serranidae), just to name a few.

The European sea bass ecology has been described in detail by Pickett and Pawson [1] and Pérez-Ruzafa and Marcos [2]. Briefly, it is a temperate water species that is distributed across the northeastern Atlantic, the Mediterranean, and the Black Sea. Larvae are planktonic, but juveniles move inshore, aggregate in brackish and estuarine areas, and can occasionally penetrate rivers. Adults are demersal, inhabiting shallow coastal waters, usually around 10m deep, but can also be found at about 100m depth. It is a

eurythermal and euryhaline species. Salinity tolerance is in the range of 0–60 ppt [3], while temperature tolerance is in the range of 5–32°C when adults [2]. Young fish form schools, but adults are less gregarious. It is a carnivorous species. Juveniles feed on zooplankton and invertebrates, and adults on worms, shrimp, squids, mollusks, and small fish [1, 2].

The European sea bass is a gonochoristic species. In the Mediterranean, males and females reach sexual maturity at two and four years old, respectively. However, the Atlantic population usually takes longer: 4–7 years for males, and 5–8 years for females. Spawning is seasonal, and takes place only once a year for a given population. Temperature and photoperiod are the two most important environmental variables that dictate when animals will reproduce. Thus, eggs are rarely found where the water is colder than 8.5–9.0°C or warmer than 15–17°C [1].

In the Mediterranean, adults start gametogenesis after the summer, and the spawning season takes place between December and April while, in the Atlantic, it takes place at the end of the spring. Females have high fecundity, producing around 200,000 eggs per kg. The eggs are pelagic, and their size is about 1.1–1.3 mm of diameter. Fertilization

is external. The time to hatching and larval development rate is temperature-dependent. Adults can grow to a length of approximately one meter, and a weight of 9–10 kg (sometimes up to 15 kg), and have a life span of up to 20 years [1].

The European sea bass is highly appreciated for the quality of its meat and, thus, is of interest for both commercial and sport fishermen, the latter especially in the North Atlantic. The demand prompted the development of its aquaculture, and it was one of the first marine fishes domesticated for intensive aquaculture production in Europe. Global capture fisheries are stable, at around 9,000 tons a year. However, aquaculture, after a slow start in the 1980s, took off in the 1990s and has been steadily increasing since then, with a production of around 160,000 tons in 2014 [4]. Aquaculture is centered on the production of pan-sized fish, and thus most fish are sold whole at a weight of 350–400 g. However, in recent years, there has been a growing interest in the production of larger animals in the range of 1–3 kg for filleting.

Females are about 30% larger in size than males. However, under culture conditions, 70–100% of the population develops as males. Further still, about one third of these males mature precociously. Together, this has made all-female (monosex) population desirable, and has created interest in exploring the possibility of controlling sex ratios (see Chapter 15). For that, it is first necessary to understand sex determination in the European sea bass, in order to devise methods to bring this process under human control.

14.2 Karyotype, Genome

The haploid European sea bass karyotype is made of 24 ($2n=48$) chromosomes, most of them acrocentric [5]. Morphological analysis of the European sea bass karyotype preparations fail to show readily distinguishable sex chromosomes, thus resembling the situation commonly found in most teleosts [6].

The nucleolar organizing regions (NORs) are located at the terminal or near-terminal sites on the short arms of chromosome pair 22 [7]. In a wild population, chromosome variation has been discovered, involving the amount and the patterns of heterochromatin distribution in one of the two smallest chromosomes of pair 24 of the mitotic complement. Difference in the distribution of C-banding patterns between the two sexes karyotypes was previously interpreted as being suggestive of a XX/XY sex-determining system [7]; however, recent published data indicated that it has a polygenic sex determination system instead [8].

The European sea bass genome sequence was made public in 2014, as a result of collaboration between several European countries [9]. It has a haploid size of 675 Mb, which is one of the smaller fish genomes sequenced to date, similar in size to the green spotted puffer, *Tetraodon nigroviridis* (≈ 400 Mb), about half the zebrafish (*Danio rerio*) genome size (≈ 1.3 Gb), and much smaller than the human genome (≈ 3.2 Gb). The total number of genes annotated upon the release was 26,719. The repetitive elements account for slightly above one-fifth of the European sea bass genome assembly, which exhibits complete synteny and large blocks of collinearity with the corresponding chromosomes of the three most closely related teleost species for which there is a chromosome-scale assembly: the three-spined stickleback, *Gasterosteus aculeatus*, the Nile tilapia, *Oreochromis niloticus*, and the green spotted puffer.

Expansions of gene families associated with ion and water regulation have been interpreted as proof of the euryhaline nature of this species [9]. Regarding recently duplicated genes, six paralogs unique to the European sea bass were identified. Among them, the nuclear receptor co-activator 5 (*NCOA5*) stands out in the context of this chapter. In the Nile tilapia, expression of this gene during larval development showed sex-linked differences [10]. Since recently duplicated genes can acquire a role in the sex

determination cascade (see Chapter 1 of this book), *NCOA5* has been suggested as an interesting candidate for the European sea bass genetic sex determination system [9]. One of the variants of *NCOA5* is upregulated in females, responsive to estradiol-17 β stimulation and downregulated by elevated temperature (Díaz, Tine, Bargelloni and Piferrer, unpublished observation).

In summary, the European sea bass genome assembly is one of the best high-quality draft genomes available for a species of economic importance for both aquaculture and fisheries. Thus, it is the grounding base for further genomic, epigenomic, and transcriptomic studies, and constitutes a valuable resource for future genetic improvement and evolutionary analyses.

14.3 Sex ratios in Farmed Populations

When European sea bass farming basic zootechnical aspects were figured out, and its industrial production (albeit in modest amounts) started in the mid-1980s, it soon became evident that the sex ratio in farmed populations was skewed toward males. Male-biased sex ratios were also common in experimental facilities in Mediterranean countries. Blázquez *et al.* [11] mentioned cultured stocks with >90% males. Similar figures have also been reported in many scientific publications, particularly those related to the study of external factors on European sea bass sex ratios, or those attempting to control the sex ratio.

Thus, starting in the mid-90s, attempts were made to promote the production of all-female stocks. As with many fish, sex ratios can be controlled in the European sea bass through the administration of sex steroids during early development, either for feminization or masculinization (see [12] for review). Masculinization can also be induced by the administration of an aromatase inhibitor [13]. However, the use of steroids is not advisable, due to concerns on environment

safety and consumer preference. Further, hormonally masculinized individuals failed to produce 100% females in their offspring [14], contrary to other species like trout, which has a XX-XY chromosomal sex determination, and where the use of sex-reverted XX “neomales” as broodstock produces 100% females – XX offspring.

It is clear that, given the small, but significant, sex ratio bias toward females in natural populations (see Section 14.4), the male-biased sex ratio found in farmed populations is due to the aquaculture setting. The values and influences of the biotic and abiotic environmental factors in a farming system are necessarily very different from the values of the same factors under the natural environment, but temperature – particularly during early stages of development – certainly has a major role (see Section 14.8). Finally, we can also say that there is no such thing as the typical European sea bass sex ratio of farmed populations, since they vary considerably but, on average, they fall in the range of 70–95% males (although figures above and below this range are possible).

14.4 Sex Ratios in Natural Populations

As sex ratios of European sea bass in farmed populations are generally heavily biased towards males, it is relevant to study the sex ratio observed in wild populations. In theory, as put forward by Fisher in 1930, sex ratios in natural populations are expected to conform to the 1 : 1 ratio, as an under-represented sex would have a higher fitness (producing more offspring per individual) and, thus, be positively selected by natural selection [15]. Many European sea bass are collected and measured in population dynamics studies related to fisheries but, unfortunately, sex ratio is not always recorded, partly because there are no clear external dimorphic sexual characters [16].

A review of existing fisheries-based studies recording European sea bass sex in natural populations was done in 2012 [17], analyzing

sex ratio data from 4,889 individuals from four studies in the UK [18–21], one in the South Atlantic in Spain [22], and four in the Mediterranean [16, 23–25]. The general finding was that, taken as a whole, the sex ratio of wild European sea bass populations is mildly, but significantly, biased towards females (59.4% females), both in Mediterranean (54.8% females) and Atlantic (61.3% females) populations.

This was confirmed by an additional study in three locations of Wales in 2015, globally showing 55.8% females in a sample of 1,147 fish [26]. Of the 16 sampling locations available combining the two studies [17, 26], a significant excess of females appears in 11 locations, the other five showing balanced sex ratios. An interesting observation, when size data are available, is that the sex ratio of the younger fish (total length (TL) < 30 cm, $n = 1314$) is balanced (52.0% females), while that of larger fish (TL > 40 cm, $n = 1811$) is strongly biased (69.5%) towards females [17]. This predominance of females in larger fish could be linked to a small extent to the larger size-at-age of females, but is more probably linked to a higher longevity of females [21].

Another important point, which may explain part of the global excess of females observed, is the fact that sampling can be biased by the time of the year, the fishing method, and the location [20, 21, 26]. Indeed, fishing methods targeting larger fish would automatically bias sex ratios towards more females. In any case, these studies show that the excess of males generally encountered in farmed populations is not a characteristic of the species, and that young European sea bass observed in natural waters generally have a balanced sex ratio, thus conforming to Fisher's theory.

One last point of interest concerning natural sex ratios is the fact that, when age class data are available, it appears that in some age classes, sex ratios are unbalanced towards females [18, 21, 25] or males [25]. It can be hypothesized that these variations are linked to environmental conditions during sex determination in the years concerned [17] and, indeed, theory shows that for a sex

determination system to have an environmental component, it is necessary that some environmental conditions provide better fitness to one or the other sex [27–29]. These observations of brood year-specific sex ratios in natural populations of European sea bass do not bring a formal confirmation of this, but at least are in line with theory.

14.5 The Genetic Component of Sex Determination in the European Sea Bass

In most vertebrates, sex is genetically determined (Genotypic Sex Determination or GSD), generally with sex chromosomes, yielding stable 1 : 1 primary sex ratios. This is not the case in European sea bass, where sex ratios in natural populations may vary according to brood years, and where the general picture in farmed populations is that sex ratio is strongly biased towards males. An alternative system is the Environmental Sex Determination system (ESD), whereby the sex of an individual is mostly determined by the environmental conditions (often temperature) during a sensitive phase of early development. However, this could not explain the observations that in the same environment, different families of European sea bass exhibit different sex ratios, suggesting an influence from the parental genotypes.

The first results suggesting this genetic variation were obtained on a series of group matings and single pair matings of European sea bass, showing variable sex ratios [30]. It was then clearly demonstrated in 2002, where sexing communally reared families of European sea bass, *a posteriori* identified by genotyping of genetic markers, revealed that there was a strong impact of the male and female parents genotypes on the offspring sex ratio [31]. The variation of observed sex ratios was wide among the 27 families tested, ranging from 3 to 79% females. Another experiment on a 2 sires \times 2 dams mating showed 20.7–68.2% females among families [32]. Considering the variation in sex ratio due to the environment,

and the fact that different families exhibit different sex ratios, a polygenic sex determination system (PSD) was postulated, whereby sex is determined by several (and likely many) independent sex factors, adding up to environmental effects [8].

This was first demonstrated in a setting of 5,893 offspring from 245 families, where proportions of females ranged from 4.7 to 46.3% in paternal half-sib families, and from 0.5 to 40.3% in maternal half-sib families [8]. The genetic effect was shown to be mainly additive, and the distribution of sex ratios among families showed that:

- 1) a simple genetic model could not explain the data – meaning that random environmental variance was required; and
- 2) with environmental variance, at least two (and possibly more) biallelic loci were needed to explain the distribution observed.

Thus, sex was modeled under the quantitative genetics framework as a polygenic threshold trait, as proposed earlier by Bulmer and Bull [27]. Under this model, the observed sex is male if an underlying sex tendency t is negative, and female if t is positive, while t follows a normal distribution with a variance equal to unity (Figure 14.1). The sex tendency t can be equally influenced by genotype and environment ($t = G + E$, with t the phenotypic

sex tendency, G the effect of genotype, and E the effect of environment).

In a given population, the observed sex ratio allows the calculation of the average sex tendency of males and females (t_m and t_f respectively – see Figure 14.1). In a group with a proportion P_f of females, the sex tendency parameters are calculated as follows, after adaptation from Bulmer and Bull [27]:

$$t_{mean} = \text{probit}(P_f) \quad (1)$$

$$t_f = t_{mean} + \frac{\phi(t_{mean})}{P_f} \quad (2)$$

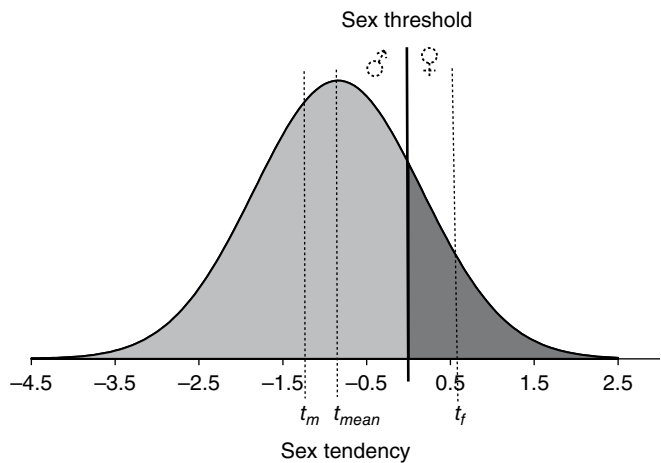
$$t_m = t_{mean} + \frac{\phi(t_{mean})}{1 - P_f} \quad (3)$$

where **probit** is the inverse of the cumulative distribution of the standard normal distribution, ϕ is the probability density function of the standard normal distribution, t_{mean} is the average sex tendency of the population, t_f is the average sex tendency of the females in the population, and t_m is the average sex tendency of the males in the population (Figure 14.1).

This modeling resulted in a high heritability estimate of sex tendency ($h^2 = 0.62 \pm 0.12$), showing that in a single (farm) environment condition, in the Atlantic population of sea bass, more than half of the observed variance

Figure 14.1 Threshold model for sex ratio in a population with a normally distributed sex tendency and 20% of females.

t_m = mean sex tendency of males,
 t_{mean} = mean sex tendency in the population, t_f = mean sex tendency of females.



in sex tendency was of additive genetic origin. This would be different in varied environments; environments would impact the average sex ratio, with possible additional genotype by environmental interactions, meaning that the relative rank of families for sex ratio may change if environmental conditions change [31].

The PSD system observed in European sea bass seems quite peculiar, but it is more and more apparent that there is a historical bias towards studying “simple” sex determination systems like GSD or ESD [33, 34] whereas, in reality, many species could have intermediate systems like PSD, with both genes and environment influencing sex. To further test for the polygenic nature of sex in European sea bass, a QTL scan was performed with 6,706 Single Nucleotide Polymorphism (SNP) markers in an F_2 population of European sea bass divergent for their sex tendency. None of the individual SNPs was significantly associated with sex, but still putative QTLs for sex determination were found at three genome-wide significant positions and six chromosome-wide significant positions on nine different chromosomes [35]. This confirms the polygenic nature of sex determination in European sea bass, which implies that it is very unlikely to develop diagnostic markers for European sea bass genetic sex, as it is only a tendency to develop as male or female, conditional to environmental conditions.

One of the important theoretical features of PSD is its supposed evolutionary instability, which implies it is expected to evolve towards GSD or ESD, depending on assumptions of the environmental variability and on the connectivity between populations determining their sexes in different environmental “patches” [27, 29, 36]. However, it has recently been shown that inter-generational variability in environmental conditions could lead to the maintenance of PSD over time [37]. It can still be expected that, in different populations of European sea bass submitted to different environmental regimes, sex determination systems may evolve differently. There is some evidence that different

populations of European sea bass yield different sex ratios when reared in the same conditions [38, 39], or may react differently to a change in environmental conditions [40], but this topic clearly deserves more attention.

14.6 The Relationship Between Sex and Growth

Sex-linked size dimorphism is observed in many fish species and, in the European sea bass, females are larger than males. This sexual size dimorphism in fish is essentially due to two evolutionary pressures: fecundity in females directly increases with size [41], and weak male-male competition is observed in most species [42]. These two pressures apply to the European sea bass. The existence of sexual growth dimorphism in farmed populations has undesirable consequences [43], especially if the sex with the highest growth does not predominate in the cultured stocks, as is the case of the European sea bass.

Sex-related growth starts to be visible before sex differentiation is complete [14, 44, 45], and must be established very early in life, as sorting the largest fish at 84 dpf (36–45 mm total length) results in a clear excess of females, compared with the general population, without further effect of additional gradings [46]. Size-grading before and during sex differentiation also results in female-dominant (faster growth) and male-dominant (slower growth) populations [47]. Sex ratio can skew up to 91% female in the large group, with just two size-gradings between 66 and 143 dpf.

Another study that tagged individual fish with nano-tags found that average weight in future females at 105 dph (590 mg mean weight, 27–53 mm total length) was already 31% higher than in future males [48]. Together, these observations indicate that sex-linked size dimorphism is present before the first histological signs of sex differentiation at 150 dph. In the Atlantic population of European sea bass, it was shown that there was a +0.50 genetic correlation of sex

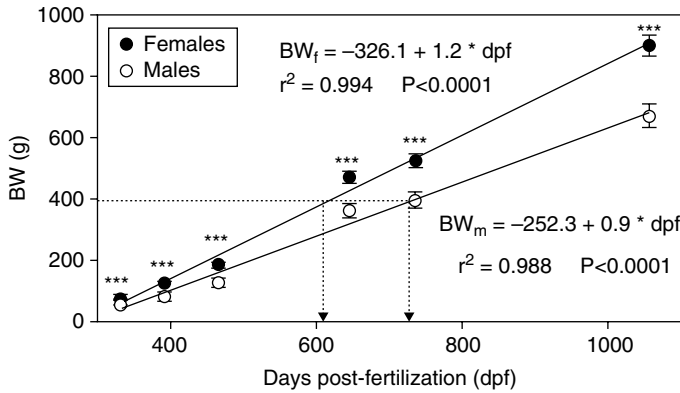


Figure 14.2 Sex-size relationship in two- and three-year-old European sea bass. Significant differences ($P < 0.001$) between sexes at each sampling time are symbolized by ***. The body weight (BW) in g as a function of age (in dpf) is shown for males (BW_m) and females (BW_f). From the linear correlation, the time of marketing (set at 400 g) was estimated to be 605 dpf for females and 725 dpf for males and is indicated by vertical arrows. Data are shown as mean \pm SEM.

Modified from Navarro-Martín *et al.*, 2009 [13].

tendency with body weight at one year of age [8], meaning that selection for growth should lead to higher percentages of females in the next generation in this population.

In another experiment, using repeated sampling and genotyping of the same families, it was shown that the genetic correlation of body length with sex tendency was 0.35 ± 0.21 (non-significantly different from zero) at 10 dpf, rose to 0.77 ± 0.16 at 90 dpf, and was between 0.46 ± 0.16 and 0.54 ± 0.14 from 238 dpf onwards [49]. This suggests that, at least in the Atlantic population of European sea bass, there may be a link between growth and sex determination. As the strongest genetic correlation occurs at 90 dpf, before the first signs of sex differentiation, it is not clear if sex has to be considered a cause or a consequence of the differential growth observed between future males and females.

One interesting point is the fact that, in an experiment where fish were kept at low temperature (13°C) during larval rearing until 146 dpf, there were even fewer females (11%) in this group than in the same fish reared at $>19^\circ\text{C}$ (a masculinizing treatment) during the same period [31]. This could be an indication that sufficient growth is needed at some time of development (besides the

positive impact on female proportion of low temperature larval rearing – see Section 14.8) for fish to develop as females. Indeed, from an evolutionary point of view, the fact that females are larger than males has fitness benefits to them in another mass spawning species, the Atlantic silverside, *Menidia menidia* [50]. If the same applied to European sea bass, this may imply that growth rate at some time of development may be beneficial to orientation towards the female sex. Such a link has, however, not been proven for the moment (see Section 14.7). Sex related growth continues to be present until the time fish reach market size, when animals are 350–400 g [45, 51] (Figure 14.2).

14.7 Influence of Manipulation of Early Growth on Sex Ratios

In the previous section, the relationship between growth and sex has been clearly illustrated. This relationship raises the question of whether it would be possible to manipulate sex in the European sea bass by manipulating growth rates. This question was specifically addressed in two different experiments by Diaz *et al.* [52].

In the first experiment, no size-grading was used, and groups of European sea bass were fed with different amounts of food, in order to create different growth rates during the period of sex differentiation, corresponding to standard length (SL) in the range of 8–12 cm. Differences in body weight at the end of the treatment were about threefold between the *ad libitum* fed group and the group whose growth rate was the lowest because of food restriction. Nevertheless, the sex ratios were in the range of 60–70% males in all groups and, thus, no differences in sex ratio were observed between groups of fish that experienced different growth rates. These results indicate that:

- 1) in contrast to what had been previously surmised [14, 53], the sex ratio in the European sea bass does not depend on the growth rates during the sex differentiation period (and, of course, does not depend either on the growth rates after sex differentiation is complete); and
- 2) that the sex ratio of a given batch is fixed before fish reach a mean of 8 cm SL [52].

Thus, for the second experiment, younger fish were used, with mean SL of ≈ 4 cm. This is before the appearance of the first known differences in aromatase expression, which takes place in the range of 5–6 cm SL [54]. In this case, fish were size-graded, and then subdivided into two groups: the fastest (≈ 6.4 cm SL) growing fish and the slowest (≈ 2.6 cm SL) growing fish. Fish in each subgroup were divided again into two additional groups. Fast-growing fish were left as such, or forced to grow equally as the initially (i.e., before size grading) slowest growing fish, by adjusting food intake. Conversely, the slowest growing fish were left as such or fed *ad libitum* to match the growth rate of the initially fastest growing fish. Highly significant differences in sex ratios were found, with more females in the groups derived from the group with the largest fish at the time of grading. This is what was expected. However, the important observation was that sex ratios were independent of subsequent growth rates.

When taken together, the data of these experiments show that different growth rates during the sex differentiation period do not affect subsequent sex ratios but, rather, that sex ratios are related to growth before the fish reach 4 cm SL. This indicates that the association between sex and growth is established well before the first signs of sex differentiation are visible. In other words, although no histological differences are visible between the gonads of future males and females, probably between 3 cm and 4 cm SL, the differences between the sexes are being established at the molecular level. Therefore, it will be important to further study the relationship between sex and growth performances at this size range.

Finally, the relationship between growth and sex could perhaps be further exploited, taking into account the possibility of combining size-grading with an appropriate temperature control. This would discard the slowest growing fish and, hence, many males. Also, the existence of catch-up growth [52] could also help to improve European sea bass culture.

14.8 Effects of Temperature on Sex Ratios

The polygenic system of sex determination in the European sea bass shows that genetics and environmental factors have an approximately equal contribution to sex ratio variance [8]. However, what it is meant by “environmental factors” is not a trivial question since disentangling the contribution of one specific, isolated, factor from the contribution of another factor is not an easy task. Of the several factors that have been claimed to influence sex ratios in gonochoristic species [55], not many have been properly tested for their influence on European sea bass sex ratios. Among these, photoperiod [44], rearing density [46], and food intake [52] did not show any clear effect. In contrast, and taking the precaution state above, the effect of temperature on sex ratios has been clearly

demonstrated in the European sea bass. It should be noted that temperature is by far the most studied single environmental factor in vertebrates, including fish in the context of temperature-dependent sex determination (TSD) [55, 56].

The effect of temperature on European sea bass sex ratios has been the focus of many studies [31, 44, 57–59]. However, contrasting results have been obtained in regard to whether more females would be obtained with lower or higher temperatures, as well as what is the best period to expose the fish to a given temperature in order to manipulate sex ratios. Taking advantage of: (1) the discovery that the sex-determining system was polygenic, with both paternal and maternal influences on progeny sex ratio [8]; (2) the existence of a single sex ratio response in fish when ecologically representative temperatures are used (higher temperature → more males) [56]; and (3) the use of different families and different periods of temperature exposure, Navarro-Martín *et al.* [51] came up with a model on the effect of temperature on European sea bass sex ratios. Under this model, we have the following considerations:

- 1) Temperatures above 17°C result in masculinization. Note that 17°C is the upper thermal limit at which European sea bass eggs are found in the ocean (see Section 14.1 above).
- 2) The thermosensitive period encompasses the first 60 days of life, when gonads are still very rudimentary. However, rather than being precisely defined at the two extremes, the available data supports the idea that starting with a maximal influence around fertilization the importance of temperature progressively diminishes, until it becomes negligible after the second month of life.
- 3) The parental influence on sex ratios is also manifested in the sex ratio response to temperature. In other words, different families exhibit different sensitivities to the masculinizing effect of temperature, an observation in support of the existence

of genotype x environment interactions [8, 31]. This means that, under “ideal” rearing conditions, without any masculinization effect of temperature (for example, by rearing the fish at, say, 15–17°C), the sex ratio of a particular brood will mostly depend on the combination of male and female determining factors inherited from the parents, plus some “micro-environmental” effects affecting fish reared in the same tank.

Thus, temperatures < 17°C normally do not induce masculinization, but simply enable the development of genetically female fish in which the sum of factors promoting female development is stronger than the sum of factors promoting male development to differentiate as phenotypic females, the actual sex ratio being mostly dependent on the genetic contribution of the parents. In contrast, high temperatures on average inhibit the orientation towards the female sex—that is, ovarian differentiation in about half of the fish that otherwise would have developed as females (Figure 14.3). In this context, what constitutes an insensitive fish to temperature has been discussed in detail elsewhere [51], but it can be summarized by saying that they are those fish that, when reared at high temperature soon after hatching, do not become masculinized (Figure 14.3). This contributes towards explaining the small number of females in farmed European sea bass stocks, which are essentially composed of males.

Additional advantages of rearing European sea bass at under 17°C during the first two months of life include the observation that it reduces the number of precocious males [51]. Furthermore, low temperature also contributes to a reduction in the incidence of haemal lordosis at harvest time, from 36% in fish reared at 20°C, to 0% in fish reared at 15°C [60]. Lordosis is detrimental for farmers, because it entails competence for food in the tanks, diminishing productivity, and because many of the lordotic fish simply are not marketable at the time of harvest.

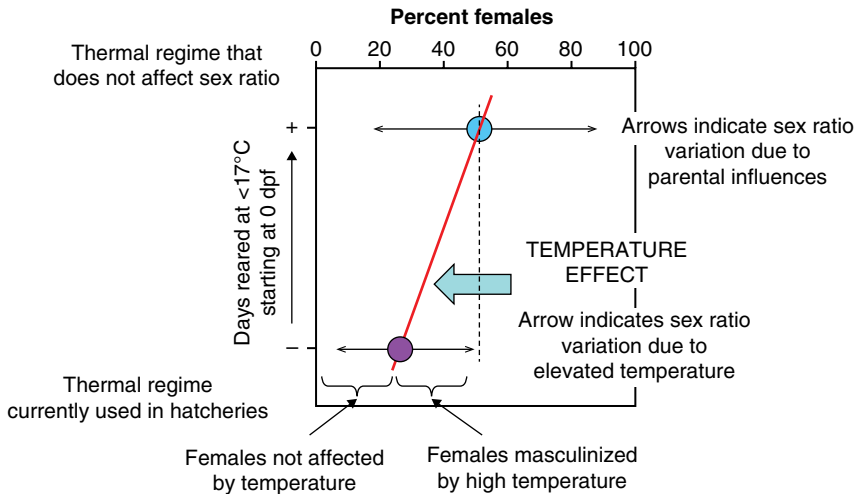


Figure 14.3 Effects of temperature on European sea bass sex ratios. Parental influences are evidenced by a wide variation in sex ratios among broods, compatible with a polyfactorial sex-determining mechanism. When many broods from different brood stock are taken together, the average number of genotypic females should be $\approx 50\%$. These females would be expected to develop as phenotypic females, provided there is no influence of temperature on sex ratios. The observed number of phenotypic females ($\approx 50\%$) under permissive temperature regimes supports this affirmation. The thermal regime currently used in European sea bass farms (i.e., < 15 days at temperatures $\leq 17^\circ\text{C}$), masculinizes more than half of the genotypic females into phenotypic males. This shifts $\approx 31\text{--}37\%$ the proportion of phenotypic males from $\approx 50\%$ to $\approx 84\%$, and results, on average, in the highly male-biased sex ratios typically observed in European sea bass farming, although in particularly sensitive batches the shift may be higher and result in essentially all-male stocks. Modified from Navarro-Martin *et al.*, 2009 [13].

Finally, one aspect also worth considering is the effect of rearing temperature on growth. As expected, fish reared at low temperature grow significantly less than fish reared at high temperature when they are about one year old. However, these differences are no longer present at harvest time [51, 60] because the European sea bass is able to exhibit compensatory growth [52]. Nevertheless, if too low temperatures are used to avoid masculinization (i.e., in the range $13\text{--}15^\circ\text{C}$) then, even accounting for subsequent catch-up growth, a superior or even similar growth at harvest is not ensured.

Based on these considerations, a patent was filed in 2008 (application no. N200802927 by Piferrer, Blázquez and Navarro-Martin entitled “Method for the thermal control of sex proportions in the sea bass”) and awarded in 2012 (patent no. ES2346122 B1). The proposed method should result, even in the absence of selection, in the production of the

highest possible number of females and, thus, higher biomass at harvest, and also in a likely decrease in the number of precocious males.

14.9 Epigenetic Regulation of Sex Ratios

Epigenetics deals with the regulation of gene functions independent of changes in DNA sequence, and the mechanisms involved include DNA methylation, modification of histones and histone variants, and the action of non-coding RNAs [61]. Epigenetics allows organisms to modify the activity of their genes in response to changes in the internal or external environment, integrating genomic and environmental information to generate a particular phenotype [62]. Epigenetics is directly involved in phenotypic plasticity [63]. Organisms in which sex

fate depends on the environment are clear examples of phenotypic plasticity, and the importance of epigenetics for sex determination in plants, invertebrates, and vertebrates has recently been reviewed [64] (see also Chapter 3 of this volume, which deals specifically with the epigenetics of sex determination and differentiation in fish).

As explained in Section 14.8, exposure to temperatures above 17°C during the first 60 days of life, which corresponds to the thermosensitive period (TSP) in this species, increases the proportion of males. However, the studies of Pavlidis *et al.* [57] and Navarro-Martín *et al.* [51] showed that temperature effects in the European sea bass are more pronounced during the first half of the TSP, when fish are smaller than 30 mm. Thus, maximum effects of temperature not only take place before sex differentiation (150 dpf; \approx 120 mm fish), but also even before the formation of the gonadal ridges at \approx 35 dpf (26–35 mm fish) [65]. This means that early temperature must be “remembered” somehow, acting on the genome or parts of it that is related to sex determination.

This has led to the hypothesis about the existence of an epigenetic mechanism activated by temperature, which could result in different levels of DNA methylation in specific loci [66]. Since a conserved effect of masculinizing temperature—which is always high in fish, but can be either high or low in reptiles with temperature-dependent sex determination—is suppression of the expression of the gonadal form of aromatase, *cyp19a1a*. This key steroidogenic enzyme was selected as the candidate for further investigation. The rationale behind this choice is that, in all non-mammalian vertebrates, estrogen is essential for ovarian development and, thus, the sexual differentiation of females [67].

Two groups of European sea bass were exposed from 0–60 days either to a low (15°C; LT) or high (21°C; HT) temperature. After this, both groups were left to grow until they were one year old at the ambient temperature. As expected, high temperature increased

the proportion of males. Gonads from males and females were dissected, and DNA was extracted and subjected to bisulfite treatment, to analyze DNA methylation in a \approx 500 bp fragment of the *cyp19a1* gene that included mostly the promoter, but also 60 bp of the first exon.

Results showed that the amount of DNA methylation in the *cyp19a1a* promoter was higher in males than in females at LT. However, at present, it is still not clear how these sex-related differences are established. Exposure to HT significantly increased *cyp19a1a* promoter DNA methylation levels in females towards levels of males, resulting in a fraction of the fish that would develop as females under a lower temperature regime developing instead as males [66]. Temperature also increased methylation levels in males but, in this case, differences were not significant, because levels in the gonads of males were already high (80%) in the LT group. Furthermore, there was a weak but statistically significant inverse relationship between *cyp19a1a* promoter DNA methylation levels and *cyp19a1* expression, the latter with lower levels in HT females when compared to LT females. This concurs with the constitutively lower levels of *cyp19a1a* expression in males, compared with females, and with the higher circulating levels of estrogens observed in females [67].

Luciferase reporter assays showed that methylation of the *cyp19a1a* promoter prevents binding of sf-1 and foxl2, two important transcriptional regulators of *cyp19a1a* activity. An interesting possibility is that differences in the methylation of the *cyp19a1a* promoter could be part of (or interact with) the polygenic mode of sex determination by parental imprinting mechanisms. This needs to be searched using different families with different constitutive levels of *cyp19a1a* methylation, and carrying out the appropriate test to check for such parental effects.

This study was the first description of an epigenetic mechanism mediating temperature effects on sex ratios in any animal. As it seems that hypomethylation of the *cyp19a1a* promoter is necessary for ovarian development,

and hypermethylation then lowers *cyp19a1a* transcription during gonadal differentiation, preventing the transformation of an undifferentiated gonad into an ovary [66].

These results fit well with the fact that *cyp19a1a* is the key enzyme for establishing the androgen-to-estrogen ratio. However, it is tempting to speculate that the effects of temperature would elicit changes in other genes which, most likely, would also be related to sexual differentiation. There are, however, two additional aspects that need to be considered. One concerns the variability in the epigenetic response of changes. Thus, the genetic component can influence the epigenome and, in turn, how it responds to environmental influences. Consequently, one would expect changes in the levels of DNA methylation of key genes, in a manner similar to the variation in the strength of different alleles of a given loci to promote a given sex tendency. This is in accordance with the view that the structure of developmental and genetic networks of sex-determining systems that emerge under natural or sexual selection are not easily predicted, and do not necessarily follow an upstream addition of initial triggers [68]. The other aspect concerns the possible inheritance of temperature effects across generations, which has been already documented in the half smooth tongue sole, *Cynoglossus semilaevis* [69]. It would be highly relevant to determine whether something similar can occur in the European sea bass.

A troubling observation recently reported by some farms is that, despite applying the thermal protocol to avoid temperature-induced masculinization, highly male-biased sex ratios are still observed occasionally. The underlying reason for this is not known, but one possibility would be the epigenetic inheritance of masculinization of brood stock that were exposed to elevated temperature some years ago, when they were in the larval stages (see Section 14.9). Research along these lines in the years to come should shed light on these relevant aspects for both our understanding of environmental influences on sex, and for possible capitalization of new findings to aquaculture production.

14.10 Selection for Sex Ratio

As sex ratio has an additive genetic basis, selection for sex ratio should be possible, with the aim to increase the proportion of females as a preferred sex with higher early growth and later puberty. Under the polygenic threshold trait framework, in natural populations, sex tendency is normally distributed and centered to zero, resulting in a 1 : 1 sex ratio (Figure 14.4.a). In a classical hatchery environment (including larval rearing > 17°C), there is an environmental negative displacement of sex tendency, resulting in a male-biased population, with a negative average sex tendency t_0 (see Figure 14.4.b, with $t_0 = -0.90$). At this point, if we mate males and females from this population to produce the next generation of fish, without intentional selection for sex ratio then, combining equations 2 and 3 in equation 4, we can figure out the average sex tendency of the parents:

$$\frac{(t_f + t_m)}{2} = t_0 + \frac{\phi(t_0)(1 - 2P_f)}{2P_f(1 - P_f)} \quad (4)$$

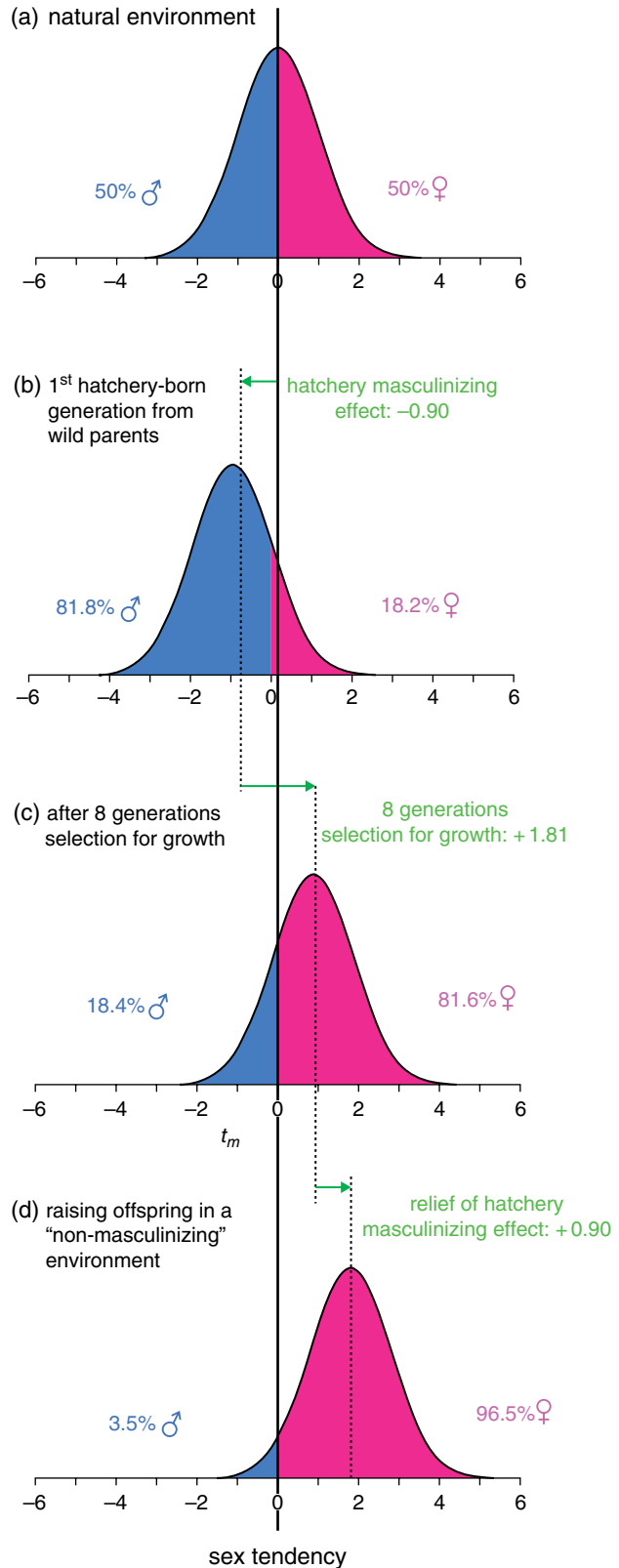
This automatically creates a selection differential between the mean sex tendency of the parents $(t_f + t_m)/2$ and the mean of the population they are sampled from (t_0) equal to:

$$\Delta S = \frac{(t_f + t_m)}{2} - t_0 = \frac{\phi(t_0)(1 - 2P_f)}{2P_f(1 - P_f)} \quad (5)$$

In a bisexual population, $0 < P_f < 1$, and $P_f(1 - P_f)$ is always strictly positive. As $\phi(t_0)$ is also always strictly positive, the sign of the selection differential will depend on the sign of $1 - 2P_f$, and then be positive when $P_f < 0.5$, negative when $P_f > 0.5$, and zero when $P_f = 0.5$. The response to selection will be $\Delta G = h^2 \Delta S$ [70], with h^2 the heritability of sex tendency, and thus selection will always tend to move the population mean toward a 1 : 1 sex ratio—conforming to Fisher's theory [15].

Thus, it can be expected that, if the environmental conditions at the hatchery are kept the same, the domestication process (i.e., producing the next generation brood

Figure 14.4 Theoretical evolution of sex tendency in European sea bass with 5% selection for growth for eight generations, combined with manipulation of the masculinization level in the hatchery environment, given the genetic parameters of sex tendency in [8].



stock exclusively from farmed brood stock) will progressively bring sex ratios to 1 : 1 in approximately eight generations [8]. This will require some time, but the fact that domesticated fish produce more females than wild fish has been experimentally demonstrated, at least in the first generation [49].

If the objective is to produce more females than males, or even tend to a female monosex population, this selection process will not be enough, and directional selection will be needed. To perform an efficient selection, the requirement is to identify, among the phenotypic males and females, those that have the highest sex tendency. This could be done directly, by sexing individuals from different families, estimating the sex tendency of males and females in each family, and choosing brood stock males and females from the best families for sex tendency. However, doing so would be extremely tedious, and would require either sacrificing (for sexing) many fish, or keeping a high quantity of fish until sexual maturity of both males and females, to be able to identify both sexes without error by stripping and/or biopsy.

Indirect ways to identify individuals with high sex tendency would, then, be a real benefit. In the case of the Atlantic population of European sea bass, where there is a positive genetic correlation of 0.50 of growth with sex tendency, individual body weight could be such an indirect indicator trait, as selecting fast-growing fish would co-select fish with a high sex tendency. Stochastic simulation of this strategy has been performed, and it showed that an equilibrium sex ratio ranging from 50–90% females can be reached in 7–8 generations [49]. This equilibrium sex ratio does not depend on the strength of the masculinizing effect of the hatchery environment, but depends on the intensity of selection for growth, on the heritability of growth, and on the genetic correlation between body weight and sex tendency.

With a selection of the 5% biggest fish at one year of age, an equilibrium close to 82% females should be reached in eight generations (Figure 14.4.c) and, if at that time the juveniles for production are reared in an environment where the masculinizing effect

of hatchery rearing is released (e.g. by using cold early rearing), an additional phenotypic displacement of sex tendency could result in a proportion of females > 95% (Figure 14.4.d). In any case, this would be a long-term process, conditional to the existence and stability over generations of the genetic correlation of sex tendency with body weight (which is not proven to date in the Mediterranean population of European sea bass).

Still, in the first generation, it has been demonstrated that offspring of growth-selected European sea bass had more females than offspring of wild or simply domesticated European sea bass [49]. One practical issue, which is not easy to resolve, is that if individuals are strongly selected based on their juvenile growth phenotype, at a time where sexing is not possible, it is likely that the selected individuals will mostly be females, since females are larger, especially in juvenile stages – while of course, males are also needed to produce the next generation.

Another possibility to select more female-prone fish would be to identify QTLs and/or to perform genomic selection for sex tendency. It has been shown that marker information could be more efficient than pedigree information to predict the sex of individuals in different families [35], but this advantage is very modest – a few percent, at most. Accumulation of data with more markers and more populations is needed to assess the real potential benefit of using molecular marker information to perform genomic selection on sex ratio in European sea bass.

14.11 Concluding Remarks

Despite important progress made in the last few years (see Box 14.1), much still needs to be learned about European sea bass sex determination. The identification of the major factors responsible for the sex tendency in a polygenic system is inherently difficult, if not impossible. This will depend on their segregation and the strength of the effect of each of the involved factors, and on our ability to disentangle genetic from environmental

Box 14.1 Key facts

The European sea bass is a gonochoristic species of the differentiated type. Sex determination is polygenic, dependent on more than two loci. The genetic and environmental contribution to sex determination is approximately of the same magnitude. Thus, in terms of sex determination, the European sea bass is a species placed midway between species with purely genetic and purely (if any) environmental sex determination. The environmental factor that has proven influence on sex is temperature. Temperatures higher than 17°C before the end of the thermosensitive period (0–60 days post-fertilization) have a masculinizing effect.

The sex of a given brood depends on maternal and paternal contributions, and also on the sex ratio response to temperature. On average, masculinization by elevated temperature ($\approx 21^\circ\text{C}$) involves about 50% of the fish that otherwise would have developed as females. There are no other environmental factors known to affect sex ratios in the European sea bass. However, since there is an association between growth and sex, selection for fast-growing fish could lead to the production of female-biased stocks. At present, the production of monosex female stocks without the use of sex steroids is not ensured.

influences – and eventual genetic by environment interactions. The development of markers linked to causal variants would undoubtedly aid in broodstock selection for breeding programs.

Another important point is the need to demonstrate correlation between females and body weight in the Mediterranean population, as done in the Atlantic population, since most of European sea bass hatcheries potentially interested in breeding are located in the Mediterranean region. Finally, there is a need to further understand the contribution of epigenetics in the integration of genetic and environmental information in this species, where both types of factors have a similar influence on sex. Particularly interesting would be the possible association between a given epigenetic makeup and the susceptibility to the masculinizing effects of heat.

Despite all the knowledge gained, right now highly biased populations, with a

predominance of females, are not being produced routinely on a commercial scale. Thus, for the moment, the combination of proper temperature regimes and selective breeding is one of the best approaches for the production of highly-female biased stocks, which would be of interest to producers, due to the superior growth of females and later maturity when compared to males.

Acknowledgments

Research at the MV lab was supported by EU projects Heritabolum (Q5CR-2002-71720) and Competus (COOP-CT-2005-017633). Research at the FP lab was supported by Government of Spain grants ref. AGL2013-41047-R and AGL2016-78710-R; Government of Catalonia grants ref. 2009SGR1050 and 2014SGR1351; and EU AQUAEXCEL (FP7) ref. 0102/06/07/20.

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15

Morphological and Endocrine Aspects of Sex Differentiation in the European Sea Bass and Implications for Sex Control in Aquaculture

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15.1 Introduction

Fish exhibit a vast array of reproductive strategies and types of sexuality, such as gonochorism, and various forms of synchronous and successive hermaphroditism [1]. Among gonochoristic species, a variety of mechanisms of sex determination have been evidenced, including monogenic, oligogenic and polygenic genetic sex determination (GSD) models [2], environmental sex determination (ESD) – with temperature being the most studied environmental factor (TSD), and various combinations of GSD and ESD systems [1, 3].

Sex differentiation refers to the development of testes or ovaries from an undifferentiated gonad [1, 4]. Understanding this process is critical in order to evaluate the results of experiments testing the role of genetic or environmental factors on sex determination and, ultimately, when developing sex control techniques for commercial aquaculture. In fish, sex differentiation is a labile process that can be influenced by external factors, allowing the manipulation of the phenotypic sex of progenies through exogenous treatments applied during a critical period of development, known as the labile period [5].

Identification and delineation of the labile period relies largely on empirical studies [3].

The labile period for TSD has been shown to overlap partly with the period when phenotypic sex can be influenced by exogenous hormonal treatments. Therefore, experimental hormonal sex reversal treatments can help locate the period of sensitivity to external factors in species displaying ESD, as well as TSD. Knowledge of the labile period allows designing protocols for the study of the physiological mechanisms and cues affecting early sex differentiation and, ultimately, developing optimal methods for sex control in farmed populations.

Research on sex differentiation and sex control has been conducted in several aquaculture species where a sexual dimorphism exists and one of the sex phenotypes is advantageous for aquaculture production (e.g. faster growth or late reproductive maturation). It has also been conducted in species that are cultured, in order to harvest a product obtained only from one sex (e.g. caviar in sturgeons, family *Acipenseridae*), or when sex control is used as a means to avoid reproduction and overcrowding in mixed-sex farmed populations, such as in several tilapias (*Oreochromis* sp) [6].

The European sea bass (*Dicentrarchus labrax*) is a species of major economic importance in the Mediterranean region. Aquaculture production reached 160,000 Tm in 2014 (www.fao.org),

making the culture of this species one of the largest marine aquaculture industries in the region. The entire life cycle has been completed in captivity, and selective breeding programs aimed at improving growth, disease resistance, and carcass quality are being developed [7], making the European sea bass a fully domesticated species, according to the criteria laid out by Teletchea and Fontaine [8].

The control of reproduction in captivity is instrumental to the success of European sea bass aquaculture, and major progress on this topic has been achieved thanks to the numerous studies conducted during the past two decades on the endocrine regulation of sex differentiation, gametogenesis, and spawning (reviewed by [9–14]). In the European sea bass, males grow slower than females and, therefore, growing monosex female populations would shorten the duration of the production cycle, making the industry more profitable [11, 15, 16]. However, the intensive culture protocols initially developed for this species were based on a rapid increase of the rearing temperature after hatching, which has been shown to induce massive masculinization of exposed fish, with sex ratios reaching, in some instances, values close to 100% males (average 75–95%) [11, 16–18].

This high proportion of males is not observed in the wild, where balanced sex ratios have been reported [19] but, rather, it seems to be an artifact due to the rearing temperature used during the larval and early juvenile stages in aquaculture, which is higher than that experienced by European sea bass larvae and juveniles in their natural environment [11]. In addition, about 20–30% of males attain puberty precociously at the end of the first year of life [20, 21] whereas, in the wild, males mature during their second year or later [10, 22].

As already shown in other aquaculture species, such as salmonids, precocious puberty induces a decrease in growth rate and an increase in feed conversion rate, because nutrients are diverted towards the production of gametes instead of somatic growth [23]. In cultured European sea bass, early puberty was

hypothesized to result in economic loss, as precocious males were shown to attain market size later than those that mature during the second year [12, 24, 25]. Considering the significant growth advantage of females and the undesirability of precocious puberty in males, monosex female populations would improve the cost-effectiveness of European sea bass aquaculture.

The genetic and environmental factors controlling sex determination must be known in order to produce monosex populations in any given species [6]. In species where the phenotypic sex is influenced by external factors, the development of effective sex control methods also requires understanding the physiological processes involved in gonad development and their chronology and, in particular, the timing and duration of the labile period of sex differentiation, so that effective protocols to achieve the desired sex ratio can be developed.

This chapter reviews the current knowledge of the process of sex differentiation and its manipulation by the use of exogenous hormonal treatments in the European sea bass. Available data on the morphological aspects of gonad ontogeny and their chronology are synthesized in the first section. The effectiveness of exogenous sex reversal treatments using steroid compounds or chemical agents impacting their metabolism is then discussed – in particular, in the context of the delineation of the labile period of sex differentiation. Finally, the recent contributions of new technologies in molecular biology and genomics to our knowledge of the fundamental processes of sex differentiation in this species are reviewed.

15.2 Morphological Aspects of Gonad Differentiation

15.2.1 Chronology and Timing of Morphological Events

The ontogeny of European sea bass gonads was first described in detail by Roblin and Bruslé [26] using histology. A second study

by Saillant *et al.* [20] updated the kinetics of the late phases of gonad development, from the end of the undifferentiated period to the stage when all males and females displayed differentiated gonads (Figure 15.1). The populations examined in the latter study were cultured under intensive conditions, and experienced faster growth rates than those sampled by Roblin and Bruslé [26]. Primordial germ cells were first detected at 23 days post-hatching (dph), when they were migrating toward the genital ridge [26]. Colonization of the gonad primordium was observed at 43 dph, and gonads remained in an undifferentiated stage for up to 12 months in some specimens. Although the number of germ cells was noted to increase progressively during this period, their proliferation, which is reported as an early indicator of active differentiation in other fishes [27], was not clearly described in these early kinetic studies.

Because sex differentiation is best described with reference to somatic length [26, 28], and the various studies reporting data on the kinetics of sex differentiation were conducted in cultured populations that experienced very different growth conditions, further discussion of the kinetics of events below is made primarily with reference to fish size (standard length –SL, in mm).

Both Roblin and Bruslé [26] and Saillant *et al.* [20] reported a precocious differentiation of females (beginning as early as 80–90 mm in SL, also noted by Papadaki *et al.* [29] during a histological study of gonad differentiation in large and small fish isolated from a population by size grading) and a small proportion of precocious males, among the largest fish at that stage. These precocious males had differentiated testicular cysts, and some even showed some spermatogenetic activity. These signs of precocious spermatogenesis were observed at sizes between 100–133 mm SL at 315 dph in the study of Roblin and Bruslé [26] but at a much younger age and smaller size in the study of Saillant *et al.* (81 mm SL, 168 dph) [20].

These differences suggest that, as aquaculture protocols improved between the two studies and led to faster growth rates, precocious

spermatogenesis was also induced earlier and at smaller sizes in fast growing fish. These observations are similar to those made by Conover and Fleisher [30], who reported that faster growth conditions (under high temperature treatments) led to an accelerated differentiation on a length scale and a labile period at smaller size in the Atlantic silverside, *Menidia menidia*. Interestingly, in both European sea bass studies [20, 26], the percentage of females did not change as fish grew larger than 90 mm SL, suggesting that ovarian differentiation is initiated at sizes close to 80–90 mm for most of the females. However, the number of females examined in both studies was very small, and this result needs to be confirmed using larger sample sizes. In contrast, the percentage of males increased over time, suggesting that undifferentiated gonads found in fish larger than 90 mm were late-differentiating testes. Accordingly, experimental populations could be sexed early by determining the percentage of females when the mean size of a population reaches 90 mm SL.

15.2.2 Juvenile Intersexuality

Several studies have reported a significant proportion of European sea bass males displaying intratesticular oocytes [20, 26, 31–33] (Figure 15.2). These signs of juvenile intersexuality were convincingly linked to ESD by Saillant *et al.* [32], who showed that, after rearing European sea bass at low water temperature for a long period, the frequency of females decreased in the same proportion as the frequency of males with intratesticular oocytes increased, indicating that these males were potential females masculinized by the prolonged exposure to low temperature conditions. These observations, coupled with the findings in the European sea bass [32] and in other species where genotypes vary in their sensitivity to the environment [3], suggest that males with no intratesticular oocytes may be non-sensitive genotypes expected to develop testes irrespective of the environmental rearing conditions, while males with oocytes are potential females who

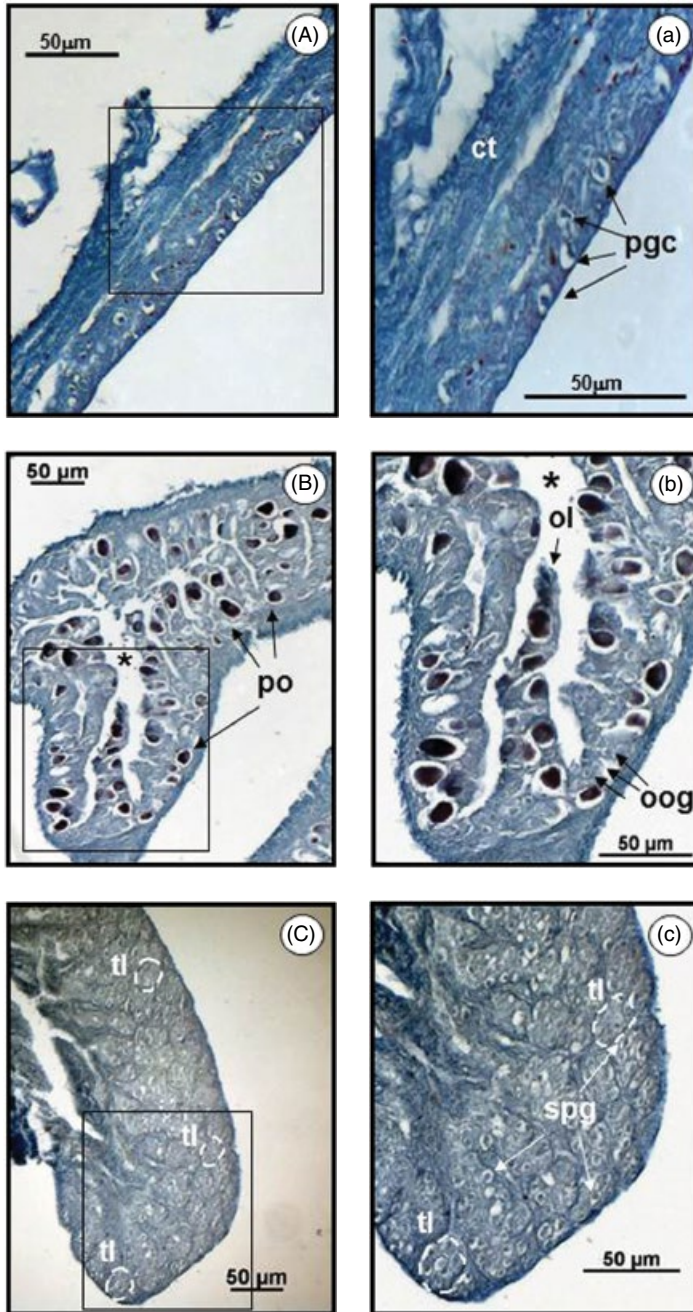


Figure 15.1 Histological sections of European sea bass gonads at early stages of differentiation.

A – undifferentiated gonad at 150 days post-hatching (dph), with the image in the insert magnified in **a**. Note the predominance of connective tissue (ct) and the scattered distribution and low density of primordial germ cells (pgc).

B – Ovary at early stage of differentiation (150 dph), with the image in the insert magnified in **b**. Note the presence of oogonia (oog) and the first differentiated perinucleolar oocytes (po). The tissue (asterisk) is organized in ovarian lamellae (ol) surrounding the ovarian cavity.

C – Differentiating testis (190 dph), with the image in the insert magnified in **c**. Note the presence of spermatogonia (spg) organized in testicular lobules (tl). Sections were cut at 7 μm thickness and stained with haematoxylin-eosin.

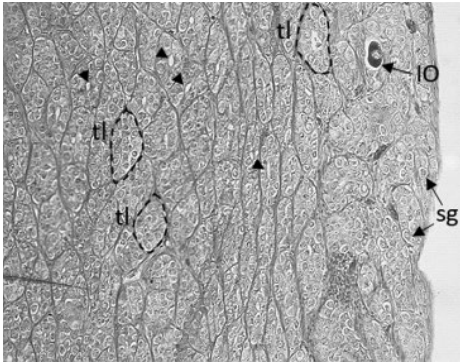


Figure 15.2 Histological section of an immature sea bass testis containing intratesticular oocytes (533 dpf). Spermatogonia (sg) appear organized in testicular lobules (tl), some with a visible lumen (arrowheads), and one intratesticular oocyte (IO) is visible at the periphery of the gonad. The section was cut at 5 μ m thickness and stained with Erythrosin-Orange G and Toluidine blue.

become male or female, depending on the environmental conditions.

The occurrence of a fraction of females, even if low, in most cultured populations suggests that some female genotypes “resistant” to environmental masculinization may also occur. Intersex males were found in three different wild populations of European sea bass (Brittany, Gulf of Biscay, and West Mediterranean, [20]) indicating that juvenile intersexuality (and sensitivity of sex differentiation to external conditions) is widespread in wild European sea bass.

15.3 Relationship Between Growth and Sex Differentiation

The relationship between early growth and sex differentiation has been studied extensively, using several approaches. Blázquez *et al.* [28] reported that females were significantly larger than males at 11 months of age. The sampled females in that study averaged 18 cm in length (fork length; FL), and all the females in the studied groups were expected to be fully differentiated, as discussed above, while a significant proportion of males were

still undifferentiated or at early stages of differentiation. Thus, this study was the first one to reveal the occurrence of an early sexual growth dimorphism in European sea bass, detectable before both sexes had fully differentiated gonads.

Later, using an individual tagging protocol, Saillant *et al.* [15] showed that males and females have the same growth rate from 13 cm SL onward, once growth rates were corrected for the effect of initial size, indicating that the growth dimorphism is restricted to the early stages of development. In the histological study of the ontogeny of European sea bass gonads conducted by Saillant *et al.* [20], early differentiating females tended to be larger than undifferentiated fish at 168 days post-fertilization (dpf) (8.1 cm mean SL), and were significantly larger than immature males and undifferentiated fish at 191 dpf (10.5 cm mean SL).

The observation that the difference in size between sexes may be established prior to the histological differentiation of gonads led to the development of two hypotheses regarding the origin of the dimorphism. The first of these was that the fastest growing individuals during early development become females, while slower growing fish become males (i.e., fast growth induces female differentiation). The second hypothesis was that the phenotypic sex is determined long before the first visible signs of gonad differentiation, and females begin growing faster than males when gonads from both sexes still appear undifferentiated based on histology criteria [20].

While the size advantage of females seemed more moderate at the very early stages of ovarian differentiation in the study of Saillant *et al.* [20], consistent with expectations under the second hypothesis, the small sample size prevented rejecting either of the two hypotheses. Experiments of early size grading strongly suggested that developing females grow faster long before morphological sex differentiation occurs [20, 29, 34]. The repeated size grading of a sea bass population, and subsequent sexing of all the resulting size fractions, revealed that most of the

females could be isolated by selecting the largest fish as early as 81 dph (38 mm SL), with very few females found in the remaining groups [18]. Other repeated size grading experiments, beginning at 66 dph [29] or 70 dph [34], yielded high proportions of females among the largest fish, and lower proportions or lack of females in the fractions containing the smallest fish.

Interpretation of the latter two experiments toward the timing of the onset of the sexual dimorphism is limited, because some of the intermediate size classes isolated during the course of the study were not sexed, which prevented determining the size of some of the females and males in the studied population. However, the results of Koumoundouros *et al.* [34] indicate that females were already slightly larger at 66–70 dph (i.e., a couple of weeks following the end of the period where sex determination is influenced by temperature). Altogether, these results led to the hypothesis that the growth rate during the late larval and post-metamorphosis periods was the trigger for ovarian differentiation, or a very early sign of the physiological processes leading to the differentiation of future females. This could be formally tested through grading fish at an earlier age (e.g., prior to metamorphosis), although size grading of fish at those ages is technically very challenging.

One experiment manipulated the feeding rate during the juvenile period, and aimed to test the hypothesis that early growth was the trigger of ovarian differentiation [35]. However, the study was inconclusive on this topic, because the feeding treatments were applied late (fish size greater than 8 cm SL), when fish had already grown past the labile period of sex differentiation. The observation that intersex males in the studies of Blázquez *et al.* [28] and Saillant *et al.* [20] were intermediate in size between males/undifferentiated and females is, however, consistent with the hypothesis that these males were potential females that did not achieve the minimum growth rate necessary to develop successfully as females.

The hypothesis that such a threshold exists, and is required for successful female differentiation, was also proposed by Vandeputte *et al.* [2], based on the observation of a positive genetic correlation between growth rate and sex ratio. Formal testing of this hypothesis using, for example, manipulations of the growth rate during the labile period of sex determination, would be useful in order to further understand this early growth dimorphism and the relationship between early growth and sex differentiation (the timing of sex differentiation of European sea bass gonads in relation to size and age is described in Box 15.1).

Box 15.1 Timing of sex differentiation in European sea bass in relation to size and age

Under current intensive aquaculture conditions, similar to those in the experiment of Saillant *et al.* [18], European sea bass gonads remain undifferentiated for a period of 4–5 months. The first signs of ovarian differentiation are observed in females at 80–90 mm SL (4–5 months of age in intensive culture conditions), which initiate ovarian differentiation within a short time window (i.e., the percentage of females showing differentiated ovaries in a population appears fixed at five months of age).

Testicular differentiation begins at the same size and age as ovarian differentiation for a

fraction of the males. These males rapidly initiate spermatogenesis, and account for up to 20–30% of the population. The remaining males engage in testicular differentiation between 5–8 months old, with less than 5% of undifferentiated gonads remaining at 250 dpf or at lengths beyond 110 mm. Females are larger than males long before morphological sex differentiation can be detected by histology. Size grading experiments have shown that females are, on average, larger than males as early as 65 dph, and that all the females in a population could be isolated in the largest size class within a group at 81 dph (38 mm SL).

15.4 Endocrine Control of Sex Differentiation

The endocrine control of sex differentiation in fish involves the coordination of the brain, the pituitary, and the gonads [6, 27, 36]. The brain appears to be the main sensor and integrator of internal and external cues, and it plays a central role in the development and function of the gonad through the production of gonadotropin-releasing hormone (GnRH), which triggers the secretion of gonadotropins (follicle-stimulating hormone, FSH and luteinizing hormone, LH) from the pituitary. These, in turn, stimulate sex steroid production from the gonads. However, the role of the brain on sex differentiation in gonochoristic fishes is still not clear [37–39].

Three forms of GnRHs have been detected in European sea bass as early as 30 dph, long before sex differentiation takes place [40]. All of these peaked in the pituitary of males by the time of histological sex differentiation, suggesting their possible involvement in this process [41]. Molés *et al.* [42] showed an increase of *gnrh* and *fsh* expression by the time of sex differentiation, with higher *fsh* levels in European sea bass males than in females, suggesting that a different neuro-hormonal regulation could be required for the differentiation of each sex in this species. However, a potential role of *gnrh* and gonadotropins in regulating earlier phases of sex differentiation, and influencing the phenotypic sex, remains to be demonstrated.

To date, a large emphasis has been placed on the endocrine regulations acting at the level of the gonad. Following the demonstration of the prominent role of sex steroids in the process of sex differentiation in medaka, *Oryzias latipes*, [5, 43], protocols for hormonal sex reversal were developed successfully in several fish species [6, 9]. Hormonal sex reversal treatments are most effective when applied during the “labile period” [44], which is defined, in this chapter, as the developmental window when the gonads are sensitive to treatments with exogenous sex steroids, or chemicals affecting the metabolism of sex steroids, resulting in

effective masculinization or feminization. This period typically precedes the morphological differentiation of the gonad.

Several methods have been used to deliver hormonal sex reversal treatments in fish, including immersion, injection, sustained-release delivery systems, and oral administration [6]. Regardless of the route of administration, successful masculinization or feminization in a species of interest requires the development of a specific protocol (the most effective treatments for hormonal sex reversal in European sea bass are summarized in Box 15.2). Key protocol elements that need to be determined include the compound used (androgen or estrogen; natural or synthetic, androgen/estrogen inhibitors), the dose, the treatment timing, and its duration.

Exogenous sex steroids or steroid-like substances, administered to European sea bass larvae and juveniles, have been shown to induce partial or complete sex reversal. Published results of experimental treatments using androgens and aromatase inhibitors (responsible for blocking the action of aromatase that results in a reduction of estrogen synthesis) for masculinization and estrogens for feminization are summarized in Table 15.1.

Initial efforts were focused on the characterization of the labile period. In a pilot study, Piferrer *et al.* [45] administered 17 α -methyltestosterone (MT) after sexual differentiation was completed, and did not observe any change in sex ratios. In a later study, an experimental design, considering androgen treatments administered earlier in development, successfully induced sex reversal [31]. In particular, the administration of MT (10 mg kg⁻¹ of food) from 126–226 dpf resulted in complete masculinization, a finding that led the authors to conclude that the latter treatment period encompassed the labile period. Longer treatments including this period also resulted in the absence of females, but induced a decrease in the number of germ cells (partial sterilization) that could be rescued after androgen withdrawal from the diets.

Studies testing the effects of the synthetic androgens MT, methyl Dihydrotestosterone

Table 15.1 Treatments used for hormonal sex reversal in European sea bass, *Dicentrarchus labrax*.

Purpose	Compound (dose ¹)	Treatment	Outcome	Reference
Masculinization	MT (10) MT (30)	Control One year old (76 days treatment)	77.8% males No change 87% males	[97]
Masculinization	MT (10)	Control 126–226 dpf 126–326 126–426 226–326 226–426 326–426	80% males 100% males 100% males 100% males* 85% males 80% males 82% males	[31]
Masculinization	DHMT (0.5, 3, 5)	Control 84–114 dph 84–144 84–174	88% males 100% males 100% males 100% males	[17]
Masculinization	MT (10)	Control 60–160 dpf 60–260 160–260	67% males 64% sterile 100% sterile 87% males	[46]
Masculinization	MDHT (10)	60–160 dpf 60–260 160–260	81% sterile 100% sterile 87% males	[46]
Masculinization	MT (10)	Control 46–66 dpf 66–86 dpf 86–106 dpf 106–126 dpf 46–76 dpf 46–86 dpf 46–96 dpf	54% males 50.5% males 74% males 100% males 100% males 59% males 79.5% males 89.5% males	[46]
Masculinization	MT (2.5, 5, 10, 20) MDHT (2.5, 5, 10, 20)	Control 110–210 dpf 110–210 dpf	82% males 100% males 100% males	[46]
Masculinization	MDHT (10) Fadrozole (100)	Control 90–150 dpf 90–150 dpf	32.5% males 100% males 100% males	[49]
Masculinization	Fadrozole (100)	Control 165–235 dph	98% males 100% males	[35]
Feminization	EE ₂ (10) E ₂ (10)	Control 48–88 dpf Control 60–260 dpf 60–260 dpf	36% females 62% females 33% females 80% females 70% females**	[47]
Feminization	E ₂ (5) E ₂ (10)	Control 226–426 dpf 226–426 dpf	20% females 17% females 30% females	[47]
Feminization	E ₂ (12.5)	Control 90–150 dph	55% females 100% females	[15]

Table 15.1 (Continued)

Purpose	Compound (dose ¹)	Treatment	Outcome	Reference
Feminization	E ₂ (12.5, 25, 50)	Control 88–148 dph	3% females 100% females	[48]
Feminization	EE ₂ (12.5) EE ₂ (25) EE ₂ (50)	Control 88–148 dph 88–148 dph 88–148 dph	4% females 96.5% females + 3.5% sterile 88.5% females + 11.5% sterile 39% females + 61% sterile	[48]
Feminization	E ₂ (10) Tamoxifen (100)	Control 90–150 dpf 90–150 dpf	67.5% females 100% females 100% females	[49]
Feminization	E ₂ (10)	Control 90–150 dph	2.5% females 90% females	[59]
Feminization	E ₂ (10)	Control 165–235 dph	2% females 94% females	[35]
Feminization	E ₂ (10)	Control 93–150 dph	50% females 100% females	Medina <i>et al.</i> , unpublished

¹ dose is expressed in mg kg⁻¹ of food.

dpf – (days post-fertilization), dph – (days post-hatching).

*Includes males and intersex for which the majority of gonadal tissue corresponds to a testis.

**Includes females and intersex fish for which the majority of gonadal tissue corresponds to an ovary.

Androgens: MT (17 α -methyltestosterone), MDHT (17 α -methylidihydrotestosterone), DHMT (1-dehydro-17 α -methyltestosterone).

Estrogens: E₂ (estradiol-17 β), EE₂ (17 α -ethynylestradiol).

(MDHT), and 1-dehydro-17 α -methyltestosterone (DHMT), clarified further the timing of the labile period for masculinization, with fully effective treatments beginning as early as 85 dpf [17, 46]. In the study of Blázquez *et al.* [46], 20 days of androgen administration (86–106 dpf) were sufficient to achieve a monosex male population, while Chatain *et al.* [17] obtained the same results with treatments lasting 30 days but at much lower doses of DHMT (0.5 mg kg⁻¹ of food), indicating that gonads were highly sensitive to treatments during the corresponding developmental window. Although a proportion of the males in the treated groups displayed intratesticular oocytes, as commonly found in the European sea bass, it is important to note that they were expected to be functional males capable of completing spermatogenesis and spermiation.

The labile period for feminization using estrogen treatments appears similar to that

reported for hormonal masculinization [11]. The first study dealing with estrogen administration in European sea bass achieved 70–80% feminization using estradiol (E₂) applied between 60 and 260 dpf at 10 mg kg⁻¹ of food [47]. However, although the treatment suppressed testicular development, it also induced sterilization in 20% of the population, suggesting that treatments should be shortened, delayed, and/or applied at lower dosages, in order to avoid adverse effects on gonad development. This study also highlighted the potential long-term impacts of exogenous estrogens on wildlife, since the exposures in treated groups had adverse effects on fecundity and gonad maturation, as evidenced by the reduction in the size of the testicular lobules in the males that did not mature [47]. When E₂ administration started at 88–90 dph, a timing consistent with the initiation of effective masculinization treatments as discussed above, all-female populations were, indeed,

obtained (90–150 dph, [15]; 88–148 dph, [48]; 93–150 dph – Medina and Blázquez, unpublished observations).

A recent study showed that treatments applied at an older age (165–235 dph) resulted in highly skewed sex ratios in favor of females, reaching values up to 94% [35]. However, the fish used in the experiment were very small at the beginning of the treatments (close to 6 cm SL, a size comparable to that of 128 dph fish in the study of Saillant *et al.* [18]), and were still at sizes where ovaries were expected to be undifferentiated and gonads potentially sensitive to exogenous hormones. The small size may have been due to slow growth prior to the experiment, which may also have delayed the labile period with reference to a length scale. Accordingly, the period of effective treatment discussed above (90–150 dph, SL between 4–8 cm in the conditions of the feminization trial of Saillant *et al.* [15]) seems to be the optimal period in European sea bass under current growth conditions in intensive culture.

The apparent delay of the labile period observed during the experiment conducted by Diaz *et al.* [35], when referring to an age scale in dph, highlight the importance of reporting fish size when describing treatment periods. As discussed earlier, fish size is a better indicator of developmental stage than age when it comes to sex differentiation and gonad development, yet most studies published to date report treatments as a function of the age in dpf or dph. Most feminization treatments tested to date employed relatively high estrogen doses and, therefore, the effectiveness of lower doses of E_2 applied during the proposed labile period should be evaluated as a means to minimize potential ulterior impacts of treatments on gametogenesis and fecundity in treated individuals, as shown by Blázquez *et al.* [47]. The success of the brief masculinization treatments applied at 85 dph discussed above suggests that short estrogen administration durations (20–30 days) focused on this specific period might also be effective, and deserve to be tested.

The use of non-steroidal compounds affecting the metabolism of gonadal steroids for sex reversal has also been explored in European sea bass. Fadrozole (Fz) is an aromatase inhibitor that blocks the aromatization of androgens into estrogens by downregulating the expression of the *cyp19a1a* gene. The administration of Fz during an early period of sex differentiation (90–150 dpf), that included the labile period for hormonal sex control, resulted in an all-male population [49]. The same result was recently obtained by Díaz *et al.* [35] with an all-male population, after treatment with Fz, during a later developmental period from 165 to 235 dpf.

In summary, the labile period of sex differentiation for hormonal masculinization or feminization in European sea bass includes the early juvenile period (approximately from 85 to 150 dph, from about 30–40 to 60–70 mm in SL under most current culture conditions), where 100% males and females have been obtained. Earlier treatments seem less effective and induced sterilization, although the effects of low dosages during earlier developmental phases would deserve to be tested.

15.5 Sex Determination and Sex Control

In aquaculture species destined for human consumption, several countries and international institutions have important restrictions for the commercialization of treated fish [50]. The direct production of monosex populations using hormonal masculinization or feminization is, therefore, not recommended for species produced for the food market, such as the European sea bass. However, in species where the sex determination system is genetic monofactorial (e.g., XX females, XY males), hormonal sex reversal treatments can be used in the parental population to produce neomales (male phenotypes with a female genotype) or neofemales (female phenotypes with a male genotype) [6, 51, 52]. In a male heterogametic

Box 15.2 Most effective hormonal treatments for direct masculinization and feminization in European sea bass

The most effective treatments for hormonal sex reversal are applied during the labile period, known as the developmental window, when the gonads are sensitive to treatments with exogenous sex steroids. This period precedes the morphological differentiation of the gonad. The requirements for effective are i) maximum effectiveness (complete sex reversal), and ii) minimum level of intervention (shortest duration and lowest dose).

Although several routes of administration have been used to deliver the different compounds to induce sex reversal in fish (typically androgens and estrogens), the alcohol evaporation method, which consists of: 1) dissolving the hormone in alcohol, 2) spraying the mixture on the pelleted food, and 3) letting the alcohol

evaporate before feeding the fish, has been the preferred route for the European sea bass.

With all these requirements, the best hormonal treatment inducing complete masculinization in this species includes the period between 86–106 dpf (20 days of treatment) using the synthetic androgen 17 α -methyltestosterone, at the experimental dose of 10 mg kg⁻¹ of food, or a slightly longer treatment (81–111 dpf) at a very low dose of methyldehydrotestosterone (0.5 mg kg⁻¹ DHMT). As for the best hormonal treatment inducing complete feminization, it includes the period between 90–150 dpf (60 days of treatment) using the natural estrogen estradiol (E₂) at the experimental dose of 10 mg kg⁻¹ of food.

system, the neomales, once identified, can be crossed with normal females to produce 100% genetic females. Similarly, in heterogametic female species, crosses of feminized ZZ animals with normal ZZ males are expected to result in an all-male population (indirect method of masculinization – see [6] for a list of species).

Initial studies of sex determination in European sea bass tested the hypothesis that a simple monofactorial model (male or female heterogamety) was operating. Based on a comparative analysis of the karyotypes of male and female specimens, Cano *et al.* [53] reported experimental evidence for a heteromorphic chromosome pair in European sea bass males, suggesting that a male heterogametic system was operating in this species. However, this hypothesis was not confirmed by the results of progeny-testing of masculinized individuals. Thirteen males obtained from a masculinized population (100% males) by androgen treatment were subjected to progeny testing via out-crossing with normal females [28]. None of the progenies obtained were all-female, which led to the hypothesis of a simple male

heterogametic genetic system being ruled out. This finding was later confirmed by the analysis of the sex ratios of gynogenetic progenies in two studies [54, 55].

In both studies, the sex ratios of gynogenetic progenies did not differ from those of progenies from the same females fertilized with a male (see Chapter 16). Similarly, a simple female heterogametic model was rejected by the results of the progeny tests of feminized individuals [56]. Sex determination in European sea bass was further documented by Vandeputte *et al.* [2], who studied genetic variation of sex ratios in 250 families and proposed a polygenic genetic model with temperature influences [11], where the final sex ratio of a cross depends on both the genotype and the temperature during early ontogenesis (see also Chapter 14).

The temperature of the rearing water is now confirmed as the primary environmental parameter inducing the severe shifts in European sea bass sex ratios observed under farming conditions [16, 32, 34, 57–62]. Therefore, potential approaches to achieve high proportions of females in farmed stocks could rely on temperature manipulations

during the thermosensitive period, following protocols similar to that described by Navarro-Martín *et al.* [16]. This approach is expected to increase the percentage of females in a population, although 100% females are not expected to be achieved using this method, considering that some genotypes show reduced sensitivity to temperature [32]. The effect of temperature on European sea bass sex ratios will not be discussed further in this chapter, as this topic is reviewed elsewhere in this volume. Another approach could involve selective breeding to increase the frequency of females under the current temperature protocols [2].

15.6 Molecular Markers of Sex Differentiation

The development and refinement of molecular biology techniques and the acquisition of genomic resources for several species has resulted in powerful new tools to study sex differentiation and maturation in fish [63]. Several molecular markers of genetic sex have been described in fish species, but these are far from being common features among teleosts [64, 65]. Because of the environmental effects on sex differentiation of the European sea bass discussed above, the evaluation of potential markers of genetic sex is challenging. On the other hand, markers of sex differentiation may provide useful information on mechanisms and pathways involved in the process.

Markers of sex differentiation are genes that show increased expression levels in differentiating gonads of a given specific sex. Several such genes have been shown to be well-conserved among species and across vertebrate groups [66, 67], and some of these markers also appear to be involved in the initial stages of gametogenesis. Molecular markers of testicular differentiation include *amh* and *amhr2* [68], transcription factors such as *dmrt1* and *sox9* [69–72], and nuclear receptors, such as *dax1* and *nr5a2*, also known as *ff1a*, [69, 71, 72].

In the European sea bass, two genes have been linked to testicular differentiation – namely, the androgen receptor beta (*arb*) [73] and the steroidogenic enzyme 11-beta hydroxylase (*cyp11b*) [74]. High *cyp11b* levels were found in males at 150 dph, when gonads were still undifferentiated at the histological level and, thus, this gene has been regarded as an early indicator of testicular differentiation [74]. A similar result was reported for *arb*, with high mRNA levels found in males at 150–200 dph [49, 73] coinciding with early stages of histological differentiation of the testes.

The gene coding for aromatase (*cyp19a1a*), which is the enzyme responsible for the conversion of androgens to estrogens in vertebrates [75], is a key player of ovarian differentiation in fish [6, 76, 77]. In European sea bass, clear sex-related differences in *cyp19a1a* gene expression and enzymatic activity were found in gonads at the time of histological sex differentiation, with higher levels in females than in males (150 dph, 85 mm SL in males and 96 mm SL in females, [78]).

A later study reported high expression levels of *cyp19a1a* in putative females prior to histological sex differentiation [74]. In this study, the sex of individual fish was predicted based on a statistical approach (canonical discriminant analysis), using length as a proxy for gonadal development, and age and *cyp19a1a* expression as predicting variables. The method allowed for the discrimination of predicted males, females, and undifferentiated fish as early as 120 dph (50 mm SL), and was subsequently used successfully to infer the future phenotypic sex ratio in histologically undifferentiated European sea bass groups [60, 74]. In addition, in fish subjected to male-inducing temperatures, *cyp19a1a* levels were consistently low in undifferentiated fish that would subsequently develop as males [59, 74].

The potential role of *foxl2* and *sf1*, which have been shown to play a key role in ovarian differentiation in rice field eel, *Monopterus albus* [79] and in northern snakehead,

Chana argus [80], was also investigated in European sea bass. Interestingly, these two genes were capable of stimulating *cyp19a1a* transcription in an *in vitro* system, suggesting a potential role in the early steps of gonadal differentiation [59].

The *vasa* gene, which encodes an ATP-dependent RNA helicase found in primordial germ cells [81], has been shown to be involved in early sex differentiation in fish. The expression levels of European sea bass *vasa* were studied during sex differentiation in male- and female-enriched populations obtained by size grading [82]. The study showed differences in *vasa* expression between the two groups as early as 100 dph (35 mm SL in male-enriched populations, and 44 mm in female-enriched populations), before any signs of histological sex differentiation were detected, with higher levels in the fastest-growing fish that contained a high proportion of future females. Because the process of sex differentiation, including the onset of germ cell proliferation, is a function of fish size, the higher *vasa* levels in the female enriched group likely reflected, in part, their larger size at the time of sampling.

Based on these results, this gene was proposed as an early marker of germ cell proliferation and ovarian differentiation in the European sea bass, although a correction for fish size would need to be determined in order to formally evaluate the use of *vasa* for the purpose of early sex identification.

15.7 Transcriptomic Studies

The gonadal transcriptome in males and females during and after sex differentiation has been studied in several fishes, including the extensively used model species zebrafish (*Danio rerio*) [83], several aquaculture species [72, 84–87]), and even primitive fish such as the coelacanth, *Latimeria menadoensis* and *Latimeria chalumnae* [88, 89]. All these studies revealed sex-related differences in the expression of several genes and

signaling pathways, thereby improving our understanding of the biological processes involved in gonadogenesis.

Studies of the European sea bass gonad transcriptome that have been completed to date aimed at investigating the effect of feeding [90] and temperature [60] on sex differentiation. The first study showed that low levels of food availability during sex differentiation significantly affected the testicular transcriptome. The main observed change of gene expression was a downregulation of genes related to protein synthesis and degradation and genes involved in immune response, possibly signaling an effort to save energy. However, genes involved in spermatogenesis were still expressed at high levels, suggesting that, despite food deprivation, fish were still capable of allocating part of their energy towards reproduction [90].

The second study showed that high temperatures during the first 60 days of life induced an upregulation of genes involved in testicular differentiation, and a downregulation of genes involved in ovarian differentiation [60], a finding consistent with the masculinizing effect of this environmental treatment [16, 59]. Other genes related to epigenetic transcriptional regulation were upregulated under elevated temperatures, although their possible role in the process of sex differentiation remains to be established [60]. These results are consistent with findings in previous studies that showed that the expression of several sex differentiation-related genes is epigenetically regulated – that is, heritable changes occur in gene expression that do not involve changes in the DNA sequence [91–93]. This is the case of the *cyp19a1a* gene discussed earlier, which is subject to temperature-induced epigenetic regulation via changes in the methylation pattern of its promoter [59].

The study of the gonadal methylome in the tongue sole, *Cynoglossus semilaevis*, also revealed that several genes involved in sex differentiation, including *cyp19a1a*, *dmrt1*, *gsdf1*, and *amh*, were differentially methylated as a result of temperature-induced sex-reversal [94],

further supporting the implication of methylation in the sex differentiation of some TSD species [93]. These results clearly show that further study of the methylome of European sea bass gonads in connection with transcriptomic studies is warranted, in order to improve our understanding of the mechanisms of TSD in this species.

15.8 Concluding Remarks

After more than three decades of research on the European sea bass reproductive function, this species has become a model for both basic and applied studies of sex differentiation and reproduction among marine teleosts. This is due to the large amount of information obtained to date on physiological and molecular aspects of sexual differentiation, morphological aspects of gonad development, and the response of European sea bass sex ratios and genotypes to environmental conditions. Studies of gonadogenesis and hormonal sex reversal, along with the results of environmental treatments that were shown to significantly impact sex ratios, indicate that the labile period of sex differentiation for hormonal sex reversal corresponds to the early juvenile period, and is preceded by the thermosensitive period of sex differentiation, although the effects of early hormonal treatments at low doses

would deserve to be tested. Mechanistic studies should continue focusing on these developmental phases.

Transcriptomic studies of European sea bass gonads during sex differentiation revealed differential expression of several genes in differentiating ovaries and testes, and led to the identification of early markers of the future gonadal sex. The recent development of a partially annotated assembly of the European sea bass genome [95], and the acquisition of transcriptome sequence databases for several tissues (see [96] for review), will greatly enhance the interpretation of expression studies. Future studies of European sea bass methylome could also add important information on the potential role of DNA methylation in regulating the expression of genes involved in TSD. These results will be very helpful in order to understand the mechanism of environmental effects on sex differentiation in this important marine teleost, and to develop protocols for sex control in aquaculture.

Acknowledgements

Research at the MB lab was supported by the Government of Spain grants ref. AGL2011-28890 and AGL2015-67477-C2, and by the Generalitat Valenciana grant ref. PROMETEO II/2014/051.

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16

The Induction of Polyploidy, Gynogenesis, and Androgenesis in the European Sea Bass

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16.1 Introduction

The natural area of distribution of the European sea bass (*Dicentrarchus labrax* L.) includes the North Atlantic, from Norway to Morocco, the Mediterranean Sea, and the Black Sea. This fish is a eurythermic and euryhaline species that lives near the coast. It is carnivorous, with a natural diet including fish and crustaceans. It has a firm white meat of exquisite flavor, and is thus appreciated as a table fish. Small- or medium-scale traditional fisheries carry out European sea bass natural captures. Due to its highly desired meat, it was one of the first target species for the development of modern aquaculture in the 1970s and 1980s in Europe. Starting around the mid-80s, the industrial production steadily increased from an annual production of less than 100 tons in 1980, to 157,000 tons in 2014 [1]. The top production countries are Turkey, Greece, Spain, Egypt and Italy.

The European sea bass is a gonochoristic species with sexual growth dimorphism in favor of females. Overall, male fish exhibit 20–40% less body weight at harvest time (\approx 18–22 months of age) than female fish. Furthermore, under current culture conditions, males attain first maturity when they are two-year-olds, and females when they are three-year-olds. However, each sex

can mature precociously one year earlier, and this may involve up to 30% of males in the population [2–4]. This is documented poorly in females, although some evidence exists that shows early puberty can also affect females in captivity [5]. This, along with the above-mentioned sexual growth dimorphism in favor of females, has prompted interest for the development of techniques to control sex ratios. As a guideline, research carried out on other species in the mid-80s, mainly in Salmonids, started on hormonal and chromosome manipulation approaches, with the aim to control sex ratios and maturation [6–13].

Chromosome set manipulation has been intensively investigated in the European sea bass, including the induction of poliploidy (triploidy and tetraploidy) and individuals with uniparental inheritance (gynogenetics – mito- and meiogynogens, and androgenetics) [14–19]. Research first focused on examining distinct experimental conditions, in order to establish optimized protocols. These protocols were based on the application of pressure and temperature shocks to retain the haploid set of chromosomes of the second polar body, that will not expel it or suppress the first cleavage of the zygote (to block the first mitotic division of the embryo's diploid nucleus). Also, the use of

UV irradiation was tested to inactivate the DNA of exposed gametes (Table 16.1). Different methods to verify polyploidy, gynogenesis, or androgenesis have additionally been applied. Furthermore, growth and reproductive performance, as well as sex ratios, have been well documented.

In spite of this, chromosome set manipulation is not routinely applied in European sea bass aquaculture. Below we summarize the current knowledge and further considerations, with emphasis on induced triploidy, gynogenesis, and androgenesis.

16.2 Induction of Triploidy

The application of chromosome set manipulation requires the proper establishment and management of broodstock fish induced to spawn. European sea bass males, and only females that exhibit oocytes with migrating germinal vesicle, are intraperitoneally injected with the luteinizing hormone-releasing hormone analogue (LHRHa). Males and females are usually injected at 5 µg/kg body weight (BW). Subsequently, only females are injected with LHRHa, at 10 µg/kg BW, four hours later, then housed with males [2, 20]. Injected fish are kept in separate tanks with one female and two males. Seventy-two hours later, at the typical rearing temperature of 12–13°C, gametes are collected by gentle abdominal massage.

Initially, in order to evaluate the quality of the gametes, 10 ml of eggs (1 ml ≈ 650–750 eggs; ≈ 1.2 mm egg diameter) are fertilized with 4 µl of sperm from 1–2 males [20, 21]. Sperm is activated by adding 2 vol of sea water/vol of eggs plus sperm. The moment of sperm activation is taken as time zero ($T = 0$). Fertilized eggs are gently but thoroughly rinsed with seawater for 30 seconds at $T = 30$ seconds, and they are immediately poured into a glass vial containing seawater. Water temperature during fertilization and before the shock is usually set at 12–13°C. Since egg quality is a critical issue for ploidy manipulation, egg quality is determined based on the

percentage of floating eggs after fertilization and egg symmetric divisions [20, 22]. Only females, in which the percentage of floating fertilized eggs with regular divisions is more than 80%, are considered suitable for chromosome set manipulation [14, 17, 18, 20].

Secondly, the optimization of shock treatments (thermal or pressure) is essential for retention of the second polar body (e.g., suppression of meiosis II), thus inducing the production triploid fish (Table 16.1). There are three important variables that must be taken into consideration: the start time of the shock after fertilization; its intensity; and its duration [14, 17, 18, 20]. When optimized conditions are used, the lower survival typically observed in triploids, in comparison to diploids, during embryogenesis and after hatching, is due to shock treatment rather than the triploid condition *per se* [23]. Regarding the type of physical shock, cold shocks using temperatures close to 0°C have been successfully used in the European sea bass [23–25], although pressure shocks have also been effective to induce triploidy [25]. Theoretically, heat shocks can also be used to induce triploidy, but the conditions for the use of this type of shocks have not been optimized, as done with cold shocks [11] (Table 16.1).

Alternatively, triploid fish can be produced by indirect methods, including the fertilization of eggs with the diploid sperm from a tetraploid male [15, 18]. Tetraploidy can be induced by inhibiting the first cell division of the zygote after chromosome duplication shortly after fertilization (Table 16.1). Tetraploidy has been induced in the European sea bass, although survival, as happens with other species, was low in most batches [15, 16, 26]. Thus, the applicability of tetraploidy and its use in triploid production are limited, because of the low larvae yields, and its performance capacity are unknown. In the European sea bass, methods to identify polyploids are available, and include chromosome count or karyotyping, selective staining of the nuclear organizer regions and cellular size [14, 23, 24] and, as in other fish, direct determination of nuclear DNA content.

16.3 Effects of Triploidy on Growth and Reproductive Performance

In the European sea bass, triploidy induction impairs meiosis in both females and males. Consequently, both chromosomal packaging and the failure of the pairing of homologous chromosomes in meiosis affects oogenesis and spermatogenesis in triploids, resulting in altered gonadal development and lower gonadosomatic index (GSI) (Figure 16.1). Ovaries of triploid females are filament-shaped, while triploid males show similar testicular development to that of diploids, although they do not produce sperm [27–29]. Thus, at two years of age, the GSI of triploid females is equivalent to only 16% that of diploids, while the GSI of triploid males is 40% lower than the GSI of diploids [27]. At four years old, the GSI in diploid females is $\approx 9\%$ and 3.6% in males, while in triploids it is $\approx 0.08\%$ and $\approx 2\%$, respectively [29]. Furthermore, while diploids produce mature gametes, triploids do not through their seventh annual cycle of life, which coincides with the sixth and fifth sexual maturation period of males and females, respectively [30].

These findings have demonstrated that, despite the longer time given to triploids for gonadal development, they are not able to reproduce, and are functionally sterile. Nevertheless, although triploidy induces both functional and morphological sterility in this teleost fish, this condition does not confer significant improvement of its growth. In fact, results in growth performance have indicated that, although one-month-old triploid larvae are larger [31], no differences exist between ploidies in 5–23 month-old animals ($2n = 172.02 \pm 2.73$ g, vs. $3n = 158.94 \pm 2.68$ g) [27, 32]. Thus, during their first 3–4 years of life, triploids grow in a similar fashion to diploids in fork length, but more slowly than diploids in body weight, even when diploids reach full sexual maturity.

Peruzzi *et al.* [32] showed that, although growth in diploids was better than in triploids up to four years of age, triploids exhibited higher gutted yield than their diploid counterparts, particularly in females. Nevertheless, it is interesting to note that when fish are four years old or above, differences in weight are no longer apparent between ploidies, suggesting that triploidy may represent a benefit for the aquaculture industry of this species, at least for the production of larger fish [30]. In this regard, a comparative study of the long-lasting impact of induced triploidy on growth and reproductive endocrinology in seven-year-old adults revealed that triploid females attained the largest sizes, particularly after the natural spawning period, when diploids might end up losing body weight due to gonadal regression. In contrast, triploids maintain regular growth rates, due to their sterility [30] (Table 16.1). Accordingly, pilot-scale evaluations need to be conducted in order to determine the use of sterile triploid fish and their optimum rearing requirements in aquaculture [18, 33–35].

16.4 Perspectives on the Use of Triploids

Traditionally, the European sea bass markets at a pan size of 350–450 g. However, markets and producers alike are in need of product diversification, fueled by an increasing demand for larger-sized fish (e.g., from 800 to 1,000 g, marketed by some companies as “Royal European sea bass”), or even for fish of >1 kg, marketed as “Imperial European sea bass.” Therefore, the production of triploids is a serious option for the production of these larger fish, since triploidy might help to assist market demand for large-sized fish, as somatic growth in triploids is steadily higher than diploids when fish are up to 3.5–4.5 kg [30].

Thus, it would be interesting to evaluate the advantage in growth of triploids according to their sex, in order to confirm that triploid females are initially the preferred

sex to maintain under farming conditions, as it has been reported in triploid rainbow trout, *Oncorhynchus mykiss* (Walbaum) [36]. In this context, it would be interesting to consider the combination of the induction of triploidy with endocrine feminization [37]. This can be achieved by the hormonal feminization of triploids or by the triploidisation of female dominant stocks (see Chapter 15).

Given all these observations, some critical aspects should be evaluated by further research before triploid European sea bass production could become a reality in fish farming facilities. A key issue would be determining the incidence of malformations, a relevant aspect for industrial production and also from an animal welfare perspective, as well as for public acceptance [18]. Although some studies suggest that triploidy is the main cause of the incidence of deformities, most studies report that the number of deformities observed in triploids seems to be related to the physical or chemical manipulations used to produce them rather than the triploid condition per se [18]. In addition, product quality, nutritional requirements, disease resistance under optimal and sub-optimal conditions, behavior, and different types of farming environments should be investigated [18, 33–35].

These considerations would help to assess the profitability of culturing triploids on a commercial scale. Furthermore, there is little knowledge on triploid fish among the public and consumers. Thus, additional information on the effect of triploidy would be of interest, in order to contribute to achieve societal acceptance as, today, companies fear that consumers would reject triploids [38]. In this sense, the recognition of triploidy as an already accepted strategy in food production (e.g., selection of triploid hybrids for producing seedless fruits), and the reduction of the interactions of cultured and wild fish populations (e.g., environmental benefits due to genetic containment), could help to increase the acceptance and the value of triploidy in aquaculture [18, 33, 39, 40].

16.5 Induction of Gynogenesis

Gynogenesis is a type of uniparental reproduction in which only the female genome transmits to the next generation. The induction of gynogenesis is feasible in lower vertebrates, particularly fish. In the European sea bass, meiogynogenetic diploids have been obtained – after fertilization with UV-irradiated sperm to trigger embryonic development – by means of cold, heat, and pressure shocks to prevent completion of meiosis II in the egg, hence restoring diploidy. Mitogynogenetic diploids have also been obtained after fertilization with inactivated sperm, and by means of similar shocks to prevent the first cleavage of the zygote, also restoring diploidy [14, 16, 20, 25, 32, 41]. The use of sperm from an unrelated species (e.g., sea bream) has also been tested after UV-irradiation to ensure no paternal contribution to the offspring [25].

Induced diploid meiogynogenesis, and mitogynogenesis, through prevention of the first cleavage of the zygote, has been obtained in the European sea bass. Survival of the produced fish depends on the type of shock, the treatment intensity/duration, and the selected parental combination (Table 16.1, Figure 16.1). Through different procedures, it has been demonstrated that these treatments are 100% effective in inducing uniparental inheritance. However, survival of the gynogenetic diploid larvae ranges from 17–35% of controls [15, 24, 41, 42], to 76% at hatching [25]. In the only case reporting the production of mitogynogenetic diploid European sea bass, survival mean was 1%, although some probing trial was up to 27% at hatching [15].

As with the induction of triploidy, the production of gynogenetic fish needs to be verified by appropriate means. This has been achieved by different methods, including cytogenetic approaches [41] that give evidence for restoration of diploidy in gynogenetic fish. However, the true assessment of gynogenetic origin is achieved by using molecular markers, such as AFLPs [43] or microsatellites [25, 42], which provide more accurate results of

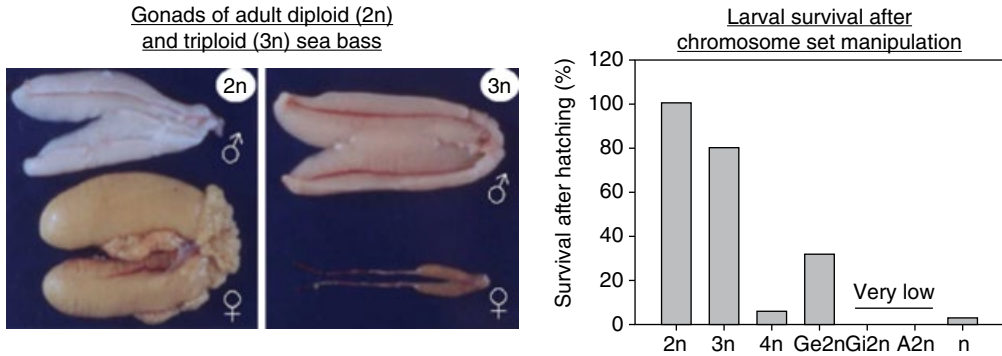


Figure 16.1 Effects of chromosome set manipulation in the European sea bass. (See inserts for the color representation of this figure.)

Left panel: Photographs of testis (above) and ovaries (below) of adult diploid (2n) and triploid (3n) males and females.

Right panel: Percentage of larval survival at hatching relative to control diploids (2n) including triploids (3n), tetraploids (4n), meiogynogenetics (Ge2n), mitogynogenetics (Gi2n), androgenetics (A2n), and haploids (n).

exclusively maternal contribution. This is, thus, the preferred option nowadays.

16.6 Effects of Gynogenesis on Growth, Gonadal Development, and Sex Ratios

In aquaculture, fish in which diploid gynogenesis has been induced are not used for production, due to their poor growth and survival, but instead are used for the creation of broodstock with desired characteristics. For example, in species with a chromosomal sex-determining system of the XX/XY (female/male) type, the induction of gynogenetic diploids is supposed to produce only females [18, 44]. These females can then be treated with androgens to produce neomales which, in turn, can be used for the hormone-free production of all-female stocks in the many species in which, there is sexual growth dimorphism in favor of females.

Nevertheless, assessing the growth of gynogenetic diploids is necessary in order to have an estimation of the time needed to reach sexual maturity and, thus, being of use in broodstock management programs. Studies on growth performance of gynogenetic diploid European sea bass have demonstrated

that no significant differences exist between the diploid control and the meiotic gynogens obtained from the same breeders, although significant differences exist among progenies originated from different breeders. This suggests that genetic and/or physiological variability exists in parental combinations [25] and, presumably, from individual females [45, 46], indicating that phenotypic growth is more dependent on maternal effects than due to the effects of the induction of gynogenesis.

In European sea bass, which has a polygenic system of sex determination [47] with environmental influences [48] [see Chapter 14], the induction of gynogenesis results in the production of both sexes. Both female and male meiotic gynogens have a normal reproductive performance similar to diploids, and so display the same onset of puberty and reproductive potential at adulthood. Thus, European sea bass gynogens undergo vitellogenesis and produce eggs with the same quality as those from control diploids after ovarian stimulation by hormonal treatment [42]. Furthermore, sperm released by meiogynogenetic males is similar to that of controls, in terms of volume, quality, and fertilization capability [42]. Thus, sex ratios in the meiogynogenetic offspring comprise both sexes,

Table 16.1 A summary of chromosome set manipulation studies in the European sea bass (*Dicentrarchus labrax*).

Manipulation	Methods	Results		References
		Survival/growth	Reproduction	
Triploidy	Heat 29°C, 25 min, 15min Pressure 55.1 MPa, 2–3 min, 5 min Pressure 58.6 MPa, 2 min, 6 min Cold 0° C, 5 min, 5 min Cold 0–2° C, 20 min, 5 min Cold 0–1° C, 15–20 min, 5 min Cold 0° C, 10 min, 5 min	3 <i>n</i> , 0.8% Sv	Full gonadal and functional sterility in both sexes	[10]
		3 <i>n</i> , 13% Sv		[58]
		100% 3 <i>n</i> , 71% Sv		[25]
		Not described		[59]
		89–90% 3 <i>n</i> , 40–50% Sv		[24]
		100% 3 <i>n</i> , 56% Sv		[25]
		95–100% 3 <i>n</i> , 80% Sv		[23]
		3 <i>n</i> = 2 <i>n</i> up to 2 yrs		[27]
		3 <i>n</i> < 2 <i>n</i> in adults up to 4 yrs		[28, 29]
		3 <i>n</i> = 2 <i>n</i> > 4 yrs and older		[30]
		3 <i>n</i> > 2 <i>n</i> in females at 7 yrs		[30]
Tetraploidy	Pressure: 81–91 MPa, 4 min, 70–90 min	Very few 4 <i>n</i> in most batches displaying 6–25% Sv, 75–94% in 9–11 day-old larvae, 4% in 46 day old fry. No tetraploid fry older than 50 days	Not reported	[15, 16, 26]
Gynogenesis (meiotic)	UV-irradiated sperm (1 : 10 diluted) at 40,000 erg.mm ⁻² plus heat 29°C, 25 min, 15 min or pressure 55.1 MPa, 2–3 min, 5 min	Ge2 <i>n</i> , Sv not described	Female and male meiotic gynogens. Normal onset of puberty and reproductive performance at adulthood at 3 yrs of age.	[10, 58]
	UV-irradiated sperm (1 : 100 diluted) at 3,300–6,600 erg. mm ⁻² plus cold 0–2°C, 20 min, 5 min	83–100% Ge2 <i>n</i> , 17% Sv		[24]
	UV-irradiated sperm plus heat 35°C, duration not shown, 3–5 min	Not described		[60]
	UV-irradiated sperm (1 : 10 diluted) at 35,000–40,000 erg. mm ⁻² plus cold 0°C, 10 min, 5 min	95% Ge2 <i>n</i> , 30–35% Sv		[41]
	UV-irradiated sperm (1 : 20 diluted, homologous and heterologous -sea bream-) at 32,000 erg.mm ⁻² plus cold 0–1°C, 15–20 min, 5 min or pressure 58.6 MPa, 2 min, 6 min	100% Ge2 <i>n</i> , 76% Sv		[25]
Gynogenesis (mitotic)	UV-irradiated sperm at 3,300 erg. mm ⁻² plus pressure shock 81 or 91 MPa, 4 min, 64–79 min	92–100% Gi2 <i>n</i> , survival of pre-larvae was 7–18% of the controls and the overall survival was very low (0.07%)	Not reported	[15, 16]
Androgenesis	UV-irradiated eggs at 0,072–0,72 erg. mm ⁻² . Egg fertilization using sperm diluted (1 : 4) plus pressure or thermal shock at 1st cleavage (specific conditions not described)	Small percentage of haploid androgenetics. UV-irradiation largely ineffective at inactivating the maternal DNA	Not reported	[19]

Abbreviations: 2*n*, diploids; 3*n*, triploids; 4*n*, tetraploids; Ge2*n*, meiogynogenetics; Gi2*n*, mitogynogenetics; Sv, survival; yrs, years. Shock treatments as values for the intensity of the shock, duration of the shock, and time after fertilization when shock is initiated. Survival was to hatching unless otherwise indicated.

although the proportions of females and males can differ considerably, according to genetic and environmental influences.

In the study of Colombo *et al.* [49], the percentage of female meiogynogenetic fish produced at different locations, compared with normal control fish, ranged from 61% to 82%. In the study of Felip *et al.* [41], a similar percentage of sexes and comparable to that of the diploid controls was observed in the two distinct progenies analyzed (Table 16.1). On the other hand, in an independent study of Barbaro *et al.* [50], only 39% of meiogynogenetics differentiated partly into males and, thus, the proportion of females was higher than usual. Furthermore, the sex ratio of the offspring from masculinized females is not female-biased, thus excluding also male homogamety [8].

Temperature influences the sex ratio in the European sea bass. In fact, a factorial mating including 253 full-sib families, and reared in a single batch to avoid any between-families environmental effects, has demonstrated that the family sex ratios are in accordance with a polygenic model interacting with environment (e.g., temperature) [47, 51]. Recently, the identification of sex ratio QTL has provided additional support to the polygenic sex determination hypothesis in this species, thus offering further opportunities for sex-ratio control in European sea bass [52].

16.7 Perspectives on the Use of Gynogenetic Diploids

As stated above, the induction of diploid gynogenesis in the European sea bass results in the presence of males and females in the offspring [14, 16]. The use of homozygous fish in several breeding experiments and/or directed individual selection, aiming to understand the genetic basis of traits of interest in aquaculture industry, would be a first step conducted in this species to improve and optimize the design of such programs. To this aim, cooperation with the

industry would need to be encouraged, as the initiation of local and/or national breeding programs in a range of countries and species could increase the use of genetically improved stocks in commercial aquaculture [39]. On the other hand, the long-term maintenance of gynogenetic fish and/or clonal founders, as well as the evaluation of their performance in comparison to regular stocks (controls), should be performed as pilot research trials, in collaboration with commercial farms, in order to increase the use of selectively bred stocks.

One particular aspect also worth mentioning is the contribution that induced gynogenesis has had in the advancement and development of genomic tools for the European sea bass. Thus, gynogenetic diploids first contributed to the development of linkage maps [54], and to the determination of the genomic sequence of this species [55]. The European sea bass is, in fact, one of the richest fish marine species in terms of genomic resources [56]. Thus, in these achievements, the contribution or the use of gynogenetic animals has been crucial. Even with the genome published, there are still new applications of the use of gynogenetic fish. Thus, it is also important to notice the usefulness of induced gynogenesis to create inbred lines. Inbred lines can aid in the determination of the contribution of epigenetic variance to phenotypic variance under a limited amount of genetic variance, as is currently done with plants [57].

16.8 Induction of Androgenesis

The inactivation of the maternal DNA is mandatory for producing androgenetics, a form of uniparental reproduction in which offspring inherits only paternal chromosomes. Eggs need to be exposed to UV light or ionizing irradiation, followed by restoration of diploidy by suppression of mitosis, using a pressure or thermal shock. Androgenesis is a useful approach for research purposes including the study of sex determination, the production of

homozygous clones, the preservation of endangered species using cryopreserved sperm, and the study of the mitochondrial genome.

Androgenesis has been achieved in some freshwater species [61], but it has not been reported in any marine species [53]. Different techniques, including individual morphology of embryos and larvae, flow-cytometry, and karyotyping, have been used to verify the genetic inactivation of the maternal genome of experimental fish. However, the exclusively uniparental inheritance (paternal) needs to be demonstrated by using molecular genetic markers. In the European sea bass, UV light has been used for egg irradiation [19] (Table 16.1).

These findings have revealed that the eggs of European sea bass, as well as other marine teleosts, present UV-screening compounds, such as gadusol, that protect eggs against UV [19]. Accordingly, alternative methods need to be explored for the production of androgenetic progenies in this marine teleost species, including interspecific androgenesis between related species showing nucleocytoplasmic compatibility. Thus, this technology still has a long way to be experimentally refined, to achieve research protocols for marine fish species, including the European sea bass (Figure 16.1).

16.9 Conclusions

Triploid, gynogenetic, and androgenetic production methods have been developed in European sea bass (Box 16.1). The effects of the induction of triploidy and gynogenesis on survival, growth and reproductive performance have been well studied. In contrast, obtaining androgenetics in sufficient amounts requires much work. In the European sea bass, the induction of triploidy results in genetic sterility in both sexes, although the effects on gonadal development are more marked in females. However, while triploids do not grow more than diploids in

Box 16.1 Triploidy and gynogenesis induction in the European sea bass

To induce triploidy in the European sea bass, the most suitable conditions are those using cold shocks. Freshly fertilized eggs are exposed to 0°C for 10 minutes, starting 5 minutes after fertilization. With these shocks, survival is typically around 80% with respect to the controls after hatching, and the rate of triploidization is typically no lower than 90%.

Heat and pressure shocks cannot be excluded as alternative methods for the induction of triploidy in this teleost. Meiotic gynogenesis should be induced by inactivating diluted sperm with UV irradiation at 35,000–40,000 erg mm⁻² before fertilization.

To restore diploidy, optimized protocols using cold shocks are recommended, similar to those applied in triploidy. To induce androgenesis, the inactivation of maternal DNA is critical. In the European sea bass, UV light has been used for egg irradiation, although alternative methods need to be explored to achieve refined research protocols for marine fish species.

regular pan-sized fish, triploids outgrow diploids in fish of 3 kg or bigger. Thus, triploidy could be of advantage in the production of large European sea bass for market and product diversification, as well as minimizing the interaction between farmed and wild populations [30].

On the other hand, gynogenetic diploids have significantly contributed to the development of genomic tools for this species, including linkage maps and the sequencing of the European sea bass genome. Gynogenetic diploids may also be useful in the creation of inbred lines to contribute to understanding the genetic basis of traits of interest, and to help disentangle the contribution of epigenetic vs. genetic variance in phenotypic variation.

Acknowledgments

We thank A. Gil, who assisted in the revision of the references of this chapter. Research at the AF lab was supported by Grants 201640I005

(Ministerio de Economía y Competitividad, MEC) and REPROBASS (Prometeo II/2014/051) from the Regional Government of Valencia, Spain. The MEC grant AGL2016-78710-R supported research at the FP lab.

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Part V

Sex Determination and Control in Centrarchidae

17

Sex Determination, Differentiation, and Control in Bluegill

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17.1 Introduction

The bluegill sunfish, *Lepomis macrochirus*, is a member of the sunfish family Centrarchidae, of the order Perciforms. It is native to North America and is distributed widely in rivers, ponds, and lakes. This species has also been introduced into other countries, such as China, Japan, and Korea [1]. Bluegill have become an economically important and high-value species, both from the perspective of their use in aquaculture, as well as their recreational value. In some Midwest states, like Ohio and Michigan, bluegill have been listed as one of the top three aquaculture species because of their desirable characteristics [2–4] for production, and the demand for them and high value in the marketplace. Bluegill and its hybrids are the one of the families that have a unique and niche market in the Midwest, middle south, and Southeastern United States. Despite this opportunity, rapid expansion of the bluegill aquaculture industry has not occurred yet. One reason in particular hindering expansion has been the relatively slow growth of currently cultured populations of this species.

For the past 20 years, research funded by the USDA-NCRAC (North Central Region Aquaculture Center) has been focused on increasing the growth rate or creating

fast-growing bluegill, with the purpose of increasing sunfish aquaculture production [5–8]. One of the most important findings from those studies is that the inherent growth rate of bluegill males is twice that of females, and males could reach commercial size (250 g) in eight months from the juvenile stage [6]. The follow-up research results through evaluating the growth performance of mostly-male groups versus mixed-sex groups indicated that male bluegill communally reared in groups were still able to grow significantly faster than mixed or mostly-female populations in commercial aquaculture settings, and the social interaction costs among males in the group did not significantly decrease growth performance of the male population [9].

Similar results are reported by Doerhoff [10], in which the top 25% were mostly-male groups (80–100% male), growing 42.3–62.3% faster than regular mixed-sex groups (48–52% male), and the males in mostly-male groups gained 50 g more than females on average by the end of the 240-day experiment. The research data and commercial practice suggested that mostly-male or all-male populations could reach market size within a year in a cage and recirculating aquaculture system [8–10]. These results support the conclusion that a monosex

culture holds considerable potential as a method to increase the efficiency and profitability of bluegill food and recreational aquaculture, by improving growth rate and eliminating the problems of prolific reproduction, precocious maturity, and their consequences.

Bluegill sunfish are of great interest to evolutionary ecologists for studies of life history, sexual selection, sex-determining mechanism, and behavioral evolution. Bluegill display an extraordinary reproductive ecology, with an extended spawning season [11–14], including: nesting colony behavior and solitary paternal care; two male reproductive strategies (parental and cuckolder males); natural interspecific (*Lepomis*) hybridization; unequal sex ratio in both natural and rearing populations; and underdetermined sex-determining mechanism [15–22].

Bluegill parental males reach maturation at seven years old, build and colonize nests, court females, defend parasitic males, and provide solitary parental care. They have a larger size than cuckolder males. At 2–3 years old, cuckolder males first adopt a “sneaker” tactic and dart into nests while the females are releasing eggs. When the sneakers reach four-plus years old, they switch to a satellite tactic and mimic the appearance and behavior of females, ejaculating between parental males and spawning females. Neither of these cuckolder tactics provide parental care [17]. Higher growth rates during the endogenous nutrition period of offspring sired by parasitic males versus offspring sired by parental males suggests a genetic difference in growth between the two types of male life histories [20]. A similar advantage in offspring sired by parasitic males, with respect to survivorship, was also found [19].

Temperature effects on sex ratio display high levels of parental, strain, or population influence [23]. In the natural population, skewed male, skewed female, and balanced population sex ratio of bluegill from different geographic locations have been reported [18, 21], indicating that temperature may be

responsible for the differences. However, the real ecology or adaptive significance of skewed sex ratios in nature still remains unclear. All of these special profiles make bluegill an excellent model to research interactions between mating system and sex-determining mechanisms on population sex ratio.

In the past 50 years, there have been many studies related to sex-determining mechanisms, sex control, and production of mostly-male or all-male populations in bluegill. Sex chromosomes could not be differentiated from autosomes cytologically [24]. In addition, no sex-specific markers were detected by screening with amplified fragment-length polymorphism (AFLP) (using 12,835 loci produced by 256 primer combinations [25]), indicating further that sex chromosomes have not yet evolved, or are at a relatively recent evolutionary origin in this species. Interestingly, male-skewed (up to 98% males) and female-skewed (up to 79.3% females) sex ratios in natural populations were both reported [18, 21]. In experimental bluegill populations, male-skewed and female-skewed sex ratios were also found frequently [14].

The complex pattern of the male-skewed sex ratios of bluegill hybrids (see Figure 19.3 in Chapter 19 of this book), cannot be explained by the current theories of sex determination. In the past 15 years, major progresses have been made in research of sex differentiation, sex determination, and monosex production in bluegill sunfish. This chapter summarizes the efforts and results. Not only is the information important for aquaculture, but it also broadens our understanding of this process beyond the specific details found within the group. The reproductive biology and ecology of sunfish is so unusually diverse that this system can provide a relatively unique example of sex-determination mechanisms, and a unique opportunity to investigate and test theoretical concepts of sex determination, ranging from evolutionary mechanisms to biochemical processes, and from genetic determination to environmental effects.

17.2 Sex Differentiation

A detailed understanding of the time of gonadal development and differentiation is critical to control sex and optimize culture. To achieve this goal, our laboratory systematically studied gonadal sex differentiation of the bluegill sunfish and its relation to fish size and age from hatching to 90 days post-hatching (dph), using a slow-growing batch (SGB) and a fast-growing batch (FGB) of fish [27].

17.2.1 Gonadal Differentiation and Development

With respect to gonadal sex differentiation, bluegill sunfish are classified as a differentiated gonochorists, in which ovaries and testes develop directly from undifferentiated gonadal tissue [27]. The key morphological events during gonadal sex differentiation in SGB are summarized in Table 17.1 and are described in detail below.

Undifferentiated gonads: at 5 dph, primordial germ cells (PGCs) were observed under the mesonephric duct (Figure 17.1a). The PGCs were arranged in groups of 10–20 cells. Morphologically, the round to slightly oval PGCs were distinguished from somatic cells by their relatively large diameter (6–8 μm) and their histological features. Their cytoplasm contained two or three round nuclei, 1–2 μm in diameter. At 25 dph, a pair of gonads appeared under the dorsal celomic epithelium (Figure 17.1b). Some germ cells were found to project into the abdominal cavity within a cord-like gonadal tissue from the dorsal celomic epithelium at 30 dph (Figure 17.1c), and a pair of gonads was present under the abdominal cavity. The nuclei of germ cells were polymorphic, often bi-lobed, with one or two large nucleoli and dispersed chromatin, in the form of an irregular meshwork in which numerous small chromatin masses were suspended. Subsequently, at 40 dph, the gonads were attached at both sides of the mesentery by a

Table 17.1 Summary of morphological events of gonadal development and sex differentiation in the slow-growing batch of bluegill sunfish. U – undifferentiated; F – female; M – male; dph – days post-hatching [27].

Age (dph)	No. of fish	TL (mm)	Gonadal stage	Sex		
				U	F	M
5	10	4.6–5.2	PGCs present	10	0	0
25	10	6.6–7.2	A pair of gonads under the dorsal celomic epithelium	10	0	0
30	10	7.0–8.8	A pair of gonads under the abdominal cavity	10	0	0
50	8	9.5–13.2	Active germ cell mitosis present in half of the individuals	8	0	0
60	5	14.0–19.2	Increases in the number of germ cells and somatic cells in the presumptive ovary	3	2	0
70	5	16.2–21.0	Elongated aggregations of somatic cells and germ cells undergoing meiosis in the ovary, increases in the number of germ cells and stromal cells in the presumptive testis	0	2	3
80	6	18.2–23.0	Complete ovarian cavity in the ovary, efferent duct anlage in the testis	0	3	3
90	7	20.0–26.0	Peri-nucleolus oocytes and fusion of anterior part of gonadal tissues in the ovary, evident efferent duct and meiosis in the testis	0	4	3

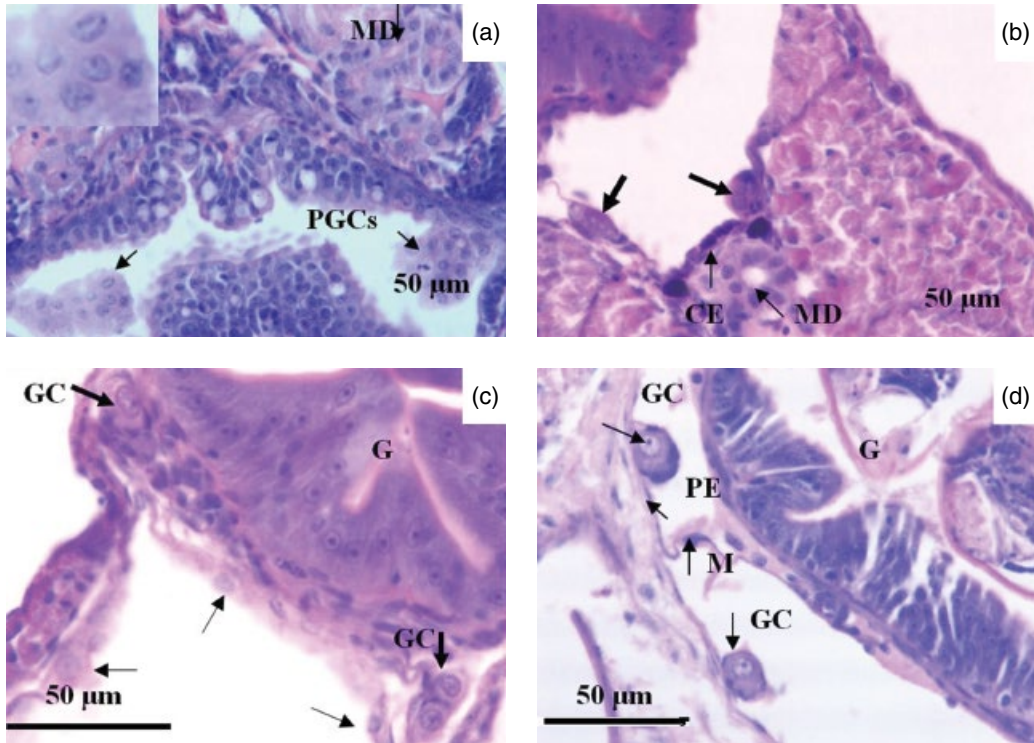


Figure 17.1 Undifferentiated gonads of the bluegill sunfish at 5–40 dph in the slow-growing batch. (See inserts for the color representation of this figure.)

- a) Clusters of primordial germ cells at 5 dph. Higher magnification of primordial germ cells (inset).
- b) Primordial gonads at 25 dph. A pair of gonadal primordia is indicated by the thick arrows.
- c) Primordial gonads at 30 dph. The migratory germ cells are indicated by the arrows, and a pair of gonadal primordia is indicated by the thick arrows.
- d) Undifferentiated gonads at 40 dph.

Abbreviations: CE – celomic epithelium; G – gut; GC – germ cells; M – mesentery; MD – mesonephric duct; PE – peritoneal epithelium; PGCs – primordial germ cells.

mesogonadium, and wrapped by the monolayered peritoneal epithelium (Figure 17.1d). One pear-shaped gonad always contained one or two primordial germ cells per cross-section. Nuclear contours of germ cells became round to ovoid, and chromatin varied from dispersed concretions of various sizes, to distinct threads arrayed in a fine peripheral meshwork. The gonads of the fish collected at 40 dph did not show any morphological characteristics indicative of a differentiating ovary or testis [27].

Ovarian differentiation: at 50 dph, two kinds of gonadal tissue were observed in different individuals. Gonadal tissue type I showed fewer cells, and all of them had characteristics similar to those observed in the undifferentiated stage. Gonadal tissue

type II consisted of two different populations of germ cells. One type of cell exhibited morphological characteristics resembling undifferentiated germ cells, as described in the previous stage, whereas the other type was undergoing mitosis (Figure 17.2a). Germ cells undergoing active mitosis were present with different features, such as smaller size, mottled nuclei with variable amounts of clumped chromatin around periphery, or a single prominent nucleoli. At 60 dph, in type II gonadal tissue, germ cells gradually multiplied in number by active mitosis, and the number of somatic cells increased, together with blood vessels appearing in the lateral region (Figure 17.2b). Somatic reorganization of the presumptive ovary began. Gonads were present in a triangular or kidney-shape

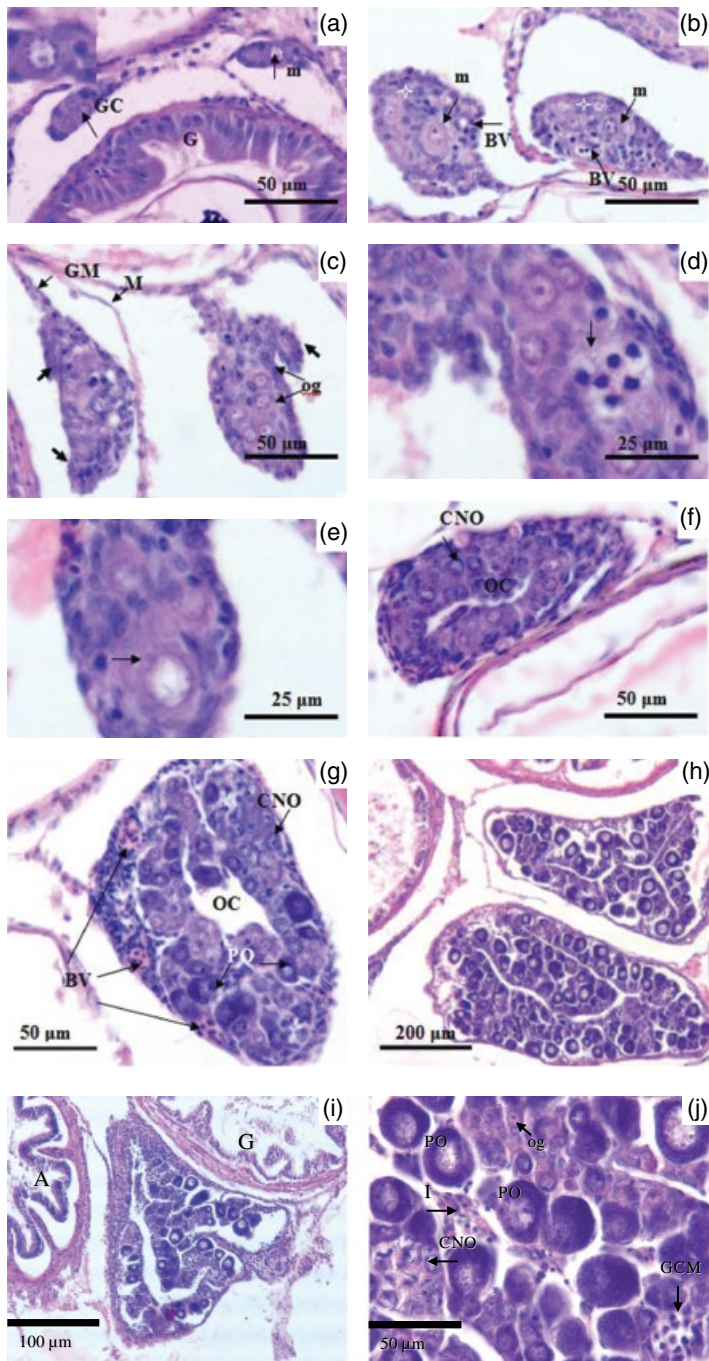


Figure 17.2 Ovarian differentiation in the bluegill sunfish at 50–90 dph in the slow-growing batch. (*See inserts for the color representation of this figure.*) a) Presumptive ovary at 50 dph, showing gonadal type I in which germ cells are undergoing early mitosis (m). Higher magnification of mitotic germ cells (inset). b) Presumptive ovary at 60 dph, showing germ cells multiplied in number, and blood vessel. The numerous somatic cells are indicated by stars. c) Initial ovary at 70 dph, showing somatic elongations. Two somatic elongations forming the initial ovarian cavity formation are indicated by the thick arrows. d) Initial ovary at 70 dph, showing germ cell nests with zygotene (bouquet) stage of oocyte meiosis (arrow). e) Initial ovary at 70 dph, showing oocyte undergoing meiosis at pachytene stage (arrow). f) Ovary at 80 dph, showing the ovarian cavity (OC) and oocytes at chromatin-nucleolus stage (arrow). g) Ovary at 90 dph, showing some oocytes at peri-nucleolus stage (PO). h) Ovary at 90 dph, showing many peri-nucleolus oocytes. i) Ovary at 90 dph, showing the fusion in the anterior part of two gonadal tissues. j) Ovarian tissue at 90 dph. Abbreviations: m – meiotic germ cell; A – anus; BV – blood vessel; CNO – chromatin-nucleolus oocyte; G – gut; GC – germ cells; GCM – germ cells undergoing meiosis at zygotene stage; GM – gonadal mesentery; I – interstitial or stromal tissue; M – mesentery; OC – ovarian cavity; og – oogonium; PO – peri-nucleolus oocyte.

when observed in sections. Gonad size and the number of germ cells increased dramatically between 60 dph and 70 dph. At 70 dph, the initial ovarian cavity formation was indicated by the presence of two elongated aggregations of somatic cells in the proximal and distal portions of the gonads (Figure 17.2c). The two elongating sheets of somatic tissue developed both upward and downward, to form a groove lateral to the gonad proper, the downward elongation from the proximal region being more conspicuous than the upward elongation from the distal region. In addition, germ cell nests with the zygotene (bouquet) stage of oocyte meiosis (Figure 17.2d), and oocytes undergoing meiosis at pachytene stage (Figure 17.2e), were observed, signifying early oogenesis. Subsequently, the outgrowths of the somatic cell aggregations had fused together to form the ovarian cavity. The ovarian cavity was completely formed by 80 dph, and oocytes at the chromatin nucleolus phase were present at this time of development (Figure 17.2f). At 90 dph, most of the ovarian gonads observed contained a few oocytes at the peri-nucleolus stage, together with a somatic layer including blood vessels, and the ovarian cavity was clearly observed in the central part of the ovary (Figure 17.2g). Numerous peri-nucleolus oocytes were found in one fish, and the anterior part of two gonadal tissues that attached at both sides of the mesentery had fused together, with the gonads coming near the anus (Figure 17.2h-j) [27].

Testicular differentiation: in contrast to ovarian development, signs of histological differentiation were not observed in the presumptive testis until day 70. The overall appearance of spermatogonia was similar to that of undifferentiated germ cells at 50 dph, referred to previously as gonadal tissue type I (Figure 17.3a). At 70 dph, they retained the original pear-like shape of indifferent gonads and were much smaller than ovaries of the same developmental stage. The most characteristic features were the presence of germ cells undergoing mitosis, and the aggregation of stromal cells

(Figure 17.3b). At 80 dph, blood vessels became evident in the dorsal region of the testis (Figure 17.3c). Moreover, a central space that became recognizable as the efferent duct anlage was present in some sections of the testes, and the unrestricted lobular organization of the testis could be distinguished. At 90 dph, some spermatogonia undergoing mitotic divisions became spermatocytes (Figure 17.3d). The onset of meiosis was noticed in the gonads of the males at this developmental stage. In contrast to ovarian development, no part of the two gonads attached at both sides of the mesentery was found to fuse together in the testis, as that in the ovary by this time [27].

17.2.2 Relationship of Gonadal Differentiation with Fish Size and Age

Generally, the time of sex differentiation in FGB, which grew significantly faster than SGB ($P < 0.05$) from hatching to the 90 dph, was earlier than that in the slow-growing batch [27]. For ovarian differentiation, the elongated aggregations of somatic cells were present at 30 dph. The ovarian cavity was completely formed at 50 dph, and the anterior part of gonadal tissues attached at both sides of the mesentery began to fuse at this developmental stage (Figure 17.4a–b), with the basophilic oocytes at the stage of meiosis present. The peri-nucleolus oocytes were observed at 60 dph (Figure 17.4c). Subsequently, numerous peri-nucleolus oocytes were found at 80 dph (Figure 17.4d). At 90 dph, the oocytes were still in the peri-nucleolus stage, while the gonad became bigger. As to the testicular differentiation, the efferent ducts were present at 70 dph (Figure 17.4e) and became evident at 80 dph (Figure 17.4f). The early meiotic activity occurred at 80 dph and became distinct at 90 dph. The anterior part of two testis tissues fused together in some fish at 90 dph. The key morphological events during gonadal sex differentiation in FGB are summarized in Table 17.2.

Gonadal differentiation of bluegill appeared to be related to size more than to age. In

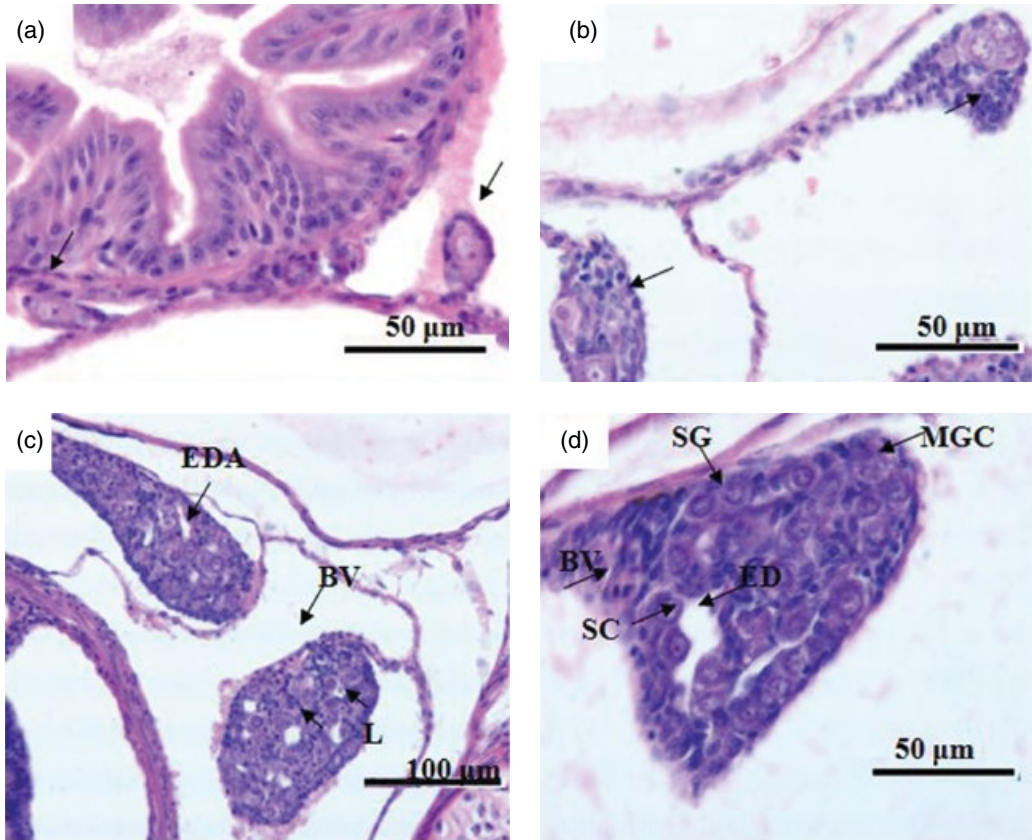


Figure 17.3 Testicular differentiation of the bluegill sunfish at 50–90 dph in the slow-growing batch. (See inserts for the color representation of this figure.)

- a) Fry at 50 dph showing gonadal type II tissue (arrows).
- b) Presumptive testis at 70 dph. The aggregations of stromal cells are indicated by arrows.
- c) Testis at 80 dph, showing the efferent duct anlage, lobule, and blood vessel.
- d) Testis at 90 dph, showing evident efferent duct, spermatogonia undergoing mitotic divisions to become spermatocytes, and the onset of meiosis.

Abbreviations: BV – blood vessel; EDA – efferent duct anlage; ED – efferent duct; L – lobule; MGC – meiotic germ cells; SG – spermatogonium; SC – spermatocytes.

general, the proliferation of germ cells and somatic cells happened in putative females between 13.2 and 16.0 mm TL (at 60 dph in SGB, and 30 dph in FGB) and the ovarian cavity was completely formed in all the female fish larger than 21.0 mm TL (at 80 dph in SGB, and 50 dph in FGB). Meanwhile, in putative males, the increases in the number of germ cells and stromal cells appeared in the fish between 19.0 and 22.5 mm (at 70 dph in SGB, and 50 dph in FGB) and the efferent ducts were present in all the males larger than 28.0 mm (at 90 dph in SGB, and 70 dph in FGB).

17.2.3 The role of *foxl2* and *cyp19a1a* Genes in Early Sex Differentiation in Bluegill

We studied early expression of *foxl2* and *cyp19a1a* and their role in early sex differentiation in bluegill sunfish. Two ovarian differentiation-related genes, *foxl2* and *cyp19a1a*, were detected at 7 dph, which is well before the onset of morphological gonadal differentiation, indicating that these genes have already played a role before sex differentiation (Shen and Wang, unpublished

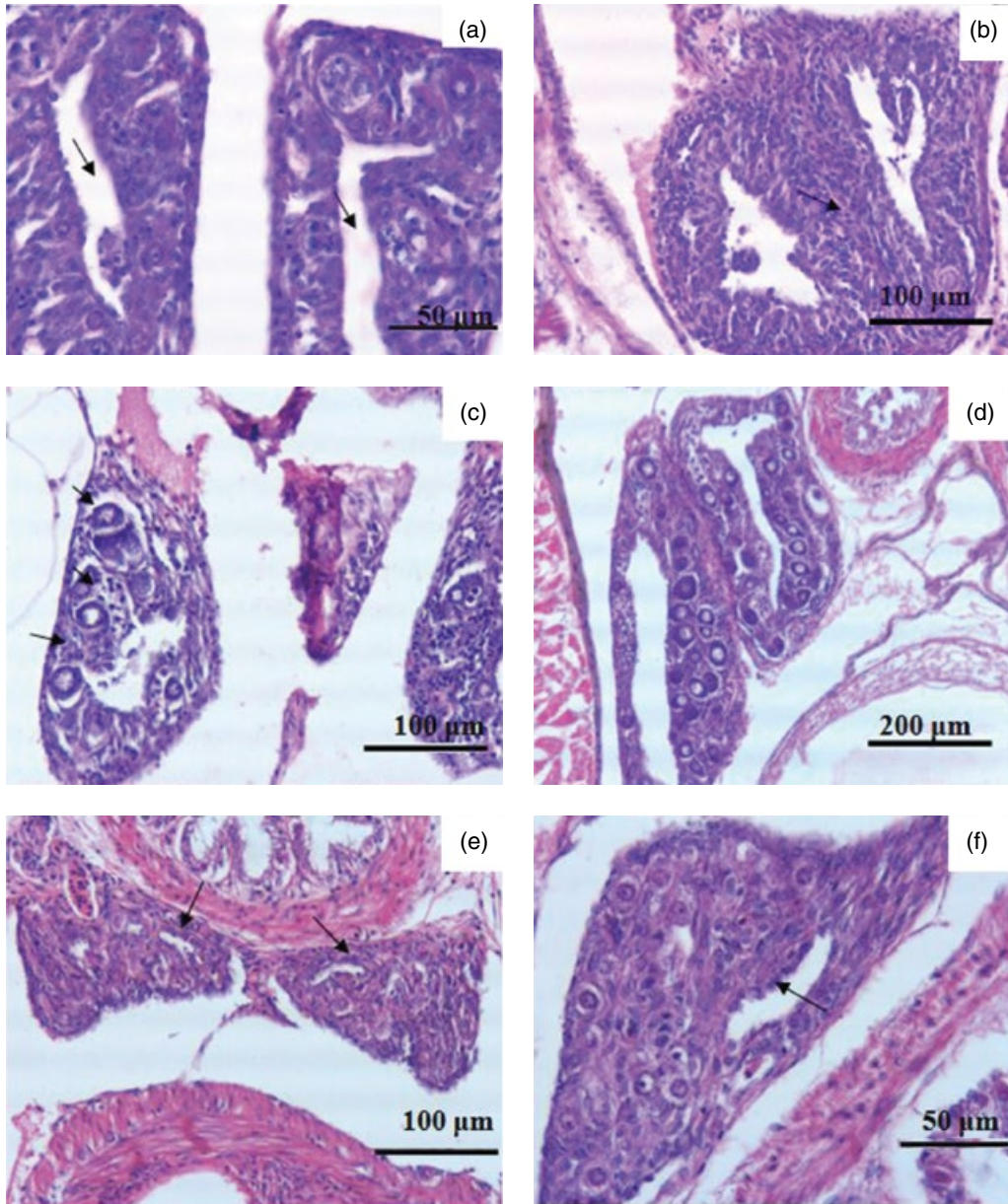


Figure 17.4 Gonadal development of the bluegill sunfish in the fast-growing batch. (See inserts for the color representation of this figure.)

- a) Ovary at 50 dph, showing the ovarian cavity.
- b) Ovary at 50 dph, showing the beginning of fusion. (c) Ovary at 60 dph, showing the peri-nucleolus oocytes.
- c) Ovary at 80 dph, showing the numerous peri-nucleolus oocytes.
- d) Testis at 70 dph, showing the efferent duct anlage.
- e) Testis at 80 dph, showing the evident efferent duct.

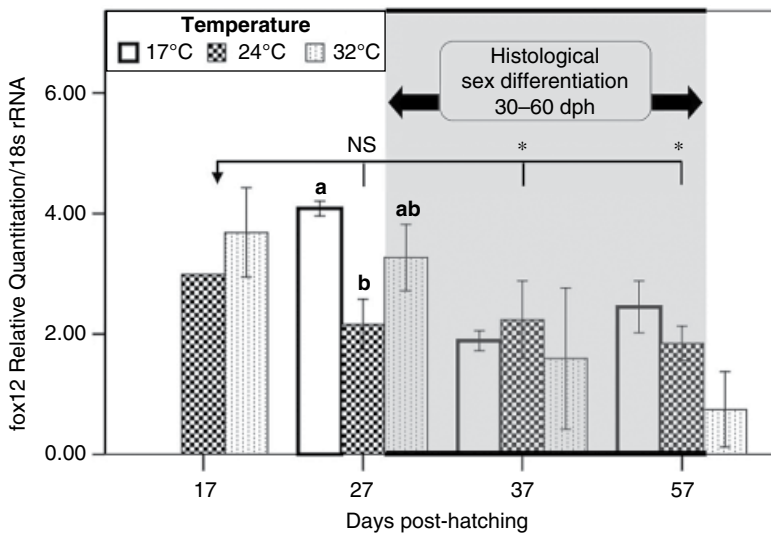
data). Temporal expression of *foxl2* prior to and during the critical period of histological sex differentiation (30–60 dph, according to [27]) in bluegill was studied. Here, we highlight the importance of the *foxl2* gene in ovarian differentiation.

The expression of *foxl2* reached its peak and was thermo-sensitive at 27 dph (highest in the low temperature group and lowest in moderate temperature group), which is just prior to the onset of ovarian differentiation. The qPCR standard curves

Table 17.2 Summary of morphological events of gonadal development and sex differentiation in the fast-growing batch of bluegill sunfish.

U – undifferentiated; F – female; M – male. dph – days post hatching [27].

Age (dph)	No. of fish	TL (mm)	Gonadal stage	Sex		
				U	F	M
30	5	14.2–19.6	Elongated aggregations of somatic cells in the ovary	4	1	0
50	5	21.0–25.5	Complete ovarian cavity in the ovary	3	2	0
60	5	22.0–27.5	Peri-nucleolus oocytes in the ovary and increases in the number of germ cells and stromal cells in the presumptive testis	0	3	2
70	5	23.5–29.5	Efferent duct anlage in the testis	0	3	2
80	6	27.5–3.40	Numerous peri-nucleolus oocytes and fusion of anterior part of gonadal tissues in the ovary, evident efferent duct and meiotic activity in the testis	0	3	3
90	7	32.0–40.0	Ovary and testis became bigger	0	4	3

**Figure 17.5** Temporal expression of *foxl2* and temperature effects in the early life stage of bluegill, normalized against 18S rRNA measured by real-time RT-PCR. Different letters in the 27 dph sampling point indicate significant difference among temperature treatments. Asterisks (*) denote significant difference between the 17 dph and 37 dph groups or the 17 dph and 57 dph groups. NS – no significance between 17 dph and 27 dph group when pooling different temperature treatments together. Gray shadow area indicates that histological sex differentiation occurs between 30–60 dph, according to our previous study [27].

exhibited a significant linear relationship between the values of threshold cycle (CT) and the gene copy number in both *foxl2* gene and *18S rRNA* gene. PCR efficiencies were both high (104.05% and 106.33%). Expression of *foxl2* increased dramatically from 7–17 dph, stabilized from 17–27 dph, then decreased significantly from 37 dph, when

we pooled all temperature treatment groups together (Fig. 17.5).

Remarkable effects of temperature on *foxl2* expression were observed at 27 dph, where *foxl2* expression was the highest in 17°C treatment and lowest in 24°C treatment. No dph-temperature interaction on *foxl2* expression was observed. Comparative analysis of the

Box 17.1 Sex differentiation in bluegill

- Bluegill is a differentiated gonochorist.
- Sex differentiation occurs earlier in females than in males.
- The gonadal differentiation in bluegill is more related to body size than to age.
- The critical period of sex differentiation in bluegill occurs between 13.2 and 16.0 mm TL.
- Histological sex differentiation is distinguishable in most fish larger than 21.0 mm TL.
- The *foxl2*/testis differentiation gene(s) ratio may trigger the direction of the gonads.

expression profile of *foxl2* in different species indicates that the expression level of *foxl2* or the *foxl2*/testis differentiation gene(s) ratio may be what triggers the direction of the gonads into the female or male pathway (key information on sex differentiation in bluegill is summarized in Box 17.1).

17.3 Sex Determination

The sex-determining mechanism in bluegill has been investigated for several decades intermittently since the 1960s. One consensus has been proposed: complicity. With added interest owing to the commercial value of the species, bluegill is considered as a good model organism to explore the processes of sex determination. Investigation efforts associated with sex determination of bluegill include: heteromorphic sex chromosomes; sex ratio investigation in natural population; natural and hormonal sex differentiation; hybridization test; gynogenesis; androgenesis; progeny test of sex-reversed brooder fish; sex-specific molecular marker; temperature-dependent sex determination, and so on.

Sex chromosomes could not be distinguished from autosomes cytologically [24, 28]. Very interestingly, highly skewed (male-dominant and female-dominant) sex ratios were consistently reported both in natural [18, 21] and experimental [26] bluegill populations. The vast majority of interspecific crosses among centrarchids in natural waters,

as well as in hatchery conditions, yielded strongly male-biased sex ratios, ranging from 48–100% [2, 29–36]. In one experiment, the majority of bluegill progenies (36 of 47) had a predominantly male sex ratio, and seven families of normal progeny contained 1–5% intersexes [11]. Furthermore, sex ratios of progeny from the mating of estrogen-treated females (true females and feminized males) and normal males were all or predominantly male or female [11]. However, researchers in that study could not be sure that these results were due to the genetic impurity of brood stock. Gynogenesis and androgenesis studies did not produce any viable offspring and, thus, have not yet offered useful information.

17.3.1 Genotypic Sex Determination (GST)

17.3.1.1 Search for Sex-Specific Markers

To provide robust molecular tools for sex-determination analyses and selective breeding programs, the AFLP technique was adopted to identify sex-specific markers using sex-type DNA pool strategy, and to construct the linkage maps using pseudo-testcross strategy in bluegill [37]. The linkage maps were constructed for the females and the males, respectively. The female linkage map consisted of 199 markers, including four co-dominant markers and one sex-specific marker (Table 17.3) [37]. A total of 183 markers were assigned to 31 linkage groups (more than three markers), which covered 1,628.2 cM in length with an average interval of 10.71 cM. The length of the linkage groups ranged from 10.5 to 122.9 cM, and the number of markers per group varied from 3 to 20 (Table 17.3) [37]. The remaining 16 markers were grouped as eight doublets. The female-specific marker Im72-519 showed 1 : 1 segregation ratio, and was located on linkage group LG 8 with a suffix “s” behind the band size, suggesting that this linkage group may be sex-related.

For the paternal map, 177 specific markers, including four co-dominant markers, were distributed onto 33 linkage groups, which covered 1,525.3 cM in length, with an average

Table 17.3 Summary of segregation markers and linkage groups of bluegill.

	Maternal	Paternal
Total number of markers scored	222	216
Distorted segregation loci	6	4
Marker number in linkage analysis	216	212
Number of markers mapped (including doublets)	192	191
Unspecific marker number	24	21
Linkage groups	31	33
Number of doublets	8	7
Average number of markers per linkage group	5.9	5.4
Minimum length of linkage group (cM)	10.5	2.3
Maximum length of linkage group (cM)	122.9	345.3
Minimum markers No. per group	3	3
Maximum markers No. per group	14	19
Average marker interval	10.71	10.59
Observed genome length (<i>Goa</i>) (cM)	1,628.2	1,525.3
Estimated genome length 1 (<i>Ge1</i>) (cM)	2,393.6	2,193.2
Estimated genome length 2 (<i>Ge2</i>) (cM)	2,292.3	2,208.2
Estimated genome length (<i>Ge</i>) (cM)	2,343.0	2,201.1
Genome coverage	69.5%	69.3%

interval of 10.59cM (Table 17.4) [37]. The maximum length and maximum marker number of male linkage groups were 345.3 cM and 19, respectively. The AFLP markers were not evenly distributed in the linkage maps. Six big marker clusters were found on five linkage groups (LG5, LG6, LG8, LG10, and LG11) of the female map [37], but the number of marker clusters on the male map was less than female. Only three were observed on three linkage groups (LG11, LG31, and LG32). Additionally, highly negative correlation between the size of the linkage group and the number of AFLP markers on the linkage groups also evidenced the uneven distribution of markers.

A total of 4,160 loci were produced, using 64 AFLP primer combinations. Only seven loci (0.17%) were observed in one sex-specific DNA pool. Among these seven loci, one and six were putatively identified as male- and female-specific markers, respectively. The subsequent amplification, using 48 unrelated individual fish (24 females and 24 males), confirmed these sex-specific markers, which were only observed in a certain putative sex. The results revealed that not all the individuals possessed all these sex-specific markers.

The numbers of sex-specific markers were only detected in 4–10 females and five males, accounting for 16.67–41.67% and 20.83%, respectively (Table 17.4). Seven out of 4,160 loci (0.17%) were identified and confirmed as sex-specific markers. The ratio is slightly low, or comparative to the studies in some other aquaculture species [38–40]. The polymorphism in the recognition sites of the restriction enzymes probably may

Table 17.4 Summary of female and male-specific markers and their percentage in 24 female and 24 male individuals, respectively.

	Female						Male
Sex-specific markers	lm56-1193	lm56-497	lm67-293	lm72-519	lm75-791	lm76-265	lm23-399
Number (percent)	7 (29.17%)	7 (29.17%)	4 (16.67%)	6 (25.00%)	10 (41.67%)	4 (16.67%)	5 (20.83%)

result in the non-appearance of these markers in some individuals. Additionally, we tried to convert these sex-specific markers into single locus markers (SCARs), but failed.

Some other studies also showed the conversion ratios were very low. For example, only one out of 15 sex-specific AFLP polymorphic markers in rainbow trout was successfully converted into an effective SCAR [39]. A lower ratio was also observed in the other salmonid species, where only one useful SCAR out of 52 AFLP markers was developed [41].

Several factors may contribute to successful conversion of AFLP markers into SCARs, such as the fragment size of AFLP markers and the levels of homogeneity between two sexes [39, 40, 42]. However, when six AFLP markers were used together for a single female identification, the success ratio reached more than 95%, which is high enough for practical use. The markers did not appear in only one individual, suggesting that the polymorphisms of the recognition sites of the restriction enzymes may be responsible for this observation. This result also indicated that high genetic diversity was presented in bluegill populations.

In the linkage analysis, only one female sex-specific marker, Im72-519, was located

on the maternal linkage group LG8. Because the progeny used in linkage analysis were too young to determine their sex, we could neither acquire more information about whether this marker inherited to her daughters or sons, nor use the sex as one morphological marker for linkage analysis. However, this result led to a weak but interesting and useful argument that the LG8 may be one sex-related linkage group.

In another study [25], we also used a different approach, with the AFLP technique, to identify sex-specific markers in bluegill sunfish, based on pooled DNA samples from known male and female individuals. A total of 12,835 loci were produced, using 256 AFLP primer combinations, including 531 (4.14%) polymorphic loci among different pools. Among the 256 primer combinations, only nine (3.52%) primer combinations yielded sex-associated amplifications across the pooled DNA samples (Table 17.5). Four AFLP loci (0.03%) were initially considered as possibly being female-specific, because they were only amplified in two female DNA pools, and another five AFLP loci (0.04%) were only amplified in two male DNA pools (Table 17.5).

However, when these loci were re-analyzed in all samples, including all individual samples composed of DNA pools, the sex-specific

Table 17.5 Summary of candidate sex-specific amplicons based on bulked samples. Loci were named with the abbreviation of the bluegill sunfish scientific name and the size of the band.

Primer combinations yielded sex-specific bands (Loci name)	Sex of DNA pools with sex-specific bands	Percent of individuals with sex-specific bands
E4/M10 (Lma429)	Female	45%
E12/M8 (Lma695)	Female	30%
E14/M6 (Lma341)	Female	25%
E15/M9 (Lma756)	Female	25%
E5/M14 (Lma870)	Male	45%
E8/M16 (Lma342)	Male	20%
E9/M1 (Lma1092)	Male	25%
E9/M3 (Lma566)	Male	25%
E14/M2 (Lma237)	Male	25%

markers were only observed in a limited number of individuals of putative sex (Table 17.5). These results revealed that, for each putative sex-specific marker, the putative sex-specific bands in the pooled DNA samples were virtually caused by the individual polymorphism.

Many studies have demonstrated that the AFLP technique, in combination with a sex-typed pool strategy, is a robust approach for identification of sex-specific markers in teleost fish [38, 39, 43, 44]. As the identity of the sex chromosomes is very labile, sex-linked genes could differ according to the species, races, or even populations. The success of the identification of sex-specific markers depends mainly on the presence of a sex chromosome, such as in African catfish *Clarias gariepinus* [45], chinook, chum and coho salmon [41], rainbow trout *Oncorhynchus mykiss* [39], or non-chromosomal genetic sex-determining mechanisms in the target species, as in the three-spined stickleback *Gasterosteus aculeatus* [38].

In contrast, failures to identify sex-specific markers have been reported in species without detectable sex chromosomes or genetic sex-determining systems, such as green spotted puffer fish *Tetraodon nigroviridis* [46], sturgeon [47], and striped catfish *Pangasianodon hypophthalmus* [48]. Despite the failure to find such markers in bluegill, our data offer useful information and brief communication for further studies targeting similar goals.

17.3.1.2 Sex-Determining System in Bluegill Sunfish

To determine the genetic mode of sex-determination in bluegill sunfish, we first sex-reversed a regular population to all-females, and then used matured sex-reversed females to cross regular males. Sex determination mode (XY, ZW or polygenic) was determined on the basis of progeny sex ratios. For example, in the case of heterogametic male mode (XY), crosses between feminized XY males and regular XY males should yield approximately 75% males. In female heterogametic systems (ZW), crosses between sex-reversed ZW males and ZW females should

Table 17.6 Progeny sex ratio from estrogen-treated females crossed with normal males.

Female ID No.	Number of progeny sexed	Progeny male %
Control	54	46.0
069 303	30	100.0
266 052	54	100.0
590 560	54	100.0
626 630	54	100.0
823 785	54	100.0
575 069	54	97.8
634 041	54	96.5
051 887	18	94.0
634 013	54	80.0
634 544	54	79.5
884 856	54	78.0
074 802	54	76.0
881 095	54	73.0
623 867	54	71.0
626 517	54	60.5

result in approximately 75% females, and crosses between sex-reversed ZZ females and ZZ males should produce all-male progeny. In polygenic systems, crosses between sex-reversed females and regular females should theoretically produce a female-biased sex ratio.

Therefore, if feminized fish produce broods of either all males or mixtures of male and female, female heterogamety is indicated. As results show in Table 17.6, predominant male (100% or close to 100%) or balanced sex ratio (close to 1 : 1) was produced from estrogen-treated females crossed to normal males in our previous work, indicating female heterogamous (ZW/ZZ) bluegill.

17.3.2 Temperature Effects on Sex Determination

We tested effects of genotype by temperatures on sex determination, and also sexual size dimorphism and growth, on two batches

of fry from different geographic populations [22]. In the first batch, sex ratios significantly deviated from 1 : 1 in 29°C and 34°C groups, in which a significantly higher proportion of males (70.64% and 66.67%) were yielded ($P<0.05$). The percents of males in 29°C and 34°C groups were significantly higher than in the 17°C and 23°C groups ($P<0.05$). In the second batch, sex ratios were not significantly different from 1:1 in all groups ($P>0.05$).

The pooled sex ratios were compared and it was found that temperature had significant effects on sex ratios in the first batch of fish ($P<0.001$), while there were no significant effects on the second batch of fish ($P>0.05$). Through histological examination, intersex fish were identified in the 17°C and 34°C groups. Rearing temperature strongly affected growth of bluegill. Fish reared at the temperature of 29°C performed best, followed by fish at 34°C, 23°C, and 17°C. No significant differences ($P>0.05$) were detected in growth of juvenile bluegill (<8.15 cm) between two sexes for any thermal treatments.

Furthermore, we studied the effect of temperature on sex ratio of more geographic strains, based on our above results [49]. In this study, effects of genotype-temperature interactions on sex determination in bluegill were further investigated using four geographic strains (i.e., Hebron, Jones, Hocking, and Missouri). In the Hebron strain, higher temperature treatment groups (24°C and

32°C) produced more males, compared with the low temperature treatment group (17°C) from 6 to 90 dph. In contrast, low temperature treatment produced more males than that of the other two higher temperature treatments in the Jones strain. No significant effects of temperature on sex ratio were detected in the other two strains. Our results from sex ratio variance in different treatment times suggested the thermosensitive period of sex differentiation exists before 40 dph.

Based on the two studies, it was concluded that genotype-temperature interactions exist in bluegill sex determination, and their coexistence suggests the interesting possibility of selecting thermosensitive genotypes in breeding programs for mostly male populations (key information on sex determination in bluegill is summarized in Box 17.2).

17.4 Sex Reversal

17.4.1 Effects of Steroids and Nonsteroidal Aromatase Inhibitor on Sex Reversal and Gonadal Structure of Bluegill

We systematically investigated the feminization of bluegill by oral administration of various doses of estradiol-17β (E₂), and evaluated their effects on the growth performance, production, and gonadal structure of sex-reversed female bluegill, at both sex-ratio and histological levels [51]. With positive

Box 17.2 Summary of sex determination in bluegill	
<p>The sex determination system in bluegill is very complicated. Sex chromosomes could not be distinguished from autosomes cytologically [24, 28]. Sex ratios of hybrids between bluegill and the other <i>Lepomis</i> species exhibit strong unidirectional male-biased sex ratio from 64–100% [2, 29–36]. Highly skewed (male-dominant and female-dominant) sex ratios have been consistently reported both in natural [18, 21] and experimental [26] bluegill populations.</p> <p>A predominant male (100% or close to 100%) or balanced sex ratio (close to 1 : 1)</p>	<p>was produced from estrogen-treated females crossed to normal males in our previous work, indicating female heterogamous (ZW/ZZ) bluegill, at least in some populations. In addition, temperature effects on sex ratio have been found in some geographic populations [50]. Based on these results, it is concluded that bluegill display genetic sex determination with a ZW/ZZ system, plus temperature effects (e.g., both genetic sex determination and temperature effects coexist in bluegill).</p>

control treatment, 30-day-old fry were fed E_2 at 50, 100, 150 and 200 mg kg⁻¹ diet for 60 days. The survival of fish in the E_2 treated and control groups was not significantly different ($P > 0.05$). The growth of the treated fish was significantly retarded during the period of treatment, while there was no side-effect detected post-treatment, and the retarded fish caught up during 120 days of culture after E_2 treatment.

All the treated groups produced 100% monosex female populations, based on the macroscopic shape of gonads, and there were no significant differences detected between any E_2 treatment and the control group in the mean GSI of females during the spawning season from June to October ($P > 0.05$). Histologically, 13.3% and 5.0% of the intersex fish were determined to come from the 50 and 100 mg kg⁻¹ E_2 treatment groups, respectively, with 6.9% and 4.1% of the gonadal area containing spermatocytes. Most of the genotypical male fish treated with exogenous E_2 developed gonadal structures histologically indistinguishable from the gonads of females. This study suggests that 150 mg kg⁻¹ E_2 is the optimal dosage for feminization in bluegill, with 50 and 100 mg kg⁻¹ E_2 being sub-optimal, and 200 mg kg⁻¹ E_2 being over-optimal.

17.4.2 Effects of Nonsteroidal Aromatase Inhibitor on Gonadal Differentiation of Bluegill

We examined the efficacy of Letrozole, a potent nonsteroidal aromatase inhibitor (AI), on gonadal sex differentiation and sex reversal in bluegill sunfish [52]. Two experiments were conducted for this purpose.

In Experiment 1, fry used in all the treatments (50, 150, 250, and 500 mg kg⁻¹ AI) and controls were from the same batch. Fry in all groups received their ration of AI feed five times a day, and mortality was monitored daily in each experimental group from 30 dph to 90 dph. After completion of AI administration, the fish were fed four times daily with a normal commercial diet. All fish were sacrificed at 210 dph for gonad samples,

and were measured to examine the growth of fish after AI diet treatments.

In Experiment 2, the same batch of fry for Experiment 1 was used, and immersions were given to the fry in their rearing tanks. The AI was dissolved in 95% ethanol to make appropriate stock solutions. Before the addition of the appropriate amount of AI, water flow to the tanks was turned off, and the water level was lowered to 10 L. The various AI stock solutions were poured into tanks to make concentrations of 250, 500, 1,000 µg L⁻¹. The same amount of ethanol was added to the control group. From 30 dph to 50 dph, fry were immersed in each AI solution on five occasions for eight hours a day, with five-day intervals between immersion treatments. At the end of each immersion period, water flow was turned back on. The fish were fed five times daily with a normal commercial diet. After completion of AI immersion, the fish were fed four times daily with a normal commercial diet, until sacrificed for gonad samples at 210 dph.

For experiment 1, the proportion of males in all AI diet treatment groups increased significantly when compared to the control group at 210 dph (Table 17.7). The proportion of males increased as AI diet dosages increased, and the 500 mg kg⁻¹ AI diet treatment had the highest proportion (70%) of males. All the ovaries from the AI diet treated groups were histologically similar to those of the control group. All of the testes in AI diet-treated groups were similar to those in the control group. The survival rates in all experimental groups were between 40–45%, and no significant differences in survival ($P > 0.05$) were detected among groups. No signs of toxicity or behavioral differences between treatment groups and control fish were observed during and after the treatment. There were no significant differences among the AI-treated and control groups at the terminal day of AI diet treatment ($P > 0.05$). After AI treatment, the body weight and total length still did not exhibit significant differences among the AI treated and control groups ($P > 0.05$) at 210 dph.

Table 17.7 Sex ratios of the bluegill sunfish fed diets containing different dosages of AI, from 30 to 90 dph. The different superscript letters indicate significant (chi-square test, $P < 0.05$) differences in proportion of males among the groups. P -values indicate differences from the theoretical 50 : 50 sex ratio (chi-square test).

AI dosage (mg kg ⁻¹)	N	Males (%)	Females (%)	P -value
Control	31	39 ^a	61	0.028
50	29	59 ^b	41	0.072
150	20	65 ^b	35	0.003
250	20	65 ^b	35	0.003
500	26	70 ^b	30	0.002

For Experiment 2, after AI immersion treatments at 50 dph, there were some gonads with initial ovarian cavity formation, indicated by the presence of two elongated aggregations of somatic cells in the proximal and distal portions of the gonads [52]. The sex ratios at 210 dph in each experimental group are shown in Table 17.8. Although the male proportion was 41% and 44% in the control and 250 $\mu\text{g L}^{-1}$ AI immersion groups, respectively, the gonads from the 500 and 1,000 $\mu\text{g L}^{-1}$ AI immersion groups showed a significant bias toward male gonads. All the ovaries and testes from the AI immersion groups were histologically indistinguishable from those of the control group.

The survival rates in all experimental groups were between 40–50%, and a relationship between administration duration and survival was not observed. At the terminal day of AI immersion treatment (50 dph), there were no significant differences among the AI-treated and control groups ($P > 0.05$). After AI treatment, the body weight and total length still did not exhibit significant differences among the AI treated and control groups at 210 dph ($P > 0.05$).

17.4.3 Summary of Bluegill Sunfish Sex-Reversal

The published reports about the bluegill sunfish sex-reversal are summarized in Table 17.9

Table 17.8 Sex ratios of the bluegill sunfish immersed into different concentrations of AI solutions for eight hours/day on 30, 35, 40, 45, 50 dph. The different superscript letters indicate significant (chi-square test, $P < 0.05$) differences in proportion of males among the groups. P -values indicate differences from the theoretical 50 : 50 sex ratio (chi-square test).

AI dosage ($\mu\text{g L}^{-1}$)	N	Males (%)	Females (%)	P -value
Control	22	41 ^a	59	0.072
250	27	44 ^a	56	0.230
500	24	67 ^b	33	0.001
1,000	24	75 ^b	25	0.001

[52]. These studies indicated that all the initial attempts to feminize by oral administration of estradiol-17 β were quite successful. In contrast, most attempts to masculinize by oral administration or immersion of androgens were unsuccessful. The criteria established by Yamamoto [53] for completing sex reversal required the administration of the hormone during the period from the undifferentiated gonad through sexual differentiation, and that the hormone be administered at an effective dose. Therefore, the most effective treatment period must be before sex differentiation.

As testicular differentiation happened later than ovarian differentiation in the bluegill sunfish, all the treatments by oral administration of E₂ for feminization conducted before testicular differentiation could successfully induce genetic male bluegill to phenotypic females [26, 51, 54]. However, androgen oral administration commonly resulted in a high percentage of intersex fish in previous bluegill sex-reversal studies (Table 17.9).

Based on our experiment for the bluegill sunfish sex differentiation, the high frequency of intersex fish might be due to the later treatment timing (18 ± 0.26 mm TL), when some fish had begun the ovarian differentiation [27]. Piferrer [55] defined the labile period as the period of time when the still sexually undifferentiated gonads are more responsive to the action of exogenous

Table 17.9 Summary of studies on sex reversal of bluegill sunfish.

Hormone	C	Initial TL/mm (age)	Final TL/mm (age)	Females (%)	Males (%)	Inter-sex (%)	Sterile (%)	Refs
Feminize								
E ₂ (Di)	100 mg/kg	15 ± 0.60 (30 d)	(60 d)	100	0			[26]
E ₂ (Di)	200 mg/kg	13.8 ± 0.60 (27 d)	(72 d)	99.3 ± 1.2	0	0.7		[54]
E ₂ (Di)	50 mg/kg	13.9 ± 1.3 (30 d)	(90 d)	80.0	0	20		[51]
E ₂ (Di)	100 mg/kg	13.9 ± 1.3 (30 d)	1.61 ± 0.14 (90 d)	93.4	0	6.6		[51]
E ₂ (Di)	150 mg/kg	13.9 ± 1.3 (30 d)	1.63 ± 0.20 (90 d)	100	0	0		[51]
E ₂ (Di)	200 mg/kg	13.9 ± 1.3 (30 d)	1.52 ± 0.15 (90 d)	100	0	0		[51]
E ₂ (Im)	1 mg/L	13.8 ± 0.60 (27 d)	(37 d) ^A	76.9 ± 3.5	20.4	2.7		[54]
DES (Im)	1 mg/L	16 ± 0.43 (34 d)	(37 d) ^B	43 ± 1.7	57.0			[26]
	1 mg/L	16 ± 0.43 (34 d)	(40 d) ^C	59 ± 6.7	41.0			[26]
Masculinize								
MT (Di)	10 mg/kg	(0 d)	(60 d)	40*	60.0*			[56]
	30 mg/kg	(0 d)	(60 d)	100*	0*			[56]
	50 mg/kg	(0 d)	(60 d)	75*	25.0*			[56]
	60 mg/kg	11.54 ± 0.31 (28 d)	23.5 ± 0.62 (58 d)	0	5.0	95		[26]
	15 mg/kg	14 ± 0.73 (28 d)	21.0 ± 2.1 (58 d)	25.7 ± 4.5	17.7	56.7		[57]
	30 mg/kg	14 ± 0.73 (28 d)	21.0 ± 1.9 (58 d)	24.7 ± 4.7	12.7	62.7		[57]
	60 mg/kg	14 ± 0.73 (28 d)	20.0 ± 2.3 (58 d)	20.3 ± 5.7	11.7	68.0		[57]
	60 mg/kg	14 ± 0.73 (28 d)	22.0 ± 3.2 (73 d)	10.7 ± 5.7	0	69.7	19.7	[57]
	60 mg/kg	14 ± 0.73 (28 d)	21.0 ± 2.7 (88 d)	8.7 ± 1.1	0	46.0	45.3	[57]

(Continued)

Table 17.9 (Continued)

Hormone	C	Initial TL/mm (age)	Final TL/mm (age)	Females (%)	Males (%)	Inter-sex (%)	Sterile (%)	Refs
TBA (Di)	50 mg/kg	11.54 ± 0.31 (28 d)	23.5 ± 0.60 (58 d)	0.3 ± 0.6	4.0	95.7		[26]
	12.5 mg/kg	15 ± 0.42 (28 d)	20.0 ± 2.8 (58 d)	17.0 ± 5.0	43.3	39.7		[57]
	25 mg/kg	15 ± 0.42 (28 d)	19.0 ± 2.8 (58 d)	17.3 ± 5.5	42.7	40.0		[57]
	50 mg/kg	15 ± 0.42 (28 d)	19.0 ± 2.9 (58 d)	23.7 ± 1.5	26.7	49.7		[57]
	50 mg/kg	18 ± 0.26 (28 d)	31.0 ± 5.2 (58 d)	24.3 ± 5.5	27.7	48.0		[57]
	75 mg/kg	18 ± 0.26 (28 d)	33.0 ± 3.8 (58 d)	20.7 ± 11.8	6.3	68.7	4.3	[57]
	100 mg/kg	18 ± 0.26 (28 d)	31.0 ± 4.4 (58 d)	19.7 ± 4.5	3.0	67.7	9.3	[57]
	50 mg/kg	18 ± 0.26 (28 d)	31.0 ± 4.3 (88 d)	10.7 ± 0.6	0	57.7	32.3	[57]
TBA (Im)	250 µg/L	16 ± 0.43 (34 d)	(37 d) ^B	15 ± 1.2	85.0			[26]
	500 µg/L	16 ± 0.43 (34 d)	(37 d) ^B	10 ± 0.6	90.0			[26]
	500 µg/L	16 ± 0.43 (34 d)	(40 d) ^C	6 ± 0.6	94.0			[26]
	750 µg/L	16 ± 0.43 (34 d)	(37 d) ^B	11 ± 2.1	89.0			[26]
	1,000 µg/L	16 ± 0.43 (34 d)	(37 d) ^B	9 ± 2.5	91.0			[26]
	1,000 µg/L	16 ± 0.43 (34 d)	(40 d) ^C	7 ± 1.0	93.0			[26]

Superscript * means the number of survivors in the experiment was insufficient for adequate statistical testing. Superscript A means fry were immersed in a 1 mg/L E₂ solution on three occasions for five hours a day with five-day intervals. Superscript B means fry were immersed in the hormone solution for five hours a day on either days 34 and 37 (two-day exposure). Superscript C means fry were immersed in the hormone solution for five hours a day on either days 34, 37, and 40 (three-day exposure).

Abbreviations: Di – diet; Im – immersion; E₂ – estradiol-17β; MT – 17α-methyltestosterone; TBA – trenbolone acetate; DES – diethylstilbestrol.

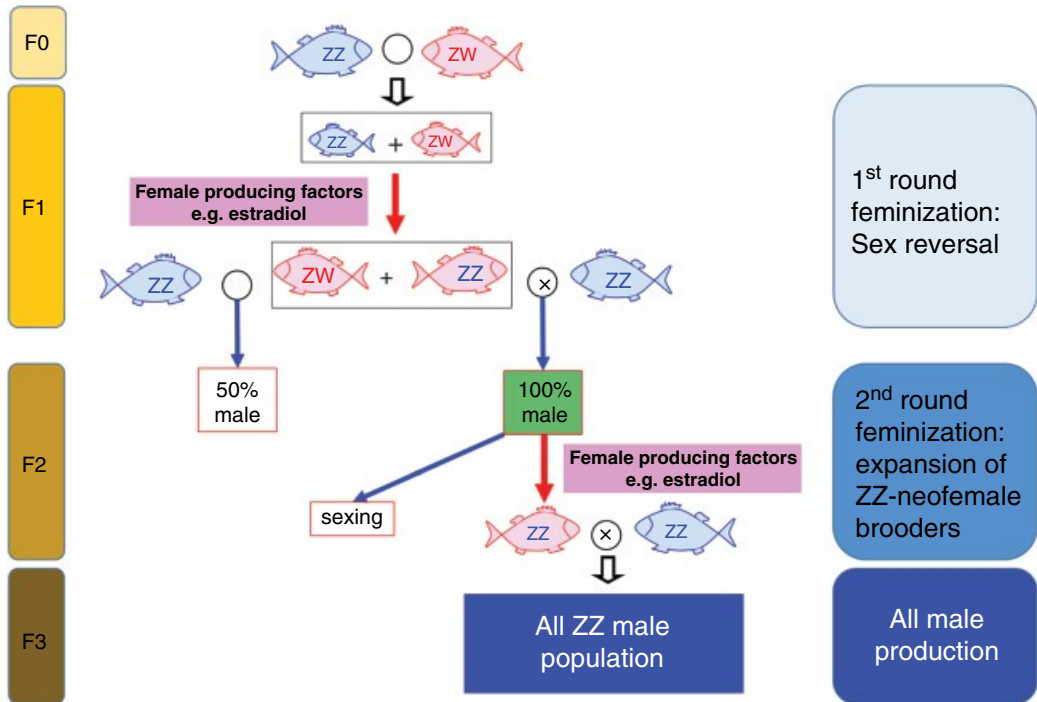


Figure 17.6 Scheme of breeding experiments for mass production of GMB broodstock in bluegill with a ZW system.

F0, F1, F2, F3 indicate each generation respectively.

steroids. Based on our findings on sex differentiation, and other previous successful or unsuccessful studies on sex reversal of the bluegill sunfish, we suggest the labile period for the bluegill sunfish sex differentiation is between 13.2 and 16.0 mm TL.

17.5 Large-Scale Production of All-Males or Mostly-Males

17.5.1 Develop GMB-Producing Brood Stock for Large-Scale All-Male Production

With the female heterogametic system (ZW), crosses involving sex-reversed and regular fish were used to develop monosex populations by our group (Figure 17.6). By feminizing some progeny from ZZ × ZZ crosses, we have also produced all ZZ-neofemale GMB (genetically male bluegill)-producing

brooders that are to generate large-scale all-male or mostly male production of bluegill for commercial monoculture (Figure 17.6).

17.5.2 Growth Performance of Genetically Male Bluegill

We tested the growth performance of genetically male bluegill with unselected bluegill stocks at the Lincoln University (LU) aquaculture facility and the Ohio State University (OSU) aquaculture facility at Piketon. At LU, three stocks (two male groups and one control) were cultured communally in multiple tanks (common gardens). Each common garden contained similar numbers of fish of all stocks at the time of stocking. The common gardens were started in April 2016, and terminated in February 2017. At the end of the above experiments, the weights of two male stocks were significantly higher

than the control group. Percentage weight increase of the two male stocks was 3–5 times ($P < 0.0001$) greater than observed for the reference Northern bluegill and Coppernose bluegill stocks, where the two male stocks increased in weight by 571% and 900%, respectively.

At the OSU Piketon aquaculture facility, two male groups and one control were cultured separately in four tanks (two replicates each). The experimental results showed:

- 1) size was much more uniform for the all-male group, and coefficient of variation (CV) for body weight ($100 \times \text{standard deviation} / \text{mean}$) was significantly lower in the male groups, compared with control groups (53.1 vs. 76.0, and 51.8 vs. 76.9 at the beginning and end of experiment, respectively);
- 2) survival of all-male groups was significantly higher than that of mixed sex groups (25.0% vs. 3.4% on average); CV for body weight is the most important determinant for survival, because we found a number of large-sized fish chased and bit small-sized fish, resulting in mass mortality;
- 3) all-males grew 16.7% faster than the mixed population in body weight during that juvenile phase, as of the writing of this chapter.

17.5.3 Establishment of Mostly-Male Groups of Bluegill by Grading Selection

We also developed a practical procedure to establish mostly-male bluegill groups through grading selection, and tested their growth against a normal population [9]. A single cohort of bluegill juveniles was cultured in a pond for a year, and then graded and divided into two mostly male groups (top 25% and top 50% of fish) and a mixed-sex control group when the fish reached a mean weight of 30.1 g. The mixed control group contained 50.0% males; the top 25% group had 75.4% males; and the top 50% group had 69.7% males. Weight gain per fish in the top 25% group was significantly greater ($P < 0.05$)

Box 17.3 Production of all-male or mostly-male bluegill sunfish

- The optimal dosage for feminization is $150 \text{ mg kg}^{-1} \text{ E}_2$ in bluegill.
- Treatment window is between 13.2 and 16.0 mm TL.
- ZZ-neofemale GMB (genetically male bluegill)-producing brooders can be produced by feminizing some progeny from $\text{ZZ} \times \text{ZZ}$ crosses.
- Mostly male bluegill populations can be established through grading selection.

than the mixed group throughout most of the experiment.

There were no significant differences detected in survival among the three groups, although the top 25% group had survival of 96.0%, compared with 90.6% and 90.5% for the top 50% group and the mixed control, respectively. The top 25% group had the highest percentage (46.3%) of fish reaching 150 g at the end of the experiment, followed by the top 50% group (28.3%), and the mixed control group (12.7%). The coefficient of variation for weight decreased in all three groups over time, with the mostly male groups maintaining lower initial and final CV values than the control group. Results indicate that mostly male bluegill groups are able to grow faster than typical mixed-sex populations, and that social interaction costs among communally reared males did not significantly decrease growth of mostly male populations in the aquaculture settings (key information on production of all-male or mostly male bluegill is summarized in Box 17.3).

17.6 Conclusions and Future Perspectives

Sex determination and sex control in bluegill have been investigated for several decades intermittently, since the 1960s. One consensus is that the sex determination

mechanism in bluegill is very complex, and it should be considered as a good model organism to explore the processes of sex determination. A predominant male (100% or close to 100%) or balanced sex ratio (close to 1 : 1) produced from estrogen-treated females crossed to normal males in our previous work suggests female heterogametes (ZW/ZZ) of bluegill. Temperature effects on sex ratio have been found in some geographic populations [50].

Based on these results, it is concluded that both genetic sex determination with the ZW/ZZ system and temperature effects coexist and contribute to the determination of sex in bluegill, at least in some populations. The near future work for sex determination and sex control in bluegill should focus on:

- 1) development of fine genetic maps to further search for the sex-determining locus

and to confirm sex-determining mechanisms, using a gynogenesis approach;

- 2) identifying sex-linked markers, making use of next-generation sequencing technologies;
- 3) a wider range of field investigations to address ecological significance and adaptation of temperature effects of sex differentiation in bluegill.

Acknowledgments

This work was supported by the National Institute of Food and Agriculture (NIFA), North Central Region Aquaculture Center (NCRAC), U.S. Department of Agriculture. Salaries and research support were provided by state and federal funds appropriated to The Ohio State University, Ohio Agricultural Research and Development Center.

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18

Sex-Determining Mechanisms and Control of Sex Differentiation in Largemouth Bass and Crappies

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18.1 Significance of Largemouth Bass and Crappies for Recreational Fishery and Aquaculture

Largemouth bass (*Micropterus salmoides*), black crappie (*Pomoxis nigromaculatus*), and white crappie (*P. annularis*) are economically important members of the Centrarchidae family. This North American native family contains a diverse clad of temperate freshwater fishes [1], many of which can grow to, or exceed, pan size [2], and have highly palatable flesh. Over the years, centrarchids have been harvested by small-scale commercial fisheries and via angling [3]. Their culture potential was realized as early as the end of 18th century [4].

With the establishment of federal and state hatcheries in the 1930s and 1940s, extensive pond culture techniques for centrarchids within black bass (*Micropterus*), crappie (*Pomoxis*), and sunfish (*Lepomis*) genera were developed [5]. Fingerlings and juveniles produced in these hatcheries were stocked into farm ponds and small impoundments, initially to alleviate the protein shortage of the World War II years and, later, for recreational purposes [6]. Fishery managers also employed hatchery-produced fingerling and juveniles for the establishment of reservoir fishery and diversification or management of existent stocks in lakes, rivers, and streams

[7]. As a result, centrarchids have become widely stocked and introduced throughout North America. They have also been transferred intercontinentally to South America, Africa, Europe, and Asia [8–10]. Through these stockings and introductions, centrarchids not only have become highly popular game fishes, but the subject of multi-billion dollars worth of recreational freshwater fishery in North America [11, 12].

Among all centrarchids, largemouth bass, which is a voracious predator, puts up a good fight and is attracted by natural and artificial lures; it has been praised as the most sought-after game fish, and is stocked more widely than the others, both in North America [3] and around the other temperate regions of the world [13, 14]. Crappies also attract a quarter of anglers' attention in freshwater recreational fishery [11]. They are usually caught for consumption [15] and could constitute a substantial part of recreational catches in some regions of the Southeastern United States [16].

North America currently proclaims centrarchids as recreational fishes, and legislation strictly regulates their recreational catches, as well as their commercial harvest [3]. Public and private hatcheries provide sufficient numbers of fingerlings and juveniles to meet the stocking demand of their recreational fishery, by using the extensive pond culture methods, developed in the first

half the 19th century. Together with this, remedial stockings, growing fee, and trophy fishing operations have increased the demand for larger centrarchids in recent years, leading the hatcheries to increase the size of fish they produce.

Additionally, there is a demand for food-size centrarchids from the ethnic and local markets, where largemouth bass and crappies are sold at higher prices than some cultured fishes [15, 17, 18], and commercialization of cultured centrarchids for human consumption has been allowed by some states [18–20]. This growing demand for larger centrarchids requires improvement of their traditional culture techniques, in order to enhance the efficiency of production and to make the culture of larger fish sustainable. Due to some reproductive characteristics, development of sex control technologies such as monosex and/or sterile culture, for them seems one of the many areas that could provide great enhancement in their production efficiency.

18.2 Reproductive Characteristics of Largemouth Bass and Crappies

Like all centrarchids, largemouth bass and crappies are gonochoristic fishes. They spawn synchronously in the spring, when water temperatures rise above 15–18°C [18, 21]. Females can spawn 1–3 times within a month period, and can mate with more than one male [22–24]. Male and female largemouth bass can mature around 160 g (22 cm) and 200 g (25 cm), respectively [25]. At high growth rates, they can reach these sizes within their first year. Early sexual maturation is more prominent in crappies, because one-year-old males and females weighing less than 100 g [26], with total lengths of 11–12.7 cm [27, 28], can spawn. Additionally, they are highly prolific. A female largemouth bass, on average, can spawn 8,800 eggs/kg [29], and crappie females weighing only 100 g can spawn up to 40–50,000 eggs [15].

Furthermore, largemouth bass and crappie males, as in other centrarchids, build nests and provide parental care to their young [30], and females in largemouth bass could voluntarily join with males in this task [24]. The longevity of parental care varies among species, but largemouth bass provide the longest parental care to their young by defending them until free-swimming fry balls start to disperse, which might take 2–4 weeks [18]. The nesting period in crappies is shorter; they quit guarding their young when they become free-swimming, but this might take 7–10 days [31]. During the nesting period, males do not leave their nest for foraging. Records indicate weight loss up to 10–30% in largemouth bass males and females by the end of the spawning season [28, 32].

18.3 Benefits of Sex Control in Largemouth Bass and Crappie Culture

The precocious maturation and prolific nature of these fishes has led to overcrowded and stunted populations, especially with crappies in small impoundments, and has provided the most challenging management issue in their recreational fishery [33–38]. These characteristics are also prone to reducing the efficiency of producing larger fish for trophy fishery and aquaculture, because they might mature in the first year, while at least a two-year culture period is necessary to grow sizes of 250 g or larger [17–19, 39].

With the onset of maturation at the first year, much of the energy needed for good growth is channeled to gamete development and reproductive activities such as nest-defending and parental care. These activities further increase the cost of reproduction in centrarchids, and divert much higher energy from growth. Furthermore, aggressive behavior and fights during the nesting period might cause injuries and wounding. These injuries, and secondary infections of wounds, might result in mortalities within hungry,

already weakened males, and can cause further losses in their production efficiency.

Confirmed, field studies also show that females in both largemouth bass and crappies live longer and reach larger sizes than males [40, 41]. Together with this, male crappies seem to grow slightly better than females for the first couple of years [42]. Either male or female, monosex or monosex/sterile culture technologies allow the selection of desired sex, and eliminate reproduction and its consequences [43]. Thus, these technologies might provide great benefits in their culture to trophy or pan sizes.

18.4 Strategies Evaluated for Sex Control in Largemouth Bass and Crappies

Fishery biologists conducted the first studies on sex control in centrarchids to solve overcrowding and stunting problems of crappies in small impoundments, and to improve the growth potential of largemouth bass for their trophy fishery. These earlier studies reported high success of exogenous steroid treatments in controlling direction of gonadal differentiation in largemouth bass, and yielded reliable triploid production procedures for both largemouth bass and crappies. Furthermore, the effects of using hybrids, triploids or triploid hybrids, and all-female bass in stockings were evaluated.

The focus of subsequent studies conducted by aquaculture biologists has been to develop more sustainable and environmentally friendly techniques for the production of monosex populations. With this aim, researchers have tried to develop and optimize the hormonal sex reversal procedures for neomale or neofemale production and persuaded gynogenesis. Moreover, they have elucidated the sex determination mechanisms of largemouth bass and black crappie by investigating the sex ratios of gynogenetic progeny, progenies of normal (not hormone-treated), and sex-reversed fish.

18.4.1 Interspecific Hybridization

Interspecific hybrids might have several characteristics that can be of benefit both in aquaculture and fisheries management. Hybrids of two species could: be sterile or have diminished reproductive capacity, due to problems in chromosome pairing and gamete production; have predominately male or female sex ratios because of opposite sex-determination mechanisms of the two species; or have higher growth rates and/or better performance in many other traits, due to dominant genetic variance and increased polymorphism [44]. Positive effects of interspecific hybridization have also been explored in centrarchids (Box 18.1).

Nevertheless, interspecific hybridization between largemouth bass and the other black basses [45–48], or between the two crappie species [49–53], has resulted in progenies with balanced sex ratios and similar fertility to their parental species. Moreover, the progenies of hybrid combinations evaluated between largemouth bass and the other popular black basses (small mouth bass, *M. dolomieu*, and Florida bass, *M. floridanus*) have shown intermediate performance to parental species in traits such as growth and survival rates [47, 54], or tolerance to handling [55].

Box 18.1 Characteristics of interspecific hybrids

- 1) Interspecific hybrids within *Micropterus* and *Pomoxis* genera are fertile and have balanced sex ratios [45–53].
- 2) Hybrids between largemouth bass and smallmouth bass or Florida bass shows intermediate performance in traits such as growth and survival rates [47–54], or tolerance to handling [55], compared with parent species.
- 3) Both F₁ hybrid crosses between black crappie and white crappie grow faster than parental species, but their growth advantage and viability declines and diminishes in subsequent generations [49–51, 53].

In contrast to black bass hybrids, F_1 hybrids between the two crappie species show higher growth rates than both parental species, but growth advantage and viability of hybrids decline beyond the F_1 generation and diminish after the F_2 generation [49–51, 53]. Crappie hybrids are also more tolerant to handling than white crappie [50], and are less vulnerable to angling than black crappie [56] (Box 18.1).

Even though crappie hybrids are fertile, faster growth of F_1 hybrids and reduced viability of subsequent generations have been suggested to be beneficial for solving the stunting problem of crappies in small impoundments [49, 57, 58]. The limited number of studies conducted on this matter so far have generally confirmed the better growth rates of F_1 hybrids than black crappie or both parental species [50, 53, 59, 60]. Nevertheless, these studies did not provide sufficient evidence of whether utilization of crappie hybrids could solve the overcrowding and subsequent stunting problem. Recruitment of hybrid crappies in these studies were highly variable, and ranged from moderate to none as in ponds stocked with parental species. Erratic recruitment, which has been another challenge for the fishery biologist dealing with crappies [61, 62], might also have complicated the outcomes of these studies.

Whatever the reason for the reported variability, it is clear that density-dependent factors could always limit growth in small impoundments, and also do so in the aquaculture setting, as long as precocious maturation and prolific reproduction are not prevented [63]. Additionally, full growth potential of crappies could not be realized without eliminating reproduction.

18.4.2 Triploidy

Since hybridization has no benefits for largemouth bass, and does not eliminate reproduction in crappies, researchers have persuaded triploidization in their further studies. Unlike diploid individuals, with two sets of chromosomes, one of each inherited from either mother or father, triploid individuals have

three complete sets of chromosomes, two of which are usually inherited from the mother. Having three sets of chromosomes usually generates sterility in males, and especially in female fish, due to problems in pairing of their odd numbered chromosomes during gamete production, and it thus eliminates reproduction and its consequences.

Garrett [64] was the first researcher who produced triploid progeny in largemouth bass. For this, he exposed fertilized eggs incubated at 22°C to 4,000–8,300 psi hydrostatic pressure for 1–3 minutes, five minutes after fertilization. Pressure shock of eggs at 5,000 and 6,500 psi for three minutes, or at 8,000–8,300 psi for one minute, yielded 100% triploid progenies, with a similar pre-hatching survival rate to control (53%) (also see Box 18.2).

Neal *et al.* [65] later repeated one of his successful treatments, and obtained 100% triploid largemouth bass by pressure shocking fertilized eggs at 8,000 psi for one minute, five minutes after fertilization. This treatment regimen again yielded a moderate hatching survival rate of 60% for triploid

Box 18.2 Effective treatment regimens for triploid induction and characteristics of triploids

In largemouth bass, exposing eggs to 8,000 psi hydrostatic pressure for one minute, five minutes after fertilization, could induce 100% triploidization, with over 50% hatching survival rates [64–65]. In crappies and their hybrids, exposing eggs to 6,000–8,000 psi hydrostatic pressures for two minutes, five minutes after fertilization, could induce 90–100% triploidization with higher hatching survival rates [71–73]. Cold shock of eggs at 5°C for 60–90 minutes, five minutes after fertilization, could also yield 90–100% triploids in crappies, but with lower hatching survival rates of 40% [69–70]. Although triploidy does not provide growth advantage for largemouth bass and crappies, it significantly reduces the size of gonads in males and especially in females [66–67, 69–70].

eggs [65]. Furthermore, these researchers compared both maturation and growth of diploids and triploids by communal stocking in a tropical reservoir. They reported that one-year-old diploid bass started to mature in the middle of the reproductive season, and the mean gonadosomatic index value ($GSI = \text{Gonad weight} \times 100 / \text{total body weight}$) was 6.8 times higher in diploid females [66]. At age two years, GSI values in both males and females were significantly higher in diploids than in triploids, and this difference was, again, more pronounced in females than males [67]. Nevertheless, they recorded no growth differences between diploids and triploids at juvenile stage or at age one or two [66, 67].

The first triploid progeny in crappies was produced in white crappie by Baldwin *et al.* [68], through heat shock of fertilized eggs five minutes after fertilization. They achieved 0–10% triploidization by administering heat shocks of 36–40°C for 1–5 minutes to the eggs incubated at 21–23°C. Cold shocks of 5°C for 45 and 60 minutes were more effective, and yielded 0–24% and 72–92% triploid progenies, with 33–40% hatching survival relative to controls. The same treatment regimen, administered for a longer duration of 90 minutes, yielded 92–100% triploid progenies in white crappie [69]. Comparison of GSI values and metabolic rates of diploid and triploid white crappie showed that GSI values in both male and female triploids were significantly lower than those of diploids, but metabolic rates of fish at both ploidy levels were similar [69].

Using the same treatment regimen, Parsons and Meals [70] produced 90–100% triploid female white crappie and male black crappie hybrids. They then compared the growth, body condition, gonadal development, and survival rates of triploid hybrid and diploid white crappies in the experimental ponds containing the other commonly stocked centrarchids (bluegill, *Lepomis macrochirus*, redear sunfish, *Lepomis microlophus*, and largemouth bass). Within three years of the experiment, they observed no crappie repro-

duction in the ponds stocked with triploid fish, and GSI values of triploid hybrid crappie were significantly lower than that of diploid white crappie, a situation that seemed particularly more pronounced in males [70]. However, they also detected no differences between growth, body condition, and survival of triploid hybrid and diploid white crappie [70].

Hydrostatic pressure of 6,000 psi for two minutes, five minutes after fertilization, was also effective to produce 100% triploid progeny in the same hybrid cross, with very high survival rate (87.3%) at hatching [71]. The North Mississippi Fish Hatchery also reports close to 100% triploidization and similar survival rates at hatching, by using higher pressures of 7,000 psi [72] and 8,000 psi (available online at: www.mdwfp.com/northmsfishhatcheries/fishhatchery/magnoliacrappie.aspx). Pressure shock of eggs at 6,000 psi for three minutes, two minutes after fertilization, yielded about 90% triploid progeny in the reciprocal hybrid cross [73].

18.4.3 Gynogenesis

Gynogenesis is the prevention of paternal inheritance by inactivation of sperm DNA, usually with UV irradiation. Individuals developed from these eggs are known as gynogens or gynogenetic progeny. Diploidy in gynogens is restored after fertilization, by retention of the second polar body (the extra set of female chromosome discarded after fertilization), or by prevention of the first mitotic division of fertilized eggs via heat or pressure shock. Gynogenesis has been used in aquaculture to elucidate sex determination mechanisms in fish, or to produce monosex female populations in species with female homogametic sex determination mechanism (XX/XY system).

For similar reasons, researchers at Kentucky State University investigated gynogenetic largemouth bass and black crappie production. First, they evaluated the various aspects of gynogen production in black crappie [74]. Their evaluation yielded that

among four different species, white bass (*Morone chrysops*) could effectively be used as heterologous sperm donors when producing black crappie gynogens. A UV dose of $1000\text{J}/\text{m}^2$ provided genetic inactivation of white bass sperm, with the least effect on its fertilizing capacity. They obtained the highest proportion of crappie gynogens with eggs incubated at 22.5°C , fertilized with UV-irradiated white bass sperm and heat shocked in water with a temperature of 37°C for 1.5 minutes, one minute after fertilization. However, the success rate of even their best treatment was low, and produced only 8.3% normal developing gynogenetic larvae.

In the second study, they produced gynogenetic progeny in largemouth bass [75]. Similar to their previous study, they used UV-inactivated (at $1000\text{J}/\text{m}^2$) heterologous sperm from white bass or striped bass (*M. saxatilis*) to fertilize the eggs. Then, they exposed eggs to 8,000 psi hydrostatic pressure for one minute, five minutes after fertilization, to restore diploidy. Nevertheless, they did not report their success rate or the survival of gynogenetic progeny.

18.4.4 Hormonal Sex-Reversal

Gonadal differentiation in fish is labile to the influence of exogenous factors such as steroids. Female or male steroids (estrogens and androgens, respectively) can functionally change the direction of gonadal differentiation in fish, and yield individuals with different genetic and phenotypic sex. These individuals are known as neomales or neofemales. Neomales or neofemales provide opportunity for more sustainable and environmentally friendly production of monosex populations, by simple breeding schemes [76, 77]. For example, all female fish in a female homogametic species can be produced by fertilizing eggs of normal females (XX females) with the sperm of neomales (or XX males). Although female and male steroids act as a gonadal sex inducer in fish, functional alteration of phenotypic sex requires administration of appropriate dosages of steroids before the commitment of primordial germ cells in

embryonic or larval gonads to male or female direction for a certain minimum duration [78, 79]. Otherwise, steroid treatments might not be effective to produce functional sex-reversal in fish, or to yield intersex, sterile, or reproductively dysfunctional fish [80–83].

With the ultimate goal of eliminating reproduction and benefitting from growth and survival differences between males and females, researchers have attempted to control the direction of gonadal differentiation in largemouth bass and black crappie. Highly successful results were reported for largemouth bass, but the efficacy of steroid treatments was inconsistent, and showed controversies across the studies. For example, Garrett [84], who tried to masculinize and feminize five-week-old largemouth bass fry (with a standard length of 20–30 mm) by oral administration of natural androgens and estrogens at doses of 50 or 100 mg/kg, reported high effectiveness of androgen treatments to induce masculinization, while estrogen treatments were completely ineffective to induce feminization (Table 18.1).

In contrast, Al-Ablani and Phelps [85] reported complete feminization in largemouth bass when they fed slightly older (40 days old) and larger (with a mean total length of 35 mm) fry with diets containing the same or higher amounts of 17β -estradiol (E_2), or a synthetic estrogen (diethylstilbestrol, DES). Porter [86] reported complete masculinization in largemouth bass by oral administration of a synthetic androgen, 17α -methyltestosterone (MT), at doses of 30–50 mg/kg to smaller (with a mean total length of 20–25 mm) and younger (exact age was not given, but fry were probably older than 3–4 weeks) fry (Box 18.3).

Arsalan *et al.* [87], who used older (40 days old) and larger (33.5 mm) fry than Porter [86], produced only a few intersex fish by oral administration of 60 mg/kg MT for similar periods. However, while oral administration of E_2 to the same cohort of fry was not completely ineffective, as in Garrett [84], it did not reproduce the success of Al-Ablani and Phelps [85], even though the treatment regimen, age

and size of fry were similar (Table 18.1). Arslan *et al.* [87] attributed these discrepancies to the proximity of gonadal differentiation in 35–40-day-old and 30–35 mm fry, to the point of no longer being responsive to exogenous steroids. Additionally, the growth rates of MT treated fish (0.18–0.22 mg/day) in their study were higher than the growth rates of E₂ treated fish (0.12–0.15 mg/day), and both had higher growth rates than E₂ treated fish (0.11 mg/day) in Al-Ablani and Phelps [85]. They thought the higher growth rates further increased the pace of gonadal differentiation in treated fish, and did not allow them to deliver pharmacologically effective doses of steroids before normal course of sex differentiation [78, 88] (Box 18.3).

In the course of his study, Porter [86] also found that the same MT treatment regimen might be completely effective or ineffective on largemouth bass (Table 18.1). Additionally, gonadal morphology of 40–54% of all treated fish did not confirm his histological classification of sex. When he evaluated the functional effects of MT treatments on 20 fish at age two years, he reported the number of MT treated fish emitting milt after hormone inductions was significantly lower than the control group. Without any further morphological or histological evaluation, he suggested that MT treatments might have caused sterilization in largemouth bass.

In contrast, Arslan *et al.* [87] used a higher dose of MT for similar durations, and reported enhanced gonadal development in the treated males, and thin gonads resembling the V-shaped testis only in intersex fish. When they evaluated the functional effects of E₂ treatments on 10 control (89–195 g) and 100 E₂ treated (77–281 g) fish at age 13 months, they reported that all fish responded to hCG injections were genetic females. Morphological and histological evaluations and GSI values of non-responding fish further revealed that all E₂ treated fish had Y-shaped ovarian morphology, but genetic males could easily be distinguished by their minute gonads. These minute gonads either contained both spermatozoa and previtellogenic oocytes, or were tightly packed with previtellogenic oocytes, or contained high amounts of connective tissue and a low number of oocytes [87]. Based on their results, and examining the age and criteria used for sexing fish across the studies, they suggested that evaluating the efficacy of steroid treatments from morphologic and barely discernable cellular features of immature gonads might be misleading, and partially responsible for the observed inconsistencies. Fry much younger and smaller than 35–40 days old and 30–35 mm should be used for complete and consistent efficacy of steroid treatments on largemouth bass [87].

Box 18.3 Effective treatment regimens to control the direction of gonadal differentiation with exogenous steroids

Further studies are still necessary to establish reliable estrogen and androgen treatment procedures in largemouth bass [87]. In black crappie, there are two reliable treatment regimens for the production of all-male populations or neomale brood stock. Fry (35–45 days old with a mean total length of 17–22 mm) could either be fed diets containing 30 mg of 17 α -methyltestosterone/kg for 30 days [93, 95], or immersed in a 1 mg/L 17 α -methyltestosterone or trenbolone acetate solutions for five hours/day every 3–5 days on 7–10 occasions [94].

Further studies are also necessary for optimization of female induction procedures in black crappie. Immersion of the same cohort of fry used in androgen treatments to a 1 mg/L 17 β -estradiol solution by using the same regimens significantly increases the frequency of females, but does not yield all-female populations [98]. Studies on control of sex differentiation with exogenous steroids are missing for white crappie.

Extended (10 weeks long) steroid treatments, initiated just after first feeding through oral administration of natural androgens and estrogens incorporated in *Artemia*, was reported to be effective to induce complete masculinization and feminization in largemouth bass [84]. However, Johnston [89], who studied the germ cells in largemouth bass, reported that primordial gonads form after first feeding (at 6–11 mm). The first sign of anatomical differentiation (ovocoel formation) can be observed in about 3–4-week-old fry with total lengths of 18–20 mm, female and male differentiation become apparent at 30–35 mm and 40–45 mm fry, respectively [89].

Although the exact timing and pace of gonadal differentiation might be somewhat different from what Johnston reported, because he did not continually monitor the growth rates of fry, the positive outcomes of steroid treatments initiated 3–4 weeks after first feeding confirm his observations in general. They also demonstrate that gonadal differentiation in largemouth bass remains responsive to exogenous steroids for several weeks after first feeding. In such a case, such an early initiation of steroid treatments with 5–7 days old and about 6 mm long first feeding fry might not be necessary. Steroid treatments initiated (probably with 18–20 mm long and 20–30 days old fry) during the somatic growth of gonads might provide sufficient time of opportunity for the delivery of pharmacologically effective doses of steroids before the normal course of sex differentiation, and can yield consistently successful outcomes and functionally sex-reversed fish [78, 90].

The timing and pace of gonadal differentiation might vary both at individual and species level [78, 91], and might not be exactly the same in all centrarchids. However, steroid treatments were consistently effective in another centrarchid, black crappie, even on fry as big as 35 mm (37 days old, Table 18.2). The high consistency and effectiveness of steroid treatments in black crappie, even on 35 mm fry, prompts the question again of

why very comparable steroid treatments produce inconsistent outcomes in largemouth bass. One possible answer to this question might be that the faster growth rates of feed trained largemouth bass during the steroid treatments (Table 18.1). Unlike largemouth bass, growth rates of black crappie, being a non-aggressive feeder, were much slower (Table 18.2). These slower grow rates probably slow down the pace of gonadal differentiation in black crappie, and allows the administration of sufficient doses of steroids before natural sex differentiation. Then again, initiating steroid treatments earlier on smaller size fry alone might not be sufficient, but control over growth rates of treated fish might be necessary for complete and consistent efficacy of steroid treatments on largemouth bass.

Our knowledge on the timing of gonadal differentiation in black crappie is based on the experimental results of steroid treatments. They demonstrated that both age [92, 93] and size [94] affect the timing of gonadal differentiation in black crappie. Androgen and estrogen treatments initiated with 35–45 days old and 16–23 mm fry were consistently effective to produce all-male or predominantly male and female populations in black crappie (Table 18.2). Androgen treatments were even effective on fry as big as 35 mm, if the growth rate was high (37 days old), but they were ineffective if the growth rate was slower (45 days old) [93, 95].

Two synthetic androgens, MT and trenbolone acetate (TBA), were used for masculinization of black crappie. Oral administration of 30 mg/kg MT for 30 days has proved to be highly effective and a reliable procedure for the production of all male populations (Table 18.2) or neomale brood stock in black crappie [93, 95]. Higher than 30 mg/kg doses of MT and/or extending the treatment period beyond 30 days, however, might increase the frequency of intersex fish [94] and probably cause feminization, due to aromatization of MT to estrogens [96].

Compared to MT, oral administration of TBA at higher doses of 50 and 100 mg/kg

Table 18.1 Reported outcomes of bath and oral steroid treatments on gonadal differentiation of largemouth bass.

Treatment	Initial age (day)	Duration or intensity	Total length (mm, \pm SD)		% Male	% Female	% Intersex	Reference
			Initial	Final				
100 mg/kg E ₂	35	42 days	20–30**	NG	46	54	0	[84]
100 mg/kg E					49	51	0	
50 mg/kg T					90	10	0	
100 mg/kg T					93	7	0	
100 mg/kg A					98	2	0	
Control		–			41	59	0	[84]
5 mg/L E ₂ *	5	70 days		NG	0	100	0	
5 mg/L E*					0	100	0	
5 mg/L T*					100	0	0	
5 mg/L A*					100	0	0	
Control		–			89	11	0	[84]
10 mg/L T*	7	70 days		NG	100	0	0	
Control		–			53	47	0	
30 or 50 mg/kg	>21–28	28 days	20–25	NG	100	0	0	[86]
MT		42 days			50–100	50–100	0	
		70 days			100	0	0	
Control		–			53–55	0	0	[85]
100 mg/kg E ₂	40	40 days	35 \pm 3.4	75 \pm 4.9	0	100	0	
200 mg/kg E ₂				73 \pm 6.1	0	100	0	
400 mg/kg E ₂				70 \pm 2.6	0	100	0	
100 mg/kg DES				65 \pm 1.0	0	100	0	
200 mg/kg DES				64 \pm 0.6	0	100	0	
400 mg/kg DES				62 \pm 2.0	0	100	0	
Control		–		86 \pm 2.9	53	47	0	[87]****
200 mg/kg E ₂	40	30 days	33.5 \pm 1.5	89 \pm 10	4.6	66.8	28.6	
		45 days		90 \pm 10	4.7	59.8	35.5	
		60 days		85 \pm 11	5.1	70.5	24.4	
60 mg MT		30 days		116 \pm 3	49.0	48.8	2.2	
		45 days		114 \pm 2	48.9	48.9	2.2	
		60 days		138 \pm 1	52.4	44.0	3.6	
1 mg/L MT		3 times***		98 \pm 10	55.4	43.9	0.7	
1 mg/L MT		6 times***		101 \pm 2	50.9	48.3	0.8	
Control		–		102 \pm 9	46.9	53.1	0	

A (androsterone), DES (diethylstilbestrol), E (estrone), E₂ (17 β -estradiol), MT (17 α -methyltestosterone), NG (not given), T (testosterone), * Administered via *Artemia* incubated in hormone solutions, ** Standard length, *** For five hours/day every three days, **** Size of all treated fish measured at 100 days of age.

Table 18.2 Reported outcomes of bath and oral steroid treatments on gonadal differentiation of black crappie.

Treatment	Initial age (day)	Duration or intensity	Total length (mm, \pm SD)		% Male	% Female	% Intersex	Reference
			Initial	Final				
30 mg/kg MT	40	30 days	23 \pm 2.2	28 \pm 3.7	71.3	23.7	5.0	[92]
60 mg/kg MT				27 \pm 3.4	90.3	7.3	2.3	
	60		24 \pm 3.4	30 \pm 3.1	57.0	41.7	1.3	
Control	40	–	23 \pm 2.2	30 \pm 1.7	51.3	48.7	0	
50 mg/kg TBA	40	30 days	17 \pm 3.1	27 \pm 1.5	72.3	25.3	2.3	[97]
100 mg/kg TBA				26 \pm 1.0	79.0	19.7	1.3	
Control		–		28 \pm 0.6	49.0	51.0	0	
60 mg/kg MT	33	30 days	26 \pm 0.6	30 \pm 0.3	52.3	47.7	0	[97]
50 mg/kg TBA				31 \pm 0.5	77.3	22.7	0	
200 mg/kg DES				29 \pm 0.4	14.0	78.5	7.5	
Control		–		30 \pm 0.5	48.3	51.7	0	
30 mg MT	37	30 days	35 \pm 0.3	55 \pm 0.8	94.7	0	5.3	[93]
Control				65 \pm 0.9	41.5	58.5	0	
60 mg/kg MT	45	45 days	20.1 \pm 1.4	37.8 \pm 4.3	48.5	28.5	23.0	[94, 98]
1 mg/L MT		10 times*		33.5 \pm 3.5	96.5	2.0	1.5	
1 mg/L E ₂		10 times*		36.8–39.5	28.0	71.0	1.0	
Control		–		39.5 \pm 3.7	57.0	42.5	0.5	
60 mg/kg MT		45 days	26.1 \pm 2.0	40.4 \pm 3.5	50.0	50.0	0	
1 mg/L MT		10 times*		37.1 \pm 3.8	59.0	38.0	3.0	
1 mg/L E ₂		10 times*		40.5 \pm 3.6	53.0	47.0	0	
Control		–		42.5 \pm 4.1	56.0	44.0	0	
1 mg/L TBA	45	10 times*	21.6 \pm 1.2	29.7 \pm 4.2	100	0	0	[94]
	40	7 times**	20.3 \pm 0.9	32.3 \pm 4.9	100	0	0	
Control		–		30.8 \pm 4.9	53.0	47.0	0	
30 mg MT	35	40 days	21.2 \pm 1.3	28.1 \pm 0.2	100	0	0	[95]
Control		–		33.9 \pm 0.4	54.5	45.5	0	
30 mg MT			16.0 \pm 1.5	33	100	0	0	
Control				NG	53.9	46.1	0	
30 mg MT			20.2 \pm 1.8	26.1 \pm 3.9	95.2	4.8	0	
Control				NG	44.9	55.1	0	
30 mg MT			20.6 \pm 1.4	29.9 \pm 4.1	100	0	0	
Control				NG	57.3	42.7	0	
30 mg MT			17.1 \pm 1.1	35.4 \pm 4.1	95	5	0	
Control				NG	38.6	61.4	0	
30 mg MT			21.1 \pm 1.2	29.5 \pm 0.5	100	0	0	
Control				NG	51.9	48.1	0	
30 mg MT	45	30 days	35 \pm 2.1	53 \pm 0.5	56.0	44.0	0	[95]
Control				NG	24.0	76.0	0	

E₂ (17 β -estradiol), DES (diethylstilbestrol), MT (17 α -methyltestosterone), NG (not given), TBA (trenbolone acetate),

* For five hours/day every 3–5 days between 45–86 days after hatching, ** For five hours/day every 3–5 days between 40–66 days after hatching.

diet yielded lower masculinization rates (72.3–79%) in black crappie [97]. However, TBA was as effective as MT at a dose of 1 mg/L, when given as short-time (five hours) periodic (every 3–5 days) baths on seven or 10 occasions (Table 18.2). Short-time periodic MT and TBA baths also proved to be a reliable and effective procedure for the production of all male populations [94] or neomale brood stock in black crappie [26].

Functional effects of MT immersions were evaluated on 20 treated (24 ± 6 g) and 35 control (28 ± 6 g) males at one year of age, by induced maturation with hCG (500 IU/kg male) and voluntary spawning in aquariums containing a control female (27 ± 5 g). Eggs hatched 50% of the time in aquariums containing a MT-treated male, while hatching occurred only 38% of the time in aquariums containing a control male [26]. Sex ratios of two out of six spawns obtained from MT-treated males demonstrated that they were actually genetic females (see Section 18.5).

Limited effort has been spent on the development of effective estrogen treatments in black crappie. In a preliminary study [97], oral administration of a synthetic estrogen (200 mg DES/kg diet) without prior feed training to fry, with intermediate growth rate, yielded highly variable degrees of feminization (59–100%, averaged at 78.5%). In the other attempt [98], the same cohort of fry (45 days old) were separated into two size classes (20.1 and 26.1 mm), before periodic E_2 baths at a dose of 1 mg/L. Short-time (5 hours/day) periodic 10 E_2 baths until 86 days of age increased the frequency of females from 42.5% to 71% in the smaller cohort, but was completely ineffective on the larger cohort (Table 18.2). Since the same treatment regimen with MT had produced a 96.5% male population in the smaller cohort, researchers suggested that the lower success of E_2 baths could be due to insufficient dose of E_2 or, most probably, due to premature termination of the treatments [98].

The functional effects of E_2 baths on 27 treated fish (25 ± 7 g) were evaluated at one year of age by hCG (1000 IU/kg female)-induced

maturation and voluntarily spawning in aquariums containing a control male (28 ± 6 g). Only two E_2 treated fish responded hCG injections, but examinations of gonads from 10 fish that did not respond to hCG injections revealed no morphological or histological abnormalities [26]. All these ovaries contained early vitellogenic oocytes, probably due to the small size of the females [26].

Further research is necessary for the development of successful and reliable feminization procedure(s) in black crappie. To my knowledge, there is no study on either feminization or masculinization of white crappie. Studies on this species will also be highly beneficial, since they tend to be even more prolific than black crappie.

18.5 Sex Determination Mechanisms in Largemouth Bass and Crappies

As in many teleosts, karyological studies have revealed no morphologically distinct sex chromosomes in centrarchids, including largemouth bass and crappies [99, 100]. No attempt has been made to elucidate the presence of sex-related genes or sequences in these three centrarchids so far. Our knowledge on their sex determination mechanisms is based on the sex ratios of natural and experimental populations, hybrids, normal and neomale progenies, and gynogenetic progeny. Natural and experimental populations of all three species and their hybrids exhibit balanced sex ratios [26, 41, 47, 50, 97, 101], suggesting that a simple dual-chromosomal sex determination system is operating in all three species and in their genera.

Gynogenetic progenies in largemouth bass contain 33.3% males [75]. Although autosomal genes or environmental influences can lead to presence of males in gynogenetic progenies [102, 103], balanced sex ratios of their experimental populations do not confirm the presence of such factors in largemouth bass. Furthermore, sex ratios of progeny from seven different normal

Box 18.4 Sex determination mechanisms

Sex ratios of gynogenetic progeny and normal pair spawns prove that largemouth bass is a female heterogametic species, with a WZ/ZZ dual chromosomal sex determination mechanism [26, 75]. Viability of super females (WW genotype), which will shorten the production procedure for all female largemouth bass populations, has yet to be proven by progeny testing.

Sex ratios of neomale progeny and normal pair spawns demonstrate that black crappie is a male heterogametic species, with a predominantly XX/XY sex determination mechanism [26, 93, 95]. Female-skewed sex ratio [95], and the presence of a simultaneously sex-reversed female within not-steroid treated fish and occasional males within neomale progenies [26], suggest that factors such as sex-modifying genes or environmental variables might have some influences on the male heterogametic dual chromosomal system of black crappie. Balanced sex ratios of hybrids between black and white crappies suggest that white crappie is also a male heterogametic species [49–52].

(not steroid-treated) largemouth bass pairs showed little variation (49–54% males) around a balanced ratio of 1 : 1 [26]. Therefore, the presence of males within their gynogenetic progeny reveals that largemouth bass is a female heterogametic (WZ/ZZ system) species (Box 18.4). Nevertheless, the viability of the WW genotype (super females), which could shorten the procedure for all-female production, remains in question, because gynogens were not progeny-tested in that study [75].

Gomelsky *et al.* [93] progeny-tested MT-treated black crappie. Only female progenies produced by two neomales suggested that black crappie has a simple dual chromosomal sex determination mechanism, and male is the heterogametic sex (XX/XY system; Box 18.4). Their later study [95] confirmed the presence of male heterogamety in black crappie, but it also reported a predominantly

female (76%) sex ratio in an experimental population (Gomelsky, unpublished data, and see [95]), which could not be explained under a simple dual chromosomal system.

Sex ratios of progeny from eight different normal black crappie pairs in our laboratory ranged between 42–56% males, and did not differ significantly from a balanced ratio of 1 : 1 [26]. However, we found occasional intersex fish within normal progenies with distinct anterior testicular and posterior ovarian portions, and a simultaneously sex-reversed female within non-hormone-treated fish that produced predominantly male (71%) progeny [26]. Additionally, progenies obtained from two neomales in our laboratory were not all females, but contained 1% and 9% males [26].

Predominantly female sex ratio of an experimental population (Gomelsky, unpublished data, and see [95]), and the presence of a simultaneously sex-reversed female within non-hormone-treated fish and occasional males in neomale progeny [26], suggest that some other factors, such as mutations in sex-determining genes [104], sex-modifying autosomal [105], and/or environmental [106] variables, might have an influence on the male heterogametic dual chromosomal system of the black crappie. A male heterogametic dual chromosomal system could also be operating the natural sex differentiation in white crappie, because both reciprocal hybrids between two crappie species repeatedly yielded a balanced sex ratio of 1 : 1 [49–52].

18.6 Conclusion and Future Projections

Triploid and monosex population technologies can help to eliminate reproduction and its consequences in largemouth bass and crappies, both for their recreational fishery and aquaculture. Treatment regimens developed for triploidy inductions so far have been highly effective and yielded 90–100% triploid progenies. However, less than 100% triploidy induction might be

problematic, especially with crappies, due to their high reproductive capacity.

Triploid F₁ crappie hybrids are beneficial for their faster growth rates, but they are also highly fertile. Then again, less than 100% triploidy induction might become problematic with hybrids, too. Monosex female technology, or combining the two sex control technologies to produce all-female triploid populations, could minimize such a risk. Effective and reliable androgen treatment regimens were developed for neomale brood stock production in black crappie, and breeding of those neomales (XX males) with normal females (XX) should yield all, or almost all, female populations in black crappie and, probably, in crappie hybrids, too. Inducing triploidy in all- or almost all-female eggs reduces the risk of reproduction due to occasional males in neomale progenies.

Unlike crappies, largemouth bass is a female heterogametic species. If WW genotype or super females are viable, a simple breeding process of WW neomales with normal females (WZ) or super females (WW) should yield all-female largemouth bass populations. Since female bass live longer and grow larger sizes, all-female or female triploid technologies might also provide a growth advantage for their fishery and aquaculture. Nevertheless, further studies are still needed to develop reliable androgen treatment regimens for neomale largemouth bass brood stock production. Initiation of steroid treatments on smaller fry (≤ 20 cm and younger than 30 days), and not allowing fast growth of fry during the treatment periods, might help to develop consistently effective steroid treatment regimens in largemouth bass.

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19

Hybridization and its Application in Centrarchids

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19.1 Introduction

The family Centrarchidae, commonly known as sunfishes, is comprised of eight genera and 38 (extant) recognized species so far (Table 19.1) [1, 2]. Four new species were identified in 2013 [2], and two provisional new species were reported in 2015 [3, 4]. The term “sunfish” usually refers to all 38 species in Centrarchidae, while some researchers use it to refer to species containing “sunfish” in their names [5], including all 13 species in *Lepomis* and four others. We use sunfish to refer to all Centrarchid species, due to its long history and popularity of usage. Extraordinary characteristics of fish in the family Centrarchidae are summarized in Box 19.1.

The sunfish family includes the most popular sport fish species, such as bluegill, black basses, and crappies in North America. In the past decades, the largemouth bass has become one of the most economically important aquaculture species in China [6, 7], with 351,772 ton production and \$549 million value in 2014 [8]. Several special features, including high incidence of hybridization in nature, complex sex-determining mechanism, sexual dimorphism, alternative reproductive tactics, solely paternal care, and morphological diversity (Box 19.1), make this clade of freshwater fishes a unique group of research objectives [1, 9–13].

The goals of this chapter are to provide a brief update of recent research on centrarchid phylogeny and phylogeography, a nearly exhaustive review of natural and artificial hybridization, an extensive analysis of hybrid sex ratios in order to propose the possible driving forces of hybridization, and to summarize the promises that sunfish hybrids hold for commercial aquaculture.

19.2 Phylogeny and Phylogeography

When we looked at the past 200 years, the phylogeny and phylogeography of centrarchid species have been challenging our understanding of speciation, hybridization, the conflict between morphology (phenotype) and genetics (genotype), and of even the definition of “species”. Many scientific names of Centrarchidae were discarded or adopted. Hybridization, introgression through connecting waters or introduction, expansion, or extinction, masked and hindered the classification and life tree of this diverse clade. Near and Koppelman [14] comprehensively reviewed species diversity, phylogeny, and phylogeography of Centrarchidae in 2009 and, therefore, we will briefly summarize new

Table 19.1 Currently recognized 40 extant centrarchid species, including two pending new species, and the number of available sequences from public database for phylogenetic analysis.

No.	Year	Scientific Name	Common Name	NCBI	Number of available sequence		
					ND2	cytb	Tmo-4C4
1	1855	<i>Acantharchus pomotis</i>	Mud sunfish	1	1	3	1
2	1936	<i>Ambloplites ariommus</i>	Shadow bass	1	1	29	1
3	1868	<i>Ambloplites cavifrons</i>	Roanoke bass	1	1	7	1
4	1977	<i>Ambloplites constellatus</i>	Ozark bass	1	1	7	1
5	1817	<i>Ambloplites rupestris</i>	Rock bass	1	1	25	1
6	1854	<i>Archoplites interruptus</i>	Sacramento perch	1	1	3	1
7	1801	<i>Centrarchus macropterus</i>	Flier	1	1	3	1
8	1855	<i>Enneacanthus chaetodon</i>	Black banded sunfish	1	1	3	1
9	1855	<i>Enneacanthus gloriosus</i>	Blue spotted sunfish	1	1	3	1
10	1854	<i>Enneacanthus obesus</i>	Banded sunfish	1	1	3	1
11	1818	<i>Pomoxis annularis</i>	White crappie	1	2	3	2
12	1829	<i>Pomoxis nigromaculatus</i>	Black crappie	1	3	3	3
13	1758	<i>Lepomis auritus</i>	Redbreast sunfish	1	1	12	1
14	1819	<i>Lepomis cyanellus</i>	Green sunfish	1	2	5	2
15	1758	<i>Lepomis gibbosus</i>	Pumpkinseed	1	1	5	1
16	1829	<i>Lepomis gulosus</i>	Warmouth	0	2	4	2
17	1858	<i>Lepomis humilis</i>	Orange spotted sunfish	1	2	4	2
18	1819	<i>Lepomis macrochirus</i>	Bluegill	1	2	7	2
19	1855	<i>Lepomis marginatus</i>	Dollar sunfish	1	1	5	1
20	1820	<i>Lepomis megalotis</i>	Longear sunfish	1	2	83	2
21	1859	<i>Lepomis microlophus</i>	Redear sunfish	1	2	6	1
22	1877	<i>Lepomis miniatus</i>	Red spotted sunfish	1	2	10	2
23	1870*	<i>Lepomis peltastes</i>	Northern sunfish	0	0	0	0
24	1831	<i>Lepomis punctatus</i>	Spotted sunfish	1	1	5	1
25	1883	<i>Lepomis symmetricus</i>	Bantam sunfish	1	1	3	1
26	2013	<i>Micropterus cahabae</i>	Cahaba bass	1	3	0	0
27	1999	<i>Micropterus cataractae</i>	Shoal bass	1	5	7	1
28	2013	<i>Micropterus chattahoochee</i>	Chattahoochee bass	1	2	0	0
29	1940	<i>Micropterus coosae</i>	Redeye bass	1	36 ^E	19	1
30	1802	<i>Micropterus dolomieu</i>	Smallmouth bass	1	15	41	2
31	1822	<i>Micropterus floridanus</i>	Florida bass	1	31	26	2
32	1940	<i>Micropterus henshalli</i>	Alabama bass	1	5	18	0
33	1949	<i>Micropterus notius</i>	Suwannee bass	1	4	5	2
34	1819	<i>Micropterus punctulatus</i>	Spotted bass	1	8	41	2
35	1802	<i>Micropterus salmoides</i>	Largemouth bass	1	20	33	1
36	2013	<i>Micropterus tallapoosae</i>	Tallapoosa bass	1	4	0	0
37	1874	<i>Micropterus treculii</i>	Guadalupe bass	1	2	5	2
38	2013	<i>Micropterus warriorensis</i>	Warrior bass	1	4	0	0
39	2015 ^Y	<i>Micropterus haiaka</i>	Choctaw bass	N.A.	0	0	0
40	2015 ^Y	Not given yet	Bartram bass	N.A.	0	0	0

*: *Lepomis peltastes* was recognized as a valid species in 2004.

^E: including sequence of all subspecies or species in pending stage.

^Y: new species proposed but not recognized to date.

Box 19.1 Extraordinary characteristics of fish in the family Centrarchidae

- High incidence of hybridization in nature
- Complex sex-determining mechanisms that cannot be explained by the current theories
- Incomprehensible sex ratio of hybrids
- Popular sexual dimorphism and males are usually larger than females
- Alternative reproductive tactics
- Solely paternal care
- Morphological diversity

findings and provide our understandings, mainly about phylogeography.

During the past two centuries, 154 different scientific names have been used to refer to centrarchid species, subspecies, hybrid, and hybrid combinations [14, 15]. In the past two decades, molecular data have been introduced to investigate the phylogenetic relationship and speciation within the Centrarchidae family. Available molecular data for phylogenetic analysis are mainly from five independent reports (Table 19.1) [2, 5, 16–18]. Mitochondrial-encoded NADH subunit 2 (*ND2*) is the only gene that has been sequenced in all centrarchids, except *Lepomis peltastes*. Interestingly, *L. peltastes* has been elevated as a valid species since 2004 [19–21] without any molecular data (Table 19.1). Bailey [19] described that *L. peltastes* had a distribution covering at least seven states in the United States, including Michigan, Wisconsin, Mississippi, Minnesota, Iowa, Illinois, and Ohio. This fish had actually long been treated as a subspecies and a “dwarf” stock of *L. megalotis*.

Interestingly, there is at least seven species-level clusters for 83 entries for *L. megalotis* when we conduct phylogenetic analysis using all 147 sequences of the cytochrome b (*cytb*) gene in genus *Lepomis* (Figure 19.1, NJ tree of *cytb*), suggesting that more groups may be elevated as species from subspecies of *L. megalotis* (Table 19.2, subspecies). *L. peltastes* may be unconsciously served as

geographic populations of *L. megalotis* in these clusters, because none of these reports recognize *L. peltastes* as a valid species [2, 5, 16, 17].

Coghill [17] reported that there was significant mitochondrial sequence divergence on either side of the Sierra de San Marcos valley in Mexico, which agrees with the analysis in the current work (Figure 19.1). Scharpf *et al.* [24] consider *L. megalotis megalotis*, *L.m. aquilensis*, *L.m. breviceps*, and *L.m. occidentalis* as valid species recently, although no comparative morphological and molecular analysis is available [14]. Nevertheless, we expect that several subspecies of *L. megalotis* will be recognized, because it is probably the most diverse clade in centrarchid species [17], and displays species-level clusters in our analysis (Figure 19.1).

It is generally recognized that there are at least two subspecies of *L. macrochirus* (Table 19.2). *L. macrochirus macrochirus* is widely distributed in North America, while *L.m. mystacalis* is limited to peninsular Florida. The inconsistency focuses on the third one, *L.m. speciosus*, which was actually described more than 160 years ago. Near and Koppelman [14] summarized the history and thought *L.m. speciosus* to be not a valid subspecies, while Eschmeyer *et al.* [15] and Scharpf [20] considered it as a valid subspecies. It is worth mentioning that bluegill is one of the most popular game fish in North America, and the most studied species in Centrarchidae but, with general recognition of subspecies, there is no morphological or molecular evidence to indicate that any subspecies should be recognized as a valid species to date.

There is also very high extent of intraspecific diversity in *Micropterus*, especially in *M. dolomieu* and *M. coosae*, based on all the 173 *ND2* gene sequences covering all 13 recognized species (data not shown) and *cytb* gene representing eight species [18]. Two subspecies of *M. dolomieu*, *M. d. dolomieu* and *M. d. velox*, have been long recognized [15, 24] (we have to mention that *dolomieu* was often incorrectly spelled *dolomieui* [15], and

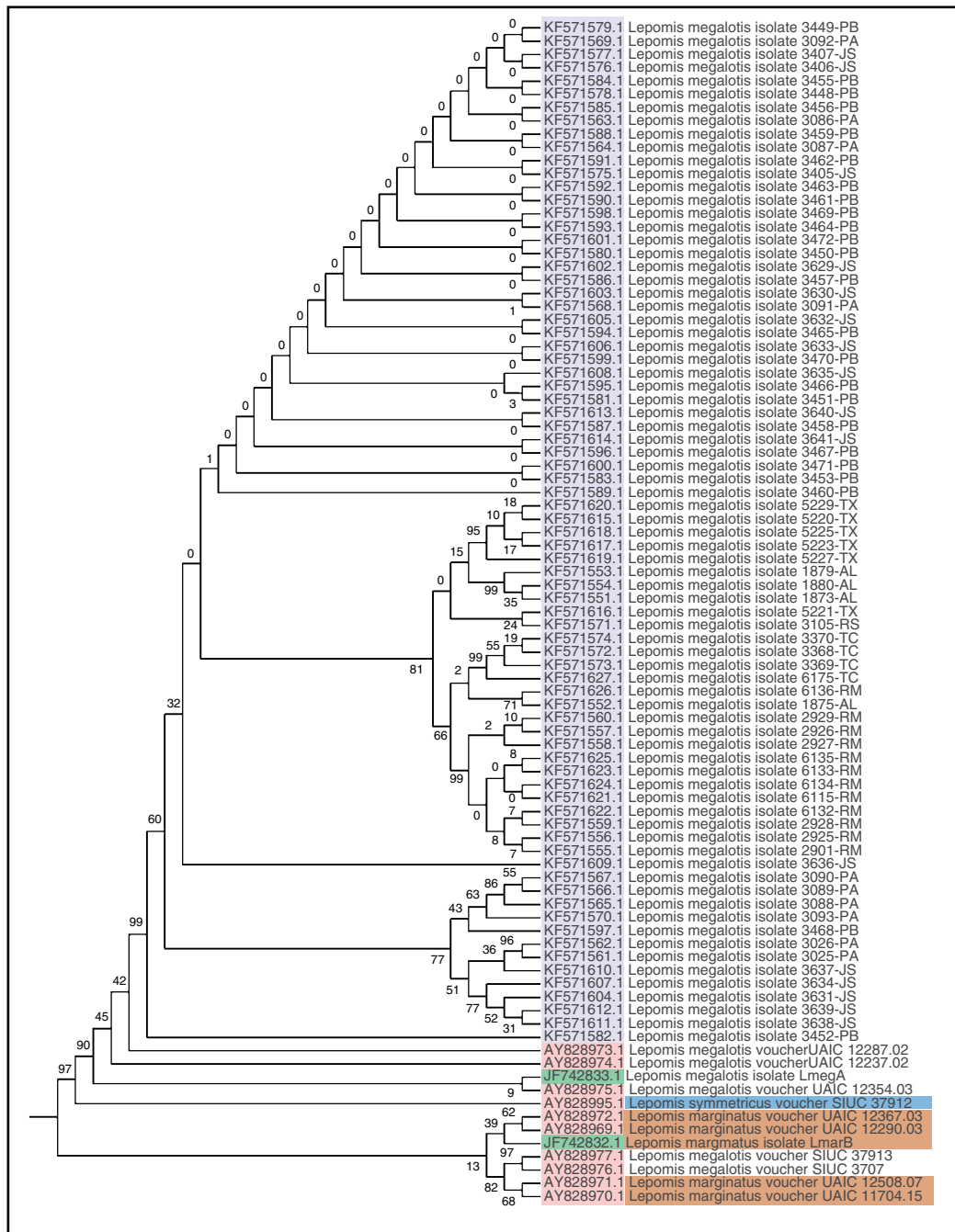


Figure 19.1 Neighbor-joining tree of all available sequence of cytochrome b (*cytb*) gene in genus *Lepomis* (Perciformes: Centrarchidae).

Figure only displays *L. megalotis* and two joined species, *L. symmetricus* and *L. marginatus*. Yellow perch *Perca flavescens* *cytb* (AF546115.1) was used as outgroup. The 148 sequence of *cytb* is available in a public collection of Genbank (<https://www.ncbi.nlm.nih.gov/sites/myncbi/18ywr7aKceeQp/collections/51735354/public/>).

Shaded or colored blocks indicate data obtained from three independent studies (left) and two joined species, *L. symmetricus* and *L. marginatus* (right). The bootstrap consensus tree, inferred from 1000 replicates, is taken to represent the evolutionary history of the taxa analyzed [22]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [22]. The evolutionary distances were computed using the Maximum Composite Likelihood method [23] and are in units of the number of base substitutions per site.

Table 19.2 Historical subspecies descriptions in Centrarchidae.

Subspecies scientific name	Common name	Recognition
<i>Lepomis megalotis megalotis</i>	Central Longear Sunfish	Rafinesque, 1820
<i>Lepomis megalotis aquilensis</i>	Rio Grande Longear Sunfish	Baird and Girard, 1853
<i>Lepomis megalotis breviceps</i>	Great Plains Longear Sunfish	Baird and Girard, 1853
<i>Lepomis megalotis occidentalis</i>	Western Longear Sunfish	Meek, 1902
<i>Lepomis megalotis convexifrons</i>	N.A.	Baird and Girard, 1854
<i>Lepomis megalotis fallax</i>	N.A.	Baird and Girard, 1854
<i>Lepomis megalotis popeii</i>	N.A.	Girard, 1858
<i>Lepomis macrochirus macrochirus</i>	Bluegill	Rafinesque, 1819
<i>Lepomis macrochirus mystacalis</i>	Bluegill	Cope, 1877
<i>Lepomis macrochirus speciosus</i>	N.A.	Baird and Girard, 1854
<i>Micropterus dolomieu dolomieu</i>	Northern Smallmouth Bass	Lacepède, 1802
<i>Micropterus dolomieu velox</i>	Neosho Smallmouth Bass	Hubbs and Bailey, 1940

N.A. – not available.

dolomieu is still used by some researchers). Intriguingly, there were no subspecies for *M. coosae*, while Bagley *et al.* [18] demonstrated comparatively high intraspecific diversity in this species in 2011. Soon afterwards, Winston *et al.* [2] reported species-level variation in morphology and mitochondrial DNA of *M. coosae*, and proposed recognition of four additional species: *M. cahabae*, *M. tallapoosae*, *M. chattahoochae*, and *M. warriorensis*. All of these five species are limited to a specific river system in Alabama and Georgia in the United States [2].

As pointed out earlier, many of centrarchid species are probably treated as subspecies [14]. As there were some speciation events dated more than 2.5 million years ago [25] and significant diversity found in nearby geographic locations [2, 17], we expect more species will be recognized, and intraspecific diversity will be identified. It is remarkably fascinating how geography isolation and local environments drive speciation of centrarchids, especially within connected water bodies. We argue that identification and classification will rely on more molecular data. Actually, there was some inconsistency between morphology and molecular data [14]. Therefore, we suggest that more

molecular data should be incorporated into analysis in future phylogeography studies.

19.3 Hybridization in Nature

Looking at the connected lines in the circular phylogenetic tree of centrarchids (Figure 19.2, circle tree), we are amazed by the extremely high incidence of hybridization in this family. Provided we exclude the newly recognized four species in *Micropterus* [2] and *L. peltastes* (not actually recognized), there are, in theory, 528 possible combinations for hybridization within the family of Centrarchidae. The exhaustive collection of hybridization, to the best our knowledge, has identified 37 natural hybridizations in centrarchids, with 22 in *Lepomis* and 11 in *Micropterus*.

Considering that, in only one case within 37, hybridization occurs between genera, intragenus hybridization is 33.3% (22/66) and 30.6% (11/36) for genus *Lepomis* and *Micropterus*, respectively, if we only consider intragenus combinations. Bolnick [26] estimated that approximately half of combinations of species have geographic overlap and possible contact, according to range

maps [27]. Therefore, the incidence of intra-genus hybridization will be over 60% for genus *Lepomis* and *Micropterus*. A comprehensive survey on natural hybridization in freshwater fishes revealed that 20.6% of hybridization was from centrarchids, out of 150 combinations representing 19 families [28], demonstrating a higher incidence of hybridization in the family of Centrarchidae than any other freshwater fish family.

As indicated in Figure 19.2, bluegill hybridize with almost all of the species in *Lepomis*, except *L. marginatus*, *L. peltastes*, and *L. symmetricus*, which are located at the extreme end of the phylogenetic tree. Interestingly, the three species located at the extreme end of the phylogenetic tree in both genus *Lepomis* and *Micropterus* did not hybridize with any species. However, based on Table 19.3, which displays search hits in the Web of Science for the topic between 1900 to 2017, we cannot rule out that the non-occurrence of hybrids in these species is due to fewer reports (less investigation or funding support).

We speculate that the incidence of hybridization was underestimated, and the reality is not as Bolnick [26] stated: “It appears safe to say that the vast majority of possible hybridizations do not occur.” First, it is highly possible that the low/no incidence of hybridization of *L. marginatus*, *L. miniatus*, and *L. symmetricus* is because of fewer investigation/samples, and it seems that the high incidence of hybridization of *L. macrochirus*, *L. cyanellus*, and *L. gibbosus* is partly because they have been intensively investigated due to their economic importance (Table 19.3). Second, Bagley *et al.* [18] reported “putative” hybrids in any of the eight species with enough samples (>10 individuals), strongly suggesting that hybridization occurs in all species. Third, it is nearly impossible to identify post-zygotic isolation of hybridization, which may produce inviable offspring [33]. Finally, yet importantly, systematic investigation of natural hybridization of centrarchids is highly challenging work, and the current information is actually comprised of patches.

The percentage of hybrids in natural populations is dependent on several factors, including species, population structure, environments, anthropogenic activities, and so on. Hybrids make up a very small proportion – up to 83% – of the local population [18, 26]. It is generally clear that molecular data play a vital role in identifying natural hybridization, especially when F_1 hybrids are viable and could produce viable offspring by themselves, or through backcross with their parents. It is worth noting, however, that it is not even possible to tell the direction or generation of hybridization using a sequence of only a few genes, while the information about direction or generation of hybridization is critical for several research fields such as speciation (a force that drives hybridization), sexual selection, and reproductive biology (see later discussion).

Centrarchids have been a very important part of recreational fisheries in North America. Quinn and Paukert [43] summarized that 19 out of 38 centrarchid species are currently of at least regional importance, based on angler preference. During the past 150 years, government institutes or agencies have made a significant impact on the composition of the local population of many species for stocking purpose [43–45]. Even though there is documentation of these translocations, the consequence of the contact of non-native species with local populations has made the already disordered situation even more complex in terms of interspecific hybridization.

19.4 Artificial Hybridization and Sex Ratio of Hybrids

Artificial hybridization of centrarchid species, mainly in *Lepomis*, has a long history. Hubbs and Hubbs [46], Ricker [47], and Childers [42] reported increased growth performance and male-skewed sex ratio of F_1 *Lepomis* hybrids, though their reports on the fertility of F_1 hybrids were inconsistent. The findings of increased growth and

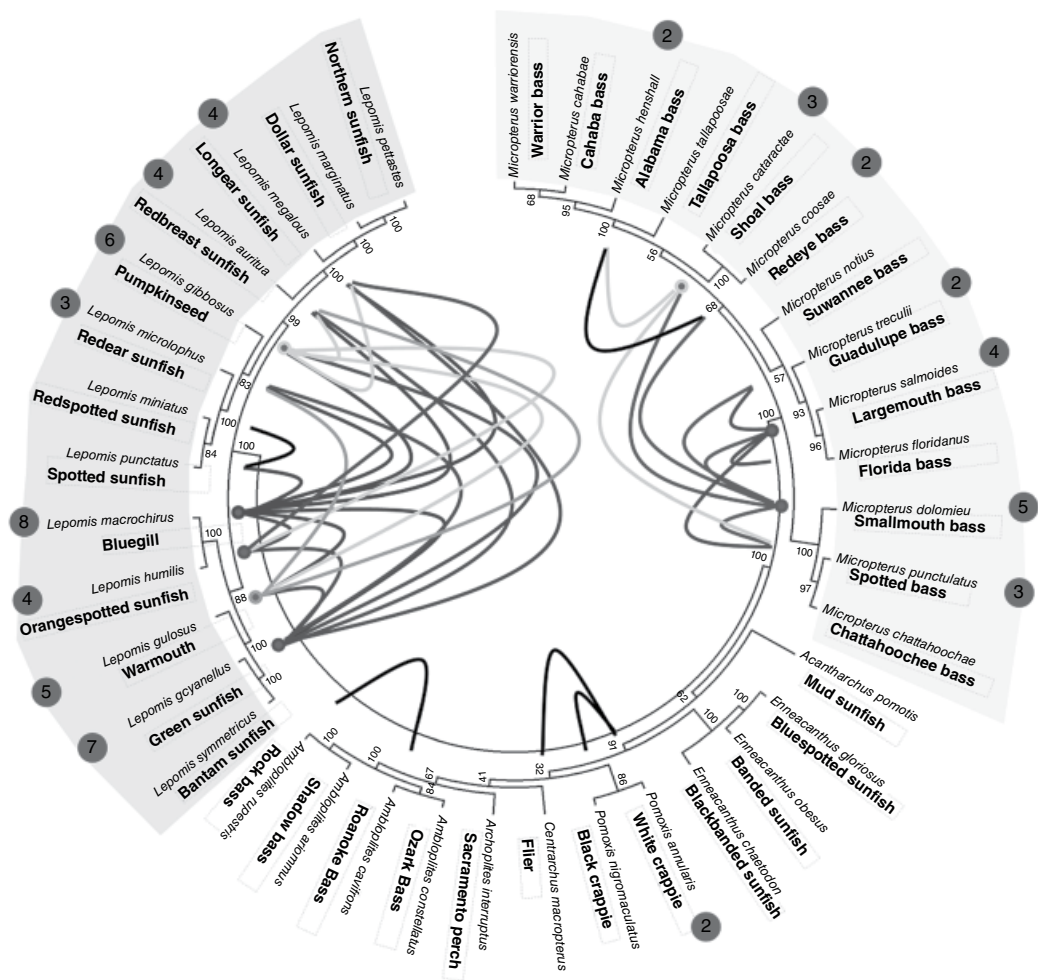


Figure 19.2 Phylogeny and hybridization in nature of all recognized centrarchid species.

The number corresponding to each species indicates the incidence of hybridization. Two shaded or colored blocks represent genus *Lepomis* and *Micropterus*, respectively. This is an exhaustive collection, to the best of our knowledge, based on published reports [14, 28–42]. This figure does not indicate the sex of parents. A neighbor-joining tree was established using Mega 6 with default parameters, using available NADH subunit 2 (ND2) genes obtained from Genbank. *Perca flavescens* was used as outgroup taxa, following Near *et al.* [5]. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed [22]. Branches corresponding to partitions reproduced in less than 50% of the bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches [22]. The evolutionary distances were computed using the Maximum Composite Likelihood method [23], and are in units of the number of base substitutions per site.

ND2 sequence for *Lepomis peltastes* is not available, and was replaced by one of *L. megalotis* because of their close relationship. Accession numbers for 38 ND2 sequences are as follows. *Lepomis. microlophus*, AY742652; *Pomoxis annularis*, AY517748; *P. nigromaculatus*, AY517752; *L. symmetricus*, AY517747; *L. punctatus*, AY517746; *L. megalotis*, AY517742; *L. marginatus*, AY517741; *L. humilis*, AY517739; *L. gulosus*, AY517737; *L. gibbosus*, AY517735; *L. cyanellus*, AY517734; *L. auritus*, AY517732; *Enneacanthus gloriosus*, AY517731; *E. chaetodon*, AY517730; *Ambloplites constellatus*, AY517729; *A. cavifrons*, AY517728; *A. ariomus*, AY517727; *Acantharchus pomotis*, AY517726; *Micropterus salmoides*, AY225744; *M. cataractae*, AY225775; *M. notius*, AY225767; *M. treculii*, AY225763; *M. punctulatus*, AY225759; *M. dolomieu*, AY225748; *L. miniatus*, AY225728; *L. macrochirus*, AY225727; *Centrarchus macropterus*, AY225726; *Archoplites interruptus*, AY225725; *E. obesus*, AY225724; *A. rupestris*, AY225723; *M. floridanus*, KJ669270; *M. henshalli*, KJ669244; *M. coosae*, KJ669241; *L. megalotis* (for *L. peltastes*), KF571702; *M. chatahoocheae*, JX502877; *M. warriorensis*, JX502873; *M. tallapoosae*, JX502871; *M. cahabae*, JX502859; *Perca flavescens*, AY225721.

Table 19.3 Search hits for topic between 1900 to 2017 in Web of Science (accessed in January 2017) and corresponding incidence of hybridization of each species in *Lepomis*.

Topic searched in between 1900-2017	Hits	Incidence of hybridization
Bluegill or <i>Lepomis macrochirus</i>	2683	8
Green sunfish or <i>Lepomis cyanellus</i>	384	7
Pumpkinseed or <i>Lepomis gibbosus</i>	756	6
Warmouth or <i>Lepomis gulosus</i>	39	5
Longear sunfish or <i>Lepomis megalotis</i>	90	4
Redbreast sunfish or <i>Lepomis auritus</i>	79	4
Orange spotted sunfish or <i>Lepomis humilis</i>	14	4
Redear sunfish or <i>Lepomis microlophus</i>	85	3
Spotted sunfish or <i>Lepomis punctatus</i>	163	2
Red spotted sunfish or <i>Lepomis miniatus</i>	12	1
Dollar sunfish or <i>Lepomis marginatus</i>	19	0
Northern sunfish or <i>Lepomis peltastes</i>	118	0
Bantam sunfish or <i>Lepomis symmetricus</i>	5	0

predominant maleness of F_1 population versus their parents greatly motivated further research and stocking trials of hybrids, especially combinations of the four popular species – bluegill, green sunfish, redeer sunfish, and warmouth (Table 19.4, sex ratio).

The collection of sex ratios of artificial hybrids, to the best of our knowledge (Table 19.4), shows that most of these sex ratios are male-predominant. It is confusing that sex ratio is male-skewed without exception, in any combination containing bluegill, as either a sire or dam (Figure 19.3, sex ratio). The male ratios of hybrids between bluegill and redeer sunfish, no matter which was the sire, were close to 100% from three independent studies.

Interestingly, Ricker [47] did not get viable hybrids of male green sunfish and female bluegill in three separate trials using conditions similar to obtain viable hybrids of the reciprocal cross, male bluegill and female green sunfish, while both Childers [42] and Whitt [35] both produced fertile F_1 and F_2 hybrids of male green sunfish and female bluegill. The only difference, from what we

can tell, is that fishes were from Indiana in Ricker's report [47] and were from Illinois in Childers and Whitt's reports [35, 42]. These results regarding the extraordinary sex ratios are beyond our current understanding of sex-determining mechanisms, including genotypic sex determination (GSD), environment-dependent SD (ESD), polygenic SD, or multifactorial SD [48–54]. Meanwhile, sex ratios of hybrids varied in some pairs, such as from 80% to 97% for male bluegill and female green sunfish, and 69% to 100% for male redeer sunfish and female green sunfish. Therefore, the most complex species in terms of sex determination are bluegill and redeer sunfish.

Haldane's rule – “when in the F_1 offspring of a cross between two animal species or races one sex is absent, rare, or sterile, that sex is always the heterozygous sex” [55] – has been used to predict the karyotype of sex chromosomes (genotype). Krumholz [56] proposed homogametic sex for males in bluegill, and heterogametic sex for females in redeer sunfish. Childers [42] speculated heterogametic sex for females in bluegill,

Table 19.4 Male percentage and parental origin of artificial *Lepomis* hybrids.

Male	Female	% male	Brood stock origin	Reference
bluegill	green sunfish	80	Illinois	[110]
		81	Michigan	[46]
		81	Missouri	[83]
		87	Michigan	[87]
		95	Mississippi	[31]
		97	Illinois	[42]
		66–78	Texas	[111]
	redear sunfish	97	Illinois	[42]
		99	Indiana	[47]
	warmouth	69	Illinois	[42]
		87	Florida	[32]
		N.S.	Florida	[32]
green sunfish	bluegill	68	Illinois	[42]
		71	Illinois	[112]
	redear sunfish	48	Illinois	[42]
	warmouth	16	Illinois	[42]
	pumpkinseed	95	Michigan	[46]
	longear sunfish	91	Michigan	[46]
redear sunfish	green sunfish	69	Illinois	[42]
		100	Illinois	[112]
	bluegill	97	Illinois	[42]
		99	Illinois	[35]
	warmouth	55	Illinois	[42]
warmouth	green sunfish	84	Illinois	[42]
	bluegill	N.S.	Illinois	[42]
	redear sunfish	N.S.	Illinois	[42]

green sunfish, and redear sunfish, and heterogametic sex for males in warmouth, based on the sex ratios of F_1 hybrids. However, as displayed in Figure 19.3, these speculations are actually conflicted, and any simple sex-determining system is not able to explain the unusually male-skewed sex ratios of *Lepomis* hybrids. To our knowledge, in any group of fish, these phenomenal results are undocumented.

In this section, we proposed four possibly interactional factors that may contribute to the male-skewed sex ratio in sunfish hybrids:

- 1) **Population- (rather than species-) based identification of sex-determining mechanism.** As proposed in Chapter 1, we should have a consideration of population, rather than species, as a fundamental unit of the sex-determining mechanism for a given species. Distinct sex-determining mechanisms in the same fish species have been reported in several cases, including Atlantic silverside, *Menidia menidia* [57], Nile tilapia, [58–60], zebrafish [50], rainbow trout [61], European sea bass [62], and bluegill [9]. In addition, individuals

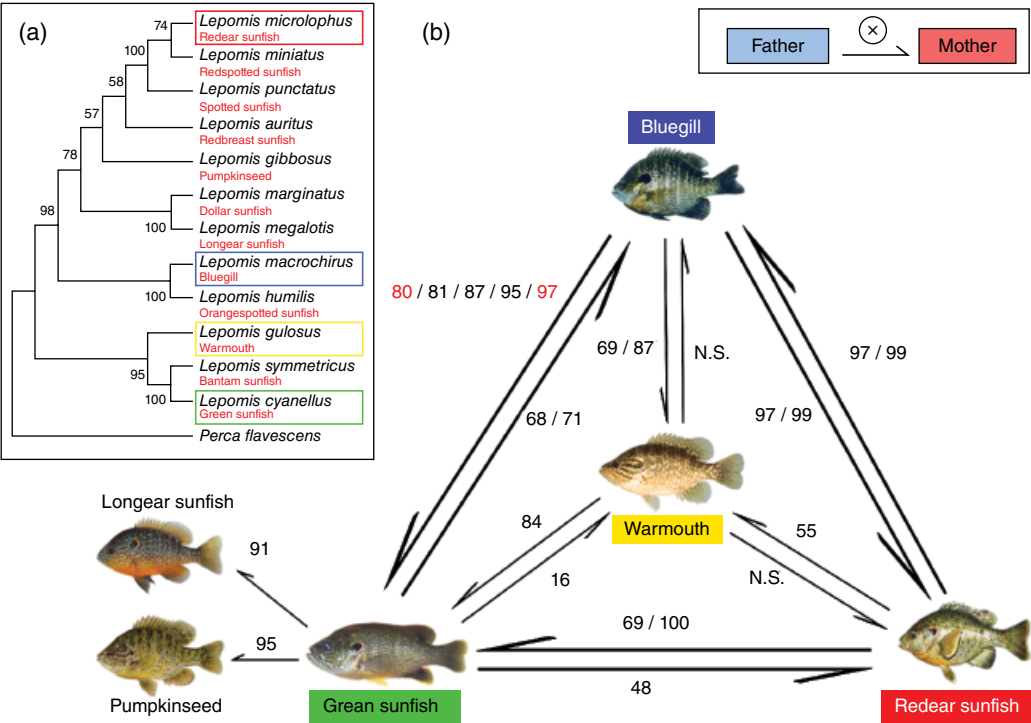


Figure 19.3 Phylogenetic tree of *Lepomis* and male ratio of artificial hybridization of several *Lepomis* species. a) Neighbor-joining tree was established using Mega 6 with default parameters, using available cytochrome b (*cytb*) gene obtained from Genbank. *Perca flavescens* was used as outgroup taxa following Near *et al.* [5]. Accession numbers are as follows: AY828996, AY828994, AY828990, AY828982, AY828977, AY828972, AY828967, AY828965, AY828963, AY828962, AY828959, AY828957, AF045357.1. b) numbers indicate male ratio for corresponding pair of species from artificial hybridization. N.S. – not survived to the stage which sex can be identified.

with TSD (temperature-dependent sex determination) and GSD were found in the same population in Atlantic silverside [63]. The sex ratio variation of hybrids of the same pair (e.g. male bluegill and female green sunfish) from different geographic locations suggests that the sex-determining mechanism may vary with populations in these sunfishes.

2) **Environment effects on sex differentiation.** Wang *et al.* [10] reported significant effects of temperature on sex ratio in one batch of bluegill fry, but no significant effects on another. We further confirmed the differential thermosensitivity of sex ratio using four geographic populations of bluegill [9]. We found that sex ratios of two populations were

sensitive to temperature, while the other two were not sensitive. Interestingly, the sex ratios of the two sensitive populations displayed opposite skewness to temperature treatments. Specifically, higher-temperature treatments produced more males in one population, while lower-temperature treatments produced more females in another population. Duffy *et al.* [63] found that the level of TSD in Atlantic silverside changes with increasing latitude, from Florida to Nova Scotia, and the length of the growing season is probably the driving force of the variation of TSD. Bluegill is a species with widespread distribution and extended spawning season from May to September [64]. It is possible that the

level of temperature-dependent sex differentiation may vary with the latitude in bluegill, though a reasonable relationship has not been established [9].

- 3) **Differential mortality.** Childers [42] reported that the mortality between fertilization and the swim-up stages was very low. According to our wet-lab experiments, mortality after the swim-up stages, specifically the transition stage of endogenous nutrition, was very high [9]. Therefore, we cannot rule out that the skewed sex ratios were not because of differential mortality, even though we did not observe a significant difference of survival among treatment groups [9].
- 4) **Parental effects.** Paternal effects on sex ratios have been reported in several taxa of animals [51] while they have not been found in fish. Chen *et al.* [65] found that the paternal methylation pattern of sex-reversed half-smooth tongue sole (*Cynoglossus semilaevis*) can be inherited to the next generation through Z-chromosomal genes. Under normal temperature conditions, there were 73% sex-reversed ZW (phenotypic male) individuals from the crosses between sex-reversed ZW males and normal ZW females [65].

In addition, a recent study reported that acquired traits of male individuals could be inherited epigenetically, through sperm RNAs and sperm RNA modifications [66]. We observed a wide range of variation of male percentage under normal temperature conditions, from 39.4% to 69.7% among different geographic strains in bluegill [9]. This variation could be the result of any abovementioned factors, or a combination of these factors.

19.5 Driving Forces of Hybridization

Centrarchidae is a clade of species with special features, including: complex sex-determining mechanisms; relatively rapid speciation; high incidence of natural

hybridization; strong sexual selection (size dimorphism; sole paternal care for offspring); and reproductive biology [1, 9, 25]. One may find some pieces of information indicating the relevance of these features with hybridization or relevance with each other. However, when we place these words together with hybridization, it looks more like a mess at the beginning. Therefore, we hope to provide relevant researchers a clear picture of the driving forces of hybridization in centrarchids (Figure 19.4).

Hybridization is the mating of females of one species with males of another species, or vice versa. In all centrarchids, males build nests and attract females during spawning season [67]. From this point, hybridization mainly relies on female choice. Therefore, hybridization is naturally associated with speciation (phenotype evolution and species recognition) and sexual selection (size disparity, coloration, and male-male competition).

The most recognized definition of “species” for sexually reproducing organisms is proposed by Mayr [68] as follows: “species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups.” Therefore, speciation is essentially the formation process of two reproductively isolated groups from a single species, and is associated with the loss of ability to interbreed with other groups. It is definitely a long and progressive process, and too long for a researcher’s lifetime. As stated by Bolnick [26], only a few species will be left if all groups capable of hybridization are reassigned, so capability of hybridization should not be used as a criterion for distinguishing species.

Within the three main clades of centrarchids, species display similarity and overlap in many aspects, including body morphology, geographic distribution, spawning periods and habitats, trophic niches, and adult body size [1, 13]. These similarities and overlaps decompose the prezygotic isolation, which is thought to be the main power of speciation events in centrarchids [38].

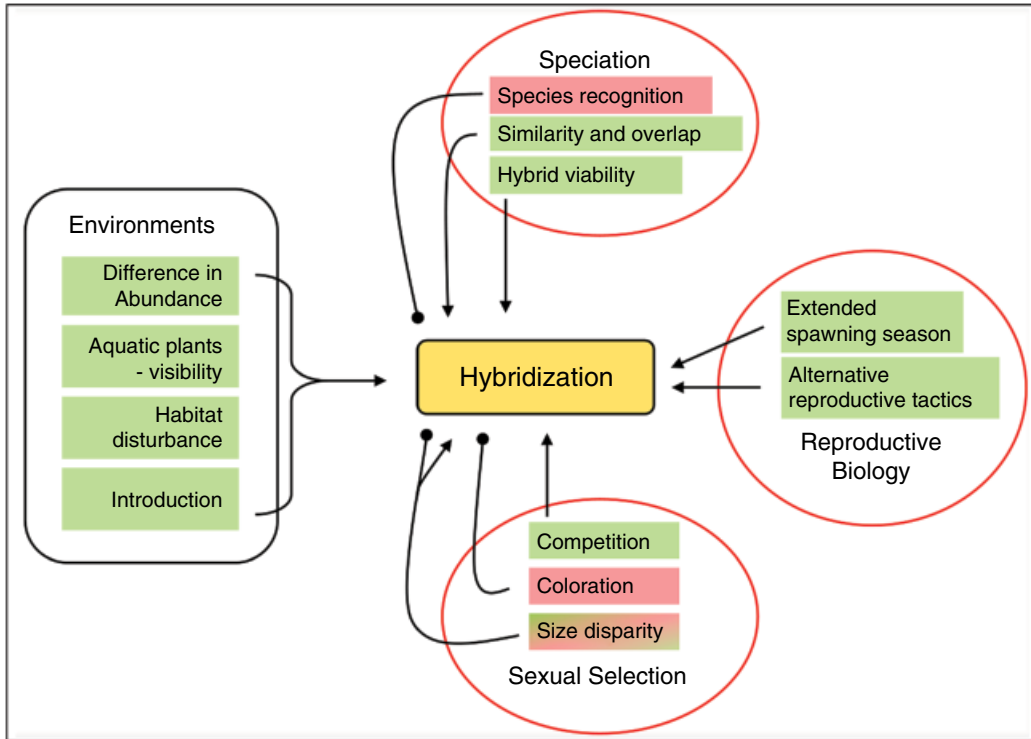


Figure 19.4 Driving forces of hybridization in centrarchids. Please find explanation in the body text. Arrows indicate promoting; solid balls indicate blocking.

Hybrid viability decreases with the divergence time of species. In centrarchids, however, hybrid viability has declined slower than most other taxa investigated so far, with a mean rate of 3.13% per million years [38]. As the long and gradual process of speciation, the decline of hybrid viability is thought to be progressive, along with diverging of population and accumulation of genetic incompatibilities [26]. Therefore, hybrid viability is probably the most important genetic background for speciation.

Phenotype evolution and species recognition, especially the accuracy of females' recognition of conspecific males, are important for hybridization. Visual cues, especially during spawning season, including extended opercular tabs, colorful spots, and brightly colored ventral surface, are probably key signals of species recognition and male quality [42, 69, 70]. Smith [13] suggests that trophic morphology and sexual

ornamentation influenced morphological diversification in centrarchids.

Within the three main clades of centrarchids, *Lepomis* displays the highest level of morphological sexual ornamentation. It is interesting, however, that *Lepomis* is the clade in which the highest incidence of hybridization is observed (Figure 19.2). Regarding size, studies found that larger species tend to be more successful maternal parents in terms of hybrid viability in *Lepomis*, using maximum body size as an indicator [38, 71], suggesting that hybrids, by the mating of large females and small males, should be more popular than other types of mating. Unfortunately, information about the sexes of parents is not available for most of the hybrids (Figure 19.2). However, female *lepomis* tend to choose larger males, for better offspring fitness [72]. Therefore, maximum body size is probably not a good indicator of the actual size of the parents, especially

in the *Lepomis* species, in which intraspecific and intrasexual size dimorphism are both significant. On the other hand, given similar morphology of small size conspecific males and large size, heterospecific males coexist; females probably prefer to choose large size heterospecific males.

Alternative reproductive tactics (ARTs) have been shown to be a driving force of hybridization in many fish species [73–75]. Garner and Neff [76] also found direct evidence that ARTs drive asymmetrical hybridization between bluegill and pumpkinseed (male bluegill with female pumpkinseed). Using microsatellite markers, they identified 13.6% larvae in pumpkinseed nests fathered by bluegill cuckolders, while no evidence showed any bluegill larvae fathered by pumpkinseed males. Cuckolders either act as “sneakers” that dart between parental males (guardian males) and females while they are mating, or act as “satellites” to deceive parental males through mimicking female characteristics [77, 78], so as to steal fertilization. These special reproductive tactics break down the female’s choice without giving the female a chance to determine mating preference and species recognition. Meanwhile, the cuckolders maybe do not distinguish species effectively, because they have to lock targets, ejaculate their sperm and escape rapidly.

Interspecific cuckoldry is probably more common when male-male competition is serious in one species, which has been observed in both natural and reared populations [9]. The incurred male-skewed sex ratio from hybridization (Figure 19.3) will, in turn, aggravate the interspecific cuckoldry. ARTs are present in four species and absent in two species in *Lepomis* [12], while currently we cannot establish any relationship between the presence of ARTs and hybridization (Figure 19.2), suggesting that ARTs is not the only driving force of hybridization in centrarchids.

An extended spawning season also provides *Lepomis* a good playground for hybridization. The evidence has demonstrated that

smaller females mature later and have a longer spawning period than larger females in pumpkinseed [79], indicating that smaller females may discount their preference during late spawning season, because ovulation is mainly induced by external cues, and ovulated eggs must be fertilized within a limited time [74].

Hybridization is often thought to be conditioned by environmental factors, including density and abundance, turbidity, aquatic plants, habitat disturbance, or introduction. Evidence indicates that hybridization tends to occur when parental species differ significantly in abundance, and rare species tend to be the female parent [37], suggesting that females may weaken preference for conspecific males, and accept heterospecific males when conspecific males are limited. Hubbs [29] found that sunfish hybridize more commonly in ponds with aquatic plants than in the ponds free of plants.

Species look similar within each clade of centrarchids [13] in terms of body shape and size, especially when visibility is reduced. In this circumstance, the opercular tabs in *Lepomis*, or discrete stripes or spots in *Micropterus* are probably the most remarkable signals, looking similar under reduced visibility. The reduced visibility challenges the female’s recognition of conspecific males, and provides cuckold male with more opportunities for stealing fertilization.

Habitat disturbance and introduction, which bring historically isolated species together, provide preconditioning for hybridization. Hubbs [29] reported that species of *Mollienisia* hybridize freely in aquaria, while they rarely hybridize in nature. During the past hundred years, centrarchid species have been introduced to different kinds of water bodies [43]. The forced re-encounters between two species of incomplete isolation provide a “paradise” for hybridization and introgression. For example, the proportion of hybrids of *M. salmoides* and *M. floridanus* increased from 2% to about 40% of the population within just four years after both species were introduced to a newly created reservoir [80].

19.6 Aquaculture of Sunfish Hybrids

Several sunfish species have become economically important as sport and food fish [9, 81, 82]. Lack of proven, profitable, and sustainable production technologies is thought to be the limiting factor of aquaculture expansion of centrarchids [81], while we think their own characteristics, such as early sexual maturity, considerable size disparity (undetermined growth), overpopulation, and strong social interaction, are also obstacles for commercial production. Some *Lepomis* hybrids display male-skewed sex ratios (Figure 19.3 and Table 19.4), as well as unverified hybrid vigor, which bring about the remarkable popularity of hybrid culturing and stocking. Meanwhile, other kinds of hybrids may also have a promising future for aquaculture and recreational fisheries.

19.6.1 Hybrid Bluegill 1 – Green Sunfish ♀ × Bluegill ♂ (GB Hybrids)

The hybrid produced by the mating of female green sunfish and male bluegill (abbreviated as GB hybrids) is the most popular sunfish hybrid. As early as 1933, Hubbs [46] found increased growth and male-skewed sex ratios in some hybrids, including the GB hybrids. Since then, GB hybrids have been overwhelmingly produced in North America until today. Even though the GB hybrids are the most common sunfish hybrids to date, we were surprised to find no controlled experiments that have tested whether GB hybrids really grow faster than their parent species, until Hayward and Wang found that GB hybrids did not actually display superior growth capacity than their parent species [83, 84].

The most cited studies, which were conducted by Childers [33, 42], Kurzawski [85], and Brunson [86], did not conclude the growth advantage of GB hybrids. For example, in 1961, Childers compared growth of

GB hybrids with green sunfish at the same density, and found that the growth of hybrids was not significantly different from green sunfish [33, 42]. Childers also concluded: “The question of whether certain F_1 hybrid sunfishes are superior to their parent species in rate of growth cannot be answered until high density populations containing equal numbers of equal-sized hybrids and parent species are studied” [33]. Other studies did some different performance evaluation of GB hybrids, rather than growth comparison between GB hybrids and their parents.

Hayward and Wang [83] conducted a series of experiments to compare growth performance between GB hybrids and bluegill. They found bluegill reached nearly twice the weight of GB hybrids in a 200-day period, starting at 30 grams, though bluegill gained less weight than GB hybrids as age-1 fingerlings. They also observed social interactions, and intrasexual and intersexual competition for food and space played a very important role in growth and size disparity. Later experiments by Hayward and Wang [84] demonstrated that male bluegill actually grew faster, and reached food-market size earlier, than GB hybrids.

While the GB hybrids are most popular, several advantages compared to their parent species are actually exaggerated. There is no research evidence showing that GB hybrids exhibit a higher acceptance of formulated feeds, a greater tolerance to poor environmental conditions, or a higher vulnerability to hook and line than their parent species. The dispersal of these unsupported advantages were mainly because some advantages of other hybrids were taken for granted in GB hybrids.

The reports associated with the fecundity of GB F_1 hybrids are inconsistent. In 1933, Hubbs and Hubbs [46] reported that GB hybrids were unable to reproduce, because males were sterile and ovaries were not well developed. Childers stocked GB F_1 hybrids in ponds, and they failed to produce an F_2 generation, while many other hybrid types

produced abundant F_2 generations in the 1960s [42]. Later studies by Laarman in 1979 reported the mean fecundity was 14,393 per female, and enough numbers of F_1 stocking successfully produce thousands of F_2 generations [87]. Interestingly, in the 2000s, Hayward and Wang [83, 84] found that gonadosomatic index of GB F_1 hybrids was much higher than bluegill, in both males and females, indicating that gonad development of GB F_1 hybrids is probably not an issue.

After a careful checking of the original reports, we are surprised. This never-verified information about the growth advantage of GB hybrids has been spread around for more than 80 years, and has caused an unnecessary popularity of GB hybrids in aquaculture and fishery stockings. As new evidence has demonstrated that there is no growth advantage of GB hybrids, we think all-male production will be the best solution for the current issues in sunfish aquaculture, including early sexual maturity, size disparity, overpopulation, and strong social interaction.

Our work in recent years suggests that bluegill display a ZW/ZZ sex-determining mode, at least in some geographic populations, through determining sex ratios of offspring sired by normal males and sex-reversed females (see Chapter 17). In addition, our most recent trials found that the survival rate is much higher, and coefficient of variation for bodyweight is lower, in all-male bluegill batches than in mixed-sex batches (Chapter 17). These results provide the bright prospect for all-male production and rearing.

19.6.2 Hybrid Bluegill 2 – Redear Sunfish ♀ × Bluegill ♂ (RB Hybrids)

As we mentioned above, the male ratios of hybrids between bluegill and redear sunfish, no matter which one was the sire, were close to 100% from three independent studies (Table 19.4). These exciting reports make RB hybrids the second most popular of the sunfish hybrids. However, no experimental

testing of the growth advantage of RB hybrids has occurred, despite their spread. In 1950, Krumholz [56] stocked 16,075 RB hybrids in 78 small ponds with different density and numbers, and only caught 722. Different survival and densities, uncontrolled rearing conditions, contamination, and great size disparity [56] made it not even possible for further statistical analysis, though the author concluded: “The hybrids were relatively larger and heavier for their length than individuals of the same age groups in either of the parent species taken from similar ponds.” The most cited study, conducted by Childers [42], did not compare growth performance of RB hybrids to their parent species.

The nearly 100% male sex ratio of RB hybrids is still attractive for rearing and stocking purposes, even though none of the advantages – such as better growth, higher acceptance of formulated feeds, greater tolerance to poor environmental conditions, or higher vulnerability to hook and line – have been experimentally verified.

19.6.3 Hybrid Crappie

Hybridization of black and white crappies in natural waters is common, and hybrids make up a high proportion in natural populations [36]. Several reports have evaluated the growth of either kind of hybrids, male white crappie and female black crappie, or male black crappie and female white crappie. Results showed that growth rate of hybrid crappie was better than their parent species, or at least comparable [88–92]. Survival of hybrid crappie was equal to that of the parent species in two studies [89, 92], while their survival was lower, especially beyond age 1, in another report [90]. Better growth performance, relative lower fecundity of F_1 hybrid crappies [90], and free mating of black and white crappies in adequate conditions, make them a remarkably promising alternative to stocking and commercial aquaculture of their parent species.

19.6.4 Hybrid Largemouth Bass

Largemouth basses have occupied an important place in recreational fisheries in North America since the 1880s [43], and have become an economically important aquaculture species in North America and China in recent years [81, 93, 94]. The two subspecies, largemouth bass, *M. m. salmoides*, and Florida bass, *M. m. floridanus*, were recognized as valid species earlier [25, 95]. Comparative evaluation of growth performance, and vulnerability to angling of the two species and their reciprocal hybrids (abbreviated as LF hybrids and FL hybrids, male first), had been evaluated in several reports since the 1970s, while the results were inconsistent [93, 96–100]. The conflicting results were attributed to the questionable identification of species, different environmental designs, different experimental geographic locations, and genotype-age interactions [93, 98]. A lot of evidence demonstrated effects of genotype-environment interactions on growth performance in many species [101–108], and genotype-environment interactions may explain at least part of these inconsistent results.

Five of the six reports found largemouth bass grew significantly faster than Florida bass and hybrids either in separate or communal ponds (Table 19.5), while Inman reported that LF hybrids grew faster than largemouth bass from age 1 to age 5 in a lake [97]. It is generally consistent that Florida bass performs the worst in terms of growth, regardless of experimental locations, growth stage, and rearing conditions. The findings on the vulnerability to angling were also contradictory (Table 19.5). It is unfortunate that none of the six reports had provided rearing conditions of experiments, specifically water temperature and water quality.

As a promising aquaculture species, some other features should also be taken into account. Williamson and Carmichael [99] conducted a series of relatively comprehensive studies to evaluate the performance of the four strains. They found that largemouth bass did better than Florida bass and the two hybrids in several aspects, including growth,

stress resistance, tolerance to ammonia, tolerance to low temperature and low oxygen, acceptance of formulated feed, and feed conversion efficiency. Carmichael *et al.* [109] also found largemouth bass was much more tolerant to low temperature than Florida bass. All of these findings strongly suggest that largemouth bass is the best strain for either recreational fisheries or aquaculture, though their performance in the recirculating system, or other rearing systems with well-controlled conditions, has not been comparatively evaluated.

19.7 Conclusion

Sunfishes are definitely an excellent clade of species to investigate speciation, phylogeography, hybridization, reproductive strategies, and the complexity of sex determination. In this chapter, we have indicated several areas in which further research will be specifically important. The complex sex-determining mechanisms in *Lepomis* are probably the most challenging work. The driving forces of natural hybridization will be the everlasting question. We are surprised to find that some unverified or incorrect information about hybrid vigor of sunfishes has been spread for a long time, even though new evidence has overthrown the previous information. This unverified or incorrect information has actually caused a misleading popularity for some hybrids, while the most negative consequence of releasing hybrids remains to be a significant risk for the natural population [26, 45].

Acknowledgments

This work was supported by the National Institute of Food and Agriculture (NIFA) and North Central Region Aquaculture Center (NCRAC), U.S. Department of Agriculture. Salaries and research support were provided by state and federal funds appropriated to The Ohio State University, Ohio Agricultural Research and Development Center.

Table 19.5 Reported experimental evaluation of growth and vulnerability to angling of largemouth bass, Florida bass, and their hybrids.

Studied Species	Origin	Experimental location	Growth stage	Rearing conditions	Growth	Vulnerability	Literature
LMB	Alabama	Alabama	age-0	separate ponds	LMB > LF hybrids = FB	FB > LMB	[96]
FB	Florida						
LF hybrids							
LMB	Texas	Texas	age-1 to age-2	Lake	LF hybrids > LMB > FB	FB > LMB > LF hybrids	[97]
FB	Florida		age-2 to age-5	Lake	LF hybrids > FB > LMB		
LF hybrids	Texas						
LMB	Texas	Texas	age-0	separate ponds	LMB > FB = LF hybrids = FL hybrids	LMB > LF hybrids > FL hybrids > FB	[98]
FB	Florida		age-1	separate ponds	LF hybrids > LMB = FL hybrids > FB		
LF hybrids	Texas						
FL hybrids	Texas						
LMB	Texas	Texas	age-0	separate ponds	LMB > FL hybrids > LF hybrids = FB	Not studied	[99]
FB	Florida			communal ponds	LMB > FL hybrids > LF hybrids = FB		
LF hybrids	Texas						
FL hybrids	Texas						
LMB	Wisconsin	Illinois	age-0	communal ponds	LMB > FL hybrids > LF hybrids > FB	Not studied	[100]
FB	Florida		age-1	communal ponds	LMB > FL hybrids = LF hybrids > FB		
LF hybrids	Illinois						
FL hybrids	Illinois						
LMB	Guangzhou, CN	Guangzhou, CN	age-0	communal ponds	LMB > FL hybrids = LF hybrids	Not studied	[93]
LF hybrids	Guangzhou, CN						
FL hybrids	Guangzhou, CN						

LMB – largemouth bass *Micropterus salmoides*; FB – Florida bass *M. floridanus*; LF hybrids – offspring of male largemouth bass and female Florida bass; FL hybrids – offspring of male Florida bass and female largemouth bass. CN – People's Republic of China.

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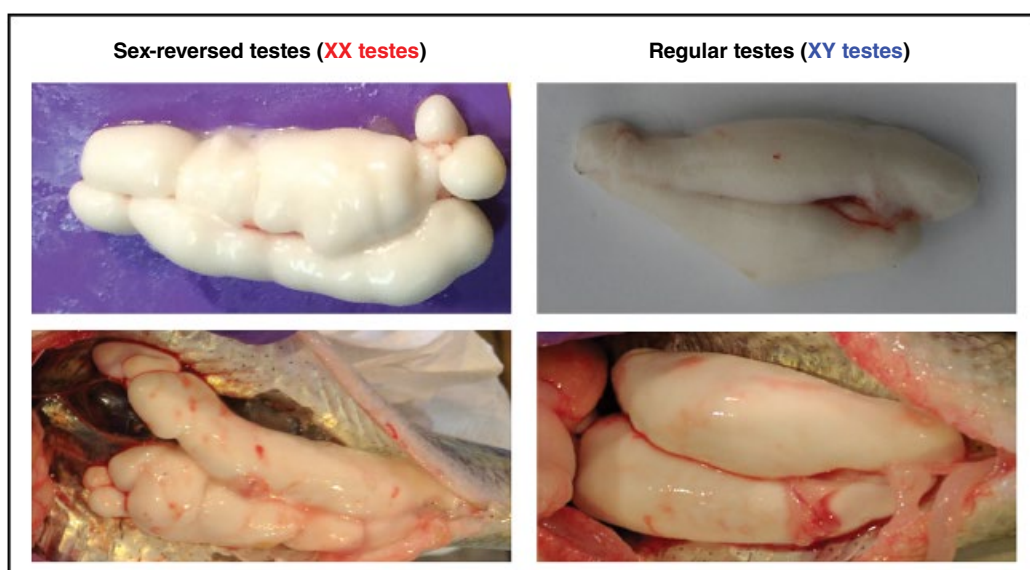


Figure 1.5 Morphological difference between the testes of nemales (XX-males) and regular males (XY-males) of yellow perch. Sex-reversed testes (left) are characterized by rough surface, cyst-like structures, indivisible single part, which have never been observed in regular testes.

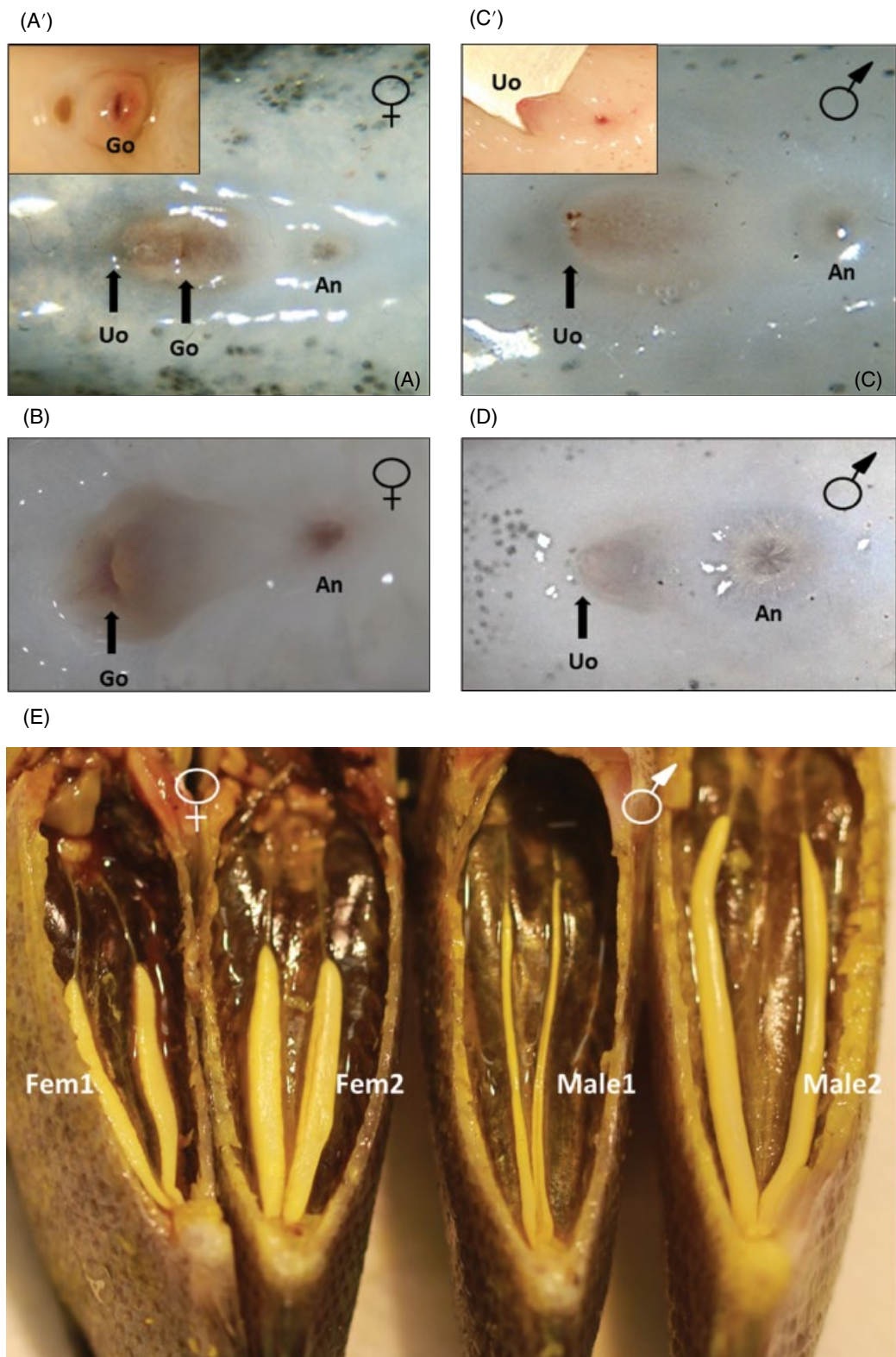


Figure 9.3 A, A', B: Females' urogenital papilla that are roundish with a horizontal genital orifice (Go), as well as the oviduct (Uo) and anus (An).

C, C', D: Males' urogenital papilla, oval in appearance, with only one oviduct and the anus.

E: Macroscopic differences between young ovaries (left) and testis (right) in shape (roundish/oval in ovaries), diameter (bigger in ovaries), and length (testis occupy the whole peritoneal cavity, whereas ovaries 2/3).

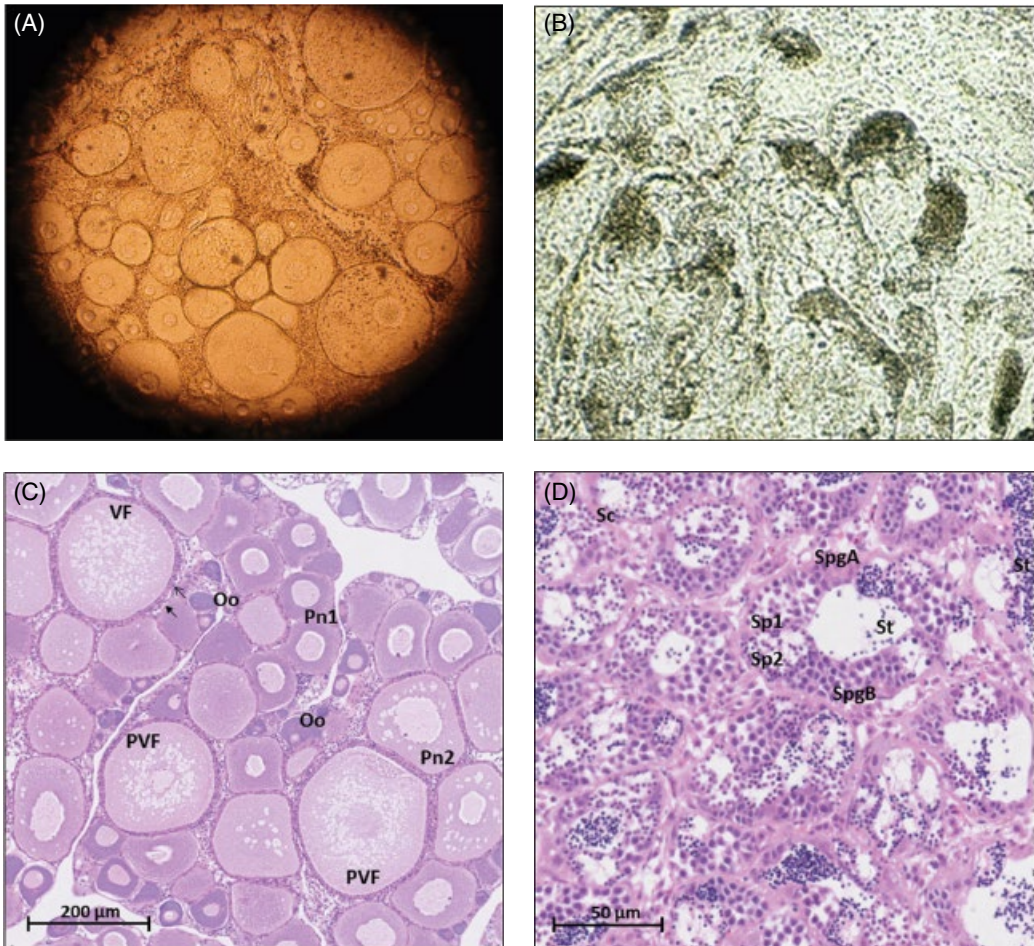


Figure 9.4 **A. Ovary squash** microscope appearance showing large oocytes and nucleus; **B. Histology of a maturing ovary** with primary oocytes (Oo), primary perinuclear oocytes (Pn1), secondary perinuclear oocytes (Pn2), previtellogenic oocytes (PVF), and vitellogenic oocytes (VF) with follicles in which the granulosa and theca cells can be distinguished (arrows); **C. Testis squash** microscope appearance with numerous small cells (spermatocytes); **D. Histology of a testis** with several tubules showing cysts of spermatocytes I (Sp1) and II (Sp2), spermatogonia A (SpgA), and slightly smaller spermatogonia B (SpgB) cells and spermatids (St).

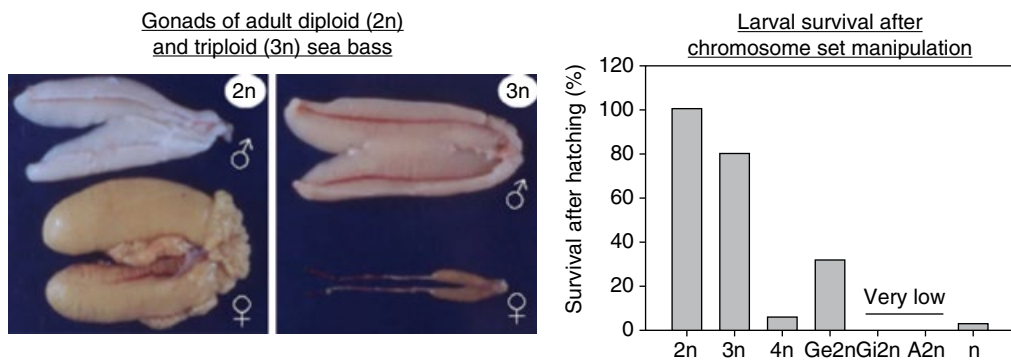


Figure 16.1 Effects of chromosome set manipulation in the European sea bass.

Left panel: Photographs of testis (above) and ovaries (below) of adult diploid (2n) and triploid (3n) males and females.

Right panel: Percentage of larval survival at hatching relative to control diploids (2n) including triploids (3n), tetraploids (4n), meiogynogenetics (Ge2n), mitogynogenetics (Gi2n), androgenetics (A2n), and haploids (n).

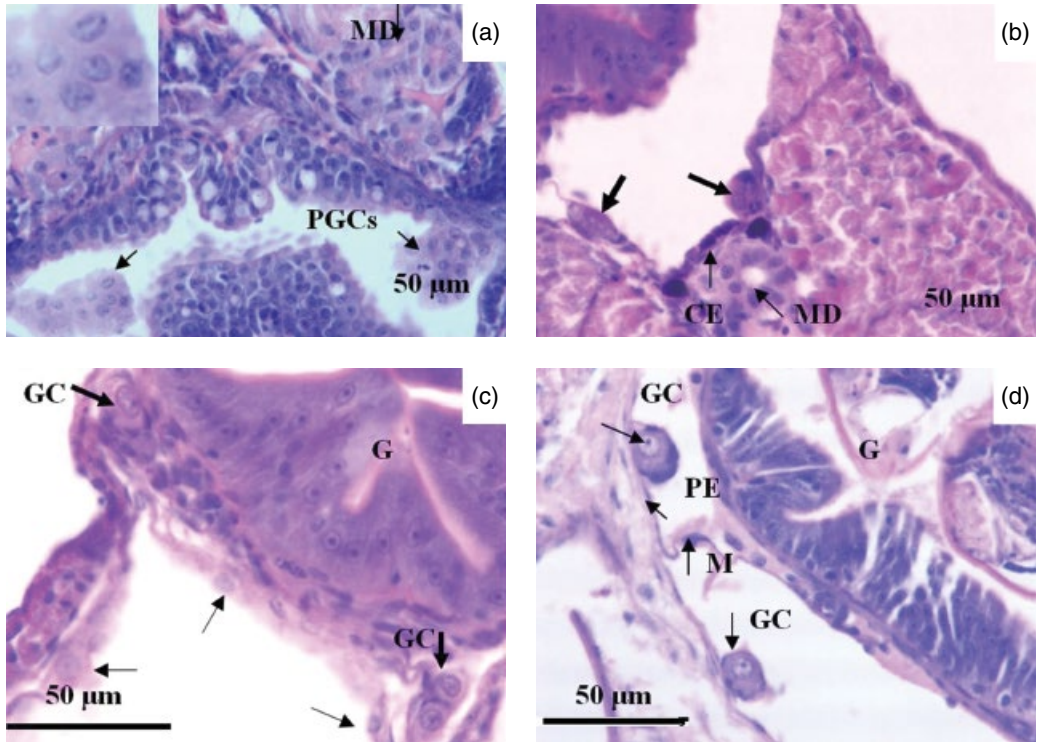


Figure 17.1 Undifferentiated gonads of the bluegill sunfish at 5–40 dph in the slow-growing batch.

- a) Clusters of primordial germ cells at 5 dph. Higher magnification of primordial germ cells (inset).
- b) Primordial gonads at 25 dph. A pair of gonadal primordia is indicated by the thick arrows.
- c) Primordial gonads at 30 dph. The migratory germ cells are indicated by the arrows, and a pair of gonadal primordia is indicated by the thick arrows.
- d) Undifferentiated gonads at 40 dph.

Abbreviations: CE – celomic epithelium; G – gut; GC – germ cells; M – mesentery; MD – mesonephric duct; PE – peritoneal epithelium; PGCs – primordial germ cells.

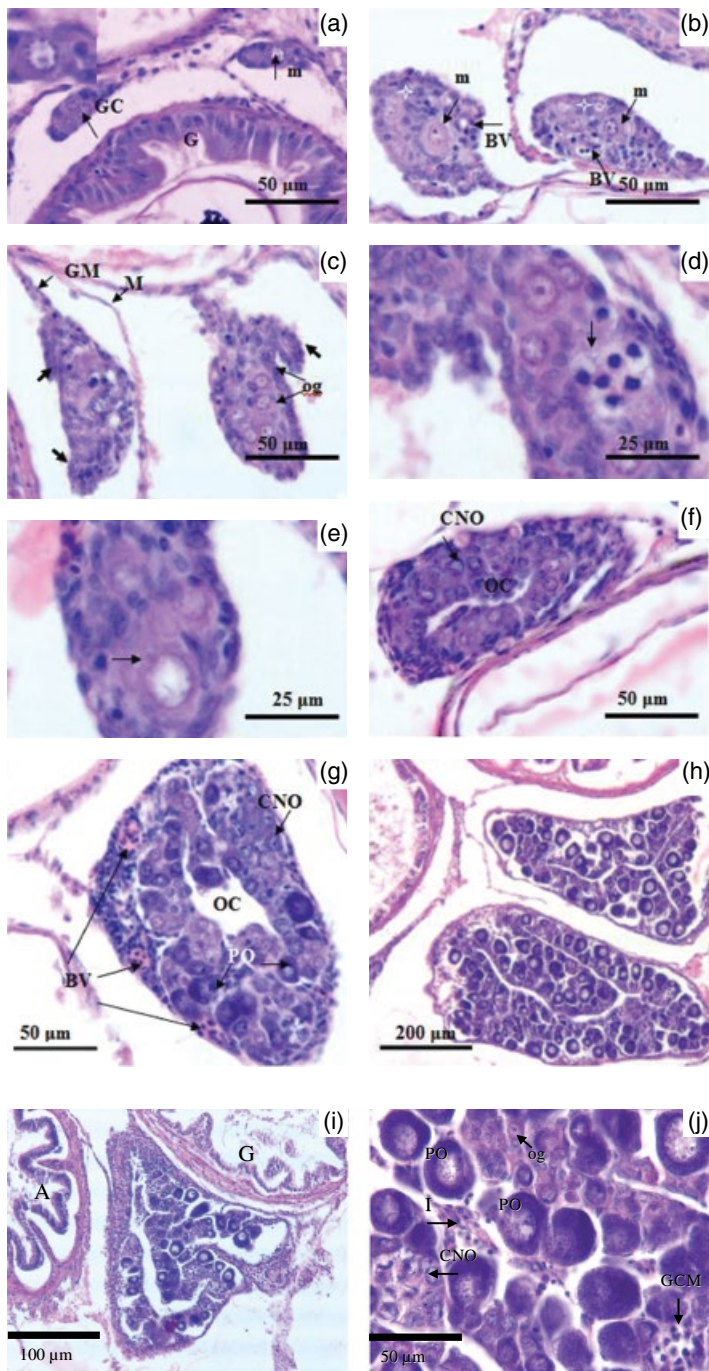


Figure 17.2 Ovarian differentiation in the bluegill sunfish at 50–90 dph in the slow-growing batch. a) Presumptive ovary at 50 dph, showing gonadal type I in which germ cells are undergoing early mitosis (m). Higher magnification of mitotic germ cells (inset). b) Presumptive ovary at 60 dph, showing germ cells multiplied in number, and blood vessel. The numerous somatic cells are indicated by stars. c) Initial ovary at 70 dph, showing somatic elongations forming the initial ovarian cavity formation are indicated by the thick arrows. d) Initial ovary at 70 dph, showing germ cell nests with zygotene (bouquet) stage of oocyte meiosis (arrow). e) Initial ovary at 70 dph, showing oocyte undergoing meiosis at pachytene stage (arrow). f) Ovary at 80 dph, showing the ovary cavity (OC) and oocytes at chromatin-nucleolus stage (arrow). g) Ovary at 90 dph, showing some oocytes at peri-nucleolus stage (PO). h) Ovary at 90 dph, showing many peri-nucleolus oocytes. i) Ovary at 90 dph, showing the fusion in the anterior part of two gonadal tissues. j) Ovarian tissue at 90 dph. Abbreviations: m – meiotic germ cell; A – anus; BV – blood vessel; CNO – chromatin-nucleolus oocyte; G – gut; GC – germ cells; GCM – germ cells undergoing meiosis at zygotene stage; GM – gonadal mesentery; I – interstitial or stromal tissue; M – mesentery; OC – ovarian cavity; og – oogonium; PO – peri-nucleolus oocyte.

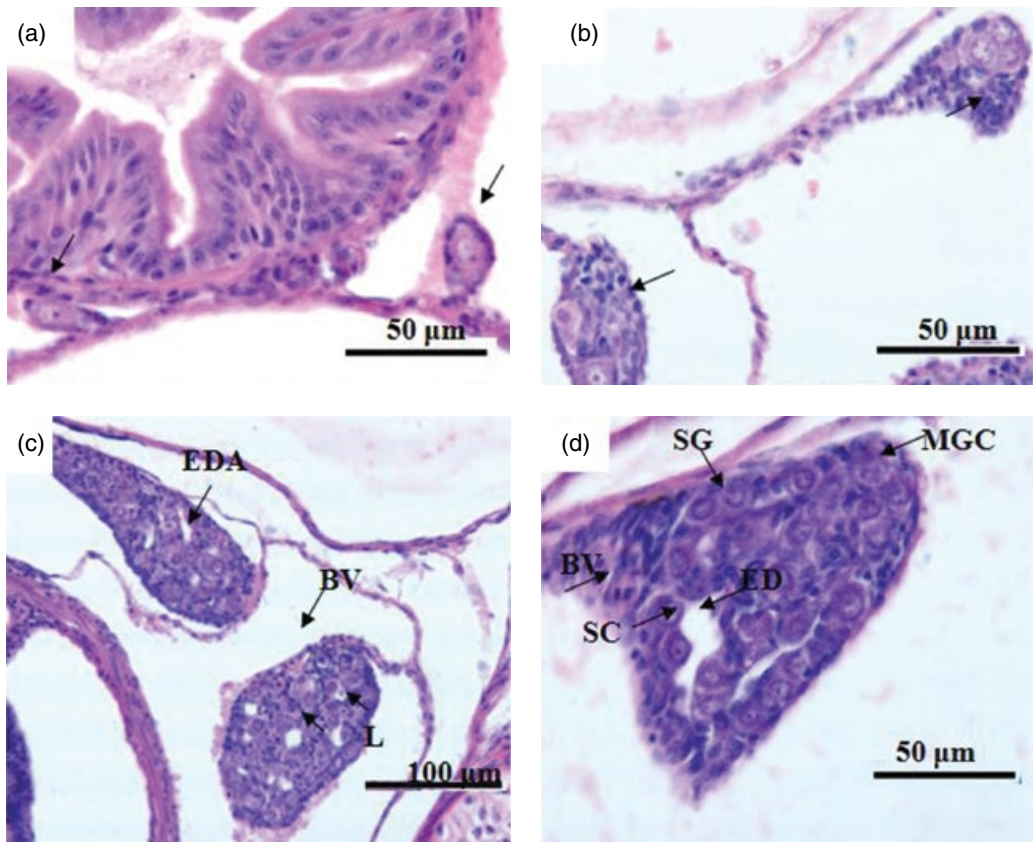


Figure 17.3 Testicular differentiation of the bluegill sunfish at 50–90 dph in the slow-growing batch.

a) Fry at 50 dph showing gonadal type II tissue (arrows).

b) Presumptive testis at 70 dph. The aggregations of stromal cells are indicated by arrows.

c) Testis at 80 dph, showing the efferent duct anlage, lobule, and blood vessel.

d) Testis at 90 dph, showing evident efferent duct, spermatogonia undergoing mitotic divisions to become spermatocytes, and the onset of meiosis.

Abbreviations: BV – blood vessel; EDA – efferent duct anlage; ED – efferent duct; L – lobule; MGC – meiotic germ cells; SG – spermatogonium; SC – spermatocytes.

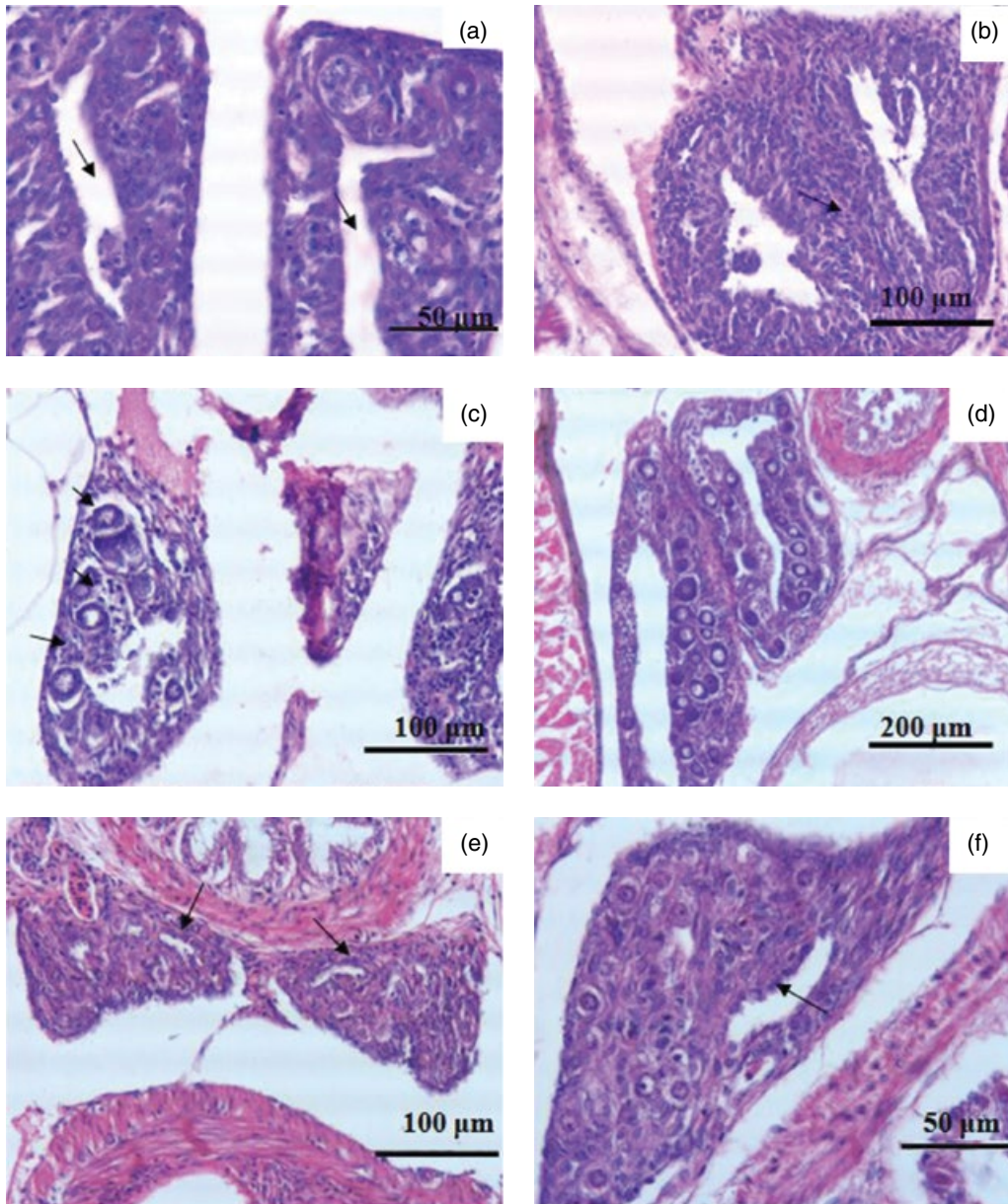


Figure 17.4 Gonadal development of the bluegill sunfish in the fast-growing batch.

- a) Ovary at 50 dph, showing the ovarian cavity.
- b) Ovary at 50 dph, showing the beginning of fusion.
- (c) Ovary at 60 dph, showing the peri-nucleolus oocytes.
- d) Ovary at 80 dph, showing the numerous peri-nucleolus oocytes.
- e) Testis at 70 dph, showing the efferent duct anlage.
- f) Testis at 80 dph, showing the evident efferent duct.

Sex Control in Aquaculture

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Volume II

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WILEY Blackwell

This edition first published 2019
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John Wiley & Sons, Inc., 111 River Street, Hoboken, NJ 07030, USA

John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK

Editorial Office

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Cataloging-in-Publication Data

Names: Wang, Han-Ping, 1958– editor. | Piferrer, Francesc, 1960– editor. | Chen, Songlin, 1960– editor.

Title: Sex control in aquaculture / edited by Han-Ping Wang, Francesc Piferrer, and Song-Lin Chen.

Description: Hoboken, NJ : John Wiley & Sons, 2018. | Includes bibliographical references and index. |

Identifiers: LCCN 2018007574 (print) | LCCN 2018014556 (ebook) | ISBN 9781119127284 (pdf) |

ISBN 9781119127277 (epub) | ISBN 9781119127260 (cloth)

Subjects: LCSH: Aquaculture—Management. | Brood stock assessment.

Classification: LCC SH155.5 (ebook) | LCC SH155.5 .S49 2018 (print) | DDC 639.8—dc23

LC record available at <https://lcn.loc.gov/2018007574>

Cover Design: Wiley

Cover Image: © Pgiam/Getty Images; © Ann and Steve Toon/Alamy Stock Photo; © Wolfgang Pölzer/Alamy Stock Photo; © Brandon Broderick/Alamy Stock Photo; © doidam10/Getty Images; © Kenneth Chamberlain for front cover; © Amaury Herpin, Chantal Cauty, Catherine Labbé for front cover (in book acknowledgement)

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To 97-year old Zhennan Wang and 90-year-old Dusheng Peng, Hong Yao, Alan and Eileen Wang – superior parents, lovely wife, and fast-growing male and female offspring that one of the editors of the book is lucky to have.

To Arianna and Núria Piferrer, the most precious all-female progeny that one of the editors of this book has ever produced.

To Youmei Li, Shengqin Xia and Ying Chen, the most important three ladies – mother, wife, and daughter of one of the editors of this book.

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Preface

This book was motivated by an increasing, strong need for the control of sex ratios and monosex production knowledge and technology by the rapid growing global aquaculture industry. Currently, aquaculture – the fastest growing food-producing sector – contributes about 50% of the world's food fish, based on the Food and Agriculture Organization (FAO) latest reports. Sex control in aquaculture serves different purposes.

First and foremost, a wide spectrum of aquacultured species show sexual dimorphism in growth and ultimate size, whereby one sex grows faster than the other or attains a larger size. Thus, there are important benefits in rearing only the fastest-growing sex or monosex production. Second, in some species, precocious maturation and uncontrolled reproduction need to be prevented. Third, some negative impacts of reproduction on product quality or disease resistance need to be prevented in some species. Fourth, in sex-changing hermaphrodites, sex ratio control can benefit broodstock management. Finally, there are some species where the gonads or gametes of females have special economic value, e.g., caviar.

Therefore, sex control for the production of monosex or sterile stocks is extremely important for aquaculture professionals and industries to improve production or to increase revenue, reduce energy consumption for reproduction, and eliminate a series of problems caused by mixed-sex rearing or sexual maturation. Incidentally, the same principles used for sex control in aquaculture can be used in population control to eliminate

undesired invasive species – an aspect that is also dealt with in this book.

The two volumes of “*Sex Control in Aquaculture*” together is composed of 11 parts and a total of 41 chapters, which have been written by leading experts in the field. Volume I consists of Parts I to V (Chapters 1–19), while the remaining Parts VI to XI (Chapters 20–41) make up Volume II.

With eight chapters, Part I is concerned with the theoretical and practical basis of sex determination/differentiation and sex control in aquaculture. These chapters provide the concepts and rationale for sex control in aquaculture, and present our current knowledge on basic aspects of the genetic, endocrine, and environmental mechanisms for sex determination and sex differentiation, including epigenetic regulation. Readers will find a detailed, most up-to-date description of the underlying mechanisms responsible for the establishment of the sexes and, hence, the sex ratios. Several chapters also provide information on chromosome set manipulation techniques, hybridization and new gene knockout, and the application of these different approaches to aquaculture. There is also a chapter on the application of sex ratio manipulation for population control (e.g., for the management of invasive species).

Parts II to XI, or Chapters 9 to 41, contain detailed protocols and key summarizing information for the sex control practice of 35 major aquaculture species or groups with sexual size dimorphism, monosex, or polyploidy culture advantages. These major aquaculture species

include Nile tilapia, blue tilapia, Mozambique tilapia, black-chin tilapia, salmonids, European sea bass, bluegill, largemouth bass, crappies, yellow perch, Eurasian perch, channel catfish, yellow catfish, southern catfish, half-smooth tongue sole, turbot, southern flounder, summer flounder, Japanese flounder, Atlantic halibut, Pacific halibut, spotted halibut, sturgeon, shrimp, prawn, Atlantic cod, malabar grouper, honeycomb grouper, large yellow croaker, rice field eel, the Japanese eel, the European eel, the American eel, and common carp.

All chapters are arranged in the same structure and format for easier reading and the extraction of useful information, but each chapter has its own unique story. Therefore, the two volumes of the book can be read cover to cover, or you can pick any chapter, depending on your interests. However, we suggest that all readers start with Chapters 1 through 8 (Part I), in order to get a comprehensive background before moving to a particular species or group of species.

In summary, the use of sex control in aquaculture is becoming one of the most important topics for both aquaculture research and the aquaculture production industry. This book synthesizes relevant and recent information on sexual development principles and sex

control practice, and emphasizes their applications for use in the aquaculture industry. It bridges the gap between theory and practice in sex control of farmed species, including new developments and methodologies used in sex determination, differentiation, monosex, and polyploidy production for aquaculture.

Thus, the book will appeal to a large audience: Scientists working directly in aquaculture research or food production will find relevant information on the principle and practical aspects of sex control in aquaculture; and scientists working with basic aspects of fish/shrimp biology, reproductive endocrinology, genetics, and evolutionary biology will find abundant information regarding sex in related species. Likewise, biologists working in the farming industry, hatchery management, fisheries, as well as related administrators, will benefit from clear and practical information on how to apply sex control in aquatic animals. Finally, young researchers and graduate students will learn about a field – the establishment of sex in fish/crustaceans and its control – with both basic and applied connotations.

May, 2018

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Acknowledgements

We thank Sarah Swanson at The Ohio State University for her efforts in chapter coordination, format review, and editing assistance. Thanks also go to Joy Bauman, Jordan Maxwell, and Bradford Sherman at The Ohio State University for their English editing. We thank Amaury Herpin, Chantal Cauty,

Catherine Labbé, and Ken Chamberlain for providing photos for the front cover.

We thank all the anonymous reviewers for their peer-review of the book chapters and constructive comments for improvement of the book quality.

Part VI

Sex Determination and Sex Control in Percidae

Sex Determination and Monosex Female Production in Yellow Perch

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20.1 Introduction

Yellow perch, *Perca flavescens*, are members of the Percidae or perch family, native to North America, and are very common in the northwest to Great Slave Lake and west into Alberta. Yellow perch are closely related, and morphologically similar, to the Eurasian perch, *Perca fluviatilis*. This species is a particularly important aquacultural and ecological species in the Great Lakes Region (GLR) and the Midwest USA.

The demand for yellow perch has remained very high in the GLR, since they are the traditional fish species used by social organizations, local restaurants, and at the Friday night fish fry dinners in many Great Lakes states. Although there are several mature aquaculture industries, such as catfish, trout and salmon, yellow perch has its unique and niche market in the GLR. One reason in particular that has hindered expansion has been the relatively slow growth of currently cultured populations of this species. Using current yellow perch strains, only 60% of the fish cultured in aquaculture operations reach market size in a normal growth cycle (16 months), with the remainder below market size. This is an inefficient use of resources, feed, and operational costs, and leads to marginal profits at best. Therefore,

improving and promoting yellow perch growth and aquaculture through selective breeding will significantly improve the profitability of fish farmers.

Historically, the supply of yellow perch has largely relied on capture fisheries in the Great Lakes. Wild harvests had declined to 11–18 million pounds per year during the 1980s and 1990s, and are currently limited to less than six million pounds per year. Except for Lake Erie and Green Bay, commercial fishing of yellow perch has been closed in the Great Lakes, due to overfishing, and quotas for sport fishing have also been greatly reduced. New viruses, such as viral hemorrhagic septicemia, will further threaten wild yellow perch populations. Increasing yellow perch aquaculture production will reduce pressure on the natural resource, thereby sustaining and improving the ecological environment and natural resource in the Great Lakes.

Yellow perch have a high nutritional value, due to their low fat and phospholipid content. This species has mild taste and firm flesh, making it appealing to both consumers and restaurant industries, and providing for a long shelf life, resistance to freezer damage, and minimal problems with off-flavor and cooking odors [1]. The health benefits of yellow perch and its history of consumer fidelity in the market place present significant

marketing opportunities for fish farmers. Farm-raised fish have a high quality value among many consumers, and the health benefits of yellow perch contribute to society as a whole by providing a safe, high quality, and healthy product.

Yellow perch aquaculture has received tremendous interest in the Midwest and elsewhere in the United States during the past 20 years, due to their high market demands, the decline of wild populations, and concern over micro-contaminant levels in Great Lakes fishes. Some major techniques for artificial reproduction [2], commercial production of feed-trained fingerlings [3], and grading and production method [4, 5], have been successfully developed, greatly facilitating the yellow perch industry.

Extensive evidence shows that yellow perch females grow significantly faster and reach larger body sizes than males in mixed populations in aquaculture practice and lab experiments. Recent data from our study shows that females also have a higher fillet yield than males (Wang, unpublished data), supporting the conclusion that a female monosex culture may have considerable potential for increasing the efficiency and profitability of yellow perch aquaculture.

Genetically improved lines of yellow perch have been developed recently, and tests at three sites at different latitudes showed that improved fish exhibited significantly higher production, higher survival, and higher growth rates than local strains. Therefore, developing neomale brood stocks and genetically fast-growing monosex female populations, using genetically improved perch, will significantly benefit the aquaculture industry. To accomplish this goal, we have undertaken research and development of large-scale production of genetically fast-growing all-female yellow perch populations, and have assessed their growth performance and production variables for many years. Here, we summarize the efforts at the Ohio State University, plus review results from other previous studies.

20.2 Sexual Dimorphism

20.2.1 Sexual Size Dimorphism (SSD)

Yellow perch display a sexual growth dimorphism, in which females grow faster and reach a larger ultimate size than males ([6] – also see details in Chapter 22). Our experiment showed that, by the end of year 1, females outgrew males by 44.62% and 45.31% in pond and tank systems, respectively, and separation started at around 30g (Chapter 22). By the end of year 2, or close to breeding season, the top 10% females were 96.38% heavier than the top 10% males, and were 18.21% longer in pond rearing conditions (Wang *et al.*, unpublished data). This sexually related dimorphic growth appears in early life stages (8–11 cm total length), is correlated with the onset of vitellogenesis and spermatogenesis [6, 7], and is stimulated by estrogen [8].

20.2.2 Sex-Specific Gene Expression Related to SSD

To examine the phenomenon of an estrogen-stimulated SSD, several tissues for sex-specific expression were analyzed from both male and female adult yellow perch [8]. The results showed that: expression of estrogen receptor- α (*esr1*) and estrogen receptor- β a (*esr2a*) was highest in female ovary and liver tissues, with low to moderate expression in other tissues; expression of aromatase *cyp19a1a* was highest in both male and female spleen tissue and oocytes, with moderate expression in male pituitary and gill tissue; and *cyp19a1a* expression was moderately high in female liver tissue with undetectable expression in male liver tissue, suggesting its involvement in sexually dimorphic growth [8].

To gain understanding into the preceding endocrine control of growth involved with SSD, Lynn [9] also examined the expression of growth-regulating genes in developing gonads in yellow perch. Young fish (102–421 days post-hatch (dph)) were sampled several times

for length, weight, and tissues over the developing period. It was found that pituitary growth hormone (gh) and liver insulin-like growth factor-1b (*igf1b*) mRNA levels were significantly correlated with growth in both sexes. There was a significant dph-sex interaction on liver *esr2a* mRNA levels, with males having higher levels than females at 379 and 421 dph, and ovarian *cyp19a1a* decreased with dph. Overall liver *esr2a* mRNA levels showed the most significant positive correlation with liver *igf1b* mRNA levels. These findings suggest that growth is correlated with increases in pituitary gh, liver *igf1b*, and liver *esr1* and *esr2a* mRNAs in juvenile yellow perch [9].

20.3 Sex Ratio

It was reported that there were highly skewed sex ratios, and more males than females in some natural lakes [10, 11]. The percentage of females in Lake Michigan was observed to be 17.0% in 1987 and 28.0% in 1988, with males accounting for 93–100% of the age-7 or older yellow perch during 1987–1997 [10], while the male-to-female ratio increased with age [11]. The male-skewed sex ratios were also reported in Eurasian perch, with only 40.6% females in Lake Majajarvi and 30.3% females in Lake Iso Valkjarvi, the two lakes in southern Finland [12].

In aquaculture conditions, some male-skewed sex ratios were observed by our group also. We sampled one-year-old yellow perch for sex ratio from different ponds and tanks during March to November 2015, and found the average male percentage was 60.1% ($N=223$) and 65.0% ($N=397$) in ponds and tanks, respectively.

20.4 Sex Differentiation and Gonadal Morphogenesis

Yellow perch were observed to have no sex-distinguishing features, with paired gonads at total length (TL) of mm 5–10 [13]. The structural difference between ovaries and

testes is observed at the very early period of sex differentiation, and the first signs of sex differentiation occur when yellow perch is 16–18 mm in total length [13]. The ovary is distinct from testis at the very beginning of sex differentiation by its larger diameter (70–90 μm) and distinct ovarian cavity, contrasted with small diameter (about 30 μm) and no internal cavity.

At this stage, the primordial germ cells are identical in size (8–10 μm) in ovaries and testes [13]. The fused single ovary and paired testis are more obvious when fish are 23–27 mm of TL, and can be identified using naked eyes, without the use of a microscope [13]. Oocytes are first observed when female perch are 35–40 mm of TL, while spermatocytes are first detected in the testes when male perch reach 80–95 mm of TL [13]. These findings suggest that differentiation of the somatic elements of the gonads precedes gametogenesis, which begins earlier in females than in males, and the mm TL is important to the attainment of a minimum size of 80–95 initial onset of vitellogenesis and spermatogenesis in yellow perch [7].

Recently, we conducted a sex reversal experiment with 17 α -methyltestosterone (MT) to examine sex differentiation age and sex reversal efficiency in yellow perch, by treating yellow perch fry at different ages (38 dph and 54–78 dph), using two dosages of 20 mg/kg and 50 mg/kg. After 60 days of treatment, 100% males were obtained in the 38 dph group with 20 mg/kg treatment (no data secured for 50 mg/kg group) while, in the 54–78 dph group, 33.33–42.86% inter-sex were found in 20 mg/kg and 50 mg/kg treatments, although some sex-reversed males were achieved in the 50 mg treatment (Table 20.1). These results suggest that sex differentiation does not complete, at least at the age of 38 dph.

Sex-differentiated and adult yellow perch have a fused single ovary, while having paired testes (Figure 20.1a). The morphological structure of the ovary is unique, and is observed in only genus *Perca* (including two other species, European perch and Balkhash

Table 20.1 Effects of various doses of 17 α -methyltestosterone (MT) on sex ratios in yellow perch.

MT dose (mg kg ⁻¹)	Start age (dph)	Mean BL at sexing (cm)	Mean BW at sexing (g)	N	Male (%)	Ovotestes (%)	Female (%)
0 (control)	N/A	11.87	21.95	11	36.37	0	73.63
20	38	13.57	29.54	8	100	0	0
20	54–78	13.4	24.56	21	42.86	42.86	14.28
50	54–78	13.39	32.15	15	66.67	33.33	0

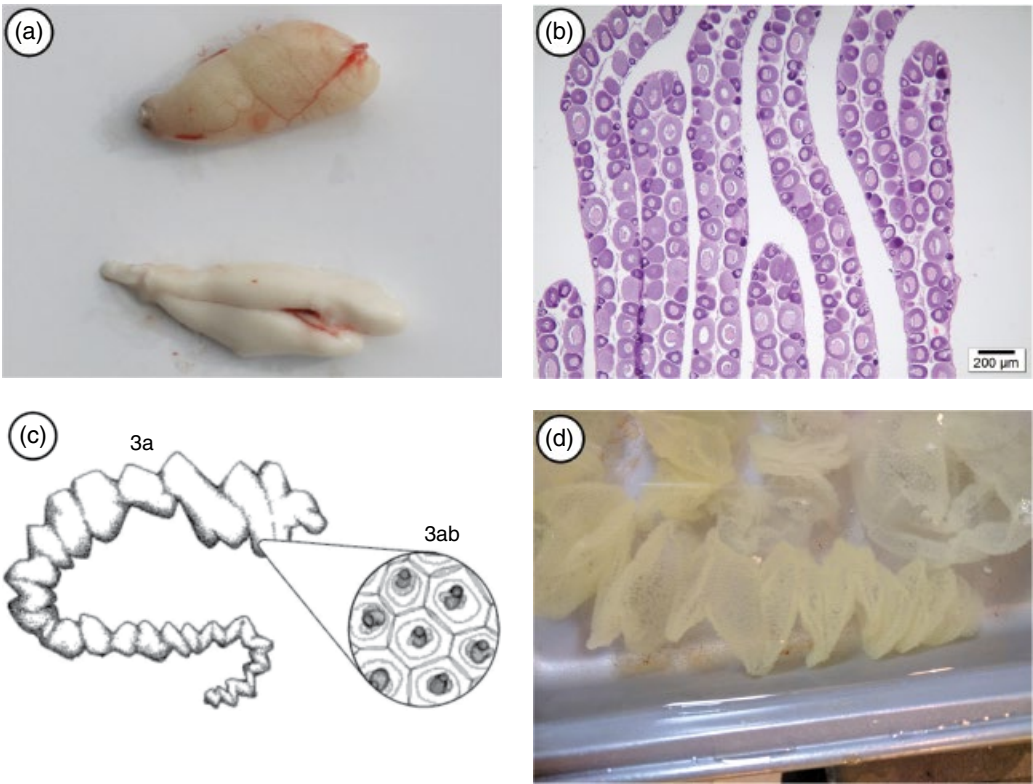


Figure 20.1 Unique ovary morphological structure of yellow perch *Perca flavescens*. (See inserts for the color representation of this figure.)

- a) single ovary vs. paired testis.
- b) ovary histology displays advanced internal organization of oocytes.
- c) egg ribbon and magnified eggs (credited to J. M. Hinshaw, North Carolina State University).
- d) egg ribbon right after fertilization.

perch, *P. schrenkii*). Another unique feature of the perch ovary is advanced internal organization (Figure 20.1b, c). This is clearly displayed when the egg “ribbon” is released (Fig 20.1c, d). The longitudinal section of the ovary is traversed by regularly organized ovigerous lamellae comprising two rows of oocytes, which is

bounded by connective tissue layers and separated by channels (Figure 20.1b). The extraordinary internal organization is the base of a hollow inside structure, surrounded by connected eggs of the released ribbon. Connective tissue across the entire egg ribbon makes each egg look like a regular or irregular hexagon,

Box 20.1 Sexual dimorphism and sex differentiation in yellow perch.

- Females outgrew males by $\approx 40\text{--}50\%$ in aquaculture systems in year 1, starting around 30g, and the separation is even bigger in year 2.
- Some estrogen-related and growth-regulating genes are involved in SSD.
- Male-skewed sex ratios were observed in both natural and culture environments.
- The first signs of sex differentiation occur when yellow perch are 16–18 mm in total length.
- Sex differentiation does not complete before the age of 38 dph.
- Sex differentiated and adult yellow perch have a fused single ovary.

which is surrounded by six regular or irregular hexagons (Figure 20.1c), even though this special feature is not displayed on histological images [14]. Therefore, we speculate that the hexagon-like eggs are the result of hardening during water absorption.

To our knowledge, the morphological structure of ovary in genus *Perca* (single ovary and advanced internal organization) is unique. From the view of evolution, the ecological significance and genomic basis of the unique structure deserve more attention. For large-scale production of all-female perch populations, the special characteristic of the ovary accelerates the entire process by one generation, since sex-reversed neomales or XX males hold a single testis. Key information on sexual dimorphism and sex differentiation in yellow perch is summarized in Box 20.1.

20.5 Sex Determination

20.5.1 Genome and Sex Chromosomes

The whole genome and RNA sequencing of yellow perch was completed in 2016 by our group. It has a genome size of ≈ 1.38 for males,

and ≈ 1.24 Gb for females (Wang and Shen, unpublished data), which is similar to the genome (≈ 1.3 Gb) of zebrafish (*Danio rerio*), and less than a half of the human genome size (≈ 3.2 Gb).

We performed the *de novo* assembly of yellow perch gonads and muscle transcriptomes in males, neomales and females, and a total of 212,180 contigs were obtained, ranging from 127 to 64,876 bp, and N50 of 1066 bp [15]. Based on the transcriptome mapping data, 93, 1,440 and 3 contigs were identified as specifically expressed in neomale, male and female respectively, while 9,476 contigs were identified as specifically expressed in gonads. When searching for sex specifically expressed ORFs, 19 ORFs were found specifically expressed in males, six ORFs were specifically expressed in neomales, and no ORF was specifically expressed in females. The number of sex-biased genes was counted in different pathway categories. However, there is no neomale- and female-biased gene involved in any pathway. Male-biased genes were involved in 29 pathways.

Of the functional pathways, two pathways involving the most genes for gonad-specifically-expressed genes were neuroactive ligand-receptor interaction and metabolic pathways. For gonad-specifically expressed genes, several pathways associated with gonadal development and sex maintenance were found, including Oocyte meiosis, GnRH signaling pathway, TGF-beta signaling pathway, Oxytocin signaling pathway, and Ovarian steroidogenesis. Additionally, TNF signaling pathway and Apoptosis pathway were found in gonads.

This study is the first report on transcriptome information in Percids, and provides rich resources for conducting further studies on understanding the molecular basis of sex determination and sexual dimorphism in fish, and for population studies and marker-assisted selection in Percids.

Yellow perch featured a karyotype of $2n=48$ with gradation in size of chromosomes, in which a pair of significantly smaller chromosomes was identified [16, 17].

20.5.2 Search for Sex-Specific Markers

We attempted to screen for potential genetic sex-specific DNA markers associated with sex in yellow perch, using the AFLP technique combined with bulked segregant analysis (BSA) [18]. In order to avoid the strain-specific or population-specific phenomenon existing in the detection of sex-specific markers, samples coming from four populations, including two wild populations and two hatchery populations, were used. Sex was identified by observation of testes and ovaries morphologically [19] or histologically [20].

The AFLP analysis of four gene pools using 256 primer combinations produced a total of 13,321 scorable bands, of which 5.17% and 4.69% were polymorphic in females and males, respectively. The total number of polymorphic bands per primer combination varied from 26 (for primer combination E9M14) to 102 (for primer combination E3M1). Four primer combinations among the 256 primer combinations produced putative female-specific AFLP fragments, and three primer combinations produced putative male-specific AFLP fragments (Table 20.2). However, when these loci were re-analyzed in all individual samples composed of the DNA pools, the sex-specific markers were observed in some individuals of putative sex, with several exceptions

(Table 20.2). These results revealed that, for each putative sex-specific marker, the putative sex-specific bands in the pooled DNA samples were virtually caused by the individual polymorphism (Table 20.2).

We were unable to identify genetic markers associated with the sex of the yellow perch using a combination of AFLP and BSA methodology. The possibility to search and develop sex identification DNA markers on a species depends on the gender determination system; thus, the lack of such markers in the search could theoretically be due to the lack of genetic sex determination mechanisms. Teleost fish exhibit complicated sex determination systems, such as a genetic sex determination system with sex chromosomes or sex determining genes as its characteristics, whereas others have a polyfactorial sex determination system (see Volume I, Chapter 1). In genetic determination models, some DNA markers have been identified in species where one sex possesses a unique chromosome or DNA sequence [21, 22].

Most successful isolation and characterization of sex DNA markers using AFLP indicated that the DNA marker always linked to the heterogametic sex chromosome [23–26]. The failure in this study may hint that yellow perch may lack a sex chromosome, or just have a incompletely differentiated sex chromosome. Furthermore, there is still no cytogenetical or molecular evidence of sex chromosomes and the sex determination system available in yellow perch so far.

Ohno [26] proposed that heterogametic chromosomes have originated from homomorphic chromosomes. During the differentiation of sex chromosomes, some molecular changes, such as mutation, insertion, or deletion events, that occur on or near the sex-specific site, may happen to DNA, as well as in chromosome architecture. Among these changes, a null allele may be responsible for some failures in finding proper DNA markers. In addition, the lack of such markers could also be due to weak correlation between the genotypic and phenotypic sex,

Table 20.2 Candidate sex-specific amplicons and population specific based on bulked samples.

Primers combination	Sex of DNA pools with sex-specific bands	Percentage of individuals with sex-specific bands
E1M4	F	50%
E8M12	F	50%
E1M7	F	40%
E4M8	F	50%
E3M1	M	70%
E3M2	M	40%
E5M3	M	40%

due to autosomal modifier genes [28], or mixed genotypes in pools, as a result of environmental sex determination [29, 30]. Moreover, yellow perch may incorporate multiple sex chromosomes, polygenic sex determination, and autosomal influence.

20.5.3 Sex Determining System

It was suggested that yellow perch belong to female homogametic sex, based on the results that all-female progenies were obtained by crossing normal females with sperm from sex-reversed intersex genetic females. Thus, the males may be heterogametic sex (XX/XY) [13].

To determine the genetic mode of sex determination in yellow perch, we used matured sex-reversed neomales, or XX males with a female genotype we have developed to cross with regular females. Sex determination mode (XY, ZW, or polygenic) was analyzed based on progeny sex ratios. In the case of heterogametic male mode (XY), crossing XX neomales with regular XX females should yield all-female progeny. In female heterogametic systems (ZW), crosses between sex-reversed ZW males and ZW females should result in approximately 75% females. In polygenic systems, crosses between sex-reversed neomales and regular females should, theoretically, produce a female-biased sex ratio. Therefore, if masculinized fish produce no monosex broods, female heterogamety is indicated. Conversely, if masculinized fish produce either all-female or mixed broods, male heterogamety is strongly indicated.

As results show in Table 20.3, mixed broods and all-female offspring [13] were

produced from MT-reversed males crossed to normal females in our previous work, strongly indicating heterogamous males (XY/XX) also. However, the many cases without producing all-female progeny, and skewed sex ratio from both natural and cultural environments, suggest the involvement of other factors (e.g., autosomal genes) in sex determination in yellow perch and Eurasian perch [13, 31]. In addition, temperature treatments (17°C and 29°C), starting from 35 dph for two months, did not affect the sex ratio of yellow perch (Shen and Wang, unpublished data). A possible role of temperature on sex determination in Eurasian perch is also not proven [31] (see also Box 20.2).

Box 20.2 Sex determination in yellow perch

- Males and females have a genome size of ≈ 1.38 and ≈ 1.24 Gb, respectively.
- Yellow perch featured a karyotype of $2n=48$, with gradation in size of chromosomes.
- Sex-specific markers were not identified using AFLP and BSA methodology.
- Mixed broods and all-females were produced from MT-reversed males crossed with normal females, indicating male heterogamous (XY/XX).
- The fact that progeny tests did not yield all-female offspring, and the skewed sex ratio observed in natural and cultural environments, suggests the involvement of other factors.
- A possible role of temperature on sex determination in Eurasian perch is not proven.

Table 20.3 Progeny sex ratio from MT-reversed males crossed with normal females.

Paired group	Female ID No.	Progeny male	Progeny female	Total	Progeny female %
3B	64807	17	15	32	46.88
3C	79548	3	26	29	89.66
3D	55623	35	14	49	28.57
3E	62521	2	34	36	94.44

20.6 Sex Reversal

As is the case for most other aquaculture species, estrogens and androgens can affect sexual differentiation, and be used to control the phenotypic sex of yellow perch ([13]; Wang and Shen *et al.*, unpublished data). Starting at total length of 20–35 mm and at a dosage of 15–120 mg/kg diet, complete germ cell sex inversion was induced in most males by administering estradiol-17 β (E₂) for 84 days [13]. However, E₂ treatment to directly produce monosex female populations, and the use of hormones in animals destined for human consumption, is not a proper direction for sex control in aquaculture. An indirect method, using androgens to produce monosex female populations, should be adopted.

For that purpose, spermatogenesis and the formation of ovotestes in females

was induced by treating fish with mg/kg diet 17 α -methyltestosterone at 1.5 to 60 for 84 days [13]. We conducted a sex-reversal experiment using MT in yellow perch, by treating yellow perch fry at different ages (38 dph and 54–78 dph), using two dosages of 20 mg and 50 mg/kg. After 60 days of treatment, 100% males were obtained in the 38 dph group with 20 mg/kg treatment (no data secured for 50 mg/kg) while, in the 54–78 dph group, 42.86% and 33.33% ovotestes were found in the treatments of 20 mg/kg and 50 mg/kg, respectively (Figure 20.2), although some sex-reversed males or neomales were achieved in the 50 mg/kg treatment (Figure 20.3; Table 20.3).

Some sections of those ovotestes are composited of intersex tissues (Figure 20.4). Therefore, the time and duration of the treatment and the doses of hormone used are keys to ensuring production of 100% of

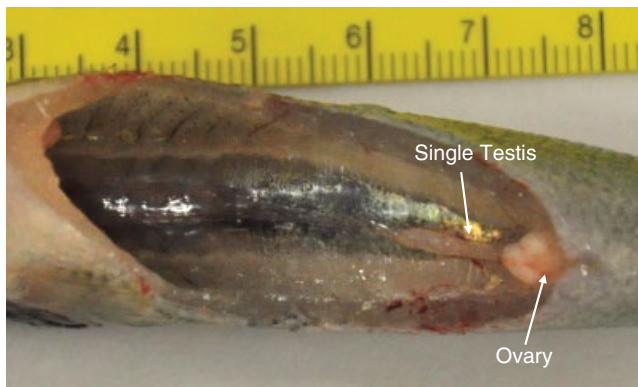


Figure 20.2 Ovotestis in the 54–78 dph group with 20 mg/kg treatment. (See inserts for the color representation of this figure.)



Figure 20.3 Sex-reversed neomales with a single testis in the 54–78 dph group with the 50 mg/kg treatment. (See inserts for the color representation of this figure.)

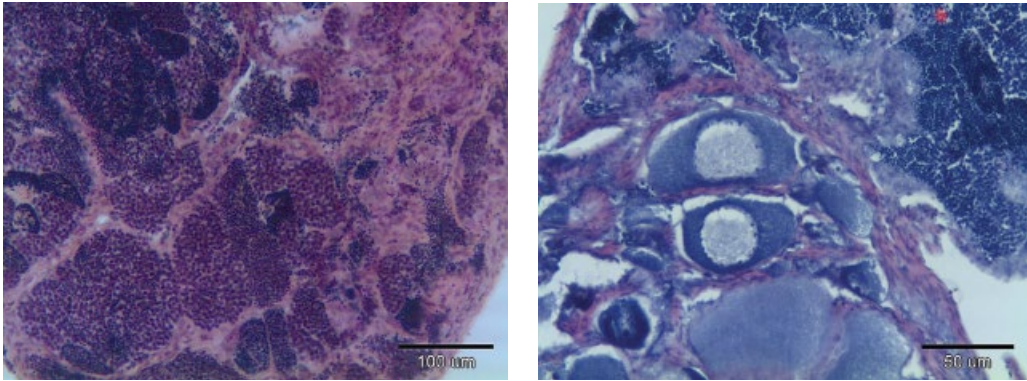


Figure 20.4 Intersex tissue in ovatestis in the 54–78 dph group with 20 mg/kg treatment. (See inserts for the color representation of this figure.)

masculinization and functional neomales in yellow perch. Fertilizing regular eggs using sperm collected from masculinized females or ovotestes could result in all-female progeny ([13]; Wang and Shen *et al.*, unpublished data). The genetic sex of treated fish or neomales in yellow perch can be easily identified by examining the morphology of gonads (e.g., the single (neomale or genetic female) vs. paired (genetic male)). This could be done as early as at 20–35 mm TL, when the onset of sex-specific gonadogenesis has already occurred [13].

20.7 Large-Scale Production of Monosex Populations Through Breeding

In past years, we used an indirect method and selective breeding to create all-female populations in yellow perch in two ways. The major advantages of the method are that the cost is almost insignificant, and no yellow perch destined for the market are treated with hormones. Key information on large-scale production of monosex populations in yellow perch is summarized in Box 20.3.

20.7.1 Creating Large-Scale Monosex Population Through Progeny Testing

For a progeny test to identify neomales or XX females, we crossed males from sex-

reversed populations with normal females, and identified those males that produced 100% or near 100% females as neomales. The sex ratios we obtained in the progeny test were 88.9–94.44% females. The small percentage of males observed in supposedly all-female populations suggested effects of other factors in the sex determining mechanism in yellow perch. The same phenomenon was reported in Eurasian perch ([31]; see Chapter 21). The identified neomales were used to cross with normal females to produce all-or mostly-female populations.

20.7.2 Creating Large-Scale Monosex Population Without Progeny Testing

During 2015–2017, we developed an approach to produce large-scale monosex populations without progeny testing. Males were killed prior to artificial fertilization, and genotypic sex was determined based on gonad morphology. The gonads of identified XX males were cut into small fragments, and milt was extracted with a syringe and was preserved on ice until fertilization. Females were examined each morning, and the egg ribbons of ready-to-go fish were obtained by abdominal stripping. Eggs were fertilized with preserved sperm from identified XX males to generate all-female populations.

20.7.3 Development of All-Female-Producing Brood Stock

By masculinizing some all-females from XX×XX crosses, we have produced all XX-neomale brood stocks, which enables the production of large-scale all-female or mostly-male populations of yellow perch for commercial monoculture (see Figure 1.3 in Volume I, Chapter 1).

20.7.4 Performance of Genetically All-Female Populations

We conducted a performance test of genetically all-females vs. a normal mixed-sex population in flow-through aquaculture systems. For the test, the all-females were communally cultured with the mixed-sex population, to avoid tank-specific environmental effects, and each individual was tagged with a PIT tag. By the end of the test in year 1, the genetically all-female group grew 26.3% faster than the normal mixed-sex group, and 60.0% faster than males on average. Pond testing of the performance of genetically all-females vs. a normal mixed-sex population is under way.

Box 20.3 Large-Scale production of monosex populations

- The time window for initiating MT treatment should be 30–38 dph.
- Effective protocol for masculinization is MT treatment of 20–30 mg/kg diet for 30 days.
- Crossing sex-reversed neomales with normal females is a preferred method to produce large-scale all-female yellow perch.
- Genetically all-females grew 26.3% faster than normal mixed-sex, and 60.0% faster than males on average.
- Gynogenesis is not recommended for producing monosex female perch, due to negative effects on the growth, survival, and reproductive development, resulting from heat or hydrostatic pressure shocks.

20.8 Chromosome Manipulation

20.8.1 Gynogenetic Approach for Monosex Populations

Gynogenesis, integrated with hormonal treatment (see Figure 1.3 in Volume I, Chapter 1), could be the fastest approach to produce large-scale all-female fish as an alternative method of sex control in perch. Methods for producing gynogenetic perch were developed in the early 1990s, and it was found that the effective methods of inducing tetraploidy were hydrostatic pressure shocks of 9,000 psi, applied at a TI of 192 minutes for durations of 16 or 24 minutes [31]. One hundred percent inactivation of paternal chromosomes was achieved through ultraviolet radiation of yellow perch sperm, with doses of 3,240–6,480 ergs/mm², and fertilized eggs by inactivated sperm had survival rates of more than 50%.

These methods could be further improved through a combination of heat and hydrostatic pressure shock treatments, or through blocking the second meiotic or first mitotic division for hydrostatic pressure shock treatments [33]. As an alternative method, it is not recommended to use gynogenesis as the first option for producing monosex female yellow perch, because:

- 1) negative effects on the growth, survival, and reproductive development of yellow perch resulted from heat or hydrostatic pressure shocks [34];
- 2) heat and pressure shock caused a negative influence on performance by increased homozygosity in some fish species [34, 35];
- 3) damage and mutations induced by irradiation, pressure or temperature shock, or chemical treatment and their negative influence on growth and performance of following generations were also observed in many other species [36, 37].

20.8.2 Triploidy Induction

Both heat and hydrostatic pressure shocks have been examined for their efficacy at inducing triploidy for production of sterile

yellow perch [34]. The most effective methods were heat shocks of 28–30°C, applied starting at 5 minutes after fertilization for 10 or 25 minutes, or hydrostatic pressure shocks of 9,000–11,000 psi, applied starting at 5 minutes after fertilization for 12 minutes [32]. The triploidy induction rates of these treatments ranged from 54–100%, and the survival of embryos was 16–18% [32], which is considered a successful case in chromosome manipulation. However, heat shocks can weaken the strands, and negatively affect incubation of perch eggs; pressure shocks can also affect egg strands, resulting in production of adhesive shortly after fertilization, and causing the strands to stick together or to the tank wall [34].

20.9 Conclusions and Future Perspectives

With estrogen-related and growth-regulating genes involved, yellow perch females grow \approx 40% to \approx 50% faster than males in aquaculture systems in year 1, and the advantage is even more pronounced in year 2. Sex differentiation in yellow perch does not complete before the age of 38 dph, and uniquely sex differentiated and adult perch have a fused single ovary. Genomically, yellow perch have a genome size of \approx 1.38 and \approx 1.24 Gb for males and females, respectively, and a karyotype of $2n = 48$ with gradation in size of chromosomes.

All-females and mixed broods were produced by progeny testing indicating male heterogamous (XY/XX). The fact that progeny tests did not produce all-female offspring, skewed sex ratio were observed in natural and cultural environments, and sex specific markers were not identified

using AFLP and BSA methodology, suggests the involvement of other factors, although the possible role of temperature on sex determination in Eurasian perch is not proven.

Crossing sex-reversed neomales with normal females is a preferred method to generate large-scale monosex female yellow perch production. Genetically all-females from this method grew 26.3% faster than normal mixed-sex, and 60.0% faster than males, on average. Gynogenesis is not recommended for producing monosex female perch, due to negative effects on the growth, survival, and reproductive development resulting from heat or hydrostatic pressure shocks.

The near future work for sex determination and sex control in yellow perch should focus on:

- 1) developing fine genetic maps to further search for the sex determining locus, and confirming sex determining mechanisms using advanced technologies;
- 2) examining the SSD mechanism through genomic (e.g., next-generation sequencing technologies), physiological and ecological approaches to take advantage of this unique model species;
- 3) further confirm whether temperature affects sex differentiation in perch.

Acknowledgments

This work was supported by the U.S. National Oceanic and Atmospheric Administration (NOAA) and Ohio Sea Grant. Salaries and research support were provided by state and federal funds appropriated to The Ohio State University, Ohio Agricultural Research and Development Center.

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21

Sex Determination and Control in Eurasian Perch

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21.1 Introduction

Eurasian perch, *Perca fluviatilis*, belong to the family Percidae, exclusively native to freshwater of the Northern Hemisphere, and to the genus *Perca*, with only three fish species: two European native, *Perca fluviatilis* (Linnaeus, 1758) and *Perca schrenkii* (Kessler, 1874) and one North American native, *Perca flavescens* (Mitchill, 1814). Both *P. fluviatilis* and *P. flavescens* are very ecologically important as top piscivores, and display significant fishery value in Europe and North America [1, 2]. Eurasian perch is an important niche market fish appreciated by the European consumers for its high flesh quality, but is still considered a “new species” in European aquaculture [3, 4]. According to FAO data (Figure 21.1), 3,618 tons of Eurasian perch were produced in Europe between 2002 and 2014, with a maximum of 512 tons produced in 2013 [5].

Eurasian Perch (from 100 to 200g body weight) are mainly produced in RAS systems (20–22°C) in Denmark, Ireland, and France, as well as in semi-intensive or extensive systems in the Czech Republic. The majority of the production is exported to Switzerland and Ireland, preferentially as 40–50 g fillets (see [6–8], for the description of Perch farming techniques; [4]).

As for other Percid fish, Eurasian perch display a sexual growth dimorphism, in which females grow about 25 to 30% faster than males [1, 9–12]. Fontaine *et al.* [11] reported that the feeding availability highly influences the sexual growth dimorphism in on-growing juveniles. Under experimental conditions, Rougeot and Mélard [13] observed that females begin to outgrow males from 30 g (170 days of rearing in 23°C). Similarly, in yellow perch, the SSD appears when females reached a total length of about 80–110 mm [9]. Therefore, the large-scale production of this species would be improved by rearing all-female populations, as they could reach commercial size long before males, about two months when rearing at 23°C [7, 14, 15].

As is the case in the majority of fish species, percid display a labile sexual development that allows modifying the phenotypic sex development towards the one of interest for production [16]. These objectives could be attained using classical methods in aquaculture, from hormonal control of sex differentiation process, to gynogenesis [17, 18]. The use of external factors may also contribute to drive the control of gender ratio.

Particularity with Eurasian perch, I am practically the only person who has studied the sex determinism mechanism and sex

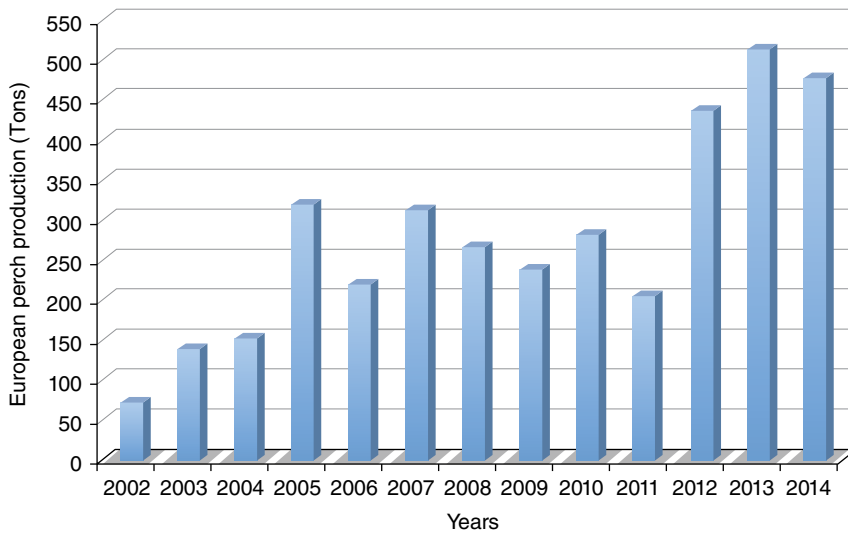


Figure 21.1 European perch production (extensive and RAS) in European countries from 2002 to 2014 (tons) [5].

control for this species. In this chapter, I will, therefore, synthesize and describe the different published and unpublished studies regarding the sex determination and differentiation process in Eurasian perch, and the different protocols that could easily be used in aquaculture to control the sex-ratio and improve the productivity of this species.

21.2 Sex Determination Process in Eurasian Perch

21.2.1 Gonad Morphogenesis

In Eurasian perch, the onset of germ cell differentiation has been reported from 10–12 mm (Mezhnin, 1978 in [1]) and less than 40 mg mean body weight, corresponding to 30 days at 17°C [19]. Perch, as other percoid fish, display an ovary with a particular morphology: rudimentary paired ovaries, fused during the early development to form a single ovary [20, 21].

21.2.2 Sex Chromosomes

Eurasian perch, as other Percoid fish, do not display any morphologically differentiated sex chromosomes, and the sex chromosomes

systems have to be studied and described using alternative and indirect methods, with retro-analysis of the sex-ratio. Therefore, three additional approaches have been used to study the sex chromosomes system in this species: the use of hormonally sex-reversed breeders; gynogenesis; and inter-specific hybridization with yellow perch.

The use of hormonal sex-reversed breeders to study the sex-determination process in fish is one of the most useful methods. In a XX female/XY males system, masculinization will produce sex-reversed XX males that will give, when crossed with an XX female, 100% of females in their progenies. In the opposite ZW female/ZZ male chromosomal system, masculinization will produce heterozygote ZW males that will give, when crossed with a normal ZW female, 25% of males and 75% of females [22].

In Eurasian perch, masculinization with 17 α -methyltestosterone (MT) was used to produce hormonally sex-reversed males breeders (see paragraph 3), and the sex-ratio of their progenies was analyzed to determine the chromosome system that governs sex determination process, based upon sex-ratio distribution [19]. From 11 artificial crosses with phenotypic males, originating from

normal families treated with MT, six progenies displayed an equilibrated sex-ratio (nearly 50 : 50) and five families displayed an all-female or nearly all-female sex ratio, from 97–100% females (Figure 21.2). Based on these results of sex ratio distribution, the authors concluded there is an XX/XY chromosomal system for Eurasian perch (Box 21.1).

Gynogenesis experimentation and retro-analysis of the sex ratio of the progeny could be applied to determine and confirm the type of sex chromosomes [23, 24]. Meiogynogens Eurasian perch juveniles were obtained by the fertilization of eggs with UV-irradiated semen (UV exposition during 160 seconds to 460 seconds), and application of heat shock (30°C, five minutes post-fertilization during 25 minutes) to restore diploidy [15, 25]. From 90–100% of gynogens were obtained, with a survival rate at hatching ranging from 3.4–41%. All of the four gynogens batches produced were 100% females (Figure 21.2). These results allow confirmation that the sex chromosomes in Eurasian perch are

the female homogamety XX/male heterogamety XY system.

Besides sex chromosomes, an autosomal influence is also suggested for Eurasian perch. Actually, some mixed-sex families (15% of the total of mixed-sex studied), which are expected to display a theoretically 1 : 1 sex-ratio, displayed an unbalanced sex ratio, significantly skewed towards males (>60%) or females (>65%) (Figure 21.2) [19]. This skewed sex-ratio could be explained by an autosomal influence. In many fish species, even in species that are considered to display a strong genetic sex determination, unbalanced sex-ratios are observed and explained by a maternal or parental effect and autosomal effect [16]. This hypothesis of autosomal influence is reinforced by the presence of unexpected males (from 1.5–3.0%) in 3/8 of theoretically all-female XX families studied.

Finally, artificial hybridization with the yellow perch, *Perca flavescens*, a species that displays female homogamety XX/male heterogamety XY sex chromosomes [9, 26],

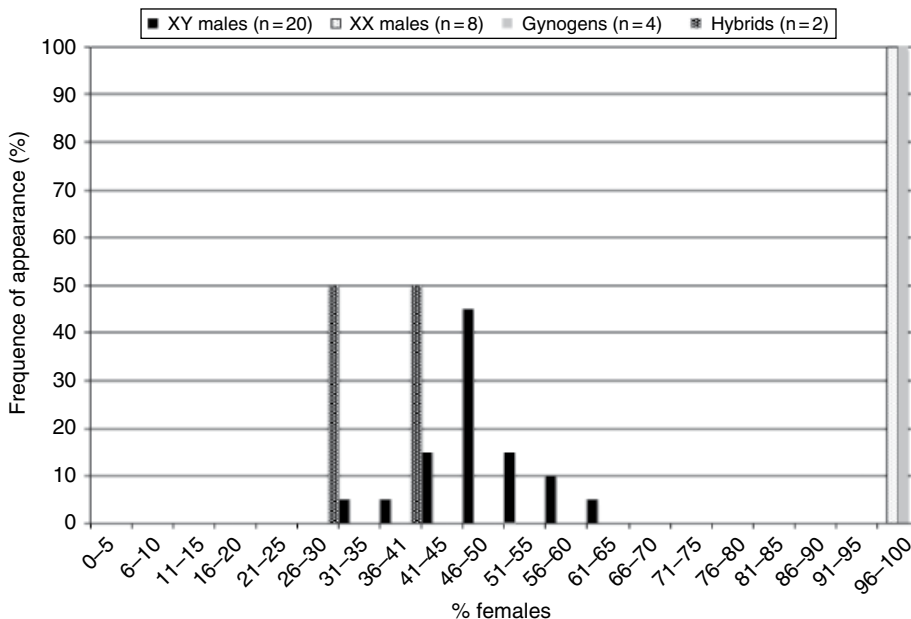


Figure 21.2 Sex ratio distribution in different Eurasian perch families. XY males – progenies from natural or artificial crosses between normal XY males and normal XX females. XX males – progenies from artificial crosses between hormonally sex-reversed XX males and normal females. Gynogens – gynogens progenies. Hybrids – progenies from artificial crosses between male yellow perch and female Eurasian perch.

Box 21.1 Sex determination and differentiation process in Eurasian perch

Sexual growth dimorphism (SSD): Eurasian perch display a sexual growth dimorphism in which females grow faster than males, about 25–30%.

Sex chromosomes: The sex determination process is under the control of sex chromosomes, with a female homogametic XX and male heterogametic XY system. Nevertheless, these sex chromosomes are not heteromorphic.

Autosomes: Autosomes are probably implicated into the sex determination process, as some significantly skewed sex-ratios are observed in normal, and in theoretically 100% female, progenies.

Sex determining genes: To date, no sex-determining genes have been described in Eurasian perch.

Sex steroids: The main sex-steroids (T, E₂ and 11KT) were maternally transferred to eggs, and used as precursors to induced initial sex differentiation of the gonads. The male differentiation process seems to be under the control of 11-oxygenated androgen 11KT, and the 11KT to E₂ ratio plays a major role in the global sex differentiation process in Eurasian perch.

Temperature effect: To date, no high temperature effect has been observed in Eurasian perch, regarding the range of temperature supported by the species.

was also used by Rougeot *et al.* (unpublished data), in order to contribute to the description of the sex chromosomal system in Eurasian perch. Cryopreserved yellow perch sperm was used for artificial fecundation of Eurasian perch eggs. Only two hybrid families (female Eurasian perch × males yellow perch) and two purebred controls (100% Eurasian perch) were obtained, with very low survival rates (2.5% at sexing at 180 days post-hatching). Both hybrids families displayed a significantly skewed sex-ratio, from 1 : 1 with 64% and 72% of males, whereas both purebred controls Eurasian perch families displayed a 1:1 sex-ratio (Figure 21.2). Eight percent of sterile fish was also reported in one hybrid family. These surprising results could reinforce the hypothesis of an autosomal influence on sex determination in Eurasian perch. Nevertheless, due to the very low survival rate, and the presence of skewed sex ratio, even in normal crosses, it is difficult to draw a conclusion.

Regarding all these results (Figure 21.2), we can conclude that Eurasian perch display a female homogamety XX/male heterogamety XY chromosomal system that governs the sex determination process. Nevertheless, the unexpected males observed in theoretically all-female populations, the significant unbalanced

sex-ratio in few “normal” families, as well as the significantly skewed sex-ratio towards males in the hybrid population, may suggested a role of autosomal factors in this mechanism (Box 21.1).

21.2.3 Sex-Determining Genes

Currently, no sex-determining genes or transcriptional factors, *Dmrt1* (*dmrt1*), aromatase (*cyp19a1a*), Sry-related HMG-box protein 9 gene (*sox9*), forkhead transcriptional factor L2 (*foxl2*), or anti-Müllerian hormone (*amh*), have been specifically studied and, therefore described, during the sex differentiation process in any Percid species (Box 21.1). Nevertheless, some available information exists regarding the sex-specific tissue expression of estrogen receptor- α (*era*) and estrogen receptor- β (*erb1*) in adults, with high expression levels in the female liver and ovaries. A higher sex-specific tissue expression level of *cyp19a1a* was also observed in female liver tissue, suggesting its involvement in sexually dimorphic growth [12].

21.2.4 Sex Steroids

In teleosts, as in all vertebrates, steroids are involved in the regulation of many biological processes, such as embryonic development,

metabolism, stress response, and immunity, as well as sex differentiation and reproduction [26]. In Eurasian perch, as in yellow perch, sex steroids have been identified as a growth regulator implicated in the sexual growth dimorphism, mainly with estradiol-17 β (E_2) considered as a growth stimulator, androgens displaying poor effect on growth [12, 28–33]. In these species with a sexually growth dimorphism towards females, sex-related growth differences emerge in the time of maturity, in relation to the increase in sex steroids levels in both males and females. Therefore, as sex steroids, mainly E_2 , are strictly implicated for SSD in perch, their implication for the sex differentiation process has been studied.

The presence of sex steroids before and during sex differentiation has been studied in many species, and their role during embryogenesis and sex differentiation is considered to be species-specific [34]. In Eurasian perch, the dynamics of the three main sex steroids, testosterone (T), E_2 , and 11keto-testosterone (11KT), were investigated during embryogenesis and the course of sexual differentiation, in mixed-sex and all-female progenies [35]. Eggs, larvae, and juveniles were regularly sampled from fertilization to hatching (D0), and from hatching to 70 days post-hatching (D70). Steroids were extracted from 3 gr sample of whole eggs, or body-mixed before the ethanol-dichloromethane extraction, and the three sex steroids (T, E_2 and 11KT) assayed by RIA.

We observed that, just after spawning, high concentrations of T (mean value: 1513.3 pg.g⁻¹), E_2 (mean value: 550.4 pg.g⁻¹), and 11KT (mean value: 1513.3 pg.g⁻¹) were accumulated in non-fertilized eggs, and we concluded that some sex steroids were maternally transferred into the eggs and could be used as precursors to induce the initial sexual differentiation of the gonads, or other organs such as brain. The detection or presence of significant levels of T, E_2 , and 11KT in embryos and developing larvae of Eurasian perch, before the histological differentiation of the gonad, strongly suggest an extra-gonadal synthesis of these sex steroid hormones.

During the entire sexual differentiation period (D2 to D70, Figure 21.3) T levels, ranging between 74.8 and 5531.3 pg.g⁻¹, were not significantly different between control and all-female groups. On the other hand, 11KT levels were six-fold higher in mixed-sex progenies (median: 431.5 pg.g⁻¹) than in all-female progenies (below the limits of assays detection), for which the 11KT level was only measurable from D40 (Figure 21.3). Surprisingly, we observed that the E_2 levels were significantly higher in the control group (median value: 725.7 pg.g⁻¹) than in the all-female groups (median : 156.2 pg.g⁻¹, Figure 21.3). Globally, we observed a general increase of T, 11KT, and E_2 levels after the onset of sexual differentiation (D35-D70) in both progenies, leading to an increase in steroidogenesis and steroid activity after the onset of gonadal differentiation (30 dph at 17°C; 18).

Finally, the 11KT to E_2 ratio was significantly higher in mixed-sex groups (1.35) than in all-female groups (0.24). Based on the important level of sex steroids from eggs to differentiated juveniles (D70), and the significant difference of 11KT to E_2 ratio between mixed-sex and all-female progenies, we concluded that sex steroids were closely implicated in the differentiation process in Eurasian perch, and that this is probably controlled by the 11KT to E_2 ratio (Box 21.1).

21.2.5 Temperature Effect on Sex Determination

As described in Section 21.2.2, the sex determination process in Eurasian perch is mainly under the control of sex chromosomes, with a female homogamety XX and male heterogamety XY system. Nevertheless, the significant skewed sex-ratio towards males or females observed in some mixed-sex families (which are expected to display a 1 : 1 sex-ratio), and the unexpected males observed in all-female XX families, strongly suggest the role of other factors in the sex determination process in Eurasian perch. Although we

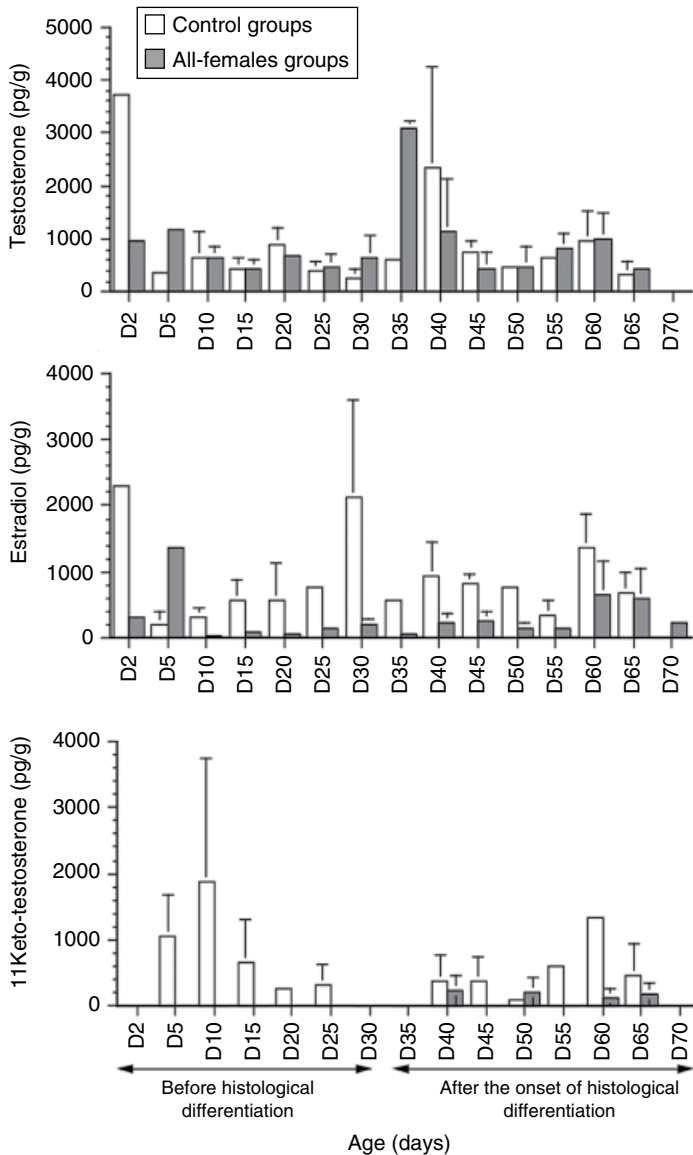


Figure 21.3 Whole-body levels of T, E₂, and 11KT (pg.g⁻¹), assessed by RIA, during larval and juvenile development in mixed-sex control (white) and all-female (greys) progenies of Eurasian perch. Values are means \pm SE. (from Rougeot *et al.*, [35]).

suspected the role of autosomes, these abnormal sex-ratios could also result from environmental factors, as temperature that could impair the normal differentiation pathway [36]. Eurasian perch display an adaptive plasticity to the environment [1], which could also be reflected through the sex determinism mechanisms, so we made two sets of experiments to test an eventual effect of high temperature on survival and sex differentia-

tion mechanisms in this species (unpublished data).

A first set of experiments was made in order to determine the lethal rearing temperature of Eurasian perch larvae that originated from two different mixed-sex families (theoretical 50 : 50 sex-ratio), first reared at 17°C in green water systems (algae, rotifera), then fed with *Artemia* and weaned at a body weight of 50 mg [6, 37]. Five hundred sexually

undifferentiated larvae (MBW < 70 mg) were pooled in 30 L cylindro-conic tanks with water from a recirculating system. Experimental rearing temperatures tested during this experiment were 20°C (control), 30, 31, 32, 33, and 34°C, applied during 30 days from 70 mg MBW. Thirty-three and 34°C were lethal for Eurasian perch larvae, as all batches died within 24 hours when the temperature reached 33°C. In other batches, survival rates ranged from 11.8 (at 32°C) to 35.2% (at 30°C).

In the second set of experiments, 2,000 sexually undifferentiated larvae (MBW < 70 mg) were reared at 32°C during the experimental period (30 days). Fish used in this experiment originated from one mixed-sex family and from one all-female family. Sex-ratio of the treated progenies were assessed at six months, when gonads were morphologically differentiated (Table 21.1). Regarding both experiments, the sex-ratio of the three mixed-sex families reared at high temperature (30, 31, and 32°C) never differed from a balanced sex-ratio 1 : 1. Similarly, the sex-ratio of the all-female family was 100% females, both in control and in treated batches.

Based on these results, we suggest that high temperatures during larval develop-

ment and sexual differentiation have no effect on the sex differentiation process in Eurasian perch. This absence of temperature effects on sex determinism mechanisms in fish is not rare, and is explained by the wide range of mechanisms involving sex determination, and the large inter- and intra-specific variation for sexual determination, resulting in a different response of each species to temperature. Nevertheless, low temperatures were not tested, because of the negative effect of low temperature on survival and growth in Eurasian perch.

In conclusion, we supported the hypothesis of an absence of effect of temperature on sex differentiation within the range of temperature tested and supported by our Belgian strain of Eurasian perch. Therefore, the skewed sex-ratio reported in mixed-sex families and unexpected males in all-female families could not be explained by a temperature effect, as it was done for many other species [36]. An autosomal influence, as we previously suggested [19], seems to be a more adequate hypothesis to explain the unexpected results of sex-ratio observed in Eurasian perch, as it was done for many other species [16].

Table 21.1 Sex-ratios (%) of Eurasian perch progenies reared at different experimental temperature during 30 days. Initial mean body weight (<70 mg). Values are mean \pm standard deviation.

Experiment	Experimental temperature (°C)	N family	N fish sexed	% males	% females
1	20 (control)	2	399	51.4 \pm 2.6	48.6 \pm 2.6
	30	2	302	48.4 \pm 3.1	51.6 \pm 3.1
	31	2	345	43.7 \pm 9.2	56.3 \pm 9.2
	32	2	131	48.1 \pm 0.7	51.9 \pm 0.7
	33	2	0	–	–
	34	2	0	–	–
2	20 (control)	1	200	51.0 \pm 5.7	49.0 \pm 5.7
	32	1	200	45.0 \pm 5.7	55.0 \pm 5.7
2	20 (control)	1	200	0	100
	32	1	200	0	100

21.3 Sex Control and All-Female Eurasian Perch Production

21.3.1 Hormonal Control of Sex

Hormonal sex control in fish may be achieved using two methods: the direct use of feminizing hormones; or the indirect used of hormonally sex-reversed breeders. Regarding European and United States legislations, the direct use of hormones on fish for human consumption is forbidden. Moreover, for experimental purposes, direct feminization, with ethynylestradiol applied in a bath or through feed, leads to 100% mortality within 48–72 hours (Rougeot *et al.*, unpublished data). Therefore, we will not elaborate on direct feminization of perch using estrogens.

Hormonal sex reversal in Eurasian Perch could be achieved within two generations: the production of hormonally sex-reversed XX males breeders in the first generation, and the production of all-female populations by artificial or natural crosses between hormonally sex-reversed males and females breeders in the second generation [19]. In the first generation, a masculinizing treatment is applied on a standard mixed-sex progenies, (theoretically 50% XX females and 50% XY males), in order to obtain an all-male population with 50% of XX males and 50% of normal XY males. The genotype of the male is then identified, based on the sex-ratio of its progenies when crossed with a normal XX female in second generation (Figure 21.4).

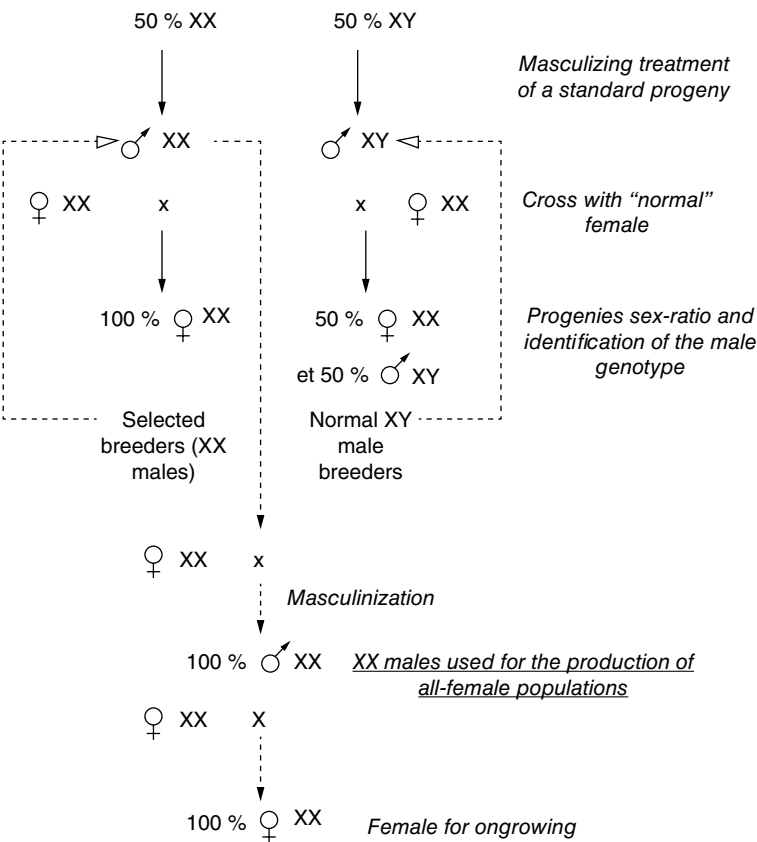


Figure 21.4 Theoretical flow diagram illustrating the different steps to produce sex-reversed XX male breeders and the production of all-female population within two generations in Eurasian perch.

21.3.2 Production of XX Males

As for other species, the success of hormonal sex reversal treatment in Eurasian perch depends on three main factors that are species-specific: the time of application; the duration of the treatment; and the doses of hormone used. In order to be effective, hormonal treatment should be applied during the labile period, the so-called hormonosensible period of sex differentiation, before the onset of germ cell histological differentiation. If the treatment is applied later, it will be less effective, or it will induce sterilization or ovotestis (reproductive organs with both ovary and testis tissues). In the same way, the increase of treatment duration would increase the percentage of female, intersex, or sterile fish [19].

The MT is the only synthetic steroid hormone used for sex reversal in percid fish and the production of sex-reversed XX males for the all-female production. MT is administered through the food. Currently, MT is first dissolved in 95% ethyl alcohol, added and mixed to the diet, and air-dried for 24 hours to allow the evaporation of the solvent, prior to the food distribution. The difficulty with Eurasian perch is the weaning period. Larvae have to be first fed with natural food (*Artemia nauplii*) from yolk resorption to 40 mg mean body weight, MBW [6, 37].

In Eurasian perch, the optimal doses of MT used for masculinization is 30–40 mg/kg food, applied before the onset of sexual differentiation of the gonads [19]. Using higher doses of MT in Eurasian perch (60 and 80 mg/kg food), we observed, in the treated progenies, fish with ovotestis (20%) or sterile fish (25%). In this species, hormonal treatment applied after the onset of histological differentiation of the gonads (>150 mg MBW) leads to a decrease of sex reversal efficiency, with up to 25% of females in the progenies. The most important parameter to ensure 100% of masculinization is the timing of hormonal treatment (usually expressed in terms of days post-fertilization, or fish body size).

In many cases, hormonal sex reversal treatments lead to various percentages of

abnormal gonad morphology. In Eurasian perch (as for yellow perch), males with a single testis are considered as XX males, as rudimentary paired ovaries fuse during early development to form a single ovary [1, 20]. In Eurasian perch, Rougeot *et al.* [19] obtained up to 20% of males with a single twisted testis with nodules, when applying a 40 mg/kg diet MT treatment on 40–70 mg MBW fish (Figure 21.5). Generally, these fish were not able to release sperm, because of the abnormal morphology of the gonads or the lack of a sperm duct. Therefore, these males should be killed for intra-testicular sperm sampling for artificial reproduction, given the same fertilization rates as stripping sperm. Nevertheless, Rougeot *et al.* (unpublished data) succeeded in obtaining 40% of XX males with a spermiduct when feeding undifferentiated Eurasian perch fry (40 mg MBW) with a lower dose of MT (5–10 mg/kg food) for

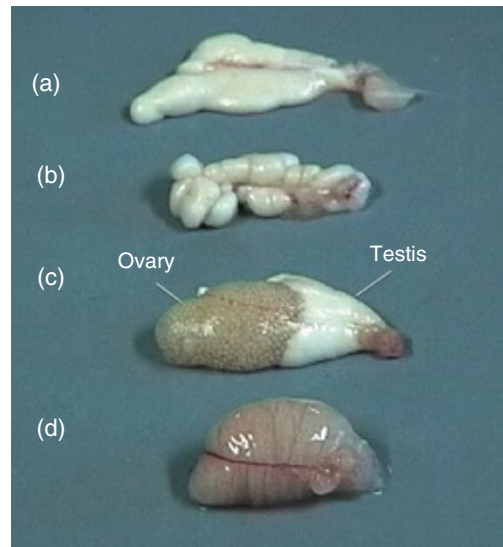


Figure 21.5 Gross morphology of Eurasian perch gonads. (See inserts for the color representation of this figure.)

- a) Double testis of normal XY male;
- b) single twisted testis with nodules of hormonally (MT) sex-reversed XX males;
- c) ovotestis with ovarian and testicular tissues of partially hormonally (MT) sex-reversed XX males;
- d) normal single ovary of normal XX female (from Rougeot *et al.* [19]).

Box 21.2 Sex-reversed males breeders Eurasian perch production

Hormone: the use of 17α -methyltestosterone (MT) is recommended for sex-reversal treatment. Hormone should be dissolved in 100% ethanol (600 ml.kg^{-1} food) and added to larval food 24 hours before feeding, in order to allow ethanol evaporation. Fish should be fed at satiety.

Initial mean body weight/age: the initial age for the application of the sex-reversal treatment is 70 mg, 36 dph (at 17°C). If the treatment is applied later, the percentage of sex-reversal significantly decreases. If the treatment is applied before, mortality is observed, due to the weaning process in Eurasian perch.

Hormonal dose: the best MT doses for sex-reversal is 5 or 10 mg kg^{-1} food, which induces 80% of functional (spermiating) XX males.

Doses higher than 80 mg kg^{-1} induces a high percentage of sterile fish.

Duration: the duration of the hormonal treatment should not be longer than 30 days. Longer treatment significantly increased the percentage of sterile fish.

Temperature: the optimal temperature for sex-reversal treatment is 23°C . At higher temperatures, mortalities appear. At lower temperatures, the duration of the treatment should be longer, because of the lower growth rate.

Sex-reversal efficiency: 100% of sex-reversal with 80% of functional (spermiating) XX males.

All-females production: natural or artificial reproduction between sex-reversed XX males and females allows production of 100% XX female progenies.

30 days treatment duration (Box 21.2). These spermiating XX males allowed the natural reproduction process in tanks.

21.3.3 Sperm Quality of Hormonally Sex-Reversed Males and Cryopreservation

Except for the difference of gonad gross-morphology (Figure 21.5), both male genotypes display similar growth curve and gonad development, and display a comparable mean body weight (MBW = 17.1 g) and GSI (7.5%) at first sexual maturity (one year). Both Eurasian and yellow perch XX males are as fertile as normal XY males, and viable progenies are obtained when these males are artificially crossed with females [9, 19]. Both one-year-old XX and XY Eurasian perch males display similar sperm density, with 32.0 and $33.7 \times 10^9 \text{ cells ml}^{-1}$ for XY and XX males respectively [38].

The sperm motility of both XY and XX males, assessed by Computer Assisted Sperm Analysis (CASA) with six sperm motility parameters (VCL, VAP, VSL, LIN, % MOT, MOC), is comparable with, for example,

87.0% (XY males) and 86.3% (XX males) of motile spermatozoa (% MOT) at 15 seconds after activation. Both male genotypes also display a similar sex steroid profile (11KT, E_2 , and T) during the natural spawning period (April–May). 11KT levels significantly decrease from $3,000 \text{ pg ml}^{-1}$ in early April to $2,000 \text{ pg ml}^{-1}$ in early May. E_2 displays a similar profile, with a peak at the end of April (about $3,500 \text{ pg ml}^{-1}$), whereas the level of T does not significantly change during the reproductive period ($1,900 \text{ ng ml}^{-1}$) [38]. All these results confirm that hormonal treatment induces a total sex-reversal process and allows fertile XX males to be obtained.

In their study on sperm motility and the fertilizing ability of frozen spermatozoa of normal XY males and XX males (XX), Rodina *et al.* [39] indicated that stripped and frozen sperm of normal XY male Eurasian perch could successfully be used for artificial reproduction, with similar results of fertilization and hatching rates to using fresh stripped sperm. On the other hand, the experiment showed that intra-testicular XX male frozen sperm displayed a significant lower percentage of motile spermatozoa, as well as a lower

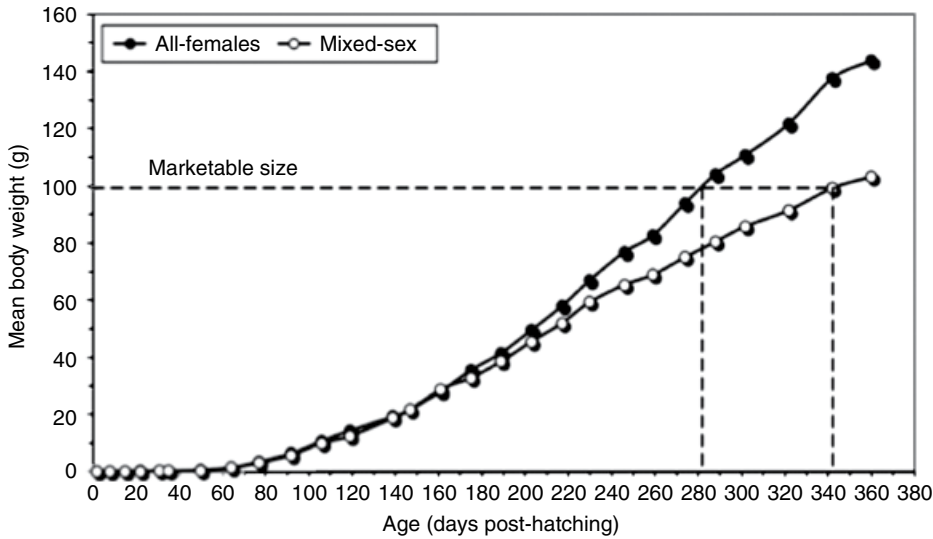


Figure 21.6 Comparative growth curve of all-female and mixed-sex juvenile Eurasian perch reared under intensive conditions in a 0.5 m³ tank in recirculating system (23°C) at an initial stocking density of 2,000 fish.m³ (from Rougeot and Mélard, [39]).

velocity than the stripped and frozen sperm of normal XY males. Hatching rate was also significantly reduced using frozen intra-testicular XX male sperm. Nevertheless, the researchers did not test the quality of frozen sperm from stripped XX males, and we can expect better results of cryopreservation, as the quality of fresh sperm is similar between both XX and XY sperm.

21.3.4 All-Female Production and Performances

All-female Eurasian perch production was obtained either by artificial crosses between normal females and intra-testicular sperm from hormonally sex-reversed males [19], or by natural reproduction in tank with functional XX sex-reversed males (Rougeot *et al.*, unpublished). All the progenies that were obtained displayed a sex-ratio ranging from 95–100% of females. In order to constitute a stock of future XX sex-reversed male breeders, an all-female progenies could be then sex-reversed with MT to produce 100% XX males, using the same protocol of sex reversal (Figure 21.4).

A comparative study [13], conducted with Eurasian perch in 0.5 m³ tanks in a recirculating system (23°C, O₂ > 6 ppm) outlined that all-female families began to grow faster than mixed-sex families from a mean body weight of 30 g (Figure 21.6). After 360 days of rearing, the difference of growth performances reached 30%. Using all-female families, the marketable size (100 g) was reached within 250 days, compared with 300 days for mixed-sex families.

21.4 Sex Control by Chromosomes Set and Ploidy Manipulations

21.4.1 Triploidization

The use of chromosome set manipulation in aquaculture is mainly interesting for the production of triploid (and, to a lesser extent, tetraploid) fish that are partially or totally sterile [22, 23, 40]. The interest in sterility relies on the possibility of increasing growth by using the energy allocated for gonadic growth to somatic growth. In triploid fish, the triplicated

chromosome sets impair the meiotic division involved in germ cell formation and, therefore, inhibits gonad development. Sterilization of females by ploidy manipulation (triploidization) would suppress the gonad development in females, and the somatic growth rate would be improved.

In Eurasian Perch, sterilization is mostly important when rearing fish in an extensive system, under natural water temperature. Low temperature during winter induces a chilling process that is necessary for the natural development of the ovary [1]. When rearing perch in RAS with constant optimal rearing temperature (23°C), the ovarian development is inhibited, females do not mature any more, and the somatic growth is enhanced (GSI: 25% at reproduction). In fish, triploidy is generally induced by inhibiting the second meiotic division and the extrusion of the second polar body, by shocking the eggs shortly after fertilization [22, 23]. Many treatments are effective in inducing the polar body retention, including: thermal (cold or heat); chemical (colchicine or cytochalasin B); or hydrostatic pressure shocks [36].

In percid fish, only heat shocks and hydrostatic pressure shocks are reported for the production of triploid fish [25, 41–43]. In Eurasian perch, Rougeot *et al.* [43] obtained from 93–100% triploids (assessed by flow cytometry analysis) using a heat shock of 30°C, applied 5–7 minutes post-fertilization for 10–25 minutes [39] (Box 21.3). The survival rate of embryos (six days post-fertilization) reached 45%, resulting in a yield of triploids above 45% among the survivors. In comparison, higher temperature (34–36°C) and short duration (2- and 5-minute) shocks allow only the production of 55% of triploids. Therefore, the yield of triploids under these conditions was only slightly above 20%, because of the lower survival (30%) and triploidization rates (55%).

21.4.2 Gynogenesis

Although gynogenesis allows production of 100% of females in one generation (see

Box 21.3 Sex control by chromosomes set and ploidy manipulations in Eurasian perch

Triploidization: A heat shock of 30°C, applied 5–7 minutes post-fertilization for 10–25 minutes, leads to 93–100% of triploids, with 45% survival rate.

Gynogenesis: Egg fertilization with UV-irradiated semen (UV exposition for 160–460 seconds) and application of a heat shock (30°C, five minutes post-fertilization for 25 minutes) to restore diploidy. This produces 90–100% of gynogens, with a survival rate at hatching from 3.4–41%. Nevertheless, gynogenesis is not recommended for all-female production, because of a lower growth rate of gynogens.

Section 21.2.2), this method is rarely used in percid fish production, for at least two reasons [24, 25]. First, the survival rates of gynogenetics progenies is often low, probably due to expression of deleterious recessive alleles or the negative effects of heat or pressure shock on the embryos survival. Second, growth performances are negatively affected by the increased homozygosity. For example, after one year of rearing in a recirculating aquaculture system at 23°C, Eurasian perch gynogens displayed a mean body weight significantly lower (108 g) than a normal diploid control group (MBW: 133 g. Rougeot *et al.*, unpublished data).

21.5 Conclusions

Eurasian perch display a strong chromosomal sex determination process with a female homogamety XX/male heterogamety XY system, with a probable influence of autosomal factors. To date, no sex-determining genes have been reported, nor temperature effect for this species. Sex steroids (11KT and E₂) are closely responsible for the sex differentiation process in this species. In Eurasian perch, the lability of sexual development, as well as the possibility of

modifying the chromosomes set, allow control of phenotypic sex development.

Using exogenous hormonal sex reversal treatment (MT), the phenotypic sex of Eurasian perch could easily be changed to obtain hormonally sex-reversed males breeders, which are as fertile as normal XY males. The production of all-female population within two generations allows improving growth performances up to 30%, compared with mixed-sex families. In the same way, ploidy manipulation – mainly triploidization – will allow sterilization of

females and, therefore, probably induce the improvement of growth performances by the reduction of gonad development (above 25% during the reproductive period), when rearing Eurasian perch under natural water temperature conditions.

Acknowledgements

I sincerely acknowledge the editors, Drs. Han-Ping Wang and Francesc Piferrer, who invited me to contribute to this book.

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22

Sexual Dimorphism in Body Size and Form in Yellow Perch

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22.1 Introduction

Sexual dimorphism is common in fish, and depends on different life history strategies [1–3]. Sexual dimorphism is regarded as the phenotypic difference between males and females, which includes size dimorphism, shape dimorphism, and color dimorphism [4]. Sexual size dimorphism commonly exhibits male-biased or female-biased phenomena in fish. The dimorphism may result from various factors depending on natural selection, such as surviving competition and reproductive strategy [5, 6].

In many species, obvious sexual size dimorphism has been observed. Sexual size dimorphism in some species is male-biased, such as in tilapia (*Oreochromis niloticus*), bluegill sunfish (*Lepomis macrochirus*), yellow catfish (*Pelteobagrus fulvidraco*), and channel catfish (*Ictalurus punctatus*) [7–10]. Large males are supposed to have a greater chance to mate with females and protect their offspring [1, 11]. Others are female-biased, such as yellow perch, rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), half-smooth tongue sole (*Cynoglossus semilaevis*), and Japanese flounder (*Paralichthys olivaceus*) [12–15]. Large females are attributed to selection for higher fecundity, while small males

could benefit from scrambling competition and reproductive success, because of their greater speed and agility [11, 16]. Research in sexual size dimorphism is important for sex control for aquaculture, and for understanding the morphological differentiation, genetic variation of populations, and the relationship with the behavior, ecology, and evolution for fisheries [5].

Yellow perch belong to the order Perciform, and are widely distributed throughout freshwater regions of North America [17, 18]. It has become one of most ecologically and commercially important species in the Great Lakes areas and the Midwestern states of the United States [17–19]. In the past several decades, the production of yellow perch has largely relied on capture fisheries, and the quantity of resources has fluctuated because of exploitation and unstable recruitment [20, 21]. Therefore, farming yellow perch is a needed approach to meet increasing market demand, and this species has become an important cultured fish in United State in the past years [17, 18]. However, the aquaculture industry of yellow perch has developed slowly, due to constraints of the slow growth rate of currently cultured strains [17, 21].

To provide high quality product and gain sustainable development in aquaculture,

breeding programs, such as Ohio Genetic Improvement of Farmed-fished Traits, have been put into effect to improve growth and resistance [17, 18]. Genetic improvement has become more crucial to increase growth rate and production in the yellow perch industry. Female yellow perch exhibit greater growth rate and larger size than males. It is very crucial to understand the mechanism of sexual growth dimorphism, in order to develop successful selective breeding programs, including sex control. The sexual dimorphism and sex determination have attracted more attention, and some important progress has been achieved [22–25]. However, due to the scarcity of obvious genital characteristics, it is difficult to determine sex by the external morphology. Currently, sex can only be visually identified during spawning season (e.g., males can be separated through sperm release).

Some breeding efforts, including all-female selective breeding, need to be based on different sexes in yellow perch. Therefore, some researchers have tried to explore important external morphology to distinguish different sexes, and have made some progress [25, 26]. However, these methods are based on external morphological criteria, especially the area of urogenital papilla. The accuracy of identification is influenced by professional skills and the degree of difference in external characters.

The objective of this work was to investigate the difference of growth performance between females and males, and sexual dimorphism in body size under different culture conditions. Furthermore, the sexual dimorphism in the differentiation of body morphology was compared. This study was devoted to establishing the identification method between sexes on the basis of the morphometrics.

22.2 Examination of Sexual Dimorphism in Body Size

We produced experimental fish in 2014 in the hatchery of the Aquaculture Genetics and Breeding Lab (AGBL), The Ohio State

University (OSU). In April 2015 (spawning season), fish from the pond in this study were collected using a seining net, and the specimens were randomly sampled. Initial weight and length were measured after anesthesia through immersion in MS-222. Each individual was sacrificed to identify the gender after the morphology characteristics were measured. The fish, except for sampling individuals, were returned to their original pond. All individuals from the tank were randomly collected at the same time. The procedure was the same as the fish from the pond.

After sampling, all fish had their gender identified by pressing the belly and checking sperm. These fish were then divided into two groups. One group, including 110 females and 116 males, were equally cultured in two one-meter diameter indoor tanks, and the other group, with 210 females and 238 males, were reared in two 2.1 m diameter outdoor tanks. Fish were kept in indoor tanks with flow-through water at 15–21°C and 14 hours of lighting per day, while fish cultured in outdoor tanks had flow-through water, at natural water temperature and photoperiod. All samples were collected at the same time, and all experiment procedures were approved by the Ohio State University Institutional Animal Care and Use Committee.

In order to compare the growth differences between genders, the following parameters were evaluated:

Sexual dimorphism weight advantage (SDWA):

$$SDWA = \frac{(W_{\text{♀}} - W_{\text{♂}})}{W_{\text{♂}}} \times 100\% \quad (22.1)$$

Sexual dimorphism length advantage (SDLA):

$$SDLA = \frac{(L_{\text{♀}} - L_{\text{♂}})}{L_{\text{♂}}} \times 100\% \quad (22.2)$$

Where: $W_{\text{♀}}$ and $W_{\text{♂}}$ are mean weights of female and male, respectively; $L_{\text{♀}}$ and $L_{\text{♂}}$ are mean total length of female and male.

Absolute growth rate of body weight (AGRW) and absolute growth rate of total length (AGRL) were calculated by the following formulae in this study:

$$\text{AGRW} = \frac{(W_t - W_0)}{t} \times 100\% \quad (22.3)$$

$$\text{AGRL} = \frac{(TL_t - TL_0)}{t} \times 100\% \quad (22.4)$$

Where: W_t and W_0 are the final and initial weight of experimental fish, respectively; TL_t and TL_0 are the final and initial length of experimental fish, respectively; t is the interval in days between initial and end time.

22.3 Examination of Sexual Dimorphism in Body Form

We collected 200 yellow perch from April to August 2015. Prior reports have shown that the phenotypic plasticity phenomena existed in yellow perch and that the phenotype was influenced by various factors such as habitat, predation, and food resource [27–30]. Therefore, in order to compare the impact of environment, fish were acquired from ponds, a small lake, and tanks

(Table 22.1). Eighty-nine of them were captured by fishing from the lake at the OSU South Centers, where fish obtained only natural food. Fifty-seven individuals were taken using a seine net from a pond where fish were fed with artificial feed twice a day. The rest of the 54 fish were obtained from the outdoor 2.1 m diameter tanks, where fish were provided commercial feed three times a day.

All fish were immediately transported to the laboratory and kept in the holding tanks. The morphology data collection and sex identification were processed within 24 hours after collection. Fish were placed in a dissecting pan on their right side, to ensure the body shape in normal condition after anesthesia. A centimeter scale was used to measure the distances, based on truss morphometric network. The truss network system is various measurements calculated between landmarks of the fish body, and is commonly used for fish body morphology [31–33]. In this study, twelve landmarks were used to determine 24 yellow perch body measurements (Figure 22.1).

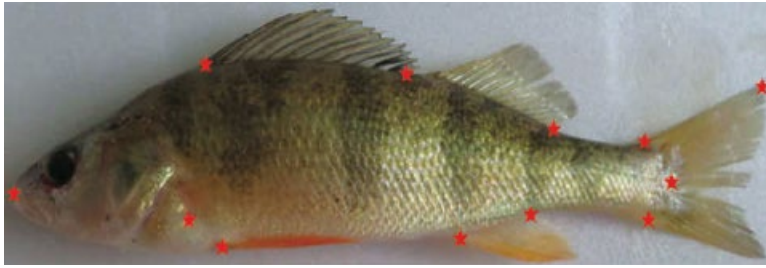
Sample basic information of yellow perch from different locations in this study is listed in Table 22.2. Each fish was sacrificed to determine the gender after the morphometric characters were measured. There were

Table 22.1 Descriptive statistics information of male and female *Perca flavescens* from different sites for studying sexual dimorphism in body form.

Site	Sex	N	Wt(g)		TL(cm)		BL(cm)	
			Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD
Lake	Male	28	26.9–137.1	86.1 ± 29.7	12.6–23.8	19.8 ± 2.7	10.6–20.7	17.3 ± 2.5
	Female	61	24.2–283.5	122.6 ± 56.9*	13.4–28.5	22.0 ± 3.6*	11.1–24.9	19.4 ± 3.3*
Pond	Male	28	13.3–156.0	56.5 ± 37.9	10.6–23.0	15.7 ± 3.4	9.1–20.4	13.4 ± 3.1
	Female	29	15.7–177.7	82.3 ± 53.6*	11.6–25.6	17.8 ± 4.3*	9.9–22.1	15.5 ± 3.9*
Tank	Male	44	16.8–119.6	49.2 ± 20.7	11.9–20.0	15.6 ± 1.7	10.2–17.2	13.3 ± 1.4
	Female	10	15.0–110.3	58.6 ± 28.3	12.0–19.2	16.0 ± 2.2	10.1–16.7	13.7 ± 2.0
Overall	Male	100	13.3–156.0	61.6 ± 32.6	10.6–23.8	16.8 ± 3.1	9.1–20.7	14.4 ± 2.9
	Female	100	15.0–283.5	104.5 ± 51.8*	11.6–28.5	20.2 ± 4.4*	9.9–24.9	17.7 ± 4.0*

*represents significant difference between male and female within system ($P < 0.05$).

(a)



(b)

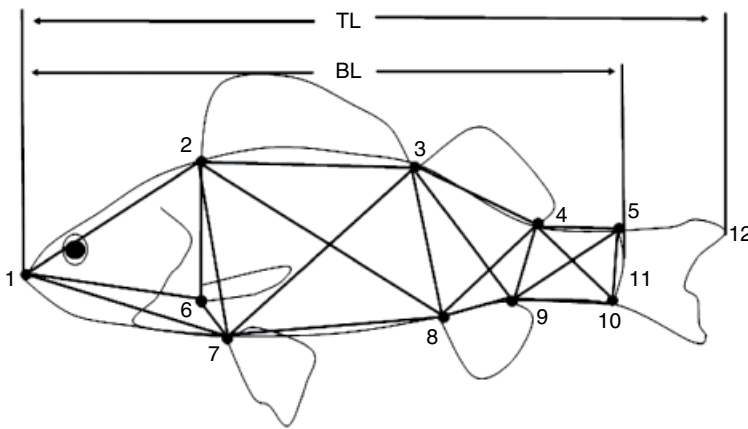


Figure 22.1 The truss network, based on 12 landmarks, was used for morphological measurement in yellow perch. The morphological characters described in this study were shown in Table 22.2. (See inserts for the color representation of this figure.)

- a) One specimen of *Perca flavescens* with 12 landmarks (stars refer to the locations of landmarks).
b) One pattern of morphometric measurements that were measured between the landmarks as lines. 1: anterior tip of snout; 2: anterior insertion of first dorsal fin; 3: posterior insertion of first dorsal fin; 4: posterior insertion of second dorsal fin; 5: dorsal origin of caudal fin; 6: bottom of pectoral fin; 7: origin of pelvic fin; 8: origin of anal fin; 9: terminal of anal fin; 10: ventral origin of caudal fin; 11: anterior margin of the caudal fin; 12: upper terminal of caudal fin.

significant correlations between morphometric measurements and fish size [32, 33]. Therefore, in order to eliminate the influence of the individual's size, all obtained morphological measurements were standardized and transformed to size-independent shape variables, according to the allometric standardized procedure described by Elliott *et al.* [34], using the following equation, before being used for analyses.

$$M_{adj} = M(Ls/Lo)^b \quad (22.5)$$

In this formula, M is the original morphometric measurement, M_{adj} is the

size-standardized measurement, Lo is the standard length of fish, and Ls is the arithmetic mean of standard length for all fish from all samples for each variable. The parameter b was estimated for each character from the observed measurement, as the slope of regression of $\log M$ on $\log Lo$, using all individuals. This standardized step can be substantial interpretation of morphology differences independent of fish size [30].

The principal component analysis (PCA) and discriminant analysis were performed using IBM SPSS Statistical Version 19.0 [35]. PCA is a weighted linear combination of

Table 22.2 Morphometric measurements of yellow perch from the landmark in Figure 22.1.

Code	Landmark	Character
X ₁	Wt	Fish body weight
X ₂	TL	From anterior tip of snout to upper terminal of caudal fin (total length)
X ₃	BL	From anterior tip of snout to anterior margin of the caudal fin (body length)
X ₄	1–2	From anterior tip of snout to anterior insertion of first dorsal fin
X ₅	1–6	From anterior tip of snout to bottom of pectoral fin
X ₆	1–7	From anterior tip of snout to origin of pelvic fin
X ₇	2–3	From anterior insertion of first dorsal fin to posterior insertion of first dorsal fin
X ₈	2–6	From anterior insertion of first dorsal fin to bottom of pectoral fin
X ₉	2–7	From anterior insertion of first dorsal fin to origin of pelvic fin
X ₁₀	2–8	From anterior insertion of first dorsal fin to origin of anal fin
X ₁₁	3–4	From posterior insertion of first dorsal fin to posterior insertion of second dorsal fin
X ₁₂	3–7	From posterior insertion of first dorsal fin to origin of pelvic fin
X ₁₃	3–8	From posterior insertion of first dorsal fin to origin of anal fin
X ₁₄	3–9	From posterior insertion of first dorsal fin to terminal of anal fin
X ₁₅	4–5	Form posterior insertion of second dorsal fin to dorsal origin of caudal fin
X ₁₆	4–8	Form posterior insertion of second dorsal fin to origin of anal fin
X ₁₇	4–9	Form posterior insertion of second dorsal fin to terminal of anal fin
X ₁₈	4–10	Form posterior insertion of second dorsal fin to ventral origin of caudal fin
X ₁₉	5–9	From dorsal origin of caudal fin to terminal of anal fin
X ₂₀	5–10	From dorsal origin of caudal fin to ventral origin of caudal fin
X ₂₁	6–7	From bottom of pectoral fin to origin of pelvic fin
X ₂₂	7–8	From origin of pelvic fin to origin of anal fin
X ₂₃	8–9	From origin of anal fin to terminal of anal fin
X ₂₄	9–10	From terminal of anal fin to ventral origin of caudal fin

correlated variables, and is commonly used to clarify the greater part of variation in the original data [36]. PCA with varimax totation was employed to enhance the interpretability of the factor analysis in this study, because the rotation minimizes the number of variables that have high loadings on a factor. Only factors with eigenvalue more than 1.00 were considered as important ones [37], and variables were tested by ANOVA at $P < 0.05$.

Discriminant analysis was performed to identify the most important measurements for differentiating sexes [38]. Stepwise

discriminant analysis, based on *wilks' lambda*, was used to evaluate the similarities between populations. The relative importance of morphometric traits in discriminating two populations was assessed using *F-to-remove* statistic (*F-to-enter*, 3.84; *F-to-remove*, 2.71). Collinearity among the variables used in the discriminant model was evaluated by the tolerance statistic. The individuals were assigned into different samples, based on resultant discriminant analysis. The classification success rate was evaluated, based on the proportion of individuals correctly reallocated into original samples.

22.4 Growth Dimorphism in Different Culture Conditions

Three different types of culture settings were put into effect to investigate sexual growth performance in this study. At the initial point, females were larger than males in the pond, whereas the males were larger than females in indoor and outdoor tanks, but the differences were not significant (Table 22.3). At 25 weeks, females were significantly larger than males ($P<0.05$) in all the experiments (Table 22.3).

The regression equations of body weight (BW) to total length (TL) of different sexes were evaluated (Table 22.4). In each of the sampling times, growth rates of females were higher than males (AGRW-female>AGRW-male; AGRL-female>AGRL-male). In the whole experiment period, the females grew faster than the males (pond: AGRW-female/AGRW-male = 1.71; Indoor: AGRW-female/AGRW-male = 1.62; Outdoor: AGRW-female/AGRW-male = 1.60). AGRW and AGRL of females and males in indoor tanks were the lowest among the three experimental groups.

All samples in this study were collected at weeks 0, 6, 12, and 25 of the experiment. The growth pattern of mean BW and TL of yellow

perch were consistent in the different settings, in which the sexual growth dimorphism was gradually exhibited (Figure 22.2). There were significant differences ($P<0.01$) in BW and TL of females and males at 12 weeks in the pond group. The mean BW was 89.1 g (mean TL 18.5 cm) for females and 68.1 g (mean TL 17.1 cm) for males in the pond group. At the same time, there were also significant differences ($P<0.01$) between sexes in the outdoor tank group. The mean BW was 65.4 g (TL 16.8 cm) for females and 50.9 g for males (TL 15.8 cm), respectively. However, BW and TL between females and males did not exhibit significant differences ($P>0.05$) in the indoor tank group at 12 weeks. At the 25th week, there were significant differences between sexes ($P<0.01$) in BW and TL for the indoor tank group. Mean BW in females and males were 85.6 g (TL 18.9 cm) and 60.1 g (TL 17.1 cm), respectively.

Throughout the experiment, the tendency of growth was consistent, and exhibited obvious advantage in body weight for females (44.62% for the pond group, 42.49% for the indoor tank group, and 48.12% for the outdoor tank group). The body weight advantage was 14.76% when the experiment was carried out for six weeks,

Table 22.3 Growth of yellow perch in body weight between genders in this study.

Week	Pond (g)		Indoor tank (g)		Outdoor tank (g)	
	Female	Male	Female	Male	Female	Male
0	46.4 ± 24.5	43.9 ± 23.1	13.7 ± 6.1	15.8 ± 7.9	13.7 ± 6.1	15.8 ± 7.9
6	66.9 ± 31.1	58.3 ± 31.9	26.1 ± 9.6	26.6 ± 12.1	28.3 ± 11.0	27.3 ± 13.8
12	89.1 ± 29.6	68.1 ± 26.2	39.5 ± 12.6	38.3 ± 16.6	65.4 ± 22.3	50.9 ± 20.1
25	158.6 ± 66.4	109.7 ± 42.8	85.6 ± 18.9	60.1 ± 18.7	146.9 ± 30.4	99.1 ± 32.4

Table 22.4 Regression equations of weight in total length of different sexes.

Sites	Female	Male
Pond	$W = 0.006660 \text{ TL}^{3.2330} (R^2 = 0.971)$	$W = 0.004628 \text{ TL}^{3.3549} (R^2 = 0.980)$
Indoor tank	$W = 0.005053 \text{ TL}^{3.3144} (R^2 = 0.981)$	$W = 0.004586 \text{ TL}^{3.3401} (R^2 = 0.949)$
Outdoor tank	$W = 0.004556 \text{ TL}^{3.3645} (R^2 = 0.988)$	$W = 0.004649 \text{ TL}^{3.3440} (R^2 = 0.979)$

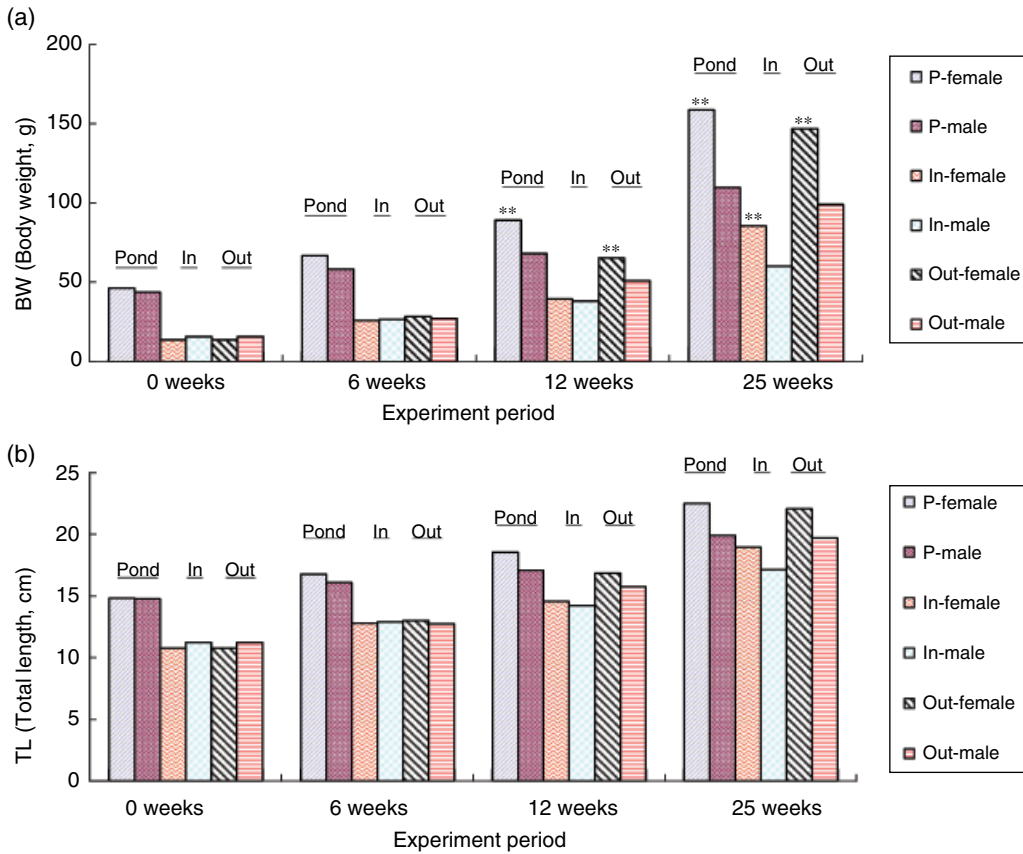


Figure 22.2 Mean body weight and total length growth in different culture conditions between female and male yellow perch:

a) Mean body weight;

b) Mean total length, base on the results of different period as given in Table 22.1.

The signal ** presents the significant level ($P < 0.01$). Pond, In and Out represent pond, indoor tank and outdoor tank experiments fish. P-female and P-male represent the female and male individuals in pond group. In-female and In-male are for female and male fish in the indoor tank group. Out-female and Out-male are for the female and male fish in outdoor tank group.

subsequently increased to 30.82% in 12 weeks, and then reached 44.62% in 25 weeks in the pond group.

With increasing time of experiment, the total length advantage of females exhibited was more obvious. At the initial point, the females' total length advantage in the pond group was small ($SDLA = 0.47\%$), whereas the advantage was obvious ($SDLA = 13.02\%$) when the experiment was finished.

The females had no initial total length advantage ($SDLA = -4.18\%$), and acquired more than a 10% advantage for the indoor

tank group (10.70%) and the outdoor tank group (11.99%).

In order to further compare the morphometric difference before and after sexual size dimorphism obviously appeared, six ratios (body length/total length, body height/total length, body width/total length, body height/body length, body width/body length, and body width/body height) were investigated. However, neither different genders (female and male) nor the same genders (female and female; male and male) exhibited an obvious difference ($P > 0.05$). The result indicated

that there were unobvious differences in these morphology characteristics when sexual size dimorphism appeared. Figure 22.3 shows the total length frequency distributions of male and female.

22.5 Morphometric Traits for Analysis of Body Form

Based on measurements and analyses of 100 males and 100 females from a lake ($N=89$), ponds ($N=57$), and tanks ($N=54$), the statistical information of yellow perch is listed in table 22.1. It should be noted that the overall size of females ($104.5\text{ g} \pm 51.8\text{ g}$) was significantly larger than that of males ($61.6\text{ g} \pm 32.6\text{ g}$, $P<0.01$). The total length distribution of samples in both males and females is shown in Figure 22.3.

All measurements of traits were highly repeatable. Nine morphological traits, including X_3 (body length, BL), X_4 (1–2), X_7 (2–3), X_{10} (2–8), X_{12} (3–7), X_{13} (3–8), X_{15} (4–5), X_{21} (6–7), and X_{22} (7–8), were positively related to total length ($b > 1.0$), whereas four traits, namely X_5 (1–6), X_6 (1–7), X_{17} (4–9), and X_{23} (8–9), showed negative relationships with the total length ($b < 1.0$) (Figure 22.4). The rela-

tionship of the remaining traits with total length exhibits a difference between male and female individuals

22.6 Principal Component Analysis

PCA of morphology characteristics was carried out in male and female populations, based on varimax totation. The results of PCA revealed that the eigenvalues of the first seven PCs in females and the first eight PCs in males were above 1.00. The first three principal components factoring the loading of male and female yellow perch are listed in Figure 22.5. Subsequently, each of the PCs explained less than 9% of the variables size-independent body morphology variables of both female and male populations, and were not interpreted.

In both sexes, the most important difference was located in the posterior body portion. For both female and male populations, the first component was correlated with the caudal peduncle significantly ($P<0.01$), indicating that caudal peduncle significantly differed between female and male individuals. In females, the second and third principal components were mainly

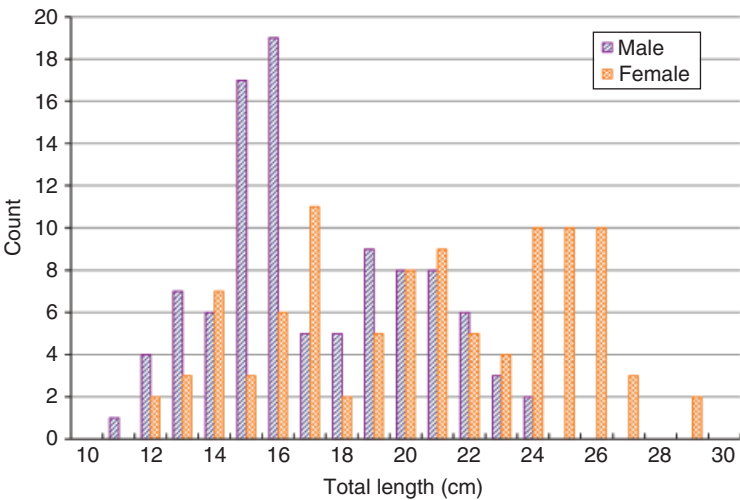


Figure 22.3 Total length frequency distributions of male and female *Perca flavescens* in this study.

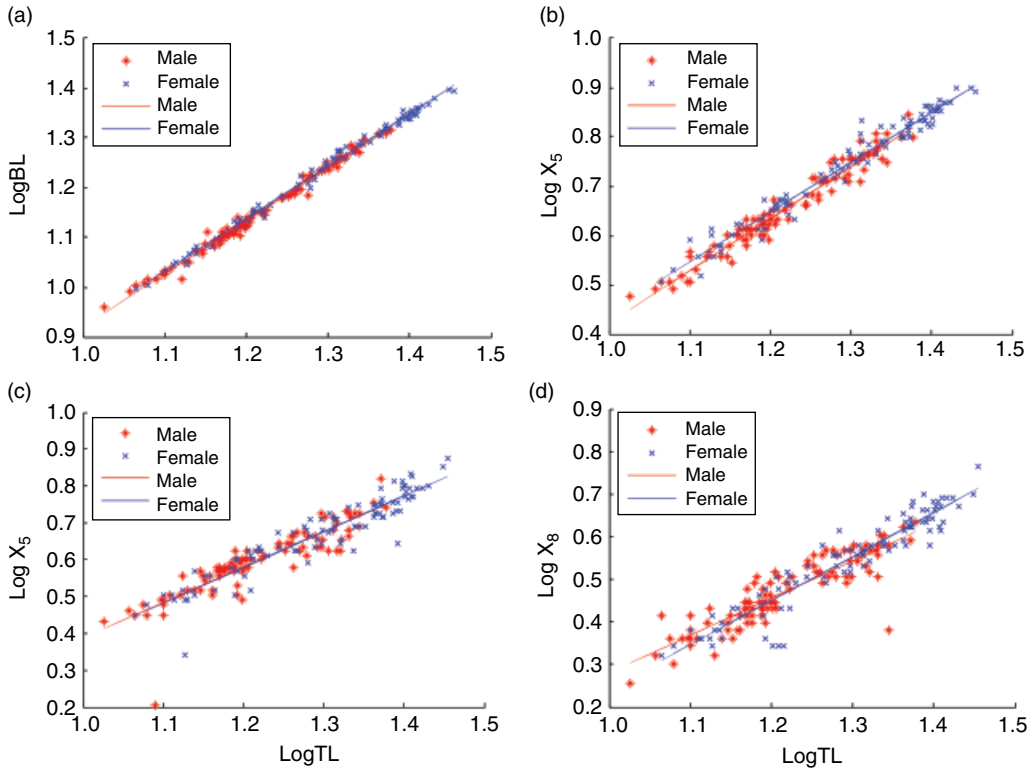


Figure 22.4 Allometric scaling relationship for BL, X₄, X₅, and X₈ for male and female *Perca flavescens*.

a) BL, Body length.

b) X₄, distance of landmarks 1–2.

c) X₅, distance of landmarks 1–6.

d) X₈, distance of landmarks 2–6.

All variables are log₁₀ transformed. The lines represent standard major axis regression slopes for different sexes.

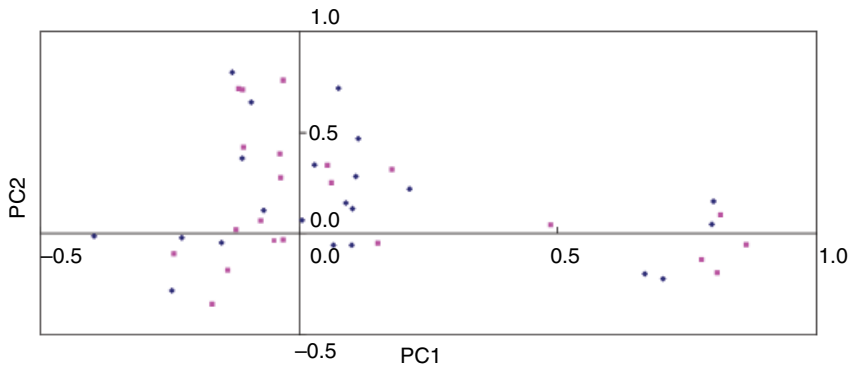


Figure 22.5 Principal component plot of morphometrics variation based on PC1 and PC2 (Square or pink represents females and Diamond or blue represents males).

Table 22.5 Principal components of males and females in yellow perch.

Item	Male			Female		
	PC1	PC2	PC3	PC1	PC2	PC3
X ₃	0.103	0.123	−0.021	0.179	0.314	−0.275
X ₄	0.102	−0.056	0.079	−0.137	−0.181	0.126
X ₅	−0.151	−0.043	0.839	−0.073	0.062	0.082
X ₆	−0.226	−0.017	0.617	−0.121	0.018	−0.030
X ₇	0.005	0.068	0.066	0.152	−0.046	0.114
X ₈	0.109	0.284	0.247	−0.106	0.425	0.527
X ₉	−0.109	0.372	0.046	0.062	0.252	0.787
X ₁₀	−0.092	0.651	0.377	−0.116	0.715	0.104
X ₁₁	0.090	0.151	0.177	−0.037	0.393	0.062
X ₁₂	−0.128	0.797	−0.065	0.055	0.336	0.688
X ₁₃	0.115	0.471	0.421	−0.108	0.710	0.211
X ₁₄	0.030	0.338	0.614	−0.029	0.755	0.162
X ₁₅	0.703	−0.222	−0.112	0.778	−0.130	−0.105
X ₁₆	0.066	−0.055	0.076	−0.030	−0.031	0.087
X ₁₇	−0.069	0.116	0.073	−0.034	0.274	−0.040
X ₁₈	0.797	0.047	−0.063	0.808	−0.194	0.168
X ₁₉	0.800	0.160	−0.150	0.864	−0.057	0.076
X ₂₀	0.214	0.223	0.245	0.487	0.042	0.252
X ₂₁	−0.245	−0.279	0.009	−0.168	−0.351	0.069
X ₂₂	0.076	0.721	−0.040	−0.047	−0.034	−0.094
X ₂₃	−0.396	−0.012	0.242	−0.242	−0.101	0.457
X ₂₄	0.667	−0.200	0.028	0.815	0.090	−0.223

shown on the posterior belly width and head height, respectively. In males, the second and third principal components were inconsistent with those of females, and were principally focused on the anterior belly width and head length characters. In females, the combination of the first two principal components explained as much as 31.347% of the variation of all variables, and as much as 28.685% of the variation in males. As a result, a principal components plot showed that most plots overlapped, and were not divided into two groups between males and females (Figure 22.5).

22.7 Discriminant Analysis

To discriminate the males and females, the stepwise discriminant analysis was performed. The discriminant function tested, based on *Wilk's lambda* values, was significant ($\lambda = 0.021$, $P < 0.001$), indicating significant differences in morphometric characters of female and male populations. The statistics results of *Wilk's lambda* values, F-values, probability, and tolerance statistics of variables used in this discriminant function, are listed in Table 22.6.

Table 22.6 Morphometric characteristics abstracted by stepwise discriminant analysis to discriminate male and female *Perca flavescens*.

Variable	Wilk's lambda	F-remove	P-level	Tolerance
X_3	0.050	255.931	<0.001	0.906
X_4	0.023	15.340	< 0.001	0.905
X_6	0.022	9.630	< 0.001	0.920
X_8	0.022	10.580	< 0.001	0.829
X_{10}	0.023	17.705	< 0.001	0.877
X_{18}	0.023	15.266	< 0.001	0.932
X_{23}	0.022	4.425	< 0.001	0.932

Seven variables were extracted to establish the objective discriminant function. The standardized canonical discriminant function, based on seven discriminating variables, was:

$$Y = 2.599X_3 + 1.255X_4 + 0.794X_6 + 0.962X_8 \\ + 1.017X_{10} + 0.837X_{18} + 0.708X_{23} - 67.316$$

(Wilks' Lambda = 0.021, $\chi^2 = 749.273$, $P < 0.01$)

The fisher's linear discriminant functions, based on the same seven variables, were:

$$Y(\text{males}) = 158.155X_3 + 67.911X_4 + 55.913X_6 \\ + 62.112X_8 + 58.649X_{10} + 52.023X_{18} \\ + 42.395X_{23} - 1838.530$$

$$Y(\text{females}) = 193.269X_3 + 84.867X_4 + 66.640X_6 \\ + 75.107X_8 + 72.394X_{10} + 63.331X_{18} \\ + 51.965X_{23} - 2748.074$$

Fisher's linear discriminant was used to test male and female populations. The discriminant analysis results showed that 100% of original grouped individuals were correctly classified, and 100% cross-validation grouped individuals were correctly classified (Table 22.7).

22.8 Perspectives and Applications

Sexual size dimorphism is widely reported in fish [2, 4, 39, 40]. Some studies have been carried out to investigate the involved mechanism of dimorphism, and devoted to improve the growth performance through selective breeding programs [9, 21, 39]. In the past decade, all-male or all-female breeding programs have made great improvements, such as those with yellow catfish, Nile tilapia, and Japanese flounder [7, 41, 42].

Yellow perch is already known to exhibit sexual growth dimorphism, with females growing faster and bigger than males. Sexual dimorphism in body size was further proved in different rearing conditions in this study. The BW exhibited significant differences ($P < 0.05$) between females and males, with the female BW ranging from 65.4 g to 89.1 g and male BW ranging 50.9 g to 68.1 g. It was further supposed, based on the three different rearing conditions, that females significantly outgrew males ($P < 0.05$), when female BW ranged from 65.4 g to 89.1 g and male BW ranged from 50.9 g to 60.1 g, and female

Table 22.7 Discriminant analysis between male and female *Perca flavescens*.

Method	Population	Predicted group membership		Discriminant accuracy (%)	Total discriminant accuracy (%)
		Male	Female		
Origin	Male	100	0	100	100
	Female	0	100	100	100
Cross-validation	Male	100	0	100	100
	Female	0	100	100	100

TL ranged from 16.8 cm to 18.5 cm and male TL ranged from 15.8 cm to 17.1 cm.

In previous studies, sexual growth dimorphism of yellow perch was initially detected, but was not significant ($P > 0.05$) when the mean female body weight and total length were 10.8 cm and 16.7 g, while mean female body weight and total length were 10.2 cm and 13.5 g, respectively [23]. However, in this study, the smallest observed female and male TL were 13.0 cm (28.3 g) and 12.8 cm (27.3 g), respectively, when the size difference was detected, which is similar to Rennie's result (13.0 cm in TL) [24]. At that size, most male individuals are mature, which supports the opinion that sexual size dimorphism is related to maturation [23, 24]. However, results obtained from different experiments showed that surroundings were not completely consistent for BW and TL in females and males when sexual size dimorphism was obviously found. It is clear that females outgrow males and are larger, indicating that sexual size dimorphism is influenced by the maturation.

To study the mechanism of sexual size dimorphism and carry out the breeding program, some experiments must be carried out on the basis of female and/or male individuals. However, it is very difficult to determine the sex from the external characteristics, because of unclear differences in secondary sexual characteristics between male and female yellow perch. To effectively perform the research procedure, the specific morphology of different sexes must be investigated fully. Sexual dimorphism with differences in body form between genders has also been proven in this study.

The most obvious differing morphology characteristic between females and males was located in the posterior trunk region, especially in the caudal peduncle shape of the fish, which was assumed to be related with swimming type, performance, and foraging strategy [43]. A longer caudal peduncle usually enhances the ability of prolonged swimming; what is more, a deeper peduncle benefits powerful sprint swimming [43].

The variability of locomotor abilities for aquatic vertebrates is usually interpreted as an evolutionary adaptation, which was tied to social purpose or swimming capabilities [44]. There are some specific performances, such as mating selection, foraging, and predator avoidance. Female yellow perch exhibited a longer and wider caudal peduncle than males, which was supposed to be an important factor of growth difference. The females were assumed to possess more powerful ability in swimming and foraging than males.

Roff (1983) [45] and Rennie *et al.* (2008) [24] supposed that having smaller males relative to females in teleosts was a selective response to increasing survival by reducing foraging activity (presuming that more activity entails more predation). However, male morphology characteristics possibly result not only in weak foraging ability, but also in growth dimorphism. It has also been proven that mature males have lower food consumption, metabolic costs, and food conversion efficiencies, compared with females [24]. As a result, females exhibit faster growth rate and larger size than males.

Phenotypes are a comprehensive outer expression of genetic manipulation, environment modification, and interaction between genetics and environment [46]. Morphometric characteristics have become an important tool to identify developmental thresholds and sexual growth dimorphism of some fishes in both males and females [16, 47]. Most studies related to sexual dimorphism have focused on sex allometric growth or sexual size dimorphism, rather than identifying their gender in fishes [48, 49].

In prior studies, comparison of morphometric traits has also proved that the ability of morphometric discriminant function could be used to correctly classify individuals, which is consistent with results obtained by discriminant function analyses with other fish species [50–52]. Malison *et al.* (2011) [25] developed an effective method to identify the different sexes by the external morphometric characteristics in yellow perch. The accuracy of this identification method

ranged from 82.7% to 97.4%, and larger size individuals had a higher proportion identified correctly. Similarly, Shepherd *et al.* (2013) [26] also developed a rapid determination method based on the external morphological characteristics. The test results showed that the accuracy was 97.3% for both sexes (98.8% for females and 95.5% for males).

There was also high correlation to these two methods by Malison *et al.* (2011) and Shepherd *et al.* (2013) [25, 26], which was above 82%. However, the two methods were established on the basis of external morphological criteria, especially the area of urogenital papilla (UGP). The successful use of the two methods mainly depends on the significant degree of UGP characteristics, and the professional skills of observers.

The classification function was set up on the basis of the morphology variations existing in female and male yellow perch, and used to identify both sexes of yellow perch. Seven discriminating variables were extracted to establish discriminant equations, and were sufficiently robust to discriminate the males and females. The discriminant functions were completely established by the measurements of body morphology traits. Moreover, the initial measurements were standardized to eliminate the influences of the individual's size. The algorithm can eliminate subjective factor influence, and can be more precisely applied in practical aquaculture.

Both standardized canonical discriminant function and Fisher's linear discriminant function were established in this study. What is more, both original and cross-validation grouped individuals were correctly classified

with 100% accuracy. The functions provided important and informative variables to differentiate male and female individuals. Other investigations have also been implemented to discriminate the different sexes in fishes based on external morphology, such as *Oreochromis mossambicus* [48] and *Oncorhynchus tshawytscha* [53]. 77.6% of the *Oreochromis mossambicus* individuals were adequately classified by the reduced discriminant function [48]. The best predictor for identifying gender of *Oncorhynchus tshawytscha* could correctly classify 96% of individuals [53].

Compared to those sex determinations models, the function obtained in this experiment provided a very high degree of accuracy, and can be used to effectively determine the gender. Therefore, the present classification function established is an effective tool to differentiate sexes, which could aid their effective breeding management and precise experiment control. However, the discriminant function was obtained on the basis of all samples being from southern Ohio, and needs to be tested further in other populations.

Acknowledgments

This work was supported by the U.S. National Oceanic and Atmospheric Administration (NOAA) and Ohio Sea Grant. Salaries and research support were provided by state and federal funds appropriated to The Ohio State University, Ohio Agricultural Research and Development Center.

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Part VII

Sex Determination and Sex Control in Catfish

23

Sex Determination, Gonadal Sex Differentiation, and Sex Control in Channel Catfish

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23.1 Introduction

The channel catfish (*Ictalurus punctatus*) is a member of the family Ictaluridae, also known as the North American catfishes. While its range originally included northeastern Mexico, the central drainages of the United States (US), and the prairie provinces of Canada, channel catfish can now be found in other regions of North America, and in multiple other countries around the world [1, 2]. Anthropogenic transfers of channel catfish outside its native range have been carried out because of its appeal as sport fish and aquaculture species. Captive channel catfish can tolerate a wide range of environmental conditions, and its processed flesh is of high quality and palatability, attributes that were key to the development of major catfish aquaculture industries in the United States [3, 4] and, more recently, in China [4].

The economic success of an aquaculture project generally depends on a combination of adequate production, cost, technological, and marketing conditions [5]. In channel catfish, a gonochoristic species, biological approaches to enhance growth and, therefore, production efficiency, have included selective breeding to generate faster growing strains [6] and interspecific crosses between female *I. punctatus* and male *I. furcatus* (blue catfish), to produce heterotic hybrids [7, 8].

Another approach to increase channel catfish production is monosex culture, as all-male progeny of channel catfish grow 10–30% faster than females [9–11]. A growth differential between young males and females has also been reported for hybrid catfish [12].

The potential of monosex male culture to improve production efficiency has prompted research into the mechanisms of sex determination, gonadal sex differentiation, and sex control in channel catfish. Knowledge of these mechanisms in channel catfish is presented here in the broader context of information generally available for teleosts. While brain sex differentiation may occur in some teleosts at approximately the same time as, and be associated with, gonadal sex differentiation [13, 14], little or no information pertaining to brain sex differentiation is available for channel catfish, and this chapter therefore focuses solely on gonadal sex differentiation.

23.2 Sex Determination

Channel catfish do not have heteromorphic sex chromosomes [15]. Consistent with female homogamety (XX), artificial gynogenesis yields all-female populations [16]. Also, when presumptive genetic male/phenotypic female (i.e., XY) channel catfish,

produced by feminizing steroid treatment, are crossed with normal males (i.e., XY), the sex ratios of the progeny are consistent with male heterogamety and with the production of viable YY males [17]. The chromosomal sex constitution, viability, and reproductive functionality of YY males was confirmed by rearing these individuals to sexual maturity, crossing them with normal (XX) females, and showing that their progenies consisted exclusively of male (XY) individuals [18]. These observations demonstrated that channel catfish sex, while labile to chemical or hormonal treatments and to extreme environmental conditions (sections 4 and 5), is normally fixed at fertilization by the dual chromosomal XX-XY sex determination system (Box 23.1).

The channel catfish was one of the first non-mammalian vertebrates surveyed for “master” sex-determining (SD) genes in the early 1990s [19, 20], shortly after novel discoveries were made with humans [21]. These surveys showed that, while sequences similar to the masculinizing SD gene of mammals (*SRY*) also exist in catfish, they are not sex-linked. More recently, a male-specific microsatellite marker was identified in the channel catfish genome [22]. PCR amplification of this marker yielded two amplicons of slightly different length. The shorter amplicon is male-specific [22] and is located within the

sex-linked region of the channel catfish Y chromosome [23], while the longer amplicon is present in the genome of both sexes [22]. It seems possible, if not likely, that the male-specific marker is closely associated with the catfish SD gene, but the identity of this gene is yet to be elucidated.

23.3 Morphological and Cytological Indices of Gonadal Sex Differentiation

In its broadest sense, gonadal differentiation begins with the formation of gonadal ridges during embryogenesis, and continues until the gonads reach full development in adult individuals [24]. Gonadal sex differentiation refers to the divergence in molecular, cytological-morphological, and physiological traits between genetic male and female gonads. Gene expression is the first step toward the establishment of observable phenotypes, and the onset of gonadal sex differentiation at the molecular level is, thus, marked by the moment when sex-linked differential gene expression first occurs. Cytologically and structurally, however, teleostean ovaries typically differentiate earlier than testes [24, 25], and channel catfish is no exception [26].

Ovarian differentiation in channel catfish reared at a temperature of 27–28°C begins about 19 days post fertilization (dpf) (Box 23.2). Proximal and distal tissue outgrowths (relative to the hilar region) are observed at this time in presumptive ovaries that will later fuse to form an ovarian cavity (Figure 23.1), and oogenesis (oocyte meiosis) is evident by 22 dpf. On the other hand, while some growth of presumptive testes takes place during the first 90 dpf, they remain histologically indifferent and, relative to ovaries, very small in size (Figure 23.2). Clear histological signs of testicular differentiation are not observed until sometime between 90–102 dpf [26]. These signs include the early organization of testicular tubules and appearance of anlagen for the outward villiform projec-

Box 23.1 Sex determination

Channel catfish do not have heteromorphic sex chromosomes. Sex in this species is established at fertilization according to a male heterogametic system (XX-XY) [17]. Also, while the sex-determining gene is unknown, a male-specific microsatellite marker was recently identified [22]. Female-skewed populations can be produced by high rearing temperatures (e.g., 34°C) [26], indicating that genetic sex can be influenced by extreme environmental conditions. Under normal conditions, however, male : female sex ratios are stable at 1 : 1.

Box 23.2 Gonadal sex differentiation

At a water temperature of 27–28°C, morphological differentiation of the ovary is first observed at 19 days post fertilization (dpf), and testicular differentiation at ≈102 dpf [26]. Full and functional reversal of genetic males into phenotypic females can be achieved by dietary treatment with estrogens or androgens. An effective feminizing treatment consists of oral administration of 60 mg 17 α -ethynyltestosterone/kg of food given to the fry for 21 days after the onset of feeding, at a water temperature of 21°C [17, 27]. However, the same treatment at a water temperature of 27–28°C also seems effective [26].

tions, typical of adult catfish testes (Figure 23.3), and mitotic proliferation of spermatogonial cells between 90 and 102 dpf (compare Figures 23.2e and 23.3c).

23.4 Signaling Mechanisms of Gonadal Sex Differentiation

23.4.1 Feminizing Signals

Dietary administration of feminizing steroids (e.g., estradiol-17 β) and most androgens (see Section 23.5 for discussion of “paradoxical sex reversal”) to channel catfish young causes functional sex-reversal of genetic males into phenotypic females [17, 27]. Indifferent gonads of genetic male catfish appear to be most sensitive to sex-reversal when steroid treatment begins at first feeding (≈10 dpf), before the onset of ovarian differentiation in genetic females (Section 23.3), and continues for a total period of three weeks (Box 23.2). These observations with channel catfish are consistent with data from numerous other studies of teleosts conducted over the last six decades [25, 28–33]. Based on this information, and as it was summarized by Guiguen *et al.* [33], the current view of gonadal sex differentiation in gonochoristic teleosts is that upregulation of the gonadal

aromatase gene, *cyp19a1a*, and the attendant increase in endogenous estrogen production, play an indispensable regulatory (stimulatory) role in the formation of ovaries.

While the information available for channel catfish is consistent with a role of endogenous estrogen in the feminization of its indifferent gonads, *cyp19a1a* expression or endogenous estrogen production have not been examined during the period of gonadal sex differentiation in this species. Given the seemingly universal role of estrogen in ovarian differentiation of teleosts, however, it is reasonable to assume that channel catfish is no exception.

High temperature yields female-skewed populations in channel catfish. Compared to the even ratio between males and females in fish reared at control temperature (27°C), the ratio at high temperature (34°C) was 1 : 1.7, nearly doubling the proportion of females [26]. No significant deviations from the control ratio were observed at low temperature (20°C). The mechanism behind these observations is uncertain. Although they could be interpreted as reflecting an increase in the intensity of, or sensitivity to, feminizing signals at high temperature, the alternative is also possible – a disruption of masculinizing signals (section 23.4.2). It must be noted, however, that temperatures required to produce biased sex ratios in channel catfish ($\geq 34^\circ\text{C}$) are extreme, and may also impair growth of the exposed fry [34].

23.4.2 Masculinizing Signals

A model describing the control of testicular differentiation in teleosts has been proposed that it is also based on *cyp19a1a* [33]. According to this model, downregulation (or in some species, absence of upregulation) of *cyp19a1a* is not only necessary, but is also sufficient for testicular formation in genetic males. This hypothesis is based on observations that sex reversal of genetic females into phenotypic males can be achieved by treatment with aromatase inhibitors or estrogen receptor antagonists [33]. Also, androgens

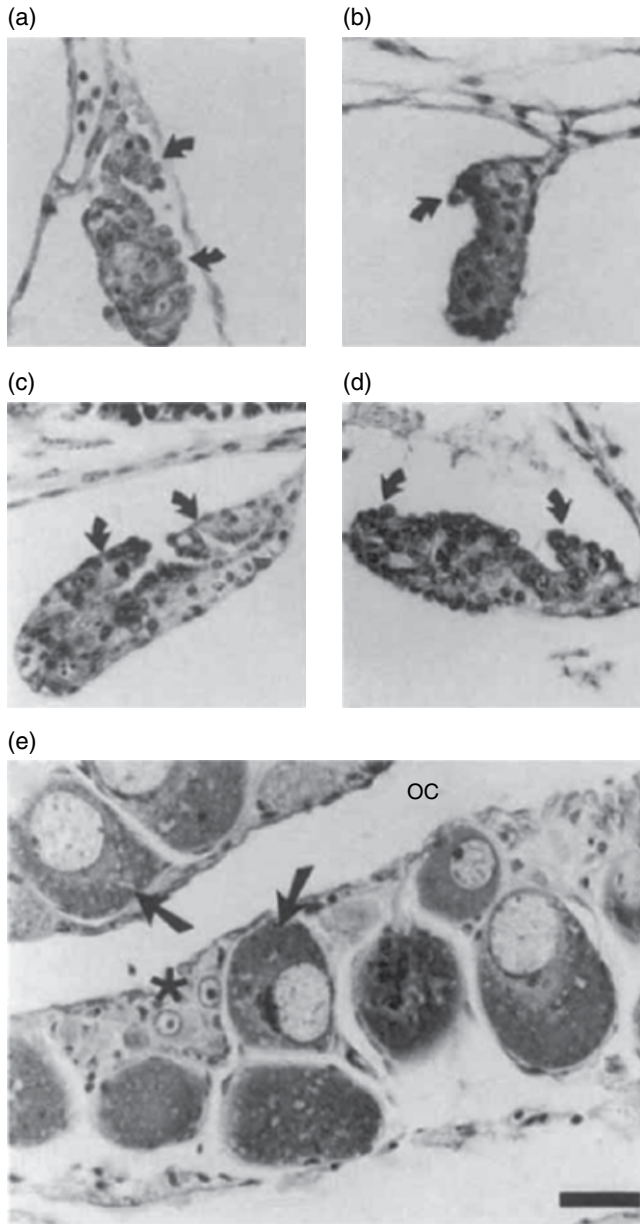


Figure 23.1 Early ovaries of channel catfish.

Day 19: gonads from individuals of a mixed-sex population (a) and from a sex-reversed female population (b) showing tissue outgrowths (curved arrows) at the proximal and distal ends. By Day 22, outgrowths had grown in size and projected towards each other in presumptive ovaries (c) and in sex-reversed ovaries (d).

By Day 90, ovaries (e) had a well-developed ovarian cavity (OC) and growing perinucleolar follicles (arrows); the ovary shown is of a female from a mixed-sex population. Oogonial nests (asterisk) are present in the germinal epithelium lining the ovarian cavity. Bar = 20 μ m.

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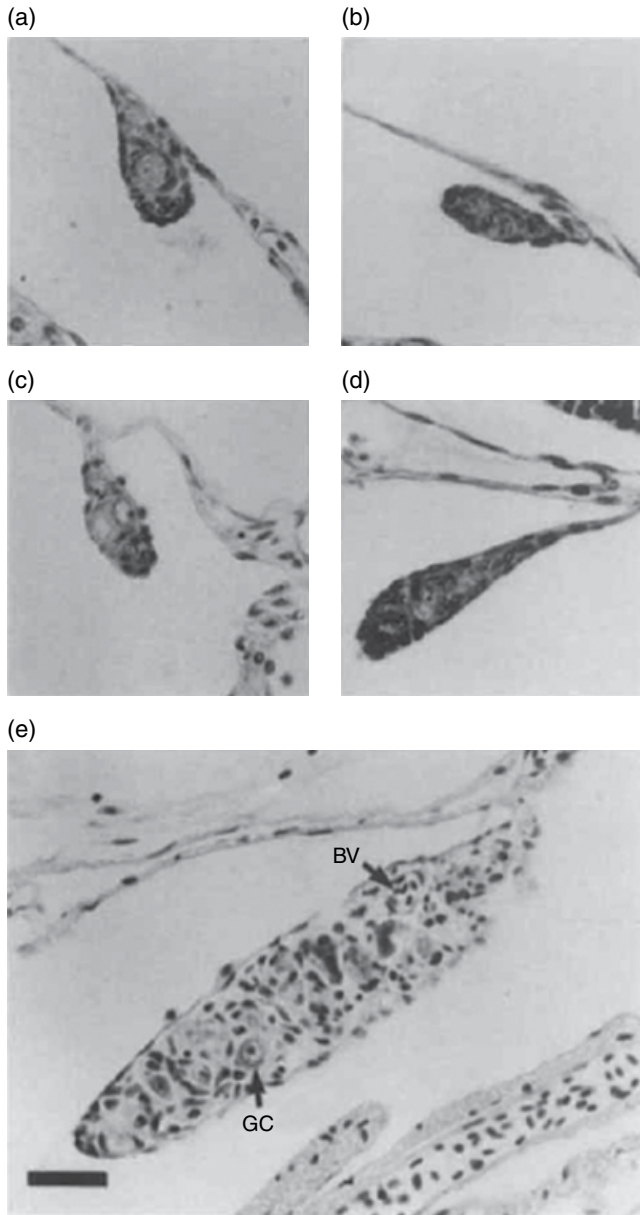


Figure 23.2 Indifferent testes of channel catfish.

a, c, e: Gonads of presumptive males from a mixed-sex population at Day 19, 22, and 90, respectively.

b, d: Gonads of genetic males at Day 19 and 22, respectively.

GC, germ cell; BV, blood vessel. Bar = 20 μ m.

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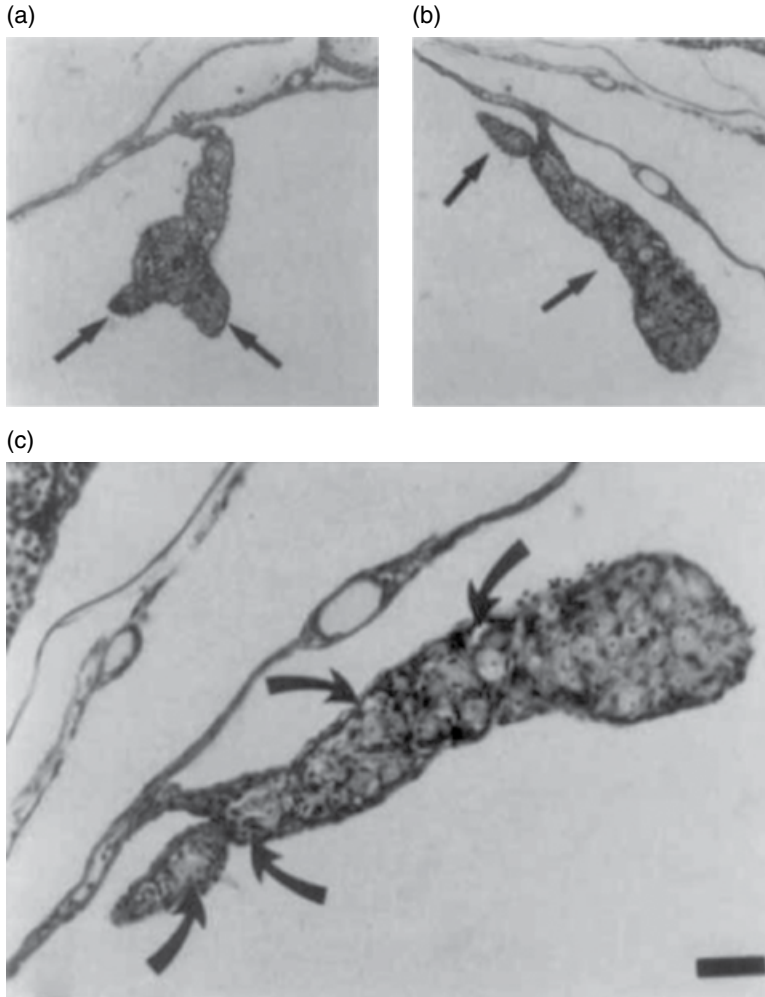


Figure 23.3 Testes of Day 102 channel catfish. The appearance of fingerlike projections (straight arrows) and occasional tubule-like cellular aggregations (curved arrows) are morphological signs of testicular differentiation.

a: Testis of fish reared at 27°C during fry stage.

b, c: Testis of fish reared at 34°C.

Bar = 40 μ m (a, b) or 20 μ m (c).

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with the ability to masculinize genetic females appear to do so by downregulating *cyp19a1a* via androgen response elements (ARE) on its promoter region [33]. Thus, the absence of feminizing signal in genetic males, or its artificial blockage in genetic females, is sufficient by itself to start the process of masculinization, making *cyp19a1a* the arbiter and executor of final decisions concerning

the sex phenotype of indifferent gonads. The signal is binary, involving up- or downregulation (of *cyp19a1a*), and so is the outcome – ovaries or testes.

This model implies that the primary, if not the only, role of the SD gene in teleosts is to inhibit *cyp19a1a* expression directly or indirectly. In this sense, the teleostean model proposed by Guiguen *et al.* [33] is similar to

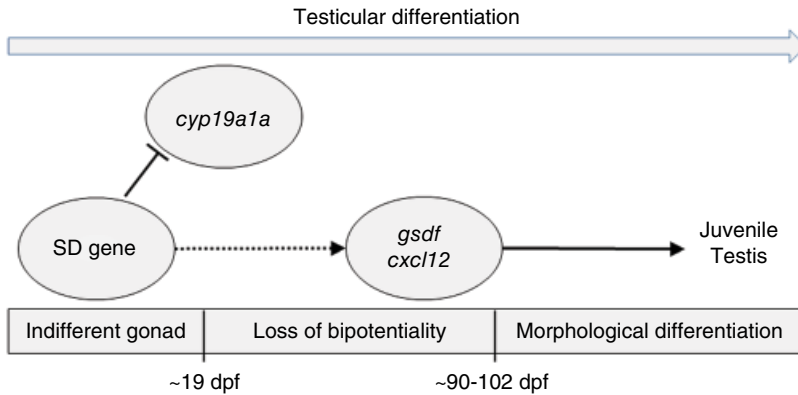


Figure 23.4 Working model of signaling mechanisms during testicular differentiation of channel catfish. In XY individuals, a signal encoded by the sex-determining gene (SD) on the Y chromosome leads to the suppression of *cyp19a1a*, and estrogen is not produced. In the absence of estrogen, the indifferent gonad becomes committed to a male phenotype at ≈ 19 days post fertilization (dpf). The gonad remains morphologically indifferent until $\approx 90\text{--}102$ dpf, when a testis-inducing signal or signals (e.g., *gsdf*, *cxcl12*) are produced, and the first signs of morphological differentiation appear. Testis-inducing signals may be regulated by the SD gene directly or indirectly (via other genes regulated by the SD gene), or may be expressed independently of the SD gene. The outcome of this signaling mechanism is juvenile testes. In XX individuals that lack the SD gene, *cyp19a1a* is not downregulated, estrogen is produced, and ovaries form.

the classical mammalian model of sex determination and differentiation [35, and references therein], where the SD gene (*SRY*) encoded in the Y chromosome leads to the male phenotype by suppressing ovarian-inducing signals (*cyp19a1a* in teleosts), while the absence of *SRY* in XX individuals leads to the female phenotype, due to the unimpeded expression of such signals. In channel catfish, and at least one other teleost (Section 23.4.3), however, this model may not fully explain the process of testicular differentiation.

In channel catfish, indifferent testes experience relatively little growth during the first 90 dpf, and clear histological signs of testicular differentiation do not appear until 90–102 dpf, nearly three months after the onset of ovarian differentiation in females (Section 23.3). This considerable time differential between ovarian and testicular differentiations led Patiño *et al.* [26] to conclude that putative testis-inducing signals, produced around 90 dpf, are necessary for testicular differentiation in channel catfish; in other words, the absence of estrogen production earlier in development is insufficient.

Moreover, because the sensitivity of indifferent testes to sex-reversal is reduced

around the time ovarian differentiation begins in genetic females [17, 26], Patiño *et al.* [26] proposed that the absence of estrogen in early development causes the still-indifferent gonads to commit to a testicular path. Thus, testicular differentiation in genetic male catfish was described in two phases [26]: an early phase, when the absence of a feminizing signal causes the indifferent testes to lose their sexual plasticity; and a late phase, when the production of testis-inducing signals actively drives morphological differentiation (Figure 23.4). It should be noted, however, that the loss or reduction in sexual bipotentiality of indifferent testes during the early phase implies a degree of differentiation, at least at the molecular level, despite the lack of clear cytological or morphological differentiation.

A recent study of channel catfish identified a considerable number of testis-biased gene transcripts during the late phase of testicular differentiation (90–110 dpf) [36]. Perhaps the most intriguing transcripts in regards to sex differentiation are those for *gsdf* and *cxcl12*. *Gsdf* is a teleost-specific gene first identified in rainbow trout,

Oncorhynchus mykiss [37]. Its product is a member of the TGF- β superfamily that is necessary for primordial germ cell (PGC) proliferation in indifferent gonads of both sexes, and for spermatogonial proliferation in juvenile testes [37]. In Japanese medaka (*Oryzias latipes*), *gsdf* is under the control of the SD gene, *dmy*, and its expression is necessary and sufficient to induce testicular differentiation [38]. In another medaka fish, *O. luzonensis*, *gsdf* on the Y chromosome (*gsdf^Y*) has, in fact, become the SD gene of this species [39].

In addition, two recent studies with Nile tilapia (*Oreochromis niloticus*) reported that *gsdf* is necessary for testicular differentiation [40, 41]. In juvenile zebrafish (*Danio rerio*), induction of autoimmunity against Gsdf near the completion of testicular differentiation severely impaired further testicular development by blocking spermatogonial proliferation and onset of spermatogenesis, but appeared to have little effect on ovaries [42]. Based on this information for other species, a likely role for *gsdf* during the late phase of testicular differentiation in channel catfish may be to induce the proliferation of germ cells that occurs at this time (Section 23.3).

Concerning *cxcl12*, its product Cxcl12 is a cytokine that, together with its cognate receptor, Cxcr4, is an important component of mechanisms regulating cell migration during embryogenesis, including PGC migration to the gonadal ridges. The receptor is located on the PGCs and the ligand is produced by the destination tissues [43–45]. Moreover, in rodents, disruption of CXCL12/CXCR4 signaling disrupts migration (but not proliferation) of spermatogonial stem cells (SSCs) to their niche on the boundary of testicular tubules (or lobules, in some teleosts), where SSC renewal occurs and differentiation begins (i.e., spermatogenesis) [46]. These observations suggest that the role of *cxcl12* during testicular differentiation of channel catfish is to participate in the early organization of testicular tubules (Section 23.3).

23.4.3 Thyroid Hormone: a New Masculinizing Signal?

Thyroid hormone (TH) seems to induce reversal of genetic female zebrafish into phenotypic males via mechanisms involving downregulation of *cyp19a1a* [47–49], but upregulation of male sex-related genes also appears to be necessary [49]. While it is unknown if TH can influence the direction of gonadal sex differentiation in channel catfish or any other teleost, there is considerable evidence indicating that TH has masculinizing activity at later stages of testicular development in multiple species from all major vertebrate taxa [50–52]. In addition, TH has recently been reported to have testicular-inducing activity in a turtle species with temperature-dependent sex determination mechanisms [53]. The dual requirement for downregulation of *cyp19a1a* and upregulation of male sex-related genes during TH-dependent female-to-male sex reversal in zebrafish is consistent with the two-phase model of testicular differentiation in channel catfish (Section 23.4.2).

23.5 Paradoxical Sex Reversal

23.5.1 Paradoxical Feminization

Most androgens tested to date have caused paradoxical feminization in channel catfish [17, 27]. The list of androgens examined includes non-aromatizable compounds, thus ruling out aromatization into estrogens as explanation for their feminizing activity [17]. Trenbolone acetate, a synthetic anabolic androgen, is the only androgen that has not shown feminizing activity in channel catfish [54]. This compound, however, does not have normal androgenic activity, either. Its main effect in young catfish seems to be the disruption of normal ovarian and testicular development [55]. Paradoxical feminization by non-aromatizable androgens is not restricted to channel catfish, and also has

been reported in Japanese medaka [56] and in another ictalurid, blue catfish [57].

The mechanism of paradoxical feminization by non-aromatizable androgens is uncertain. These androgens are unlikely to act via estrogen receptors, as channel catfish estrogen receptors are highly specific, and have negligible affinity for androgens [58, 59]. Also, androgens typically suppress the activity of the *cyp19a1a* genes that bear AREs in their promoter region [33]. It was recently reported, however, that androgens can directly upregulate the brain-type aromatase gene (*cyp19a1b*) in the pituitary of ricefield eel (*Monopterus albus*) via ARE, while estrogens have no effect [60]. The study with ricefield eel also reported that the use of ARE to activate *cyp19a1b* is tissue-specific [60]. Thus, a hypothetical scenario can be proposed to explain paradoxical feminization of indifferent gonads in male channel catfish, where an aromatase gene, either *cyp19a1a* or *cyp19a1b*, is artificially activated by exogenous androgen via ARE. Further research is clearly needed to understand the phenomenon of paradoxical feminization in channel catfish.

23.5.2 Paradoxical Masculinization

Genistein is a major phytoestrogen present in plant material such as soybean meal, a common ingredient of fish diets. As the term “phytoestrogen” implies, prominent side-effects of genistein are associated with its known feminizing properties. In fact, a study with Japanese medaka showed that treatment with genistein can partially sex-reverse males into intersex individuals [61]. A more recent study with channel catfish yielded the opposite results. Green and Kelly [62] reported that the proportion of males in experimental populations increased as the concentration of genistein was increased in the diet. This conclusion may be correct if the reference for the comparison is only the females. When intersex individuals are considered, however, the clearest overall trend was for the proportion of intersex individuals to increase at the expense of females, while

the overall proportion of males generally did not change (see Figure 23.3 in Green and Kelly [62]).

Despite these nuances of interpretation, female catfish seem to be partially masculinized by genistein [62]. While the mechanisms of this paradoxical masculinization in channel catfish are unknown, it is relevant to note that anti-estrogenic properties of genistein have been reported in mammals [63]. Therefore, the possibility cannot be ruled out that this seemingly unique example of paradoxical masculinization in channel catfish is, in fact, associated with the anti-estrogenic properties of genistein. Curiously, genistein-induced feminization of male medaka [61] and masculinization of female catfish [62] were incomplete in both cases (yielding intersex condition but not full sex reversal), suggesting the occurrence of relatively complex physiological responses to this compound.

23.6 Integrated Model of Signaling Mechanisms

Based on general knowledge [33] and information specific to channel catfish presented in the preceding sections, an integrated working model of sex determination and gonadal sex differentiation can be formulated for this species. In this model, the absence of the SD gene in genetic females (XX) allows the feminizing gene *cyp19a1a* to be upregulated, and the consequent increase in estrogen production is sufficient to initiate morphological differentiation of ovaries. Conversely, the presence of the SD gene in genetic males (XY) leads to testicular differentiation in two phases – early and late. In the early phase, the SD gene acts to prevent the upregulation of *cyp19a1a*, and the sexual bipotentiality of indifferent testes is, consequently, reduced or lost. In the late phase, testis-inducing signals are produced and are required to initiate morphological differentiation (see Figure 23.4). Administration of exogenous estrogen (or feminizing androgens) to genetic males presumably overrides

the effects of late-phase, testis-inducing signals, and leads to a female phenotype.

A recent study describing a two-step regulatory model of testicular differentiation in zebrafish, where inhibition of *cyp19a1a* and production of masculinizing signals are both required under certain conditions [49], is consistent with, and provides support for, the proposed channel catfish model. The recent identification of candidate gene products for the role of late-phase, testis-inducing signals [36] represents significant progress towards an understanding of signaling mechanisms of gonadal sex differentiation in channel catfish.

Important questions remain unanswered by empirical evidence, or are unexplained by the proposed catfish model (Figure 23.4). First and foremost, identification of the SD gene is necessary to determine the signaling pathways associated with the two phases of testicular differentiation. Of particular interest would be to determine if testis-inducing signals of the late phase are directly or indirectly regulated by the SD gene or, perhaps, not even regulated by this gene. Also, are these signals produced *de novo* during the late phase, or do their levels increase gradually through the entire process until they reach threshold values in the late phase? In genetic females, does the feminization process initiated by endogenous estrogen simply involve the unimpeded induction of an ovarian phenotype, or do downstream testis-inducing signals (e.g., late-phase signals) have to be actively suppressed, as reported for mammals [35, and references therein]? Lastly, a question that is also still open for other teleosts is, what drives the early upregulation of *cyp19a1a* prior to gonadal (morphological) sex differentiation?

The channel catfish model also brings attention to an old question: Morphologically, why do ovaries differentiate earlier than testes in teleosts? In the catfish model, the answer would be simple – namely, because estrogen is the first major phenotypic sex-inducing signal produced in either sex, while

phenotypic testis-inducing signals are not produced in genetic males until later in development (Figure 23.4). The magnitude of the temporal dissociation between ovarian and testicular differentiations may be relatively exaggerated in channel catfish, but earlier ovarian differentiation is the norm among gonochoristic teleosts [24, 25].

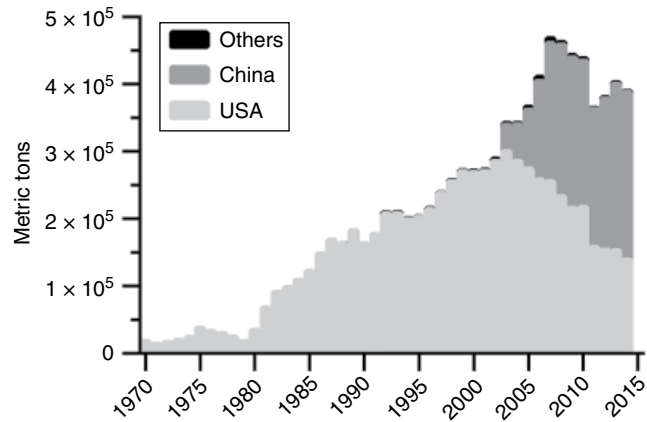
23.7 Sex Control

23.7.1 Current Status

There are no methods presently available for the direct masculinization of channel catfish (Box 23.3). Procedures used for the production of monosex male catfish have relied on feminizing steroid treatment, selective crossings, and progeny testing over several generations [17, 27]. Among the various feminizing steroid treatments tested to date, 60 mg 17 α -ethynyltestosterone/kg of food, given during the first 21 days after yolk sac absorption, has reliably led to the complete and functional feminization of genetic male catfish [17, 27]. Although it seems likely that estradiol-17 β may be more effective than feminizing androgens such as 17 α -ethynyltestosterone, a full dose-response study with estrogens has not been conducted.

Fish treated with feminizing steroids (F_0 generation) [27] were raised to adulthood, and females from these populations were crossed with normal males [17]. Sex ratio analysis of the F_1 generations allowed the identification of the sex-reversed F_0 females (XY), and also suggested the presence of viable YY males among the F_1 progenies [17]. Phenotypic males from the F_1 progenies were raised to adulthood and crossed with normal females (XX), and YY individuals were identified as those that produced all-male (XY) F_2 progenies [18]. In this manner, a stock of YY males was produced that yields monosex male populations when crossed with normal females (Box 23.3). While sex-reversed YY females can be produced by treatment of YY males with feminizing steroids, these females

Figure 23.5 Global production of channel catfish. Time-series data were extracted from databases maintained by the Food and Agriculture Organization (FAO), using FAO FishStatJ [67]. These data include production values reported through the end of 2014. Yearly production values are the sum total of all reporting countries (others, minor producers).



are physiologically impaired, and yield gametes of poor quality [18].

The absence of heteromorphic sex chromosomes and, until recently, the lack of genetic sex markers in channel catfish, have made progeny testing the only option available to determine the chromosomal sex constitution of individual fish. This is a time- and labor-consuming process, and is therefore a costly option. For these reasons, despite experimental demonstrations that monosex male seed production is technologically feasible for channel catfish, this procedure has not been applied at commercial scales.

Box 23.3 Sex control

Because of their faster growth, all-male progenies are desirable in channel catfish culture. A combination of feminizing steroid treatment, selective crossings, and progeny testing over several generations was successfully used to produce a brood stock of YY males that yields all-male progeny when crossed with normal females [17, 18, 27]. Nevertheless, this procedure is time-consuming and labor-intensive, and has not been applied at commercial scales. The recent discovery of a male-specific DNA marker [22] may facilitate the development of cost-effective methods for YY brood stock production.

23.7.2 Future Outlook

Males of channel catfish grow significantly faster than females [9–11], and this fact continues to justify research into the economic benefits of monosex male culture. This is especially relevant to the US catfish industry. Commercial production of channel catfish in the United States experienced a strong period of growth from the early 1980s through the early 2000s, but production levels have been contracting just as strongly since about 2003 (Figure 23.5). Reasons for this remarkable turnaround include increased production costs (primarily feed and fuel) and competition from lower-priced foreign catfish imports [64, 65].

While, at a global scale, the production of channel catfish continued to increase through 2007–2008, due to a rapidly growing industry in China, global production seems to have already plateaued, and may even be showing signs of a decreasing trend (Figure 23.5). The development of more efficient production management and technologies is essential for the US catfish aquaculture industry to remain competitive [66], and the same could be said of catfish projects elsewhere. The recent discovery of a male-specific genetic marker in channel catfish [22] provides a useful screening tool for the development of cost-effective protocols to produce all-male seeds at commercial scales. Affordable all-male seed have

the potential to increase catfish production efficiency, and maintain the vitality of the US catfish industry.

Acknowledgements

I thank Drs. Ricardo Hattori, Carlos Strüssmann, and Terrence Tiersch for their useful critique of an early draft of this manuscript. This manuscript was prepared under

the auspices of a seed grant from Texas Tech University and São Paulo Research Foundation. The Texas Cooperative Fish and Wildlife Research Unit is jointly supported by US Geological Survey, Texas Tech University, Texas Parks and Wildlife Department, The Wildlife Management Institute, and US Fish and Wildlife Service. Any use of trade, firm, or product names is for descriptive purposes only, and does not imply endorsement by the US Government.

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24

Sexual Size Dimorphism, Sex Determination, and Sex Control in Yellow Catfish

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The yellow catfish, *Pelteobagrus fulvidraco*, is an important aquaculture fish species widely distributed in the fresh water areas of China, including rivers, lakes, and reservoirs [1]. It is favored by a large number of consumers in China, due to its delicious meat, high nutritional value, and no intermuscular bones besides the spine [2]. With the progress of technologies for artificial breeding and genetic manipulation, the yield of yellow catfish in China has seen an annual increase of about 15% in recent years, and reached 330 thousand tons in 2014, according to the reports of China Fishery Statistical Yearbook (Figure 24.1). Here, we will introduce the genetic basis of sex determination and its application in the artificial production of yellow catfish.

24.1 Sexual Dimorphism and Sex Determination

24.1.1 The Phenotype and Molecular Mechanism of Sexual Size Dimorphism

Sexual size dimorphism is commonly defined as the growth difference between male and female individuals. People have observed significant size dimorphism between male and female yellow catfish during long-term

field surveys and aquaculture practices (Figure 24.2) [3]. Under the same culture conditions, the growth rate of males is about 50% and 1–2 times larger than females in the first year and second year, respectively [4]. The explanation for this phenomenon may be that the males mature later than the females, so that the nutrient substance in males is able to transform into body composition and body weight during maturation.

The body weight is usually controlled by the somatic growth and food intake in vertebrates. In teleosts, somatic growth is greatly regulated by hormones secreted from the neuroendocrine system, including growth hormone (GH) and its primary downstream mediator, insulin-like growth factor (IGF), which integrate into a GH/IGF axis expressed in the hypothalamus-pituitary-gonad axis [5, 6], while food intake is usually regulated by hormones such as ghrelin and leptin, which regulate appetite and energy balance [7].

The GH/IGF axis has been shown to control growth rate in multiple fish species. Sexual dimorphic expression of *GH* has been observed in European eels [*Anguilla anguilla* (L.)]. Compared to the males, a higher expression of *GH* in the female eels has been detected, and explains the faster growth rate in females [8]. In tilapia, the males grow

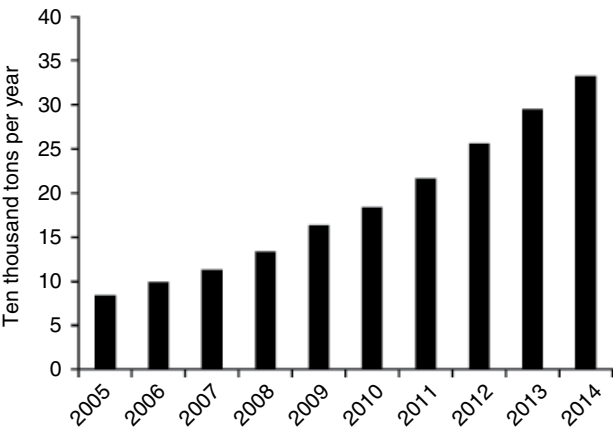


Figure 24.1 The annual yield of yellow catfish in China between 2005–2014.

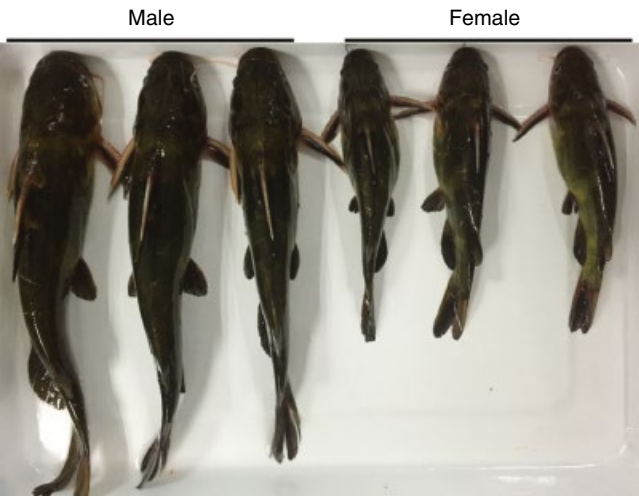


Figure 24.2 The body size difference between male and female yellow catfish. (See inserts for the color representation of this figure.)

much faster than females, and MT treatment has been shown to elevate expression of GH/IGF axis genes and promote body growth [9]. Therefore, the expression of *GH*, *IGF-1*, and *IGF-2* genes is characterized in yellow catfish [10]. *GH*, *IGF-1*, and *IGF-2* are mainly expressed in the hypothalamus and pituitary, hypothalamus and liver, pituitary and liver of adult yellow catfish, respectively. These three genes have been detected to have higher expressions in the main tissues of males than in females. In male larval fish, expression patterns of *GH*, *IGF-1*, and *IGF-2* gradually increased, whereas their expression was inconsistent in female larval fish. Interestingly, expressions of *GH*, *IGF-1*, and

IGF-2 were significantly higher in male fish than in female fish during larval growth. 17 α -methyltestosterone (MT) treatment resulted in upregulation of *GH*, *IGF-1*, and *IGF-2* mRNA in female larvae, and down-regulation of these genes in male larvae, suggesting that MT exerted androgenic effect to promote the GH/IGF signaling in females, while overdose of androgenic hormone inhibits the GH/IGF axis genes in males. 17 α -ethynylestradiol (EE₂) treatment has been shown to significantly reduce the growth of yellow catfish, whereas body weight and body length were not obviously changed after MT treatment [11], which could be explained by the simultaneous activation of

GH/IGF axis gene expressions in females and inhibition of their expressions in males. In conclusion, MT treatment could neither be used to induce sex-reversal of XX females, nor to enhance the yields of yellow catfish.

As potent stimulators of pituitary growth hormone secretion, *ghrelin* and its functional receptor, growth hormone secretagogue receptor (GHSR), are involved in the regulation of food intake and body weight gain. *Ghrelin* is a brain-gut peptide, originally identified in the stomach. In yellow catfish, higher expression of both *ghrelin* and *GHSR* were detected in hypothalamus and gut of adult male fish than in adult females, as well as being higher in male larval fish than in female larval fish. After MT treatment, the expression of *ghrelin* and *GHSR* was initially upregulated in both female and male larval fish, whereas expression of *ghrelin* decreased as the treatment time prolonged. However, *GHSR* mRNA was consistently upregulated in both sexes.

During the short- and long-term fasting and re-feeding periods, the expression of both *ghrelin* and *GHSR* was significantly induced in male juveniles, compared with female juveniles, suggesting that male juveniles may have a better appetite and energy intake than female juveniles during fasting [12]. All data demonstrate that sexual size dimorphism in yellow catfish is probably caused by the sex difference in expression of *ghrelin* and *GHSR* that regulate feeding and food intake, and GH/IGF signaling that controls body growth.

24.1.2 Sex Determination System

Meiotic gynogenesis, a method widely used in the study of sex determination, was performed on female yellow catfish with inactivated sperm of *Leiocassis longirostris*. The results indicated that 96.3% of the gynogenetic progenies were females, while around 50% were females in the control groups that were generated by crossing male and female yellow catfish. The evidence of this female homogametic system in yellow catfish suggested that it has an XY

sex-determining type [4]. In most XY sex-determining fish species, including medaka (*Oryzias latipes*) and yellow catfish, the gonad development process starts with formation of the bipotential gonad, which then differentiates into testis or ovary. For ovary differentiation of yellow catfish, initial ovarian cavity, primordial germ cells (PGCs) with condensed chromatin and a small number of oocytes of 7–9 μm in diameter were observed in the primordial gonad at 12 days post-hatching (dph), while numerous oocytes were detected around the ovarian cavity at 16 dph. For testis differentiation, spermatogonia were distinguishable from PGCs as early as 20 dph, and emergence of vas deferens and seminiferous lobules was observed at 57 dph [3, 13]. These data suggest that sex determination should occur before 12 dph in yellow catfish.

In fish species, sex determination is usually controlled by both genetic and environmental factors. High temperature (32°C) significantly increases the percentage of males in yellow catfish [13]. Sexual dimorphic expression of aromatase P450 genes has been detected in the hypothalamic-pituitary-gonad axis of yellow catfish, which is regulated by 17 α -methyltestosterone (MT) treatment. The expression level of *cyp19a1a* is significantly higher in ovary than in testis, and is reduced in ovary after MT treatment [14]. Non-steroidal aromatase inhibitor, letrozole treatment results in an increase of male proportions in a dose-dependent manner, and a stimulation of spermatogenesis, showing a greater amount of spermatozoa and enlarged lobule lumens in yellow catfish, compared with control.

However, oral administration of MT had no obvious effect on the ratio of males, whereas a small percentage of intersex fish was produced [15, 16]. It is noteworthy that the genetic sex of experimental fish treated by high temperature or letrozole was unknown. Therefore, a systematic investigation on producing XX males should be performed in the future by using either a single factor or a combination of several factors, including high temperature, sex hormones, or sex hormone inhibitors (Box 24.1).

Box 24.1 Glossary of key terms

Type of sex determination in yellow catfish:

This belongs to the XY sex-determining system and is controlled by both genetic and environmental factors. However, the sex-determining gene has not been revealed yet.

Sex differentiation in yellow catfish:

Ovary differentiation and testis differentiation were initiated at about 12 days post-hatching (dph) and 20 dph, respectively.

24.2 Sex control and All-Male Production

Because male yellow catfish grow faster than females, the cultivation of all-male populations is highly desirable, and will greatly improve the yield and economic benefits. Monosex populations have been successfully produced in many fish species through several approaches, including inter-specific hybridization, temperature control, artificial gynogenesis, and a combination of sex-specific markers and biotechnological approaches [17, 18]. Here, we will introduce the progress and technique for massively producing all-male yellow catfish.

24.2.1 Production of YY Super-Male

XX/XY male heterogametic system is the main sex determination system in fish species. Creation of YY super-males is a key step to produce an all-male population. YY super-males do not naturally exist, but can be artificially created by combining sex reversal and gynogenesis technology, or by crossing sex-reversed XY females with XY males in several fish species. As early as 1963, Yamamoto reported the production of YY in medaka [19]. Subsequently, YY super-males were produced in some other fish species, including *Carassius auratus* var. [20], *Oreochromis mossambicus* [21, 22],

Oreochromis niloticus [23, 24], *Oncorhynchus mykiss* [25], *Ictalurus punctatus* [26], *Poecilia reticulata* [27], *Cyprinus carpio* L. [28], and *Puntius conchonius* [29]. YY super-males can survive and be fertile in these fish species. However, there are two reports that YY cannot survive in both *Betta splendens* and *Cichlasoma nigrofasciatum* [30, 31].

After hormone-induced sex reversal, using *Artemia* larvae as the carrier of estrogen (Box 24.2), an XY female yellow catfish was generated and carried out with artificial gynogenesis. Finally, YY super-males were created (Figure 24.3). In order to identify the YY male genotypes, the male fish should be killed to obtain the sperm and progeny testing performed [4]. However, this procedure is laborious, time-consuming, and cannot be employed in massive production of all-male yellow catfish.

24.2.2 Establishment of YY Female

To achieve large-scale breeding of all-male populations, a large number of YY super-male fish need to be produced. Theoretically, the best way is to create YY physiological female yellow catfish by hormonal-induced sex reversal technology. Large-scale production of YY super-male fish can then be achieved by mating YY super-males with YY physiological females. To date, only a few cases of production of YY females have been reported in fish species, including *Poecilia reticulata* [27], *Oreochromis niloticus* L. [32], and *Ictalurus punctatus* [33, 34], which was possibly due to the very low survival rate or infertility of YY females.

It is noteworthy that only one percentage of progeny could survive when YY super-males were crossing with YY females in channel catfish [33]. In Nile tilapia, the YY males could be feminized after feeding with a hormone-treated food [32]. However, no sex reversal was observed in YY individuals after exposure of embryos to either 17 β -estradiol or 17 α -ethynylestradiol, while sex reversal rates of XY progeny were induced up to 61%

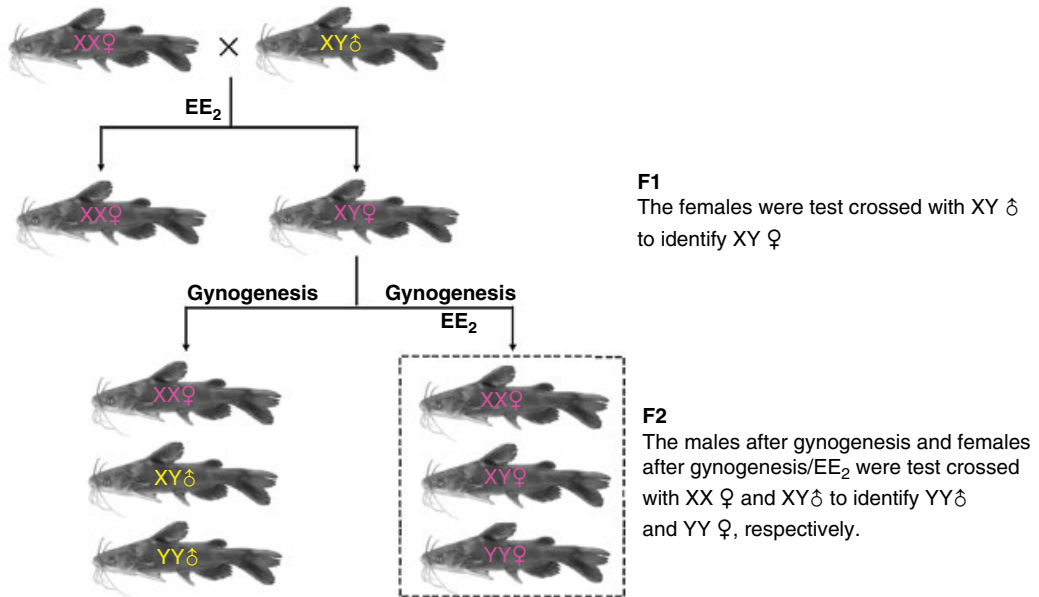


Figure 24.3 A schematic diagram of production of YY♂ and YY♀ by an integration approach of gynogenesis and EE₂ treatment. These genotypes were identified by test crosses.

Box 24.2 Glossary of key terms

The optimal treatment for sex control in yellow catfish:

Nine-dph larvae were fed three times per day with the filtered Artemia that was soaked in 200 µg /L EE₂ for 1.5 hour. The rearing temperature is about 24–25 °C. When EE₂ was applied to larvae for 40 days, the sex reversal rate of female to male was higher than 95%.

and 91% under the same conditions [35, 36]. Therefore, the feminization of YY super-male is the vital step to produce a large population of YY super-male yellow catfish.

Until now, there have been only two successful cases for the large scale production of monosex populations through development of YY male technology. Nile tilapia was the first reported instance of feminization of YY genotypes and mass production of YY males through mating YY males with YY females [32]. The second instance was yellow catfish. The larval yellow catfish generated by mating XY males and females were fed with

estrogen-treated Artemia, and YY females were identified by progeny testing (Figure 24.3). Fortunately, YY physiological females are viable and fertile [37]. However, the breeding cycle is very long, and it is a laborious process to maintain the experimental fish separately after cross-testing.

24.2.3 Development of Sex Chromosome-Linked DNA Markers

To avoid cross-testing and killing the male fish, an approach to accurately and rapidly identify the genetic sex of YY super-males is urgently required. Recently, a number of sex-specific DNA markers have been identified in different fish species, including SSR (simple sequence repeats), SNP (single nucleotide polymorphism), AFLP (amplified fragment length polymorphism), RAPD (random amplified polymorphic DNA), and QTL (quantitative trait locus) [38]. These genetic markers provide us with a clue to screen specific sequences of sex chromosomes that would offer a highly efficient approach to identify the genetic sex of yellow catfish.

The tail fins of gynogenetic XX female, XY male, and YY individuals were sampled, and genomic DNA was extracted. AFLP is a highly precise molecular marker to characterize the genomic difference. To identify sex-specific markers in yellow catfish, the AFLP technique and bulked segregant analysis (BSA) were employed. A total of 256 EcoRI/MseI-based AFLP primer combinations were used to screen the genomic differences between six samples, including single DNA sample and bulked DNA sample of XX, XY, or YY yellow catfish [39]. As a result, two Y-linked and four X-linked AFLP fragments were screened out.

Sequence analysis revealed two pairs of allelic genes, *Pf33* and *Pf62*, whose flanking sequences were further cloned and analyzed. Based on the polymorphisms and variations in the sequences, four Y-linked or X-linked SCAR primer pairs were designed and converted into Y-linked and X-linked SCAR markers (YSM and XSM). Consequently, the YSM and XSM were successfully applied to identify the genetic sex of XX, XY, and YY. Accordingly, a novel and simple PCR-based technique to assist production of YY super-males and all-male populations was established in yellow catfish. Using this technique, all-male yellow catfish have been approved as a novel variety – “yellow catfish all-male No. 1” – by the National Certification Committee for Aquatic Varieties.

Actually, the screened sex-linked SCAR markers were designed according to the nucleotide difference between X and Y chromosomes, and the primers of *Pf62-X* and *Pf62-Y* have only two nucleotides different. Since allelic polymorphism usually exists among various populations from different geographic regions, the above sex-linked SCAR markers were only tested in one artificial breeding population as reported, and may not be suitable for other populations of yellow catfish. For this reason, genome walking was performed to clone the flanking sequence of *Pf62-X* and *Pf62-Y*, and significant genetic

discrepancies, including a large number of single nucleotide polymorphisms and some small segment deletions, have been detected between the 8102bp *Pf62-Y* sequence and 5362bp *Pf62-X* sequence.

Based on the small segment deletions between X and Y chromosome allele sequences, three pairs of primers were designed to efficiently identify XX females, XY males, and YY super-males in both an artificial breeding population and a wild population from Dongting Lake, in Hunan province, China. Using the XY1-F and XY1-R primer pair, two different size fragments (X-fragment: 955bp, Y-fragment: 826bp) were amplified in genomic DNA samples from four other wild populations, including Liangzi Lake, Hong Lake, Chang Lake, and South Lake in Hubei province [40]. Since the detection efficiency is 100%, the PCR detecting method with XY1-F and XY1-R primer pair serves as a highly stable and efficient method for genetic sex identification by both scientists and companies working on yellow catfish.

24.2.4 Mass Production of XY All-Male

Accordingly, we have designed a rapid and efficient flow chart to massively produce all-male yellow catfish. As shown in Figure 24.4, the XY physiological females were produced from sex reversal progeny by EE₂ treatment and identified by the XY1 marker. From the mating progeny of the reversed XY female and normal XY male, 25% of the progeny were identified to be YY super-males by the XY1 marker, while the YY physiological females can be induced from the EE₂ treated YY fingerlings by sex-specific marker identification. Moreover, YY super-males can be produced continually from the mating of the YY physiological females and YY super-males. Finally, the YY super-males can be used to mate with XX females for commercial mass production of all-XY males.

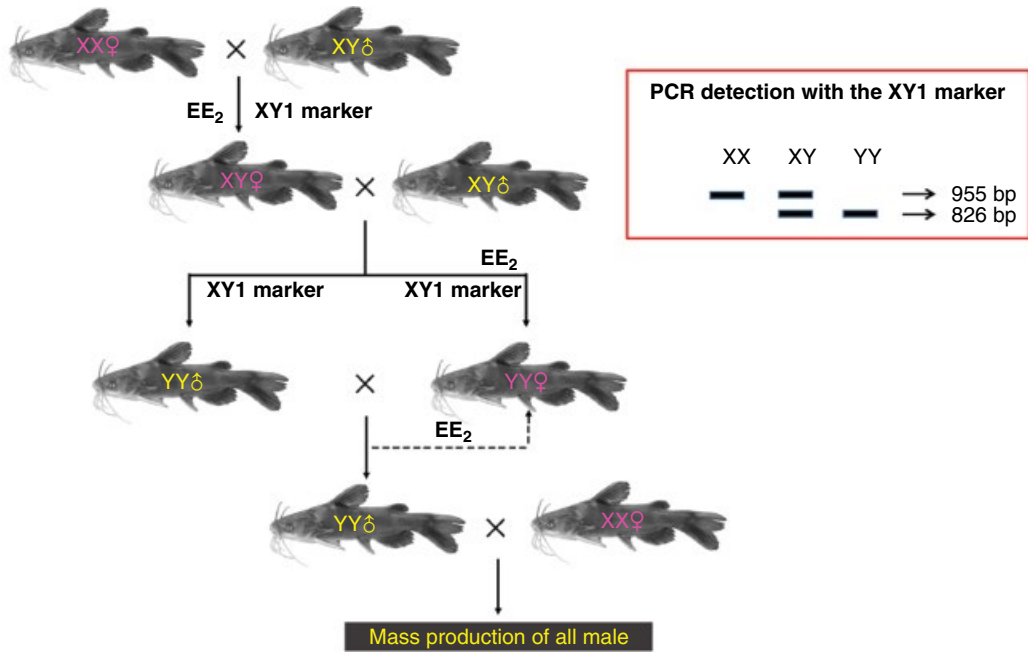


Figure 24.4 A schematic diagram for massive production of all-male yellow catfish. The genetic sex was identified by PCR method with the XY1 marker.

24.3 Genetic Mechanism of Sex Determination

24.3.1 Identification of Sex-Biased mRNA and miRNAs in the Testis and Ovary

In order to investigate the molecular mechanism of sex determination and differentiation in yellow catfish, comprehensive transcriptome analysis for XX ovary, XY testis, and YY testis of yellow catfish was performed [41–43]. Several genes related to male determination and testis differentiation, such as *dmrt1*, *sox9a1*, *fshr*, *cyp17a*, *ARA-α*, and *piwi*, have been revealed to have higher expression level in testis than in ovary. In contrast, *gdf9*, *vasa*, *sf1*, and *nanos*, which are related to female determination and ovary differentiation, have higher expression in ovary than in testis [42, 43].

Comprehensive miRNA transcriptome analysis was also performed on XX ovary, XY testis, and YY testis of yellow catfish, to

identify sex-biased miRNAs [44]. Totally, 384 conserved and 113 novel miRNAs were identified. Among them, 322, 372, and 348 conserved miRNAs, and 68, 82, and 82 novel miRNAs were expressed in XX ovary, XY testis, and YY testis, respectively. Multiple most abundant miRNAs, such as miR-146a / -21 / -462 in XX ovary and miR-7 g / -200a / -200b in XY testis and YY testis, had more than a two-fold difference in expression between testis and ovary. MiR-462 has only been detected in fish species. Interestingly, members of miR-200 family, including miR-200a, -200b, -200c, and their star sequences, had male-biased expression in yellow catfish.

24.3.2 Differential Gene Expression Between XY and YY Testis

Fish species are low vertebrates, and have a very complex sex determination system, with XX/XY and ZZ/ZW sex chromosomes.

YY super-males do not exist in the natural world, but have only been artificially created in several fish species, including yellow catfish, Nile tilapia, *Oreochromis niloticus*, and rainbow trout, *Oncorhynchus mykiss*. Significant differences in gene expression and morphology have been detected between XY and YY testis in rainbow trout [45, 46]. Thus, XY male and YY super-male provide a unique model to study fish testis development and spermatogenesis.

Hematoxylin and eosin (HE) staining was performed on testes of XY and YY yellow catfish with the same age and similar body size. Compared to the XY testis, the YY testis has a larger spermatogenic cyst, more spermatid, and less spermatocyte in the spermatogenic cyst, suggesting a higher degree of sexual maturity in YY super-male than in XY male. Intriguingly, the expression of miR-141 and miR-429 are higher in the XY testis than in the YY testis, and their expressions are significantly induced in testis when treated by a high dose of 17 α -ethynylestradiol (EE₂), which will impair testis development and spermatogenesis. In humans, expression of miR-141 and miR-429 significantly increases in the testicular tissue of asthenozoospermia and oligoasthenozoospermia patients, compared with normozoospermic men [47, 48]. In conclusion, the expression of miR-141 and miR-429 is negatively correlated with the progression of testis development and spermatogenesis in both human and fish species.

Comparative transcriptome was further performed to reveal differentially expressed genes (DEGs) between XY and YY testis. 1235 and 1146 unigenes displayed significantly higher expression in YY testis and XY testis, respectively. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was carried out, and indicated that PI3K-AKT and G protein-coupled receptor (GPCR) signaling pathways were activated in YY testis, compared with XY testis. The PI3K-AKT signaling pathway, which is stimulated by many kinds of growth factors specifically binding to receptor tyrosine kinase (RTK)

or G protein-coupled receptors (GPCR), is required for normal sperm activity and male fertility [49, 50]. Multiple members of RTK signaling, including spleen tyrosine kinase (Syk), colony stimulating factor 1 receptor (Csf1r), prolactin receptor (Prlr), β 1-Integrin (Itgb1), and β 2-Integrin (Itgb2), have higher expression in a YY testis than in a XY testis. Moreover, Kiss1r (GPR54), somatostatin receptors (Sstr), glutamate receptor 5 (GRM5), glutamate receptor AMPA 2b (gria2b), glutamate receptor AMPA 4a (gria4a), and prolactin receptor (PRLR), which are associated with G protein signaling, were significantly upregulated in the YY testis.

Relative low expression of miR-141/429 in YY testis might be correlated with high level of sexual maturation in testis. MiR-141-3p and miR-429b-3p were predicted to target 31 and 11 YY enriched DEGs, respectively. For example, Itgb2 and gria2b, factors involved in the PI3K-AKT and GPCR signaling pathways, were predicted targets of miR-141-3p, and validated by dual-luciferase reporter assays. The Tgf β signaling pathway has been revealed to be involved in male sex determination in fish species, including *Odontesthes hatchery*, *Takifugu rubripes*, and *Oreochromis niloticus* [51–53]. AMH and Tgf β 1 were potential targets for miR-141-3p and miR-429b-3p, respectively.

24.4 Prospectives

24.4.1 A Prospective Way to Improve the Quality of All-Male

The YY super-male yellow catfish used for production of the novel variety “yellow catfish all-male No. 1” is actually the offspring of one fish. After several generations, the quality of this variety has degenerated. Previously, genetic selection breeding was performed on four wild populations of yellow catfish, from Hunan and Hubei provinces [54]. The selected XY progeny, with excellent growth traits, can be used to create more families of

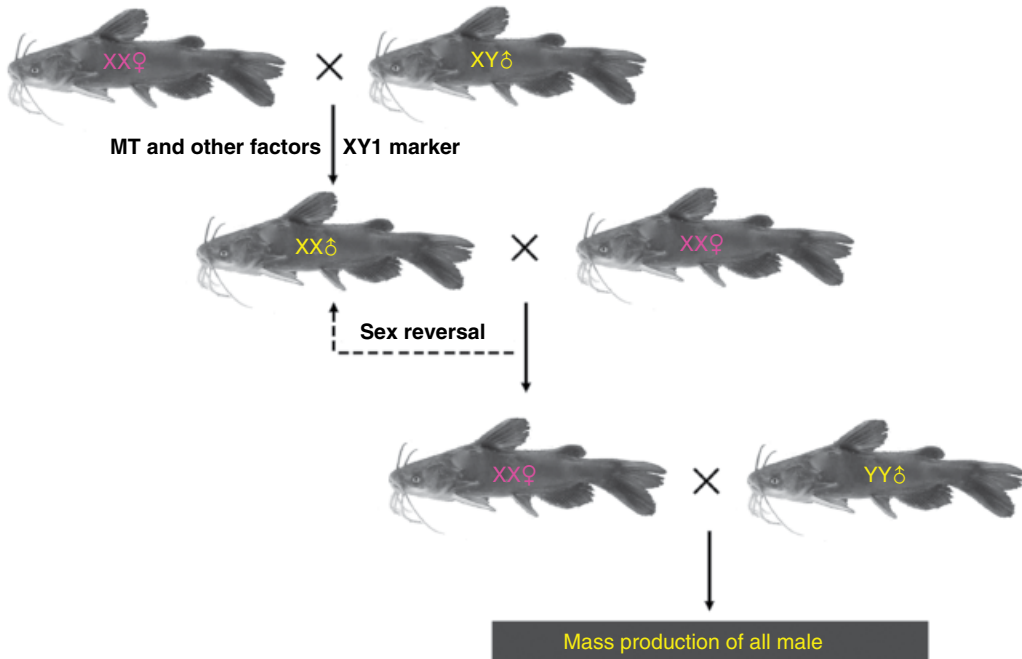


Figure 24.5 A schematic diagram of quality improvement of all-male yellow catfish. In each generation, the parents with excellent growth traits were selected. The genetic sex was identified by PCR method with the XY1 marker.

YY yellow catfish. Growth performance of the offspring is greatly determined by the quality of their mothers.

After crossbreeding between XX females and XY males with excellent growth traits, their XX female progeny could be used to produce XX physiological males by hormone (or other factors)-induced sex reversal, and then were selected by the Y chromosome-specific marker (YSM) and X chromosome-specific marker (XSM). Further, the batch XX physiological males can be obtained from the hormone-treated offspring produced by mating between the selected XX male and XX female with good growth traits. Finally, mass production of all-females can be produced by the mating of XX males and females (Figure 24.5).

Accordingly, we have proposed an improved approach for mass production of all-male population in XX/XY sex determination system fish, by crossing the YY super-males and XX females that were selected to have

good growth performance. As a traditional and preponderant research field [55], we believe that sex control breeding will be a significant contributor to sustainable aquaculture [56, 57].

24.4.2 The Future Direction on Studying the Molecular Mechanism of Sex Determination

Most of the sex-determining genes, such as *Dmy*, *sox3*, and *gsdf* were firstly characterized from the BAC library and identified as male-specific genes in *Oryzias latipes*, *Oryzias dancena*, and *Oryzias luzonensis*, which were originally screened as sex-related genes linked to sex-specific markers or a genomic DNA resource [58–60]. We have constructed a BAC library of a YY super-male individual, and screened out BAC clones in which Y-linked fragment *Pf62-Y* was located. After sequencing of the selected BAC clone, the

local BLAST comparison was run based on the genomic data of BAC clone and transcriptome data previously obtained [41, 42]. Finally, several candidate sex-determining genes have been identified.

Moreover, the screened BAC clones could be used as probes to perform fluorescence *in situ* hybridization (FISH), and identify sex chromosomes in yellow catfish (data not shown). The X and Y chromosomes could be separated and collected by several techniques, such as flow cytometry sorting and laser

microdissection, as previously described [61, 62]. Then, two DNA libraries corresponding to X and Y chromosomes could be established and sequenced, to search for male- and female-specific genes.

Recently, genome editing approaches, such as TALEN and CRISPR/Cas9, have been applied to elucidate the functions of various genes in aquaculture fish species [57]. These also could be used to reveal the function of the sex-determining gene and sex-specific genes identified.

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Mechanisms of Feminization and Sex Differentiation in Southern Catfish

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The southern catfish (*Silurus meridionalis* Chen, 1977), which is widely distributed in the Yangtze River basin in China, belongs to *Siluridae*, Siluriformes. It is an important economic fish in China because of its large size, nutritional values, high fecundity, fast growth, and resistance to diseases. It is a single-spawning fish, with a spawning season limited to March and April, which is different from that of the multi-spawning fish [1]. It takes 3–4 years for the female, and 2–3 years for the male fish, to be sexually mature [2]. The sex ratio of the feral Southern catfish is about 1 : 1, and the females grow faster than the males.

Interestingly, a survey on the sex ratio of the fry obtained by artificial fertilization from several institutes revealed that they are always 100% female (Table 25.1) [3]. In order to find out the reason for this feminization, the sex determination and differentiation of the catfish were studied. These studies presumed that micro-environmental changes during artificial insemination and later development, gynogenesis induced during artificial propagation, and effect of environmental estrogenic substances on early sex differentiation might be responsible for this feminization.

25.1 Mechanisms of Feminization in Southern Catfish

25.1.1 Feminization by Micro-Environmental Changes?

Compared with the natural spawning process, the feminization of the fry was probably induced by the changed micro-environment during artificial propagation. These environmental changes include conditions adopted during fertilization, during hatching, after hatching, and so on. Under both aquaculture and laboratory conditions, artificial propagation of the catfish was usually performed with the dry insemination method. Briefly, mature semen and eggs were collected from the male and female, respectively. Then the semen was directly added into the egg pile and mixed in a uniform manner. The main differences between the dry insemination method and natural spawning were the high concentration of H⁺, or the low pH value, which might have killed the Y sperms specifically, or the high concentration of semen, which might have prevented the Y sperms from fertilizing during the process of artificial insemination [3].

Table 25.1 Sex ratios of catfish obtained by artificial propagation from 1995 to 2007.

Year	Source of fish	Age	Total	Female	Female rate (%)
1995–2003	Aquatic science institute of Chongqing	8 dph to 2 years	1700	1700	100
1999	Dengta propagation base of Chongqing	1 year	50	50	100
2001	School of Aquatics of Sichuan	5 months	100	100	100
2004	Deyang propagation base of Tongwei Co Ltd	3 years	89	89	100
2005–2007	Southwest University of China	8 dph to 6 months	2000	2000	100

dph = days post-hatching

To investigate these possibilities, the pH value of ovarian fluid was measured, and the fluid with the eggs was diluted, using water from the natural spawning site, by 10, 10², and 10³ times. Then, artificial propagation was performed using the wet insemination method to mimic the natural spawning process (eggs were diluted with water, then the semen was added into the water). Undiluted eggs with ovarian fluid were used as controls. The pH of the undiluted ovarian fluid was 6.25; after dilution by 100 times, the pH rose to 7.85 – very close to the pH value (7.51–7.99) of the water from the spawning site. However, the hatched fry of all the groups treated, as well as those of the control group, all remained female when they were checked three months after hatching [4], suggesting that the change of pH value has no influence on sex ratio during artificial insemination.

Other environmental changes, such as water temperature, water sources, and feed, also took place during and after hatching, and might have influenced the sex ratio under aquaculture and laboratory conditions. It is well known that the temperature influences the sex ratio in many species in lower vertebrates [5–9]. Higher temperature favored the female sex in the channel catfish (*Ictalurus punctatus*), which is closely phylogenetically related to the southern catfish [10]. Thus, the possible influences of the water temperature on the sex ratio of the catfish were investigated.

The time of ovarian cavity formation in the Southern catfish is around 12 days post-hatching (dph), and it becomes very clear around 25 dph, through histological observation at 24°C. Therefore, the fry were treated between 5–25 dph. The fry are usually reared at around 24°C under laboratory and aquaculture conditions while, in nature, the water temperature during its reproductive season is 16.8–23.6°C. Therefore, the fry were cultured in 10 groups (200 fry/group), with a temperature of 15°C, 18°C, 21°C, 24°C, 28°C and 32°C, respectively [11]. The fry were fed to satiation with nematodes twice a day, and 10 samples were collected from each group once a week. The sex ratios were checked at three and six months after hatching. The different types of hatching water, including the river water at the natural spawning site, aerated tap water and well water, and different feed, including natural nematodes, man-made feed, and algae, were also investigated. However, fry in all groups were all females, indicating that these environmental changes did not influence the catfish sex ratio during and after hatching.

25.1.2 Feminization by Gynogenesis

It is well known that there are cases where natural gynogenesis is part of the reproductive strategy in some fish species, which can also cause an all-female population. The environmental changes mentioned above are not responsible for catfish femi-

nization in aquaculture conditions, and the feminization might result from gynogenesis occurring during artificial propagation conditions. To this end, artificial induction of gynogenesis was carried out, and cytological observations on the fertilization biology during artificial insemination were performed under laboratory conditions [12].

In a typical gynogenesis process, the sperm with fragmented genomic DNA does not enter into the egg, or the male pronucleus does not fuse with the female pronucleus. However, by cytological observation of the insemination process of the catfish, a similar fertilization process to other teleost species was found in the southern catfish [12]. It revealed that the catfish undergoes its normal fertilization process, even under artificial propagation conditions. Moreover, the second polar body was found to be released at about 20 minutes after insemination (mai) at 24°C [12]. With the fusion of the female and male pronuclei at 45–50 mai, it is unlikely for gynogenesis of the catfish to occur during artificial propagation.

Heat shock method has been used to induce artificial gynogenesis [13]. However, even under the optimal conditions [15 min ultraviolet light illumination on the sperm and one minute of heat shock (41°C) from 5 mai], the gynogenetic rate still remained extremely low [13]. All these data suggested that no gynogenesis occurs during artificial propagation, unless artificial induction is applied.

25.1.3 Complete Feminization by Feeding *Limnodilus* spp

Endocrine-disrupting chemicals (EDCs) have been found ubiquitously distributed in natural waters, including in industrialized areas and in remote environments [14–16]. They enter the bodies of humans and wildlife mainly through the food chain [17]. EDCs exert their effects by interfering with endogenous hormone action, and can affect male and female reproduction [18, 19].

Feminization of animals by EDCs has been observed in all classes of vertebrates including fish, amphibians, reptiles, birds, and mammals [20–25]. High concentrations of EDCs were reported in rivers in Europe, America, Africa, and Asia [26–34]. Fish (such as rainbow trout, common carp, flounder, bream, sharp tooth catfish, and black basses) exhibited feminization, intersex, and contamination of EDCs under natural conditions [28–42].

The southern catfish fry used in experiments of feminization induction were obtained by artificial propagation, using parental fish raised in laboratory or directly captured from the Jialing River. The fry for the observation were cultured in large tanks with a recirculating aerated fresh water system. Water temperature ranged from 22°C to 24°C, pH was 7.0–7.5, and total ammonia-nitrogen was 0.05–0.15 mg/L. Dissolved oxygen concentration was monitored and maintained at minimal 7 mg/L throughout the experimental period. The fry were under natural photoperiod throughout the experiment.

The fry were first divided into two groups, one fed with *Limnodilus* spp. (treatment group, collected from the wild), and another fed with commercial fish diets SSs (Shengsuo, Shandong, China) (control group). The experiments were repeated three times with *Limnodilus* spp. from three different tributaries of the Jialing River. Treatment was from 3–90 dph. All fish were reared under the same conditions except for food. To determine the treatment length duration needed for feminization, the experimental fish were further divided into two groups, fed with living *Limnodilus* spp. for 15 and 30 days, respectively, then commercial diet was resumed till 90 dph.

Different food (including *Limnodilus* spp.) and time treatment were applied to the catfish. In addition, EDCs in *Limnodilus* spp., an annelid worm collected from wild contaminated small streams, was detected by LC–MS (Liquid chromatography-mass spectrometry). It suggested that feeding of

Table 25.2 Sex ratio of southern catfish fed with different diets examined at 90 dph (days post-hatching). F = fadrozole, TAM = tamoxifen, E₂ = estradiol-17β, MT = 17α-methyltestosterone treatment dosage: F, 100 mg/kg; TAM, 25 mg/kg; F + TAM, 100 mg/kg + 25 mg/kg; E₂, 25 mg/kg; MT, 50 mg/kg [62]

Diets	Detected number			Female number			Female (%)		
	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3
<i>Limnodilus</i> spp. (for 15 dph)	200	200	200	137	141	146	68.5	70.5	73
<i>Limnodilus</i> spp. (for 30 dph)	200	200	200	200	200	200	100	100	100
<i>Limnodilus</i> spp. (for 90 dph)	200	200	200	200	200	200	100	100	100
Heat inactivated <i>Limnodilus</i> spp.	200	200	200	200	200	200	100	100	100
Artificial diets with EDCs cocktail	200	200	200	200	200	200	100	100	100
Commercial diets	200	200	200	94	105	111	47	52.5	55.5

Limnodilus spp. resulted in complete feminization of the southern catfish, which has a 1 : 1 sex ratio in wild conditions. Furthermore, HPLC analysis showed that the extraction of *Limnodilus* spp. contained EDCs, including bisphenol A (BPA), diethylstilbestrol (DES), 4-tert-octylphenol(4-t-OP), and 4-nonylphenol(4-NP), which were further confirmed by LC–MS (Box 25.1).

Southern catfish feeding using commercial diets sprayed with EDCs cocktail also resulted in 100% female, whereas the control fish displayed approximate 1 : 1 sex ratio (Table 25.2). *Limnodilus* spp. fed fish displayed similar serum VTG levels and estradiol-17β and gonadal *sfl* (Steroidogenicfactor-1), *foxl2* (winged helix/forkhead transcription factor gene 2), *cyp19a1a* (cytochrome P450, family 19, subfamily A, polypeptide 1a), *dmrt1* (doublesex/mab-3 related transcription factor 1) expression levels to those of female control [43].

Box 25.1 Glossary of key terms

Feminization in Southern catfish:

Neither micro-environmental changes nor gynogenesis changed the sex ratio during artificial propagation in southern catfish. Feeding of *Limnodilus* spp. resulted in complete feminization of southern catfish, which has a 1 : 1 sex ratio in wild conditions. It demonstrated that EDCs in *Limnodilus* spp. cause southern catfish feminization by affecting aromatase expression and endogenous estrogen level.

25.2 Sex Reversal in Southern Catfish

25.2.1 Female-to-Male Sex Reversal

It is well known that estrogen plays a pivotal role in the sex differentiation of lower

vertebrates, including fish [44–54]. The estrogen might also be the key to resolve the problem of sex reversal.

Sex reversal by the blockage of estrogen production and function was performed. As the key enzyme catalyzing the conversion of androgen to estrogen, aromatase (encoded by the *cyp19* gene) expression is highly related to ovarian differentiation. Inhibition of its expression and enzymatic activity resulted in reduced estrogen production and subsequent female-to-male sex reversal, which has been reported in zebrafish (*Danio rerio*) [9], Nile tilapia (*Oreochromis niloticus*) [50], coho salmon (*Oncorhynchus kisutch*) [55], bastard halibut (*Paralichthys olivaceus*) [56], rainbow trout (*Oncorhynchus mykiss*) [57], and golden rabbitfish (*Siganus guttatus*) [58]. Therefore, the maintenance of aromatase expression and activity might be prerequisites for ovarian differentiation and development in these species.

To induce female-to-male sex reversal, the catfish fry were treated with drugs, including: the aromatase inhibitor fadrozole (F, 100 mg/kg); the androgen methyltestosterone (MT, 50 mg/kg); the estrogen receptor antagonist tamoxifen (TAM, 25 mg/kg); and a combination of F and TAM (100 mg/kg + 25 mg/kg). These drugs have been reported to be effective in inducing female-to-male sex reversal in many species [9, 58–60].

The treatment was performed from 5–25 dph at 24°C, the critical period for southern catfish sex differentiation. After treatment, the fry were reared with control feed. The expression levels of several genes involved in sex differentiation were checked at 65 dph. The catfish were dissected, and the sexes were distinguished at 130 dph. The results showed that 56%, 70%, and 80% sex-reversed

males were obtained in the F, TAM, and F + TAM groups, respectively (Table 25.3). The gonads of the other fish in these groups were observed as retrogressive ovaries or ovotestis [62]. The studies revealed that either reduction of estrogen and/or inhibition of estrogen function resulted in female to-male sex reversal in catfish, as reported in other fish species [50, 55, 56], suggesting that estrogen played a decisive role in southern catfish sex differentiation. These results also indicated that feminization of southern catfish might have been caused by those factors that are able to downregulate aromatase expression and estrogen production, while the exact reason remains unknown (Box 25.2).

25.2.2 Sex Reversal by the Blockage of Estrogen Production and Function

In contrast to other fishes [59, 60], it was found that androgen (including ethynyl testosterone and MT) treatments, at the dosages of 10, 25, and 50 mg/kg, failed to induce sex reversal in the catfish both morphologically and histologically [11]. However, this is not the first report showing that MT treatment could not induce sex reversal in fish. One acceptable explanation is that MT might have been converted to estrogen by aromatase, because it is an aromatizable androgen.

In the channel catfish, neither aromatizable nor non-aromatizable androgen could induce female-to-male sex reversal. However, androgen can induce sex reversal in some other species, such as the African catfish (*Clarias gariepinus*), the Nile tilapia [59], and rainbow trout [61]. Recent reports have showed that it is probably due to the downregulatory role of androgen in the expression of steroidogenic enzymes, especially aromatase, resulting in a

Table 25.3 Sex ratios of the southern catfish fry treated with drugs.

	F	TAM	F + TAM	E ₂	MT	Control
Number of fish treated	100	100	100	100	100	100
Number of males	56	70	80	0	0	0
Male rate (%)	56	70	80	0	0	0

Box 25.2 Glossary of key terms**Female-to-male sex reversal in southern catfish:**

The fry were treated with drugs, including: the androgen methyltestosterone (MT, 50 mg/kg); the aromatase inhibitor fadrozole (F, 100 mg/kg); the estrogen receptor antagonist tamoxifen (TAM, 25 mg/kg); and a combination of F and TAM (100 mg/kg + 25 mg/kg). The treatment was performed from 5–25 dph at 24°C. MT treatment could not induce sex reversal in southern catfish. The results showed that 56%, 70%, and 80% of sex-reversed males were obtained in the F, TAM, and F + TAM groups, respectively.

decrease in estrogen production [59, 61], as in the case of aromatase inhibitor treatment (1,4,6-androstatriene-3,17-dione or fadrozole) [57, 62]. These results again support the conclusion that it is estrogen that plays a key role in the gonadal sex differentiation, while androgen has no direct effects in non-mammalian vertebrates [57, 63]. It seems that whether female-to-male sex reversal can be induced or not is probably dependent on the suppression of aromatase, and not on the type of androgen (aromatizable or non-aromatizable).

To understand the molecular mechanism of sex reversal in the southern catfish, the expression level of *cyp19a* was monitored in the MT (50 mg/kg), F (100 mg/kg), and control groups at 25 dph. The results revealed that the expression of *cyp19a* in the F-treated group was significantly downregulated, while in the MT group, *cyp19a* expression remained similar to that of the control group [62]. On the other hand, E₂ treatment was able to upregulate *cyp19a* expression in the gonad according to the previous results [62]. In contrast, *cyp19a* expression in the gonad did not show any significant change after MT treatment compared with the control in this experiment. Therefore, it seems that the aromatization of MT may not be responsible for the failed sex reversal.

It is necessary to test whether the non-aromatizable androgens, such as 11- β ketotestosterone or 11- β hydroxyandrostenedione, can induce sex reversal in the southern catfish, in order to completely understand why MT treatment cannot induce female-to-male sex reversal.

25.3 Sex Differentiation in Southern Catfish

25.3.1 Time of Sex Differentiation

With the normal female and sex-reversed male fry obtained by F and TAM treatment at 24°C, histological observation of gonadal development was investigated. Gonads from the control group and the TAM group were dissected and fixed in Bouin's solution at 120 dph, according to the method described [62]. Female and male gonads were indistinguishable histologically until 8 dph (24°C), when the female gonad started to form two processes ventrally (Figure 25.1a, b) and gradually fused to form an ovarian cavity at about 12 dph (Figure 25.1c). In contrast, in the sex-reversed gonad, no ovarian cavity was observed during all the gonadal differentiation stages (Figure 25.1 d, e, f).

Mitosis of germ cells in the female gonad began around 29 dph, and reached the fast proliferation period at around 35 dph, while meiosis of the female gonad was not observed until 55 dph [4]. However, mitosis of germ cells in the male gonad began at around 55 dph, and reached the fast proliferation period as late as 83 dph; the meiosis of the male gonad was not observed until 130 dph [4]. These data revealed that female and male gonads were rather similar before 8 dph, while significant differences could be observed after that time, such as the ovarian cavity of the female. Meanwhile, the initiation of both germ cell proliferation and meiosis in ovary was much earlier than in testis (see also Box 25.3).

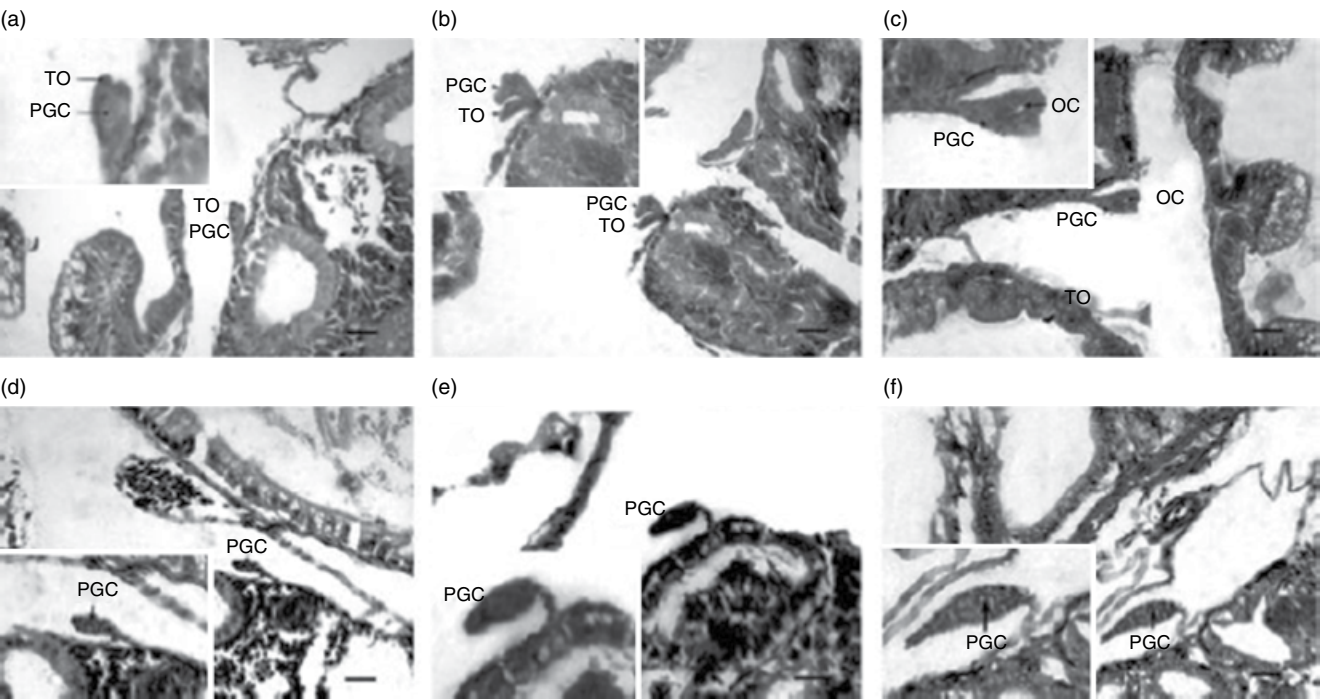


Figure 25.1 Histological observations of the gonadal development of the southern catfish. Female gonadal sections were prepared and observed at: a) 8; b) 10; and c) 12 days post-hatching (dph), at 24°C. Male gonadal sections were prepared and observed at: d) 8; e) 12; and f) 14 dph at 24°C. Inset: the magnified gonad region. Scale bar: 50 μm. PGC – primordial germ cell, OC – ovarian cavity, TO – tissue outgrowth.

Box 25.3 Glossary of key terms**Sex differentiation:**

Sex differentiation is initiated at about 12 dph by histological observation. Mitosis of germ cells in the female gonad and in the male gonad begins around 29 and 55 dph, respectively. The results strongly indicate that the two types of *cyp19s* and *foxl2* favor female differentiation, while *dmrt1* favors male differentiation in southern catfish.

25.3.2 Genes Involved in Sex Differentiation

Estrogen is the natural inducer of ovarian differentiation in many species [44, 46, 47, 50–52, 64]. The level of estrogen in the gonad during early stages of sex differentiation determines the direction of gonadal differentiation in non-mammalian vertebrates, including fish. Aromatase is the key enzyme responsible for estrogen synthesis, catalyzing the conversion of androgen to estrogen. Many reports have also demonstrated that aromatase is involved in fish gonadal differentiation [7, 51, 64, 65] and oocyte maturation [88].

Transcription factors, such as *dmrt1* and *foxl2*, have also been reported to be involved in vertebrate sex determination and differentiation, including in fish [61, 66–71]. These genes were found to be expressed sex-dimorphically in the gonads during the early stages of sex differentiation, with higher expression of *dmrt1* in the testis and *foxl2* in the ovary. Additionally, *dmrt1* mutations [72] and *foxl2/wnt4* (wingless-related MMTV integration site 4) knockout [73] have even been reported to cause sex reversal in mammals. It has also been reported that high *foxl2* expression is related to the upregulation of *cyp19* gene expression, thus resulting in increased estrogen production [61, 74] (see also Box 25.3).

To elucidate the molecular mechanisms of sex differentiation and feminization in southern catfish, the expression profiles of *cyp19a*,

cyp19b, *foxl2*, *dmrt1a*, and *dmrt1b* in adult fish, and their expressions after the aforementioned drug treatments, were studied [62]. Tissue distribution analysis revealed that *cyp19a* was mainly expressed in the gonads, while *cyp19b* was expressed in both brain and gonads in both sexes of the adult fish and, meanwhile, *foxl2* was found to be mainly expressed in the brain, pituitary, and gonads.

Higher expression of these genes was observed in females than in males. *Dmrt1a* and *b* were also higher in testis than in ovaries, in contrast to *foxl2* and *cyp19s* [62]. At 65 dph, high expression of *cyp19s* and *foxl2* (Figure 25.2b–d), but low expression of *dmrt1a* and *b*, were observed in the ovary of the female catfish fry (Figure 25.2a). However, the expression patterns of *dmrt1*, *foxl2*, and *cyp19a* in the gonads were completely reversed during drug-induced (F and/or TAM) female-to-male sex reversal (Figure 25.2a–c). At the same time, levels of *foxl2* and *cyp19b* were upregulated with E_2 treatment (25 mg/kg), similar to the situation found in rainbow trout [79], and downregulated under F and/or TAM treatment in the brain (Figure 25.2b, d). It suggested a possible positive feedback regulation of estrogen on *foxl2* expression in the gonad, and the influence of estrogen on sex differentiation in the brain-pituitary-gonads axis [3].

Gth α , *fsh β* , and *lh β* were found to be expressed not only in the pituitaries of both sexes, but also in the ovary. However, no expression was found in the testis. In gilthead sea bream (*Sparus aurata*) and zebrafish, the expression of *gth α* , *fsh β* , and *lh β* in the gonad has also been reported [76, 77]. Using all-female catfish fry, it revealed that *gth α* and *fsh β* were expressed in the female gonad from 25 dph, while *lh β* was expressed from 40 dph during the sex differentiation period in the catfish [78].

During the transition period of female-to-male sex reversal after TAM treatment, the expression levels of the three *gth* subunits were all downregulated in the gonad (ovotestis) when measured at 65 dph [78]. In rainbow trout, the expression of *fsh β* coincided

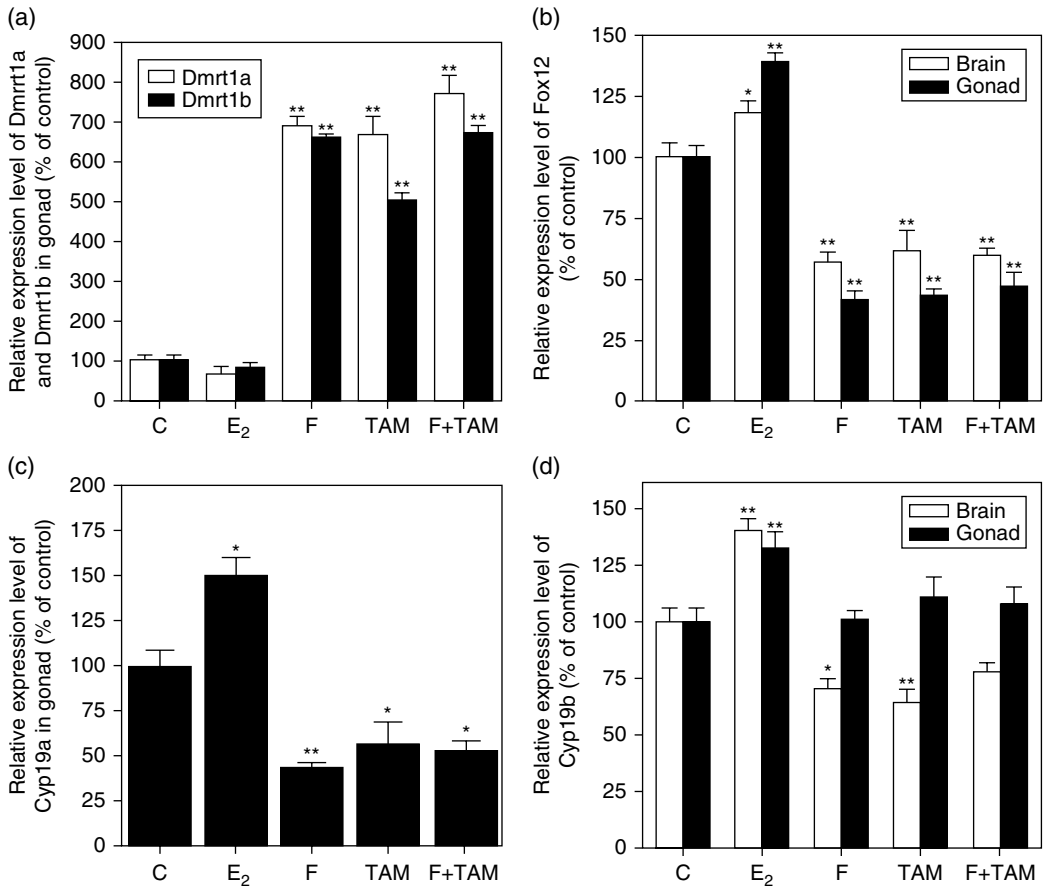


Figure 25.2 Changes in expression in southern catfish of: a) *dmrt1a* and b) *foxl2*; c) *cyp19a*; and d) *cyp19b* after E₂ (17β-estradiol), F (fadrozole), TAM (tamoxifen), and F+TAM (fadrozole + tamoxifen) treatment in the brain and gonad. Results obtained by semi-quantitative RT-PCR are expressed as mean values ± SEM from five individual fishes.

*P < 0.05; **P < 0.01 compared with the respective controls using one-way ANOVA. C – control.

The dosages of the drugs used are described in Section 25.2.2 (modified from [62]).

with the onset of oocyte meiosis in the female differentiating gonad [79]. This might be attributed to the possible positive feedback of estrogen on *gth* subunit expression. Since there is no proof that gonadotropin secretion is clearly active at these stages of development, further investigations are needed to demonstrate whether *fshβ* is involved in the ovarian differentiation in teleost fish.

In addition, *Limnodilus spp*-fed catfish, complete feminization of southern catfish, displayed similar serum VTG and estradiol-17β levels, and gonadal *cyp19a1a* *sf1*, *foxl2*, *dmrt1* expression levels, to those of female control. These results demon-

strated that EDCs in *Limnodilus spp* cause southern catfish feminization by affecting endogenous estrogen level and aromatase expression [43].

Taken together, the results strongly indicate that the two types of *cyp19s* gene and *foxl2* gene favor female differentiation, while *dmrt1* favors male differentiation in southern catfish. Moreover, the results on gene regulation further confirm the pivotal role of estrogen in southern catfish sex differentiation, possibly in two different pathways: direct action on downstream genes involved in ovarian differentiation, and possible positive feedback actions of transcription factors, such as *foxl2*, and

steroidogenic enzymes, such as aromatase, on both gonad and brain levels.

25.4 Future Directions

25.4.1 Genetic Sex Determination (GSD) or Environmental Sex Determination (ESD)

Although much has been done to clarify the sex differentiation and feminization of the southern catfish, more questions remain to be resolved. What is the sex-determining gene of the southern catfish? Why does androgen not induce sex reversal in the catfish? Is this feminization caused by environmental sex determination (ESD), or by genetic sex determination (GSD)? Answers to these questions are important in order to gain a complete understanding of the sex determination, mechanism of feminization, and differentiation. It is necessary to identify the sex-specific DNA markers in southern catfish.

Catfish fry feeding with *Limnodilus spp*, an annelid worm collected from wild contaminated small streams, and commercial diets sprayed with EDCs cocktail, also result in 100% female, whereas the control fish display an approximately 1 : 1 sex ratio. Furthermore, HPLC analysis shows that the extraction of *Limnodilus spp* contains EDCs, including bisphenol A (BPA), diethylstilbestrol (DES), 4-tert-octylphenol(4-t-OP), and 4-nonylphenol(4-NP).

Several environmental factors were not responsible for the catfish feminization, but the feminization was probably caused by ESD, because estrogen is the natural inducer of ovarian differentiation in teleosts, including the southern catfish. The importance of estrogen in southern catfish ovarian differentiation has been confirmed.

Blockage of estrogen function (TAM treatment) and/or estrogen production (F treatment) resulted in partial or complete female-to-male sex reversal. However, MT failed to induce sex reversal, in contrast to the tilapia [59] and medaka (*Oryzias latipes*) [80].

To elucidate the mechanism, the southern catfish androgen receptor (AR) was isolated. The catfish AR is longer by 19 amino acids at the C-terminus than those from other vertebrates, including fish [81]. Further study is needed to clarify whether the C-terminus extension of AR is related to the phenomenon of MT being unable to induce female-to-male sex reversal.

25.4.2 Sex-Determining Gene and Sex Determination Cascade

Sex-determining genes are the master switches controlling the sex determination and differentiation of vertebrates. Most of these sex-determining genes were originally screened as sex-related genes linked to sex-specific markers or genomic DNA resources, such as *dmy* (the DM domain gene on the Y chromosome), *sox3* (sex determining region Y-box 3), and *gsdf* (Gonadal soma-derived factor), identified as male-specific genes in *O. latipes*, *O. dancena* and *O. luzonensis* [82–84].

SRY (the sex-determining region on the Y chromosome) and *dmy* have been the only sex-determining genes isolated in mammals and two strains of medaka [82, 85], but neither the *dmy* nor *SRY* homolog has been isolated in any other fish species, including the southern catfish. In the Nile tilapia, it has been proven that transcription factors *foxl2* and *dmrt1* determine the direction of sex differentiation, probably through direct or indirect regulation of the aromatase gene [75, 86], inferring that the regulation of aromatase might occupy a decisive position in fish sex differentiation.

More investigations on the regulation mechanisms of the aromatase gene are necessary. However, the gene cascade or networks controlling sex differentiation and determination are complicated, especially in fish, with additional fish-specific whole-genome duplication during evolution. Many genes execute their functions by direct or indirect regulation of other genes. Recently, genome editing approaches, such as CRISPR/

Cas9 and TALEN, have been applied to elucidate the functions of various genes in aquaculture fish species [87], and also could be used to reveal the function of sex-specific and sex-related genes identified. Isolation of

the sex-determining gene in the southern catfish, and elucidation of the sex determination cascade, will finally help to completely understand the feminization mechanism in this species.

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Part VIII

Sex Determination and Sex Control in Flatfishes

26

Genomic and Epigenetic Aspects of Sex Determination in Half-Smooth Tongue Sole

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26.1 Introduction to Sex Determination of the Half-Smooth Tongue Sole

The half-smooth tongue sole (*Cynoglossus semilaevis*), which belongs to Cynoglossidae of the Pleuronectiformes, is a large, commercially valuable flatfish that is distributed in Chinese coastal waters. Due to its delicious taste, enriched nutrition, fast growth, easy domestication, and natural resource depletion, the half-smooth tongue sole has become a promising and economically important marine finfish species for aquaculture in China. Currently, the annual production of the tongue sole in China maintains at more than 10,000 tons, and is valued at about 1.5 billion RMB.

The half-smooth tongue sole has a complex sex determination mechanism, including both genetic sex determination (GSD) and environmental sex determination (ESD). For the GSD, both chromosome karyotype analysis and artificial gynogenesis induction have confirmed that tongue sole has a female heterogametic sex determination system (ZW/ZZ) [1–3], which contains a total of 42 chromosomes, including 20 euchromosome pairs and a sex chromosome pair (chromosome Z and W) [2].

The half-smooth tongue sole exhibits distinct growth and morphology dimorphisms.

Females grow much faster than males, and the body length and weight of females are two to four times those of males (Figure 26.1) [4, 5]. Therefore, females have a higher commercial value than males in the aquaculture market.

Despite the primary GSD by sex chromosome inheritance, high temperature can induce a sex-reversal of genetic females to phenotypic males, and fertile ZW phenotypic males (pseudomales) are produced, representing an ESD mechanism in this species. It is observed that, at normal rearing temperature (22 °C), ~14% of genetic females (ZW) naturally sex-reverse to pseudomales. When exposed to a higher temperature of 28 °C during a critical period of gonad differentiation that determines whether the gonad develops towards testis or ovary, the rate of sex-reversal significantly increases to ~73%, demonstrating that the sex of tongue sole can be changed by environmental conditions [6]. Interestingly, the sex-reversed “pseudomales” can mate with normal females and produce offspring that inherit the ability of sex reversal from genetic female to phenotypic male. Even under normal rearing temperature of 22 °C, the rate of sex reversal of the F1 generation of pseudomales is as high as ~94% [6].

All of these features indicate that tongue sole has a complex sex determination system,

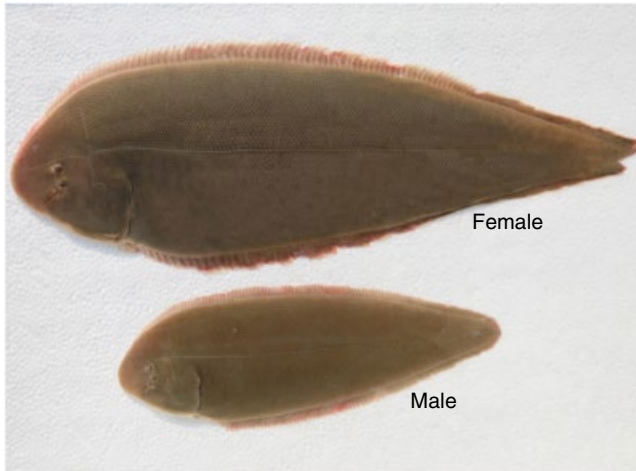


Figure 26.1 Half-smooth tongue sole at two years old. Top: female; bottom: male (adopted from [5]).

with a plasticity between genetic and environmental factors. Therefore, the half-smooth tongue sole is an excellent model to study the sex determination mechanisms in fish. The study of sex determination mechanisms will reveal critical genomic components and their functions for sex determination. It will also provide insights into the evolution of sex chromosome systems, as well as fundamental knowledge to support the development of sex control and selective breeding technology, with the purpose of increasing the ratio of females in the farming population, which implies the improvement of production and profitability in the aquaculture of tongue sole.

26.2 Genomic Foundation of Sex Determination in the Half-Smooth Tongue Sole

26.2.1 Whole-Genome Sequencing

The great commercial value of tongue sole has promoted its artificial farming and the development of breeding technology. Due to the distinct sex-associated growth differences, a critical goal of breeding programs is controlling the sex ratio and improving the

female proportion in the culture population. Although an increasing number of studies have unraveled much knowledge of the genomic basis of the sex determination in the tongue sole, a reference genome sequence will shed light on the genomic basis and evolving mechanism of economy-related traits of this flatfish, and will strongly promote the development of refined genomic breeding tools for sustainable and intensive farming of the tongue sole.

An initial study for getting the whole genome sequence of the tongue sole was the construction of high quality bacterial artificial chromosome (BAC) libraries [7] and BAC-based physical map [8]. In the physical map, a total of 29,709 clones were assembled into 1,485 contigs, with an average length of 539 kb and a N50 length of 664 kb. The estimated physical length of the assembled contigs was 797 Mb, representing approximately 1.27 coverage of the half-smooth tongue sole genome [8], which provides a useful resource for future integration with linkage map and whole-genome sequence assembly.

In 2014, by combining high throughput sequencing with genetic and physical maps, Chen and his colleagues completed the whole-genome sequencing of a ZZ male and a ZW female tongue sole, respectively, and produced a high quality chromosome-scale genome assembly [6]. The genome assembly

of *C. semilaevis* spans 477 Mb with a scaffold N50 of 867 Kb, representing the first genome sequence of flatfish worldwide [6].

To facilitate the genome assembly, two types of genetic maps were constructed. A population of 92 offspring of a wild male and a cultured female was used for simple sequence repeat (SSR) genetic mapping. Another 216 individuals were used for single nucleotide polymorphism (SNP) genetic mapping. The SSR and SNP genetic maps for the half-smooth tongue sole were then constructed by linkage analysis, comprising

942 and 12,142 SNP markers, respectively [6]. Using SSR and SNP markers, together with BAC sequences of tongue sole constructed previously, more than 93% (445 Mb) of the sequences in the genome assembly have been anchored and ordered on 22 chromosomes, including 20 autosomes and the sex chromosome Z and W (Table 26.1).

The average GC content of the tongue sole genome was 40.8%, comparable to that of other teleosts such as medaka (40.5%). The repetitive elements in the tongue sole

Table 26.1 Number of markers and total scaffold size for each chromosome. We anchored Z-linked scaffolds from male assembly and autosomal scaffolds from female assembly onto Z chromosome and autosomes (chr1 ~ 20) (adopted from [6]).

Chr.	# SSR	# RAD-tag	Contig		Scaffold		Source	No. of Genes
			#	Len. (bp)	#	Len. (bp)		
1	81	1,184	2,410	32,791,084	53	34,529,112	Female	1,487
2	40	810	1,227	19,259,417	29	20,052,734	Female	911
3	29	484	1,189	15,467,848	25	16,253,993	Female	596
4	85	323	1,263	19,377,156	31	20,014,501	Female	846
5	43	89	1,147	18,609,661	29	19,279,693	Female	706
6	30	825	1,270	18,113,957	29	18,841,016	Female	978
7	54	54	993	13,185,383	15	13,814,722	Female	613
8	53	642	2,144	28,615,567	37	30,153,790	Female	1,395
9	50	454	1,314	18,790,677	31	19,618,599	Female	1,029
10	46	777	1,507	20,081,642	33	21,015,569	Female	1,037
11	42	949	1,428	19,676,390	34	20,528,432	Female	1,022
12	40	517	1,349	17,485,432	35	18,398,590	Female	745
13	43	865	1,518	20,959,882	34	21,922,143	Female	892
14	50	1,288	1,782	27,668,722	47	28,847,931	Female	1,228
15	46	703	1,478	19,132,837	32	20,094,621	Female	761
16	40	430	1,252	17,874,443	29	18,785,820	Female	809
17	38	246	1,333	15,583,495	25	16,472,647	Female	984
18	28	639	1,092	14,404,870	22	15,207,555	Female	783
19	33	553	1,108	17,115,378	24	17,747,288	Female	847
20	34	226	1,036	14,355,002	18	15,234,830	Female	881
Z	37	53	2,044	20,757,346	26	21,915,962	Male	930
W	NA	NA	2,436	13,020,023	306	16,461,726	Female	320
Total	942	12,111	32,320	422,326,212	944	445,191,274	NA	19,800

genome was analyzed using a combination of *de novo* prediction and homology search. In the genome of the half-smooth tongue sole, there are only 5.85% transposable elements (TEs), which are in the range of compact fish genomes, but significantly lower than that of mammals such as human (TE sequences represent 45% of the genome). A low diversity of DNA transposons and retrotransposons was observed, and the genome has few long terminal repeat retrotransposons [6]. Additionally, 674 tRNA genes, 104 rRNA genes, 285 microRNAs, and 434 small nuclear RNAs were annotated in the genome of tongue sole [6].

Several gene prediction methods, including *ab initio* prediction, homology search, and transcript mapping were used to predict genes in genome of tongue sole. As a result, a reference gene set of 21,516 protein-coding genes was identified, 94% of which were annotated in the Swiss-Prot or Gene Ontology database [6].

Based on the reference gene set of tongue sole, and those from fully sequenced teleosts, including medaka, stickleback, *Takifugu*, *Tetraodon*, and zebrafish, 2,426 single-copy gene families were identified, using human and chicken as outgroups [6]. The single-copy genes were then used to reconstruct a genome-wide phylogenetic tree (Figure 26.2),

showing that tongue sole appeared relatively independent of other clades.

The single-copy genes were also used to date the divergent time of flatfish with other bony fish at about 197 million years ago with PAML package [9]. Moreover, the orthologous and paralogous relationships were built between the genome of tongue sole and medaka, stickleback and zebrafish, allowing the establishment of the conservation of synteny between genomes of the sequenced bony fish and the reconstruction of the karyotype of their last common ancestor.

In addition to the two rounds of whole-genome duplications (WGDs), which occurred in the vertebrate evolution, a third genome duplication (TGD) event in a common ancestry of the teleost fishes was considered to play important roles in the evolution of fish species. A total of 2,733 paralogous genes in the genome of tongue sole were clustered into paralogous chromosomal regions, distributed over 21 tongue sole chromosomes, as a result of the TGD (Figure 26.3) [6]. The conserved syntenic blocks and genomic rearrangements in comparison to other fish and vertebrate genomes were detected, suggesting the relationship of such rearrangements to the phylogenetic tree of the genomes being compared. According to this, the evolutionary history of

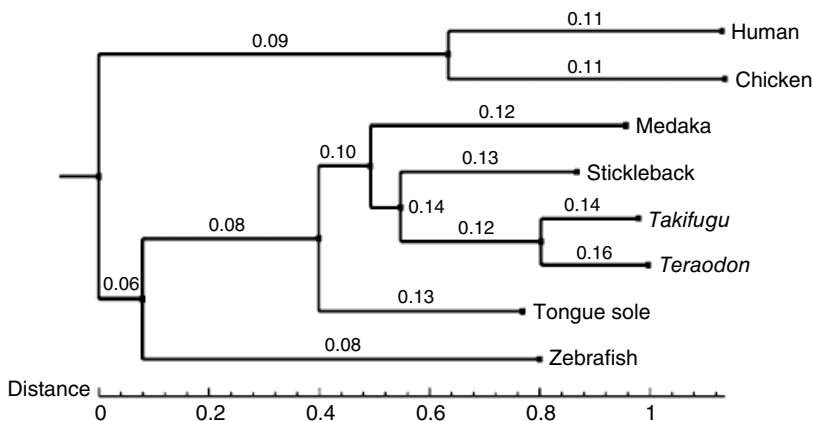


Figure 26.2 Phylogenetic tree using all single-copy orthologs from tongue sole, zebrafish, medaka, stickleback, *Takifugu*, *Tetraodon*, human, and chicken. The branch length represents the neutral divergence. Numbers on the branch represent the dn/ds. The posterior probabilities (credibility of the topology) for each inner branch are all 100% (adopted from [6]).

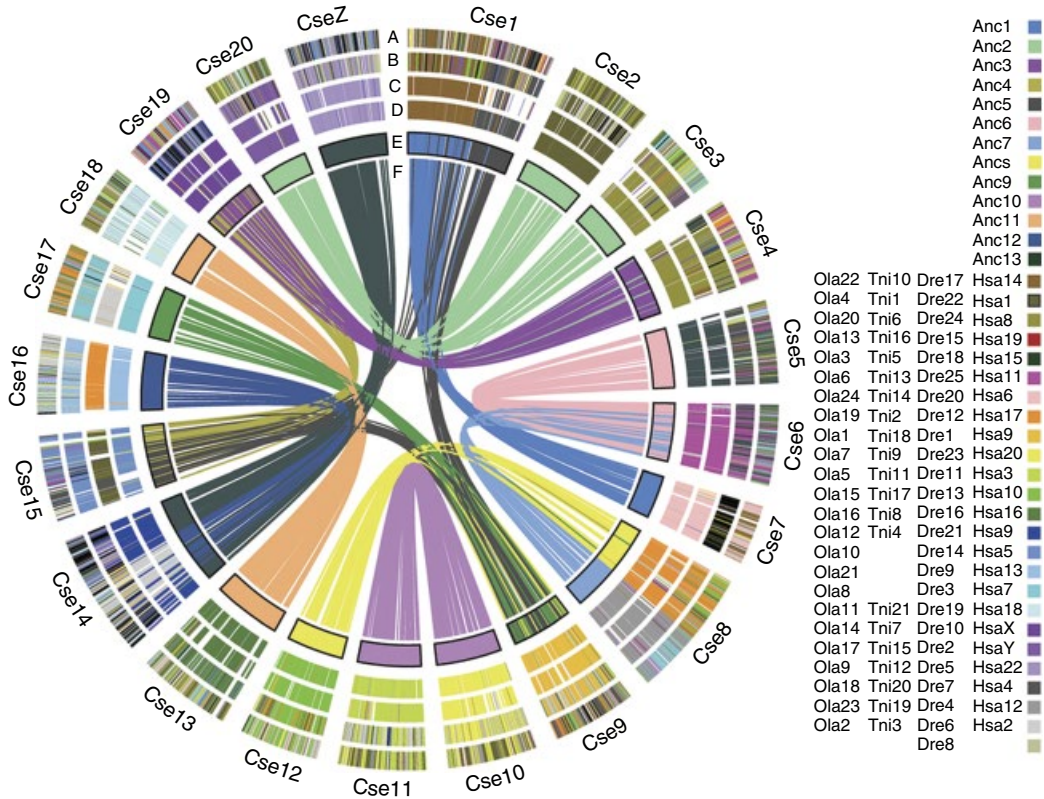


Figure 26.3 Teleost genome evolution. WGD in the tongue sole and orthology in the medaka, *T. nigroviridis*, zebrafish and human genomes. (See inserts for the color representation of this figure.)

The arcs of concentric circles represent each tongue sole chromosome (Cse1–Cse21 and Z). A–D represent tongue sole chromosomes painted with different colors according to the location of the orthologs in the human (Hsa), zebrafish (Dre), *T. nigroviridis* (Tni), and medaka (Ola) genomes. A 100 kb region around a gene is painted in the same color. E represents tongue sole chromosomes painted by the corresponding ancestral chromosomes (Anc1–Anc13). In F, each line joins duplicated genes at their respective positions (Cited from [6]).

the tongue sole genome was reconstructed at the chromosomal level, and it is deduced that there was a reduction in the chromosome number of 21 in tongue sole after separation from the medaka lineage, which has 24 chromosomes, because of three major lineage-specific chromosomal fusion events [6].

26.2.2 Genomic Organization and Evolution of Sex Chromosomes

Tongue sole has a classical sex chromosome-based genetic sex determination system.

Box 26.1 Sex determination system of Tongue sole

C. semilaevis has a heterogametic sex determination system with (ZW/ZZ) sex chromosomes, which have a young evolutionary history of 30 Mya. *Dmrt1* was identified as the male-determining gene, which has a functional copy only present in the Z chromosome and is highly expressed in males during the critical gonadal development period and persists at high levels during testis development, but is suppressed in females by epigenetic regulation [6].

Intensive investigation of the genomic architecture and evolution mechanism of the sex chromosomes is crucial for the elucidation of the GSD mechanism, and allows the identification of candidate locus and genes for sex determination. The whole genome sequencing and assembly provides the opportunity to intensively survey the complete structure, functional features, and evolutionary history, of the sex chromosomes of the half-smooth tongue sole.

Males of tongue sole have two copies of the Z chromosome, and females have a single Z chromosome and a female-specific W chromosome. Theoretically, under the same sequencing depth, the coverage of Z-linked sequences in males should be twice that in females. The W-linked sequences are in female assembly only, and their sequencing depth should be half of the average coverage of autosomes in females. Using this method, Z- and W-linked scaffolds were identified, with a length of 23.3 and 16.4 Mb, respectively [6]. The Z-linked sequences were further confirmed by quantitative PCR in ZZ and ZW individuals. Notably, the Z chromosome sequence in tongue sole is the full sequence of the Z chromosome, except that reported in birds.

The Z and W chromosome of the tongue sole showed a high level of homology to the chicken Z and W chromosome. Phylogenetic analysis revealed that the sex chromosome pairs of the tongue sole evolved from a pair of ancestral vertebrate protochromosome, which is represented by an autosome in other fish lineages. This ancient chromosome has also been independently pressed into service as a sex chromosome in a lizard (a gecko) and a mammal (platypus), suggesting that some chromosomes are intrinsically good at the job [10].

The sex chromosome consists of a pseudo-autosomal region (PAR) and non-PAR structure. The PAR is at the telomeric ends of both sex chromosomes, containing 22 protein-coding genes and one pseudogene (Table 26.2). These genes are highly conserved, and neither sequence divergence nor apparent dosage

compensation have been observed. Therefore, the PAR of sex chromosomes still pair in female meiosis, and normally cross over.

In contrast, there are 297 genes in the remaining region (non-PAR) of Z and W chromosomes, but showing some sequence divergence. In this region, the Ks value (number of synonymous substitutions per synonymous site) is stable at around 0.15, which is higher than that of 0.0115 in the autosomal region and 0.0188 in PAR, suggesting a reduction or even absence of recombination [6]. Moreover, the uniformity in distribution of Ks values for the Z-W gene pairs in the non-PAR of sex chromosomes indicates a single evolutionary level. No evidence has shown that transposition of genes or chromosome segments occurs between autosomes and sex chromosomes [6].

The gene density on Z chromosome (42 genes/Mb) is comparable to the average value of autosomes (46 genes/Mb). However, the gene density on the W chromosome (19 genes/Mb) is lower than any of the autosomes. In contrast, the density of repeat sequences of both Z and W chromosomes is much higher than that of the autosomes. The most abundant type of repeat sequences on Z and W chromosome are DNA transposons (36.1% of all interspersed repeats) and LINE elements (31.4% of all interspersed repeats), respectively. In addition, compared to Z and autosome, the W chromosome has much more TEs (29.94%, compared with 13.13% on Z and 4.33% on autosomes) and pseudogenes (19.74%, compared with 3.54% on Z and 2.48% on autosomes) [6].

The age of mammalian Y chromosome and avian W chromosome is estimated to be hundreds of millions of years [11]. During the evolving period, the degeneration of Y and W chromosome has been dramatic, and massive genes have been lost. In tongue sole, 907 genes have been attributed to the Z chromosome, but the non-PAR of the W chromosome has a total of 317 protein-coding genes (297 plus 20 genes that are not shared with the Z chromosome), which is about one-third of that on the Z chromosome [6].

Table 26.2 PAR genes and protein function. We identified 22 protein-coding genes and one pseudogene on PAR, and inferred their function by BLAST searching against SwissProt (E-value < $1e^{-5}$) and kept the best hit. Furthermore, we presented the human ortholog loci, if any (adopted from [6]).

Gene ID	Scaffold	Functional	Gene name	Human chr.	Protein
CSZ00000142.4	scaffold589	Yes	Pbx3	9	Pre-B-cell leukemia transcription factor 3
CSZ00000940.4	scaffold589	Yes	Unknown		
CSZ00000660.4	scaffold589	Yes	FAM125B	9	Multivesicular body subunit 12B
CSZ00000791.4	scaffold589	Yes	LMX1B	9	LIM homeobox transcription factor 1-beta
CSZ00000041.4	scaffold589	Yes	ZBTB34	9	Zinc finger and BTB domain-containing protein 34
CSZ00000311.4	scaffold589	Yes	Angptl2	9	Angiopoietin-related protein 2
CSZ00000543.4	scaffold589	Yes	Stat2	12	Signal transducer and activator of transcription 2
CSZ00000762.4	scaffold589	Yes	Hmcn2	9	Hemicentin-2
CSZ00000433.4	scaffold589	Yes	Ncs1	9	Neuronal calcium sensor 1
CSZ00000899.4	scaffold589	Yes	ADAMTS13	9	A disintegrin and metalloproteinase with thrombospondin motifs 13
CSZ00000859.4	scaffold757	No	Pbx3	9	Pre-B-cell leukemia transcription factor 3
CSZ00000288.4	scaffold757	Yes	Unknown		
CSZ00000490.4	scaffold757	Yes	GAPVD1	9	GTPase-activating protein and VPS9 domain-containing protein 1
CSZ00000020.4	scaffold757	Yes	C9orf172	9	Uncharacterized protein
CSZ00000272.4	scaffold757	Yes	SYN1	X	Synapsin-1
CSZ00000758.4	scaffold757	Yes	Vgll4	3	Transcription cofactor vestigial-like protein 4
CSZ00000040.4	scaffold757	Yes	slc20a1a	2	Sodium-dependent phosphate transporter 1-A
CSZ00000664.4	scaffold757	Yes	Dtx1	11	Protein deltex-1
CSZ00000508.4	scaffold757	Yes	RASAL1	12	RasGAP-activating-like protein 1
CSZ00000897.4	scaffold757	Yes	RASAL1	12	RasGAP-activating-like protein 1
CSZ00000423.4	scaffold757	Yes	DGCR6	22	Protein DGCR6
CSZ00000596.4	scaffold757	Yes	Slc7a4	22	Cationic amino acid transporter 4
CSZ00000458.4	scaffold757	Yes	RNF34	12	E3 ubiquitin-protein ligase RNF34

In birds, only 26 genes were found to express on the W chromosome, whereas the Z chromosome has about 1,000 genes. In mammals, there are only 40–80 intact genes on the Y chromosome, but more than 1,000 genes on the X chromosome. The difference in the ratio of gene number on sex chromosomes indicates a relatively recent divergent time of sex chromosomes in tongue sole and, thus, the evolution of the W chromosome has not progressed to the stage where most of the genes have disappeared, like in human and birds. According to evolutionary analysis, we estimated a mean divergent time of the Z and W chromosomes of tongue sole at about 30 Mya. Due to the relative short evolutionary history, the degeneration of the W chromosome has not reached a degree where most genes have mutated. Thus, in the non-PAR of the W chromosome in the tongue sole, there are still many original genes.

26.2.3 Male Sex-Determining Gene *dmrt1*

In tongue sole, the phenomenon of temperature-sensitive sex reversal promises to elucidate the sex-determining mechanism. The naturally sex-reversed pseudomales (ZW) are viable, and the F1 generation of pseudomales (ZW) can sex-reverse to pseudomales under normal temperature. Molecular marker identification has demonstrated that all the progeny of ZW pseudomales inherit the Z chromosome from their sex-reversed fathers. In addition, the DNA methylation pattern on the Z chromosome of the second-generation pseudomales is consistent with their paternal pattern, indicating that the inheritance of DNA methylation status of some special loci on Z chromosome plays a role in the inheritance of sex reversal [6]. Therefore, in tongue sole, sex determination is maybe operated through a Z-encoded mechanism that determines male development.

A male sex-determining gene, *dmrt1* (doublesex and mab3-related transcription factor

1) is found on the Z chromosome whereas, on the W chromosome, there is only an inactive pseudogene (Figure 26.4a). *Dmrt1* specifically expresses in male germ cells and pre-somatic cells of the undifferentiated gonad at the sex-determination stage and persists at high levels during testis development, so may take over a master sex-determining role (Figure 26.4b, d). The high expression of *dmrt1* in males is accompanied by DNA demethylation in its promoter region (Figure 26.4c) whereas, in females, the promoter of *dmrt1* is highly methylated and its expression is inhibited.

Dmrt1 has been validated as the male-determining gene in birds, and has been found to map on Z chromosome, express in male embryos, and overexpress on female-to-male gonadal transformation [12]. *Dmrt1* has also spawned a novel sex-determining gene recently in medaka fish and *Xenopus laevis* [12]. Recently, using TALEN genome-editing technology, we produced ZZ *dmrt1* mutants in tongue sole, which developed ovary-like gonads, and the spermatogenesis was disrupted (Figure 26.5) [13]. In addition, we observed that the *dmrt1*-deficient ZZ fish grew faster than ZZ male control, providing the functional evidence that *dmrt1* is the master sex-determining gene and has application potential for enhancing male growth in tongue sole [13].

26.2.4 Other Sex-Related Genes

In organisms harboring GSD mechanisms, males and females have different genomic locus or genes that specify their sexual morphology, which is often accompanied by chromosomal differences. The tongue sole has a ZW chromosome system, and the sexual differentiation is attributed to a main gene of *dmrt1*, with a polygenic system including a number of other following genes taking effect in this process. A number of genes that are conserved in the sex determination system have been identified to be similarly involved in tongue sole. Using transcriptome sequencing/quantitative PCR, epigenetic analysis, and functional verification, the gene expression

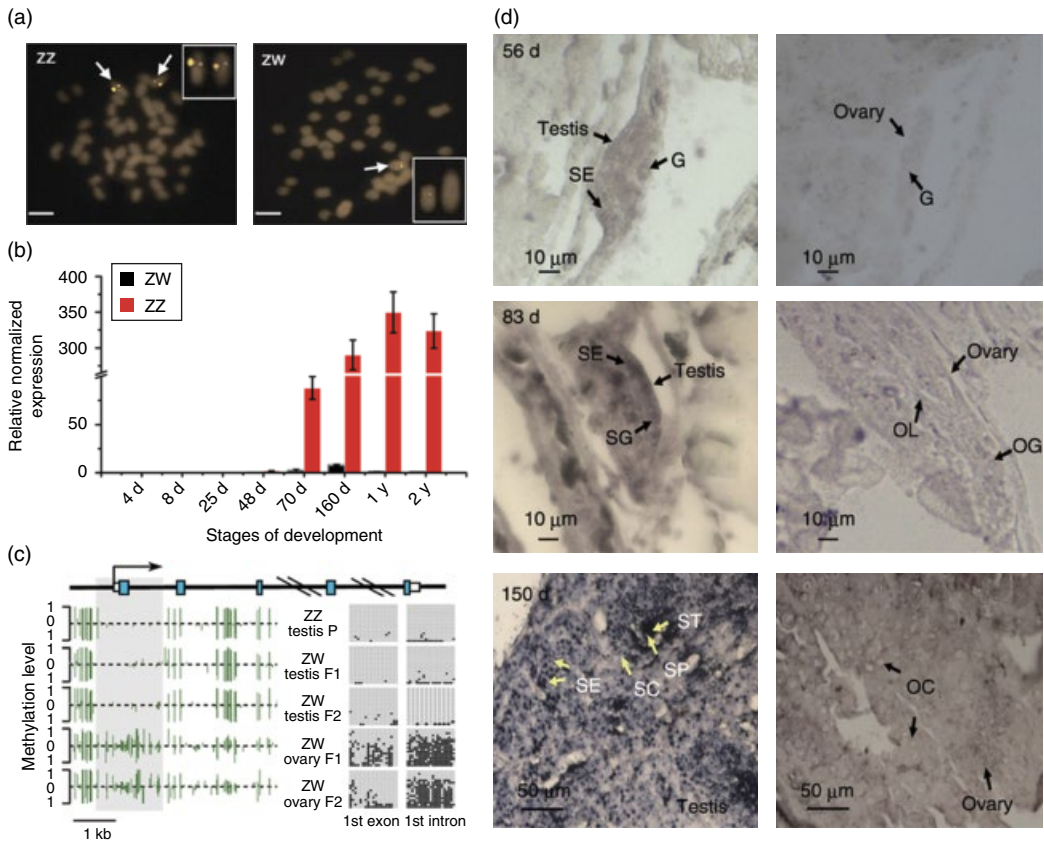


Figure 26.4 Characterization of *dmrt1* in tongue sole. (See inserts for the color representation of this figure.)

- a) *dmrt1* BAC FISH analysis of tongue sole chromosomes, showing a double signal in males and a single signal in females. BAC clone Hind012D10-3J, which contains the full-length *dmrt1* gene, was labeled and used to probe male (ZZ) and female (ZW) chromosome spreads. Scale bars: 5 μ m.
- b) RT-PCR analysis of *dmrt1* during developmental stages in female (black bar) and male (red bar; see color plate) tongue sole. The data are shown as the mean \pm s.e.m. ($n=3$).
- c) Methylation status across the differentially methylated region (DMR) of *dmrt1* in the gonads of an adult WZ female, a ZZ male and a WZ female, compared with male sex-reversed fish. The schematic diagram at the top shows the genomic structure of *dmrt1* in tongue sole. Exons are depicted as blue boxes (see color plate), and the 3' and 5' UTR regions are indicated by white boxes. The black arrow indicates the direction of the *dmrt1* gene from transcriptional start site. Also shown is the methylation level of each cytosine, indicated by a green line (see color plate), identified on both DNA strands throughout the *dmrt1* gene in female and male fish. The gray shadow indicates the DMR. Open and filled circles represent unmethylated and methylated cytosines, respectively, validated by TA clone and Sanger sequencing. ZZ testis P – testis of the male parent; ZW testis F1 – testis of a pseudomale in the first generation (temperature induced); ZW testis F2 – testis of a pseudomale in the second generation (untreated); ZW ovary F1 – ovary in the first-generation female; ZW ovary F2 – ovary in the female offspring of a pseudomale.
- d) Specific expression of *dmrt1* in testis. Gonad *in situ* hybridization using the antisense RNA probe of *dmrt1* – performed in tongue sole larvae at 56, 83 and 150 days during the gonad-development stage. G – gonium; OG – oogonium; OL – ovarian lamellae; OC – oocyte; SG – spermatogonia; SC – spermocyte; SE – sertoli cell; SP – spermatid; ST – spermatozoa.
- (Cited from [6]).

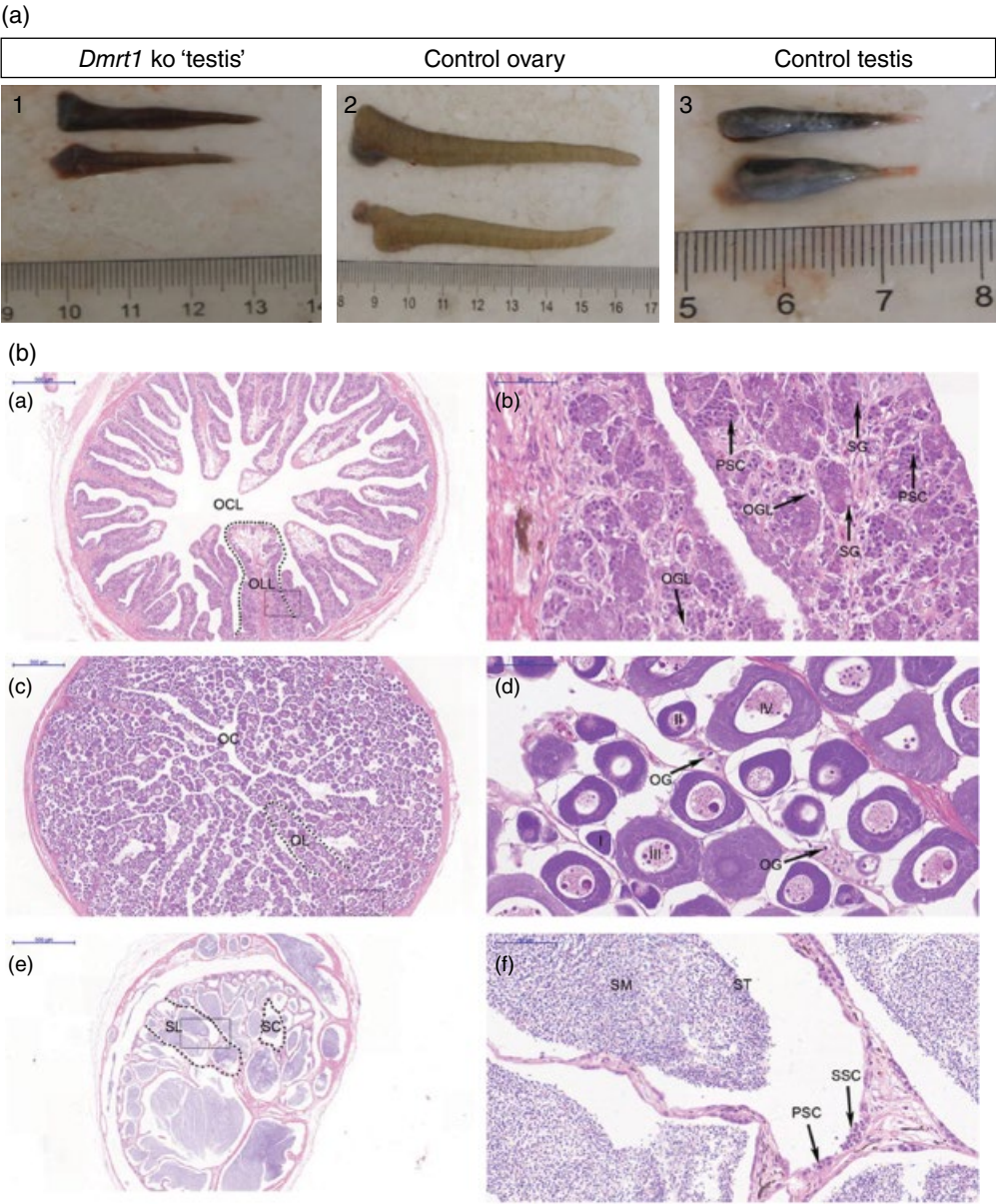


Figure 26.5 Effects of *dmrt1* disruption on gonad phenotype, sex differentiation. (See inserts for the color representation of this figure.)

a) Gross morphology of gonads from approximately one year old fish: (1) *dmrt1*-deficient “testes”; (2) wild-type ovaries; (3) wild-type testes.

b) histology of gonads from approximately one year old fish: (a) *dmrt1*-deficient testis. The development of testis is ceased. The shape of the *dmrt1*-deficient testes in transverse sections is similar to control ovaries, and there are structures resembling ovarian cavity and ovarian lamella in the gonad of the mutant male fish. Ovarian cavity-like (OCL); ovarian lamella-like (OLL); (b) large magnification of frame area in (a). No secondary spermatocytes, spermatids and sperm are observed. Oogonia-like (OGL); spermatogonia (SG); and primary spermatocytes (PSC). (c) Ovary of control female, including ovarian cavity (OC), ovarian lamella (OL); (d) large magnification of frame area in (c). Four stages of oocytes: stage I–IV and oogonia (OG). (e) Testis of control male. seminiferous lobuli (SL), seminiferous cyst (SC); (f) larger magnification of frame area in (e). Secondary spermatocytes (SSC), spermatids (ST) and sperm (SM). Scale bar is shown in the figures.

(Cited from [13]).

pattern, epigenetic regulation, and molecular function of many sex-related genes in the half-smooth tongue sole have been identified, suggesting their specific roles and regulative mechanisms in the genetic sex determination system and sex shift process responding to environmental stimulation.

The gene family of *dmrt* contains a large number of transcription factors having a highly conserved function in sexual development in animals. Multiple *dmrt* gene homologues have been discovered in tongue sole, among which *dmrt1* displays the typical features of a sex-determining gene, which is also the critical gene that responds to environmental change and triggers the gonadal reversal in half-smooth tongue sole [14]. As a transcription factor, the recombinant DMRT1 protein of tongue sole might regulate the expression of several sex-related genes. It was observed to suppress the expression of *cyp19a* and *foxl2* gene, but increase the transcript level of *sox9a* [15]. Two other members of *dmrt* gene family, *dmrt3* and *dmrt4*, show a distinguished higher expression in males than females, but no evidence can indicate that these two genes are critical for sex reversal [16, 17].

Sox9a and *foxl2* genes also encode transcription factors. In the tongue sole, the expression of *sox9a* gene is significantly regulated in the period of sex differentiation. Moreover, its transcript level in gonads of pseudomales is higher than that in normal females. Thus, the *sox9a* gene might have a close link with sex reversal, sex differentiation, and cell differentiation of embryos [18]. *Foxl2* is highly abundant in females than in males, suggesting that it may play a role in sex determination and sex reversal [18].

Cytochrome P450 aromatase (P450arom) is an enzyme responsible for the conversion of androgen to estrogen. Two types of P450 aromatases, including P450aromA and P450aromB, have been found in tongue sole. Both the P450 aromatases are encoded by *CYP* genes, whereas P450aromA and P450aromB belongs to the gonadal and brain P450arom subfamilies, respectively. The

P450aromA has a significantly high degree of expression in ovaries, but this is less in testis, and it is not present in other tissues [19].

In contrast, the expression level of P450aromB mRNA is high in the brain and gill, but lower in gonad and skin. However, the P450aromB transcript is downregulated in the brain of sex-reversed pseudomales after treated by methyltestosterone or at high temperatures [20]. These results suggest that the P450aroms are involved in gonad development and sex differentiation in this fish. Moreover, when incubated at high temperature during the early developmental stage, the promoter region of *cyp19a1a*, the coding gene of P450arom, was found to be up-methylated in ZW/ZZ testes, compared with ovaries, suppressing the expression of this gene and inhibiting the development of male gonad [14].

Anti-Müllerian hormone (Amh) is a glycoprotein belonging to the transforming growth factor β superfamily, which has been confirmed to play a major role in the development of reproduction system in vertebrates. In tongue sole, the expression level of the *amh* gene increases in the gonads of males and pseudomale offspring, but does not change in females, indicating that the *amh* gene is required for sex reversal and plays a role during reproductive development [21].

Three homologous genes of growth arrest and DNA-damage-inducible protein 45gamma (Gadd45g) have been identified in the half-smooth tongue sole. Characterization and expression analysis suggested that Gadd45g1 may be necessary for sex differentiation in the early stage of gonad development, and then both Gadd45g1 and Gadd45g2 play a major role in embryonic development by maintaining ovary development and female characters [22]. The expression pattern of *gadd45g3* indicates that it is a gender-related gene that is necessary for testes maturation, and is involved in sex determination prior to gonadal differentiation [23].

The gene family of Wilms' tumor suppressor (*wt*) is also important for sex differentiation. *Wt1a* is expressed in multiple tissue types and is more highly expressed in the

gonad. Notably, expression in the testes is significantly higher than that in the ovaries and gonads of sex-reversed fish. Among these, the lowest expression has been found in the gonad of sex-reversed fish. However, we cannot conclude that it is the key gene-controlling gonad differentiation until more detailed functional evidence is obtained [24].

Ftz-f1 gene is characterized to be highly abundant in gonad, kidney, brain, and head-kidney, but weak in other tissues in the half-smooth tongue-sole. Its expression level in females is higher than that in males. Moreover, the transcript of *ftz-f1* is specifically expressed in embryo, but absent in larvae, indicating that the *ftz-f1* gene may function in the organogenesis in the half-smooth tongue sole [25].

The expression level of *ubc9* gene is significantly higher in the temperature-induced pseudomales than in normal females and males, so it may also have been regulated in the sex reversal process [26].

The sex determination, differentiation, and gonad development in the half-smooth tongue sole is a polygenic system, and a number of genes have been identified to function in these pathways. Elucidation of the expressions, regulations, and interactions of the sex-related genes will provide insights into the sex determination mechanism of the tongue sole, and will offer the target molecules for the development of sex control technologies.

26.3 Role of Epigenetic Regulation in Sex Determination and Sex Reversal in the Half-Smooth Tongue Sole

26.3.1 Epigenetic Regulation Mechanism of Sex Determination

26.3.1.1 DNA Methylation and Gonadal Differentiation

Genome-wide comparisons of DNA methylome were performed using BS-Seq on bisulfite-converted DNA extracted from

gonad samples of different types of tongue sole, including normal females (P-ZWf), pseudomales (P-ZWm), and their F1 generation of pseudomales (F1-ZWm) and females (F1-ZWf), as well as normal males (ZZm). As a result, a total of ≈ 171 Gb methylome data were produced, with an average sequencing depth of 22 per strand for each sample, covering $\approx 90\%$ of the genomic cytosines, most of which are in the CpG context [14]. The methylation status of CpGs is different throughout the genome; CpGs in exons, introns, and repeat region have a relatively higher methylation level (>0.75), while hypomethylated CpGs (methylation level <0.25) were abundant in gene promoters and CpG islands [14]. Transcriptome was also analyzed to assess the gene expression profiles of these samples, which verified that, generally, the expression level is negatively correlated with the DNA methylation status in the gene promoter region.

Moreover, the chromosome-level epigenetic analysis also showed the methylation level was overall higher in testes than in ovaries. It was observed that the global methylation patterns of testes were highly similar in pseudomales (P-ZWm), normal males (ZZm) and pseudomale offspring (F1-ZWm), and the three testis samples were significantly different from ovary samples in methylation status, with a methylation level about 10% higher in testes than in ovaries, except for the two sex chromosomes. The Z and W chromosomes have an upregulated methylation status in ovary samples, especially on the W chromosome, probably because there is a high and moderate percentage of transposable elements on the W and Z chromosomes, respectively [14].

Pair-wise comparison of differentially methylated regions (DMR) on the genome showed that there was a high concordance in DNA methylation region among three testis samples (with 160 kb of DMR, 0.040% of the genome), or between two ovary samples (with only 60 kb of DMR, 0.015% of the genome). In contrast, differentially methylated regions are much larger between testes

and ovaries, up to an average of ≈ 15 Mb (4% of the genome) [14]. Therefore, in sex-reversed pseudomales, the global methylation pattern has been modified to be the same as that in normal males.

In addition, genes containing the testes/ovaries DMR in their promoter regions were identified to be associated with development, morphogenesis, and reproduction. Enrichment analysis of gene function showed that a number of biological processes are regulated by DNA methylation modification in the gonadal differentiation process. Genes up-methylated in ovaries were overrepresented in the biological processes of development and morphogenesis, including reproductive structure development, female gonad development, and oogenesis and spermatogenesis. In testes, genes that are up-methylated were enriched not only in developmental processes, but also in the biological processes of responding to stimulus (e.g., cellular response to steroid hormone stimulus), signal transduction (e.g., steroid hormone mediated signaling pathway), and biological regulations [14].

Furthermore, the changes in the methylation patterns in testes, compared with ovaries, are closely associated with gonad differentiation in tongue sole. We have verified that all of the offspring of pseudomales inherit the Z chromosome exclusively from their fathers. Interestingly, they also inherit their paternal methylation patterns on the Z chromosome, which has been changed by environmental stimulation, leading to the development of the male gonad. The next generations do not have any reprogramming mechanism of epigenetics to erase or reset the transgenerational methylation status, but stably maintain the paternal methylation pattern in lifetime, thus developing into functional males [14]. The stable transgenerational inheritance of both the Z chromosome and Z-methylation pattern from paternal pseudomales can explain why the next generation can spontaneously sex-reverse to pseudomales without any environmental induction.

26.3.1.2 DNA Methylation in Genes in Sex Determination Pathway

Genes in sex determination pathways appear to be strongly conserved throughout different vertebrate species. The genomic methylation analysis allowed us to evaluate the role of epigenetic regulation in genes in the sex determination pathways. A total of 58 sex-related genes were analyzed, among which 16 genes ($\approx 28\%$) displayed strikingly different methylation pattern between testes and ovaries (Figure 26.6A). This data is significantly higher than that of $\approx 14\%$ over the whole genome, indicating that genes in the sex determination pathways are the major targets of substantial methylation modification during sexual reversal.

Dmrt1 has been identified as the male-determining gene in tongue sole, with a functional copy only located at the Z chromosome. *Dmrt1* specifically displays a high expression during critical gonadal differentiation and sex determination periods in males, and persists at a high expression level during testis development [6]. The high expression of *dmrt1* in male gonads is correlated with an extremely low methylation status at a DNA methylation site in its promoter region. However, in females, up-methylation increasingly occurs at the critical sex determination stage, resulting in its transcriptional silence (Figure 26.6B). Moreover, when incubated at high temperature, the hypermethylation pattern in genotypic females can be eliminated and the gene expression of *dmrt1* is activated, which triggers the undifferentiated gonad developing toward testis [14]. These features indicate that in tongue sole, *dmrt1* not only takes over the role of master sex-determining gene, but also is critical in the sex reversal cascade upon environmental induction through epigenetic regulation.

In addition to *dmrt1*, several downstream conserved genes in the sex determination pathways are regarded to be critical for gonad differentiation in a wide range of vertebrate species, such as *amh* in the male cascade, and *foxl2* and *rspo1/wnt4* in the females [27, 28]. In tongue sole, some homology of these genes

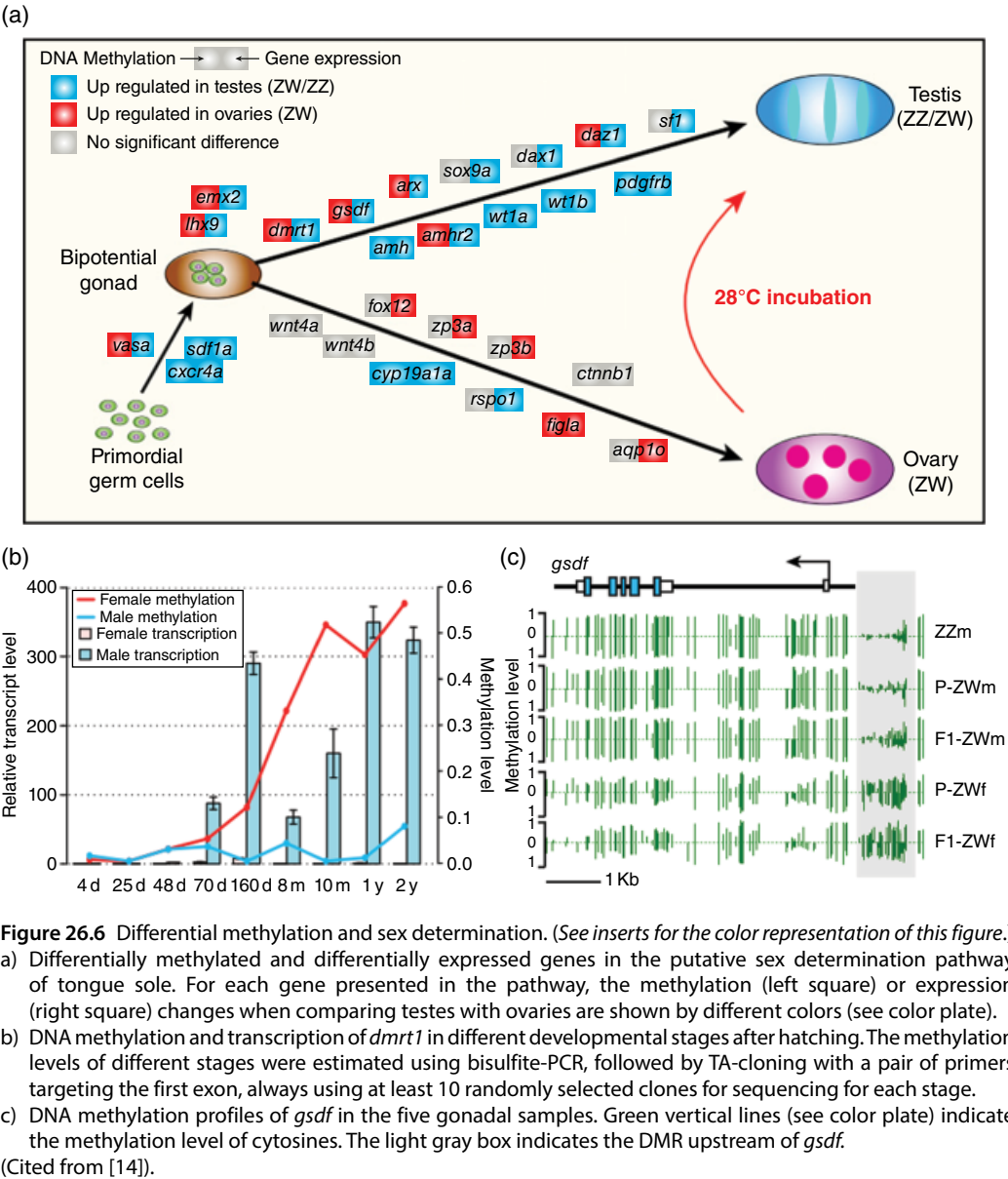


Figure 26.6 Differential methylation and sex determination. (See inserts for the color representation of this figure.)
a) Differentially methylated and differentially expressed genes in the putative sex determination pathway of tongue sole. For each gene presented in the pathway, the methylation (left square) or expression (right square) changes when comparing testes with ovaries are shown by different colors (see color plate).
b) DNA methylation and transcription of *dmrt1* in different developmental stages after hatching. The methylation levels of different stages were estimated using bisulfite-PCR, followed by TA-cloning with a pair of primers targeting the first exon, always using at least 10 randomly selected clones for sequencing for each stage.
c) DNA methylation profiles of *gsdf* in the five gonadal samples. Green vertical lines (see color plate) indicate the methylation level of cytosines. The light gray box indicates the DMR upstream of *gsdf*.
(Cited from [14]).

was also regulated by epigenetic modification. *Gsdf* (gonadal somatic cell derived factor) has been identified as the downstream gene of *dmY/dmrt1Y* in the sex determination cascade in medaka [29]. In tongue sole, it exhibited a significantly high expression level in testes and up-methylated status in ovaries (Figure 26.6C), which is consistent with the expression and methylation pattern of *dmrt1*

during the sex-sensitive period in gonad development early in life. Methylation differences between testes and ovaries were also observed in *amh* (anti-Müllerian hormone), *amhr2* (the receptor of *amh*), *wt1a* and *wt1b* (potential activators of *amhr2*) [14]. It appears that epigenetic regulation of a few core genes in the sex determination cascade can mediate the gene expression in sex determination

pathways and affect the sex-specific morphological and physiological traits.

26.3.2 Epigenetic Regulation Mechanism of Sex Reversal

Compared with normal males (ZZ) of tongue sole, pseudomale (ZW) individuals have one less Z chromosome and one more female-specific W chromosome synchronously. How to balance the expression dosage of genes in sex chromosomes is critical for the development of pseudomales after sex reversal. Comparison of the gene expression profiles on the Z chromosome, the W chromosome, and autosomes in females versus males, indicated that tongue sole has an incomplete dosage compensation mechanism by upregulation of female genes [6], resembling the pattern of dosage compensation in birds [30].

On the W chromosome, a total of 317 protein-coding genes are annotated, 86% of which have a paralog copy on the Z chromosome. Since the evolution period of the ZW chromosome system in tongue sole is only 30 Mya, the genes on the two sex chromosomes contain few sex-linked mutations, and the W-genes have a high identity with their counterparts on the Z chromosome. Therefore, genes on W chromosomes are generally harmless to male development, and their expression may even compensate the dosage inadequacy of their paralogs on the Z chromosome [14].

Moreover, on the Z chromosome, there is one region (from 13.6–15.6 Mb) specifically enriched with dosage compensated genes (Figure 26.7 A). This region is diverged from the W chromosome, containing a significantly low number of paralog genes with the W chromosome. By upregulation of gene expression in ZW testes, genes in this region achieve an equal expression level with that in normal male (ZZ) testes (Figure 26.7 B-D). Interestingly, this region has a high density of cytosine in hypermethylated status (Figure 26.7 A).

The differentially methylated regions between pseudomale and normal male are

also enriched in this region, suggesting that DNA methylation might play an important regulative role in the dosage compensation in this restricted region [14]. Although a compensated gene enriched region and dosage compensation mechanism have also been found in chicken [31], there are no significantly homologous relationships between the two regions [14], suggesting an independent evolution in chicken and tongue sole.

Genes in this unique dosage compensation region in pseudomale testes are related to male and testes development. For example, *Piwi2* is a member of the *piwi* family of genes that exhibits conserved functions relating to transposon silencing during spermatogenesis [32]. It displays a moderate degree of dosage compensation in ZW testes, but no compensation has been observed in ovaries [14]. Similarly, *Pik3r1* (phosphoinositide-3-kinase, regulatory subunit 1[p85 alpha]), which plays a central role in the self-renewal division of spermatogonial stem cells [33], displays a high degree of dosage compensation whereas, in ovaries, its expression is highly inhibited [14].

On the other hand, the pseudomales need to suppress the expression of genes in favor of female development, or detrimental to male development. *FIGLA* (factor in the germline alpha) gene, a germ cell-specific basic helix-loop-helix (HLH) factor required for ovarian follicle formation [34], has been found to locate on the W chromosome without functional paralogs on the Z chromosome or autosomes. It has two types of transcripts by alternative splicing. One splicing form is specifically transcribed in ovaries, which contains the HLH DNA binding domain that is critical to the formation of ovarian follicles. Another splicing form lacks the HLH domain, and is only expressed in pseudomale testes. The different splicing forms in ovaries and testes are tightly associated with different DNA methylation status, exhibiting a high level of methylation in ovaries, but no methylation in pseudomale testes [14]. Thus, the W-linked female-beneficial gene is expressed specifically in

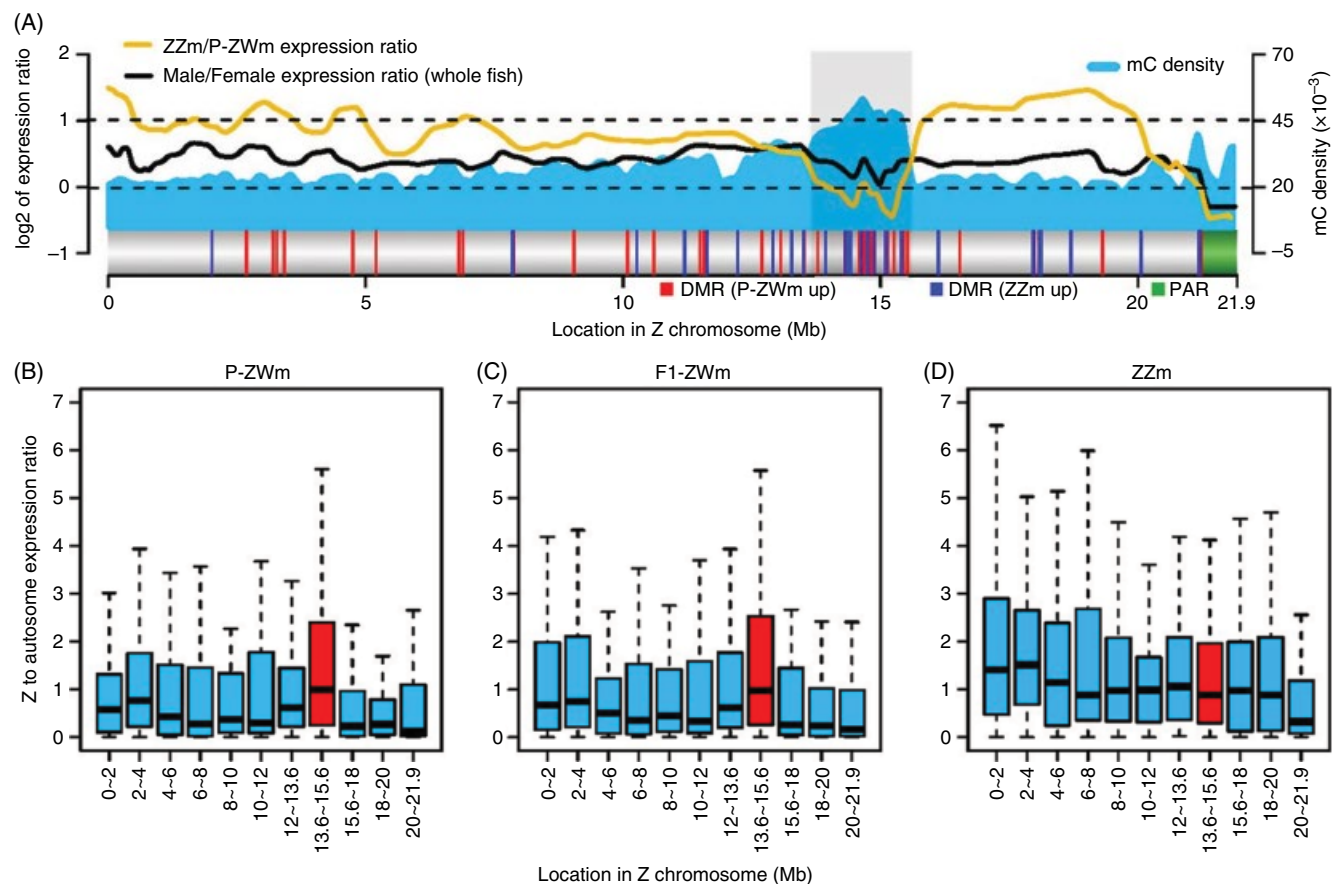


Figure 26.7 Dosage compensation of the Z chromosome in pseudo-male testes.

(A) Methylated cytosine (mC) density (5 kb window), log₂-transformed expression ratios (running averages of 20 genes), and DMR profiles of the Z chromosome. The light gray box indicates the outstanding dosage-compensated region where DMRs were concentrated ([red vertical lines] (see color plate), DMRs that were up-methylated in P-ZWm [blue vertical lines] (see color plate), DMRs that were up-methylated in ZZm, and the green block (see color plate) indicates the pseudoautosomal region (PAR) where Z and W chromosomes still pair in meiosis. Only 22 genes were annotated in PAR. Z-chromosomal to autosomal gene expression ratios (Z : A) in P-ZWm (B), F1-ZWm (C), and ZZm (D). The dosage compensation region (light gray box in A) is plotted in red. For each Z interval, the expression level of each Z-gene was first divided by the median expression level of all autosomal genes, then the Z : A ratios in each interval were plotted. (Cited from [14]).

ovaries in a special transcript form, but is suppressed in testes in another splicing form, ingeniously regulated by DNA methylation. Therefore, the expression of some genes can be regulated by differential methylation to alternative splicing forms.

In summary, in the tongue sole, multiple epigenetic regulation mechanisms are involved in the development of sex-reversed individuals. Firstly, on the Z chromosome of pseudomales, dosage compensation occurs in a unique region with a high level of methylated cytosines. The expression level of dosage-compensated genes is equal to that in normal males. Secondly, some W chromosomal genes are suppressed in pseudomales by methylation regulation. In addition, the incompletely differentiated ZW chromosome system, where both the two chromosomes have highly conserved paralog genes, also contribute to the plasticity in GSD and ESD.

26.4 Conclusions and Future Directions

The half-smooth tongue sole has both GSD and ESD systems. With a sex chromosome type of ZW/ZZ, a male-determining gene,

dmrt1, has been identified in the Z chromosome. In addition, a number of sex-related genes may also function in the sexual differentiation process. Epigenetic regulation has been verified as playing an important role in both the sex determination and sex-reversal of the half-smooth tongue sole. In the future, more genomic analysis and functional validations should be performed, to identify other important genes and regulatory pathways in sex determination, such as female-determining genes, using genome editing and other tools. More research is also needed to figure out the detailed mechanisms of sex determination and sex reversal.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (31730099), AoShan Talents Cultivation Program Supported by Qingdao National Laboratory for Marine Science and Technology (No.2017ASTCP-OS15), and Taishan Scholar Climbing Project of Shandong of China.

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27

Sex Identification and Control in Half-Smooth Tongue Sole

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27.1 Isolation of Sex-Specific Marker and Identification of Genetic Sex

Half-smooth tongue sole has ZZ/ZW sex chromosomes and exhibits significant sexual dimorphism [1, 2]. Accurate identification of genetic sex is a pivotal step for developing sex control techniques and exploring the high-female fry in tongue sole, which can in turn achieve healthy, and sustainable development in the aquaculture industry. Sex-specific molecular markers represent one of the techniques with the most potential for genetic sex identification. Sex-specific markers have been identified in several fish species, including medaka (*Oryzias latipes*, *O. curvinotus*) [3–5], four species of salmon (*Oncorhynchus keta*; *O. gorbuscha*; *O. kisutch*; *O. tshawytscha*) [6], 3-spined stickleback (*Gasterosteus aculeatus* L.) [7], rainbow trout (*Oncorhynchus mykiss*) [8–10], African catfish (*Clarias gariepinus*) [11], and platyfish (*Xiphophorus maculatus*) [12]. However, all the markers are male-specific, and no female-specific markers have been reported in the fish. In this section, we have summarized the exploitation of molecular markers in half-smooth tongue sole, the first female-specific molecular markers in fish, and their application in aquaculture.

27.1.1 Discovery of Female-Specific Amplified Fragment Length Polymorphism Marker and Identification of ZZ Male and ZW Female

27.1.1.1 Technical Principle

Amplified fragment length polymorphism (AFLP) marker is a type of dominant DNA marker based on restriction enzyme digestion and PCR amplification [13]. The AFLP technique contains the following steps: the genomic DNA is first digested by two restriction enzymes, one being common, while the other enzyme is rare. Then, the digested fragments are ligated with adaptors. According to the adaptor and restriction cutting sequence, primers are designed and used for pre- and selective PCR amplification. After two rounds of PCR screening, the sex-specific AFLP fragments are cloned, sequenced, and transferred to the sequence-characterized amplified region (SCAR) marker, which is used as a quick, efficient method for genetic sex identification.

Combined with RFLP reliability and PCR efficiency, AFLP possesses several advantages, including high polymorphism, stability, and sensitivity. Moreover, this method is not dependent on the availability of whole-genomic data and, thus, can be widely applied in genetic diversity analysis, genetic

linkage map construction, and sex-specific marker selection.

27.1.1.2 Methods and Applications

In 2007, Chen and his colleagues discovered seven female-specific AFLP markers in half-smooth tongue sole for the first time, and one of them was converted into SCAR marker (CseF382) for genetic sex identification [14]. The brief experimental procedure follows:

- 1) *Tissue DNA extraction*: A small piece of tissue ($\approx 50 \mu\text{g}$) was picked up from tongue sole and homogenized with $300 \mu\text{L}$ lysis buffer, then another $300 \mu\text{L}$ lysis buffer containing proteinase K and RNaseA (both freshly added to a final concentration of $100 \mu\text{g/ml}$) was added and incubated at 55°C for 90 minutes. After phenol-chloroform extraction, the supernatant was picked up, and DNA was precipitated with ethanol. The DNA was stored in TE buffer, and the quality and concentration were assessed by agarose electrophoresis and spectrophotometer.
- 2) *AFLP analysis for genomic DNA*: AFLP analysis was performed according to the manufacturer's procedures (Li-Cor, Lincoln, NE) with some modifications. In brief, genomic DNA was digested with *EcoRI* and *MseI*, and the resulting fragments were ligated with specific adapters. The ligated product was diluted 1 : 10 and used for two rounds of PCR amplification. The first round amplification was performed using preselective primers with the following conditions: 94°C for 5 minutes; 20 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for one minute, and extension at 72°C for one minute. The products from the first round were diluted 1 : 45 and used as a template for the second round PCR, which followed the same PCR condition, except that the selective primers were used in this round. The amplified fragments were separated on an automatic DNA sequencer (Li-Cor4300) and analyzed by SAGA software (Li-Cor) [14].
- 3) *Selection of sex-specific marker*: This stage included two steps – sex-specific marker screening and sex-specific marker cloning. In sex-specific marker screening, the AFLP patterns were analyzed, based on the electrophoresis. In this study, seven female specific AFLP markers, designated as CseF382, CseF575, CseF783, CseF464, CseF136, CseF618, and CseF305, were identified, and their specificity was further confirmed by performing large-scale experiments (Figure 27.1). These PCR fragments were purified and ligated into pMD18-T, and the recombinant vector was transformed into Top 10 competent cells. The transformants were screened by PCR and the positive clones were sequenced. Five sequences (CseF382, CseF783, CseF464, CseF136, CseF305) were clarified among the seven sex specific markers [14].
- 4) *Establishment of sexual identification method in half-smooth tongue sole*: Based on the five sequenced AFLP fragments, specific PCR primers were designed, and the products were analyzed by agarose gel. All five AFLP fragments only existed in the female samples, but not in male samples, suggesting the successful establishment of a sexual identification method.

Sex identification of tongue sole during the cultivation and breeding process, especially in the early developmental stage, was crucial for shortening the breeding cycle, improving breeding efficiency, and thus enhancing productivity. Taking the advantage of the established method, three of the five AFLP markers (CseF382, CseF783, CseF305) were applied in genetically sexual identification, where female individuals showed one band, but males showed no bands. For example, CseF382 could generate $\approx 350 \text{ bp}$ band in ZW females, while producing no band in ZZ males. It was first confirmed in 15 males and 13 female fish, and then tested in 59 male and 59 female fish. The results indicated its positive detection in all female fish, while there was no detection in 58 of the male fish (Figure 27.2) [14].

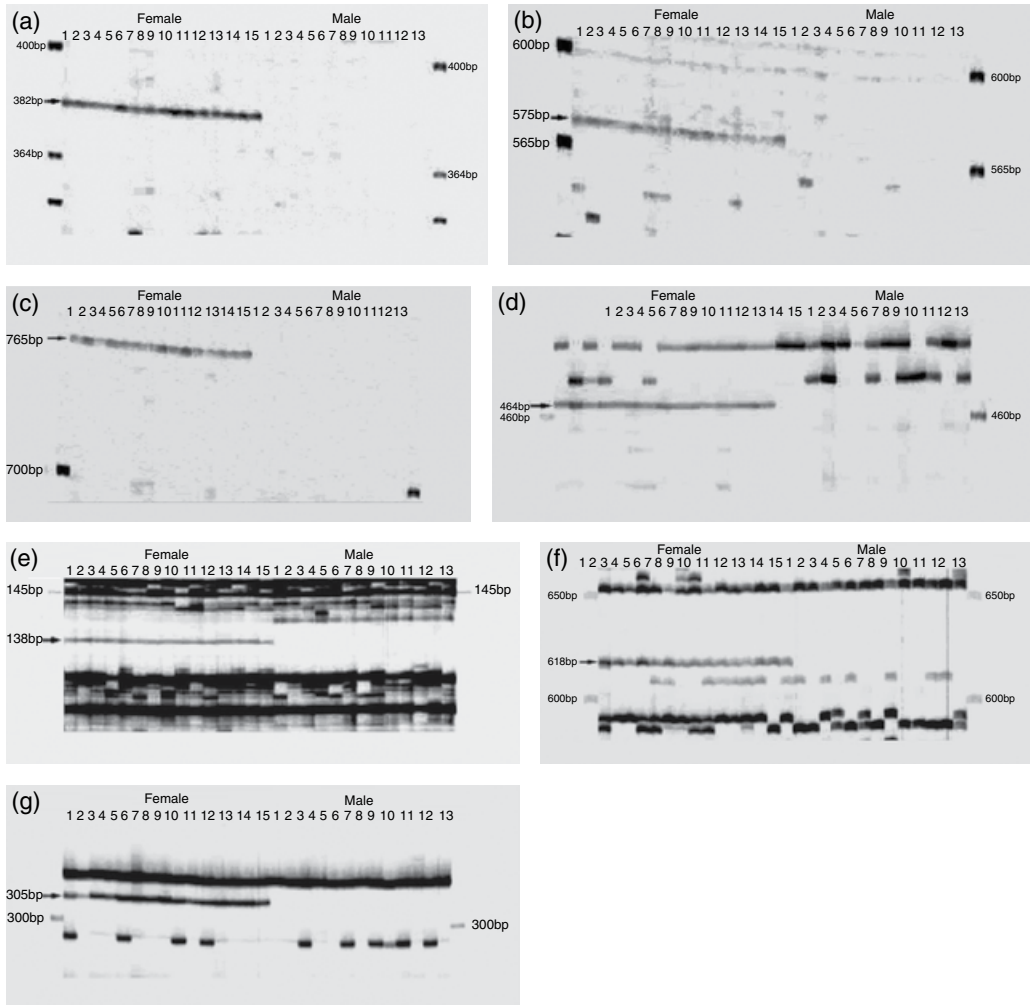


Figure 27.1 Seven female-specific AFLP markers: (a) Cse F382 (E-ACT/M-CAA). (b) CseF575 (E-ACT/M-CAA). (c) CseF783 (E-ACT/M-CAA). (d) CseF464 (E-AGC/M-CTG). (e) CseF136 (E-AGC/M-CTG). (f) CseF618 (E-ACA/M-CAG). (g) CseF305 (E-ACC/M-CTA). The primer combinations used for obtaining the markers are indicated in brackets. Cited from [14].

With CseF382, around 10,000 fish were screened and among them ZZ males were selected as parental fish. In this way, more than 10 million fry were produced with the increased female ratio (see Section 27.1.3). Other AFLP for other AFLP markers, CseF783 was used to test sex ratio in cultured population. In 36 individuals, there were positive bands in 15 fish and no band in 21 fish, indicating that the ratio of female/male is roughly 1 : 1. CseF305 was tested in four pseudomale individuals (by high

temperature treatment), and all of them showed positive bands (data not shown).

27.1.2 Discovery of Sex-Specific Simple Sequence Repeat Marker and Identification of ZZ Male, ZW Female and WW Superfemale

27.1.2.1 Technical Principle

Although AFLP markers can be used for genetic sex identification, their dominant

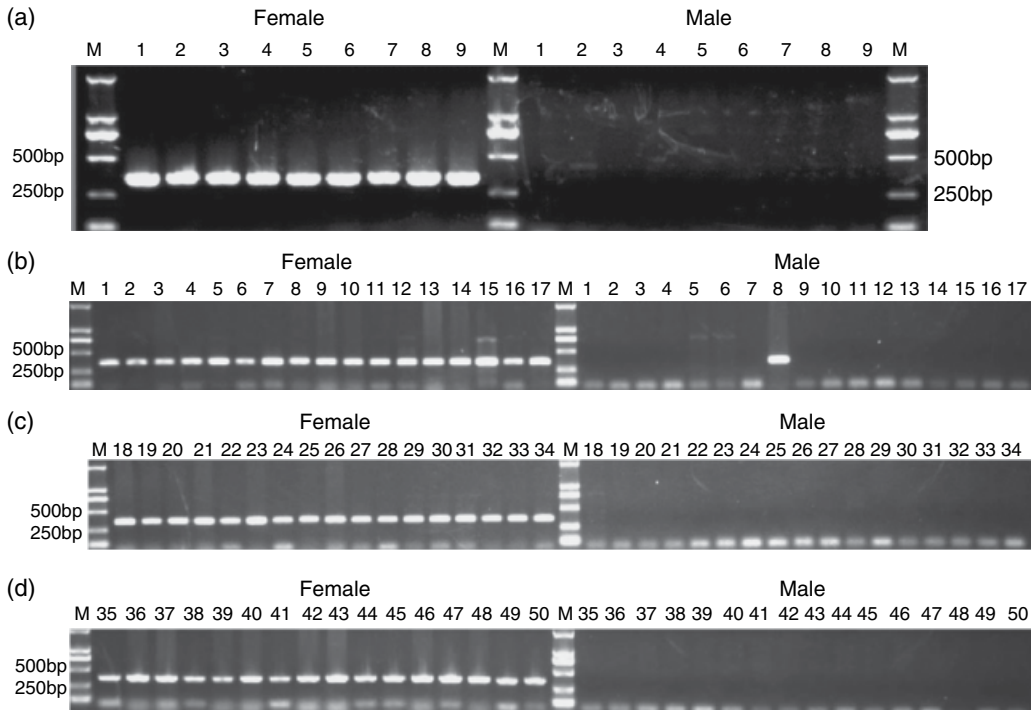


Figure 27.2 Agarose analysis of PCR products from CseF382 in females and males. (a) DNA from nine female individuals and nine male individuals were examined. (b–d) DNA from an additional 50 female individuals and 50 male individuals were examined. Cited from [14].

characteristic makes them unable to distinguish between homozygous and heterozygous fish. For example, the female-specific AFLP marker can identify ZW females and ZZ males, but shows the same results for ZW female and WW superfemale fish. Therefore, new co-dominant molecular markers are required for ZW female and WW super-female identification. In this principle, a simple sequence repeat (SSR) marker, also known as a microsatellite marker, has emerged into our sight. SSR markers have the advantages of high polymorphism, co-dominance, and genomic abundance, so they have been developed as a new generation of molecular markers, and widely applied in linkage map construction, comparative genome analysis, genetic diversity evaluation, and breeding technique development [21–23].

27.1.2.2 Methods and Applications

The technical process mainly consists of three experimental steps:

- 1) *Screening sex specific SSR markers from the tongue sole genome*: The genome of half-smooth tongue sole was deciphered in 2014 [16], where the male and female individuals were subjected to *de novo* sequencing by SOLEXA technique. Sex-specific SSR markers can be screened by simply comparing male and female genome, and then the primers are designed in the differential SSR flank area.
- 2) *Experimental confirmation for sex specificity*: Using genomic DNA from male or female fish as a respective PCR template, the PCR reaction was performed according to the following PCR program: predenaturation at 94°C for 5 minutes; 33 cycles of denaturation at 94°C for

30 seconds, annealing at 56–58°C for 30 seconds, and extension at 72°C for 30s; additional extension at 72°C for five minutes. The PCR products were separated by 6% denatured polyacrylamide gel electrophoresis, and visualized via silver staining. The SSR markers displaying different patterns in males and females (e.g., existence only in one sex or a different number of bands in different sexes) were selected as candidates.

- 3) *Purification, cloning, and sequencing of sex-specific SSR markers*: These bands were precisely cut and purified with gel extraction. The purified products were ligated into pMD18-T, and the recombinant vector was transformed into Top 10 competent cells. PCR were used for screening positive transformants to sequence. The obtained sequence was subject to alignment analysis in Genbank to check whether it was a new SSR marker.

The genomic comparison between males and females led to the discovery of 159 sex-specific SSR markers [24], which are categorized into two types. In type I, there is one band in female fish, but no band in male fish, whereas in type II there is one band in male fish, but two bands in female fish. Among

those markers, one SSR marker designated CseF-SSR1 was confirmed to be closely linked to sex. This marker belongs to type II, where the 206bp band was amplified from ZZ male genomic DNA, while the 206 and 218bp bands were obtained from ZW female samples (Figure 27.3).

With this feature, CseF-SSR1 has been applied in WW superfemale identification. When mitogynogenesis was performed in half-smooth tongue sole, the mitogynogenetic embryos were examined with CseF-SSR1. The individuals with two bands were identified as ZW genotype, while individuals producing only 206bp or 218bp were considered to be ZZ or WW, respectively. Preliminary analysis of the mitogynogenetic embryos found that four of 39 individuals showed only 218bp bands (Figure 27.4).

Before the prevalence of SSR markers, the AFLP marker was widely used for sexual identification. However, as a dominant molecular marker, the application of AFLP was largely limited for its inability to distinguish ZW females and WW superfemales. In addition, AFLP usually exhibits one band in female fish, but no band in male fish, easily leading to false-negative readings if the DNA extraction quantity is low. In contrast, SSR markers are co-dominant markers, which

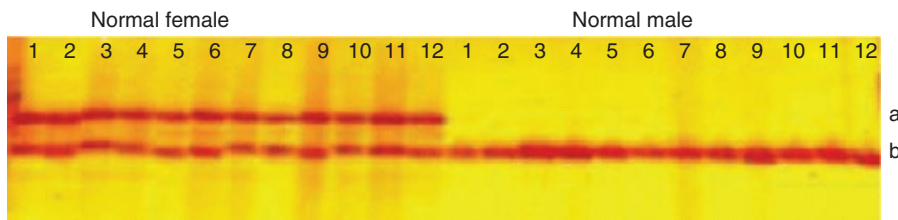


Figure 27.3 Identification of sex-specific SSR marker (CseF-SSR1) in half-smooth tongue sole. Cited from [17].



Figure 27.4 Genotype of mitogynogenetic half-smooth tongue sole at embryonic stage by using sex-specific CseF-SSR1 markers. Cited from [17].

can distinguish ZW females and WW super-females by showing different band patterns.

Meanwhile, the type II SSR marker could produce bands in all genotypes (one band in ZZ or WW genotype, two bands in ZW genotype), thus eliminating the false-negative readings. In terms of technical level, there is no obvious shortcoming for SSR-based sex identification technique, so it has great applicable potential in the tongue sole industry, not only in gynogenetic fry and parental fish screening, but also in sex control technique and high/all-female stock breeding.

27.1.3 Application of Sex-Specific Markers in Production of Fry with High Female Proportion

Due to their fast growth, high female fry are favored in tongue sole aquaculture, while female fry account for only about 20% in culturing population. The main reason is as that, in half-smooth tongue sole, the sex-reversal phenomenon occurs, and genetically female fish easily become phenotypic male fish, designated as pseudomale (ZW), when water temperature is high (above 24°C). The pseudomale fish have male growth characteristics, and can develop mature testes, producing functional sperm, while their offspring are prone to become pseudomale [15, 16], which altogether leads to 70–90% males in culturing population.

This situation undermines the enthusiasm of aquaculturists, and is disadvantageous for industrial development, so high female breeding technology is urgently required in this field. Based on sex-specific markers, high female breeding technology has been successfully developed, mainly including the following steps:

First, the sex-specific SSR markers were discovered and transferred into SCAR markers. Second, a rapid and precise identification technique was established, which is usually dependent on DNA isolation and PCR technique. Third, the male fish were subjected to screening; ZW pseudomales

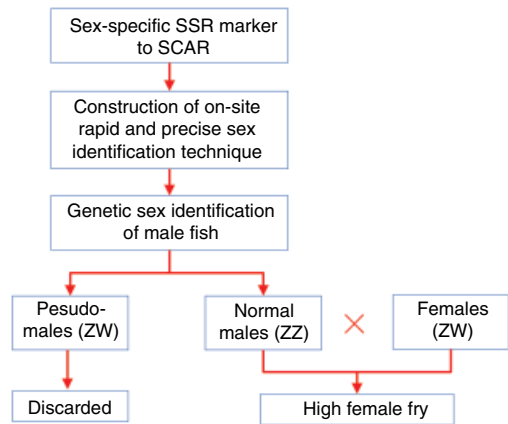


Figure 27.5 Road map of the high female breeding technology. SSR – simple sequence repeat; SCAR – sequence-characterized amplified region. Cited from [25].

were discarded while ZZ males were kept as parental fish to mate with ZW females.

Using this technique, the female ratio in offspring can be largely increased (Figure 27.5). One application example is as follows: more than 3,500 normal male fish were selected as parental fish after screening over 4,500 individuals, by which the female ratio in the population was increased from less than 20% to above 40%.

27.2 Artificial Propagation

27.2.1 Technical Principle

Under natural conditions, half-smooth tongue needs more than two years to attain gonadal maturity, and the reproductive cycle is only once a year (in September or October). Given the social benefit of half-smooth tongue sole in aquaculture, much research has focused on artificial control of the culturing conditions, including temperature and light cycle, to stimulate maturity and spawning [26]. However, the strict requirements on the artificial environment makes the precise control rather difficult, and the effect is not very satisfactory, with low spawning and

fertilization rates. Most of all, in this model, unfertilized eggs cannot be obtained, which constrains the subsequent development of sex control technique (i.e., artificial gynogenesis). Thus, establishment of artificial propagation technique, especially the synchronous development and spawning by hormone induction, would greatly facilitate the development of artificial gynogenesis and large-scale breeding in half-smooth tongue sole.

27.2.2 Methods and Applications

Artificial induction of reproduction using hormone in half-smooth tongue sole was reported by Yang *et al.* [27], and the protocol consists of three major steps:

- 1) *Selection of parental fish.* The sexually mature fish (female, three-year-old; male, two-year-old) were selected and cultivated with the density of three individuals per square meter, including one female and two males. Starting from 2–3 months before the artificial propagation, the temperature was increased from 17–19°C to 22–23°C, and the light period was increased from eight hours to 16 hours. The female fish, with bulging gonads and a sense of fulfillment by squeezing, were selected for hormone induction. The male fish were only selected when semen flowed out by slight squeezing.
- 2) *Hormone and dosage selection.* Half-smooth tongue sole showed high sensitivity to all tested hormones. After experimental comparison, luteinizing hormone releasing hormone A3 (LHRH-A3) and human chorionic gonadotropin (HCG) were selected for induction, and injected with the specified dosages (HCG: female, 50–120 IU/kg; male, 300–500 IU/kg; LRH-A3: female, 0.4–2.0 µg/kg; male, 2–5 µg/kg).
- 3) *Determination of the disposing duration.* The disposing duration indicates the time period from hormone injection to collecting the eggs. It can significantly vary with regard to the hormone types

and water temperatures. When the water temperature was 23°C and the reactive time of HCG and LRH-A3 were 39–48 hours and 35–43 hours, respectively, the artificial propagation could reach the optimized effect. Under these conditions, the gametes were collected and stored for fertilization. Since the establishment of the method, it has been successfully applied in several industries, and widely used in the tongue sole breeding process.

27.3 Artificial Gynogenesis

Gynogenesis is an important approach to control sex ratio in fish aquaculture [28]. The production of gynogenetic diploid includes two major steps: sperm inactivation and maternal chromosome set diploidization. Sperm inactivation indicates that the genetic material of sperm is inactivated by physical radiation (e.g., γ-ray, X-ray, UV), chemical treatment (ethylene urea, toluidine blue), or micromanipulation (removal of masculonucleus), but the motility and penetrating capability are still maintained in order to fertilize. The resulting zygote contains only maternal genetic material, and the individual usually displays developmental impairment – so-called haploid malformation – so chromosome set diploidization is required for further survival [18].

Based on the induction time of chromosome set diploidization, the artificial gynogenesis can be classified into two types: meiogynogenesis and mitogynogenesis. In meiogynogenesis, the diploidization process in the gynogenetic embryo is performed before the second meiosis, in order to block the release of a second polar body, while in mitogynogenesis, it is done before the first cleavage to achieve diploidization. Artificial gynogenesis has been widely applied in freshwater fish species, while this technique was only developed in a few marine fish, including turbot, Japanese flounder, red sea bream, and sea bass [28–31]. Furthermore, all these

marine fish species employ a male heterogametic sex-determining system, where the male has XY sex chromosomes and female has XX sex chromosomes. As a marine fish that employs a female heterogametic system (ZW/ZZ), half-smooth tongue sole is the first reported marine fish that succeeded in gynogenesis.

27.3.1 Meiogynogenesis

27.3.1.1 Technical Principle

During the second meiosis cycle, the second polar body is released from the oocyte to form the mature egg. The extrusion can be inhibited by cold shock or hydrostatic pressure and, based on this principle, meiogynogenesis is induced to achieve the diploidization. However, due to the chromosome crossing-over in the first meiosis, the meiogynogenetic diploid is heterozygote. Meiogynogenesis has been studied in some freshwater fish, including several carp species, turbot, and southern flounder [29, 32, 33]. Here, we explore the inducing conditions of meiogynogenesis and establish this technique in half-smooth tongue sole. Its potential application in all-female fry production is also discussed in this section.

27.3.1.2 Methods and Application

In 2009, the meiogynogenesis technique in half-smooth tongue sole was established by Chen *et al.* [18], and mainly involved five experimental steps:

- 1) *Gametes collection.* After the artificially induced spawning, half-smooth tongue sole eggs were collected by squeezing the female fish abdomen, and were then stored for further use. Two types of sperm were used for meiogynogenetic induction. The homologous sperm was collected by squeezing male tongue sole abdomen, while heterologous sperm was from our cryopreserved bank, as previously described [34]. Several fish sperm, including sea perch, Japanese flounder, turbot, and summer flounder, were tested,

and the sea perch sperm exhibited the best inducing effect.

- 2) *Sperm inactivation.* The homologous and heterologous sperms were subjected to the same inactivating procedure. In brief, 100 μ l semen was diluted 1 : 10 with MPRS solution and exposed under 80–110 J/cm² UV light, then mixed with 3–6 ml tongue sole eggs and incubated under 22–23°C. The fertilization rate, hatching rate, and fry morphology were inspected. The feasibility of using sea perch sperm for heterologous fertilization was emphatically studied. It was found that eggs fertilized with normal sea perch sperm or inactivated sea perch sperm, without cold shock, resulted in either death or failure of hatching, respectively. When fertilization was performed with inactivated sea perch sperm and cold shock treatment, 2.5% embryos could continue the hatching process and survive when checked at 48 days postfertilization (dpf). These findings suggest that only the diploid produced by gynogenesis could survive by the heterologous fertilization (Figure 27.6).
- 3) *The initiation time of cold shock.* In this method, cold shock was employed for inhibiting second polar body extrusion and inducing chromosome diploidization. The eggs were submerged into 2°C or 4°C sea water for 30 minutes at different time points postfertilization (2–9 minutes), then the fertilization rate, haploid rate, and diploid rate were calculated. From the data, we can see that the induction of diploidization ranged from 3.5–6.5 minutes postfertilization, but the peak of induction rate appeared between 4.5–5.5 minutes (Figure 27.7a).
- 4) *Determination of shock temperature and duration.* After the initiation time, the optimum temperature and duration of cold shock were also investigated. Cold shock was conducted at different temperatures ranging from 2–8°C, and the gynogenetic diploid rate was assessed. Gynogenetic diploids could be produced

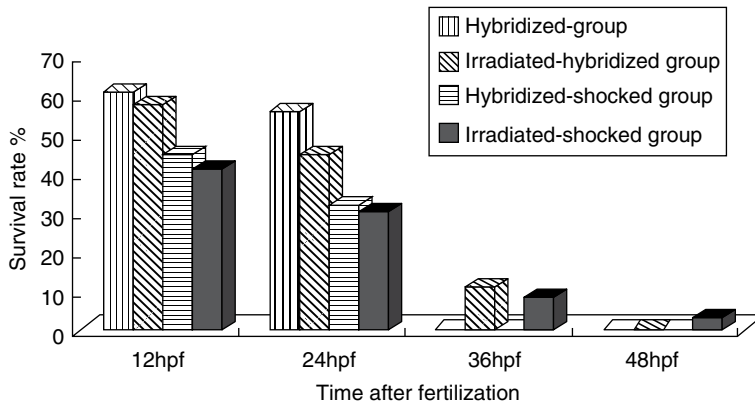


Figure 27.6 The survival rate of gynogenetic embryos and fry by different treatment of irradiation and cold shock. Cited from [18].

in the range of 3–7°C, while the highest induction rate occurred at 5°C (Figure 27.7b). Cold shock were also performed with five time durations (15, 20, 25, 30, and 35 minutes), and then the diploid induction rates were assessed. It was found that gynogenetic diploids could be produced in 15–30 minutes, but the induction rate between 20 and 25 minutes was significantly higher (Figure 27.7c).

- 5) *Karyotype analysis*: Chromosomes of gynogenetic embryos or fry were prepared according to the previously described method [18]. The number of chromosomes in sea perch and half-smooth tongue sole significantly differed (sea perch, $2n=48$; half-smooth tongue sole, $2n=42$); the karyotype analysis would provide the direct evidence whether the fry was derived from gynogenesis. In the examined fry, 42 chromosomes were identified, indicating that these fry were produced by gynogenesis. Moreover, some embryos were observed to have two huge chromosomes (WW genotypes) (Figure 27.8). These data altogether support successful induction of diploid by gynogenesis.

Since the meiogynogenesis method was established, it has been used many times to successfully obtain fry and mature fish.

However, the diploid induction rate is only about 0.3–2.8%, which is lower than the diploid induction rate in other reported marine fish. For example, in Japanese flounder gynogenesis activated by homologous sperm, the diploid induction rate is around 34% [28]. Subsequently, Piferrer and his colleagues performed gynogenesis in turbot, and the diploid induction rate was 10% [29]. However, a low diploid induction rate was also observed in some reports. In southern flounder, the induction rate was only 2.1% when homologous sperm was used, and this rate was 1.6–11.4% for heterologous sperm (*Mugil cephalus*) activation [33]. Similarly, the low diploid induction rate in Atlantic halibut gynogenesis was observed in the following research [35].

This is the first report of gynogenesis in half-smooth tongue sole. We have taken advantage of the sperm bank in our lab, and sperms of various species have been tested for their feasibility in half-smooth tongue sole gynogenesis. Sea perch sperm, with their superior performance, were chosen to produce gynogenetic fry and mature fish. The successful application of heterologous frozen sperm in gynogenesis widens the applied range of cryopreserved sperms and provides the new perspective for fish sex control and genetic manipulation. For example, incomplete inactivation of homologous sperm always led to indistinguishability between

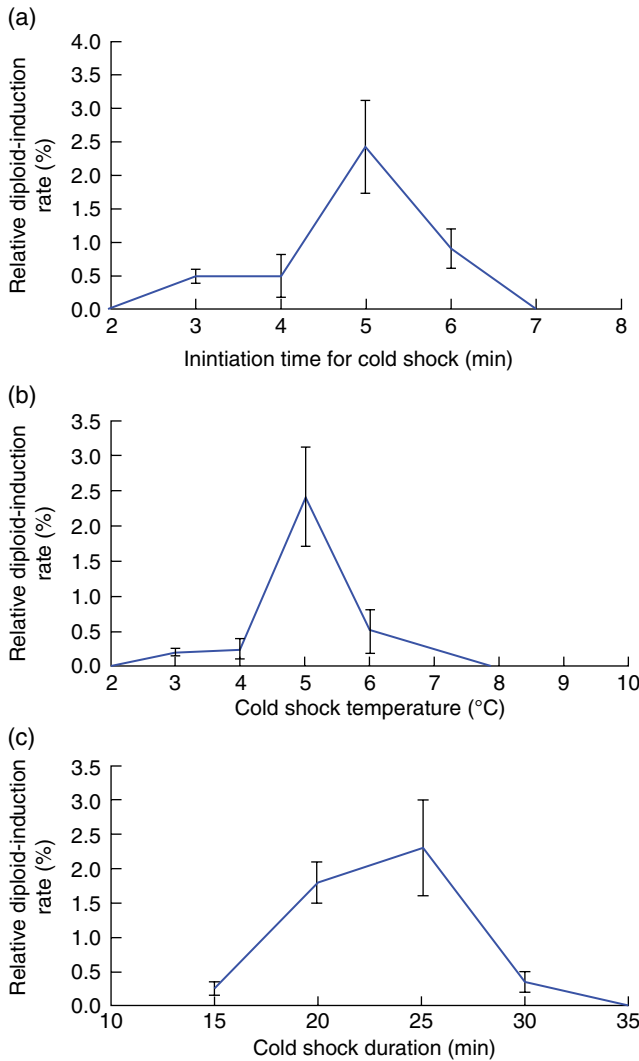


Figure 27.7 Screening for the optimal initiation time of cold shock (a), shock temperature (b), and duration (c) on the gynogenesis induction rate in half-smooth tongue sole. Cited from [18].

gynogenetic and normal fertilized fish but, with the heterologous sperm, only gynogenetic diploid fish are able to survive and, in this way, this obstacle can be overcome. Moreover, the usage of cryopreserved sperm can reduce the seasonal limitation and guarantee a consecutive supply of sperm material, which is believed to have great value if applied in aquaculture.

However, as high frequency of crossover occurs in the first meiosis, the meiotogynogenetic diploid maintains a high level of heterozygosity in a number of loci [36]. For example, in the 124 gynogenesis fry activated

by sea perch sperm, all five female fish were identified as ZW type. These data suggest that it is difficult to generate homozygous individuals by meiotogynogenesis, so WW superfemale induction should rely on the development of mitogynogenesis technique.

27.3.2 Mitogynogenesis

27.3.2.1 Technical Principle

Unlike meiotogynogenesis, mitogynogenesis induces the diploidization process by inhibiting the first cleavage, so the diploidization is derived from the replication of one

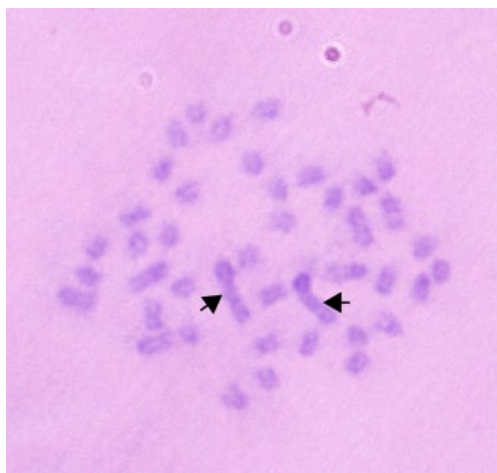


Figure 27.8 Karyotype of gynogenetic WW embryo; two huge WW chromosomes are indicated by arrows. (See inserts for the color representation of this figure.)

chromosome set. Because of this, mitogynogenetic individuals are also known as double-haploids and, in theory, they should exhibit homozygosity at all gene loci. Mitogynogenesis has great potential in the application of many aspects, such as genetic mapping, production of all-female fry, and elimination of recessive deleterious genes [37]. However, due to the induction difficulty and low survival rate, its application is largely limited. Ever since the 1990s, great effort has been made for artificial mitogynogenesis in marine fish, but only a few species have successful examples, such as Japanese flounder, red sea bream, and sea bass [28, 30, 31]. Here, we report the mitogynogenesis technique in half-smooth tongue sole, and discuss the potential application in the flatfish industry.

27.3.2.2 Methods and Applications

In 2012, Chen and colleagues established the mitogynogenesis technique in half-smooth tongue sole [17]. This method adopted the hydrostatic pressure for diploid induction, and mainly consisted of the following experimental steps:

- 1) Gamete collection and sperm inactivation were performed according to the previously described method [17].

- 2) *The initiation time of induction.* Hydrostatic pressure was employed for chromosome diploidization. The eggs, at different time points postfertilization (15.5–42.5 minutes), were placed into a hydrostatic pressure machine and treated with 60–70 MPa pressure for 4–6 minutes. The diploid can be induced in the range of 21.5–24.5 minutes and the peak value arose at 21.5 minutes, so the optimum initiation time was set between 21.5 and 24.5 minutes after fertilization.
- 3) *Determination of pressure intensity and duration.* The optimal intensity and duration for hydrostatic pressure treatment was examined. Different pressure conditions (50–80 MPa) were tested and, under each pressure condition, three time points (2, 4, and 6 minutes) were examined. After the comparison of the induction rate, the optimal pressure intensity was between 65–70 MPa and the efficient duration was between 4–6 minutes.
- 4) *Homozygosity examination.* Ten pairs of SSR markers were used to test the homozygosity of 24 mitogynogenetic fry, and the homozygosity rate ranged from 73.91–87.50%, with an average value of 80.54%. The screening results by five SSR markers are shown in Figure 27.9. Because the releasing time of the second polar body showed individual differences, a small part of the heterozygote still existed, which might be derived from meiotogynogenesis.
- 5) *Confirmation of genetic sex.* AFLP marker CseF382 was used for genetic sex identification, and four embryos were identified as female among 39 mitogynogenetic embryos. To further clarify their genotypes (ZW or WW), sex-specific SSR marker CseF-SSR1 was used, and confirmed all four embryos as WW genotype.

Gynogenesis is an important technique that has been reported in fish breeding. However, most studies focused on meiotogynogenesis and freshwater fish, and only a few reports concentrated on mitogynogenesis of marine

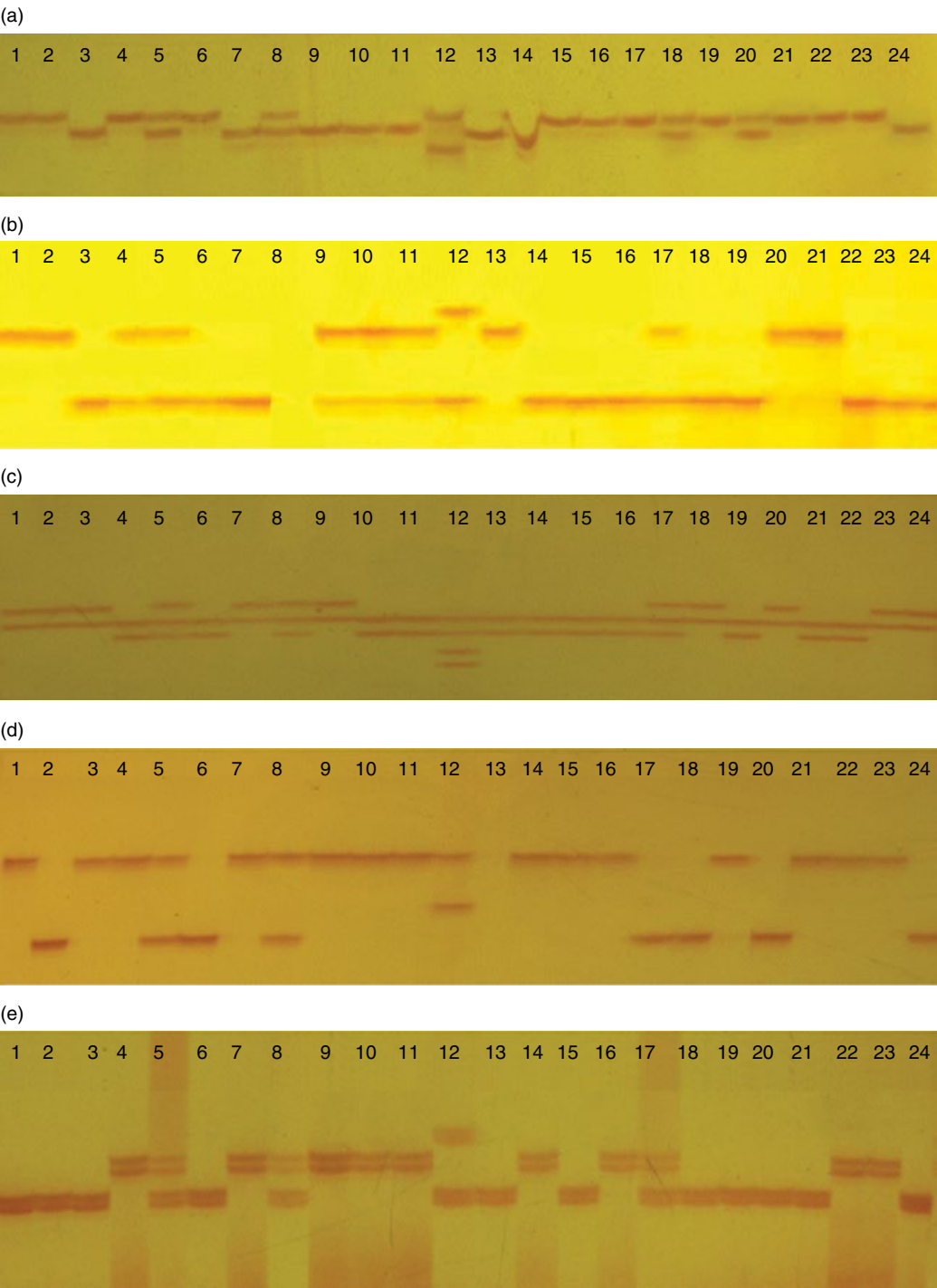


Figure 27.9 Five SSR markers were used in the analysis of mitogynogenetic half-smooth tongue sole: cyse147 (a), cys215 (b), newcys17 (c), newcys56 (d), and cyse105 (e). Cited from [17].

fish [28, 30, 31]. In addition, none of the reported gynogenesis was established on the fish that have a ZW sex determination system. Here, we developed a mitogynogenesis induction technique in half-smooth tongue sole, and successfully obtained a mitogynogenetic diploid, which is the first report of mitogynogenesis in fish of ZW genotype [17].

Compared to meiotogynogenesis, the homozygosity of mitogynogenetic tongue sole was significantly higher. Moreover, the WW embryos were detected in the embryonic stage. These data provide technical support for pure line development and sex control in tongue sole. However, there were still some points to be improved, and the induction rate was found to be low (0.19%) compared to the previous study (Japanese flounder, 34%; turbot, 10%). This might be attributed to the two following reasons:

- 1) Fish that have ZW sex chromosomes might show different characteristics during the mitogynogenetic process, compared with the XY fish.
- 2) The eggs of half-smooth tongue sole are very sensitive, which might result in the low induction rate.

With the CseF-SSR1 marker, four mitogynogenetic embryos were identified as WW genotype, indicating that WW superfemales could survive until the embryonic stage, as shown in Figure 27.4, but whether it can continue to develop needs further investigation.

27.4 Polyploid Induction

27.4.1 Technical Principle

Polyploid refers to an individual whose somatic cells have three or more chromosome sets. Polyploid production is an important breeding approach in aquaculture. For example, triploid has an imbalanced chromosome set, which usually leads to synapsis failure and incomplete gonad development. Despite being adverse to reproduction, triploids may avoid the growth arrest and

meat quality decline that usually occurs at the gonad developmental stage, so the production of all-triploid stock would benefit productivity in aquaculture. However, the triploid is sterile, so its maintenance needs tetraploid, which is used to cross with diploid to produce triploid, and can be also stabilized by self-crossing.

Since the 1970s, successful triploid and tetraploid production has been reported in many fish species, including black sea bream, Japanese flounder, rainbow trout, and so on [38–40]. Moreover, even an interspecific tetraploid was produced by red crucian carp and common carp [41]. These studies provided a solid foundation and abundant experience for developing the technique in half-smooth tongue sole. In this section, we have reviewed the triploid and tetraploid production of half-smooth tongue sole [19, 20].

27.4.2 Methods

Fish release their eggs at the metaphase of the second meiosis, and complete the fertilization *in vitro*, followed by the second polar body release and the first cleavage, so the polyploid can be induced by inhibiting the second polar body release or the first cleavage which, in turn, results in triploid and tetraploid production.

Similar hydrostatic pressure is used for polyploid induction as in mitogynogenesis, so here it is briefly summarized. For triploid, the optimal initiation time is five minutes postfertilization, and treated under 36 MPa pressure for four minutes. For tetraploid, the optimal initiation time is 21.5 minutes postfertilization and treated under 40 MPa pressure for 4.5 minutes (for both treatments, the temperature is 23°C). The resulting polyploid is analyzed by inspecting the chromosome numbers ($2n=42$). Under the optimal conditions, 100% triploid and 68.3% tetraploid rate was observed in the fry. The successful production of polyploid has laid the foundation for all-triploid stock production in half-smooth tongue sole.

Box 27.1 Summary of key information	
Type of sex determination: Half-smooth tongue sole combines genetic (GSD) and environmental sex determination (ESD). It has ZZ/ZW sex chromosomes [1, 2], and <i>dmrt1</i> has been identified as a male-determining gene. Under high temperature (28°C), the genotypic ZW female can be sex-reversed to phenotypic male, designated as pseudomale [14, 15].	Artificial gynogenesis: Techniques for meiogynogenesis and mitogynogenesis induction have been developed in half-smooth tongue sole. Cold shock is used in meiogynogenesis induction and egg is subjected to 7°C at 4.5–5.5 minutes postfertilization for 20–25 minutes. For mitogynogenesis induction, the egg is subjected to hydrostatic pressure (65–70 MPa) at 21.5–24.5 minutes postfertilization for 4–6 minutes [17, 18].
Sex-specific markers: Two types of sex-specific markers, the amplified fragment length polymorphism (AFLP) marker and simple sequence repeat (SSR) marker, were discovered in half-smooth tongue sole. AFLP is used to distinguish ZZ and ZW genotypes, while SSR is able to distinguish ZZ, ZW, and WW genotypes [16].	Polyloid induction: Triploid and tetraploid are induced by hydrostatic pressure. For triploid induction, the treatment initiates at five minutes postfertilization under 36 MPa for four minutes. For tetraploid induction, the treatment initiates at 21.5 minutes postfertilization under 40 MPa for 4.5 minutes (both treatments are under 23°C) [19, 20].

Key information about sex determination and sex control in this species is summarized in Box 27.1

27.5 Future Perspective for Sex Control in Half-Smooth Tongue Sole

In the past several years, it has been shown that the newly emerging OMICS platform can provide massive data at one time, opening a big data era in the biotechnology field. Benefitted by abundant resources, sex control technique in fish has been greatly accelerated. The OMICS data facilitates the exploration of new molecular markers, and also offers new insights into the molecular mechanism of some phenotypic phenomena – for example, the offspring has been found to exhibit a rather high male ratio, and methylome analysis has indicated that the epigenetic regulation might play an important role in this phenomenon. Offspring

maintains methylation patterns from the pseudomale father, and this epigenetic inheritance makes the offspring prone to be sex-reversed [15]. Given this, the SSR marker is used to eliminate ZW pseudomale, while ZZ males are kept for crossing. With this method, the female ratio of the offspring has been obviously improved.

Besides the traditional technique, new rising techniques also provide alternative solutions for sex control (e.g., genomic editing). Since the deciphering of the half-smooth tongue sole genome in 2014, functional analysis has identified a series of genes participating in sex determination and differentiation, such as *dmrt1*, *tesk1*, *figla*, and *neurl3* [16, 42–44], which lay foundation for in-depth functional dissection. Combined with the genomic editing technique, it would be interesting to generate the knockout individuals and check their phenotypic behavior, which will provide valuable data for understanding the mechanism and, in turn, facilitate the establishment of sex control technology.

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28

Reproduction and Sex Control in Turbot

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28.1 Introduction to Turbot Biology and Aquaculture

Turbot (*Scophthalmus maximus*) is a marine flatfish of the family *Scophthalmidae* (Order Pleuronectiformes). This species has a demersal lifestyle, inhabiting sandy or rocky bottoms in a range of 20–100 meters depth. It is characterized by a flat and almost circular body with cryptic color, which varies to imitate the substrate, allowing an effective camouflage (Figure 28.1) [1]. Turbot has a carnivorous narrow prey-spectrum diet [2], and has one of the highest growth rates among flatfish, growing about 30 cm in the first three years of life. This species is mainly distributed on the continental shelf of the northeastern Atlantic Ocean, from Morocco to the Arctic Circle, but it also occurs in the Baltic, Mediterranean, and Black Seas [3, 4].

Turbot aquaculture started in the 1970s in Scottish inland farms, and was subsequently introduced in France and Spain, becoming one of the most important farmed fish in Europe. Nowadays, this species is mainly cultured in Spain, France, and Portugal, but there are also smaller producers in the Netherlands, United Kingdom, Iceland, and Denmark [1]. In 2014, 11,067 tons of cultivated turbot were produced in Europe, Spain being the major producer, with

7,808 tons [5]. Furthermore, in the last decade, turbot has also been introduced into other countries, using imported juveniles from Europe. A turbot production of 67,000 tons in China and 107 tons in Chile was reported in 2013 [5].

Although in nature the spawning of this gonochoric species occurs between April and June in the Mediterranean region, and between May and August in the Atlantic region [6], in captivity, gametes can be obtained all year by means of controlled rearing temperatures and photoperiod [7]. However, sperm must be stripped since, in captivity, spawning does not happen spontaneously. On the other hand, females undergo ovulatory cycles with an approximate period of 70–90 hours, producing 5–10 million pelagic and spherical eggs per season [1, 6]. Larvae can be reared on semi-intensive (2–5 larvae/L) or intensive culture (15–20 larvae/L); they are initially symmetric, but at about 10–20 days post-fertilization (dpf) metamorphosis occurs, and the right eye moves to the left, producing its characteristic asymmetry [8]. During 2–3 months after hatching, fish are nursed in small tanks. Fry are then graded to avoid size dispersion, and are communally maintained in circular tanks with open-circuit pumped seawater or recirculation systems [6, 9].



Figure 28.1 The turbot (*Scophthalmus maximus*).
Photo courtesy of Jorge Fernández-Urcera.

Turbot farming productivity greatly depends on fry quality, rearing temperatures, and on how successfully the main pathogens are controlled [9, 10]. An important drawback for turbot production is size dispersion. This species shows one of the largest growth rate dimorphisms between sexes described in farmed marine fish, which also continues after sexual maturation [11]. Females grow significantly faster than males from eight months post-hatch, reaching commercial size 3–4 months before males, when they are around 20–24 months old. Furthermore, females reach sexual maturity around three years old, one year later than males. Sexual maturity results in diminished growth, higher mortality and susceptibility to diseases [12]. For these reasons, the turbot industry is interested in producing all-female populations. Thus, expanding the knowledge about sex determination and gonad differentiation mechanisms in turbot is important in order to improve production of this species.

28.2 Sex Determination in Turbot

Sex determination (SD) and sex differentiation are the basic mechanisms underlying the resulting proportion of males and females in a population (sex ratio). In turbot, measuring

Box 28.1 Genetic architecture of sex determination in turbot

Turbot shows a standard karyotype of $2n=44$ chromosomes and $NF=48$ chromosome arms, with no sex-associated chromosome heteromorphism. The main sex-determining (SD) region is located at linkage group 5 (LG5), corresponding to a ZZ/ZW system, although another three minor SD-QTLs, at LG6, LG8 and LG21, have been reported. The position of the sex-determining gene (SDg) has been estimated at 2.6 cM from SmaUSC-E30, a microsatellite that shows the strongest association to sex; hence, it has been used to develop a molecular tool for precocious sex identification. In the vicinity of this marker, several SDg candidates, such as *lhx9*, *bcar3*, and *dmrt2b*, have been detected. Furthermore, temperature is a minor factor influencing sex determination.

this population parameter is not straightforward, since sex cannot be identified until the time of maturation. Due to the higher growth rate of females, the turbot industry has promoted an active investigation to understand the sex determination mechanism in this species (Box 28.1).

The first studies on turbot SD were focused on examining putative sex-associated chromosome heteromorphisms, through cytogenetical methodologies applied on mitotic chromosomes. Conventional banding techniques, fluorochrome and restriction-endonuclease banding revealed a standard karyotype of $2n=44$ chromosomes and $NF=48$ chromosome arms, and no sex-associated heteromorphism (morphological differences between both members of a chromosome pair) was detected [13, 14]. The synaptonemal complex of spermatocytes and oocytes was also studied, to visualize the process of chromosome synapsis in the much more stretched meiotic chromosomes (11 times longer on average), but neither size heteromorphism, nor atypical bivalent pairing, was observed [15].

Chromosome set manipulation techniques and sex reversal through hormonal treatments can provide clues to the SD mechanism [16]. Cold shock treatments and DNA sperm inactivation were refined in turbot to obtain triploids and gynogenetic progenies, respectively, as potential aquaculture products [11, 17]. Also, hormonal treatments were adjusted to obtain progenies from sex reversed individuals [18, 19]. Since sex ratio in turbot is around 1 male (M): 1 female (F) [12], and no remarkable interfamilial differences are observed in sex ratio in farms (Ana Riaza, Stolt Sea Farm S. A., unpublished results), a simple genetic mechanism seemed to be operating (XX/XY or ZZ/ZW).

Cal *et al.* [20] obtained 75% and 100% females in two gynogenetic families, suggesting a XX/XY mechanism (female homogamety), since all-female offspring progenies would be expected under a XX/XY system. However, crosses between hormonal-treated gynogenetic males with normal females produced 35% males, consistent with a female heterogametic model (ZZ/ZW) [18]. Furthermore, the sex ratio of crosses of hormone sex-reversed individuals regarding untreated controls mostly fitted to a ZZ/ZW mechanism (female heterogamety) [19], although some data deviated from this model suggested that other minor genetic and environmental factors may be involved. In fact, these authors reported a certain influence of temperature on turbot SD [19].

Finally, triploid progenies consistently showed a higher proportion of females (3F : 1M), which would support a ZZ/ZW mechanism, since a 1 : 1 sex ratio would be expected under an XX/XY system, assuming similar viability of XXX and XXY offspring [21]. In a ZZ/ZW system, the higher proportion of females would be related to the distance of the SD gene (SDg) to the centromere (≈ 10 cM in turbot; [22]), which would facilitate crossing-over in the first meiotic prophase, giving rise to ZW gametes. These gametes would give rise to females, assuming the dominance of the W chromosome, thus increasing female

proportions [23]. Taken together, the hormonal sex reversal and chromosome set manipulation data discussed above were not fully conclusive, but suggested a ZZ/ZW system with other minor factors involved.

The first turbot genetic map, integrated by 242 anonymous microsatellites distributed in 26 linkage groups (LG), was reported by Bouza *et al.* [24]. The density of this map increased in the following years, reaching up to 485 markers ordered in 24 LGs [22], and integrating all previous mapping data [25–27]. Also, centromeres – key cytogenetic structures to understand meiotic segregation – were localized in the turbot map [28]. Important progress in understanding the SD architecture of turbot was achieved when the genetic map was applied to identify sex-related quantitative trait loci (QTL) in five full-sib families and to detect sex-associated markers in a wild population. The main SD region was located at the proximal end of LG5 (close to the centromere), whereas other suggestive QTLs were detected at LG6, LG8, and LG21 (Figure 28.2).

A strong sex-associated microsatellite at LG5, SmaUSC-E30 (6.0 cM from centromere), allowed correct sexing of 98.4% of the offspring in four out of the five families analyzed [23]. This marker was demonstrated to be closely linked to SDg, because it showed significant association with sex in a wild population. Furthermore, this marker revealed that the sex of progenies was dependent on the allele received from the mother, strongly supporting a ZZ/ZW mechanism [23].

Recently, this marker was revealed to be part of the *fxr1* (fragile X mental retardation 1) gene [29], which has been related to female gametogenesis in pig [30]. The SmaUSC-E30 microsatellite was used to develop a molecular tool for precocious sex identification in turbot [31], and it is currently used by industry, as detailed below in Section 28.4.5 [32].

The sex-related QTLs were later re-evaluated using a denser turbot genetic map [22], and the main sex-related QTL was confirmed at LG5, with the SmaUSC-E30 marker, the strongest associated, explaining up to 86.1%

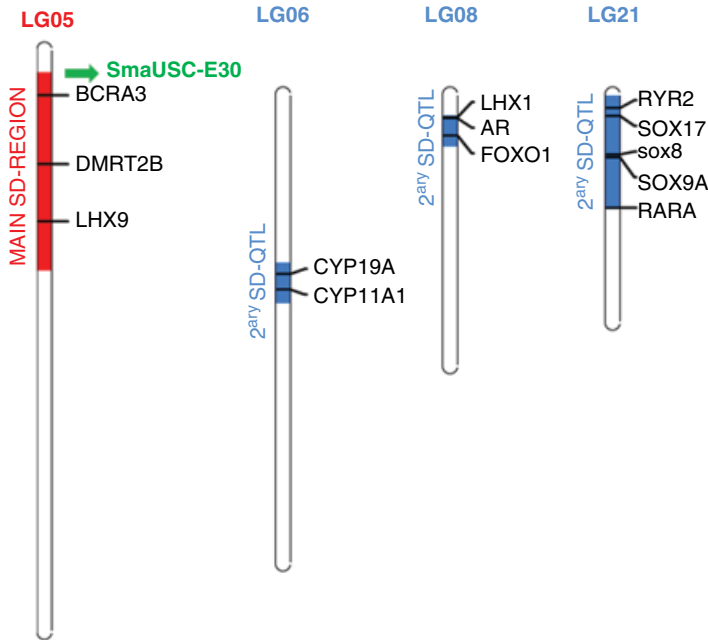


Figure 28.2 Representation of the main sex-determining region at LG5 and the minor SD-QTLs at LG6, LG8, and LG21, along with the most relevant genes detected by mining the turbot genome.

of the phenotypic variance. The number of highly associated markers largely increased at LG5, facilitating the refinement of the sexing molecular tool, and the relevance of the SD-QTLs at LG6 and LG8 was reinforced, moving from suggestive to significant level [22]. However, other data obtained from experiments testing different rearing temperatures suggested that environmental factors may also play a role in turbot SD [33], as previously suggested by Haffray *et al.* [19]. Recently, the application of a high-density genetic map (6,647 single nucleotide polymorphisms (SNP) [34] to look for sex association revealed a rather similar picture, with one major QTL and two other minor ones, although their correspondence with previous genetic maps could not be established.

Parallel research, using genomic screening without previous sequencing information, was done by applying RAPDs (Random Amplified Polymorphic DNA) to look for sex-associated markers in turbot. Two female (*SmaFe1* and *SmaFe2*) markers and one male (*SmaM1*) from pooled samples were identified using population and family data.

The combined use of these markers enabled the researchers to correctly sex 90% of males and 83.3% of females [35].

This methodology was also used to analyze sex associated markers in males and females of inbred gynogenetics, interesting material to compare ZZ vs WW individuals, at least for long stretches of the sex chromosome pair. A female RAPD marker was identified containing a microsatellite locus closely linked to *foxl2*, a key gene involved in aromatase regulation at the initial steps of gonad differentiation [36]. All these sex-associated RAPD markers were located in the turbot map, together with other SD candidate genes: *amh*, *dmrt2*, *cyp19a1a*, *cyp19a1b*, and several *sox* genes, a family including transcription factors involved in sex determination and differentiation, but none of them was positioned at the main SD region [36, 37]. Nonetheless, some of these genes were located within the confidence interval of the minor SD-QTLs: *cyp19a1b* at LG6, and *sox9* and *sox17* at LG21 [37].

Since none of the SD candidates previously tested was located at the main SD region, a

fine mapping approach was carried out in order to identify other sex-related genes in this region. The position of the SDg had been estimated to be at 2.6 cM from SmaUSC-E30 by Martínez *et al.* [23]. Based on comparative mapping with model fish, six genes were identified and successfully mapped to that region, including *sox2*, a strong SDg candidate previously related to gonad differentiation [38, 39]. Unfortunately, none of these candidates showed association with sex at population level, being discarded as the turbot main SDg [29].

However, this approach allowed increasing marker density on this region, enabling researchers to refine the genomic position of the SDg (between 10.1 and 16.3 cM), and to get new insights on the turbot SD system [29]. Thus, the few discordances observed between genetic and phenotypic sex corresponded to males with a female genotype, suggesting incomplete dominance of a new ZZ/ZW SD system [29].

The recent origin of the turbot SD system was also supported by the apparent lack of recombination suppression between Z and W chromosomes, and the existence of a well-established XX/XY system at LG21 with male suppressed recombination in the brill (*Scophthalmus rhombus*), a congeneric species that will hybridize with turbot [29]. The available data so far suggest an ancient XX/XY mechanism in the genus *Scophthalmus* that would have recently shifted towards a ZZ/ZW in the turbot, similarly to what has been reported in other fish groups [40]. Nevertheless, this hypothesis should be contrasted with additional data.

The turbot genome was recently sequenced, enabling a more refined analysis of the SD region through gene mining around the SD-QTL [41]. This approach identified suggestive candidates at the main SD region, such as *lhx9*, *bcar3*, and *dmrt2b*. *Lhx9* was reported to be involved on mouse gonad formation [42, 43]. *Dmrt2b*, on the other hand, is a member of the Doublesex and mab-3 related transcription factor family which, along with *dmrt1*, has been involved in sex determination in different vertebrates [44]. Furthermore, multiple

genes involved in sex differentiation were identified within the confidence intervals of the secondary SD-QTL: *cyp19a1b* and *cyp11a1* at LG6; *ar*, *lhx1*, and *foxo1* at LG8; and *ryr2a*, *sox17*, *sox8*, *sox9*, and *rara* at LG21 (Figure 28.2). Several of these genes, such as *ar* and some members of the *sox* family, have been extensively studied in fish, because of their involvement in gonad differentiation processes [45–49].

28.3 Sex Differentiation in Turbot

Nowadays, sex determination and sex differentiation are not viewed as two strictly separated processes, especially in those taxa with unstable sex determination systems, like fish [32, 50, 51]. Instead, sex in many species is currently regarded as a quantitative threshold trait mimicking a single gene effect, established by a network of different interacting genetic and environmental factors at the beginning of gonad development. Therefore, studying this network of interactions during early sex differentiation is critical for understanding how sex is established.

In turbot, female and male gonads are histologically distinguishable around 100 dpf (≈ 7 cm) so, hence, sex must be established earlier (Box 28.2). The first gene expression change along turbot gonad development is

Box 28.2 Main features of gonad differentiation in turbot

One of the first changes denoting the start of gonad development in turbot is the increased expression of *gsdf* between 60–75 days post-fertilization (dpf). Global gene expression analyses indicate 75–90 dpf as the time corresponding to the onset of sex differentiation which, histologically, is first observed at ≈ 100 dpf in fish of ≈ 7 cm total length. At 90 dpf, analysis of the combined expression of just three genes – *cyp19a1a*, *vasa*, and *amh* – allows > 90% correct sexing of juveniles

an increase of *gsdf* (gonadal somatic cell derived factor) between 60 and 75 dpf [33]. This gene is involved in early sex differentiation in several teleost species, acting directly downstream of the SD mechanism in medaka (*Oryzias latipes* [52]) or Nile tilapia (*Oreochromis niloticus* [53]). Moreover, *gsdf* is the sex-determining gene in the Indian ricefish, *Oryzias dancena* [54]. However, *gsdf* does not show dimorphic gene expression during early gonad development in turbot; it seems to have a role in this process, but not in SD in this species.

The first genetic differences between male and female gonad differentiation are observed at 90 dpf, the following assayed time point and, therefore, this period (75–90 dpf) is considered the onset of sex differentiation, and critical to understand how the fate of the gonad is determined. At this stage, differential expression between sexes is observed for three genes: *cyp19a1a* and *vasa* in females; and *amh* in males [33]. The expression of these three genes alone was found to be able to correctly sex about 90% of the fish at 90 dpf. Also, the expression of these three genes, along with that of *gsdf*, significantly increased in both sexes from 75 to 90 dpf.

The connection between these genetic factors seems to be the germ cells, key in early sex differentiation and, maybe, even in SD in fish. The presence of a sufficient number of these cells at a given point in development has been reported to be critical for female sex determination in species like zebrafish (*Danio rerio*) [55], medaka [56], stickleback (*Gasterosteus aculeatus*) [57], and Prussian carp (*Carassius gibelio*) [58]. Thus, in general, a higher number of germ cells are associated, during early gonad development, with ovarian differentiation in teleosts [59, 60].

Gsdf is supposed to have a proliferating effect over germ cells and, in fact, one of the other genes, *vasa*, is a germ cell marker, which supports an increase of germ cells between 75–90 dpf [33]. Despite the fact that *gsdf* does not show a dimorphic expression, somehow germ cells seem to proliferate faster in females, as suggested by the higher gene expression of *vasa* at 90 dpf in this sex.

A possible explanation is that *amh* (antimüllerian hormone), more expressed in males at 90 dpf, inhibits germ cell proliferation in this sex. In fact, *amhY*, a copy of *amh*, is the sex-determining gene in the Nile tilapia [61] and the Patagonian pejerrey (*Odontesthes hatcheri*) [62] and, in the latter, it is hypothesized to regulate germ cell proliferation determining male sex differentiation.

Further, a receptor of *amhrII* is the sex determination gene in fugu (*Fugu rubripes*), where females have a defective version of this receptor, making them insensitive to *amh* [63]. The loss of function of *amhrII* in medaka by mutation has been shown to cause an excessive proliferation of germ cells and male-to-female sex reversal [64]. Therefore, there appears to be enough evidence to link the role of *amh* with the inhibition of germ cell proliferation in fish.

The balance between germ cell proliferators and inhibitors in this network at the onset of sex differentiation seems to be critical for the establishment of a male or female phenotype in fish. Germ cells have been involved in the maintenance of *cyp19a1a* expression in zebrafish female gonads [55]. This gene catalyzes a key step at the onset of gonad differentiation, transforming androgens into estrogens and, thus, it is regarded as the main feminization factor along all vertebrate taxa.

In summary, the absence of *amh* expression would result in a large number of germ cells which, in turn, would maintain high levels of *cyp19a1a*, leading to the development of a female gonad. Conversely, a high expression of *amh* would inhibit germ cell proliferation and the subsequent downregulation of *cyp19a1a*, leading to the development of a male gonad. Understanding this network of early differentiation and the regulation of the factors involved is essential to figuring out sex determination in teleosts and, as outlined above, in turbot.

The onset of sex determination between 75 and 90 dpf in turbot is supported by both qPCR [33] and microarray analyses [65]. Gonads remain mostly undifferentiated between 75 and 90 dpf but, after that (105 dpf

onwards), gonads develop rapidly and the differences between males and females rapidly increase. Expression analyses, using microarrays ranging from undifferentiated (75 dpf) to juvenile turbot (485 dpf), suggest that, after 90 dpf, turbot gonads differentiate towards an “in differentiation” male-like stage, genetically very similar to adult male gonads. Conversely, females rapidly abandon this male-like stage, exhibiting strong signals of female differentiation even at 135 dpf in some cases [65], and revealing female gonad development sooner (≈ 5 cm in total length in females vs ≈ 10 cm in males).

However, this study also showed that individuals of the same sex, age, and size could show different gonad developmental stages, which could be the result of genetic variation underlying the onset, or the rhythm, of sex differentiation. This is an interesting observation, since delaying sex differentiation could improve production by avoiding the undesired effects of gonad maturation (decreased growth or poorer flesh quality).

These studies, using qPCR and microarrays, revealed the involvement of many genes in turbot sex differentiation for the first time, many of them already studied in other species (*foxl2*, *ctnnb1*, *sox19*, more expressed in females, or *sox9*, *sox8*, *dmrt3* in males). Also, new insights were obtained about the gonad development process, including the involvement of epigenetic and splicing mechanisms, or the role of some immune related genes like the interferon regulatory factor 7, upregulated in males [33, 65]. Further, in the microarray study, 56 genes not previously related to sex differentiation in fish were associated for the first time to female (44) or male (12) gonad development [65].

The differences between mature male and female gonads in turbot (and also in other organs like brain and liver) were first studied using cDNA-AFLPs (Amplified Fragment Length Polymorphism) and qPCR [66] and, more recently, other studies using RNA-seq have been published [67, 68]. RNA-seq enabled the identification of *dmrt1* for the first time in this species, being upregulated in males, while other members of the *dmrt* family, like *dmrt2*

or *dmrt5*, were more expressed in females [68]. *Dmrt1* has been verified as a male sex determination gene in Chinese tongue sole (*Cynoglossus semilaevis*), the other flatfish species with a sequenced genome [69]. A paralogue of *dmrt1*, *dmY*, is the sex-determining gene in medaka, the first reported SDg in fish [70], and is directly involved in the regulation of germ cell proliferation. Future work on this gene could shed light on early sex differentiation and the establishment of phenotypic sex in turbot (Figure 28.3).

28.4 Sex Control in Turbot

Turbot exhibits sexual dimorphic growth, females outgrowing males from eight months post-hatching onwards. The resulting size dispersion represents a drawback for turbot production. In addition, females achieve sexual maturity later, delaying the drawbacks associated to maturation [12]. Thus, development of techniques for obtaining all-female populations will maximize the profit of turbot aquaculture [32]. For this reason, besides understanding the genetic basis of sex determination, several other approaches have been carried out to test the effects of temperature, hormone, and chromosome manipulation on turbot, with the aim of controlling sex ratio. All the information gathered on the factors influencing sexual development, the timing of gonadal development, and the labile period, will facilitate the effectiveness of these treatments.

28.4.1 Environmental Control

Environmental factors can play an important role in sex determination. The environment can interact with genetic factors during early sex differentiation, modifying its expression which could, in turn, change the balance of the sex differentiation network toward the production of males or females. Although several environmental variables can potentially disturb sex ratios (i.e., salinity, pH, stocking density, or social factors), the most important one is temperature which, so far, is the only one reported to have some effects on turbot sex determination [71].

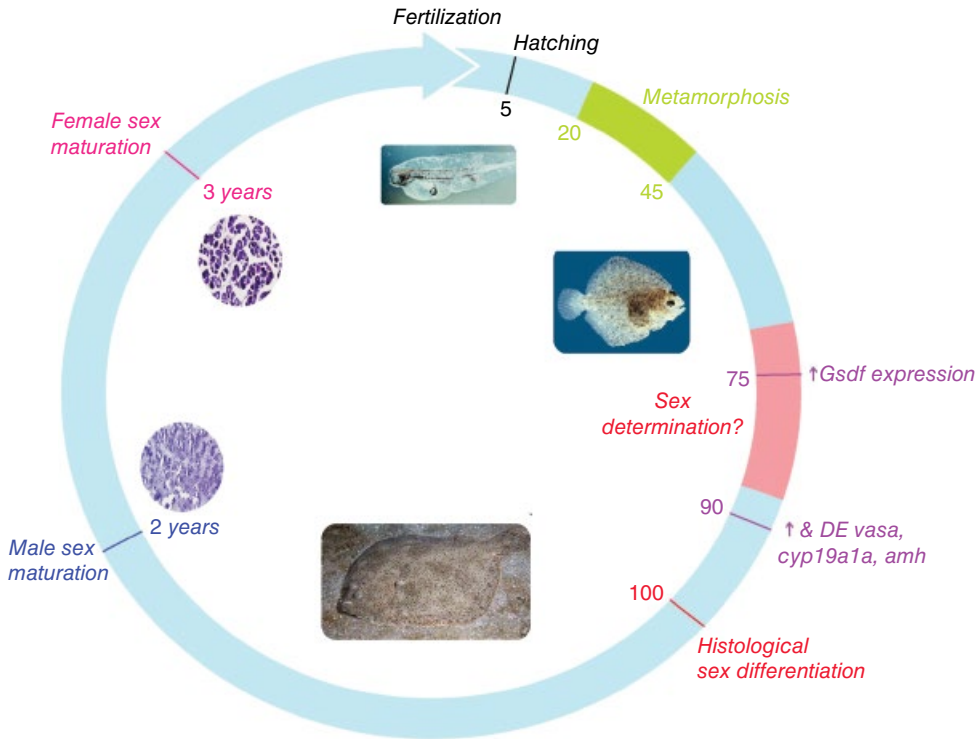


Figure 28.3 Representation of the main genetic, histological, and physiological events during sexual differentiation in turbot.

In a first study using eight different turbot families, two families showed male-biased sex ratios, but the effects of temperature were not consistent across families, suggesting the existence of genetic-by-environment (temperature) interactions, the higher proportion of males being obtained at high or low temperatures, depending on the family [19]. A recent study using 15 families showed that, overall, low temperatures ($\approx 15^{\circ}\text{C}$) have a feminizing effect, while high temperatures ($\approx 21^{\circ}\text{C}$) have a masculinizing one (Hernández-Urcera *et al.*, manuscript in preparation), and this could be explained by the upregulation of *ctnnb1* at lower rearing temperatures [33].

However, this general trend was not always observed and, as in the previous study by Haffray *et al.* [19], genetic-by-environment interactions seem to occur; temperature effects are dependent on the family and, hence, are determined by the genetic

background. Yet, when all the families are considered as a single population, the effect of temperature on sex ratio is significant, and lower temperatures could be used in farms to increase the proportion of female offspring. Importantly, temperature treatments were carried out from the end of the metamorphosis period (≈ 30 dpf) until the appearance of the first morphological differences between male and female gonads (≈ 100 dpf) – that is, including the sensitive period where the gonadal fate is determined (Hernández-Urcera *et al.*, manuscript in preparation).

Lower temperatures reduce growth rate of the fish. However, when low temperature-reared fish are returned to normal temperatures, a compensatory growth takes place, resulting in no significant differences at the time of marketing, as has been observed in fish [40]. This seems to occur in turbot (Hernández-Urcera *et al.*, manuscript in preparation).

In an effort to understand the effects of temperature, gene expression was studied in a family showing a higher proportion of females at low temperature. In this family, genetic males (tested with the SmaUSC-E30 marker tool) became phenotypic females more frequently as temperature decreased. Several genes showed differential expression, depending on rearing-temperature. In this study, the most interesting finding was the expression increase of *ctnnb1* in males as temperature decreased [33]. This gene is at the bottom of the *wnt* pathway [72], and is critical for female sex development in many vertebrate species. Higher expression of *ctnnb1* has also been related to female-biased sex ratios in oyster (*Crassostrea gigas*; [73]). Therefore, *ctnnb1* and the *wnt* pathway are good candidates to explain female-biased sex ratios in turbot and in other species.

The effects of other environmental factors on sex ratio have not been explored in turbot although, for example, those related to stocking density or social status do not seem to be operating in this species, since there is a good correspondence between genetic and phenotypic sex at farming conditions.

28.4.2 Hormone Treatments

Administration of steroid hormones is the most widespread method for changing sex ratio, due to its straightforward application at a commercial scale and its consistency for producing monosex populations [74]. Although control of sex differentiation through the

administration of hormones has been achieved in a wide variety of fish, the type and timing of these treatments vary largely between species. Usually, fish are much more sensitive to the effects of steroid treatment in the labile period, when the gonad is still undifferentiated [75, 76]. As mentioned above, turbot gonads remain mostly undifferentiated up to 75–90 dpf, and female differentiation usually precedes that of males [33, 65].

Data about hormonal treatments for sex reversal in turbot are still scarce, being limited to research studies focused on the analysis of the SD system. The first reports proposed 17 β -estradiol treatment supplied through diet for feminization [76]. Some years later, Baynes *et al.* [18] reported sex reversion of gynogenetic fish to fertilize normal females. They concluded that the most effective treatment begins approximately 800–1,000 day-degrees (d.d.) post-hatch, after moving the fish from live feeding to an inert diet. The fish reared at 12°C were fed with a diet containing methyltestosterone at a concentration of 1 mg/kg of food over a period of 400 d.d. (Table 28.1) [18].

Haffray *et al.* [19] produced androgen-treated males (ATM) and estrogen-treated females (OTF) to analyze the sex ratio in several families obtained by crossing control parents with either ATM or OTF reversal parents. ATM and OTM were produced with oral treatments of 17 α -methyltestosterone and 17 β -oestradiol, respectively (Table 28.1). Treatments consisting of 3 mg/kg concentration of feed during 500 d.d. (day-degrees [°C])

Table 28.1 Hormonal treatments for sex reversal in turbot.

Purpose	Steroid and concentration	Timing and duration ¹	Rearing temperature	References
Masculinization	17 α -methyltestosterone, 1 mg/kg of feed	800–1,000 d.d. post-hatch, 400 d.d.	12°C	[18]
	17 α -methyltestosterone, 3 mg/kg of feed	35 dpf, 500 d.d.	13.5–15.0°C	[19]
Feminization	17 β -estradiol, 3 mg/kg of feed	35 dpf, 500 d.d.	13.5–15.0°C	[19]

¹ d.d. – day-degrees (°C).

at a rearing temperature between 13.5°C and 15°C were the most efficient for producing 100% sex reversal. Maturation of treated animals tested at three and five years was very similar to controls [19].

In Europe, the application of hormones to commercial grow-out food fish is prohibited, so their use is limited to sex reversal of brood fish. Steroids are permitted for sex control during early development of fish in the legislation of many countries, but this practice sometimes provokes consumer rejection [74, 75]. In turbot, hormonal treatments have been used for the production of neomales (phenotypic males with genetic female constitution), in order obtain all-female progenies, as detailed below [76].

28.4.3 Triploidy

Triploids are individuals containing three chromosome sets, usually two from the mother and one from the father. Induction of triploidy in the turbot was developed in order to obtain sterile populations and, thus, to avoid the undesirable effects associated with sexual maturation. Sexual maturation usually results in lower growth rate, higher incidence of diseases, and changes in the organoleptic properties of the edible parts [74, 77]. In turbot, triploidy was first induced applying hot thermal shock (25–31°C) to fertilized eggs [78], but survival rate was very low.

Later, Piferrer *et al.* [17, 79] accomplished survival rates of ~60% regarding untreated controls, and achieved triploidy rates higher than 90% on average across families. This protocol was based on cold shocks between –1 and 0°C, applied 5–6.5 minutes after fertilization, for 20–25 minutes (Table 28.2) [17, 79]. Triploidy validation in turbot has been traditionally performed by chromosome counting ($2n = 44$; [13, 17]), nucleoli counting [17], erythrocyte size measurement [79], and flow cytometry [80]. All these techniques are invasive and show different limitations but, recently, a simple method based on microsatellite markers, selected

by their high polymorphism and distance to centromeres, demonstrated 100% accuracy and can be performed on a very small piece of tissue [81].

Triploidy skews the sex ratio in turbot, increasing the proportion of females. Cal *et al.* [21] reported a ratio 1 M : 3 F for triploids, significantly different from the 1 M : 1 F usually observed in diploids. In turbot, the higher female proportion in triploids represents an additional advantage to their sterile condition since, as outlined before, females largely outgrow males.

Growth, survival, and gonadal development of triploid turbot were also analyzed to evaluate their performance for aquaculture production [21]. Although growth was similar for both ploidies during the juvenile phase and puberty, adult triploids grew significantly more than diploids, with marked differences after each spawning season due to energy redirection from gametogenesis to somatic growth. On average, triploids exhibited, on average, a weight 11.4% higher than diploids from 24–48 months of age; a survival 8% higher than diploids, due to the lack of post-spawning mortality; and the gonads were significantly smaller and rudimentary than in diploids, demonstrating functional sterility [21].

Recently, other studies have compared gross body morphology and skeleton characteristics [82], as well as muscle hardness at post-mortem stages [83], and no differences were observed between turbot triploid and diploid full-sibs. The good performance of turbot triploids represents an opportunity to improve production and, additionally, their sterility makes them useful for avoiding the impact of farming on wild populations from escapees or intentional releases, which could compromise the viability of natural resources.

28.4.4 Gynogenesis

Gynogenesis was investigated in turbot to ascertain the mechanism of sex determination, and to evaluate its usefulness for

obtaining all-female populations in commercial farming. Gynogenesis in turbot was induced by activating eggs with its own UV-inactivated DNA sperm (homologous fertilization) [11, 20, 84], or using the sperm from other species (heterologous fertilization), such as Atlantic halibut (*Hippoglossus hippoglossus*) [85], Japanese flounder (*Paralichthys olivaceus*) [86], and the red sea bream (*Pagrus major*) [87].

To make gynogenetic embryos viable, diploidy is restored by blocking the extrusion of the second polar body (meiotic gynogenesis), or by blocking the first embryonic cleavage (mitotic gynogenesis) through thermal or pressure shocks. The parameters used for obtaining gynogenetics in turbot are detailed in Table 28.2. As in other species, diploid gynogenetics are not useful for turbot production because of their low viability. Turbot gynogenetics show reduced hatch rate and survival during the first year [11, 20, 84, 87].

However, thereafter, and up to three years, their viability is similar to that of untreated controls [21].

The decreased viability of gynogenetics is in part related to handling and treatments of eggs in a species which, additionally, shows low larval viability [9], and also to a high degree of inbreeding, resulting in gynogenesis. Inbreeding increases the opportunity for the manifestation of lethal recessive alleles, as previously demonstrated in turbot [28]. Accordingly, mitogynogenetics ($F=1$) show a much lower hatching rate and a higher proportion of abnormalities than meiogynogenetics ($F \approx 0.33$) [87].

Validation of the gynogenetic condition in turbot has been usually performed using microsatellite markers, by confirming their exclusive maternal inheritance [88]. Alternatively, gynogenesis has been verified by karyotype analysis, counting NORs

Table 28.2 Experimental conditions for obtaining triploid and gynogenetic turbot.

	Sperm inactivation ¹	Shock conditions ²	Results ³	References
Triploids	No	cold shock: 0°C, 20 min, 5 maf	90% 3n, 80%	[17]
	No	cold shock: -1 to 0°C, 25 min, 6.5 maf	95–100% 3n, 60%	[75]
Meiogynogenetics	Scophthalmus maximus sperm, UV, 30,000 erg mm ⁻² , 1 : 10	cold shock: -1 to 0°C, 25 min, 6.5 maf	100% G2n, \approx 10% at 6 months and \approx 90% from 9–36 months	[11, 20]
	Paralichthys olivaceus sperm, UV, 36,000 erg mm ⁻² , 1 : 50	cold shock: 1°C, 25 min, 6 maf	39.58% G2n, \approx 30% at 8 dph	[82]
Mitogynogenetics	Pagrus major sperm, UV, 6480–7200 erg mm ⁻² , 1 : 20	hydrostatic pressure shock: 75 MPa, 6 min, 85–90 maf	\approx 1.46% G2n, \approx 18% since 1 until 40 dah and \approx 62% from 40–60 dph	[83]

¹ Indicated are: the species of sperm origin, the type of radiation, the dose used, and the dilution of sperm.

² Indicated are: the type of shock, its intensity, duration, and time of start.

³ Indicated are: the yield in triploids (3n) or gynogenetic diploids (G2n) and percent survival relative to diploid controls. dph – days post-hatch; maf – minutes after fertilization; Mpa – MegaPascal.

(nucleolus organizer regions) after silver staining banding, and by flow cytometry [11, 86, 87]. In turbot, both meiotic [11, 20, 86] and mitotic [87] gynogenetics have been obtained.

The exclusive female constitution of gynogenetic genomes provides valuable information to study the SD system. In all studies with turbot meiogynogenetics, a higher proportion of females were obtained in the families analyzed: 75% and 100% [20]; 69% and 90% [18]. As in triploids, deviation from the 1 : 1 sex ratio is likely related to the occurrence of crossing-over between the SDg and the centromere, producing ZW individuals and, thus, increasing the frequency of females.

Mitogynogenetics represents an interesting material for investigating the SD genetic mechanism, because fully homozygous ZZ and WW individuals are produced, facilitating the sequencing and assembly of both chromosomes of the sexual pair for identifying genetic differences associated with sex. Gynogenetics have also been used for research purposes and, for instance, the first genetic map was constructed using an haploid family, because each embryo represents a meiotic product, thus facilitating the analysis of linkage [24]. Also, diploid gynogenetics were used to locate centromeres in the turbot map, by using half-tetrad analysis [28].

28.4.5 Molecular Tool for Sex Identification

Nowadays, the technology for obtaining all-female turbot juveniles is supported by the identification of the genetic sex using the microsatellite SmaUSC-E30. As mentioned above, this marker allows a sexing efficiency of $\approx 98\%$ and explains 86.1% of the phenotypic variance [22, 23]. Accordingly, it has been used to develop a molecular tool for precocious sex identification in turbot [32]. Since sex cannot be identified in this species until maturation, this tool is used in breeding programs to

identify the sex of selected individuals, as well as to facilitate the production of all-female populations.

Since females are the heterogametic sex in turbot (ZZ/ZW system), a three-generation pedigree is required to obtain all-female populations (Figure 28.4). In the first cross, neomales are produced by methyltestosterone treatment. The cross of these individuals with normal females enables superfemales (WW) to be obtained. In a final step, these superfemales are crossed with normal ZZ males, producing the desired all-female progeny.

The difficulty of this protocol relies on the progeny tests required to identify the ZW neomales among the hormone-treated larvae (ZZ or ZW), and the WW superfemales among the female offspring in cross II (ZW or WW) [32]. This procedure is extremely laborious, since the analysis of sex ratio in the progenies requires waiting until fish maturation – at least two or three years in turbot. Furthermore, sex can only be visually identified 4–6 months after hatching in sacrificed offspring.

For this reason, the development of a molecular tool for precocious sex identification has represented a major breakthrough for turbot production. In this way, genotypic sex can be assessed after 4–6 months, by simply obtaining a fin clip for DNA extraction – a non-invasive procedure for the animal. This methodology allows saving a minimum of five years for the production of all-female progenies [32]. However, this tool still presents some limitations, since production of 100% females is not always achieved, because of other minor genetic and environmental factors affecting turbot sex. Furthermore, since SmaUSC-E30 is a marker linked to the SDg but is not sex-specific, it is necessary to know its association with sex for each family, following a marker-assisted selection (MAS) strategy. The distance between the marker and SDg also makes feasible crossovers between them breaking association.

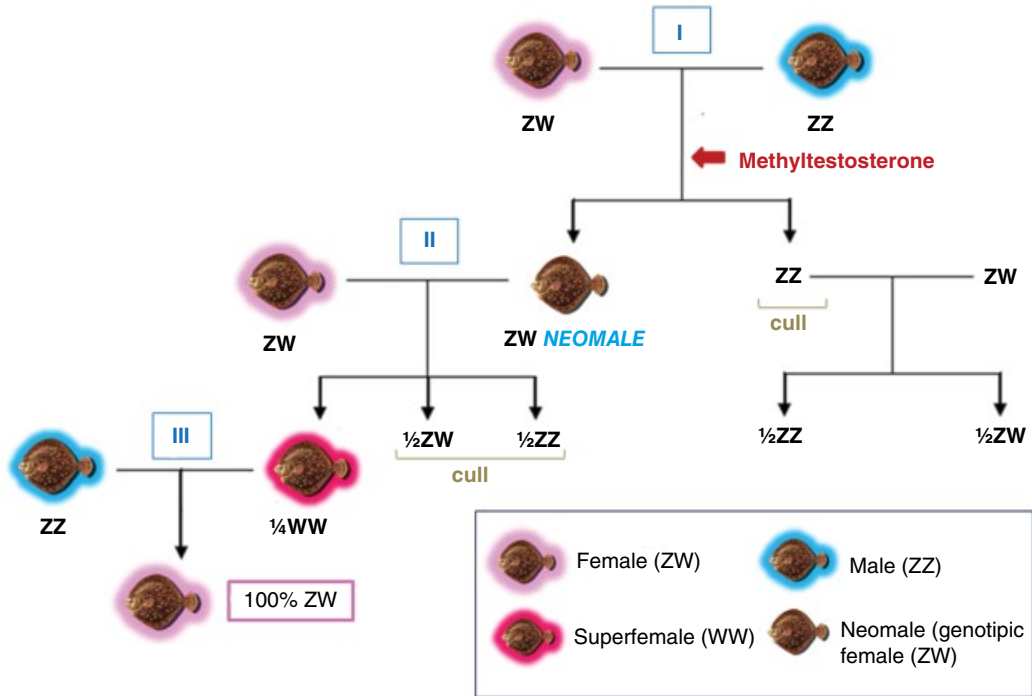


Figure 28.4 Scheme for obtaining all-female populations in turbot.

28.5 Concluding Remarks and Future Perspectives

A great advance has been achieved on sex determination and differentiation in turbot, as well as on their application for sex control in farms. However, the final identification of the SDg remains elusive, likely because genetic differences between males and females at this region are very small, and no recombination suppression occurs, as corresponds to a recent SD system. In a ZZ/ZW system like that of turbot, 100% females can only be obtained by hormone treatments and through a three-generations pedigree.

The molecular tool developed for precocious sex identification in turbot has greatly speeded up this process. Although this methodology is being used by turbot companies, with encouraging results, some limitations still remain. In order to improve this methodology, or to develop hormone-free methods to produce monosex populations for turbot culture, the new genetic markers

developed in the SD region are being incorporated into the molecular tool, to increase the number of available informative markers. It should be stressed that, with this strategy, turbot used for marketing have never been exposed to steroid hormones.

Currently, several ongoing projects are trying to elucidate the architecture of the SD region and to identify the SDg in turbot. A RAD-seq screening is being performed on a high number of families (≈ 40) by genotyping $\approx 25,000$ SNPs. Also, WW superfemales obtained through hormone sex reversal parents are being sequenced and compared with normal ZZ males, using the turbot genome as reference, in order to identify the SDg and the underlying SD mechanism of turbot. If finally achieved, this would be one of the first fish species with a ZZ/ZW mechanism whose master gene is identified and, additionally, it would represent a new scenario for appropriate managing of wild and cultured stocks, and for producing all-female populations by industry.

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Sex Control in Southern and Summer Flounder

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29.1 Introduction

29.1.1 Life Histories of Southern and Summer Flounder

Southern flounder (*Paralichthys lethostigma*) and summer flounder (*Paralichthys dentatus*) are high-value flatfishes native to the coast of North America. Southern flounder inhabit rivers, estuaries, and ocean waters along the mid-Atlantic and Gulf coasts of the United States, from approximately North Carolina to northern Mexico, while summer flounder inhabit estuarine and shelf waters along the entire Atlantic coast of the United States [1–3] (Figure 29.1). Ranges of these flounder species overlap between North Carolina and Southeast Florida. They are typically distinguished by five distinct oscillated spots on the dorsal (pigmented) side of summer flounder, and more diffuse spots and blotches in southern flounder [2].

Adult flounder migrate from estuaries during the fall and winter to spawn in ocean waters. The reproductive strategies of southern and summer flounder are similar. The spawning season typically begins in December, with fish first spawning in the northern limits of their natural range of the

Atlantic Ocean, and by late January to February in the southern limits of their range [1, 2]. This timing is accurate for both species of flounder on the Atlantic coast, although southern flounder that reside in the Gulf of Mexico typically begin spawning earlier (October–December). Additionally, instead of migrating from estuaries to the ocean, these southern flounder move out into Gulf waters to spawn [3, 4]. Immediately after spawning, adults typically return to coastal estuaries and rivers.

Flounder eggs are buoyant, and will float for 2–3 days until hatching is complete. Larval flounder feed on zooplankton in off-shore waters for 30–60 days, and then undergo metamorphosis before migrating into rivers and eventually settling into estuarine nurseries [5, 6]. Controlled studies indicate that 50 day old juvenile southern flounder can tolerate salinities as low as 5 ppt, while older juveniles can even tolerate fresh water [7]. During metamorphosis, one eye of the flounder will migrate to join the other eye so, when complete, they will both be located on the same side of the head. Southern and summer flounder are considered “left-eyed” or “left-sided” flounder since, following metamorphosis, both of

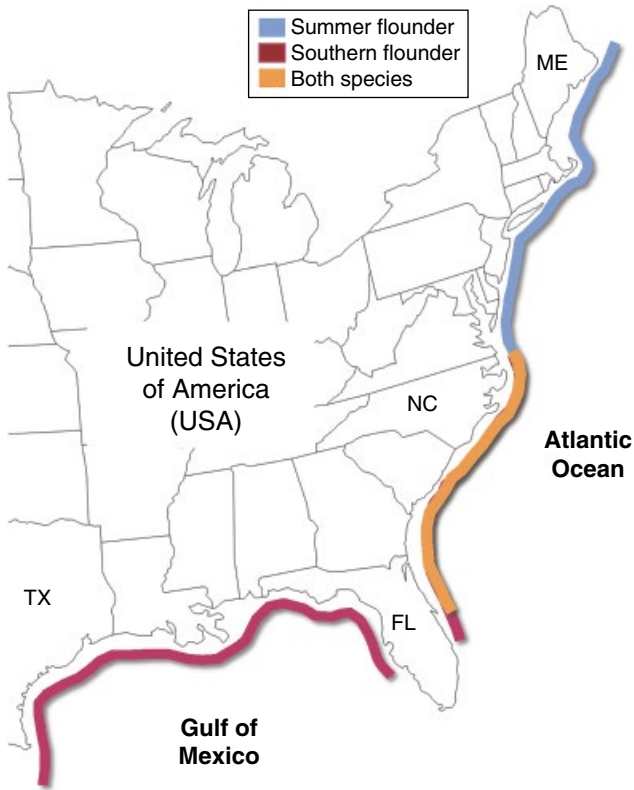


Figure 29.1 Primary ranges of southern flounder (*Paralichthys lethostigma*) and summer flounder (*Paralichthys dentatus*). Ranges of summer and southern flounder overlap along the western Atlantic coast for the United States from approximately North Carolina (NC) to Florida (FL). Map of the eastern United States obtained from: <http://d-maps.com/>

their eyes are on the left side of their head. This feature of the flounder development is a valuable adaptation for a benthic life.

29.1.2 Commercial Aquaculture

Declining natural populations, and wide temperature and salinity tolerances of juveniles and adults, make both southern and summer flounder versatile candidates for intensive culture in inland, as well as in coastal, areas of the Southeastern United States or other countries [8]. Also, advances in commercial cultivation of other flatfishes, such as Japanese flounder (*Paralichthys olivaceus*) in Asia [9, 10], and turbot (*Scophthalmus maximus*) in Europe [11], continue to stimulate interest in commercial production of other flatfish species [12]. However, the economic feasibility of culturing southern and summer flounder is yet to be fully demonstrated.

There have been attempts to transfer culture technologies for these species to

growers in the United States, although there is currently little to no commercial production [13, 14]. The same holds true in China, where these species were introduced for aquaculture research and development around 2002. Currently, there are only a small number of farms producing these species in China.

There is still ecological and economic demand for southern and summer flounder fisheries and, as of 2015, southern flounder were updated to “near threatened” on the IUCN Red List. This could ultimately lead to more interest in aquaculture for production and/or stock enhancement as populations continue to decline.

29.1.3 Sexually Dimorphic Growth

Typical of flounder of the genus *Paralichthys*, southern and summer flounder exhibit sexually dimorphic growth, where females grow 2–3 times larger in size than males [15, 16]. For summer flounder, the age at which

sexually dimorphic growth is first observed is 12 months [15]. Because of minimum size restrictions on commercial harvest of southern flounder, the majority of harvested flounder are female, and are usually caught within the first three years of life [16]. Male southern flounder, in fact, rarely attain body sizes greater than the current minimum size restriction of 350 mm in North Carolina.

Growth rate is one of the major factors influencing profitability of flounder aquaculture, so cost and time associated with grow-out to harvest size must be reduced to gain competitiveness in the global marketplace [15]. Towards this end, research efforts have focused on producing all-female southern and summer flounder stocks, thereby eliminating production of slower-growing males [17].

29.1.4 Sex Determination

Flatfishes exhibit either genotypic sex determination (GSD) or a combination of genotypic and environmental sex determination (ESD) [18, 19]. Among most flatfishes, including Paralichthids, GSD tends to follow the XX/XY sex chromosome system, albeit that evidence indicates a ZW/ZZ system may occur in some species (for review see [18, 19]). Like their congener, the Japanese or olive flounder (*P. olivaceus*), southern and summer flounder have been demonstrated to exhibit ESD [18]. Interestingly, this phenomenon appears to be limited to the XX genotype, where factors in the external environment (not limited to temperature) can influence sex ratios.

If conditions are suboptimal for development of a particular sex and fitness differs between sexes, then it is advantageous to have mechanisms to alter phenotypic sex [20]. Studies have shown that individuals are sensitive to temperature extremes, tank color, and exogenous cortisol when exposed during the critical period of sex determination and differentiation ([18, 21]; and see also relevant sections below). Although XX flounder are genotypically female, they can become sex-reversed and develop as phenotypic males. Meanwhile, the XY geno-

type does not appear to be influenced by environmental factors, and all individuals of this genotype develop as phenotypic males.

Here we discuss sex determination, sex differentiation, and methods for controlling sex ratios of southern and summer flounder, with a goal of improving production of these species for aquaculture.

29.2 Larval Development and Sex Differentiation

29.2.1 Embryonic and Larval Development of Southern Flounder

Based on studies in the United States and China, when water temperature and salinity are maintained at 16–19°C and 32–33 ppt, respectively, southern flounder larvae exhibit melanophores by 36 h and hatch at 48–55 hours post-fertilization [22, 23]. By 4 dph, the yolk sac has been completely absorbed and the larval mouth is open. By 14 dph, the total length (TL) is 5.15 ± 0.50 mm, and melanophores have spread across the body. By 20 dph, the TL is 6.17 ± 0.65 mm, and a crown-like dorsal fin has typically appeared. Metamorphosis begins on day 26 and is completed by ≈ 45 dph. By 50 dph, the TL is 16.42 ± 2.35 mm, and there is no significant difference in morphology relative to adults, except for the pigmentation, which is lighter than that of adults [22].

29.2.2 Sex Differentiation and Sexual Maturity of Southern Flounder

The process of sex determination and differentiation varies among flatfish species, and may be influenced by both genetic and environmental factors [18]. A detailed understanding of early sexual differentiation and its timing is critical for development of methods for sex control and optimization of culture of both southern and summer flounder (see [18, 19] for review).

The formation of clusters of germ cells and the ovarian cavity are regarded as the

distinguishing cytological and anatomical features of ovarian differentiation. Luckenbach *et al.* [24] found that female southern flounder develop an ovarian cavity from 75–100 mm TL. Interestingly, this feature is considerably delayed relative to that observed for the Japanese flounder, where an ovarian cavity is seen in fish as small as 40 mm TL. The smallest fish that possessed primary oocytes in the early perinucleolus stage was 115 mm TL. Testicular differentiation appeared to be delayed, relative to ovarian differentiation [24]. In presumptive testes, the formation of seminiferous tubules was first observed in fish ≈ 100 mm TL. Spermatogonia remained quiescent (i.e., meiosis was not initiated) until most fish were over 100 mm TL. Overall, the gonads of southern flounder > 120 mm TL had initiated meiosis, and sex could be clearly distinguished by histology [24].

In another study of southern flounder cultured in China, results were similar [25]. Clusters of oogonia appeared at 85 dph or at 59 ± 3.0 mm TL, indicating onset of ovarian differentiation. A presumptive ovarian cavity appeared at 71 ± 3.6 mm TL (100 dph). Ovary differentiation was completed around 180 dph (134 ± 12 mm TL). Indicative of the beginning of testicular differentiation, seminiferous tubules appeared at 160 dph (68 ± 5.6 mm TL). At 200 dph (87 ± 9.3 mm TL), the testis began to develop spermatogonial clusters of cysts and formed seminal lobules, major cytological features of testicular differentiation. Testis differentiation was completed by 240 dph (103 ± 11 mm TL).

Male southern flounder typically reach sexual maturity by 300–400 g (250 mm TL) at one or two years of age, while females reach maturity at two years old, or around 800–1,000 g (350 mm TL) [3]. Females typically spawn small batches of 100,000 eggs per kg body weight over several days. Based on controlled spawning work, the number of eggs released per female at any one time is relatively low, compared with other types of fish of similar body weight. However, total egg production is similar if all egg batches are combined [3, 6]. After spawning, southern

flounder typically migrate back to the rivers and estuaries from which they came during the spring months. Populations in the Gulf have been observed returning to Texas bays from February to April [2]. The fish will remain there until the fall, when they move offshore again to spawn.

29.2.3 Embryonic and Larval Development of Summer Flounder

Cleavage in summer flounder, as in other teleosts, is meroblastic. The first cleavage of the blastodisc cytoplasm takes place approximately 2.5 hours after fertilization (all times based on 14°C rearing temperature), yielding two equal-sized blastomeres [26]. Subsequent divisions occur approximately every 1.5–2.0 hours. The blastula stage begins 7.5–10.0 hours after fertilization. The beginning of epiboly, occurring at 15.0–26.5 hours after fertilization, marks the onset of gastrulation. From 27–36 hours after fertilization (cephalization stage), the raised anterior-posterior axis becomes more clearly defined, with distinct rostral and caudal regions. At the cranial regionalization stage (47–57 hours after fertilization), optic vesicles gradually develop, and the primary brain vesicles begin to differentiate. At 59 hours post-fertilization, the tail tip and the yolk sac further separates, and movement and heartbeat of the embryo are observed. At 85.0 hours after fertilization, the embryo hatches [26].

The larval mouth and bipartite gut open as active feeding begins ≈ 3 –12 dph [26]. Subsequently, the notochord flexion stage occurs from 12–25 dph, with the notochord tip bending 35–40° at the end of this stage. At 30–65 dph (around 13 mm TL), metamorphosis occurs and the right eye migrates to the left side of the head [19, 26].

29.2.4 Sex Differentiation and Sexual Maturity of Summer Flounder

There is no published detailed assessment of gonadal sex differentiation in summer flounder, although studies indicate that it occurs

during a window similar to that of southern flounder. King *et al.* [15] concluded that morphological sex differentiation in summer flounder occurs from 60 (undifferentiated gonad) to 120 mm TL (differentiated gonad). Colburn *et al.* [27] reported that gonadal differentiation was completed in fish reared at 21°C and 26°C by 227 dph (\approx 150 mm TL), but not until 336–376 dph in those reared at colder temperatures (\approx 160 mm TL).

Summer flounder reach sexual maturity around two years of age, and the L50 (i.e., the size at which 50% of individuals are sexually mature) is 246 mm TL for males and 322 mm TL for females [28]. The wide range of maturity indices for female summer flounder during the spawning season suggest that they have non-synchronous maturation and a relatively protracted spawning season. Maturity indices and spawning time of female summer flounder peak in October–November in the Mid-Atlantic Bight (i.e., Cape Cod, Massachusetts to Cape Hatteras, North Carolina) [29]. Male indices, on the other hand, peak in September and are lowest in April. Fecundity of female summer flounder is considered relatively high, as the number of eggs produced per gram of total female weight (i.e., relative fecundity) ranges from 1,077–1,265.

29.2.5 Early Markers of Sex Differentiation in Flounder

It is important to understand and to be able to manipulate the sex of flounder, for producing both faster-growing all-female stocks for aquaculture, and appropriate sex ratios for stock enhancement. Gross morphology cannot be used to determine the sex of flounder, and histological identification of flounder sex can be reliably done only when animals are >120 mm TL. Considering that flounder sex is determined not only by the genetics of the fish, but also by the environment, it is critical that we gain a better understanding of what controls this process. There is interest in identifying early markers of flounder sex both for determining the mechanisms underlying sex determination

and differentiation in these species, as well as determining the window over which this process is susceptible to exogenous manipulation (i.e., sex control).

Sex differentiation in flounder can be strongly influenced by steroid hormone manipulations and endogenous steroid patterns during sexual development, which is consistent with a key role for these hormones. As such, many studies to date have examined the rate-limiting enzyme in the conversion of estrogens to androgens, aromatase or *cyp19a* [19]. In fishes, different aromatase genes are predominantly expressed in the gonad (*cyp19a1a*) and brain (*cyp19a1b*) [18]. Gonadal expression of *cyp19a1a* increases and remains elevated during sex differentiation in those individuals that will develop as females, while expression remains low in those individuals destined to become male [18, 30, 31].

Another, and perhaps earlier, marker of female sex differentiation is forkhead transcription factor L2 (*foxl2*), which is responsible for promoting transcription of aromatase, and is expressed primarily in the gonads of developing females, and not in males, during sexual differentiation [21]. By contrast, Müllerian-inhibiting substance (*mis*, also known as anti-Müllerian hormone or *amh*) shows a pattern of expression opposite to that of *cyp19a1a* and *foxl2*, with levels rising during testicular differentiation and declining during ovarian differentiation [21] – hence serving as a marker of male development.

Work using these biomarkers has demonstrated that the critical window for phenotypic sex differentiation is based on length, and is thought to occur between 30 and 65 mm TL in southern flounder [18, 21, 30, 32]. A study in summer flounder, however, suggests that the window of molecular sex differentiation may begin at an earlier size, at ≈ 15 mm TL [33]. Overall, gonadal expression patterns of *cyp19a1a*, *foxl2*, and *mis* allow flounder to be sexed much earlier than 120 mm, aiding in research aimed at identifying conditions that regulate sex determination and differentiation in Paralichthids.

29.3 Sex Control in Southern Flounder

29.3.1 Gynogenesis

29.3.1.1 Meiotic Gynogenesis

It is important to note that, among the Paralichthids, specific genotypic sex-determining mechanisms have only been elucidated for Japanese flounder. Investigations have shown that Japanese flounder utilize an XX/XY system of sex determination with homogametic females (XX) and heterogametic males (XY) [9, 34]. Studies in southern flounder suggest they utilize a similar system [21] (see Section 29.5), which underscores the importance of producing gynogenetic, all-XX stocks for aquaculture.

Gynogenesis is a form of asexual reproduction in which female eggs are activated by male sperm, but no male genetic material

is contributed to the offspring (i.e., eliminating any possible contribution of the Y-chromosome in XX/XY species). Effective methods for induction of diploid gynogenesis are a critical first step toward potential all-female production of flounder fingerlings (Figure 29.2).

In some teleost fishes with an XX/XY system of sex determination, a 100% genotypic female population can be created through meiotic gynogenesis (meiogynogenesis). First, one must effectively exclude the contribution of chromosomes from the heterogametic sex, in this case the flounder sperm. In 2004, Luckenbach *et al.* [17] found that the lowest UV dosage that produced optimum results for inactivating the chromosomes contained in sperm was 70 mJ/cm², regardless of the semen-to-egg ratio (Figure 29.3). Specifically, at this dose, all hatched embryos exhibited haploid syndrome, and did not sur-

Monosex Production of Flounder for Aquaculture

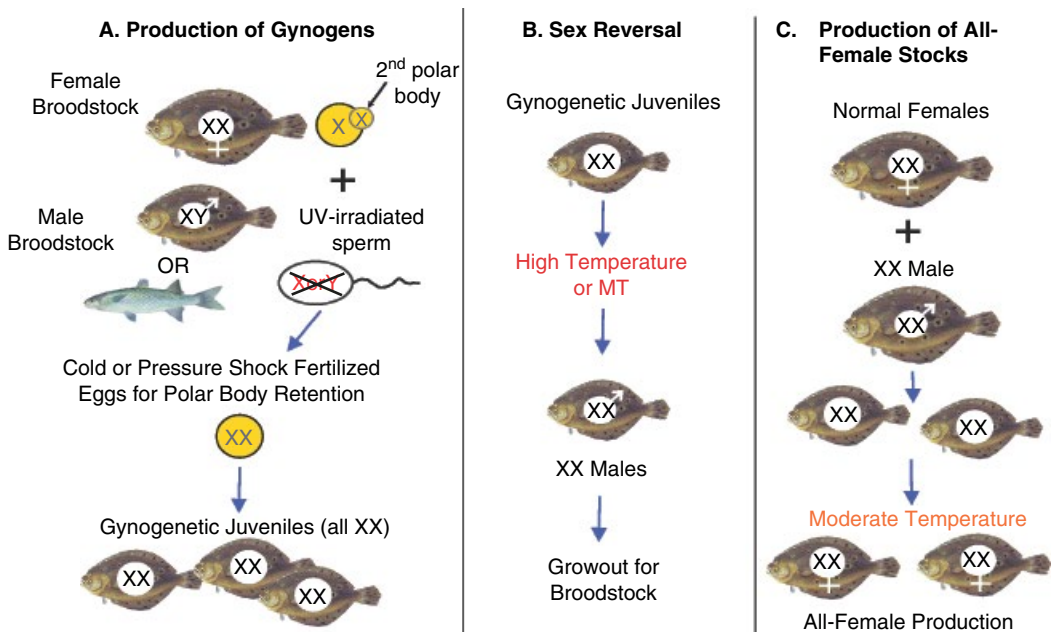


Figure 29.2 Schematic overview of indirect sex control for monosex, all-female production of flounder.

Shown are methods for producing gynogenetic, all-XX juveniles (A), sex reversal of gynogenetic juveniles into XX males for use as future brood stock (B), and routine production of all-female fingerlings by crossing normal female brood stock with XX-male brood stock (C).

Figure modified from that published by Luckenbach [57].

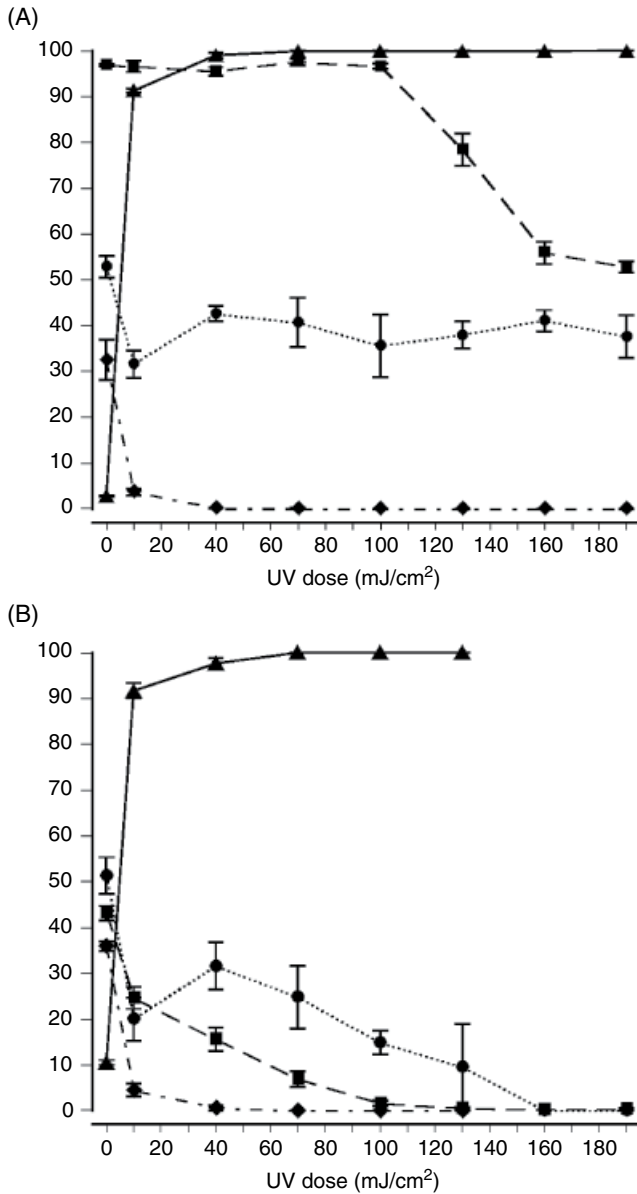


Figure 29.3 Optimization of the UV dose for induction of gynogenesis in southern flounder eggs.

Flounder semen was pooled from three to five males, diluted with Ringer's, and UV-irradiated at dosages ranging from 0–190 mJ/cm². Upper (A) and lower (B) semen to egg ratios (0.9 : 4.0 ml versus 0.3 : 4.0 ml) were tested in separate experiments. Data represent triplicate determination of percent fertility (■), hatch (●), haploid syndrome (▲), and survival ≈ 36 hours after hatching (◆) for each treatment.

Figure modified from that published by Luckenbach *et al.* [17].

vive post-hatch; however, egg activation and hatch rates were maximal (Figure 29.3).

Ultimately, meiogynogenesis in southern flounder could be successfully induced by activating egg development with the UV-

irradiated southern flounder sperm (70 mJ/cm²) or striped mullet sperm (50 mJ/cm²) for 3–4 min in seawater, and then subjecting the eggs to cold-shock in 0–2°C seawater for 45–50 min for retention of the second polar

body [17] (Figure 29.2). This polar body, which is typically extruded post-fertilization, contains a full maternal set of chromosomes (including the X-chromosome), and maintains diploidy of the embryo when retained.

Subsequent studies demonstrated that southern flounder meiogynogenesis could be induced by heterologous sperm of several other species: black sea bass [36]; sea perch [37]; red sea bream [38]; and black porgy [39]. In addition, methodology was developed for inducing second polar body retention in southern flounder eggs, via pressure shock instead of cold shock [36].

Work conducted in China showed that body morphology of larvae was not significantly different between meiogynogens produced by homologous or heterologous sperm or between meiogynogens and diploid controls [40]. Ploidy analysis also showed that the DNA concentration of meiogynogens was not different than controls [40], similar to previous work in the United States with measurements of erythrocyte nuclei [17]. Genetic analysis of microsatellite loci Pa1e11 showed exclusively maternal alleles, suggesting a 100% gynogenetic performance [37]. Except for two expected homozygotes, the genetic analysis with microsatellite loci Pa1e11 indicated that there were 23.08% heterozygous offspring, indicating that the recombination events were between the locus and the centromere [37].

After continuous culture of meiogynogens at 23°C for one year, the female proportion was determined to be 42.8% [40]. Thus, the female proportion of meiogynogens was not 100%, suggesting that other environmental factors might affect sex determination of southern flounder, or that the presumed XX/XY genotypic sex determination system may be more complex.

As discussed below, there is now compelling evidence that environmental factors such as temperature and tank color, as well as treatment with exogenous cortisol, influence sex ratios and cause masculinization of XX genotype flounder [21] (see section 29.5 below). Importantly, Mankiewicz *et al.* [21]

showed that sex-reversed meiogynogens (XX male) brood stock crossed with female brood stock yielded 91% phenotypic females, closely approximating the 100% that one would expect with the XX/XY genotypic sex determination system.

29.3.1.2 Other Ploidy Manipulations

Production of cloned Japanese flounder has been successful in Japan, and has improved genetic selection of this species for aquaculture [9]. Production of cloned populations of southern and summer flounder, through mitotic gynogenesis (mitogynogenesis), would also greatly enhance sex control research with these species, as having mitogynogens would open the future for many new genetic studies, and fix genetic traits within brood stock lines.

In southern flounder, the timing of the first mitotic division varies between 63–88 minutes post-fertilization (mean 76.25 ± 2.95 minutes) [41]. The duration from the time the first egg started mitosis until the last egg sampled began mitotic divisions ranged from 8–40 minutes (mean 22.88 ± 3.11 minutes). There was no correlation between temperature and the beginning of the first mitotic division. However, only one trial was conducted, and the percentage fertility was 44.7% and hatch was 0% [41]. Clearly, further studies are required to establish southern flounder mitogynogens.

Induction of triploidy has been achieved in numerous freshwater and marine fish species [42]. The main objectives of triploidy are potential reproductive sterility and associated growth benefits. In theory, sterility avoids metabolic costs of sexual maturation and, as a result, somatic growth continues in triploid fish, with maintenance of flesh quality during the period when diploids sexually mature [43]. Limited triploid work has been conducted in southern flounder to date. The use of cold shock for triploid induction was tested and validated by Luckenbach *et al.* [17], but no attempt was made to rear the triploids beyond hatch.

Other cold-shock triploid trials were conducted by Xu *et al.* [39], with percentage

fertility ranging from 19.9–53.3% and hatch ranging from 3.96–10.68% [41]. Meanwhile, percentage hatch of the diploid control was 18.0–42.7%. Larvae hatched approximately two days after fertilization. Presumptive triploids were reared through metamorphosis, although growth was poor after metamorphosis and survival low (<1%).

29.3.2 Temperature Induced Sex Control

Southern flounder inhabit the east coast of the United States, ranging from approximately North Carolina to Florida, and in the Gulf of Mexico from Florida to northern Mexico, with a break along the southern portion of Florida (Figure 29.1). Populations of southern flounder flanking the Florida peninsula are thought to have minimal interbreeding, due to their physical separation. This separation may have led to the localized adaptations affecting temperature-dependent sex determination (TSD) that occur in domestic southern flounder [41]. Although studies on sex ratios of wild southern flounder populations are extremely limited, it has been suggested that they are 1 : 1 in North Carolina, and potentially female-skewed in Texas [44].

In the laboratory, 1 : 1 sex ratios were observed for southern flounder reared in North Carolina at constant temperatures of 23°C in fresh water, beginning at 40 mm TL. However, when juvenile southern flounder were grown at 18°C or 28°C for 245 days, a significantly higher proportion of males were produced (78% males at the low temperature; 96% males at the high temperature) [24]. In a more recent study, juvenile southern flounder were exposed to 14°C, 18°C, 22°C, 26°C, and 32°C seawater from 40–160 dph [25]. The larval body length at 40 dph ranged from 16.5–17.1 mm.

Temperatures of 26°C and 32°C produced a higher proportion of males (66.3% males at 26°C; 72.5% males at 32°C). Low temperature (14°C, 16°C) also caused a slightly higher proportion of males, albeit not significantly different (58.8% males at 14°C; 63.8% males at

16°C). Fish raised at 22°C produced 1 : 1 sex ratios [25]. These two studies demonstrate that a 22–23°C rearing temperature may best promote female sex differentiation in southern flounder, at least in populations originating from North Carolina.

Studies with Texas populations of southern flounder suggest they may have a different TSD threshold, relative to North Carolina populations [32]. The highest proportion of females were obtained in Texas fish reared at 18°C, while higher temperatures progressively skewed ratios toward males [32]. This difference in the TSD threshold between North Carolina and Texas flounder suggests that the populations may have a TSD response adapted to their local environment.

Such latitudinal differences in TSD have been shown in Atlantic silverside (*Menidia menidia*), where more northerly latitude populations exhibit strict GSD with little influence of temperature, and southerly latitudes exhibit a lesser degree of GSD and a higher degree of TSD [45–47]. It should be cautioned, however, that because the temperature studies in southern flounder from Texas and North Carolina were not conducted in the same experiment, it is possible that environmental factors other than temperature could have influenced the results. Hence, a future experiment should directly compare southern flounder from different latitudes.

Toward the goal of all-female production (Figure 29.2), southern flounder meiogynogens of the presumed XX genotype were sex-reversed to males by high temperature (28°C). Upon reaching maturity, an *in vitro* cross was conducted between the spermiating gynogens and a wild-caught female. Survival of F1 larvae was >90% to first feeding, and 33.5% through metamorphosis at day 34 [36]. If southern flounder utilize a genetic XX/XY system of sex determination, it would be expected that this cross of meiogynogenetic, sex-reversed male (XX) and wild female (XX) would produce all-XX progeny, similar to that observed in Japanese flounder [9]. Indeed, when progeny

were raised at the permissive temperature of 23°C, 91% phenotypic female progeny were produced [21]. This strongly supports an XX/XY system for southern flounder, with some influence of environmental conditions on a low proportion (<10%) of the XX-genotype fish. As discussed below, this could be anything in the rearing environment that is perceived as a stressor and leads to increased cortisol levels.

29.3.3 Hormone Treatment for Sex Reversal

Strategies of sex control are categorized as direct or indirect. The direct method for producing all-female stocks often uses estradiol-17 β (E₂) to directly induce feminization during the labile period of sex differentiation, and achieves this goal within the same generation [48]. The indirect method for producing all-female stocks often uses androgen treatment to generate phenotypic XX males (or neomales), which can be ultimately grown to sexual maturity and crossed with normal females to produce all-genotypic female stocks.

Direct and indirect sex control has been effective in Japanese flounder [49]; therefore, the use of sex steroids as a means to alter phenotypic sex of southern flounder has been examined. Juvenile southern flounder from 40–160 dph, reared at 20 \pm 0.5°C, were exposed to MT and E₂ via immersion at concentrations of 0, 20, 60, 80, or 100 μ g/L, respectively. The larval body length at 40 dph ranged from 16.4–17.1 mm TL. MT treatment produced significantly higher proportions of males (51.6% males at 0 μ g/L; 78.5% males at 60 μ g/L; 83.4% males at 80 μ g/L; 87.3% males at 100 μ g/L). Treatment with E₂, on the other hand, did not significantly increase the proportion of females (53.4% females at 0 μ g/L; 58.2% females at 20 μ g/L; 66.8% females at 60 μ g/L; 71.3% females at 80 μ g/L; 71.8% females at 100 μ g/L) [25]. The average length of fish from the MT and E₂ treatment groups at 160 dph were 83.6 \pm 7.3 mm and 78.6 \pm 6.8 mm TL,

respectively, and significantly lower than that of controls (87.0 \pm 7.6 mm TL).

These results suggest that MT is useful for masculinization of southern flounder, but that E₂ may not be a promising approach for all-female production. Dietary treatment (i.e., during a later period of development) with E₂ has not been attempted, and is something that should be investigated in the future. Interestingly, studies show that genistein, a phytoestrogen derived from soy processing, can feminize southern flounder when incorporated in the diet during the sex determination period [50].

Meiogynogens in southern flounder produced in China were also treated with 1 mg/mL MT immersion for 12 hours/day for 60 days, starting from the post-metamorphosis stage (45 dph). After culturing the treated fish for one year at 17.4°C, 200 individuals were sampled for sex identification, and the male ratio was 95.3%. Additionally, meiogynogenetic post-metamorphic southern flounder (45 dph) were fed pellets containing MT at a dose of 30 mg/kg feed (at 4% body weight per day) for 60 days. After culturing them for one year, sex identification showed that 97.5% males were produced [40]. The sex-reversed meiogynogens are now useful for routine production of all-XX genotype southern flounder in China.

29.4 Sex Control in Summer Flounder

29.4.1 Meiotic Gynogenesis

Colburn *et al.* [27] found that summer flounder meiogynogens could be produced by activating eggs with UV-irradiated (70 mJ/cm²) black sea bass sperm, and applying a six-minute pressure shock (58,600 kPa) two minutes post-fertilization. Other studies, from Yang *et al.* [51], found that meiogynogens could also be induced in summer flounder by activating eggs with UV-irradiated sperm (80 mJ/cm², homologous sperm or heterologous sea perch sperm) at five minutes after

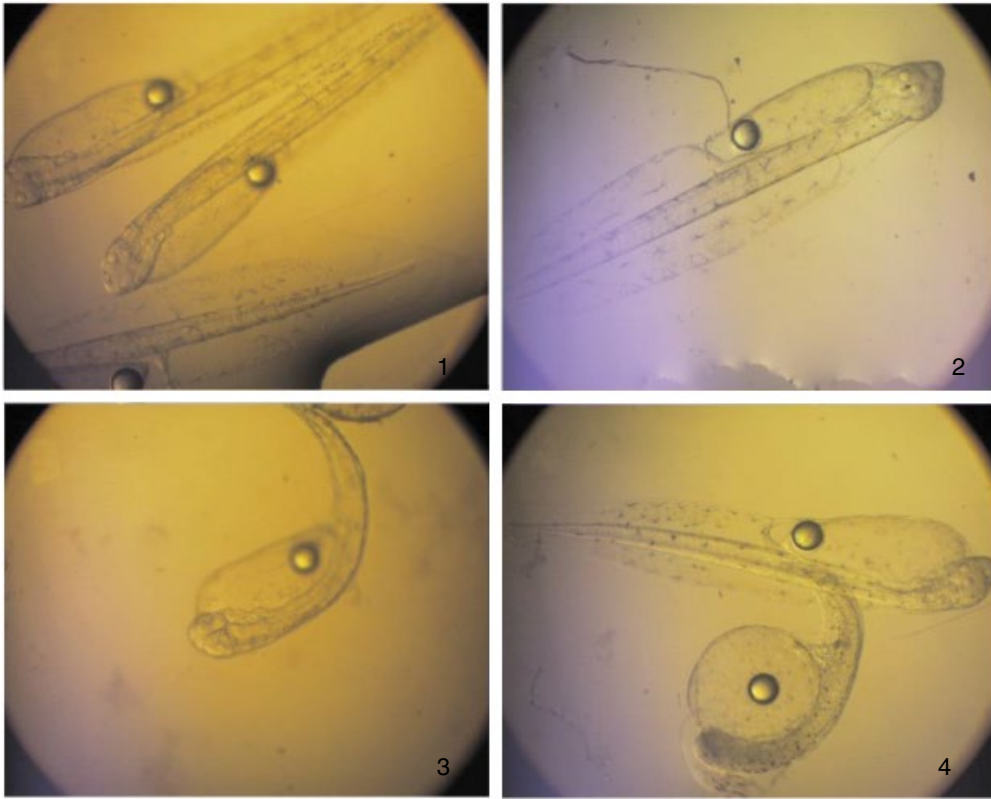


Figure 29.4 Larvae morphology of gynogenetic haploid and diploid in summer flounder. (See inserts for the color representation of this figure.)

1: normal diploid; **2:** gynogenetic diploid induced with homologous sperm; **3:** gynogenetic haploid induced with heterologous sperm; **4:** gynogenetic diploid (upper) and haploid (lower) induced with heterologous sperm.

Figure reproduced from that published by Yang [37].

fertilization in seawater at 18°C, and then subjecting the eggs to cold shock in 3°C seawater for 45 minutes. Rates of diploid gynogenesis using homologous or heterologous sea perch sperm were $32.66 \pm 7.03\%$ and $28.00 \pm 6.48\%$, respectively.

Ploidy analysis showed that the DNA concentration of the diploid meiogynogens was the same as diploid controls [51]. Karyotype analysis in embryos and larvae of meiogynogens demonstrated high accuracy in the estimation of ploidy level. The number of chromosomes was in accordance with their expected ploidy level; embryos resulting from eggs fertilized with UV-irradiated sperm and not cold-shocked were haploid (24 chromosomes), while putative gynogenetic diploids showed the standard summer flounder karyotype ($2n = 48$ chromosomes).

The morphology of diploid meiogynogens induced with homologous or heterologous sperm was similar to that of diploid controls, and different from that of haploids, the latter showing deformities (curled body: Figure 29.4), as previously reported for southern flounder [17]. These results indicate that meiogynogenesis can be successfully induced using homologous and heterologous sperm and pressure or cold shock.

Further analysis using microsatellite locus Pade12 showed that the genotypes of 39 putative meiogynogens exclusively showed maternal alleles (Figure 29.5). For microsatellite loci Pade1 and Pade22, the two expected homozygotes and a variable proportion of heterozygous offspring were found, indicating recombination events between the locus and the centromere. Genetic analysis of microsatellite

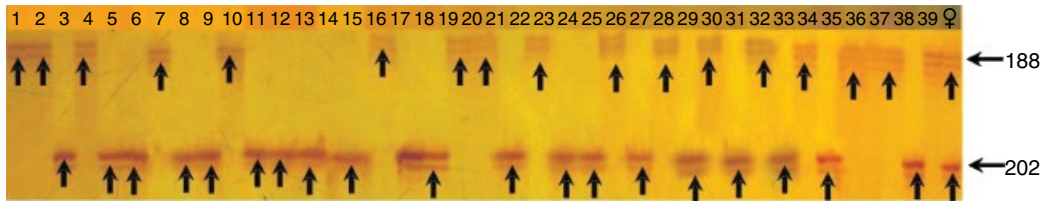


Figure 29.5 Homozygosity analysis of meiogynogenetic diploids in summer flounder using microsatellite locus Pade12.

1–39: putative meiogynogenetic diploids; ♀: female parent.
Figure reproduced from that published by Yang [37].

locus Pade6 showed higher homozygosity in meiogynogens than normal diploids in summer flounder [40]. After continuous culture for one year, of 100 meiogynogens, 38 were female and 62 were male [40].

Spermatozoa characteristics from normal summer flounder and meiogynogenetic fish masculinized by exposure to male-determining temperatures ($\geq 21^{\circ}\text{C}$; XX males) were further compared using computer-assisted sperm analysis [52]. Sperm concentration was lower for the meiogynogens, but not when normalized to body weight, and swimming characteristics were similar for meiogynogens and normal males. In fertilization trials using pooled eggs from two females, sperm from normal and meiogynogenetic males had equal fertilization success, but fewer embryos survived that were produced from meiogynogenetic sperm (36.3% versus 46.6%). Twenty-four hour survival of hatched larvae was equal for both groups ($>96\%$).

Sperm collected from meiogynogenetic males was used to fertilize eggs from seven domesticated female brood stock during commercial production. Mean fertilization and hatch were $56.0 \pm 6.8\%$ and $32.7 \pm 8.9\%$, respectively, resulting in the production of 304,450 larvae. Unfortunately, sex ratio data were either not assessed or not reported for the offspring in this study.

29.4.2 Temperature Induced Sex Control

When summer flounder meiogynogens and controls were reared under a low temperature

regime (12°C gradually increased to 20°C) for up to 376 dph, the female proportion was higher in meiogynogens (62.5%) than in controls (22.6%) [27]. Highly male-skewed proportions (96.1–100%) were produced at higher temperatures for both meiogynogens and controls, indicating a strong effect of temperature on phenotypic sex.

Offspring produced from crosses with meiogynogenetic, sex-reversed males and normal females were raised at 14°C , 16°C , or 18°C for ≥ 300 days, or 12°C for 30, 60, or 120 days, and then transferred to a male-determining temperature (21°C). In all cases, the fish developed predominantly as phenotypic males ($\geq 74\%$). Additionally, offspring produced from crosses with normal males and females were reared at 15°C , 17°C , and 19°C for 111, 227, and 278 days, and then transferred to 21°C . Most fish ($\geq 92.1\%$) developed as phenotypic males, irrespective of rearing temperature or duration of exposure [53]. Therefore, gynogenetic summer flounder, cultured throughout the period of sex determination and differentiation, can be easily phenotypically sex-reversed to males by exposure to a relatively high water temperature.

Phenotypic sex of summer flounder is highly influenced by temperature [27, 40, 54]. However, based on the temperature studies with meiogynogens, it remains uncertain whether summer flounder utilize an XX/XY system, as nearly pure populations of phenotypic females have yet to be produced when meiogynogens are reared over a wide range of temperatures ($12\text{--}21^{\circ}\text{C}$), including one

that would presumably favor retention of the female phenotype. Thus, it is likely that environmental factors other than temperature masculinize these fish [21] (see Section 29.5). Also in the wild, the sex ratio of young-of-year summer flounder is often biased toward males, with >70% males sampled in some years, indicating that sex-reversal to the male phenotype could be occurring in natural populations [29, 54].

Interestingly, in one study, milt was obtained from ovulating females during routine captive brood stock spawning, and both ovarian and testicular tissues (i.e., intersex gonads) were observed in histological sections from juveniles reared at different temperatures [54]. Therefore, protandry or some other form of sexual plasticity in summer flounder cannot be ruled out.

29.4.3 Hormone Treatment for Sex Reversal

To date, there are no published studies for direct induction of female development in summer flounder using E_2 exposure, although data are available on uptake, accumulation, and depuration of E_2 in larvae and juveniles [55].

As previously mentioned, the indirect method of sex control for producing all-females uses temperature or androgen treatment to produce XX males, which are then crossed with normal females to produce all-XX populations (Figure 29.2). Two methods have been used to sex-reverse summer flounder meiogynogens [40]:

- 1) Meiogynogens were immersed for 12 hours/day with 1 mg/mL MT, for a period of 60 days, starting at metamorphosis;
- 2) Meiogynogen larvae ($n = 200\text{--}300$) were fed pellets at a rate of 4% body weight/day, for 60 days with MT doses of 30, 60, or 90 mg/kg feed. Fish were cultured for one year post-treatment, after which 30–100 individuals (TL > 150 mm) were sampled for sex identification. The male proportion for all groups was 100% [40].

These data show successful masculinization of summer flounder via MT treatment. Again, when these XX males are ultimately crossed with normal females (Figure 29.2), all-female summer flounder stocks are possible if the sex determination system is XX/XY, and female differentiation is not overridden by environmental factors.

29.5 Other Factors Influencing Sex

While flounder have been shown to exhibit TSD, other environmental factors may also influence sex differentiation. In southern flounder, background color appears to influence sex ratios. When southern flounder are raised through the period of sex determination and differentiation in black, grey, or blue tanks (at 23°C), a significantly higher proportion of males are observed in blue tanks (95%), compared with black and grey tanks ($\approx 50\%$, using mixed-sex stocks) [21]. This masculinization in blue tanks is also associated with increases in endogenous cortisol, the primary stress hormone in fishes. When an all-XX cohort of southern flounder (produced via meiogynogenesis) is fed cortisol, a dose-dependent increase in the number of males was seen. The 0 mg/kg cortisol produced 91% female and 9% male, the 100 mg/kg cortisol treatment 29% females and 71% males, and the 300 mg/kg cortisol yielded 13% females and 87% males [21].

These data suggest that cortisol is a key regulator of sex determination and differentiation in this species, possibly through regulation of aromatase [21], as is suggested for Japanese flounder [56]. Whether this holds true for summer flounder remains to be determined. Nonetheless, it seems plausible that suboptimal environmental conditions that may elicit a stress response, or increase cortisol, may lead to male-skewed populations. Hence, it will be important to

identify the optimal environmental conditions that best promote the development of faster-growing female southern and summer flounder for culture.

Key information for the sex control of southern flounder and summer flounder is summarized in Box 29.1 and Box. 29.2.

29.6 Conclusions and Future Directions

There has been significant progress in developing an understanding of the mechanisms of sex determination for southern and summer flounder. Their overarching genotypic system

Box 29.1 Summary of key information for southern flounder sex control	
<p>Sexually dimorphic growth:</p> <p>Female southern flounder grow 2–3 times larger than males. In the wild, males rarely attain body sizes greater than the current minimum size restriction for harvest (350 mm total length; TL) within the first three years of life.</p> <p>Sex determination:</p> <p>The sex determination mechanism has not been fully resolved, although evidence supports an XX/XY-type system, with XX-genotype individuals easily sex-reversing to phenotypic males due to environmental effects, at least in captivity. There may also be latitudinal differences in the temperature-dependent sex determination mechanism between southern flounder of different origins. The male sex-determining gene has not been identified.</p> <p>Gonadal sex differentiation:</p> <p>Phenotypic sex can be distinguished for most fish > 120 mm TL via gonadal histology. Early features of ovarian differentiation are typically observed from 60–100 mm TL (≈85–100 days post-hatch; dph) and ovarian differentiation is completed by 120–150 mm TL (≈180 dph). In putative males, seminiferous tubules are observed as early as 70 mm TL, denoting the beginning of testicular differentiation. Testicular differentiation is typically completed by 120 mm TL (≈240 dph). Gonadal levels of <i>cyp19a1a</i>, <i>foxl2</i>, and <i>mis/amh</i> mRNA have been used as early markers of sex differentiation.</p>	<p>Gynogenesis:</p> <p>Meiogynogens were successfully produced by UV-irradiating either homologous or heterologous sperm at a dose of 70 or 50 mJ/cm², respectively, using this for egg activation for 3–4 minutes in seawater, and then subjecting the eggs to cold shock in 0–2°C seawater for 45–50 minutes. Although results have varied among labs and studies, phenotypic female proportions as high as 91% have been obtained for southern flounder meiogynogens.</p> <p>Environmental effects:</p> <p>A so-called ‘U-shaped curve’ was reported with both low and high rearing temperatures inducing male-skewed sex ratios and a moderate temperature producing a 1 : 1 sex ratio. Studies also show that background color influences sex determination, with blue color, but not grey or black, inducing male-skewed ratios. Evidence suggests that this phenomenon may be mediated by the stress hormone, cortisol.</p> <p>Hormone treatment:</p> <p>Immersion of metamorphosis stage meiogynogens (45 dph) in 1 mg/ml 17α-methyltestosterone (MT) for 12 hours/day for 60 days, or dietary treatment with MT at 30 mg/kg feed, produced 95.3 and 97.5% males (i.e., neomales), respectively. Estradiol-17β (E₂) immersion at doses of 20–100 µg/L did not significantly increase female proportions relative to the control.</p>

Box 29.2 Summary of key information for summer flounder sex control.**Sexually dimorphic growth:**

Females grew 1.4 times larger than males by 15 months post-hatch, and were projected to be twice as large by harvest at 23 months. The age at which sexually dimorphic growth begins is around 12 months post-hatch.

Sex determination:

The sex determination mechanism is less clear for summer flounder, but thought to be XX/XY, with strong masculinizing environmental effects on the XX genotype. The sex-determining gene(s) are unknown.

Gonadal sex differentiation:

Limited work has been conducted, but morphological sex differentiation is thought to occur from 60–120 mm TL. Gonadal expression of *cyp19a1a* has been used as an early sex marker.

Gynogenesis:

Meiogynogens were produced by activating eggs with UV-irradiated (70–80 mJ/cm²) homologous or heterologous sperm and

applying a six-minute pressure (58,600 kPa) or cold shock (3°C) for 45 minutes, beginning 2–5 minutes post-fertilization. When meiogynogens and controls were raised under a low temperature regime (12°C gradually increased to 20°C), the female proportion was higher in meiogynogens (62.5%) than in controls (22.6%).

Environmental effects:

In the wild, the sex ratio of young-of-year summer flounder is often biased toward males, with >70% males sampled in some years. In aquaculture, most offspring (≥92.1%) produced from normal male and female crosses develop as phenotypic males when reared at temperatures higher than 15°C.

Hormone treatment:

Immersion of metamorphosis-stage meiogynogens in 1 mg/ml MT for 12 hours/day for 60 days, or dietary treatment with MT at a dose of 30–90 mg/kg for 60 days, each produced 100% males (i.e., XX males). Direct sex control with E₂ has not been attempted.

appears to be XX/XY, with putative XY individuals always developing as males, and putative XX individuals being extremely sensitive to environmentally induced masculinization. Development of methods for induction of meiogynogenesis has led to the generation of putative all-XX populations for both species. In southern flounder, up to 91% female meiogynogens have been attained by identifying conditions that maintain the female program while, in summer flounder, the highest female proportion reported to date is 62.5% (with 22.6% females for co-reared controls). Overall, data for summer flounder suggest a potentially greater sensitivity to environmental factors compared to southern flounder but, of course, nothing is known regarding possible influences of other environmental factors (e.g., tank color) that could be confounding results.

Because of the propensity of putative XX flounder to sex-reverse in response to environmental factors, the development of methods for consistent production of all-phenotypic female stocks will continue to be a goal for aquaculture. Specifically, it will be important to identify: what factors naturally drive female sex differentiation; environmental factors that override this process, giving rise to testicular differentiation; and precisely when exposure to these environmental factors must be avoided in the aquaculture setting. The possibility of whether similar female-to-male sex reversal naturally occurs in the wild, and evolutionary advantages to such a mechanism, remains to be determined for Paralichthids. Finally, genetic and/or epigenetic differences in the ESD response in flounder will also be an interesting avenue for future research.

Acknowledgments

We thank Sun L.X. at Shandong Agricultural University for his considerable help with data collection and English editing. A portion of the research outlined herein was

supported by grants to R. Borski from the Saltonstall-Kennedy Program of the National Marine Fisheries Service (NA14 NMF4270047) and the University of North Carolina Sea Grant College Program (R/16-SFA-2).

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30

Gynogenesis and Sex Control in Japanese Flounder

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30.1 Introduction

The Japanese flounder, *Paralichthys olivaceus*, is an economically important marine fish that is distributed along the coasts of far-eastern Russia, Japan, Korea, and China. Because of the low level of metabolism of this bottom-dwelling species, it is possible to culture individuals in terrestrial tanks supplied with relatively small amounts of seawater, or in semi-enclosed recirculating tanks [1]. In recent years, with the improvement of culture techniques, Japanese flounder aquaculture has become a major enterprise in East Asian countries.

In Japan, in addition to culturing in terrestrial tanks, Japanese flounder has been artificially released for more than 30 years, and it has become one of the most successful species for cultivating fisheries. In China, research on artificial breeding and development of culture technologies for Japanese flounder were started in 1959 and, by the 1990s, these had become well established. Currently, it is a major cultivated marine flat-fish species in China, with culture production estimated to be 30,000 tons per year.

Although the Japanese flounder has become one of the major species in aquaculture, the parent fish that are used for seed production are still either directly captured from the sea, or are derived from the progeny

of wild-caught parents. However, despite many generations of culturing, the growth rate of this fish is still slow, and its feed conversion efficiency is low. Thus, the fish's growth is insufficient to meet the needs of farmers. In order to increase aquaculture production, the breeding of new varieties of Japanese flounder that are adapted to intensive farming, and that are fast-growing and disease-resistant, has become an urgent need within the aquaculture industry.

A difference in the growth rate of male and female animals is frequently observed in nature. In the Japanese flounder, the growth rate of females is significantly higher than that of the males. For one-year-old male and female Japanese flounder cultured under the same conditions, males have an average body weight of approximately 400 g whereas, in females, it is approximately 500 g, which means that, at this age, females are 20% heavier than males. By the time the fish reach two years of age, the average body weight of males is approximately 800 g, whereas that of females is some 1.5 times heavier, at approximately 1200 g [1]. Thus, increasing the number of females using sex control technology has considerable potential for improving the yield and economic benefit of Japanese flounder culture.

The Japanese flounder has a male heterogametic (female XX, male XY) sex-determination system [1]. To obtain all-female populations in

fish species such as this, artificial gynogenesis is an easy and effective technique, because no influence of sperm containing a Y-chromosome is expected, and resultant progeny with all-maternal inheritance should develop as females [2].

In this chapter, we outline the progress that has been made in artificially induced gynogenesis and sex control in the Japanese flounder. We also describe the prospects for future studies.

30.2 Artificially Induced Gynogenesis

Teleost eggs are normally ovulated at the metaphase stage of the second meiosis (M II). At this point, the eggs are physiologically mature, and are spawned outside of the female in ambient water, to receive sperm for fertilization. Such reproductive traits in most teleosts provide the potential for chromosome manipulation, such as induced polyploidy, androgenesis, and gynogenesis.

Gynogenesis is a type of parthenogenesis, whereby homologous or heterologous sperm penetrate into the egg and trigger embryogenesis, but the sperm nucleus does not fuse with an egg nucleus to form a zygote. Thus, gynogenetic individuals inherit only maternal genetic information. In teleosts, natural gynogenesis is found in the Amazon molly, *Poecilia formosa* [3], crucian carp, *Carassius auratus langsdorfii* [4], and also in local populations of the loach, *Misgurnus anguillicaudatus* [5]. These species spawn unreduced diploid or triploid eggs, which are then activated by sperm from the same or a closely related species [6].

On the basis of the mechanism of natural gynogenesis, the first step in the artificial induction of gynogenesis in fish involves genetically inactivating the nuclear DNA of sperm by irradiation. The haploid or diploid intact eggs are then activated by these irradiated sperm. When haploid eggs are used, the embryos must be treated with physical or

chemical shock to recover diploidy. Induced gynogenesis can be divided into meio- and mitogynogenesis, depending on the time at which the shock is administered. If release of the second polar body is inhibited, the resultant embryo will be meiogynogenetic diploid. Mitogynogenetic diploid eggs can be obtained by shock treatment, around the metaphase of the first mitosis [6].

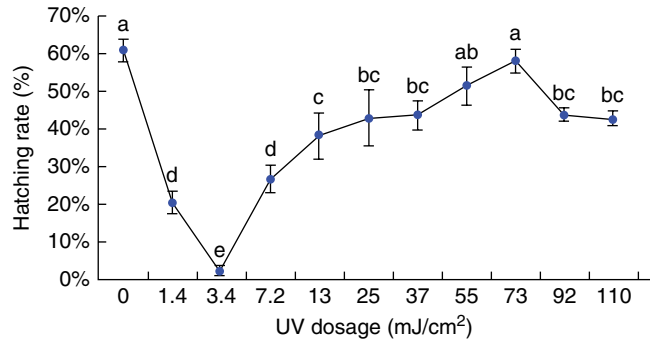
30.2.1 Induced Meiogynogenesis in the Japanese Flounder

The first attempt to induce meiogynogenesis in the Japanese flounder was made in Japan by Tabata *et al.* [7], who were then followed by Yamamoto [8]. Induced meiogynogenesis has also been reported in China [9, 10].

Before the activation of eggs, sperm from the same or different species is diluted to 1/50–1/100 with Ringer's solution, and is then genetically inactivated using UV irradiation. In practice, sperm from a different species, such as the red sea bream *Pagrus major*, is preferred, to prevent the survival of non-gynogenetic fishes [11]. The sperm of red sea bream exhibits a significant Hertwig effect after irradiating with UV (Figure 30.1). The embryo has the lowest hatching rate when the UV dosage is 3.4 mJ/cm², but this increases with increasing UV dosage. The highest hatching rate is achieved when the UV dosage is 73 mJ/cm², and ploidy analysis by flow cytometry has shown that the embryos are all haploid [9]. Thus, 73 mJ/cm² is used as the optimum irradiation dosage in our practice.

Restoration of diploidy by inhibition of second polar body release is achieved by cold-shock treatment (0–4°C for 45–60 minutes, from 2–5 minutes after insemination) or hydrostatic pressure treatment (600 kg/cm² for 6 minutes, from 2 minutes after insemination) [1]. After a series of tests for initiation and duration at a water temperature of 0 ± 0.5°C, we determined that the best conditions for meiogynogenetic induction in Japanese flounder are 45 minutes of continuous cold shock, beginning at 3 minutes

Figure 30.1 Results of irradiating the sperm of red sea bream, *Pagrus major*, using a UV irradiation gradient. Modified from Liu *et al.* [9].



after insemination [9]. However, heat-shock treatment is not effective in inhibiting release of the second polar body [7].

Cytological observation show that mature eggs are at the metaphase of the second meiosis when inseminated with UV-irradiated sperm. After the beginning of cold-shock treatment, the previously visible spindle disappears, and chromosomes at the center of the metaphase plate are condensed. This condition continues during the cold-shock treatment and several minutes thereafter. The release of the second polar body is blocked, and it develops into a female-like pro-nucleus. This then fuses with the female pro-nucleus to generate a diploid zygotic nucleus, after which the egg undergoes its first mitosis. Consequently, the haploid female chromosome set of the egg is doubled by the inhibition of second polar body release. From the time of insemination to early cleavage, the UV-irradiated heterospecific sperm nucleus remains condensed [2].

The meiogynogenetic diploids have a higher level of homozygosity than the normally fertilized diploids [12]. Furthermore, they also have a higher genetic similarity to the female parent, and also among meiogynogenetic diploid individuals, when compared with normally fertilized diploids. The second generation of meiogynogenetic diploids have higher genetic similarity than the first generation [12]. For the third generation, the average similarity index between offspring within a family is from 0.9838 to 0.9918, whereas as that between dam and offspring is from 0.9923 to 0.9968, and that

between families is 0.9714 to 0.9810 [13]. These results indicate that artificially induced successive meiogynogenesis not only increases the homozygosity of individuals, but also the genetic similarity of offspring within and between families. Accordingly, artificially induced successive meiogynogenesis is an effective method for establishing inbred lines in fish.

30.2.2 Induced Mitogynogenesis in the Japanese Flounder

Induced mitogynogenesis in fish is a type of parthenogenesis, whereby the ploidy of gynogenetically activated haploid eggs is restored by inhibition of mitotic cleavage. The mechanism underlying the artificial induction of mitogynogenesis has previously been explained in terms of blockage of the first cleavage, and formation of doubled nuclei without cell division [14–16]. In rainbow trout, *Oncorhynchus mykiss*, Zhang and Onozato found that heat shock or hydrostatic pressure did not inhibit the first cleavage, owing to the regeneration of the bipolar spindle, but it did inhibit the second cleavage by forming a monopolar spindle during the second cell cycle. Thus, the chromosome set was doubled [17].

Cytological studies of artificially induced mitogynogenesis in Japanese flounder also support the theory of Zhang and Onozato [18–20]. Theoretically, the progeny hatching from mitogynogenetic eggs are homozygous, and are named doubled haploids (DHs). Because of the complete homozygosity, DHs are ideal material, not only for

genomic study, but also for breeding practice [6]. However, in fish, research on DHs in the context of breeding has developed slowly, due to the low induction and survival rates. The highest yield of mitogynogenetic DHs previously reported (23%, calculated as the survival of larvae at hatching relative to a diploid control) was obtained in rainbow trout, by using heat shock to suppress egg mitosis [21]. It has been reported that the survival rate of DH zebrafish is 4–20% [22], whereas with DH induction in medaka, only four out of 10 hatched fry survive to adulthood [23]. In the rosy bitterling, survival of DHs between hatching and 30 days post-hatching (dph) was 5.5% [24] whereas, in a study of DH salmon, only six out of 98 first-feeding DH amago salmon survived until the spawning season, two years later [25].

In the Japanese flounder, the first attempt to induce DH was carried out by Tabata and Gorie [11]. However, although they optimized the induction conditions, they obtained a rate of mitogynogenetic diploid induction of only 2.7–5.3%. Yamamoto [8] also reported the successful induction of mitogynogenetic DHs, with a frequency of hatched larvae to total eggs used of 2.34%–8.88%, which is also low. Using DHs, homozygous and heterozygous clone lines can be established [1, 8]. In China, we also succeeded in inducing mitogynogenetic DHs and clones in Japanese flounder [26]. However, the induction rate we achieved was not notably different from that obtained previously.

Recently, we found that the higher the level of homozygosity in the female parent, the higher the rate of mitogynogenetic DH induction will be [27]. This finding raised the possibility of increasing the rate of mitogynogenetic DH induction, using eggs spawned by females with high levels of homozygosity, and also the potential for massive production of DHs, which is necessary due to the low viability of DHs. In 2015, we succeeded in massively producing mitogynogenetic DHs in Japanese flounder, and at six months post-induction, there were 10,766 DHs surviving. Using eggs from heterozygous clonal females,

we obtained a hatching rate of $34.33\% \pm 8.45\%$ (hatched larvae to total eggs used), and an abnormality rate of $3.33\% \pm 0.58\%$ (abnormal larvae to the total hatched larvae) [28]. This is the first time that DHs have been massively produced in the Japanese flounder, and it has laid the foundations for the extensive use of DHs in this species.

Among the progeny that hatch following artificial induction of mitogynogenesis, there is often a certain percentage of heterozygous diploids [29–32]. Thus, it is important to detect the true homozygous DHs from the heterozygous diploids. In the Japanese flounder, putative mitogynogenetic DHs have been identified with homozygosity at a single locus, the diagnostic isocitrate dehydrogenase (IDH) allozyme locus, which has a high gene-centromere recombination rate [11]. However, this single locus test is insufficient to estimate complete homozygosity.

We therefore used 21 maternally heterozygous microsatellite markers, which are located in the telemetric region of chromosomes, to identify the homozygosity of putative mitogynogenetic DHs in Japanese flounder. These 21 markers cover 16 out of the total 24 linkage groups in the Japanese flounder. The results indicated that 75% (58 out of 77) of individuals were homozygous at all 21 loci [26]. Now, to verify the mitogynogenetic DHs in practice, we selected 24 maternally heterozygous microsatellite markers with a high recombination rate (0.9–1.0) that cover all the linkage groups of Japanese flounder [28].

Several genetic linkage maps have been constructed, and quantitative trait loci (QTL) have been mapped in Japanese flounder [33–35]. Shao *et al.* (2012) constructed a linkage map, in which 12,712 high-confidence SNPs were assigned to 24 consensus linkage groups, and nine positive QTLs, forming two main clusters for *Vibrio anguillarum* disease resistance, were detected [34]. By using 165 DHs that hatched from a single female, a genetic linkage map, with 24 linkage groups containing 574 genomic microsatellites (type II SSRs) and expressed sequence tag-derived markers (EST-SSRs), was constructed. The length of

the linkage map was estimated as 1270.9 centiMorgans (cM), with an average distance between markers of 2.2 cM.

On the basis of this genetic linkage map, the QTLs that control skeletal traits of the Japanese flounder were also mapped [35]. Many of the microsatellite markers show segregation distortion in DHs. By modifying the conditional probabilities of QTL genotypes on the distorted flanking markers, Bayesian model selection is used to dissect the genetic architectures of traits such as body weight and morphological characters. Forty-two main-effect QTLs and 59 pairs of interacting QTLs were identified. Among these QTLs, the largest interacting QTL accounts for 25.20% of the phenotypic variance for body weights. Furthermore, many QTLs show pleiotropic effects [36].

As the Japanese flounder has an XX-XY sex determination system, the DHs hatched from mitogynogenetic eggs should theoretically all be female. However, in practice, in addition to females, a certain percentage of males, together with sex-undifferentiated individuals, are observed. In one DH population that hatched from one female parent, the percentages of females, males, and undifferentiated individuals were 56%, 38%, and 6%, respectively [37]. The gender differentiation in female DHs was later than that in females resulting from normally fertilized eggs. In contrast, in male DHs, the time of gender differentiation was essentially the same as that in normal males. With the development of gonads, the degeneration of ovaries and testes are both observed.

Inbreeding has significant effects on fertility-related traits, particularly in females [38]. In the Japanese flounder, sterile female DH individuals have also been observed. The GSI of six-year-old sterile individuals ranged from 3.77% to 4.17% (compared with 8–11% for fertile individuals). Histological sections indicated that sterile ovaries are arrested at stage III of ovary development (Figure 30.2B, D), whereas fertile ovaries develop to stage V (Figure 30.2A, C).

By genotyping 198 polymorphic microsatellite markers in sterile and fertile DHs, four markers were found to be significantly associated with fertility [39]. In order to identify the major genes and study the regulatory mechanism in sterile gonads, the gonad transcriptomes of sterile and fertile female DHs of Japanese flounder were analyzed by high-throughput sequencing. A total of 1,225 differentially expressed unigenes were identified, including 492 upregulated and 733 downregulated genes. Gene ontology and KEGG analyses showed that genes showing significant upregulation in sterile gonads, such as *cyp11a1*, *cyp11b2*, *cyp17*, *cyp21*, *hsd3 β* , *bcl2l1*, and *prrl*, are principally correlated with sterol metabolic process, steroid biosynthetic process, and the Jak-stat signaling pathway. The significantly downregulated genes were primarily associated with immune response, antigen processing and presentation, cytokine-cytokine receptor interaction, and protein digestion and absorption. Identification of genes showing significantly different expression will provide further insights into DH reproductive dysfunction, and also into oocyte maturation processes in teleosts [40].

30.3 Production of Clones

30.3.1 Production of Homozygous Clones

A homozygous clonal line can be established by inducing meiogynogenesis in DH females, and has a coefficient of inbreeding of $F = 1.00$. Individuals with such a high coefficient of inbreeding are ideal for use as experimental animals, which are commonly used for research purposes in several fields, including medicine, biology, and environmental toxicology. These animals possess desirable characteristics, such as a clear genetic background, and genetic uniformity among individuals from the same family.

The traditional approach to preparing experimental animals is continuous full-sib

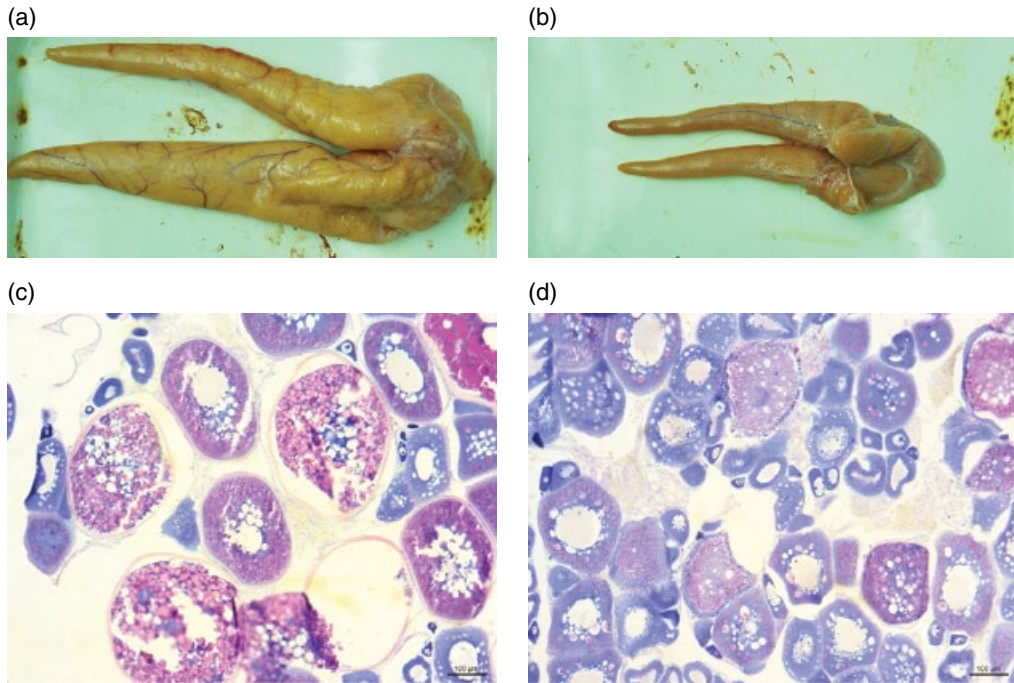


Figure 30.2 The shape and histological sections of fertile and sterile gonads of doubled haploid Japanese flounder, *Paralichthys olivaceus*. (See inserts for the color representation of this figure.)

A: Shape of fertile gonad; B: Shape of sterile gonad; C: Histological section of fertile gonad; D: Histological section of sterile gonad.

mating for at least 20 generations [41]. By using the full-sib mating approach, hundreds of inbred lines of rodents have been established and used commercially [42]. When full siblings are used for mating, the theoretical coefficient of inbreeding is $F=0.986$ [43]. Although the coefficient of inbreeding is close to 1.00, a genetic variance of approximately 2% remains within the family. When compared with full-sib mating, establishing experimental animals using gynogenesis has the advantages of being less time-consuming (only two generations) and having a higher coefficient of inbreeding ($F=1.00$). By using this method, clonal lines have been successfully established in zebrafish, *Danio rerio* [14], medaka, *Oryzias latipes* [23], common carp [30], Nile tilapia [38, 44], amago salmon [25], ayu, *Plecoglossus altivelis* [45], red sea bream [46], and Japanese flounder [1].

In the Japanese flounder, the first attempt to induce homozygous clones was carried

out by Dr. Eiichi Yamamoto. In his outstanding research, he established several homozygous clonal lines and studied their embryonic development, survival, growth, and genetic identity [8]. He also outlined a technical breeding program that uses homozygous clonal fish for genetic improvement [1]. This has laid the foundations for the application of clones in the aquaculture of Japanese flounder.

In China, two homozygous clonal lines of Japanese flounder were established by inhibiting extrusion of the second polar body of eggs from mitogynogenetic DHs, and the homozygosity and genetic identity of these clones were verified using 21 polymorphic microsatellite markers [47]. Due to the all-femaleness of homozygous clonal lines, meiogynogenesis was again induced in order to produce second-generation clones of Japanese flounder (Figures 30.3, 30.4). Twenty-four microsatellite markers, which covered all the linkage

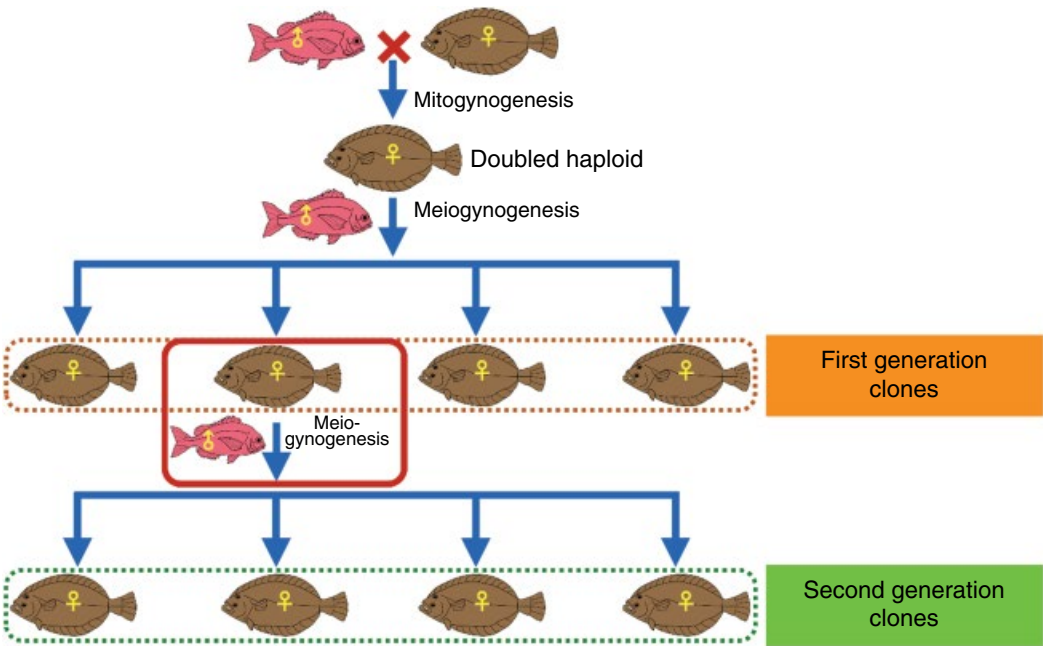


Figure 30.3 A schematic flow of the production procedure for second-generation clones of Japanese flounder, *Paralichthys olivaceus* [48].

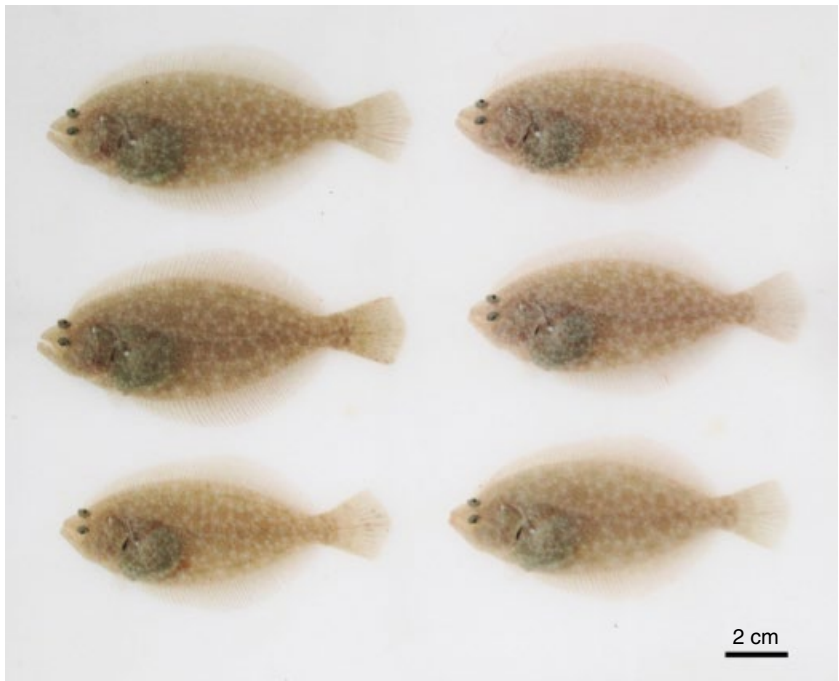


Figure 30.4 A picture of the second-generation clones of Japanese flounder, *Paralichthys olivaceus*, at three months after hatching.

groups of the Japanese flounder, were used to determine the homozygosity of the second-generation clones; no heterozygous loci were detected. Restriction site DNA-associated sequencing at the genomic level also confirmed the homozygosity and clonality of the second-generation clones. Furthermore, these second-generation clones had a smaller coefficient of variation for body shape indices at 210 days old and showed a higher degree of similarity in body characteristics among individuals when compared with intact controls [48].

Using the homozygous clones, we studied differences in the hematological indices of clonal and normal Japanese flounders, and found that aspartate aminotransferase (AST) was significantly higher in clonal Japanese flounders than in normal Japanese flounders ($P < 0.01$). This revealed that these homozygous clones could be used as a liver disease model [49]. The homozygous clones were also applied in a study of the acute toxicity of mercury (Hg^{2+}). A comparison of the tolerance and consistency of death between clonal and normal Japanese flounders indicated that the clones were more sensitive to Hg^{2+} , and had a higher consistency of death [50].

As the issue of marine pollution has become prominent in recent years, studies on the impact of polluting materials on the environment have become more important. Fish are widely used in such studies. However, the majority of experimental animals used have been non-standard fish. The genetic background of these fish was not clear, and the genetic similarities were low, which reduced the repeatability of the results. The use of standardized experimental animals in future research can effectively overcome these problems, and will improve the accuracy of such studies. The homozygous clonal Japanese flounders we produced could meet the criteria of an experimental animal, and could be used in studies of marine environmental pollution.

30.3.2 Production of Heterozygous Clones

Crosses between different female and male DHs or clonal lines can be used to produce heterozygous clones (Figures 30.5, 30.6). Heterozygous clones are free of lethal recessive genes and often show heterosis, in terms of viability and growth-related traits, relative

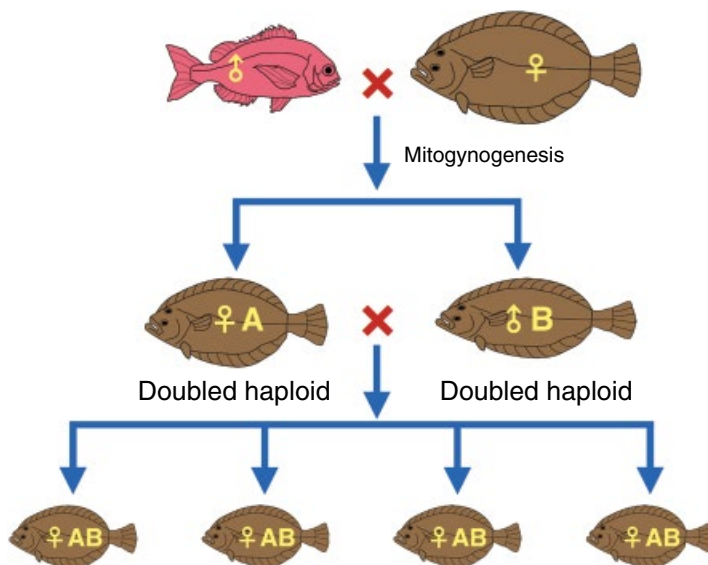


Figure 30.5 A schematic flow of the production procedure for heterozygous clones of Japanese flounder, *Paralichthys olivaceus*.

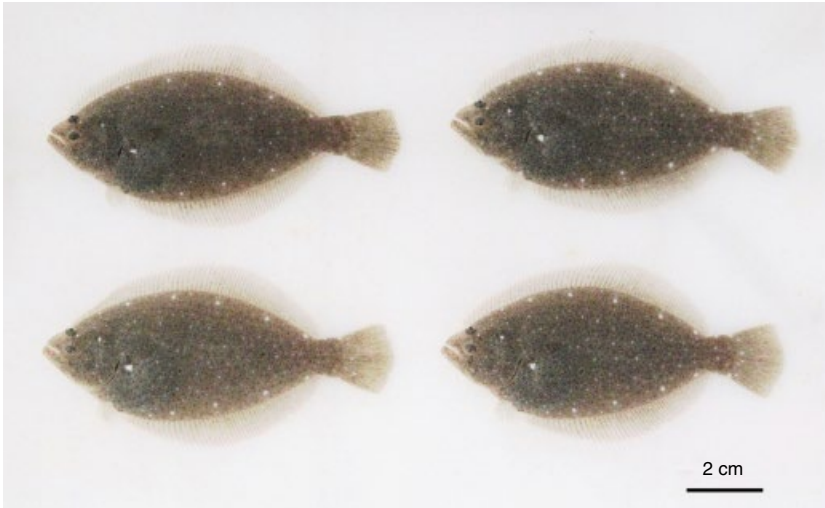
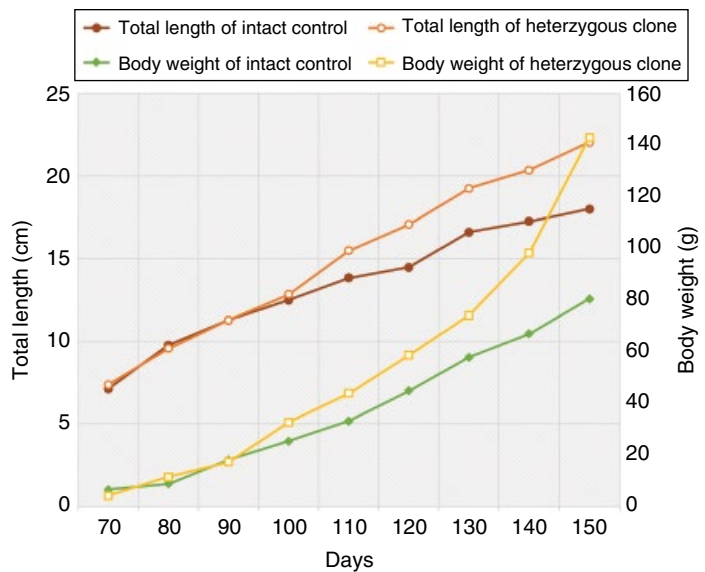


Figure 30.6 A picture of the heterozygous clones of Japanese flounder, *Paralichthys olivaceus*, at three months after hatching.

Figure 30.7 A growth curve for one heterozygous clonal line of Japanese flounder, *Paralichthys olivaceus*, from 70–150 dph.



to homozygotes [6]. In the Japanese flounder, several heterozygous clonal lines have been produced [8, 51]. In 2013, one of the heterozygous clonal lines that we produced displayed high heterosis in growth rate, which was 77.66% higher than the intact control for the trait of body weight at 150 dph (Figure 30.7).

In order to study sex differentiation, sex ratios, and growth differences under different culture water temperatures, heterozygous

clones from one family were grouped and reared in water of 16°C, 19°C, 22°C, 25°C, 28°C, or 31°C from 35–110 dph. The water temperature had a significant effect on the growth of heterozygous clones. With the exception of the water at 31°C, we found that from day 35 to 50, the higher the water temperature, the faster was the fish growth. However, after day 50, until the end of the experiment, the 25°C group grew faster than the 28°C group and other groups (Figures 30.8A, B).

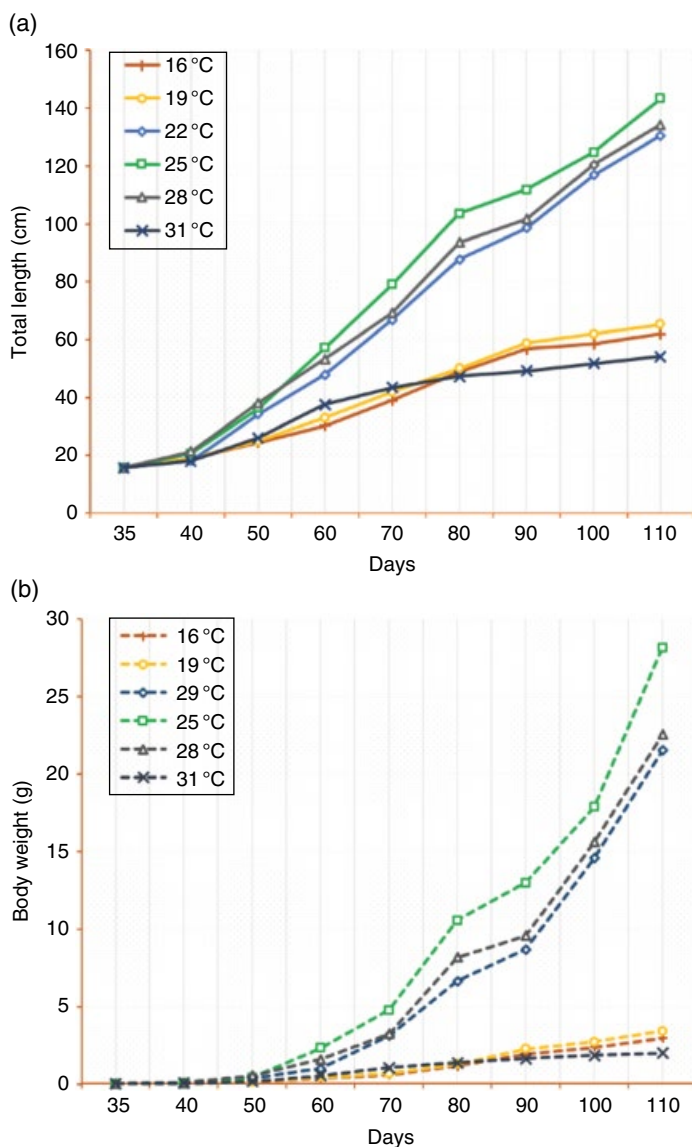


Figure 30.8 The total length and body weight of heterozygous clonal Japanese flounder, *Paralichthys olivaceus*, in different water temperatures from 35–110 dph. A – total length; B – body weight. Modified from Tang *et al.* [52].

From 16°C to 28°C, the higher the temperature, the faster was the sex differentiation, whereas gonad development was arrested in the 31°C group. The heterozygous clones cultured in water of different temperatures were all female, as determined by histological observation (Table 30.1), which suggested that high water temperature had no effects on sex reversal [52]. These results contrast with those from a previous study on temperature-induced sex

reversal in Japanese flounders (see Section 30.4). The results of this study indicated the possibility of selecting a new variety that is suitable for culturing at a relatively high temperature (25°C), with high growth rate, while retaining all-femaleness. The stability of gender in heterozygous clones at high temperature also provides a new perspective for studying the mechanism of sex determination and differentiation in the Japanese flounder.

Table 30.1 Sex ratios of heterozygous clonal Japanese flounder, *Paralichthys olivaceus* in water of different temperature [52].

Group	No. of fish sampled at 90 dph	No. of females	No. of males	Undifferentiated
16°C	30	28	0	2
19°C	30	30	0	0
22°C	30	30	0	0
25°C	30	30	0	0
28°C	30	30	0	0
31°C	30	0	0	30
Total	180	148	0	32

Both homozygous and heterozygous clones are valuable for aquaculture application and basic research. Because specific traits are fixed, the homozygous clones are more suitable for detecting the mechanisms that control certain traits, and can also be used as brood stock. Heterozygous clones have more advantages for commercial culture, due to their excellent performance.

30.4 Sex Control

In Japanese flounder, manipulation of sex differentiation characters offers the possibility of producing all-female seedlings that are favorable for commercial culture, owing to the higher growth rate of females compared with males. In Japan, Yamamoto used a sex steroid (17 α -methyltestosterone) or temperature (27.5°C) to induce the sex-reversal of genetic females to functional males. All-female eggs were produced on a large scale by natural spawning in tanks containing normal females and pseudo-males [1, 8].

In China, by using a combination of artificially induced meiotogynogenesis and sex reversal, we produced a new variety of Japanese flounder, named “Beiping No. 1” (Figure 30.9). We first optimized the parameters for temperature treatment to induce pseudo-males. On the basis of histological sections, we made observation on sex differ-

entiation and gonad development. At 30 dph (total length: approx. 9.7 mm), the primordial gonad was formatted. At 46 dph (total length: approx. 27.3 mm), the primordial germ cells could be observed, but sex was still undifferentiated; at 50 dph (total length: approx. 23.7 mm), the gonad differentiated into testis; at 95 dph (total length: approx. 96.0 mm), the gonad developed into stage I testis; at 65 dph (total length: approx. 56.1 mm), the gonad differentiated into ovary; at 105 dph (total length: approx. 127.8 mm), the gonad developed into stage I ovary.

According to the characteristics of sex differentiation, we started the temperature treatment at 25 dph, and the water temperature was increased from 16°C to 19°C, 22°C, 25°C, 28°C or 31°C. After 75 days of culture, the water was restored to normal ambient temperature. The results indicated that the 16°C and 19°C groups had a female ratio of more than 80%, whereas the 28°C group had a male ratio of more than 80%. The gonads of individuals in the 31°C group were un-differentiated, and it was impossible to identify the gender. Thus, in our experience, culturing fish at 25 dph at 28°C for 75 days represent the optimum conditions for pseudo-male induction [53].

The pseudo-males and normal females were then reared in the same tank for spawning eggs. Theoretically, progeny that

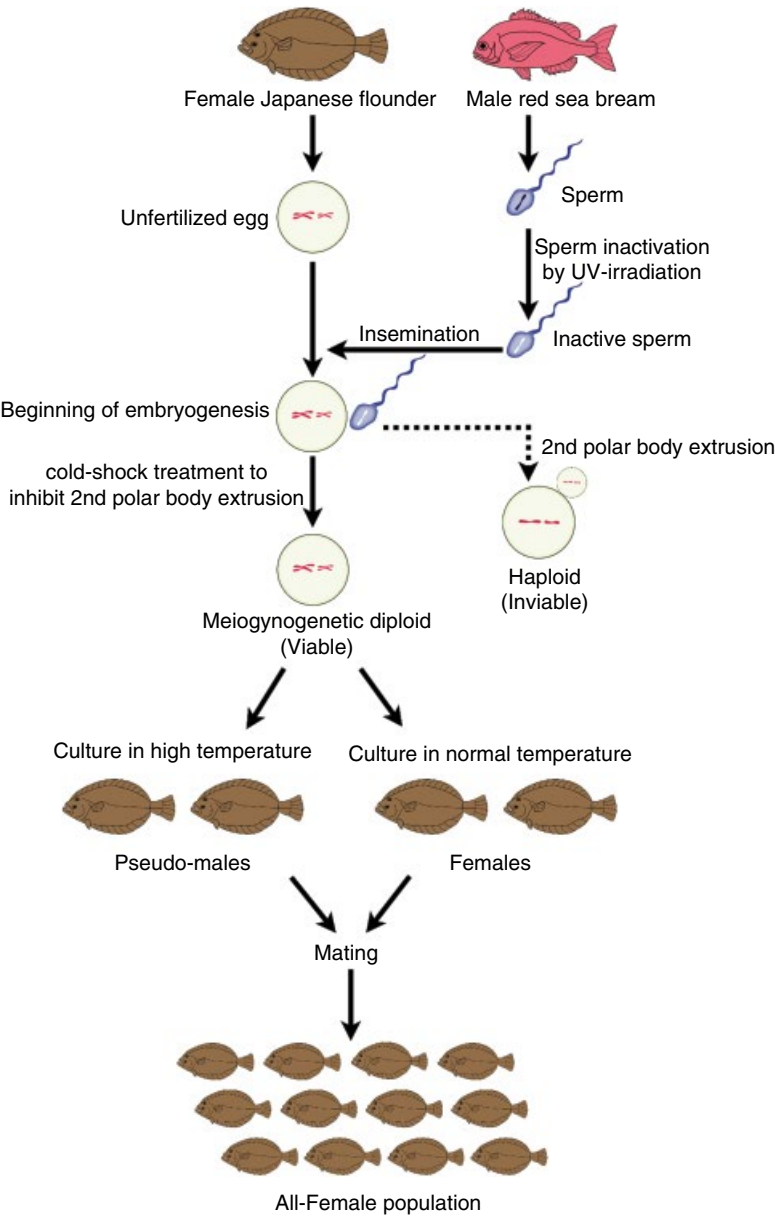


Figure 30.9 Illustration of the breeding technology of the new all-female variety of Japanese flounder, *Paralichthys olivaceus* "Beiping no. 1."

hatch from these eggs will all be female; however, due to the effects of some environmental factors, some individuals could change their gender from female to male. Nevertheless, the female ratio of the progeny is high at 90%. In addition to a high female ratio, the "Beiping No. 1" variety has

advantages such as similar body shape, high survival ratio, and 20% faster growth than the unselected fish. On the basis of our experience with "Beiping No. 1," we bred a new variety of Japanese flounder, "Beiping No. 2," which is a single hybrid. The founders of this variety

were artificially induced by gynogenesis, and then selected using the pedigree-breeding method. Finally, following selection, a single hybrid was obtained by the hybridization of two excellent gynogenetic founder families. This single hybrid shows heterosis and higher genetic similarity when compared with unselected Japanese flounders. The female ratio of “Beiping No. 2” is 90%, two times higher than that of the unselected fish, and the growth speed is 35% higher than that of unselected fish.

Using the single hybrid method, two inbred lines are hybridized to select improved varieties, and this is one of the most important methods used in crop breeding. Using this method, we produced, for the first time in fish, a single hybrid based on gynogenesis. The effects of the single hybrid method are significant, and this method has incomparable superiority to the other breeding methods used for fish [54].

Key information about sex determination and differentiation of Japanese flounder is summarized in Box 30.1.

30.5 Perspectives

After many years of study on the induction of gynogenesis and sex manipulation in Japanese flounder, we proposed the gynogenesis breeding system for fish.

The gynogenesis breeding system comprises all-femaleness, improved breeding, and cloning. All-femaleness refers to the induction of gynogenesis and pseudo-males, and facilitates large-scale production of feminized seed through the mating of females and pseudo-males. This method is particularly effective and necessary for species in which the female is larger than the male. Improved breeding refers to the selection of excellent gynogenetic and pseudo-male families and hybrids to produce single hybrids, and then selecting the optimal hybrid combination. Single hybrid is a term used in crop breeding, and refers to the offspring produced by the hybridization of two inbred lines. Most of the new varieties of corn are bred using the single hybrid method. One obvious feature of single hybrids is heterosis, whereas others are the uniform specifications and strong vitality. These latter two features are essential for animal breeding. In fish, the genetic similarity of a meiogynogenetic family is equivalent to 8–10 full-sib hybrid generations.

By using the gynogenesis method, we can considerably shorten the breeding cycle, whereas traditional breeding methods are associated with both huge economic (e.g., breeding grounds, labor costs) and time (generations) costs. The single hybrid method applies not only to species in which the female is larger than the male, but also

Box 30.1 Key information about sex determination and differentiation of Japanese flounder

Type of sex determination:

The Japanese flounder has an XX/XY genetic sex determination system; however, the sex determination gene has not been detected. Its sex differentiation is unstable, which could be because of the influence of environmental factors [1].

Beginning and end of sex differentiation:

At 30 dph (total length: approx. 9.7 mm), the primordial gonad is formatted. At 50 dph (total

length: \approx 23.7 mm), the gonad differentiates into testis; at 95 dph (total length: \approx 96.0 mm), the gonad develops into stage I testis. At 65 dph (total length: \approx 56.1 mm), the gonad differentiates into ovary; at 105 dph (total length: \approx 127.8 mm), the gonad develops into stage I ovary.

Induction of pseudo-male:

Culturing fish at 25 dph at 28°C for 75 days, and the expected male rate is more than 80% [53].

to species in which the differences between females and males are not significant. This method is also effective because of the obvious heterosis and uniform specifications of hybrids, which could meet breeding targets. Cloning refers to the initial production of homozygous clones by gynogenesis or androgenesis, and then crossing the female and male clones to produce heterozygous clones. The heterozygous clones can maximize the heterosis of traits that may be of interest to commercial aquaculture.

The gynogenesis breeding system may not only be applicable to flatfish, but could also be used for other types of fish, including freshwater fish. This not only is an effective way to select new varieties for aquaculture, but also provides ideal materials for genetic mapping and genomics research.

Acknowledgments

The authors wish to express their thanks to all the members of the Beidaihe Central Experiment Station, Chinese Academy of Fishery Sciences, headed by Prof. Ligeng Yang, for their hard work over the duration of this study. This study was financially supported by the earmarked fund for Modern Agro-industry Technology Research System (CARS-47), the National Key Technology R&D Program (2006BAD01A12017), the Transformational Fund of Agricultural Scientific and Technological Achievement (2006GB23260365), the Special Fund for Agro-scientific Research in the Public Interest (200903046) in China, and the State 863 High-technology R&D Project (2012AA10A408-5).

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31

Sex Determination, Differentiation, and Control in Atlantic Halibut and Pacific Halibut

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31.1 Introduction

Although the term “halibut” is associated with many flatfish species, it has historically and most commonly been used to refer to two congeneric right-eyed flounder species (family Pleuronectidae): the Atlantic halibut (*Hippoglossus hippoglossus* L.) and the Pacific halibut (*H. stenolepis* Schmidt). Both are demersal marine species, inhabiting temperate regions of roughly the same latitudes in the North Atlantic and North Pacific, respectively. Capture fisheries for Atlantic halibut showed a steady decline from 20,000 metric tonnes in 1960 to 3,300 tonnes in 1998, but have since gradually recovered to 7,300 tonnes in 2015 [1]. The collapse of the capture fisheries spurred interest in Atlantic halibut aquaculture in the late 1990s, with peak production of 2,900 metric tonnes in 2011, but farmed production has since declined to 1,300 metric tonnes by 2015, coincident with slowly recovering capture fisheries [2]. Capture fisheries for Pacific halibut have been characterized by cyclical collapses and recoveries, most recently with a steady decline from 44,000 tonnes in 2002 to 19,000 tonnes in 2015 [1]. To date, there has been no commercial aquaculture production of Pacific halibut.

There is abundant evidence that female Atlantic halibut grow faster as juveniles, and

mature later (and at a larger size) than males in aquaculture systems, therefore making female the desired sex for production purposes, in order to minimize time to harvest [3]. Fisheries data for wild-caught Pacific halibut have shown a temporal change in sex ratio of harvested animals, from approximately 1 : 1 in 1985, to a strongly female-biased sex ratio now. This is apparently due to slowing growth rates in both sexes, and subsequent increasing disparity in the age at which the sexes are harvested, to the extent that age-related mortality may be limiting the numbers of males that survive to harvestable size [4].

In contrast to aquaculture, where female-biased halibut populations would be seen as a positive attribute, a shift to female-biased harvests of wild fish could precipitate a rapidly increasing rate of population decline, especially if those females have not reached sexual maturity. The interest in sex control in halibut is, therefore, very different for the two species: for Atlantic halibut, the goal is to develop methods to produce all-female populations for aquaculture whereas, for Pacific halibut, the goal is to determine what is driving the slowing growth rates of wild halibut and, therefore, skewing population sex ratios. In either case, information is needed on the genetic basis of sex determination, as well as the developmental stage at which undifferentiated gonads begin proceeding down the

pathway to becoming ovaries or testes. In addition to providing basic knowledge that can be applied to fisheries management, this information is needed to design effective treatments for changing functional sex for aquaculture applications [5].

31.2 Sex Determination

The fact that Atlantic halibut have a female-homogametic sex-determining system (Box 31.1), equivalent to the XX/XY mammalian system, was determined by examining sex ratios in experimental populations produced through uniparental maternal inheritance (i.e., gynogenesis) [6], a tool commonly used to identify the genetic mechanism of sex determination in fish [7, 8].

In order to produce gynogenetic populations of Atlantic halibut, milt was first collected from mature males, diluted in a non-activating extender solution (i.e., Atlantic halibut seminal plasma obtained by centrifugation of fresh milt, and then stored at -80°C for a minimum of two days prior to use), and then exposed to UV radiation. The optimum radiation dose is one that does not wholly destroy the ability of spermatozoa to swim and activate embryonic development, but is sufficient to cause conformational changes in DNA structure that prevent mitotic duplication of paternal chromosomes in the developing embryo.

This optimum dose was determined for Atlantic halibut through a series of

experiments that always began with standardizing milt density by dilution to 3.7×10^{11} spermatozoa/mL in extender. The first experiment assessed three further dilutions (1 : 20, 1 : 40, and 1 : 80 – i.e., 18.5, 9.25, and 4.625×10^9 spermatozoa/mL, respectively) at each of seven UV doses (spanning 0 to 1382 mJ/cm^2). This was followed by two experiments to minimize UV dose, both using only 1 : 80 dilution: one testing six doses from 0–432 mJ/cm^2 , and the second testing nine doses from 0–173 mJ/cm^2 .

In all cases, diluted milt was spread in a thin layer in a glass dish and mixed with a magnetic stir bar during UV exposure. This is necessary because UV radiation has poor penetrating power through water. Diluted milt was kept cold by conducting the entire procedure in a cold room. A germicidal UV lamp (254 nm) was used for irradiation, and actual radiation intensity at the surface of the milt sample was confirmed using a UV meter. From these experiments, it was determined that the optimum milt treatment is dilution to 4.625×10^9 spermatozoa/mL followed by a UV dose of 65 mJ/cm^2 [6].

Milt treated in this way yields gynogenetic haploids when used for *in vitro* activation of embryonic development in eggs. Although haploids often progress through early embryonic development, they do not survive for long. Restoring viability in gynogens requires the creation of diploids, generally through the use of hydrostatic pressure treatment to block the completion of meiosis in the maternal genome and, thereby, retain the haploid second polar body. This protocol results in the production of gynogenetic diploids (i.e., fish that have the correct diploid chromosome number, but with their entire chromosome complement inherited from the mother).

In order to determine the optimum time at which to apply hydrostatic pressure treatment for retention of the second polar body in Atlantic halibut, experiments were conducted using treatments of 5 minutes at 58.6 MPa applied at 5, 15, or 25 minutes post-fertilization (mpf) and incubation at 5°C , using

Box 31.1 Sex determination

Sex determination is clearly genetic (female homogametic) in Atlantic halibut, with no evidence of any environmental component. Sex determination is less well understood in Pacific halibut, but there is some evidence for a female-heterogametic system. A number of sex-linked loci have been identified in both species, but no sex-determining genes have been found as yet.

untreated milt [6]. Retention of the second polar body in this case should yield triploids, which are easy to identify, and serve as a useful marker of treatment success. Although fertilization rate was not significantly different among control and pressure treatment groups, the highest absolute fertilization rate (71%) was obtained in the groups that were pressure-treated 15 minutes post-fertilization (mpf), compared to 68 % for the control groups and 62–63% for the two other pressure-treated groups.

Survival rates also were not significantly different among treatments, but the best result among the pressure-treated groups was, again, for the ones treated at 15 mpf (47%, compared with 48% for the controls and 31% for both other pressure-treated groups). Triploid yields from these treatments were 0% for the controls and 95%, 88% and 93% for the 5–10, 15–20 minutes, and 25–30 minutes pressure treatment groups, respectively.

Having optimized these treatments, gynogenetic diploid populations of Atlantic halibut were produced by diluting milt to 4.625×10^9 spermatozoa/mL, exposing this diluted milt to a UV dose of 65 mJ/cm^2 , then using the UV-treated milt to activate development in eggs and, finally, pressure-treating the eggs for 5 minutes at 58.6 MPa, beginning 15 mpf and incubation at 5°C. Standard culture methods for Atlantic halibut were then used to rear the fish.

Genotyping with microsatellite DNA markers was used to confirm the absence of the paternal genome in fish identified as gynogenetic diploids. All gynogenetic fish were confirmed to be females, both when assessed by histology when they were nine months old and, again, by macroscopic examination of the gonads when they were 21 months old [6]. Given the way by which these populations were produced (exclusion of the paternal genome and duplication of the maternal genome), this provides clear evidence of a female-homogametic sex-determining mechanism in Atlantic halibut. This was subsequently confirmed by showing that 100%

female offspring result from crossing functionally masculinized genetic females (neomales) with normal females [9, 10].

There has been no definitive study to confirm the sex-determining mechanism in Pacific halibut. However, combined data for allelic variation in three sex-linked microsatellite DNA loci are suggestive of a female-heterogametic sex-determining system in this species [4], equivalent to the W/Z/ZZ avian system and in contrast to the clear female-homogametic system in Atlantic halibut (Box 31.1). If correct, this means that there has been rapid divergence in the sex-determining systems of these two closely related species.

31.3 Sexual Differentiation

There is no published description of sexual differentiation in Pacific halibut. In Atlantic halibut, anatomical and cytological differentiation of the gonads has been detailed [11]. Their study used formalin-fixed fish, and the following size measurements therefore likely underestimate the size at which specific processes associated with sexual differentiation of the gonads occurs *in vivo*.

Germ cells are present in larvae as small as 10 mm fork length (FL), and primordial gonads can be seen by the end of the yolk-sac stage, when fish have reached 20 mm FL. At this time, the gonads are located in the posterior peritoneal cavity, below the kidney, and are

Box 31.2 Sexual differentiation

Ovarian differentiation is apparent in Atlantic halibut shortly after metamorphosis, with the appearance of an enclosed ovarian cavity by 38 mm FL, and is completed by 92 mm FL with the first appearance of primary oocytes. Differentiation of the testes begins when fish are 75 mm FL, and is completed by 100 mm FL. No information is available on the timing of sexual differentiation in Pacific halibut.

mostly comprised of connective tissue. They remain morphologically and cytologically undifferentiated during metamorphosis but, by 38 mm FL, an enclosed ovarian cavity, which is taken as definitive evidence of anatomical differentiation of the ovaries, is present (Box 31.2). This corresponds to the size at which the postlarvae have completed metamorphosis and have adopted the juvenile, benthic lifestyle. Oogonia are present by this stage, but with no evidence of meiotic division. The growing ovaries then begin to extend into the caudal muscle wall and, by 92 mm FL, oocytes are clearly evident (Box 31.2).

Differentiation of the testes occurs later in males, with the first signs of morphological differentiation at around 75 mm FL, and clusters of spermatogonia present by 100 mm FL (Box 31.2). There was no evidence of intersex gonads in this study, thereby indicating that the Atlantic halibut is a differentiated gonochoristic species (i.e., with undifferentiated gonads developing directly into ovaries or testes rather than through intermediate stages).

Photomicrographs included in the study by van Nes and Andersen [12] showed the formation of an ovarian cavity by 9 mm FL, and the presence of clusters of spermatogonia by 10 mm FL in Atlantic halibut, indicating that morphological differentiation of the gonads not only occurs simultaneously in both sexes, but also much earlier than suggested by Hendry *et al.* [11]. The reason for this discrepancy between studies is unknown, and was not commented upon by the authors.

A study of expression patterns of *cyp19a*, which encodes ovarian cytochrome P450 aromatase, found that individuals could be separated into two groups throughout early development (hatch to metamorphosis), with some individuals having high whole-body levels of mRNA, and others having none detectable [13]. The principal role of this enzyme is to convert endogenous testosterone to the feminizing hormone estradiol-17 β (E₂), thus mediating ovarian differentiation of the undifferentiated gonads. Thus, despite the sex of the fish being unknown, it is

tempting to equate apparent presence/absence of *cyp19a* expression at this time (i.e., during the period preceding and including when Hendry *et al.* [11] described morphological differentiation of the ovaries) with the sex of the fish. However, the proportion of each temporal sample that expressed *cyp19a* was generally higher than expected (i.e., 50% in just two of the seven developmental stages sampled between hatch and metamorphosis, and 70–80% in the others [13]).

A similar study [14] did not find this *cyp19a* presence/absence effect in similarly sized fish at the same life history stages as van Nes *et al.* [13], although there was a huge variation in expression among individuals (>1000-fold difference between lowest and highest). However, they observed that the ratio of abdominal *cyp19a* to *cyp19b* (i.e., the brain cytochrome P450 aromatase gene) increased in approximately half the population after ovarian differentiation, in fish between approximately 35–60 mm standard length (SL). Although it is possible that this difference in ratio of *cyp19* paralog expression was related to sexual differentiation, this could not be confirmed, because the sex of the fish was not known.

The only other study to examine *cyp19a* expression during sexual differentiation in Atlantic halibut examined the effect of rearing temperature during sexual differentiation on gene expression and sex ratio [12]. In this case, mRNA levels were measured at 15 mm SL (start of exogenous feeding), 25 mm SL (weaning onto prepared feeds, and close to the completion of metamorphosis), and an intermediate stage. As in the other studies, the sex of the fish was not known at the time of sampling, but average *cyp19a* expression levels were significantly higher at the latter two developmental stages in the treatment group that was subsequently shown to have the highest proportion of females (49%), compared with the two other treatment groups (42% and 38%).

Similar to these *cyp19* expression studies, Bizuayehu *et al.* [15, 16] examined expression

patterns of microRNAs (small non-coding RNA molecules associated with posttranscriptional regulation of gene expression) during early development, including sexual differentiation, in Atlantic halibut. Their first study [15] showed that, as expected, many microRNAs change their expression patterns prior to, during, and after sexual differentiation, but there was no indication of a bimodal pattern that might indicate a sex-specific role for any of them in the development of ovaries or testes.

A follow-up study [16] took advantage of fish that had been treated with a synthetic androgen or an aromatase inhibitor for six weeks, beginning when the fish were 30 mm TL ([17]; see section 31.4 for details). In this case, fish were sampled at the end of the treatment, and again two weeks later. The expression of one specific microRNA (miR-202-3p) was shown to be significantly lower in the gonads of untreated females than in untreated males at both times, and was also significantly higher in untreated females at the second sampling compared with the first. The expression of miR-202-3p in the gonads of treated fish (both synthetic androgen and aromatase inhibitor) was not significantly different from either untreated males or females at either sampling stage. This would indicate that elevated androgen levels, either alone or in conjunction with lowered estrogen levels, remove the suppression of gonadal miR-202-3p expression around the time of sexual differentiation.

31.4 Sex Control

The interest in halibut sex control comes from two quite different perspectives: for Pacific halibut, it is to develop a better understanding of what is driving temporal shifts in the sex ratio of commercially harvested wild populations [4] whereas, for Atlantic halibut, it is to develop protocols for the production of all-female populations for aquaculture, in order to take advantage of their faster growth compared to males [3, 9].

In species where female is the homogametic sex, as is the case for Atlantic halibut, crosses between neomales and normal females can be used to produce all-female populations, with neomales produced by exposing fish to masculinizing agents at the time of ovarian differentiation [5, 8]. Three studies have used this approach to produce Atlantic halibut neomales (Box 31.3). The first of these [18] used the synthetic androgen 17 α -methyl-dihydrotestosterone (MDHT) as the masculinizing agent, by incorporation in the diet at either 1 or 5 mg/kg, and then feeding it to juveniles for 45 days, beginning when they were 30 mm FL (i.e., starting when their gonads were still undifferentiated and ending after formation of the ovarian cavity) [11]. Histological examination of the gonads of these fish 41 days posttreatment showed every sampled fish to be developing normal testes – that is, 100% sex reversal of genetic females was achieved with each MDHT concentrations, compared with 47% male population in the control.

However, when these fish were reassessed almost a year later, small numbers of females were found in the MDHT treatment groups. Although this suggests that early sexing by histology was not completely accurate, all males appeared to be developing normally, including presumptive neomales. Some of these males were subsequently used to create separate families from each individual, by collecting its milt and using it to fertilize a separate batch of eggs and, in this way, neomales were identified by demonstrating that only female progeny occurred in the families that they had sired [9]. Not only does this

Box 31.3 Sex reversal

The optimum treatment for masculinization of Atlantic halibut for the production of neomales is by feeding MT (5 mg/kg feed), MDHT (1 mg/kg feed), or fadrozole (100 mg/kg feed) for six weeks, beginning when fish are 30 mm FL or 40 mm TL. Sex reversal of Pacific halibut has not been reported.

provide further conclusive evidence of an XX/XY-type of sex-determining mechanism in Atlantic halibut, it also shows that neomales are fully functional, and can be used for breeding. Neomale Atlantic halibut have been used for several years by a Canadian company (Scotian Halibut Ltd.) to produce all-female halibut populations for commercial aquaculture [9].

A second study aimed at producing neomales [17] also used feeds containing masculinizing agents but, in this case, either the synthetic androgen 17 α -methyltestosterone (MT) or the aromatase inhibitor fadrozole. The rationale for testing the latter compound was to replace treatments that use exogenous steroids, such as MT or MDHT, with one that results in different levels of endogenous steroids, specifically by blocking the feminizing effects of estrogens produced *in vivo* via the cytochrome P450 aromatase pathway.

Treatments began when fish were 30 mm total length (TL), and continued for either 60 days (first experiment, fadrozole at 500 mg/kg only) or 42 days (second experiment, MT at 5 mg/kg or fadrozole at either 100 or 700 mg/kg). Treatment efficacy was determined by macroscopic examination of the gonads of fish sampled approximately five months later, when the fish exceeded 150 mm TL, and again by histology approximately 10 months (first experiment) or five months (second experiment) after that, when average fish length exceeded 300 mm. These two assessment methods gave very similar results – namely, 100% male populations with MT treatment and 93–100% male populations with fadrozole treatments, compared with 42–54% male for the control populations. Although none of these males were used for breeding purposes, they appeared to be developing normally, and many were strippable.

The third study to produce Atlantic halibut neomales [10] used in-feed MDHT treatments at 10 and 5 mg/kg, in both cases beginning when fish were 40 mm TL and then continuing for either three or six weeks, respectively. Treatment success was determined by sexing fish histologically when they

were one year old. Both treatments resulted in a significant change in sex ratio, compared with the control population (52% male), increasing to 70% male and 97% male, respectively. Progeny testing from four males derived from the 5 mg/kg treatment group revealed all-female offspring sired by two of them (i.e., neomales) and 1 : 1 sex ratio for the offspring from the two others (i.e., normal males).

From a brood stock management perspective, neomales should, ideally, be functionally indistinguishable from normal males, in order to facilitate the collection of their milt. However, in the absence of sex-specific genetic markers, the only way to identify neomales within a population that also contains normal males is to examine the sex ratio of each individual male's offspring. This "progeny-testing" approach was used for commercialization of all-female Atlantic halibut production, due to the lack of sex-specific markers at the time [9]. Finding reliable sex-specific markers has been challenging in flatfish, and those that have been identified vary considerably among species in their chromosomal locations [19]. Restriction-associated DNA (RAD) sequencing has since been used to identify a sex determination locus in Atlantic halibut and, on this basis, to develop a panel of single-nucleotide polymorphism (SNP) markers to accurately predict sex in this species [10].

In the case of Pacific halibut, a survey of spatial and temporal genetic variation identified three sex-linked microsatellite DNA loci that, when used simultaneously, accurately predicted sex in 92% of the sampled populations [4]. Further research with these same markers has confirmed their reliability for determining the sex of Pacific halibut, with a high degree of accuracy [20]. Interestingly, none of these three loci showed sex-linked patterns in Atlantic halibut, in spite of this species being the origin of the primer sequences used [4].

As an alternative to the neomale approach for producing all-female populations of Atlantic

halibut, Hendry *et al.* [18] also tested the efficacy of dietary E_2 at 10mg/kg for the direct feminization of fish, using the identical experimental approach as for MDHT. This was done because the sex-determining mechanism was unknown at the time the research was conducted and it was, therefore, uncertain whether the neomale approach would be an effective way to generate all-female populations.

Although E_2 treatment did change the sex ratio of the population (three females for every male, compared to 1 : 1 in the control), this approach of direct feminization was not pursued any further once it became apparent that neomales could be used to create all-female populations [9]. However, if Pacific halibut do, indeed, have a female-heterogametic sex-determining system, equivalent to the avian WZ-female/ZZ-male system, then the production of functionally feminized males (“neofemales”) should allow the production of all-male populations, by crossing neofemales (i.e., ZZ genotype) with normal males [5]. Alternatively, if the desired goal is all-female populations, this could be achieved through a two-step process beginning with the production of neomales (WZ genotype), crossing them with normal females, and then selecting the “superfemales” (WW genotype, theoretically $\frac{1}{4}$ of the population) for breeding with normal males [5].

Although neomales are routinely used in aquaculture for the production of all-female populations [5, 8], the fact that it requires the administration of synthetic compounds has led to the search for more “organic” methods for manipulating sex ratios. Of particular interest in this regard is the fact that several flatfish species exhibit temperature-dependent sex determination (TSD), whereby the incubation temperature during sexual differentiation of the gonads affects the sex ratio of the population [21].

Two studies have attempted to manipulate sex ratio in Atlantic halibut in this way. In the first, fish were reared at 7°C, 10°C, or 13°C for the growth interval from 15–25mm SL [12]. Increasing temperature appeared to have a masculinizing effect, with the proportion of

females within the populations declining from 48.8% at 7°C, to 41.6% at 10°C, and 37.7% at 13°C. However, this effect was not statistically significant. A subsequent study, which reared fish at 7°C, 12°C, or 15°C through a later growth interval, from 29–80 mm TL, found no evidence of a temperature effect on sex ratio, with none of the treatments giving a significant difference from the others or from 1 : 1 [22].

Given the earlier onset of *cyp19a* expression in Atlantic halibut, it may be that the latter study, and perhaps even the former, began treatment too late to effect functional sex reversal. However, it is also possible that the colder environment inhabited by Atlantic halibut, compared with flatfish species which exhibit TSD, does not allow any adaptive advantage to TSD in nature [21, 22].

31.5 Conclusions

Atlantic halibut have been confirmed to have a female-homogametic sex-determining system by demonstrating that: (1) milt obtained from genetic females yields all-female offspring when used to fertilize eggs from normal females; and (2) gynogenetic diploids are always female. Furthermore, histological studies have shown that Atlantic halibut is a differentiated gonochoristic species, with ovarian differentiation preceding that of the testes. Ovarian differentiation appears to begin during the transition from yolk absorption to exogenous feeding, and is completed by the time the fish have metamorphosed into the benthic phase. Feeding juveniles synthetic androgens or aromatase inhibitors around this time can be used to produce functionally masculinized genetic females, and the milt obtained from such fish yields all-female offspring.

This approach has been commercialized for Atlantic halibut, providing one of the few examples where neomales have been integrated successfully into traditional aquaculture breeding programs. Compared with Atlantic halibut, little is known about sex determination or sexual differentiation in

Pacific halibut. However, they appear to have a female-heterogametic sex-determining system.

Acknowledgements

My contributions to the study of sex control in Atlantic halibut were only possible through team work that involved dedicated

graduate students and government scientists, and with the generous support of government funding agencies and industry partners. I especially want to thank my students Chris Hendry, Vikki Hughes, and Harald Tvedt, research colleagues Debbie Martin-Robichaud, Makoto Matsuoka, and Mike Reith, and industry partners Brian Blanchard and Melissa Rommens (both with Scotian Halibut Ltd.).

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Sex-Specific Markers, Gynogenesis, and Sex Control in Spotted Halibut

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32.1 Introduction

Spotted halibut (*Verasper variegatus*), a rare marine flatfish species with right eyes, is mainly distributed along the coastal areas of the Western Pacific Ocean, such as Northern China, Korea, and Japan [1–3]. This species has been broadly recognized as a promising candidate for aquaculture and fisheries resource enhancement in North Asia, due to its high commercial value [4]. The wild population status of spotted halibut is reported to be critical, with the amount harvested decreasing year by year [5]. Under wild conditions, the juveniles live in shallow intertidal zones, while the adults migrate to deeper sea water to prepare for maturation [6]. In practice, spotted halibut show sexual growth dimorphism where the females grow faster and their body size is bigger than the males (the body length of females could be up to 53 cm) [7]. Thus, it will be beneficial for aquaculturists to have the ability to develop an all-female stock in aquaculture, as it could make the body size of individuals similar, and increase economic incomes [8].

Monosex female strains have been generated in several aquatic species, such as chinook salmon (*Oncorhynchus tshawytscha*) [9], silver barb (*Puntius gonionotus*) [10], Mozambique tilapia (*Oreochromis mossambicus*) [11], brook

trout (*Salvelinus fontinalis*) [12], and Japanese crucian carp (*Carassius cuvieri*) [13]. In addition, a number of fish species are reported exhibiting sexual dimorphism between sexes [14], such as half-smooth tongue sole (*Cynoglossus semilaevis*) [15] and Japanese flounder (*Paralichthys olivaceus*) [16].

Molecular sex identification techniques are considered to be essential for production of a monosex population, but the genetic difference between sexes need to be clear in advance. By now, sex-specific markers have been found in several fish species, such as half-smooth tongue sole [17, 18], medaka (*Oryzias latipes*) [19], yellowtail fish (*Seriola quinqueradiata*) [20], catfish (*Pseudobagrus ussuriensis*) [21], and Pacific bluefin tuna (*Thunnus orientalis*) [22]. These sex-specific markers will facilitate the production of monosex strains of the above species.

A genetic linkage map is considered a valuable molecular tool for studies on comparative genomics mapping [23, 24], quantitative trait loci analysis [25, 26], and molecular marker-assisted selection [27, 28], as well as for sex determination [29]. Genetic linkage maps have been constructed in many aquatic species, and have facilitated research on sex differences and differentiation, such as in Atlantic halibut (*Hippoglossus*

hippoglossus) [30], rainbow trout [31], half-smooth tongue sole [32], and mud crab [33]. Among current popular molecular markers, the AFLP system could generate a number of polymorphic fragments for one time, so it is considered a good marker for genetic map construction in non-model organisms. Further, microsatellite markers are also thought to be an ideal system for mapping, due to their abundant, codominant, good transferability, and high polymorphism in animals.

Artificial gynogenesis is a powerful technique, which could generate monosexual populations by manipulating chromosome sets of animals with a XX-XY sex-determining system [34]. Meiotic gynogenesis is broadly employed in practice, because it is relatively easier to operate than mitogynogenesis [35]. Heterologous sperm is popular for induction of gynogenesis, because it could ensure that the live progeny are real gynogenetic diploids. In fact, meiotic gynogenetic diploids are not purely homozygous, and they usually show residual heterozygosity because of the recombination events [36]. For example, a total of 82% of meiotic gynogenetic diploids in Japanese flounder were reported to be heterozygous [37]. Hence, it is very important to evaluate the genetic diversity of induced diploids.

In this study, we first identified female-specific AFLP markers and developed a molecular sex identification method, then we constructed a male genetic linkage map, using AFLP and microsatellite markers. Finally, we developed an artificial meiotic gynogenesis method for spotted halibut. These findings should lay a good foundation for sex determination and sex control in flatfish.

32.2 Methods and Applications

For sex-specific marker isolation [38], a total of 148 individuals were collected, with weights ranging from 350–700 g. The phenotypic sex

was identified using a histological section and microscopic inspection of gonad tissues. Fifty offspring from an interspecific hybrid family (female barfin flounder and male spotted halibut) were sampled for testing the inheritance of sex-specific markers. Genomic DNAs were isolated from fin tissue using the traditional phenol-chloroform extraction method, and the concentration of DNA was adjusted to 100 ng/μl. The AFLP assay was performed using the instruction described in reference [39], with minor modification.

Two kinds of endonucleases, *EcoRI* and *MseI*, were used to digest the genomic DNA, followed by adding specific adapters to DNA fragments. The fragments were pre-enriched by PCR amplification with pre-amplification primer mixture. Further, the second PCR amplification was carried out with 64 selective AFLP primer combinations. Finally, PCR products were separated by 6% denaturing polyacrylamide gel. We were interested only in those bands present in a single sex, but absent in the other sex. These target bands were cut down from the gel with a razor, then put in a TE buffer. They were recovered by PCR amplification with the corresponding primers, and sequenced in biology company.

For genetic linkage map construction [40], an F₁ family was generated using one female barfin flounder and one male spotted halibut. A total of 81 offspring and both parents were sampled for linkage mapping. Genomic DNA was extracted by phenol-chloroform protocol. A total of 439 polymorphic microsatellite loci were genotyped in 83 specimens. For AFLP assay, *EcoRI* and *MseI* endonucleases and a total of 58 selective primer combinations were used. In this study, the pseudo-testcross strategy was used for map construction. The linkage relationships among molecular markers were calculated using *JoinMap* 3.0 software [41]. Markers which were in accordance with the expected Mendelian ratios were used for linkage analysis. A critical logarithm of odds (LOD) score threshold ≥ 3.0 was referenced for marker

assignment for different linkage groups. Linkage groups were drawn by *MapChart* 2.1 software [42].

The expected genome size (G_e) was estimated using the formula: $G_e = (G_1 + G_2)/2$ [43]. The expected genome size was equal to the total length of the revised linkage groups [44]. The observed map length (G_{oa}) was the total length of groups, triplets and doublets, and the estimated coverage of the genome (G_{oa}) was calculated as: G_{oa}/G_e .

For development of the gynogenesis method [45], we first placed the candidate females into a pool with constant photoperiod and water temperature for two months before the experiment. Eggs were obtained by massaging the abdomen of naturally mature females. The sperm of sea perch (*Lateolabrax japonicus*) was UV-irradiated with a dose of $30\text{--}50\text{ mJ cm}^{-2}$, as described [46]. We then set up two assays to find out the appropriate condition for induction of gynogenetic diploids.

For assay A, five groups of eggs were fertilized with 0.4 ml UV-irradiated sea perch sperm separately, and then put with cold sea water (-1°C) at 1, 3, 5, 7, and 9 minutes after fertilization for 75 minutes, respectively. For assay B, the five groups of fertilized eggs were cold shocked with cold sea water (-1°C) for 5 minutes after fertilization for 30, 40, 65, 75, and 90 minutes respectively. The eggs were then incubated in 1–1 incubators with sea water of 11°C . The survival rate of embryos at the 2–4 cells stage, and four days after hatching, compared with the original number of eggs, was calculated as the fertilization rate and survival rate. All experiments were performed three times.

Based on the optimized method (see Section 32.5), large-scale gynogenetic diploids were produced. A total of seven batches of eggs were induced to produce gynogenetic fry. The number of induced pre-hatching embryos and gynogenetic larvae were analyzed for each batch of eggs. The AFLP-based molecular sexing technique created previously was employed to identify the genetic

sex of 19 gynogenetic diploids. The genetic diversity of 20 gynogenetic diploids and 22 normal individuals was evaluated by microsatellite markers. The differences among groups were assessed by analysis of variance analysis (ANOVA). Genetic diversity indexes were calculated using *POPGENE* 1.31 software. Significances for multiple tests were corrected by sequential Bonferroni procedure [47].

32.3 Isolation of Sex-Specific AFLP Markers

Based on the microscopic inspection of histological sections, 88 female and 60 male specimens were identified. AFLP genotyping results showed two bands were present in all tested females, but were absent in all but three males (Figure 32.1), which suggested that both AFLP markers are female-specific in spotted halibut.

After sequencing, we found that the lengths of both bands were 533 and 218 bp respectively, so we named them as VevaF533 and VevaF218 separately. There was no sequence similarity between both bands, which indicated they were two different markers. Furthermore, no homology was observed between both markers and the known sequences from the GenBank database. Both sequences were submitted to GenBank under the accession numbers of FJ467937 and FJ467936. We tried to determine if there was a homology of VevaF218 in barfin flounder, but the results showed that no corresponding fragments were identified. Hence, VevaF218 is a species-specific AFLP marker which could discriminate female spotted halibut from barfin flounder. The inheritance assay (Figure 32.2) in an interspecific hybrid family indicated that VevaF218 marker was not present in any offspring, suggesting that this female-specific marker is passed on from female to female.

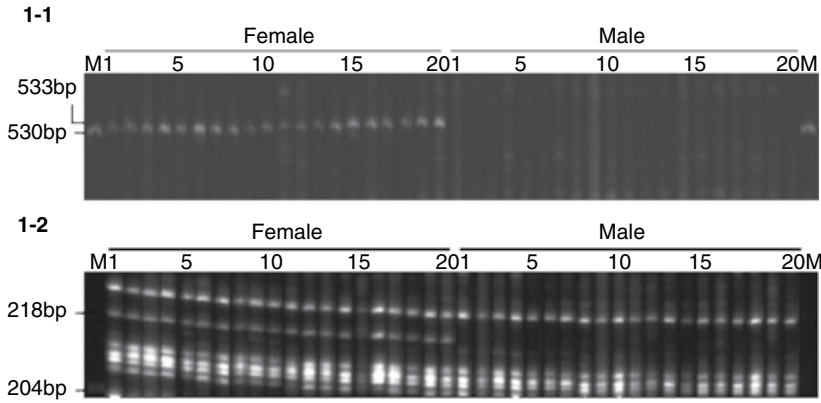


Figure 32.1 Two female-specific AFLP markers amplified using the primer combination M-CAG/E-ACC (1–1) and M-CAT/E-AGG (1–2). M, 50–700 bp sizing standard (cited from [38]).

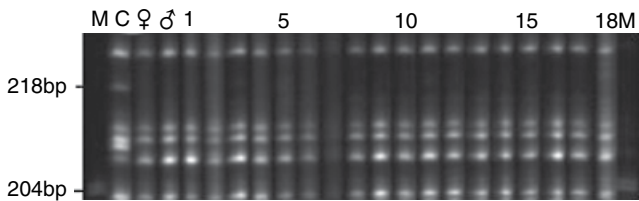


Figure 32.2 The amplification of the marker VevaF218 in an interspecific hybrid family. M, 50–700 bp sizing standard; C, positive control; ♀, the mother barfin flounder; ♂, the father spotted halibut; 1–18, the fingerlings (cited from [38]).

32.4 Construction and Characterization of a Genetic Linkage Map

Out of 439 genotyped microsatellite markers, 90 showed segregation in male spotted halibut, and 92 showed segregation in female barfin flounder. Thirteen and nine microsatellite markers significantly deviated from Mendelian ratio in male spotted halibut and female barfin flounder, respectively. Fifty-eight AFLP selective primer combinations were employed to genotype the mapping family, which generated 284 segregating bands. Of these segregated bands, 131 segregated from father to progeny, and 135 segregated from mother to progeny. Only 13 and 9 bands significantly deviated from the Mendelian ratio in male and female parents.

The male spotted halibut genetic map contained 24 linkage groups, 48 AFLP loci, and 38 microsatellite loci. Sixteen genetic groups included three or more markers. The maximum number of markers per group was seven, and the minimum number of markers

per group was two. All molecular markers were relatively evenly distributed on the linkage map, and no marker clusters were found (Figures 32.3, 32.4). The map of male spotted halibut was 625 cM in length, with a mean value of 10 cM between marker pairs. The lengths of linkage groups ranged from 1.3–52.5 cM, with a mean value of 26.0 cM. The expected genome size of spotted halibut was 1115 cM, with a coverage rate of 56% by the observed genetic linkage map.

32.5 Development and Evaluation of Gynogenesis Technique

Fertilization experiments showed that when spotted halibut eggs were fertilized by unirradiated or irradiated sperm of sea perch, the hybridized or haploid fry had a high malformation rate and died within four days. In contrast, the gynogenetic fry could survive over four days when eggs were fertilized by irradiated sperm of sea perch and had

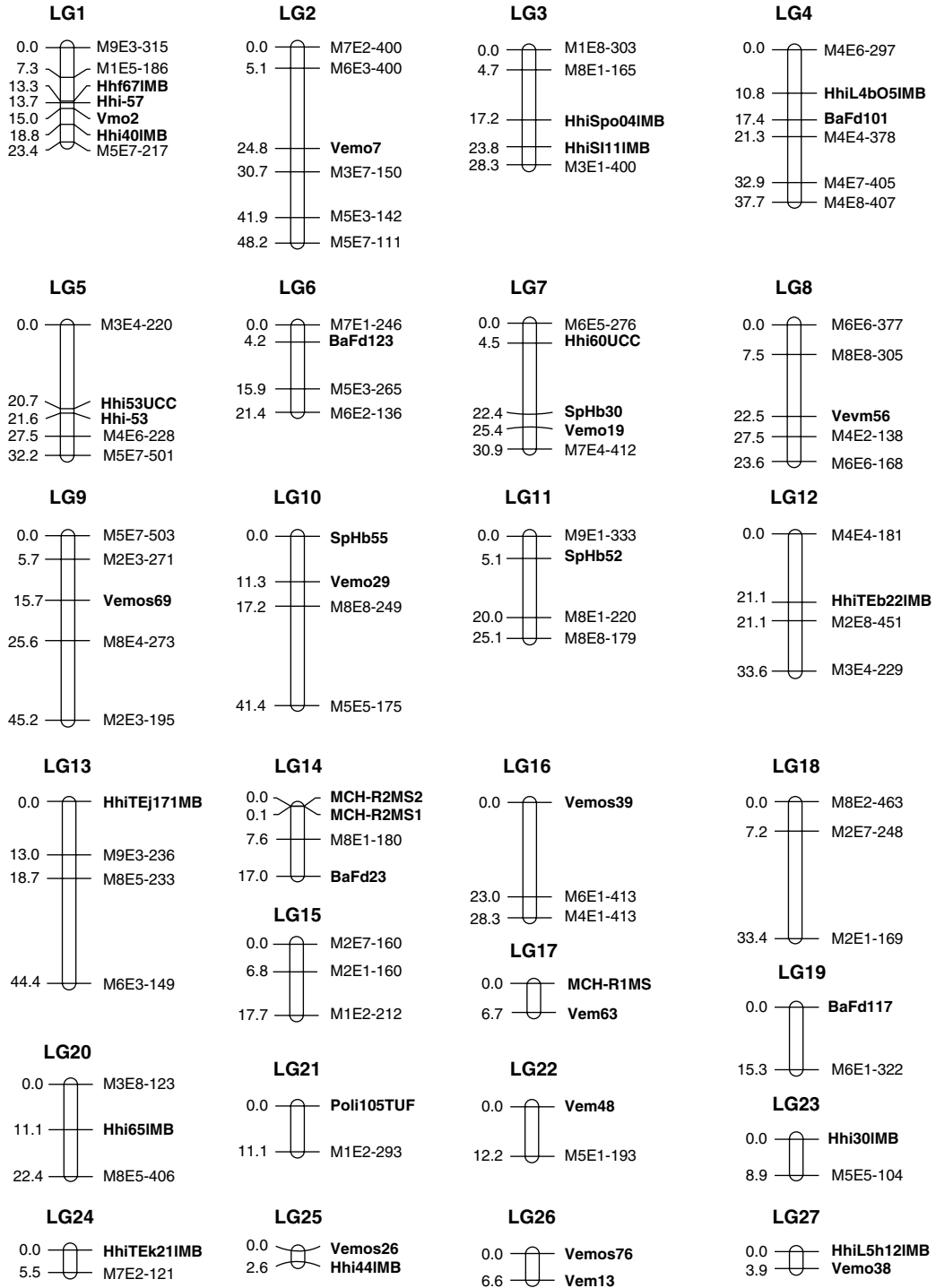


Figure 32.3 Female genetic linkage map of barfin flounder, based on AFLP and microsatellite markers. Markers are shown on the right of each group, and the genetic distance is listed on the left in Kosambi units (cM). AFLP markers are named after the primer used and the fragment size. Microsatellite markers are in bold (cited from [40]).

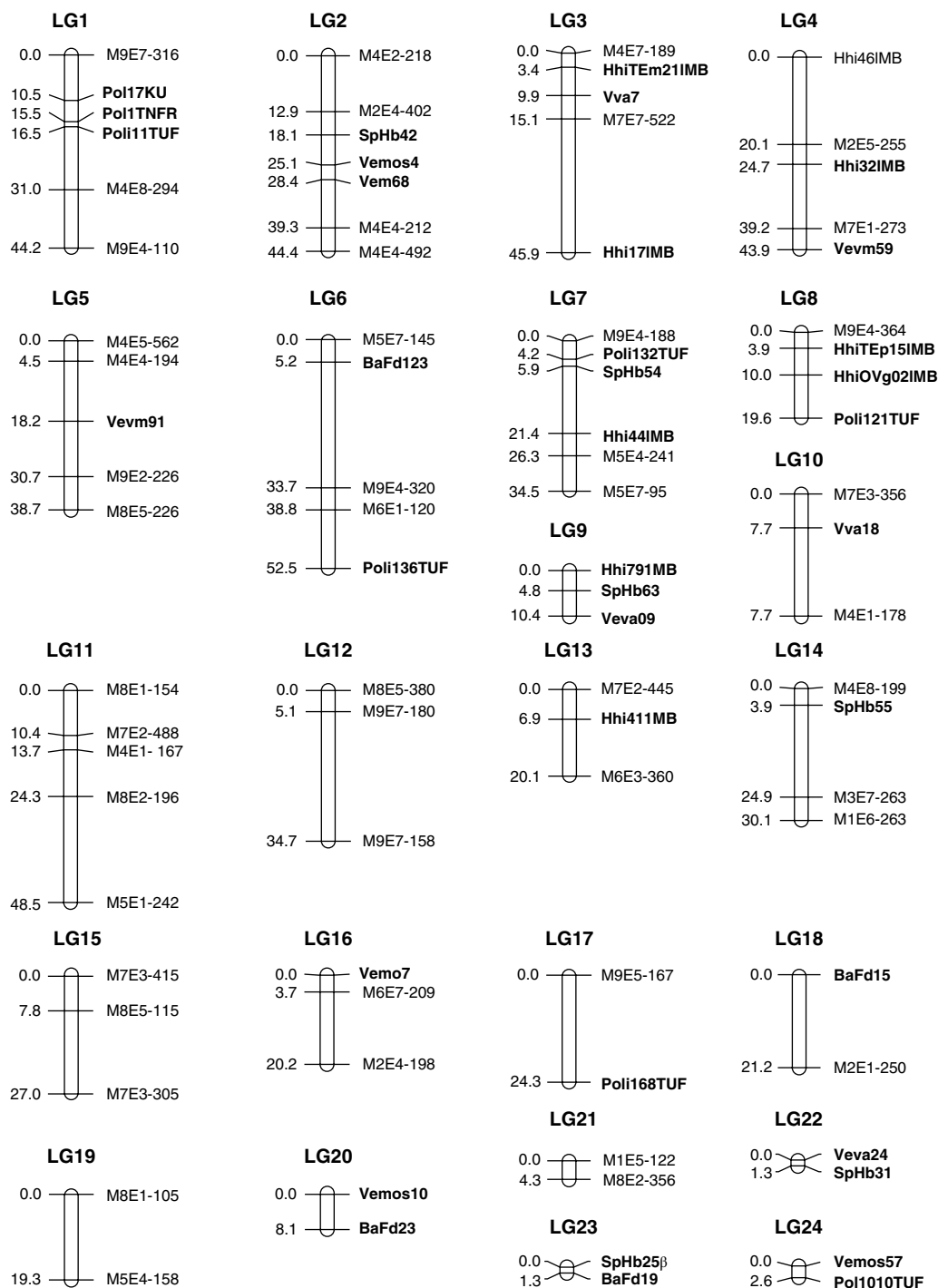


Figure 32.4 Male genetic linkage map of spotted halibut, based on AFLP and microsatellite markers. Markers are shown on the right of each group, and the genetic distance is listed on the left in Kosambi units (cM). AFLP markers are named after the primer used and the fragment size. Microsatellite markers are in bold (cited from [40]).

undergone cold shock. The highest survival rate of gynogenetic diploids (17.1%) was present only when cold shock took place at -1°C for 60 minutes and at 5 minutes after eggs were fertilized. When cold shock was set up at 9 minutes after fertilization, no survival was found (key information on sex determination and gynogenetic induction in spotted halibut is summarized in Box 32.1).

Using this optimized method, we found 85,100 induced pre-hatching embryos in large-scale production activity, with the rate of induced pre-hatching embryos in fertilized eggs between 2.0–18.5%. We finally obtained 15,200 diploids, which showed normal morphology and survived for several months. We identified the genetic sex of gynogenetic diploids by using the AFLP-based molecular sexing method developed in this study previously. The results showed that, out of 19 individuals, 17 were genetically female, while the other two individuals failed to be identified because of the poor DNA quality.

Meanwhile, we assessed the genetic diversity of these individuals, which showed that the observed heterozygosity at microsatellite loci Veva08 was zero in gynogenetic diploids, but was 0.565 in normal population. Totally, the average observed heterozygosity at seven microsatellite loci was 0.404 in the gynogenetic population, while it was 0.724 in the normal population. This result indicated that the homozygosity of the gynogenetic population

had been quickly increased. Moreover, the mean allele number of microsatellite loci was 4.5 and 7.1 per locus in gynogenetic and normal populations, respectively, and many alleles were lost in gynogenetic diploids.

32.6 Comparison of Current Technologies

AFLP technology can generate a large amount of genetic data in a short time without genome information in advance; hence, it has been used to identify sex-specific markers in several aquatic animals, such as half-smooth tongue sole [17], Pacific bluefin tuna [22], and rock bream (*Oplegnathus fasciatus*) [48]. In this study, we first isolated female-specific molecular markers from spotted halibut, using the AFLP fingerprint technique. Two female-specific AFLP markers were successfully identified, but both of them were also present in three phenotypical males. A possible explanation could be that these three individuals were naturally sex-reversed individuals that were still genotypic females. The sex chromosomes of fishes evolved on parallel pathways, and their sex determination is flexible with respect to evolutionary patterns among genera and families [49]. Besides, the recombination event between DNA loci and the sex determination region is thought to be the other potential reason.

There are multiple sex determination mechanisms in aquatic animals, in some cases even under the same genera. The presence of female-specific markers or DNA sequences suggests a female heterogamety mechanism (female: ZW; male: ZZ). The female-specific AFLP markers and female heterogamety were identified from half-smooth tongue sole [17]. In this study, none of the progeny inherited the VevaF218 marker, suggesting that the female-specific marker is inherited from mother to daughter, and further indicating that VevaF218 marker might be present on the sex chromosome of females. This result suggested that the sex determination mechanism of spotted halibut is female

Box 32.1 Key information

Female-specific AFLP markers were isolated, which suggested a ZZ/ZW sex determination system.

The genetic map of spotted halibut contained 24 linkage groups, which was 625 cM in length with an average value of 10 cM between marker pairs [40].

The protocol for gynogenetic induction was developed and optimized as follows: eggs were first fertilized by irradiated frozen sperm of sea perch, and then shocked by cold water (-1°C) for 60 minutes at 5 minutes after fertilization.

heterogametic. Moreover, the inheritance patterns of sex-linked DNA have also been reported in chinook salmon and rainbow trout [50, 51].

In this study, a first genetic map was constructed for male spotted halibut using pseudo-testcross strategy and AFLP and microsatellite markers. For the AFLP assay, the proportion of polymorphic bands is relatively lower than that in turbot (*Scophthalmus maximus*) [52], sea cucumber (*Apostichopus japonicus*) [53], and bluegill sunfish (*Lepomis macrochirus*) [54], which may be the result of a relatively low genetic diversity of spotted halibut [55].

The good applicability of cross-species of microsatellite markers was confirmed in this study. Forty-eight microsatellite loci from Atlantic halibut (*Hippoglossus hippoglossus*) and 22 from Japanese halibut (*Paralichthys olivaceus*) had good application in spotted halibut. The segregation pattern of markers significantly deviating from Mendelian ratio was often observed in genetic linkage analysis. In common carp (*Cyprinus carpio*), the deviation proportion was 16.5% for AFLP markers [56], whereas it was 85% in European flat oyster (*Ostrea edulis*) for AFLP markers [57], and 30% in zhikong scallop (*Chlamys farreri*) for microsatellite markers [58]. However, the proportion of deviated markers in our study was 8.3% for AFLP markers and 12.1% for microsatellite markers, respectively.

A number of factors may result in segregation deviation, such as small sample number, errors in genotyping, non-random segregation, selective pressure, and competition of gametes [58–60]. Furthermore, segregation deviations of markers may result from close linkage relationships between markers and deleterious genes, or chromosomal regions which could affect gametogenesis, fertilization, and embryogenesis [61].

A total of 24 genetic linkage groups were constructed in male spotted halibut. However, a previous study reported that there were 23 haploid chromosomes in spotted halibut [62]. Ideally, the number of linkage groups is supposed to be equal to the number of haploid

chromosomes. The mismatch between numbers may be caused by the existence of genetic gaps in linkage groups. As was found in this study, the differences between numbers of linkage groups and haploid chromosomes were also reported in some other animals, such as European sea bass (*Dicentrarchus labrax*) [63], sea urchin (*Strongylocentrotus nudus* and *S. intermedius*) [64], sea cucumber [53], and bluegill sunfish [54]. In addition, a relatively even distribution of AFLP and microsatellite markers was found in this linkage map, and no marker clusters were identified, which may be related to the low marker density and limited mapped markers in this genetic map.

In this study, artificial meiotic gynogenesis technology of spotted halibut was developed using UV-irradiated cryopreserved sperms of sea perch. So far, heterologous sperm has been used for induction of gynogenesis in many fish species, such as large-scale loach (*Paramisgurnus dabryanus*) [65], turbot [66], sterlet (*Acipenser ruthenus*) [67], and tench (*Tinca tinca*) [68], but little information about induction of diploid gynogenesis by heterologous frozen sperm is available [46]. In this work, the haploid chromosomes were induced for diploidization in cold sea water of -1°C for about 40–75 minutes at 5 minutes after fertilization. The start time of cold shock is affected by some factors, and it was different among fish species. For example, it is 2–4 minutes after fertilization for sarpunti (*Puntius sarana* (Hamilton)) [69], 6.5 minutes for turbot [70], and 20 minutes for shortnose sturgeon (*Acipenser brevirostrum* (Lesueur)) [71].

Sex determination mechanism of fish species is not only controlled by genetic factors, but also influenced by environmental factors [72]. The average ratio of females of gynogenetic diploids was observed to be 87.2% in honmoroko (*Gnathopogon caeruleus*); meanwhile, 3.0–35.3% of males were included [73]. In this study, the gynogenetic diploids were preliminarily determined to be genetical female by the molecular sexing technology.

It was reported that if there is no crossover, the heterozygous loci would change to homozygous because the segregation of allele was blocked [36]. However, with the existence of crossovers, the heterozygous loci would reach 67% [74]. In the present study, the genetic diversity of gynogenetic diploids was assessed by microsatellite markers. The results showed an abundant recombination rate, with the average H_O being 0.40. Similarly, the recombination proportion was found to be high in gynogenetic diploids of Japanese eel (*Anguilla japonica* (Temminck and Schlegel)) [75] and large yellow croaker (*Pseudosciaena crocea* (Richardson)) [76]. In contrast, the

tested loci were observed to be homozygous in two meiotic gynogenesis groups in grass carp (*Ctenopharyngodon idellus*) [77]. Further, a higher recombination rate was found in females than in males at the centromeric region [30].

Acknowledgments

This work was supported by the State High-Technology R & D Project of China (863) (2006AA10A403), the Taishan Scholar Project of Shandong Province of China, and the National Program for Support of Top-Notch Young Professionals.

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Part IX

Sex Determination and Sex Control in Sturgeon

33

Sex Determination in Sturgeon

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33.1 Introduction

From the 19th century on, sturgeon populations have decreased rapidly, and nowadays sturgeons are among the most endangered freshwater fish (IUCN, 2006). All 24 species of the family Acipenseridae are listed in the Red List of endangered species, and international caviar trade is controlled by CITES (CITES Conf. 12.7 Rev. CoP13). Over-fishing for caviar production, water pollution, and the destruction of the natural habitat have evoked this dramatic decline over the last century [1–3]. Nowadays, illegal fishing continues, and illicit trade of mislabeled caviar is rarely uncovered [4–6], but several restoration programs worldwide give cautious hope that some populations may recover [7–10].

In sharp contrast, global sturgeon aquaculture has grown remarkably since the 1990s but, only at the turn of the millennium, has it taken off exponentially [11]. Currently, China accounts for more than 85% of the meat production worldwide, followed by Russia and the EU (FAO database, 2016). Bronzi and Rosenthal (2014) estimated a total annual production of approximately 51,500 tons of sturgeon. Caviar production from fisheries is irrelevant, while it is estimated that aquaculture production will be more than 260 tons

by 2012 [5]. Here, the EU is the largest producer, with 70 tons (Italy 30 tons, France 28 tons, Germany 12 tons).

Considering the annual sturgeon meat production in China, current caviar production of 56.6 tons is expected to increase rapidly in the near future. On the other hand, one should bear in mind that caviar is a high-priced product, and product quality standards of caviar, as well as sustainable production, will not be achieved by current sturgeon farms without changes in the operational procedures. Conservative production forecast an increase in caviar production to 500–750 tons in the next years, depicting extraordinary growth within the aquaculture sector [11].

The caviar produced from sturgeon is one of the most valuable fish products in the world [11, 12]. Despite the recent developments in aquaculture production, demand is far from being met by current availability. Late maturity, a reproductive cycle regularly encompassing several years, and frequently observed reproductive dysfunctions challenge the economic efficiency and impede the development of the industry. Accordingly, an ideal production system would grow females only, thereby reducing the costs and maximizing profit per production unit.

Although sex is determined early in life, and cytological differences can be noted at an early stage after histological analysis (see Section 33.2), these methods serve research and development purposes only, and are not employed in commercial practice (see Section 33.4).

The lack of external sexual dimorphism in sturgeon only allows for the selection of females using invasive or non-invasive techniques to assess internal anatomical differences in older fish. Depending on the species and the method applied, sexing is carried out after years of on-growing (Table 33.1), when the tremendous growth of the oocytes during the accumulation of yolk in vitellogenic females allows a reliable identification of sex (shape and structure of the ovary). Methods currently used do not offer a high grade of diagnostic safety, and the distinction between males and immature, pre-vitellogenic females is problematic in routine screening.

In those species with late maturity, such as *Acipenser transmontanus* or *Huso huso*,

this is even more relevant than in early-maturing species, such as sterlet, *A. ruthenus*, or Siberian sturgeon, *A. baerii*, due to the variability in the maturation of late-maturing species. Consequently, sexing is carried out repeatedly, to improve diagnostic safety and reduce the risk of sexing immature females as males. Thus, considering the variability of maturation in sturgeon, the labor costs and expenditure involved for unexpected on-growing and multiple sexing may easily threaten profitability. Rearing system, water quality, feeding intensity, diet and temperature may delay maturation and, thus, affect the efficiency/accuracy of sexing further.

In sturgeon, the sex seems to be genetically determined as suggested by a 1 : 1 male-to-female ratio (see Section 33.3). So far, no sex chromosomes have been identified and, with regard to the sex determining gene or genes, information is limited to those players that channel sex-specific differentiation (see Section 33.2). An exclusive, ultimate marker or gene inherited by one of the sexes

Table 33.1 Age of sexing compared to the age at harvest for caviar production, referring to the two most important methods – invasive endoscopy and non-invasive sonography in sturgeon aquaculture. Literature data and information of commercial caviar farms were used to compile estimations on common practice. Age (+) refers to a significant older age of at least three months.

Species	Harvest	Endoscopy	Sonography	References
Sterlet <i>A. ruthenus</i>	3–4	1(+)-2	1–2	a [120, 121]
Siberian sturgeon <i>A. baerii</i>	4–6	1(+)-2	1(+)-2	a, b [105, 108, 122]
Starry sturgeon <i>A. stellatus</i>	6–8	2–2(+)	2–3	a, b [120, 121]
Russian sturgeon <i>A. gueldenstaedtii</i>	6–8	2–2(+)	2–3	a, b [69, 120, 121, 123]
White sturgeon <i>A. transmontanus</i>	7–10	3–3(+)	2(+)-3	a Chebanov (pers. comm)
Beluga <i>H. huso</i>	9–15	3(+)-4	3(+)-6	a [120, 121, 124]

a Fischzucht Rhönforelle GmbH & Co., Gersfeld, Germany
b Attilus GmbH, Jessen, Germany

is unknown. Consequently, the genetic sex determination system is far from being understood in sturgeon. Evidence for environmental or social sex determination has not been reported in sturgeon, suggesting that the basis of sex determination is purely genetic [13].

Since the rearing of males contributes significantly to the production costs (up to 30% of the total costs, EU CRAFT project 1999-72183), genetic identification of the sexes at an early life stage, based on PCR techniques, could substantially reduce costs and ensure economic sustainability, fueling future development of the industry. Not surprisingly, there have been several studies aimed at the identification of such a sex-specific marker(s) [13–18]. Recent genome projects on sturgeon will hopefully advance knowledge on sex determination and improve current sexing techniques (see Section 33.4).

Strategies to control sex in farming and allow the development of all-female stocks are promising (see Section 33.5), but are still not used on a commercial scale, due to the long life cycle of sturgeon. Undoubtedly, sex-specific genome markers will speed up the exploration of all-female stocks. Although one could argue that market demand for sturgeon meat is huge, supporting the current practice, improved sexing technology undoubtedly represents a competitive advantage for those farms involved in caviar production and, more importantly, allows for better efficiency in caviar production and, thus, improves sustainability.

One needs to keep in mind that sturgeon meat production is rather a consequence of farming activities aiming for caviar, rather than a goal itself. Also, production figures of sturgeon meat are rather low, compared with other commodities, such as Atlantic salmon (2.2 million tons in 2015) or sea bass (150,000 tons in 2015). Thus, sturgeon meat is a niche market and, at least in the EU, is often unprofitable if not marketed directly.

Consequently, sturgeon aquaculture is driven by the caviar industry, rather than the meat market. Reflecting on the past, the traditional market absorbed over 3,000 tons [11] and, along with market segmentation, will provide the impetus for innovation of sexing technology.

33.2 Sex Determination and Differentiation

33.2.1 Cyto-anatomical Gonad Differentiation

In sturgeon, the undifferentiated gonad develops directly either into a testis or an ovary. Dependent on species (Table 33.2), shortly before hatching, primordial germ cells (PGCs) begin to actively migrate along the mesentery of the embryo towards the developing gonad (Figure 33.1). This migration can easily be observed for at least three months *in vivo* by simply labeling the vegetal hemisphere of the 1–4 cell stage with fluorescein isothiocyanate (FITC)-dextran [22]. Days after hatching, PGCs settle in the gonad primordium, which soon thereafter develops into a conspicuous genital ridge [21–28]. The proliferation of PGCs and differentiation of a lamellar gonad takes place within the first months, but morphological sex differentiation is only observed after several months. At this stage, notched epithelium of the ovary, in contrast to a smooth appearance of the testis epithelium, allows first identification of the sex in histological slides (Figure 33.1). Still, this diagnostic characteristic assigned to the differentiating ovary does not allow the differentiation between undifferentiated ovaries and differentiating testis.

Several months later, nests of oocytes are observed. At the same time, tubular structure of the testis becomes pronounced, but meiotic stages cannot be observed at that time. The timing of cytological sex

Table 33.2 Cyto-anatomical sex differentiation in commercial sturgeon species (Figure 33.1).dph – days post hatching; PGCs – primordial germ cells; dpf – days post-fertilization.

	Migrating PGCs	Gonadal ridge with PGCs1	Lamellar gonad with proliferating PGCs (PGCs2)	Germinal area advanced PGCs2, first blood vessels	Morphological sex differentiation ^a (gonad anatomy)	Proliferation of germ cells	Meiotic stages	Oocyte nests tubular testes	References
Adriatic sturgeon <i>A. naccarii</i>	1.5 (6.5 dpf)–10 dph	16–30 dph	60 dph	105 dph	180 dph	females: 210 dph males: 292 dph	females: 292 dph males: no meiotic stages until 594 dph	oocyte nests: 594 dph tubular testes: 594 dph	[23, 24]
Russian sturgeon <i>A. gueldenstaedtii</i>	1 dph	18 dph		44–80 dph	70–115 dph	females: 115 dph males: 197 dph	females: 197 dph males: 420 dph	oocyte nests: 266 dph tubular testes: 439 dph	[25, 26]
Sterlet <i>A. ruthenus</i>	3.5 (1.5 dpf) dph	9–27 dph	20*–40 dph	78*–120 dph	90*–240 dph				[27, 28]
Siberian sturgeon <i>A. baerii</i>	1 dph	14 dph		44–80 dph	115 d	females: 115 dph males: 197 dph	females: 197 dph males: 420 dph d	oocyte nests: 266 dph tubular testes: 439 dph	[25–28, 39]
Shortnose sturgeon <i>A. brevirostrum</i>		26 dph			180 dph	females: <43 m males: 43 m)	females: 43 m males: approx 4 a	oocyte nests: tubular testes: approx. 4 a	[73, 125]

^afemales: notched gonad (gonadal epithelium), males: smooth, continuous gonad
^{*}unpublished data Güralp & Pšenička

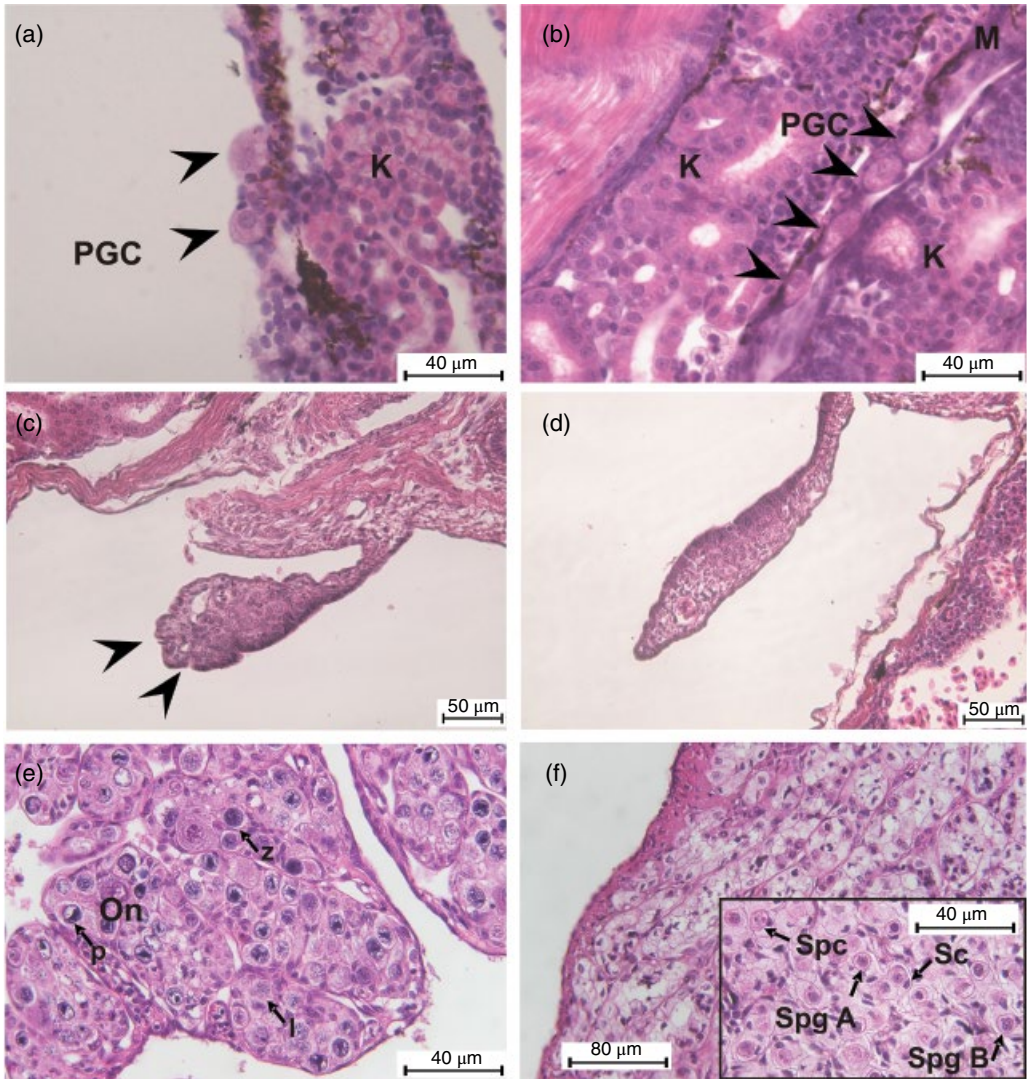


Figure 33.1 Early cytological germ cell and anatomical gonad differentiation in sterlet *Acipenser ruthenus*, from germ cell migration, genital ridge formation to anatomically distinct gonads. (See inserts for the color representation of this figure.)

- PGCs (arrowhead) with a high nucleus/cytoplasm ratio (25–30 μm) migrating within the genital ridge of the embryo 5 dpf (sagittal longitudinal section);
- PGCs observed along the dorsal mesentery (M) between the right and left kidney (K) in 28 dpf larvae (coronal longitudinal section);
- anatomical differentiation of the ovary 82 dpf indicated by notches/folds (arrowheads) of the columnar epithelium compared to a;
- “smooth” epithelium without notches, indicative of a male gonad (juveniles were 11.8 cm and 9.8 cm);
- ovary of a 9-month-old female with nests of oocytes (On) at different meiotic stages (l-leptotene, p-pachytene, z-zygotene respectively);
- testis of a 10-month-old male with spermatogonia A and B (Spg A, Spg B), spermatocysts (Spc) and sertoli cells (Sc). HE staining.

determination is species-specific, but modulated by rearing conditions such as temperature, feeding and rearing density, as in other fish species [23–25, 27–29]. Interestingly, it has been shown that embryos become sensitive towards sex steroids [24], indicating that hormone-sensitive sex differentiation may occur around hatching. Nevertheless, most studies on hormonal feminization were carried out at a later stage. Thus, the time frame for hormone-induced sex reversal, often referred to as the window of physiological sex determination, seems to be relatively wide, depending on the route (oral, immersion) of application [26, 30–32].

33.2.2 Sex Determining Genes Controlling Early Cyto-Anatomical Sex Differentiation

In several fish species, such as medaka and zebrafish, PGC proliferation seems to play an important role in sex determination. Here, apparently, female PGCs develop faster than male PGCs [33–36]. This may, in fact, represent the earliest step of cytological sex determination. Nevertheless, other species, such as goldfish or loach, do not reveal such a role in early sex differentiation [37, 38]. So far, sturgeon have not been studied in detail.

The differentiation of PGCs into oocytes or spermatocytes is controlled by a cascade (or network) of transcription factors, enzymes, and hormones regulating somatic, as well as germ cell differentiation in a sex-specific manner. Each of those may be regarded as an upstream switch or downstream differentiator in sex determination and differentiation [20]. Assessing these genes not only provides a better understanding towards the mechanism of sex determination, but can also be used to establish sensitive biomarkers that allow an early identification of sex. Undoubtedly, in contrast to sex determination in mammals, variety in the sex determining cascade among fish is huge [19, 20]. Currently, this mechanism of sex determination and early differentiation is poorly understood in sturgeon (Figure 33.2). Still, using

gene expression approaches, several key players involved in other vertebrate species have been identified in sturgeon, several exhibiting a sexually dimorphic expression pattern in the gonad [14, 39, 40].

Often, results on candidate gene expression such as *dmrt1* are inconsistent. Several studies address gene expression in juveniles, sub-adults or adults, rather than assessing candidate genes during the critical period of sex determination. As a consequence, samples analyzed comprise several cohorts of developmental stages (e.g., spermatogonia, spermatocysts, spermatids), which may mask expression of a stage-specific candidate gene. Also, spermatogenesis and oogenesis in fish is a recurring process, but early sex determination may as well be a one-time process. Presumably, only a few of the genes potentially involved have been identified so far [15, 17, 41, 42], and others remain to be discovered, as hypothesized in other species [20].

As described, phenotypic sex determination is observed within the first year in females, whereas male-specific differentiation seems to take place thereafter (Table 33.2). Therefore, in contrast to species exhibiting a fast sex determination, sturgeon provide a better model to study the succession of processes regulating early gameto- and gonadogenesis, allowing for an improved sequential resolution.

In vertebrates exhibiting genetic sex determination, one or a couple of interlinked, sex-specific master gene(s) control upstream regulators of the SD cascade (i.e., pro-testis: *amh*, *sox9*; pro-ovary: *sf1*, *foxl2*), which initiate early sex determination before steroidogenesis-related factors (i.e., *srd5a2*, *cyp17a1*, *aro*) set the delicate balance between androgens and estrogens critical for the ongoing sex differentiation [20, 43, 44]. In contrast to the diversity of master genes ultimately determining sex, the subsequent male and female specific differentiation pathway is rather conserved among non-mammalian vertebrates [20, 44–46]. It has to be noted that it is difficult to assign upstream transcription factors to either sex determination or differentiation, but dimorphic expression of these

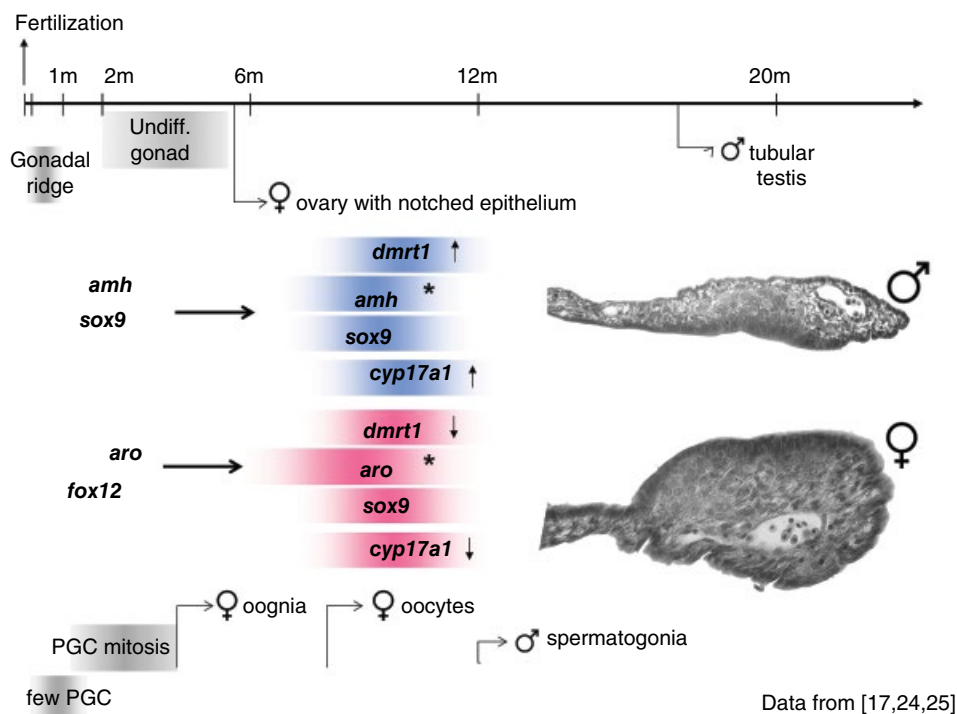


Figure 33.2 Scheme of the molecular and cyto-morphologic differentiation in Siberian sturgeon with two groups of genes with opposite expression pattern of pro-testis (*sox9*, *amh*) and pro-ovarian factors (*foxl2*, *aro*) in undifferentiated 3–4-month-old juveniles, and sex-specific expression of *dmrt*, *amh*, *sox9*, *cyp17a1* in male and *dmrt*, *aro*, *sox9*, *cyp17a1* in female gonads (see also Box 33.1).
↑ higher compared to ↓ expression, * exclusively expressed in one sex.

Box 33.1 Genes involved in early sexual differentiation in sturgeon (Figure 33.2)

amh – anti-Müllerian hormone
aro/cyp19 – aromatase, estrogen synthetase
cyp17a1 – steroid 17alpha hydroxylase
dmrt1 – doublesex and Mab-3 related transcription factor 1
foxl2 – forkhead box transcription factor L 2
sf1 – steroidogenic factor 1
sox9 – transcription factor sox 9 (SRY box 9)
srda5a2 – 5 alpha reductase

factors ultimately leads to the cyto-anatomical sex differentiation. Streamlined, molecular sex determination/differentiation precedes cyto-anatomical sex differentiation and initiates steroidogenesis in a sex-specific manner.

Here, doublesex and mab-3 related transcription factor 1 (*dmrt1*) has a pivotal role in

testis differentiation [43] and, as duplicated gene *dmY*, gave rise to the male-specific master gene in medaka [47]. Action of *dmrt1* is not just limited to the initiation of testis differentiation, but also accounts for a life-long inhibition of the “anti-testis” pathways involving *foxl2* and *wnt4*. Thereby, *dmrt1* is essential to suppresses reprogramming of Sertoli cells into granulosa cells [48–50].

Common among vertebrates, male differentiation involves an early, dimorphic expression of *dmrt1*, *sox9*, *dax1*, *tbx1*, and *amh* [51]. During early ovarian differentiation, *foxl2* has been reported as one of the earliest markers of ovarian differentiation in several vertebrate species. In Nile tilapia, for example, *foxl2* stimulates *aro* expression in the granulosa cells (ovarian aromatase), initiating the steroidogenesis-driven period of ovarian development [52]. Thereafter, *aro* regulates conversion

of androgens into estrogens in the ovary, dominating the female pathway.

In sturgeon, the role of *dmrt1* in sex determination of males remains unclear, due to highly inconsistent results reported. In Siberian sturgeon, molecular sex differentiation preceding cyto-anatomical sex differentiation by approximately four months has been reported. Here, *amh* and *sox9* were presumably involved in male differentiation, whereas *cyp19a* and *foxl2a* seemed to be upregulated in undifferentiated females [39]. Unexpectedly, *dmrt1* revealed a reciprocal expression to *amh* and *sox9*, similar to genes involved in female differentiation at this age (90–120 dph). Nevertheless, at this time, males can be considered undifferentiated.

A peak in *dmrt1* observed 330 dph (11 months) preceding the first detectable differentiation in males may, thus, suggest a similar role in early testis differentiation, as observed in other vertebrate species, congruent with a later differentiation in males reported in several sturgeon species (Table 33.2). In 9-month-old Russian sturgeon, neither of the two *dmrt1* copies identified revealed a dimorphic expression in the undifferentiated gonad [14]. Again, *cyp19a* and *foxl2* were upregulated in presumptive females. Unfortunately, no older stages were assessed here. Low expression of *dmrt1*, similar between specimen, has been also reported in Russian sturgeon until 50 dph [53]. Thereafter, expression increased exponentially between 100–200 dph when morphological differentiation in females, but not in males, is commonly observed (Table 33.2, Figure 33.2).

After 500 dph, up-regulation was male-specific, suggesting a similar role of *dmrt1*, as in other vertebrates. In sub-adults and adult sturgeon, male-specific over-expression of *dmrt1* was observed in the testis of 16-month-old Siberian sturgeon [54, 55]. Again, early sex differentiation, including first morphological differentiation of the gonad, proliferation of germ cells and meiotic stages (even oocyte nests in females), are usually observed before (Table 33.2). A male-specific upregulation of *dmrt1* has also been reported in 3–4-year-old

juvenile Chinese sturgeon and sub-adult maturing shovelnose sturgeon. Considering the multiple germ cell stages in the testis, such expression may not necessarily be attributed to the advanced stages of spermatogenesis, but be associated with stem cell renewal.

Despite its fundamental role in early testis differentiation, including proliferation of male germ cells and inhibition of the female pathway [49, 50, 58], *dmrt1* is also required for female germ cell differentiation in mice and claw frogs [59, 60]. Thus, differential, sex-specific functions along gonad differentiation in both sexes, as well as asynchronous development during early gonad differentiation in males and females, may contribute to inconsistent results reported in sturgeon. Similar to *dmrt1*, dimorphic expression of *foxl2* has also been confirmed in adult and sub-adult short-nose, Chinese and Russian sturgeon [41, 42, 57]. Since cohorts of different germ cell stages have been observed in the ovary of sturgeon [61–64], it seems plausible, that *foxl2* is upregulated in early stage cohorts, rather than in the abundant cohort of maturing follicles. Hence, future studies need to differentiate stage-specific expression, rather than analyzing entire tissue samples, at least in older fish.

Recently, RNA binding protein dead end (*dnd*) has been identified in Chinese sturgeon, *Acipenser sinensis* [65]. *Dnd* is expressed exclusively in germ cells of both sexes. Abundant expression in spermatogonia tends to decrease towards late spermatogenic stages, whereas expression in females is higher in primary oocytes than in oogonia. This is particularly interesting since *dnd*, in zebrafish, is crucial for PGC migration and survival [66, 67], and is specifically expressed by PGC in chicken [68]. As a conclusion, *dnd* may serve as an additional candidate.

33.3 Genetic Sex Determination (GSD)

In most fish species, sex is primarily determined by genetic mechanisms [43] but, so far, the ultimate sex determining master gene has

only been identified in some species, including medaka (*dmY* – a male-specific paralog of autosomal *dmrt1*) and pejerrey (*amhY*). Many studies have suggested that sex in sturgeon is mainly genetically determined, as suggested by the sex ratio of 1 : 1 mostly observed, and lack of evidence for environmental sex determination [69, 70]. Nevertheless, it has been recognized that selection tends to establish a balanced sex ratio most likely close to 1 : 1 [71, 72], irrespective of whether sex is determined purely genetically or not. In fact, slightly biased sex ratios shifted towards females (<60%) have been reported for commercial (“untreated,” “normal”) stocks in Shortnose sturgeon [73] and Russian sturgeon [69], but neither environmental nor social factors seem to influence the inheritance of sex. Rather, it has been suggested that sex-biased survival under intensive aquaculture conditions may modulate sex ratio slightly [69].

Approximately 10% of fish species studied have a cytogenetically distinct sex chromosome [74]. In sturgeon, no heteromorphic sex chromosome has been found, which may partly be explained by the inability of traditional cytogenetic techniques to identify small-scale differences, such as those reported in puffer fish, *Takifugu rubripes* [75], or three-spine stickleback [76]. For example, a single amino acid change of Ser in *dmrt1* to Thr in *dmY* established a species-specific master gene in medaka *O. latipes*, illustrating that genetic sex determination may be based on the tiniest differences on the genome level [40]. Consequently, depending on the level of sex-specific genomic differences, identification of a genomic sex marker may literally be like looking for a needle in a haystack. Furthermore, the complexity of the sturgeon genome, characterized by a high number of chromosomes (particularly small micro-somes) and species-specific levels of ploidy, complicates analysis [77, 78].

Several studies addressing gynogenesis suggest a chromosomal mechanism with a sex-related gene(s) located on a specific chromosome, rather than a polygenic sex determination in which sex-determining

genes are found throughout the entire chromosome set [73, 79, 80]. Further evidence is based on gynogenesis, suggesting female heterogamety (ZZ-ZW) according to the sex ratio observed (18–35% males: 65–82% females [73, 79–83]; see also Box 33.2). Still, it cannot be ruled out that other chromosomal or polygenetic factors modulate sex ratio, in particular in the context of variability between females among the studies.

Approaches used to extract genomic sex markers comprise the screening of male and female genome by DNA fingerprinting techniques [13, 17, 84], candidate gene approaches targeting those genes that are involved in the sex determination cascade in other species or are differentially expressed in sturgeon [14, 16, 17], and subtractive strategies looking for male or female-specific sequences in the genome [17, 85]. Recent NGS-based efforts target both extraction of differentially expressed candidate genes (transcriptomics), and subsequent evaluation on the genome level, as well as genome projects, where male and female genomes are comparatively analyzed to extract sex-related genomic markers.

Despite the recent efforts, neither a sex-specific marker, nor the ultimate sex determining master gene, has been identified in a sturgeon species. Further, considering SD in other fish taxa, it seems likely that the mechanism of SD may vary between species, or even between populations of sturgeon. For example, the male-determining gene *dmY* (*dmrt1bY*) of *O. latipes* (ZZ-ZW) is only present in *O. curvinotus* (located on the Y chromosome, XX-XY system). In most of the other *Oryzias* species, GSD remains to be

Box 33.2 Genetic sex determination – evidence and conclusions

Despite the lack of heteromorphic sex chromosomes in sturgeon, GSD is supported by a stable 1 : 1 sex ratio observed in aquaculture, irrespective of environmental conditions, and a sex ratio observed in gynogenetic offspring, which suggests female heterogamety (ZZ-ZW mechanism).

identified [86]. Thus, identification of sex markers in one sturgeon species will probably allow sexing in other species.

During normal reproduction in sturgeon aquaculture, individuals with unusual ploidy level have recently been observed that actually develop into maturing fish [87, 88]. In the wild, detection of autopolyploidization has not been feasible so far, due to limited genetic diversity impeding microsatellite analysis [88]. Thus, it remains to be clarified whether autopolyploidization occurs at a comparable frequency during natural spontaneous reproduction, or is a result of currently practiced reproductive technology using hormone therapy to induce final maturation.

Using flow cytometry and microsatellite genotyping, evidence has been presented for a maternal origin of spontaneous autopolyploidization during controlled reproduction in aquaculture [89]. Surprisingly, since sturgeon possess multiple micropyles (species-specific; between 2–52) at the animal pole that may function as potential entry sites for multiple sperm [90] and polyspermy, this may thus explain abnormal ploidy levels. Indeed, polyspermic fertilization has been observed at high concentrations of sperm in sturgeon, and sperm concentration has to be adjusted carefully in routine reproduction [91, 92].

However, recent data suggests that failure to exclude the second polar body results in autopolyploidization [89]. The occurrence of unidentified males or females with such autopolyploidization within a sturgeon stock, and subsequent sampling for studies aiming at the identification of genetic sex markers, will corrupt analysis strategies based on the common assumption of a sex-specific marker, and may explain the failure of research studies in the past. Recent findings demonstrate that such a scenario is not so improbable as one may assume. In White sturgeon aquaculture, five out of 10 families revealed spontaneous autopolyploidy, which was observed in up to 33% of the individuals within a family [88].

33.4 Sexing in Aquaculture

There are large-scale markets for sturgeon meat in Russia and China, but not in Europe, where a large part of the sturgeon are produced. Also, the prices of caviar, ranging between 300 € and above 2000 €, are at least 100–500 times higher than for meat. Consequently, the primary objective of sturgeon farming is caviar production. However, if caviar prices drop, marketing of small sized fish as meat may be economically advisable [93]. In current farming practice, individuals are sexed as early as possible, to sort out females for on-growing (Table 33.1). Males are subsequently sold as medium-sized fish to the meat market. Therefore, sexing is a key step in sturgeon aquaculture, which is currently only feasible for older fish.

Despite recurring efforts to identify external characteristics that allow sexing in sturgeon [94–96], criteria presented have little differentiating power. Often, females have a slightly more robust confirmation [95], but only a few authors claim to have criteria to segregate sex via morphological features [96, 97]. Here, only assessed on a few individuals, sex-specific shape of the urogenital opening (Y shape in males, O shape in females) was reported in three commercial species – *A. oxyrinchus*, *A. transmontanus* and *A. brevirostrum* [96]– but was undeterminable in others [96,98]. However, it is accepted that external criteria do not provide sufficient accuracy and, thus, should only be used to confirm other sexing techniques.

Nowadays, in sturgeon aquaculture, sexing may be performed with a variety of invasive (biopsy samples followed by visual inspection or histology, endoscopy, blood/plasma analysis) and non-invasive techniques (sonography, near infra-red spectroscopy). However, these methods are labor-intensive and, more importantly, often bear the risk of injury or stress to the fish, and can only be used on large individuals after years of rearing (Table 33.1). In terms of accuracy, surgical biopsies allowing histological analysis can be

considered the gold standard, but this is labor-intensive and stressful for the fish. In several countries, the application is tightly regulated, due to animal welfare concerns and may require specific professional training. Novel techniques based on plasma hormones, gene expression and comparative proteomic analysis have also been assessed, but are far from being applied on a commercial scale, particularly with regard to the costs [13].

Analysis of blood plasma parameters was successfully used to determine sex and stage of gonad maturity, originally including testosterone (T), 11-ketotestosterone (11-KT), estradiol (E_2) and vitellogenin or, correlated, Ca^{2+} [99, 100]. Optimistically presented as an alternative to biopsies, the method was optimized, reducing the number of diagnostic parameters and, subsequently, the costs [101]. However, analysis is costly and, if at all, only used to select females for hormonal induction of spawning, or to exclude atretic individuals, rather than for sex determination in younger individuals [13]. Once more, biopsies assessing the position of the nucleus/germinal vesicle, or used to score germinal vesicle breakdown, are by far faster, easier to perform and cheaper [102].

An extensive study on shovelnose sturgeon, *Scaphirhynchus platyrhynchus*, reported that endoscopy is more effective and reliable, compared with sonography and blood chemistry [103, 104]. In contrast to ultrasonography, images obtained allow an identification of sex without requiring a lot of experience and training [94]. Still, limited discrimination has been reported in earlier stages, when the fat and gonad tissue of immature ovaries cannot be distinguished, and resemble testis in appearance [94, 103, 104]. Since identification and staging are often more reliable, endoscopy may be carried out as final confirmation if sonography is ambiguous [103, 105].

Currently, by far the most frequently used method by the industry is sonography. Here, sex is assigned due to the brightness compared to the adjacent tissues (echogenicity), graininess (echotexture), and uniformity of

the gonadal tissue. The ovary is identified as grainy and heterogeneous in overall echotexture, with irregular margin indicating ovarian folds, whereas males appear bright, homogenous, with a fine echotexture and continuous margins, indicating ovarian folds (Figure 33.3).

Differentiating power highly depends on the maturational stage of the fish. In general, false sexing is much higher in immature females, as reported in pre-vitellogenic or early vitellogenic (F2, F3) females [106]. Also, high deposition of fat impedes the analysis, since the mesenteric fat (e.g. intermediate brightness between ovarian and testis tissue) cannot be securely differentiated from gonad tissue at that stage and, thus, is interpreted as the characteristic appearance [106] of a male gonad. Usually, in the course of vitellogenesis, fat reserves are rapidly depleted but, if the feed is excessively provided, abnormal fat deposition may not just interfere with the caviar quality, but may also present an obstacle during sexing.

It is difficult to estimate the ratio of correct identification, since most commercial farms recruit long-experienced workers, and scientific trials do probably not always represent this level of experience [103, 105–107]. Also, accuracy highly depends on the fish (maturational stage, nutrition) examined as outlined, and studies are difficult to compare. Commonly, fish are also successively scanned and, in case of doubt, reared longer until sexing is considered convincing. Still, correct identification has been reported to range between 70–90% [103, 105–108]. Recent results on non-invasive staging using near infrared spectroscopy are promising, and may be used to follow maturation and optimize harvest, in addition to other methods [109].

33.5 Control of Sex – All-Female Stocks?

The culture of monosex populations in aquaculture is an ultimate goal in caviar production, reducing the costs for rearing males

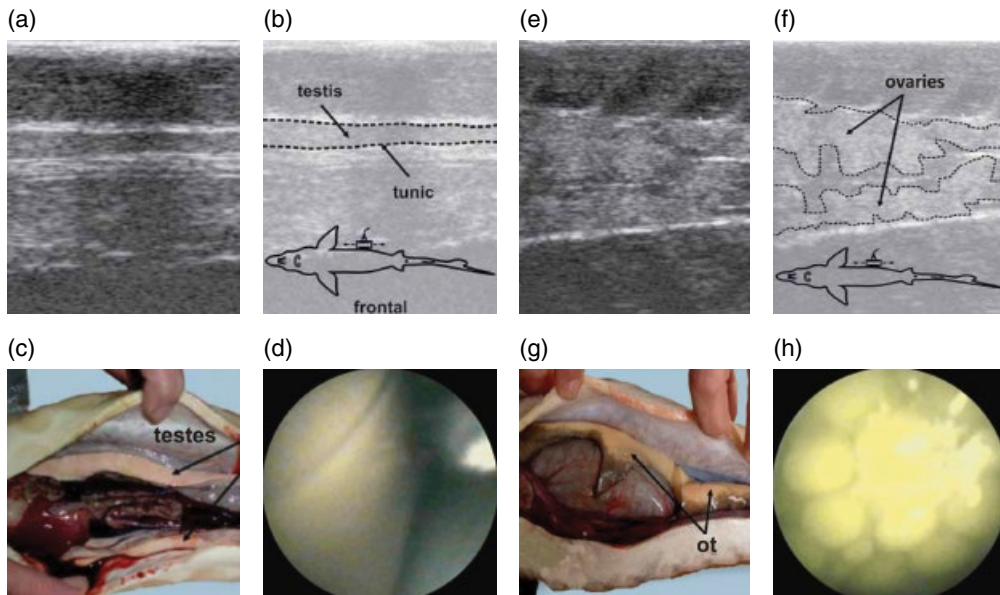


Figure 33.3 Current sexing methods in sturgeon aquaculture, illustrated in a male (a–d) and a female (e–h) Russian sturgeon. The testes (a, b) appear as a homogenous tissue strand with smooth margins, compared to the irregular form of the fine grained ovarian tissue (e, f) using sonography. The irregular form of the ovigerous lamellae can be observed macroscopically (g), whereas the margin of the testes appears rather smooth and continuous (c). By endoscopy, small oocytes can be observed (h). ot- ovarian tissue From: Chebanov, M.S. and Galich, E.V. (2010). *Ultrasound diagnostics of sturgeons. FSGTSR, Krasnodar. Izdatel' stvo Prosvesheniye-Yug*. 135 pp. (See inserts for the color representation of this figure.)

until they can be sexed. The most promising strategy to produce all-female stocks is gynogenesis (Figure 33.4), which limits the inheritance of DNA to the maternal parent. As a first step, this involves the inactivation (destruction) of male DNA, usually achieved by UV treatment (mostly UV-C) but, at the same time, maintain sperm viability. Motile, genetically “blank” sperm is subsequently used to activate the eggs, triggering the finalization of meiosis and initiating the exclusion of the second polar body.

Subsequently, exposure of activated eggs to temperature or pressure shock restores diploidy, either applied shortly after activations by inhibiting the exclusion of the second polar body (meiotic gynogenesis), or later by suppressing the first mitotic division (mitotic gynogenesis). In contrast to higher vertebrates, viable offspring (called gynogens) have been produced by gynogenesis in fish, including sturgeon [73, 80,

110–113]. Due to the recombination during meiosis I (crossing over), meiotic gynogens considered “heterozygous” compared to homozygous gynogens obtained after mitogenic gynogenesis (Figure 33.4).

The female has been suggested as heterogametic (ZW) and males as homogametic sex (ZZ), due to the sex ratio of meiotic gynogens observed. The percentage of males ranged between 18–50% in *A. transmontanus* [80], 19% in Siberian sturgeon [81], 28% in ship sturgeon, *Acipenser nudiiventris* [114], 20% in paddlefish, *Polyodon spathula* [79], and 20–30% in hybrid bester [82], confirming a common sex determination mechanism (but not a sex determining master gene – see above) among Acipenseriformes. Also, these findings suggest that the sex determining region segregates independently from the centromere during meiosis I (Figure 33.4) prior to the exclusion of the first polar body [80, 81]. Furthermore, van Eenennaam

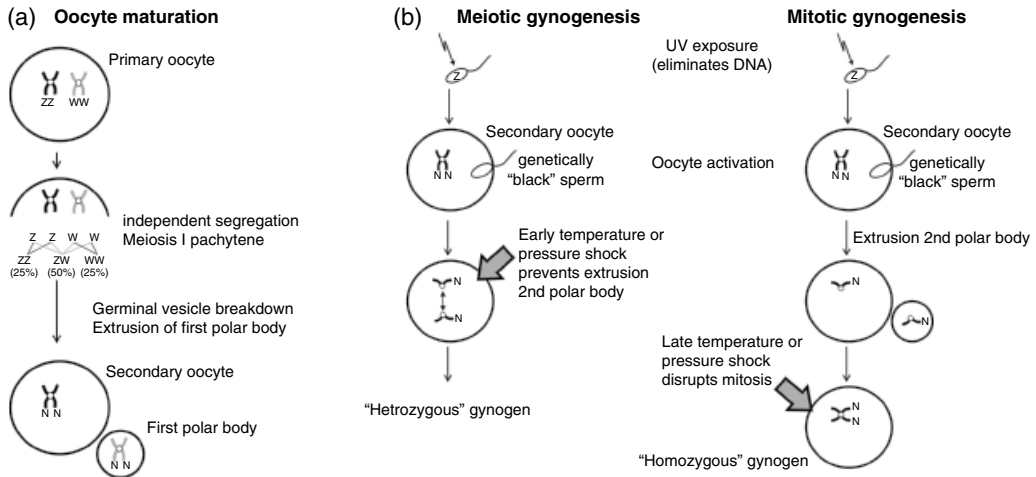


Figure 33.4 a) Oogenesis in heterogametic female sturgeon involves independent segregation of the sex determining region (here labeled Z – male, W – female) during meiosis I.

b) independent segregation of the sex determining region (not specified here and labeled N) during meiosis I results in "heterozygous" gynogens, if temperature or pressure shock is applied shortly after activation, whereas late shock preventing mitotic cell division results in "homozygous" gynogens (mitotic gynogenesis).

reported a percentage of 14% males upon triploidization, as one would expect for a ZW-ZZ sex determination system with triploid genotypes of 1/6 ZZZ (male) : 2/3 ZZW (female) : 1/6 ZWW (female).

Upon fertilization, sturgeon eggs become sticky, and de-adhesion treatment has to be carried out to prevent clumping [115], approximately within 5–10 minutes [73]. Mineral silt, Fuller's earth, and tannin are most commonly used in farming, but each has its shortcomings and may increase mortalities [115]. Due to the fact that activated eggs need to be shocked rapidly for rediploidization (retention of the second polar body), treatment needs to effectively counteract adhesion in a short time. Among treatments recommended for hatchery use, tannin treatment has been reported as the fastest, providing effective deadhesion within 40 seconds [115]. In contrast, clay can only be used during gynogenesis if eggs are incubated in clay during shock application. Recently, hypochlorite has been reported to be as effective as tannic acid (40 seconds), revealing evidence for improved hatching rates [116]. The retention of the second polar body is subsequently achieved either by

application of high pressure, cold or heat shock (Table 33.3). Using inactivated sperm from a different species, a similar yield of gynogens has been reported, but may be more efficient to monitor the effectiveness of DNA destruction and sort out potential hybrids from gynogens according to the differences in ploidy [82, 114, 117–119].

Considering female sex as the heterogametic sex, and independent segregation from the centromere as described, one would expect genotypes of 1/4 ZZ (males) : 2/4 ZW (females) : 1/4 WW (super females) upon meiotic gynogenesis. If these super females are viable and can be reared as brood stock, reproduction with normal males would consequently provide all-female stocks.

Alternatively, heterogametic females can be sex-reversed by hormonal treatment. Using such neomales for reproduction with females would similarly provide super females that can be used for the generation of all-female offspring. Again, it has not been demonstrated that super females survive and mature. Comparing both strategies, the latter requires the maturation of the neomales for the generation of super females and, thus, involves an additional maturation.

Table 33.3 Details on protocols used for meiotic gynogenesis in sturgeon species (summarized according to optimal treatment of respective study), specifying sperm inactivation, shock treatment to retain the 2nd polar body, de-adhesion treatment (if applied) and hatching rate.

Species	Sperm inactivation	Shock	Antiadhesion	Hatching rate	Ref.
Shortnose sturgeon <i>Acipenser brevirostrum</i>	2–5 minutes UV _{254 nm} (120–330 mJ/cm ²)	580 bar for 5 minutes	sterile river silt	low	[125]
Siberian sturgeon <i>Acipenser baerii</i>	2 minutes UV (13.5–29 mJ/cm ²)	36°C for 2 minutes		3–18%	[81, 111, 112]
Sterlet <i>Acipenser ruthenus</i>	2–5 minutes in 100 μM AMT ¹ + 3 minutes UV ₃₆₀ (90 mJ/cm ²)				[126, 127]
	45 s UV _{260 nm} (200 mJ/cm ²)				[77]
Sterlet <i>Acipenser ruthenus</i> (bester sperm only)	2 minutes UV (13.5 mJ/cm ²)	34°C for 2 minutes		19–25%	[110, 128]
Bester hybrid <i>A. ruthenus</i> × <i>H. huso</i>	7 minutes UV _{254 nm} (210 mJ/cm ²)	34°C for 3–6 minutes	none (using attached eggs)	16–50%	[82]
Ship sturgeon <i>Acipenser nudiiventris</i>	1 minutes UV _{254 nm} (28 J/cm ²)	2.5°C for 30 minutes cold shock	not specified	60%	[114, 118]
Starry sturgeon <i>Acipenser stellatus</i>	90–120 seconds UV _{254 nm} (43 J/cm ²)	3°C for 10 minutes cold shock	not specified	20–30%	[83]
White sturgeon <i>A. transmontanus</i>	180 seconds UV _{254 nm} (216 mJ/cm ²)	34°C for 1–5 minutes 3°C for 15–30 minutes cold shock	none (using attached eggs)	low	[80, 113]

¹aminomethyl-4,5,8-tri-methylpsoralen (AMT)

Considering the late maturation in sturgeon species, establishing all-female brood stock, involving backcrossing for validation, is a long-term strategy that has not been carried out, and should explicitly target first those sturgeon species with a shorter maturation, such as sterlet and Siberian sturgeon.

Also, next to the low yield of gynogenesis, low survival of gynogenic embryos and larvae have been reported [73, 113]. In general, gynogens appeared to be weaker than control diploids, exhibiting erratic swimming and loss of equilibrium, as well as a higher incidence of deformities [73]. Upon dissection, fluid-filled

cysts associated with the internal organs have been observed. As a conclusion, although promising, super female brood stock has not been established, but offers a visionary perspective for caviar production, as it is possible to bypass sexing and avoid rearing of males.

33.6 Conclusions

Undoubtedly, the prime goal of sturgeon farming is caviar, rather than meat. Thus, early sexing is a necessity, to assure competitiveness and to optimize current technology

in the future. Current sexing methods can be successfully applied in maturing fish, but the differentiating power increases with the age and, subsequently, the maturational stage of the fish. Although sex is primarily determined by genetic mechanism(s), and key factors of the sex determination cascade in other vertebrates reveal a sex-specific expression pattern in sturgeon, the ultimate sex determining gene(s) remains to be identified.

Despite the rapid progress in sequencing technology, and some recent whole-genome projects in sturgeon, no sex-specific marker has been identified in any of the 24 sturgeon species so far. Undoubtedly, such a marker would not only allow early sexing, but would also support the development of gynogenetic all-female stocks, which seems

currently unrealistic, due to the long generation time of most commercial species. Nevertheless, particularly in a high-priced, valuable food product such as caviar, such technology requires acceptance among the consumers.

Acknowledgements

Martin Pšenička and Hilal Güralp were supported by the Ministry of Education, Youth and Sports of the Czech Republic, projects CENAKVA (CZ.1.05/2.1.00/01.0024), “CENAKVA II” (LO1205 under the NPU I program), and by the Czech Science Foundation (17-19714Y).

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34

Hybridization and Polyploidization in Sturgeon

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34.1 Introduction

Sturgeon (order Acipenseriformes) is one of the oldest fish groups, having evolved more than 200 Mya (million years ago) at the end of the Triassic Period [1]. The evolution of the order is inherently connected to autopolyploidization and/or allopolyploidization events [2], resulting in some of the most remarkable diversities in chromosome number and genome size among vertebrates [3]. Chromosome numbers are ≈ 120 , ≈ 240 , or ≈ 360 , and genome size ranges from 2.44 pgDNA nucleus⁻¹ in beluga, *Huso huso* L. 1758 [4], to 13.78 in shortnose sturgeon, *Acipenser brevirostrum* (Lesueur 1818) [5] (see Box 34.1).

Sturgeon are prone to interspecific hybridization under natural conditions [6, 7], and sturgeon hybrids are commonly used in aquaculture [8, 9]. Hybridization in sturgeon may occur: between species with the same chromosome number, resulting in hybrids of the same karyotype as the parent species [10]; between species differing in chromosome numbers, producing hybrids with karyotype intermediate to the parent species [11]; and among those hybrids and pure species [12]. In addition, individuals with spontaneously increased genome size and chromosome numbers have recently been identified among cultured stock of several sturgeon

species [13–20]. These phenomena further expand the already substantial genome plasticity of sturgeon.

All 27 sturgeon species occur exclusively in the northern hemisphere. Seventeen species are classified as critically endangered [21], most populations of which continue to decrease, and extinction of some seems highly probable [22]. Declines in catches over the past 40 years have led to the development of sturgeon aquaculture, originally for reintroduction, but more recently for caviar production [8]. Sturgeon farming is currently a rapidly growing branch of aquaculture, with China recognized as the leader in meat and caviar production, followed by Italy, France, Russia, and the United States [8].

To meet the market demand for sturgeon products, aquaculture techniques have been gradually developed [23], but sex control has not been widely implemented, although it could potentially bring significant economic benefit to the industry. The caviar produced by sturgeons is a highly profitable product and, hence, females are more valuable in aquaculture. Sturgeon are gonochoristic, but none of the species exhibit external sexual dimorphism, and sex chromosomes have not been identified. Females can be identified only by examination of differentiated gonads at 3–5 years old at the earliest, depending on the species; thus, males can only be removed

from production after a lengthy rearing period, reducing profit by about 30% [24]. This has encouraged the search for early gender identification and sex control techniques. Several promising procedures have been introduced experimentally, but none has been fully implemented on a commercial scale.

This review aims to summarize current knowledge of polyploidization and hybridization, with respect to sex determination in

sturgeon. Techniques for control of sex are presented, and their feasibility for employment in sturgeon aquaculture is discussed.

34.2 Chromosome Manipulation

34.2.1 Gynogenesis

Gynogenesis has been suggested to be a suitable tool for sex control in aquaculture [30], as

Box 34.1 Sturgeon ploidy levels

While reported sturgeon chromosome numbers and DNA content of cell nuclei are relatively constant in the literature, ploidy levels are the subject of discussion. Currently, two scales of ploidy level are recognized in sturgeon: the *evolutionary scale*, arising from several polyploidization events in sturgeon evolution and referring to ancient ploidy levels [25]; and the *functional scale*, arising from significant functional re-diploidization in the sturgeon genome [26, 27], the process by which the gene dosage of a polyploid species is reduced but the original chromosome number and DNA content are maintained [28]. Understanding the phenomenon of sturgeon ploidy level is desirable for readers of this chapter, because ploidy levels, and thus the number of homologous or homeologous chromosomes and their pairing, play an important role in meiosis and in sex

differentiation. The ploidy levels of sturgeon species are presented in Table 34.1. For clarity, we include the identifier of ploidy scale at each mention of ploidy. We use functional scale in this chapter.

Glossary

Autopolyploidization – duplication of the same or very similar genomes within a species. All duplicated chromosomes are homologous, generally paired as multivalents in meiosis [29].

Allopolyploidization – hybridization of two species with distinct genomes, concomitant with genome duplication resulting in homeologous sets, each consisting of homologous chromosomes of the same original genome. Homologs are generally expected to pair with each other, but not with homeologs [29].

Table 34.1 Ploidy levels in sturgeon according to two scales.

Group	Ploidy scale		Chrom. number	DNA content
	Functional	Evolutionary		
A*	Diploid (2 <i>n</i>)	Tetraploid (4 <i>n</i>)	≈120	≈4 pg N ⁻¹
B†	Tetraploid (4 <i>n</i>)	Octaploid (8 <i>n</i>)	240–270	≈8 pg N ⁻¹
C‡	Hexaploid (6 <i>n</i>)	Dodecaploid (12 <i>n</i>)	≈360	≈12 pg N ⁻¹

**Acipenser nudiiventris*, *A. oxyrinchus*, *A. ruthenus*, *A. stellatus*, *A. sturio*, *Huso huso*, *Polyodon spathula*, *Psephurus gladius*, *Scaphirhynchus* sp., *Pseudoscaphirhynchus* sp.

†*A. baerii*, *A. dabryanus*, *A. fulvescens*, *A. gueldenstaedtii*, *A. medirostris*, *A. mikadoi*, *A. naccarii*, *A. persicus*,

A. sinensis, *A. schrenckii*, *A. transmontanus*, *H. dauricus*

‡*A. brevirostrum*

well as a valuable experimental approach for investigating sex-determining mechanisms in fish [31]. Induction of gynogenesis involves activation of the egg by irradiated sperm, and genome re-duplication by retention of the second polar body (meiotic gynogenesis), or genome duplication of the zygote during initial stages of cleavage (mitotic gynogenesis). Because successful mitotic gynogenesis has not been reported in sturgeon, this section deals only with meiotic gynogenesis.

In aquaculture, meiotic gynogenesis is worthwhile for production of all female progeny, especially in species with the female homogamety (XX) sex determination system [32]. In species exhibiting female heterogamety (ZW), meiotic gynogenesis produces ZZ males, WW “superfemales,” and/or ZW females, depending upon the rate of recombination of the sex-determining genes and the centromere during meiotic prophase I [33, 34]. If the sex-determining genes segregate independently of the centromere, gynogenetic progeny of ZW female consist of 1/6 males (ZZ) and 5/6 females (2/3 ZW + 1/6 WW).

In sturgeon, meiotic gynogenesis has been studied with respect to both sex control and sex determination. The first attempts were carried out in the former Soviet Union in Russian sturgeon, *Acipenser gueldenstaedtii* (Brandt and Ratzeburg, 1833), beluga, and sterlet, *Acipenser ruthenus* L. 1758, with almost total mortality of the obtained gynogenetic larvae [35]. In the 1990s, more promising results were achieved in several American acipenserids, with the hatching rate of viable gynogenetic larvae reaching around 11% in shovelnose sturgeon, *Scaphirhynchus platyrhynchus* (Rafinesque, 1820) [36], 21% in white sturgeon, *Acipenser transmontanus* (Richardson, 1836) [37], and 22% in American paddlefish, *Polyodon spathula* (Walbaum, 1792) [38]. More recently, a 29% hatching rate of gynogenetic progeny was obtained in American paddlefish [39].

In an extensive study of three Ponto-Caspian sturgeon species, Recoubratsky

et al. [40] found survival of stellate sturgeon, *Acipenser stellatus* Pallas, 1,771 gynogenetic offspring to be $\approx 36\%$ at the pre-larva stage and 33% of the pre-larvae at six months post-hatching. Yield of Russian sturgeon gynogenetic pre-larvae was $\approx 19\%$, with 4% of pre-larvae surviving to six months. Almost all gynogenetic embryos of sterlet died after heat shock [40]. A hatching rate of 28% gynogenetic offspring was obtained by Hassanzadeh Saber *et al.* [41] in stellate sturgeon, and successful production of gynogenetic progeny of sterlet was described by Fopp-Bayat *et al.* [42] with 19–25% hatching rate.

A hatching rate of 22% was achieved in meiotic gynogenesis of the currently most frequently cultured sturgeon species, Siberian sturgeon, *Acipenser baerii* (Brandt, 1869), and 108 gynogenetic specimens from 1,180 larvae reached age 3+ years [43]. In ship sturgeon, *Acipenser nudiiventris* (Lovetsky, 1828), meiotic gynogenesis yielded a 61% hatching rate [44]. Meiotic gynogenesis was also successfully induced in functional hexaploid shortnose sturgeon, with low hatching rate and low survival to five months post-hatching [45]. In bester, the hybrid of beluga female and sterlet male, the most commercially exploited sturgeon hybrid, meiotic gynogenesis resulted in hatching rates from 1–49% [46].

With few exceptions, the reported hatching rates in sturgeon gynogenesis were lower than those in gynogenesis of other cultured fish species. Survival of the juvenile gynogenetic progeny, as well as their growth performance, was significantly lower than that of juveniles from non-treated eggs. Hence, if gynogenesis is to be used in sturgeon culture, more effective protocols must be developed.

34.2.1.1 Inactivation of Male Genome

The genome in spermatozoa is usually inactivated using ultraviolet C (UV-C) irradiation. A wavelength of 254–260 nm provides the maximum level of absorption by DNA. It is difficult to generalize an optimal dosage of UV-C for sturgeon sperm genome

inactivation, as it may be influenced by individual differences in spermatozoon density. Reported doses vary from 120 J m^{-2} [40] to 2500 J m^{-2} [39]. Lebeda *et al.* [47] showed that a dosage of around 200 J m^{-2} was sufficient for inactivation of the male genome in Siberian sturgeon. This dosage is likely to be realistic for other species. Lower UV-C doses may result in incomplete inactivation of the genome, while high doses can damage the spermatozoon motility system and/or acrosome, which is essential for activation of sturgeon oocytes [48].

Generally, sperm should be diluted before irradiation according to spermatozoon density. Commonly used dilution ratios range from 1 : 4 to 1 : 19, sperm : diluent (seminal fluid or 0.45–0.7 % NaCl solution). Diluted sperm should be spread in a thin layer and irradiated by short, high intensity UV, rather than longer-duration, less intense, radiation [47]. To minimize possible light-dependent DNA restoration of UV-inactivated DNA by photoreactivation [49], the workspace should be illuminated by red light, irradiated sperm should be stored in light-protected containers, and bright illumination should be avoided at the beginning of incubation of gynogenetic embryos [40, 50].

Irradiation by UV-C affects the spermatozoon motility system and acrosome [51, 52]. Treatment with 4'-aminomethyl-4,5,8-trimethylpsoralen, followed by UV-A irradiation, has been reported to show low influence on spermatozoon motility, while ensuring sufficient inactivation of DNA [53]. This might represent a more moderate approach to sperm irradiation and be a possible substitute for UV-C treatment, as UV-A has been shown to have little or no effect on sperm fertilization capacity [54].

In sturgeon gynogenesis, it is useful to use heterospecific sperm of a species with a different ploidy level from that of the female species. Simple measurement of relative DNA content (ploidy level) of resulting progeny will allow evaluation of sperm irradiation without requiring molecular genetics analyses. Ploidy levels will differ among

gynogenetic progeny, offspring resulting from spermatozoon genome inactivation only, and offspring resulting from failure of sperm irradiation or from no treatment. This is also applicable to assessing the results of external shock treatment (Table 34.3).

34.2.1.2 External Shock

The parameters of external shock used in sturgeon meiotic gynogenesis are summarized in Table 34.2. Primarily, heat shock has been utilized for retention of the second polar body, using temperatures from $34\text{--}37^\circ\text{C}$. The optimal time for initiation of shock is $0.22\text{--}0.28\tau_0$ or 15–18 minutes post-activation. The τ_0 value refers to the duration of one mitotic cycle of synchronous cell division related to water temperature, and is more accurate than time post-activation [55]. Optimal duration of heat shock is 2–3 minutes. These treatment parameters are effective for retention of the second polar body, with a success rate of 94–100%.

Application of cold shock for retention of the second polar body in meiotic gynogenesis has been investigated in stellate sturgeon [41] and ship sturgeon [44]. Applied temperatures and duration of shock were 3°C for 60 minutes and 2.5°C for 30 minutes, respectively. The cold shock resulted in a hatching rate of 61% in ship sturgeon [44] and 28% in stellate sturgeon [41]. While the hatching rate of stellate sturgeon was similar to reports of other studies of sturgeon meiotic gynogenesis, that of ship sturgeon was considerably higher.

In fish, cold shock generally ensures higher percentages of viable gynogenetic progeny than does heat shock [32]. It is difficult to draw conclusions about value of cold shock in sturgeon. More research and, especially, more detailed studies of progeny from cold shock treatment than that of Hassanzadeh Saber and Hallajian [44], is needed to confirm efficacy of cold shock meiotic gynogenesis in sturgeon.

Surprisingly, only a single study has reported on the use of hydrostatic pressure shock to inhibit second polar body release in

Table 34.2 Treatment used in sturgeon meiotic gynogenesis and sex ratio of progeny, when available.

Female species	Male species	Re-duplication treatment ⁺			Sex ratio (%)		Reference
		Initiation	Treatment	Duration	♀	♂	
American paddlefish	shovelnose sturgeon	18 mpa/18 °C	35 °C	2 minutes	80.2	19.8	[58]
white sturgeon	white sturgeon	15 mpa/16 °C	34 °C	3 minutes	82	18	[37, 59]
bester	bester	15 mpa/15 °C	34 °C	3 minutes	70–80	20–30	[46]
shortnose sturgeon	shortnose sturgeon	20 mpa/13 °C	58.6 MPa	5 minutes	65	35	[45]
Siberian sturgeon	Siberian × Russian sturgeon	18 mpa/15 °C	37 °C	2 minutes	81	19	[43]
ship sturgeon	Siberian sturgeon	10 mpa/15 °C	2.5 °C	30 minutes	73.3	27.7	[44]
stellate sturgeon	Persian sturgeon	0.3 τ_0	35 °C	2 minutes	0	100	[60]
stellate sturgeon	stellate sturgeon	10 mpa/20 °C	3 °C	60 minutes	No data		[41]
stellate sturgeon	stellate sturgeon	0.25–0.35 τ_0	37 °C	2.5 minutes	No data		[40]
stellate sturgeon	Russian sturgeon	0.25–0.35 τ_0	37 °C	2.5 minutes	No data		[40]
sterlet	bester	18 mpa/15 °C	34 °C	2 minutes	No data		[42]
shovelnose sturgeon	American paddlefish	16 mpa/18 °C	35 °C	2 minutes	No data		[36]
American paddlefish	Amur sturgeon	18 mpa/18 °C	37 °C	2 minutes	No data		[39]
Russian sturgeon	sterlet	0.25–0.35 τ_0	37 °C	2.5 minutes	No data		[40]
Russian sturgeon	Russian sturgeon	0.25–0.35 τ_0	37 °C	2.5 minutes	No data		[40]

⁺ = treatment used for retention of the second polar body; mpa = minutes post-activation.
 τ_0 = duration of one mitotic cycle of synchronous cell division relative to water temperature [61].

sturgeon [45], although pressure shock is assumed to result in higher survival of offspring, compared to thermal shock [32]. The primary challenge in using pressure shock in sturgeon is the stickiness of the activated eggs, which must be fully eliminated before the eggs are transferred into the pressure chamber. Pressure treatment for meiotic gynogenesis must start 15–20 minutes post-activation [45], so commonly used techniques requiring at least 40 minutes for elimination of egg stickiness are not suitable. This difficulty can be overcome by application of 0.05–0.1 % tannic acid [56] or 0.03% sodium hypochlorite for 40 seconds [57], immediately after egg activation and fertilization are completed. In our experience, placing eggs into a commonly used de-adhesion solution (clay, NaCl, urea) in the interval between tannic acid treatment and pressure shock application, and for 30 minutes after shock, provides better incubation results and easier hatching.

Sturgeon gynogenesis represents a useful experimental technique, but its applicability to sturgeon aquaculture is questionable. With the average hatching rate of gynogenotes around 20%, and the survival of hatched larvae to adulthood around 10%, 10,000 eggs will provide in the neighborhood of 200 gynogenetic individuals, including 160–170 females available for further breeding or processing. Among these, 30–40 would be WW superfemales and 120–130 would be ZW females. Identification of WW superfemales, their viability, gonad development, and overall performance in controlled conditions, are important issues for further study. If they were viable and fertile, breeding with natural males would provide all-female populations, assuming female heterogametic sex determination in sturgeon (Box 34.2).

Pressure shock might hold the greatest potential for successful sturgeon meiotic gynogenesis, especially for mass application in aquaculture, but detailed investigation of the shock parameters, at least in commonly cultured sturgeon species, should be addressed in future studies.

34.2.2 Androgenesis

Androgenesis could serve as a tool for conservation of endangered species, as it allows restoration of live animals derived from spermatozoa only. Studies of interspecific androgenesis (using heterospecific sperm) may also aid in understanding interaction between the heterospecific nucleus and egg cytoplasm. There has been no report of viable androgenetic sturgeon progeny resulting from protocols commonly used in teleosts – egg genome inactivation, monospermic fertilization, and shock resulting in genome duplication of the zygote during the initial stages of cleavage. This may be attributed to the greater sensitivity of sturgeon eggs to treatment applied prior to insemination, greater sensitivity of the zygotes to shock treatment at the beginning of mitotic division, or greater sensitivity of sturgeon to high levels of homozygosity. As sturgeon are of polyploid origin, their genome expresses naturally higher heterozygosity than does that of diploids. Therefore, sturgeon may be more sensitive to reduced heterozygosity, a situation unlikely to occur under natural circumstances. Extreme depression of fertility and reduced size has been observed in entirely homozygous polyploid plants [62–64].

To overcome the problem of high homozygosity in androgenotes, Russian researchers, led by Anna S. Grunina and Alexander V. Recoubratsky, developed a protocol of dispermic androgenesis in sturgeon. The method includes genetic inactivation of eggs, their insemination with concentrated sperm to facilitate polyspermy, and heat shock to facilitate the fusion of sperm pronuclei [65]. Presumption of possible dispermic fertilization in sturgeon comes from the unique feature of sturgeon oocytes. Sturgeon oocytes differ from those of other Actinopterygii in possessing numerous micropyles, located in the region of the animal pole on the surface of the egg chorion [55]. Hence, several spermatozoa can move into micropyles concurrently, resulting in potential polyspermic fertilization.

Box 34.2 Sex determination in sturgeon

The sturgeon does not exhibit external sexual dimorphism. Molecular cytogenetic studies have not revealed significant chromosomal difference between males and females [78]. All analyses conducted to identify sex-specific markers have, thus far, failed, and no reliable marker for discrimination of sex in sturgeon is available. Mainly based on the sex of meiotic gynogenetic progeny, in which females and males have been usually reported in ratio 65–82% : 18–35% (Table 34.2), sturgeon are presumed to have female heterogametic sex determination (ZW females and ZZ males).

However, contradictory reports can be found in the literature. Entire male populations have been observed in gynogenetic progeny of stellate sturgeon, whereas control offspring comprised both sexes at the age of six months [60]. This substantially differs from results of other studies of gynogenetic sturgeon.

The available data suggest female sex heterogamety in many sturgeon species. However, studies published thus far do not preclude the possibility of concurrent male heterogamety, or of more complex autosomal factors influencing sex in sturgeon.

However, Pšenička *et al.* [48] reported that, following fusion of the first spermatozoon with egg cytoplasm, a fertilization cone is created at the entry site, to prevent fusion of another spermatozoon with the egg cytoplasm. Other micropyles are plugged by cytoplasmatic processes, and supernumerary spermatozoa are agglutinated by the content of egg cortical granules that is released into the newly formed perivitelline space. This process is presumed to prevent natural polyspermy in sturgeon [48]. Whether this mechanism is effective in irradiated eggs with inactivated female genome has not been investigated, and the most recent findings suggest that polyspermic fertilization may occur naturally in sturgeon (M. Pšenička, manuscript in preparation).

The inactivation of the egg genome in dispermic androgenesis is performed by X- or γ - ray irradiation at 220 Gy, followed by fertilization of inactivated eggs by sperm diluted at 1 : 10, rather than the commonly used 1 : 100, which should ensure dispermic fertilization [65]. Subsequently, heat shock at 35–37 °C for 2–2.5 min applied at 1.4–1.6 τ_0 causes fusion of the pronuclei of two spermatozoa [66]. The restoration of the diploid state of androgenetic progeny, by fusion of two sperm nuclei of different males, allows a heterozygosity level similar to that of normal progeny. If the spermatozoa originate from

the same male, the coefficient of inbreeding is 0.5.

Employing the above described protocol, viable dispermic androgenetic offspring were reported to be produced using conspecific gametes in Siberian sturgeon, Persian sturgeon *A. persicus* (Borodin, 1897), Russian sturgeon, stellate sturgeon, and beluga [66–68]. Dispermic androgenesis was also investigated between different sturgeon species to produce nucleocytoplasmic hybrids. Viable progeny was obtained using species with the same ploidy: diploid stellate sturgeon \times beluga; tetraploid Persian sturgeon \times Russian sturgeon; and Russian sturgeon \times Siberian sturgeon [65, 69]. However, not all combinations of species with the same level of ploidy will produce viable nucleocytoplasmic hybrids.

The development of androgenetic hybrids of closely related stellate sturgeon and sterlet or beluga was arrested at early stages of embryogenesis [69]. Similarly, interspecific dispermic androgenotes of species differing in ploidy (diploid \times tetraploid and vice versa) were found to be non-viable, with development ceasing at various stages of embryogenesis [69–71]. The viability of nucleocytoplasmic hybrids is probably driven by compatibility of the nucleus and cytoplasm in the hybrid combination.

Resulting problems might be partially overcome, as hybrids of sturgeon species

with same ploidy are fertile (see below). Use of eggs of fertile hybrids and sperm from one of the parent species of the hybrid might ameliorate the effect of nucleocytoplasmic incompatibility. In these cases, half the cytoplasm of hybrid eggs is derived from the genome of the species of sperm that was used for androgenesis. Hence, the nucleocytoplasmic incompatibility of egg cytoplasm and sperm nucleus is half that encountered using egg and sperm of two different pure species. The potential feasibility of this approach for overcoming nucleocytoplasmic incompatibility in nucleocytoplasmic hybrids was reported in interspecific androgenesis of common carp, *Cyprinus carpio* L 1758, and Prussian carp, *Carassius auratus gibelio* Bloch 1782 [72, 73].

Successful dispermic androgenesis has also been conducted using cryopreserved sperm of stellate sturgeon [74], Siberian and Russian sturgeon [75], sterlet, and beluga [76]. Such an approach might be highly valuable in sturgeon, allowing restoration of a species when only its cryopreserved sperm is available. Techniques of cryopreservation of sturgeon sperm has been well developed [77], and cryobanking of sperm of critically endangered sturgeon should become standard practice for their conservation via *in vitro* culture.

Despite the undoubted usefulness of dispermic androgenesis, the approach remains a topic of controversy among sturgeon researchers. Successful dispermic androgenesis has not been reported by any researcher other than Grunina and Recoubratsky, although its original publication was more than 30 years ago. Utilization of dispermic androgenesis techniques would be highly desirable for both research and conservation, and the described procedures are straightforward and within the reach of most laboratories.

34.2.3 Polyploidization

34.2.3.1 Meiotic Polyploidization

Induced polyploidy, particularly triploidy, is a commonly used technique in fish and

mollusc aquaculture, mainly to ensure sterility. Sexual maturation usually results in decreased body growth, higher incidence of disease, increased aggression, causing injuries and fish losses, and negative changes in organoleptic properties of the edible portions [79]. Hence, preventing sexual maturation of cultured fish before they reach market size has important economic benefits.

Triploidization in finfish culture is mostly obtained by meiotic polyploidization, via retention of the second polar body using pressure or thermal treatment. Resulting triploids are sometimes referred to as “maternal triploids,” as two-thirds of their genome is of maternal origin. Paternal triploids may be produced by using diploid spermatozoa of a fertile tetraploid male, but the value of such an approach is limited by low survival rates and growth performance of induced tetraploids of naturally diploid fish species.

In sturgeon, the process of triploidization and its results vary, depending on the ploidy of species used (Table 34.3). As in other diploid fish, triploidization of functional diploid sturgeon results in functional triploids, and such triploids are likely to be sterile, due to interference in chromosome pairing during meiosis. However, this assumption has been confirmed only in triploid bester females [46], and more evidence is lacking in the literature.

Induced retention of the second polar body in a diploid egg fertilized by a diploid spermatozoon, both from functionally tetraploid species (analogous to triploidization in diploids), produces functionally hexaploid offspring. Therefore, it is different from triploidization in the true sense of the word. Hence, we use term “meiotic polyploidization,” referring to doubling of the maternal chromosome set by retention of the second polar body, irrespective of the original ploidy of the species. Functional hexaploids, originating from meiotic polyploidization of functionally tetraploid species, possess six sets of homologous chromosomes (four from the mother and two from the father). Hence,

an even number of homologous chromosomes are likely paired for meiosis phase I. Such hexaploids are likely to be fully fertile, similar to spontaneous hexaploids (Box 34.3) of functionally tetraploid white sturgeon [13, 18, 80] and Siberian sturgeon [15].

Triploids of functionally diploid sturgeon species and hexaploids of functionally tetraploid sturgeon species can be easily produced using the protocols described for meiotic gynogenesis (see above), omitting sperm irradiation [37, 43, 46]. This approach has resulted in a higher hatching rate and overall survival of triploids of bester [46] and hexaploids of white sturgeon [37] and Siberian sturgeon [43], compared with gynogenetic progeny of those species. The triploid offspring of bester comprised 60–73% female [46], and hexaploids of white sturgeon were 86% female [59].

The fertility of spontaneous sturgeon hexaploids, better hatching and survival rates of induced sturgeon hexaploids compared to gynogenotes, and the potentially high ratio of

females produced, suggest that meiotic polyploidization of tetraploid sturgeon might be superior to gynogenesis as a tool to increase the number of females in cultured populations. Such functional hexaploids are more suitable for processing than for breeding, because their back-crossing with tetraploids results in pentaploid progeny, with most likely reduced fertility or full sterility. Establishment of brood stock containing both hexaploid males and females might avoid the necessity for meiotic polyploidization. However, there is currently no available information on overall performance, fertility, and sex ratio of offspring of hexaploid individuals derived from functionally tetraploid sturgeon species.

34.2.3.2 Mitotic Polyploidization

Mitotic polyploidization is accomplished by application of thermal or pressure shock to duplicate the zygote genome during initial stages of cleavage. It is sometimes known as tetraploidization, referring to the

Box 34.3 Spontaneous polyploidy in sturgeon

Spontaneous polyploidization is defined as naturally occurring duplication of one or more complete sets of chromosomes in a single individual. It has been reported in artificially propagated sturgeon [13–20, 80], with retention of the second polar body confirmed to be the cause in white sturgeon [80] and Siberian sturgeon [14]. Spontaneous polyploidization in functionally tetraploid sturgeon species results in fertile functionally hexaploid individuals. Backcrossing of these spontaneous hexaploids to tetraploid individuals produces fully viable functionally pentaploid offspring [14, 18, 80]. These individuals likely have reduced fertility, since their chromosomes cannot pair during meiosis prophase I, due to the odd number of chromosome sets. Such impairment interferes with gonad development and gametogenesis, similar to what is observed in triploid individuals.

Currently, most cultured sturgeon originate from tetraploid species. The occurrence of fertile spontaneous polyploid individuals among tetraploid brood stock can negatively affect reproductive capacity, by producing sterile pentaploid offspring, thus reducing caviar production and the overall efficiency of the facility. Spontaneous polyploidization in sturgeon is presumed to result from a prolonged interval between hormone stimulation and ovulation or stripping, causing eggs to over-ripen, as shown in bester [17] and several other fish species [84–87]. To avoid spontaneous polyploidy in cultured sturgeon, hormone stimulation of maternal fish should be planned with attention to establishing and maintaining optimal thermal conditions for the species [56]. Eggs should be stripped and fertilized immediately after ovulation, and the ploidy level of all fish should be determined before their inclusion in reproduction.

ploidy of obtained progeny. Analogous to triploidization, the expression “tetraploidization” is appropriate only for diploid species. The use of the term tetraploidization to refer to natural tetraploids or other ploidy levels is incorrect since, for example, suppression of the first cleavage of a tetraploid zygote results in octaploidy, and not in tetraploidy (Table 34.3). Therefore, “mitotic polyploidization” is a more accurate term.

A protocol for induction of mitotic polyploidization in sturgeon has been recently developed by Lebeda and Flajšhans [81]. The authors identified the optimal time of initiation, temperature, and duration of thermal shock for mitotic polyploidization of functionally diploid sterlet and functionally tetraploid Siberian sturgeon. The highest hatching rate of mitotic polyploids was obtained at 37°C for two minutes, applied 56 minutes and 59 minutes post-activation in sterlet (31%) and Siberian sturgeon (34%), respectively. Before shock treatment, fertilized eggs were held in clay suspension at 16°C [81]. The initiation of shock corresponded to

female pronuclei formation and the beginning of its migration, 0.88–1 τ_0 [55]. Mass application of the optimized mitotic treatment resulted in 12% hatching in sterlet, and produced 67% mitotic polyploid individuals. The larvae showed high mortality, with only 41 surviving to nine months, among which 34.15% were mitotic polyploids [81].

Fertile mitotic polyploids of sturgeon species would, hypothetically, produce gametes of the same ploidy as those of their natural conspecifics: diploid gametes from mitotic tetraploids of diploid species, and tetraploid gametes from mitotic octaploids of tetraploid species. In fish, spermatozoa of mitotic polyploids usually exhibit reduced fertilization ability [82], and mitotic polyploid females may produce aneuploidy or unreduced eggs [83]. The lower fertility of mitotic polyploids is usually attributed to dramatic genome size changes and alteration in the nuclear : cytoplasm ratio. However, this may not necessarily be the case with sturgeon mitotic polyploids, as sturgeon exhibit high genome plasticity [14]. No information with

Table 34.3 Ploidy levels of progeny resulting from different treatments of sperm and/or eggs, with respect to ploidy of parental individuals. Female and male genome contribution to resulting ploidy is in parentheses (female : male). All ploidy according to the functional scale.

Sperm		Normal			Inactivated genome*		
Eggs		Intact	Meiotic treatment†	Mitotic treatment‡	Intact	Meiotic treatment†	Mitotic treatment‡
Female	Male						
2n	2n	2n (n : n)	3n (2n : n)	4n (2n : 2n)	1n (n : 0n)	2n (2n : 0n)	2n (2n : 0n)
2n	4n	3n (n : 2n)	4n (2n : 2n)	6n (2n : 4n)	1n (n : 0n)	2n (2n : 0n)	2n (2n : 0n)
4n	4n	4n (2n : 2n)	6n (4n : 2n)	8n (4n : 4n)	2n (2n : 0n)	4n (4n : 0n)	4n (4n : 0n)
4n	2n	3n (2n : n)	5n (4n : n)	6n (4n : 2n)	2n (2n : 0n)	4n (4n : 0n)	4n (4n : 0n)

*gynogenesis
†retention of the second polar body
‡genome duplication of zygote during initial stage of mitotic cleavage

respect to fertility of mitotic polyploid sturgeon is currently available. They are likely fertile, as they possess an even number of chromosome sets. Their gametes could, therefore, potentially be used for more effective androgenesis and, possibly, mitotic gynogenesis.

34.3 Hybridization

Generally, interspecific hybrids are non-viable or, if they survive, their gonad development is significantly influenced by functional incompatibility of multiple interacting genes. Among fish, interspecific hybridization is not rare [88]. It occurs in natural populations [89], and is occasionally used in fish breeding [90]. The sturgeon is an exception among fishes, as many of its hybrids are commonly utilized in aquaculture [9, 23]. Similar to other fish hybrids, sturgeon hybrids are reared mainly for better performance compared with parent species (hybrid vigor).

The female-to-male ratio in sturgeon hybrids is 1 : 1, so hybridization is not a suitable tool for sex control. The best, a hybrid of beluga female and sterlet male, is the most common sturgeon hybrid in aquaculture. Other commonly cultured hybrids are crosses of the Adriatic sturgeon, *Acipenser naccarii* × Siberian sturgeon; the Russian sturgeon × Siberian sturgeon; and the kaluga, *Huso dauricus* × the Amur sturgeon *Acipenser schrenckii* – or their reciprocal hybrids.

Many sturgeon hybrids are fertile and are occasionally used for production of backcross hybrids. The best can be crossed back to its parental species [91]. Fertility of sturgeon hybrids is presumed to be dependent on the ploidy of the parent species. It is generally considered that sturgeon hybrids resulting from crosses of species of the same ploidy exhibit the ploidy of the parents and are fertile [10], while hybrids of species differing in ploidy levels exhibit a ploidy intermediate to those of parents [15, 92], and are sterile or only partially fertile. However, this

is a generalization not supported by some firm evidence.

Flajšhans and Vajcová [92] described functionally aneuploid sturgeon brood stock, and referred to these individuals as evolutionary pentaploids and heptaploids. They also hypothesized that evolutionary pentaploid (functionally $2.5n$) specimens may have originated in aquaculture from an intentional or accidental backcross of evolutionary hexaploid (functional triploids) with evolutionary tetraploid (functionally diploid) species, and the evolutionary heptaploid (functionally $3.5n$) specimens may have originated from a similar backcross of the evolutionary hexaploids to an evolutionary octaploid (functionally tetraploid) parent.

Fertile male hybrids of sterlet × kaluga, species with differing ploidy, were reported by Rachek *et al.* [93] (cited in Vasil'ev *et al.* [12]). The sterlet is a functional diploid with ≈ 120 chromosomes [94], and the kaluga is a functional tetraploid species having 250–270 chromosomes [95]. Their hybrid is a functional triploid with 185–195 chromosomes, and would be presumed sterile. Rachek *et al.* [93] observed that the males of this hybrid were able to produce sperm, and confirmed the fertilization ability of this sperm by producing backcrosses with sterlet and kaluga females. These results showed potential fertility of hybrids of species differing in ploidy.

Recently, we investigated gonad development in several sturgeon interspecific hybrids, and compared the results with gonad development in purebred species of the same age. Hybrids of species with the same ploidy (sterlet × beluga and Siberian × Russian sturgeon) exhibited normally developed gonads, similar to those seen in purebred specimens. In contrast, hybrids of species differing in ploidy (sterlet with Siberian and Russian sturgeon) did not display fully developed gonads. The results demonstrated that gonad development is influenced by genetic origin and ploidy of the sturgeon hybrids, and were consistent with full fertility of hybrids of species with the same ploidy. Sterility of females, but

possibly limited fertility of males, has been suggested for hybrids of species differing in ploidy [96].

These recent findings suggest different levels of fertility in sturgeon hybrids. The general assumption of sterility of hybrids of sturgeon species differing in ploidy, and the consequent lack of concern with respect to their escape from farms, should be seriously reconsidered. As a precaution, we suggest that all male sturgeon hybrids should be assumed to be potentially fertile.

34.4 Induced Sex Reversion

Sturgeon have an extensive period of gonad differentiation, with wide variation in onset both among species and within populations. They undergo gonad differentiation at six months to three years, with female differentiation preceding that of the male. Full maturity and first spawning is reached at 4–20 years, depending upon species, as well as geographic and environmental factors. The histological structure of the gonad is similar in all sturgeon species examined thus far. Detailed information about sex differentiation in sturgeon can be found in Chapter 33 of this book.

Treatment with sex steroids is a widespread technique for monosex production in fish culture [31], but it has not been commercially utilized in sturgeon, although several experimental studies have been conducted. Treatment with estradiol dipropionate resulted in incomplete feminization in sterlet [97]. Administration of an intraperitoneal capsule of 5 mg 17 α -methyltestosterone (MT) changed the sex ratio of a population of juvenile American paddlefish [98]. In bester, a diet including 10 mg kg⁻¹ body weight and 25 mg kg⁻¹ body weight of Estradiol-17 β (E₂), provided at 14–31 months of age, resulted in incomplete feminization, while MT at the same doses failed to induce masculinization. In contrast, 1 mg kg⁻¹ body weight E₂ and MT, fed from

3–18 months, induced feminization and masculinization, respectively [87].

In shortnose sturgeon, feminization was obtained by feeding on E₂ at 10, 25, 50, and 100 mg kg⁻¹ of body weight, starting from 5–7 months old. Fish fed 10 mg kg⁻¹ actively consumed the diet, and showed survival rates similar to a control group. Higher doses of E₂ were associated with decreased activity, and fish exhibited pathological changes in the liver and kidney [99]. Grandi *et al.* [100] showed a single eight hour immersion of Adriatic sturgeon embryos in 400 μ g l⁻¹ E₂ at 6.5 days post-fertilization to result in 70% females. The same treatment, applied at 1.5 days and 10 days post-hatching, did not change sex ratio from 1 : 1 [100]. In stellate sturgeon, intraperitoneal injection of E₂ at 5 mg kg⁻¹ body weight at three-week intervals from five months to 190 days, produced feminization without significant effect on growth and survival [101].

The results show that sex reversal via hormone treatment is feasible in sturgeon, as in many other fish species. Based on available research, hormone treatment in feed, starting at approximately three months of age, at doses of 1 mg kg⁻¹ to 10 mg kg⁻¹ body weight, is sufficient to ensure sex reversal with little or no effect on survival and growth. Application of a capsule into the body cavity or intraperitoneal hormone injection is prohibitively labor-intensive for mass production. On the other hand, sex reversal treatment during the embryonic period may be more efficient.

There are no available data on the early sex differentiating pathway in sturgeon, but the results of Grandi *et al.* [100] indicate that the gonadal differentiation pathway might occur in the brain or in primordial germ cells, prior to development of the gonad, similar to recent suggestions in other fish [102, 103]. Hence, hormone treatment should likely be administered before physiological sex determination in germ cells. Because the period is difficult to detect by observing the morphology of the gonad, more research on origin and migration of PGCs [104, 105], as well as on early sex

differentiation, is required to optimize timing of sex reversal treatment in sturgeon.

34.5 Conclusions and Future Perspectives

Undoubtedly, effective techniques for sex control could be of significant benefit to sturgeon aquaculture. Production of all-females, or at least a high proportion of females, in cultured populations would increase the profitability of sturgeon farms focused on caviar production. Despite considerable effort, such a program is not currently available. Meiotic gynogenesis provides a higher proportion of females in many sturgeon species studied thus far, but overall efficiency is low, due to poor survival and performance of gynogenetic offspring. Meiotic polyploidization of functionally tetraploid species to produce functionally hexaploid progeny could be a suitable method of increasing the ratio of female offspring, but much more research is needed to confirm this.

Androgenesis, implemented via dispermic fertilization, may be feasible for restoration of critically endangered sturgeon species, but shows no potential to be used for sex control in aquaculture. Commonly utilized interspecific hybridization in sturgeon aquaculture has no effect on the sex ratio. Fertility/sterility of sturgeon interspecific hybrids is influenced by their genetic origin and ploidy. Thus, it is not possible to make a general statement regarding the

sterility of hybrids of sturgeon species differing in ploidy.

Sex reversal via hormone treatment may be applicable to sturgeon, but more research is required before its wide implementation. Further research efforts focused on sex control in sturgeon should be devoted to:

- i) application of pressure shock for retention of the second polar body, at least in the most common commercially exploited species;
- ii) identification of WW superfemales among gynogenotes, and investigation of their performance;
- iii) determination of the sex ratio and performance of functional hexaploids resulting from meiotic polyploidization of functionally tetraploid sturgeon species;
- iv) the feasibility of influencing sex in early embryogenesis, for example, by manipulation of PGCs or with hormone treatment.

Acknowledgements

The present study was financially supported by the Japan Society for the Promotion of Science (JSPS), KAKENHI Grant number 14F04751. The grant provided Postdoctoral Fellowship for Overseas Researchers to Miloš Havelka (ID No: P14751). The Lucidus Consultancy is gratefully acknowledged for English correction and suggestions.

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Part X

Sex Determination and Sex Control in Crustaceans

35

Sex Control in Cultured Decapod Crustaceans

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35.1 Sex Determination and Differentiation in Decapod Crustaceans

Crustaceans, a largely diverse group containing over 65,000 species, inhabit most aquatic niches, where they are essential components of the marine food web [1]. They also support aquatic ecosystem well-being in their vital roles as scavengers and cleaners. Additionally, recent decades have witnessed a significantly increased demand for crustaceans in the global food market [2] that, in turn, has motivated efforts to increase yield and profit by optimizing crustacean aquaculture methods. While some of these optimization efforts have analyzed water quality, feed, genetics, and veterinary measures, others in the animal husbandry field have focused on the use of monosex crustacean populations, because of their inherent aquacultural advantages over mixed cultures. This scenario has elevated the demand for monosex populations, due not only to commercial considerations [3, 4], but also to ecological applications [5] and to concerns about sustainability. Therefore, understanding the mechanism that controls sex determination and sex differentiation in crustaceans is of global importance.

Crustaceans exhibit a wide array of reproductive strategies (Table 35.1). Gonochorism, the most common decapod crustacean reproductive strategy, in which male and female individuals are maintained dioeciously [6], constitutes most of the cases investigated thus far, as exemplified in this chapter by the giant freshwater prawn *Macrobrachium rosenbergii*. In decapod crustaceans, however, the gonochoristic model can reflect atypical complexity, insofar as it can include the formation of multiple male morphotypes [7] and even intersexual phenotypes [8]. For instance, the Australian red-claw crayfish, *Cherax quadricarinatus*, is a gonochoristic decapod crustacean that, in addition to having separate male and female phenotypes, also displays a fixed intersexual form, comprising individuals that, genetically, are females but, functionally, are males [9].

A more complex strategy is represented by hermaphroditism, which can be either simultaneous or sequential. Simultaneous hermaphroditism, or the concurrent functioning of an individual as both a male and a female [10, 11], can be seen in *Lysmata debelius* and *L. amboinensis*, in which each individual is able to function as either a male or a female, with a lapse of a few days between the corresponding sexual activities [12]. Sequential

Table 35.1 Different types of reproductive strategies and their representative decapod species.

Reproductive form	Strategy	Representative species
Gonochorism	Male and female are developed separately	<i>Macrobrachium rosenbergii</i>
Sequential hermaphroditism	Protandry (Male → Female)	<i>Pandalus platyceros</i>
	Protogyny (Female → Male)	Not reported yet*
Simultaneous hermaphroditism	Function with both male and female sexual organs	<i>Lysmata amboinensis</i>
Intersexuality	Genetically female which function as a male	<i>Cherax quadricarinatus</i>
Parthenogenesis	Female reproducing asexually to yield all-female clone	<i>Procambarus fallax forma virginalis</i>

*Protogynous crustacean species have only been found in isopod species; to date, no protogynous decapod species have been reported [18].

hermaphroditism occurs when an individual can change its sexual function during its lifetime [10], such as is the case of the northern spot shrimp, *Pandalus platyceros*. A protandric hermaphrodite decapod crustacean native to the North Pacific Ocean, *Pandalus platyceros*, exhibits three consecutive life stages: each animal begins its life as a functional male, and then undergoes a transitional stage that is followed by its transformation into a functional female [13].

At the opposite end of the decapod crustacean reproductive strategy spectrum is a case of parthenogenesis, involving an asexual reproductive strategy, in which females give rise to viable offspring without the requirement of fertilization [14]. Such a case was described in Marmorkrebs (*Procambarus fallax forma virginalis*), which produce females from unfertilized eggs to create an all-female population [15, 16].

Not surprisingly, the wide array of reproductive strategies found in crustaceans, coupled with their above-mentioned global importance as a viable source of nutrition, has rendered Crustacea one of the earliest evolutionary arthropod groups [17] in which sex determination and differentiation have been studied.

The general sequence of events that ultimately lead to mature, sexually differentiated animals in gonochoristic species (Figure 35.1) begins, upon the formation of the zygote,

with genotypic sex determination. Based on karyotype analysis, most crab species are assumed to bear the X/Y inheritance system, wherein the female is homogametic (XX genotype) and the male is heterogametic (XY genotype) [19]. However, in most decapods studied thus far, clear evidence of visually different sex chromosomes is yet to be found. This lack of evidence can be explained by the fact that decapods are known to have a large number of tiny chromosomes [20] that are difficult to pair to reveal which of them are actually the sex chromosomes.

On the other hand, according to progeny tests, studied decapod species, except for most crabs [19, 21], are assumed to possess the W/Z model of inheritance, in which the male is homogametic (ZZ genotype) and the female is heterogametic (WZ genotype) [9, 20, 22]. Genotypic sex determination in species that deviate from the gonochoristic scheme is yet to be found. For example, in hermaphrodite species in which sexual shifting between genders happens routinely throughout their life history, and in parthenogenetic species in which no males are found, it is likely either that sex chromosomes do not play a crucial role in the sexual determination process, or that they may not even exist. Genotypic sex determination is also absent in *Daphnia magna*, in which sex is determined solely by environmental effects [23].

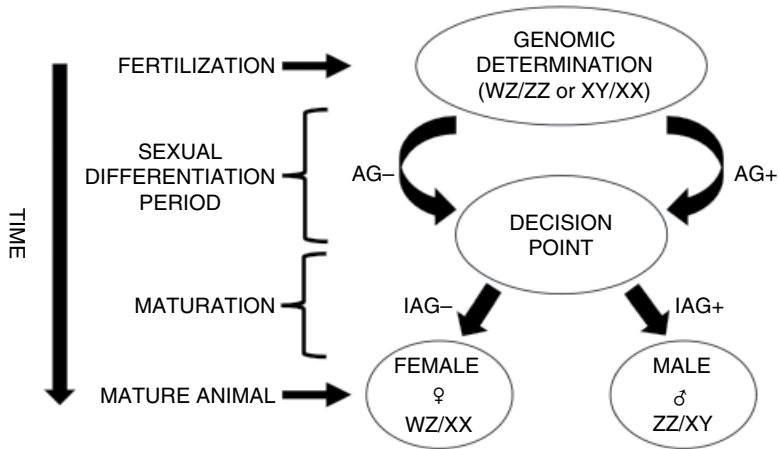


Figure 35.1 General sequence of events from fertilization to maturation in gonochoristic species bearing either the W/Z or the X/Y model of inheritance.

Genomic sex determination is followed by a differentiation period, prior to a decision point at which, if the AG has been formed, the insulin-like androgenic gland hormone (IAG) is secreted to cause maleness. If the AG has not been formed, then no IAG is secreted, and the animal develops into a mature female.

The sex determination process is followed by a short sexual differentiation period, during which several factors act to control the processes that drive gonad and secondary sexual character differentiation. It is suggested that a key factor in these processes is the androgenic gland (AG), a unique male crustacean organ that functions as a major endocrine switch, since its presence induces development of the male reproductive system, while its ablation in males promotes feminization [24–26].

The AG was first described by Cronin [27], who termed it an “accessory gland,” devoid of any function that could be inferred from direct observation. Charniaux-Cotton’s subsequent experiments on amphipods led her to suggest for the first time that the AG is a major player in male sexual differentiation [26]. In her experiments, implantations of AG-free testicular tissues in females were ineffective, while implantation of the AG not only induced the development of masculine characters in females – it also inhibited vitellogenesis [28].

A few years later, AG grafting in a female isopod resulted in the loss of a typical female secondary sexual character that, instead, was transformed into the corresponding

male-like feature [29]. In the decapod Australian red-claw crayfish *C. quadricarinatus*, AG implantation in females did not cause their complete sex reversal into males, but it did increase their aggressiveness and, in the presence of other females, they exhibited typical male courtship behavior [30].

As stated above, the same species demonstrates a rare case of genetically female individuals that function as males – which manifests in a small fraction of the *C. quadricarinatus* population whose members permanently bear both male and female genital openings (termed intersex individuals [9, 31, 32]). Following AG ablation in these intersex individuals, not only was male-like behavior reduced (aggressiveness and mating with females) but, also, the female reproductive system was activated (i.e., induction of vitellogenesis), while the male reproductive system was inhibited (i.e., inhibition of spermatogenesis) [32–35].

The manifestation of a permanent intersexual form in *C. quadricarinatus* reflects the degree of sexual plasticity found in crustaceans and, although it has been investigated in several works, a reasonable explanation for its existence has not been offered. In the giant freshwater prawn, *M. rosenbergii*, females

implanted with an AG exhibited evidence of masculinization [36], while AG ablation (andrectomy) caused the sex reversal of males to females. Re-implantation of the AG into andrectomized animals reversed the effects of ablation [37].

While the corresponding effects of the presence or absence of the AG are well described above, because the AG is the major determinant in the sexual differentiation process, the specific genes, hormones, and AG factors involved in this process will be described in the next section.

35.2 Regulation of Decapod Sexual Development

Upon zygote formation, the determination of genotypic sex occurs through the expression of sex differentiating genes that promote masculinization or feminization during embryonic development. In addition, those genes may directly or indirectly control the development of the AG. In decapod crustaceans, the genes that may drive sexual differentiation are numerous. Examples include *fem-1*, which was initially found in *Caenorhabditis elegans* [38], but whose homolog was found in the decapod *M. nipponense* [39]. The common pancrustacean genes *transformer-2* (*tra-2*) [40] and *doublesex* (*dsx*), first found in the fruit fly *Drosophila melanogaster*, control sexual differentiation. The latter gene is being alternatively spliced to produce different sex-specific *dsx* proteins [41]. A homolog to *tra-2* was found in the Chinese white shrimp *Fenneropenaeus chinensis* [42], and in the transcriptome of the decapod *M. rosenbergii*, in which a *dsx* homolog was also found [43].

Another example is the *dsx* and *mab-3* related transcription factor (DMRT), which is expressed in the testis of the Chinese mitten crab, *Eriocheir sinensis* [44]. In *D. melanogaster*, most of the genes on the male X chromosome are upregulated by the male-specific lethal (*Msl*) complex [45] and, in

decapods, a homolog to the *Msl3* gene was recently discovered in *M. nipponense* [46]. However, specific genes that form the controlling bridge between genetic sexual determination and AG development which, in turn, regulates the induction of masculinization in decapods, have yet to be found.

The next level in the regulatory hierarchy of sexual differentiation requires hormones and AG factors that directly control differentiation of the gonads and of secondary sexual characters. The IAG [47] is the most prominent AG factor responsible for inducing masculinization, including male gonad differentiation, the development of secondary male characteristics and the maintenance of masculine behavior. Since the discovery of the first decapod IAG in the Australian red-claw crayfish *C. quadricarinatus* [24], the IAG peptide has been found in all 20 species of the decapod groups investigated thus far [31, 48, 49], including the crustacean groups that comprise the bulk of aquaculture industry crops (i.e., prawn [50], lobster [51], crab [52], crayfish [24], and shrimp [53]). These and similar results suggest that the IAG-mediated sexual differentiation mechanism is conserved among decapod species.

Among the secondary male characters that are controlled by AG factors (i.e., IAG) is the development of the *appendix masculina* (AM) on the second pleopod. Development of the AM was proved to be correlated with AG cell activity, based on findings that AG-implanted females generated AM [36], and that IAG silencing in loss-of-function experiments prevented its regeneration [50]. In *C. quadricarinatus*, AG-implanted females exhibited male characteristics, such as the red patch on the propodus, and male-like pleopod shape and setation. On the other hand, the vitellogenesis process in those animals was inhibited [54].

In the giant freshwater prawn *M. rosenbergii*, three male morphotypes, representing different reproductive behaviors and secondary male characteristics, are well known [55]. AG-secreted factors were found to be essential to male morphotypic differentiation

based on AG ablation, which inhibited the appearance of morphotypes [56]. On the other hand, the injection of AG cell suspension into post-larvae WZ animals (supposed to develop into mature females) resulted in full sex reversal and the development of mature males exhibiting the typical male morphotypes [57]. In the same species, temporal knockdown of *Mr-IAG* in post-larvae ZZ animals (supposed to develop into mature males) not only inhibited masculinization, but also resulted in viable females [58].

In conclusion, AG activity in general, and the IAG hormone in particular, have proved to be key regulators of masculine sex differentiation, based on findings that its activation or silencing results in the appearance or regression, respectively, of primary and secondary male sex characters.

The AG functions within the eyestalk-androgenic gland-testis endocrine axis [59]. The X-organ, situated in the eyestalk, produces specific neuropeptides that mediate growth and reproduction activity, which are stored in, and secreted from, the sinus gland. Among other functions, they are thought to regulate AG activity, including IAG synthesis and secretion [59–61], which is supported by observations of hypertrophied and hyperplastic AG (hAG) [59, 62] and of the over expression of AG factors [63, 64] in eyestalk-ablated males. Further support of their regulation of AG activity comprises findings that reductions in the transcript levels of two prominent eyestalk-derived neuropeptides, gonad-inhibiting hormone (GIH) and molt-inhibiting hormone (MIH), significantly increased IAG expression (over 450%). These results also suggest that GIH and MIH negatively regulate the expression of IAG [61].

Another eyestalk neuropeptide, crustacean female-specific hormone (CFSH), was discovered to be highly expressed in the females, rather than in the males, of two crab species, *Callinectes sapidus* and *Carcinus maenas*. In these species, CFSH knockdown altered the normal anatomy of the female reproductive and mating system, suggesting that CFSH plays a role in female

phenotype development processes [65]. Female sexual differentiation, however, is commonly regarded as the default process, due to the wide consensus that the AG is a major factor in male sex differentiation. The recent findings by Zmora and Chung [65] of CFSH in crabs, therefore, may challenge the dogma claiming that female sex differentiation is the default process, caused solely by the absence of AG and IAG.

CFSH was also discovered in the Eastern rock lobster, *Sagmariasus verreauxi*, but its expression in the eyestalk ganglia was found to be similar in males and females [66], a result that dictates the need for further study of the physiological role of this hormone. In contrast, the wide understanding of the function and mode of action of the prominent conserved mechanism of IAG among decapods has paved the way for manipulations of sexuality in crustaceans. Such manipulations not only enable sex ratios to be altered in a certain population, they even facilitate the production of monosex populations, as will be elaborated on in the following sections.

35.3 Monosex Aquaculture of Decapod Crustaceans

For aquacultural purposes, monosex crustacean populations have distinct advantages over mixed cultures, because the males and females of most decapod species exhibit dimorphic growth patterns that cause variations in harvest size. Dimorphic growth can be attributed to a variety of parameters, including behavior, specific growth rate following maturity [67], and food conversion ratios [68, 69]. Some control over these parameters can be gained by using a monosex population, in which the absence of reproduction may effectively direct most of the energy of each individual to somatic growth [70]. As such, demand for monosex populations is rising, driven in part by commercial considerations [3, 4] such as yield improvement [71].

In addition, monosex populations are also sought as the ideal providers of certain ecological services – for example, sustainable pest control [5]. Prawns can be introduced into an aquatic niche to prey on pest snails [72], or on parasite-containing snails that are hazardous to humans [73]. For such pest control, a male monosex population is preferable, because of their larger body size, because they do not tend to migrate to the estuary to spawn like females do and, without females, they will be unable to reproduce and overrun the niche – a scenario with potentially devastating consequences for the niche ecosystem.

Intuitively, the gender chosen for culture when considering the use of a monosex population to obtain improved aquaculture yields should be that which grows faster and reaches larger size at harvest – both parameters that vary by species. Thus, male monosex populations have proved to have a higher mean weight at harvest in species exhibiting male superiority, such as prawns [67, 71, 74], crayfish [75–77], lobsters [78], and crabs [21], while monosex female populations are preferred in shrimp species that exhibit female superiority, such as *L. vannamei* and *P. monodon* [3, 79].

In species with male superiority (such as the freshwater prawn *M. rosenbergii*; [80]), however, it was recently suggested that all-female populations may be preferable over all-male cultures under high stocking densities, due to the lack of aggressive and territorial behavioral patterns in the former [70]. As a consequence, aquaculture based on female monosex populations could be intensified, to further increase both yield and profit [57, 74, 81, 82]. The homogenous size that females exhibit at the end of the grow-out season, an additional benefit to their use in aquaculture, reduces the need to perform manual partial selective harvests during the grow-out period [71, 81, 83, 84].

In conclusion, the increasing demand for monosex crustacean populations, either all-male or all-female, has generated the need to develop novel biotechnological tools to achieve the desired outcomes.

35.4 Commercial Biotechnologies to Achieve Monosex Aquaculture of Decapod Crustaceans

Traditionally, regardless of whether they were grown for research or aquaculture purposes, crustacean monosex populations have been generated by manual sorting [71, 85]. Labor-intensive, and of questionable reliability, manually distinguishing between the genders during the early crustacean developmental stages is too difficult to guarantee the production of a 100% monosex population. Moreover, the rising demand for monosex populations dictates the need to develop effective and sustainable biotechnological tools to enable the efficient and rapid generation of such populations.

Commercial biotechnologies to achieve monosex populations in aquaculture were initially developed for fish [86–93]. Today, some of the ideas first used in fish – primarily, sex reversal during the early developmental stages – are implemented in decapod crustaceans [57, 58, 80, 94]. As described in the previous sections, the IAG sexual differentiation switch is suggested to be a mechanism that is universal to all decapod species. Hence, sex manipulation via the IAG mechanism could be achieved by intervention, after genotypic sex determination but before the sexual differentiation period has concluded.

In most decapod crustaceans, which are assumed to bear the W/Z model of inheritance [9, 20, 22], monosex male populations may be obtained by a two-step procedure (Figure 35.2A). The first step comprises male (ZZ genotype) sex-reversal into a ZZ genotype female, termed a “neofemale.” The second step includes crossbreeding neofemales with normal males, to achieve an all-male population [58, 80] (Box 35.1).

On the other hand, to achieve all-female monosex populations in those species requires the use of a three-step procedure (Figure 35.2B). The first step comprises the sex reversal of females into “neomales” bearing the WZ genotype, followed by crossing

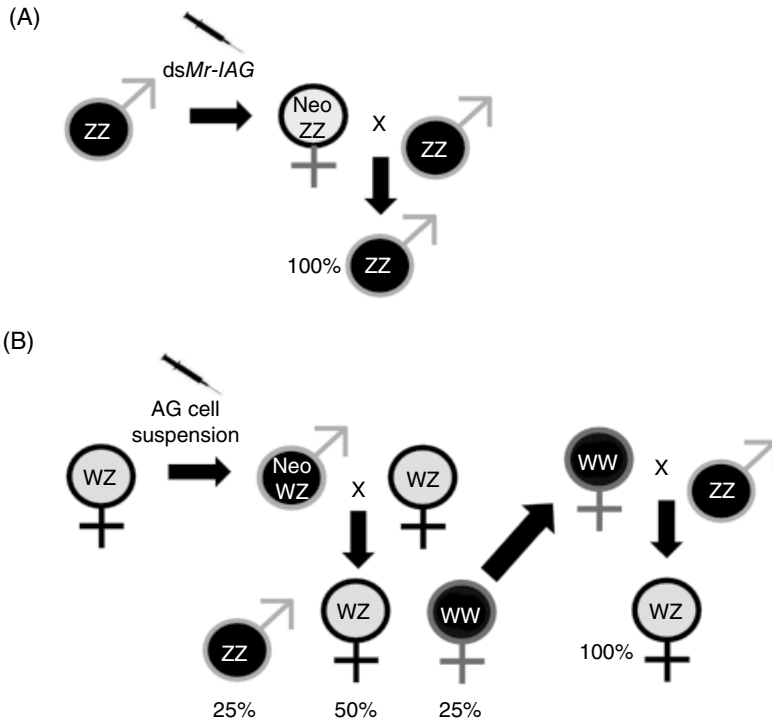


Figure 35.2 Scheme of crustacean monosex population production (modified from [57]).

A – Generation of an all-male population, using neofemales produced by silencing *Mr-IG* with *dsMr-IG*.

B – Generation of an all-female population, using neomales produced by a single injection of AG cell suspension.

Box 35.1 From decapod sex determination to monosex populations

Most decapod genotypic sex determination bears the W/Z model of inheritance, except for crabs, which have been found to display the X/Y model.

Early sexual differentiation occurs between fertilization and the point at which, if the androgenic gland is formed, then the animal develops into a mature male; however, in its absence, the animal develops into a mature female.

In the giant freshwater prawn *Macrobrachium rosenbergii*, 60 days post-metamorphosis is the window of opportunity for manipulating the sexual differentiation process.

M. rosenbergii monosex populations are achieved through a first step comprising sex reversal of males into neofemales or females into neomales, to create all-male or all-female populations, respectively.

Monosex biotechnologies in *M. rosenbergii* are implemented via the temporal knock-down of *Mr-IG* through injection of *dsMr-IG* (RNAi), or by a single injection of androgenic gland cell suspension to achieve neofemales or neomales, respectively.

neomales with normal females (WZ) to yield progeny in which 25% are WW females. Ultimately, WW females should be crossed with normal males (ZZ) to achieve all-female populations [57]. In most crab species, which are assumed to bear the X/Y model of

inheritance [19, 21], the procedures for obtaining monosex populations (male or female) will probably be the opposite of those described above.

The main challenge in producing a monosex population in crustaceans is the

generation of the neomale or the neofemale, because after a fertile “neo-animal” is obtained, the remainder of the work comprises simple crossbreeding. For example, to obtain all-male populations of the giant freshwater prawn *M. rosenbergii*, the sex reversal of males (ZZ) into neofemales was achieved through microsurgical AG ablation in post-larvae males [80, 95]. Although all-male populations were successfully produced using this procedure, the complexity of the microsurgery performed in the post-larvae resulted in low survival rates [80].

It was, therefore, suggested that the sex reversal procedure of *M. rosenbergii* males into neofemales could be improved by using temporal gene silencing via RNA interference (RNAi) [96]. The target gene for silencing was the prominent AG factor *Mr-IAG*, which was silenced by injecting ds*MR-IAG* [58], using an easy-to-perform and efficient (86%) [58] biotechnological procedure to obtain neofemales that produce all-male populations. Additionally, insofar as *Mr-IAG* expression is knocked down without genomic modification, it is suggested to be a safe procedure that does not involve genetic modification [58].

The first step in the production of the *M. rosenbergii* all-female population (i.e., generating neomales with the WZ genotype) was initially performed through the implantation of ablated AG in juvenile females [36]. Neomales (WZ) were crossbred with normal females (WZ) to yield the expected progeny ratio of 1 : 3 (males : females) [94]. This procedure provides strong support for the theories that the AG induces masculine characters, and that *M. rosenbergii* bears the W/Z

model of inheritance. Also in this case, however, the surgical procedures were difficult to perform, and the mortality rate of the implanted animals was high ($\approx 90\%$; [94]).

Recently, the production of *M. rosenbergii* neomales was markedly simplified by the development of a process based on a single injection of suspended hAG cells [57]. This biotechnology comprises the injection of juvenile females (age 60 days post-larvae or younger) with a suspension of ≈ 2000 hAG cells that was produced from endocrinologically manipulated males. WW females, from the progeny of neomales and normal females, were isolated after validation by genomic sex-specific markers [47], and crossed with normal males (ZZ) to yield all-female populations. Moreover, the fecundity of the abovementioned WW females, according to brood somatic index (BSI) [58], did not significantly vary from that of normal females, thus suggesting that this method holds promise as a highly applicable and simple biotechnology for the commercial mass production of all-female decapod crustaceans in aquaculture [57].

While the biotechnological tools to achieve monosex crustacean populations were invented for and applied to *M. rosenbergii*, due to the universality of the IAG-switch in *Crustacea*, it is suggested that these are, in fact, universal tools that can be easily tailored to other decapod species. Moreover, additional determinants of early sexual differentiation may be potential candidates for manipulation, but determining whether they are also applicable will require further, thorough, basic study of key factors and genes along the sexual differentiation cascade.

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36

Sex Reversal and Determination and Sex Control in Shrimp and Prawn

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36.1 Introduction

This review covers pioneer and recent advances on the knowledge of sex determination in commercially farmed prawn and shrimp, where control of sex is an important profitable factor in aquaculture because of a natural sex dimorphism. We have made our best effort to redirect the reader toward many articles that contain reviews and what would be necessary to achieve in further research, discussed from our personal perspective.

Fisheries and aquaculture of lobsters, shrimp, prawn, crabs, and crayfish constitute an enormous economic industry around the world for human consumption. Modern aquaculture of prawn and shrimp is always acquiring knowledge to improve production. Control of sex and growth have been two of the main challenges in the aquaculture industry, and a long-term intellectually puzzling achievement for biologists.

In decapods and, thus, in commercially-reared shrimp and prawn, one sex grows larger than the other, because of a genetic sexual dimorphism [1]. Males grow larger than females in freshwater species such as the Malaysian giant river, *Macrobrachium rosenbergii* de Man [2, 3], the red claw crayfish, *Cherax quadricarinatus* von Martens [4], and Australian yabbies such as *Cherax destructor*

Clark, *Cherax albidus* Clark, and *Cherax rotundus* Clark [5, 6].

In penaeids, the opposite occurs: females grow larger than males, previously reviewed in [7], in such species as the Pacific white shrimp, *Litopenaeus vannamei* Boone [8], the Kuruma prawn, *Marsupenaeus japonicus* Bate [9], the giant tiger prawn, *Penaeus monodon* Fabricius [10], the Indian prawn, *Penaeus indicus* H. Milne-Edwards [11], and the Chinese shrimp, *Fenneropenaeus chinensis* Osbeck [12]. The size of prawn and shrimp determines the selling price in the market, so male monosex or female monosex culture, respectively, make a better profit at harvest.

Prawn and shrimp are decapods, and males have a gland that is attached to the distal vas deferens, called the androgenic gland (AG), which secretes a hormone involved in male sex differentiation, spermatogenesis, and the development and maintenance of sexual characters [13–16]. Sex reversal techniques in freshwater prawn and crayfish are based on the removal (andrectomy) or implantation of this gland (see Box 36.1). The sex determination system for each species has been elucidated through sex reversal, followed by breeding and progeny testing, which are explained further ahead in detail, and are the principle of control of sex for aquaculture purposes. In marine shrimp, sex reversal has not been achieved but, in two species, sex

Box 36.1 Glossary of terms

Andrectomy: refers to the removal of the ejaculatory bulb (sperm duct) in freshwater prawns or terminal ampoule in penaeids, distal vas deferens, and AG attached to tissue, either by pulling them out from the coxa of the fifth pereopod with fine tweezers or by microsurgery.

Neofemales: prawns that have a female phenotype, but genetically are males.

Neomales: prawns that have a male phenotype, but genetically are females.

Intersex: an organism that bilaterally contains an ovary and testis, and externally it may have male or female structures.

has been skewed to females through the induction of triploid shrimp. In prawn and shrimp, the female is the one that determines sex and, therefore, a WZ/ZZ sex determining system is established.

Thus, we have two scenarios regarding sex in decapods. The first is a steady primary sex determination system with female heterogamety (a sex gene or genes somewhere at the W-chromosome), and the second involves a hormone secreted by a gland that differentiates the individual into a male (presumably, from a gene or genes at the Z-chromosome or at autosomal chromosomes). In theory, there must be a connection between these two scenarios but, until now, the molecular sex-mechanism that precedes the AG-development to elicit maleness, or that precedes and overrules a male-AG sexual pathway to elicit femaleness, remains unknown.

What could be the nature of this connection? One hypothesis points to what is known about sex determination in worms, but mainly in insects. Next generation sequencing has emerged as a powerful tool to study genomes and, recently, several genes involved in sex determination in the nematode *Caenorhabditis elegans*, and in the fruit fly *Drosophila melanogaster* Meigen, have been identified in prawn and shrimp. This is not entirely surprising, because crustaceans may

have colonized the land as insects [17]. A recent study of the phylogenetic history of arthropods supports that hexapods (terrestrial insects) are more closely related to aquatic crustaceans than to myriapods (centipedes and millipedes), and that these two are most distantly related to chelicerata (spiders and scorpions) [18]. This phylogenetic relationship may explain the existence of genes in the genome of prawn and shrimp (and other crustaceans) that are involved in the sex determination mechanism in insects. However, their function in decapods remains to be investigated.

36.2 Sex Reversal Techniques and Male Monosex Aquaculture in Freshwater Species

Aquaculture techniques in freshwater prawn, such as *M. rosenbergii*, began in the early 1960s, and the red claw crayfish *C. quadricarinatus* in the late 1980s [19].

36.2.1 Sex Reversal in *M. rosenbergii*

Monosex farming in *M. rosenbergii* began in the late 1980s, when manual sexing was the only way to separate males, but it was an impractical approach [3]. Sex-reversal techniques in *M. rosenbergii* were well-established for sex control, and all-male monosex culture is feasible nowadays. Boxes 36.2 and 36.3 describe pioneer sex reversal techniques in the prawn *M. rosenbergii*.

36.2.2 Sex Reversal in Crayfish

In *C. quadricarinatus*, intersexuality relates to sexual plasticity, and occurs having half of the genital organ as male and the other half as female, but secondary external characters are masculine on both sides. In the internal male side and externally, the AG maintains its effect of maleness whereas, on the other side, the absence of this gland allows the differentiation into an ovary, but up to previtellogenic stage – basically, an arrested ovary [25, 26].

Box 36.2 Sex reversal of male to female (to obtain neofemales) in *M. rosenbergii*

Neofemales were obtained by andrectomy of vas deferens in the youngest males, having around 1 g of body weight (around 60 days after metamorphosis), just when the gonophore complexes started to develop, and the gonads had not differentiated into testis. The prawns were then reared to adults [20, 21]. However, survival of neofemales was low under this procedure (1.3%). Thus, a second step was applied by crossing neofemales with males to obtain all-male progeny, and performing andrectomy in an earlier stage (between 20–30 days after metamorphosis), ensuring and increasing the number of sex-reversed prawn, and shortening the time to maturation [22].

In male to female sex reversal, a study in intersex *C. quadricarinatus*, andrectomy of distal vas deferens, along with AG tissue, showed in parallel a regression of male reproductive organs and the onset of vitellogenesis and functional ovaries. Additionally, an inhibition of male aggression during reproductive behavior occurred [25, 27].

In species such as *Procambarus clarkii* Girard [28], *C. destructor* [29], and *C. quadricarinatus* [30–32], within others, female to male sex reversal has been tested, using implantation of AGs or injections of a crude homogenate of terminal vas deferens and attached glandular tissue. Results have shown the development of male sexual characteristics, a change to male sexual behavior, partial or total inhibition of vitellogenesis, and a decrease of functional female reproduction.

In some species of crayfish, sex reversal could be overruled, because inter-specific hybrids among them showed all-male, or mostly all-male, progeny [33, 34]. Additionally, some male hybrids from specific crosses are sterile [34]. Therefore, inter-specific crosses require further research, and may lead to a genetic improvement for crayfish male monosex aquaculture.

Box 36.3 Sex reversal of female to male (to obtain neomales) in *M. rosenbergii*

Masculinized females were obtained by the implantation of one or two AGs. The tissue was surgically inserted inside the ventral commissure, between the cephalothorax and abdomen, in immature and mature females. Results showed that 81% of prawn had appendixes masculine and male gonophores connecting with vasa deferentia. Additionally, in the anterior region of the gonad, there were spermatogenic lobules, and the ovary was regressed with inhibition of vitellogenesis [23]. In another study, active male function and near-complete reversal of secondary sexual characteristics depended on the size and age at implantation of AG tissue into very young putative females. Recipients had 6.5–7.5 mm in carapace length around 30 days after metamorphosis. After growing, these neomales retained female gonophores, but were otherwise indistinguishable from normal males [24].

36.2.3 Sex Reversal Techniques Changed from “Manual” to Molecular Biotechnology

Modern biological research, such as RNA interference (RNAi), permits the post-transcriptional silencing or knockdown of a gene that may play a key role in sex differentiation, allowing to analyze or to corroborate its function. Briefly, the principle lies on the *in vitro* synthesis of double-stranded RNA (dsRNA) of a knowing gene sequence (such as the insulin-like AG precursor of the AG-hormone), which is injected into the organism tissues. Once in the cellular cytoplasm, dicer enzymes bind and cleavage dsRNA in small fragments (short interfering RNA, siRNA), which are recognized by the RNA-induced silencing complex (RISC). RISC complexes separate dsRNA, degrade the sense strand, and recognize and align by complementarity the antisense strand into the messenger RNA transcript (mRNA). Once incorporated, an RISC protein,

Argonaute, activates and cleaves the target mRNA, thus interfering with gene expression [35]. See review in decapods in [36].

As an alternative approach to the morphological identification of young males of *M. rosenbergii*, a female specific sex-linked marker allowed an early sex identification of prawn, and the genetic confirmation of neofemales [37]. Alternative to the highly-qualified expertise needed when performing one-by-one andrectomy by micro-surgery, the insulin-like androgenic gland hormone transcript was knockdown in *M. rosenbergii* from an early stage after metamorphosis [38, 39]. Methods are described in Box 36.4, which also includes this technology applied in crayfish [40].

Box 36.4 RNA interference for sex reversal in freshwater prawn

After periodically injecting male young *M. rosenbergii* at a dosage of 5 µg Mr-*LAG* dsRNA/g body weight, the therapy produced full and functional neofemales. When crossed with normal males, these produced all-male progenies, which represented novel biological, technical, and applied approaches. Importantly, the intervention procedure was within a certain time frame after metamorphosis, and male sexual characteristics were regularly monitored. The long-term assay of dsRNA injections (twice a week) were prolonged for nine months [39].

In *C. quadricarinatus*, after biweekly injections over a period of 25–30 weeks with Cq-*LAG* RNAi (1 µg dsRNA/g body weight), intersex prawn showed an empty sperm duct, degenerating testicular lobules, arrested spermatogenesis, and AG cells hypertrophied, possibly to compensate for low hormone levels. Additionally, pleopods presented feminized characteristics; the ovaries were larger than those of intersex control prawn, and were yellowish, due to the accumulation of yolk protein, thus, feminizing male-related phenotypes [40].

36.3 Sex reversal Techniques in Penaeids

There are no reports of successful sex-reversal by andrectomy or AG-implantation assays in young shrimp. Marine shrimp aquaculture techniques began in the early 1970s, and nowadays the most important species are *P. monodon*, and *L. vannamei* [19]. There are two rare cases of hermaphroditism in *L. vannamei* that could be related to inbreeding in farming [41]. Sexual dimorphism becomes significant at a certain size during rearing, which does not always conform with economic models of production, such as intensive shrimp farming.

Intensive *L. vannamei* culture has gained attention [42], mainly to prevent emerging diseases [43]. Although intensive systems produce high yields per cubic meter, shrimp may attain a mean size without a significant difference in gender; in *P. monodon* and *L. vannamei*, sexual size dimorphism starts from about 9–10 g, and becomes significant around 17 g [8, 10, 44]. Nevertheless, as intensive rearing technologies advance, and genetic selection programs apply, intensive monosex culture may contribute to higher yields and profitability. The major producers of *P. monodon* include Thailand, Vietnam, Indonesia, India, the Philippines, Malaysia, and Myanmar, whereas *L. vannamei* is a worldwide cultured species [19].

36.4 Sex Determination

Sex determination in crustaceans is primarily through sex chromosomes [45, 46]. In bisexual *Artemia franciscana* Kellogg, the recessive eye-white phenotype is partially sex-linked to the putative sex W-chromosome [47–49]. Studies showing distinguishable sex chromosomes are scarce [50]. In shrimp and prawn, there is no evidence of environmental sex determination; only one report discusses it [24] and, so far, studied species have a steady WZ/ZZ sex determination system, as reviewed [7, 36].

36.4.1 Sex Determination System in Freshwater Species, and Interspecific Hybrids

Sex-reversal in *M. rosenbergii* began in the early 1980s, and progeny testing in the early 1990s [20–24], giving the first achievements in sex control that were applied to prawn aquaculture. Crossing sex-reverted prawn with normal prawn elucidated a WZ/ZZ sex determination system in this species; WZ-neomales crossed with WZ-females skewed the progeny towards females [24], while ZZ-neofemales, crossed with ZZ-males, produced all-male progeny [21, 22]. This biotechnology is currently used in India, Thailand, and Vietnam [51].

In *C. quadricarinatus*, a WZ/ZZ sex determination system was determined after crossing WZ-females with ZZ-males, and between WZ-intersexes and WZ-females, which gave the expected 1 : 1 and 3 : 1 sex proportions, respectively. Therefore, WZ-intersexes were phenotypically functional males, but genotypically WZ-females [52]. All these studies confirm a WZ/ZZ primary sex determination mechanism.

In freshwater and marine interspecific hybrids, Haldane's rule has to be understood as a background, and this is presented in Box 36.5.

In crayfish, intra-subspecies crosses, and the inter-subspecific cross between male

Cherax destructor albidus Clark and female *C. destructor destructor*, gave the expected 1 : 1 sex proportion. However, the reciprocal inter-subspecific cross gave a consistent 3 : 1 male-to-female in three families, and all-male in one family. The authors suggested a different mechanism of sex determination between the two subspecies [5].

A hypothetical alternative suggestion may indicate Haldane's rule because of hybrid incompatibility, where the female *C. destructor albidus* is rare or unviable.

The idea of female heterogametic sex (WZ/ZZ) in penaeid shrimp followed Haldane's rule, after observing progeny skewed to male, from a hybrid cross between female *P. monodon* and male *P. esculentus* Haswell [55].

36.4.2 Sex Determination System in Penaeid Species (Triploid and Tetraploid Shrimp)

In penaeids, sex reversal and progeny testing have not been achieved and, therefore, there is no evidence of a sex determination system using these techniques in a particular species. Nevertheless, genome mapping shows sex-linked markers on the maternal genome in *M. japonicus* [56], *L. vannamei* [57], and *P. monodon* [58], suggesting a WZ/ZZ sex determination mechanism.

Chromosome-set manipulations for the induction of triploid organisms started to

Box 36.5 Haldane's rule

Haldane's rule (observed in nature by John Burdon Sanderson Haldane in 1922) states that, in a hybrid, when one sex is absent, rare or sterile, that sex is always the heterozygous sex [53]. Haldane's rule could be related to the evolution of sex chromosomes involving favorable and faster substitution of recessive or partially recessive sex-linked alleles fixed by selection, with some damaging effect as non-viability or sterility, and epistasis between them and autosomal loci. This suggests an intermediate stage of evolution,

where heterogametic sterility or non-viability appears in only one reciprocal cross and, therefore, predicts the following events in speciation:

- 1) hybrids of both sexes are viable;
- 2) the heterogametic sex is sterile or inviable in one reciprocal cross;
- 3) the heterogametic sex is sterile or inviable in both reciprocal crosses;
- 4) both sexes are sterile or inviable in both reciprocal crosses [54].

apply in fish and shellfish industries during the 1980s and 1990s, respectively [59]. In penaeids, it began by the end of the century (see review in [60]). The first report of induction of triploidy in penaeids was in *L. vannamei*, using cold shocks to retain the second polar body [61]. Ironically, non-viable triploid juveniles have been produced in this species [62], apparently due to mosaicism during early embryo development [63]. Nevertheless, viable triploid induction has been achieved in *F. chinensis* [64–66], *M. japonicus* [67–69], and *P. monodon* [70–72], with a plus of partial or total sterility [72].

In *P. monodon*, after retention of the second polar body, the triploid sex proportion was two females : one male [70]. In another study, it was one female : 1.625 males [72]. In contrast, sex in *F. chinensis* was skewed to females in a 4 : 1 proportion [64], stating by [73] that the presence of male offspring implied that the sex determination system of penaeid shrimp might be more complicated than the simple X/Y or Z/W system. *M. japonicus* gave all-female triploid shrimp, and proposed WWZ-female and ZZZ-male genotypes; if the female W-chromosome was over-dominant and the ZZZ genotype was not viable, then all-female triploid shrimp were produced [68].

There are alternative suggestions to the above statements. First, the sex proportion skew to females in triploid shrimp suggests a WZ/ZZ determination system, otherwise the sex proportion would be 1 : 1 (half XXX female and half XXY male) under a XX/XY sex determination system. Second, male triploid does exist [64, 66, 70, 72] and, therefore, the unviable, or lethal ZZZ-male genotype lacks support. Third, as proposed in [74], a hypothesis can be established based on recombination between sex chromosomes. This is between the centromere and the sex-determining region (or locus), which is shown in Figures 36.1–3, and explained in Box 36.6.

In the case of triploid shrimp, after retention of the first polar body, triploid *M. japonicus* gave a sex proportion of 16 females : one

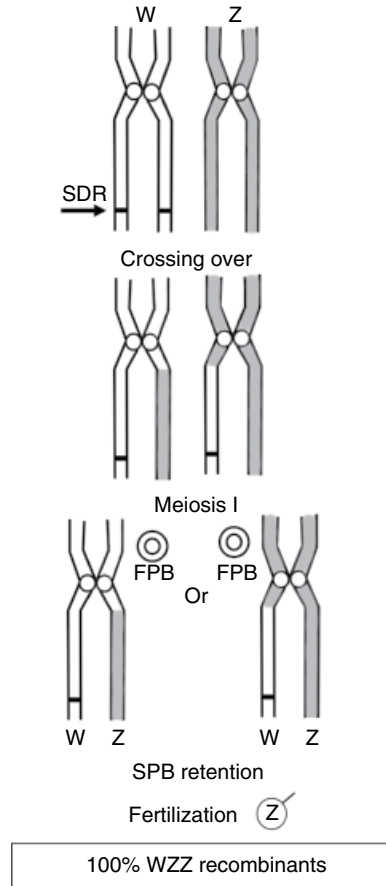


Figure 36.1 Hypothetical location of a sex-determining region (SDR) at the W-chromosome in *M. japonicus*. In the event of a crossing over, the farther this region is from the centromere, the higher the possibility of being included within the chromatid exchange, producing 100% triploid female WZZ recombinants.

FPB – first polar body; SPB – second polar body.

male [69], and triploid *F. chinensis* 4 : 1 [64]. Here, the presence of few males could result from rare non-recombination events. As an example, WZ-recombinants occur in *Artemia parthenogenetica* Bowen and Sterling. The diploid restoration happens during meiosis II, by retaining the second polar body; however, a previous crossing over is obligated, giving all-female progeny (100% WZ recombinants). Eventually, when an error occurs during meiosis, or the sex-region does not recombine, a rare fertile male

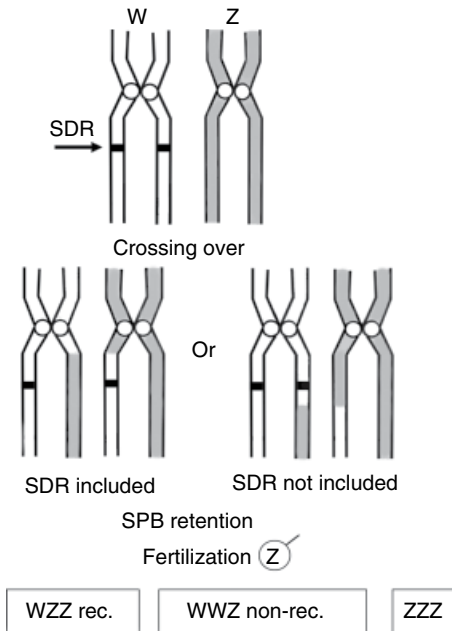


Figure 36.2 Hypothetical location of a sex-determining region (SDR) at the W-chromosome in *F. chinensis*. In the event of a crossing over, this region would be mostly included, producing WZZ recombinants (rec.), but also resulting in female WWZ non-recombinants (non-rec.) and few male ZZZ.

SPB – second polar body.

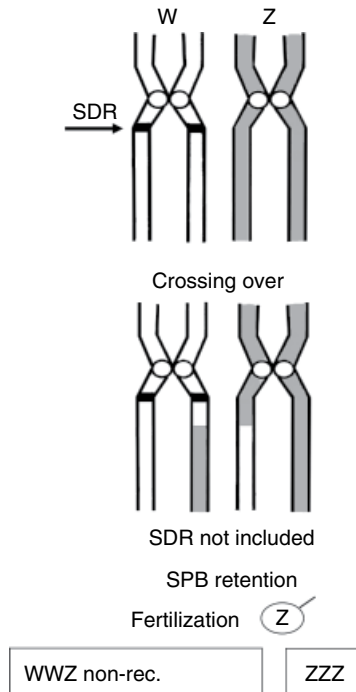


Figure 36.3 Hypothetical location of a sex-determining region (SDR) at the W-chromosome in *P. monodon*. In the event of a crossing over, this region is never included within the chromatid exchange, producing female WWZ non-recombinants (non-rec.) and males ZZZ.

SPB – second polar body.

Box 36.6 Recombination

After the induction of triploid shrimp with the retention of the second polar body, a 1 : 1 sex proportion is expected, where about half would be females (WWZ) and about half would be males (ZZZ), but only if there were not WZ recombinants during meiosis I. This statement assumes that achiasmatic meiosis does not occur, or would be rare [74], and has three hypothetical scenarios:

- Figure 36.1 one crossing over at pachytene exchanged non-sister chromatids, including the W-sex-determining region, resulting in all-female WZZ recombinants triploid shrimp, and suggesting that this sex region

should be far away from the centromere at the *M. japonicus* W-chromosome.

- Figure 36.2 if the sex determining region is relatively far away, it will frequently be included within the crossover, resulting in mostly female WZZ recombinants, female WWZ non-recombinants, and few male ZZZ, which could explain the 4 : 1 sex-proportion in *F. chinensis*.
- Figure 36.3 independently of recombination, the sex determination region should be much closer to the centromere, with a smaller possibility to recombine and, therefore, it never does (or, exceptionally, it would), which could explain the nearly 2 : 1 or 1 : 1.6 sex proportion in *P. monodon*.

appears [75]. Parthenogenesis may be the result of selection acting for another attribute in the heterogametic sex involving asexual reproduction [76]. Other examples involving sex recombination are found in fish, as the Nile tilapia, *Oreochromis niloticus* L. [77], and *O. mossambicus* Peters [78], having both an XX/XY system, and the blue tilapia, *O. aureus* Steindachner, having a WZ/ZZ system [79].

Induction of tetraploid shrimp has been unsuccessful. The rational logic to produce triploid shrimp is to perform crosses between viable tetraploid shrimp and diploid shrimp. However, after comprehensive research on the induction, no viable larvae have been produced [80–82], and experimental treatments resulted in lethal cytological defects during embryogenesis [63, 83, 84]. The importance of producing tetraploid shrimp is the further natural production of triploid shrimp, without the adverse effects of direct triploid treatments on growth and survival, plus a clearer analysis of the sex proportion.

As final comments, induction of triploids produces sterility in shrimp that could be important for genetic protection [60]. A clear advantage of triploid female in *M. japonicus* and *F. chinensis* should be kept in mind for further research in the still unviable *L. vannamei*, which will require new research protocols. If tetraploid shrimp remain unviable, then the growth of triploid shrimp under specific treatments of induction will require more research.

36.5 Sex Determination Mechanisms in Insects

In the fruit fly *Drosophila melanogaster*, sex determination depends on the balance of female determinants on the X chromosome and male determinants on the autosomes. The germline shows an XX-female, whereas an XY is in male, where the Y-chromosome is only required for functional spermatogenesis. In the female, germ cell development

requires three genes: (*ovo*), ovarian tumor (*otu*), and Sex-lethal (*Sxl*), which are inactive in the male germline (see review in [85]).

Sxl belongs to the family of RNA-binding proteins [86], and it is involved in the female-specific process of meiotic recombination [87], and mitosis of early germ cells [88]. The germline interacts with the surrounding somatic gonadal cell, which also influences sex differentiation (reviews in [85, 89]). Thus, *Sxl* is the sex determination master switch, and it controls somatic sexual development [85, 89, 90]. In the somatic embryo, there could be a double or a single dose of X chromosomes. If there is double (XX-female), *Sxl* is expressed and transcribes sex-specific mRNAs by alternative splicing, in which one encodes a functional SXL protein that activates a female transformer (*tra^F*) by a single SXL binding site at the *tra^F* pre-mRNA, and SXL also represses the activity of male-specific-lethal-2 (*msl-2*), involved in male-specific dosage compensation.

The presence of *tra^F* and transformer 2 (*tra2*) proteins regulates the mRNA alternative splicing of doublesex (*dsx*), producing a female-type transcriptional factor *dsx^F*, by binding to *dsx^F* pre-mRNA, and encoding the DSX^F protein, which will follow a female development. However, if there is a single dose of X chromosomes (XY-male), the sex-lethal gene (*Sxl*) is turned off; thus, male mRNAs are transcribed with a premature stop codon, originating a non-functional *Sxl* peptide, and so there is neither SXL nor *tra^F* proteins to regulate female *dsx* splicing. Instead, male-type transcriptional factors *dsx^M* are produced, which encode male DSX^M proteins. A dosage compensation is then activated, which increases gene expression of the single X-chromosome to equalize as an XX-female, followed by male development, including the second target of *tra* in the nervous system, fruitless (*fru*) involved in the regulation of sex-specific behavior (from reviews in [85, 89, 91]).

In the Mediterranean fruit fly, *Ceratitis capitata* Wiedemann, *Sxl* Sex-lethal is

conserved, but not sex-specifically regulated [92]. The gene *Cctra* has structural and functional homology to *tra*, which seems to start an autoregulatory mechanism in XX embryos, providing continuous *tra* female-specific function [93]. In the olive fruit fly, *Bactrocera oleae* Rossi, the gene *Botra* is orthologous to *tra*, splicing and encoding female functional polypeptides [94, 95]. In contrast, in the honey bee, *Apis mellifera* L., females are heterozygous at a single locus harboring the complementary sex determiner (*csd*) gene, whereas haploid bees and homozygotes are males.

The gene *feminizer* (*fem*) is an upstream component of *csd*, and encodes a domain involved in RNA splicing, sharing the same domain arrangement of *tra*, and a motif with *tra* of *C. capitata*. Therefore, *csd* may control sex-specific splicing of *fem*, and produces a functional protein in females. In males, a spliced variant contains a non-functional peptide, suggesting that the *fem* gene is the master switch by heterozygosity at *csd* of the sex determination pathway in this species [96]. *Sxl* is also highly conserved in other insect species [86, 97, 98].

36.5.1 Sex Determining Insect Genes Identified in Class Branchiopoda; Daphniidae

In *Daphnia magna* Straus, a *tra* ortholog gene (*dmagtra*) exists; however, this gene is not involved in environmental sex determination [99]. Nevertheless, a *dsx* homolog (*DapmaDsx1*), develops male characteristics; after knock-down in male embryos, a female ovary develops and matures, whereas the ectopic expression in female embryos results in male-like phenotypes. Therefore, this gene acts as a key regulator of the male phenotype during environmental sex determination. There is another *dsx* gene (*DapmaDsx2*) with similar structure, but clear phenotypic changes are not induced after silencing it [100]. In *Daphnia pulex* Leydig, there are two *Sxl* gene variants reported [101].

36.5.2 Sex Determining Insect Genes Identified in Macrobrachium Nipponense

Two *Sxl* gene variants (*Mnsxl1* and *Mnsxl2*), have 56–67% identity to insects and 51% and 64% to crustaceans such as *D. pulex* and the copepod *Lepeophtheirus salmonis* Krøyer, respectively. Both genes are expressed during embryo development, and decline at the zoea stage, before gradually increasing at post-larval stages. Additionally, testis and ovary have the lowest expression patterns [102]. Other homologous genes have been identified, such as *tra-2* (*Mntra-2*), which shares homology with *tra-2* in *Penaeus monodon* [103]. A homologous of *tra*, fruitless (*fru*), found in testis [104], has a male dosage compensation – male-specific lethal 3 homolog (*Mnmsl3*) – with a high expression in testis [105].

36.5.3 Sex Determining Insect Genes Identified in Penaeids

In *P. monodon*, *tra-2* (*PmTra-2*) has higher expression levels in testes and ovaries than other organs, and a testis-specific transcript 1 (*PmTst1*) [106]. The orthologous *tra-2* has also been identified in *F. chinensis* (*FcTra-2*), with three splicing variants – *FcTra-2a*, *FcTra-2b*, and *FcTra-2c*. The latter has the highest expression in ovaries whereas, during larval development, it increases its expression from mysis stages, remaining during post-larvae development [107]. In *M. japonicus*, two variants of the *Sxl* gene exist (*Pjsxl*), where the longest contig has 61% identity to *D. pulex Sxl*. *Pjsxl* expression has been identified from six hours post-spawning embryos.

Additionally, six variants of the orthologous *tra-2* have been identified, of which three had the highest identity for *PmTra-2*, while the other three aligned with the 3'untranslated region of *FcTra-2a* [108]. Moreover, *dsx* (*Pjdsx*) had a low level of expression after spawning, increasing from six hours up to the nauplius stages, and then maintaining at low levels during post-larva and adult ovaries and testes [109]. Therefore,

the complete *Drosophila* sex determination pathway (*Sxl*; *tra*; *dsx*) has been identified in the kuruma shrimp, long before the organogenesis of the genital organs [108, 109]. The *Sxl* gene has also been identified in *L. vannamei* [110]. Two versions of the male-specific lethal gene (*msl*) were identified in *M. japonicus* [108].

36.5.4 Sex Determining Insect Genes Identified in Crabs

The *Sxl* gene has been identified in the Chinese mitten crab, *Eriocheir sinensis* H. Milne Edwards (*Esxl*), showing the highest expression at the zoea stage larvae, and higher in testis and hepatopancreas than the ovary. Two splice variants were found without sex-specific expression in both genders [111].

36.6 Sex Determination Mechanisms in *C. elegans*

In the nematode *C. elegans*, the determination of sex depends on the ratio of X chromosomes to sets of autosomes. Hermaphrodites have XX (2X : 2A = 1.0), and males have XO (1X : 2A = 0.5) ratios. Most somatic tissues and organs differ from each other in anatomy and physiology, and there is a distinctive behavior in each of them. All these features are because of the differential activity of a “global” sex determination regulatory pathway, including control of the X-chromosome dosage compensation, by reducing transcription of X-linked genes in XX animals by one-half. Therefore, the number of X chromosomes controls sexual differentiation throughout the soma (reviewed in [112–114]).

The male cascade pathway begins with high expression of the upstream regulator *xol-1* (*XO lethal 1*), promoted by autosomal signal elements *sea-1* and *sea-2*, (signal element on autosome), which are transcriptional regulators in XO males. Thus, the inhibitory effects of X-signal elements from XX-hermaphrodites on *xol-1* do not overcome the positive expression of autosomal

signal elements. Then, *xol-1* remains active and inhibits *sd*c (sex-determination and dosage-compensation defect) genes that are active in XX animals.

In the next cascade step, the autosomal gene *her-1* (hermaphroditization) secretes a protein that binds to, and negatively regulates, the transmembrane receptor product of *tra-2* (sexual transformer). Thus, with *tra-2* off, *her-1* activates the *fem* genes (feminization) that produce three proteins: FEM-1, FEM-2, and FEM-3 and, together with CUL-2 (Cullin-2-like ubiquitin ligase), these form a complex that inactivates *tra-1* to bring out male development [112–115].

In XX animals, the double dose of X-signal elements represses *xol-1*. The genes that encode for these elements are *fox-1* (feminizing gene on X) and *sex-1* and *sex-2* (signal element on X). As a result of *xol-1* being off, the female pathway proceeds to regulate both somatic sex determination and X-chromosome dosage compensation, through *sd*c genes (*sd*c-1, *sd*c-2 and *sd*c-3), which encode a sex-specific SDC protein complex that controls somatic and germline sex by transcriptional repression of the *her-1* gene. Then, as a result of *her-1* being off, the product of *tra-2* is activated, inhibiting FEM proteins in hermaphrodites and, consequently, activating *tra-1* to bring out hermaphrodite development [112–115].

Therefore, in essence, *tra-1* is the terminal regulator, in which its activity is sufficient to trigger hermaphrodite development, and it contributes to the maintenance of *xol-1* repression, whereas the loss of its activity specifies male development, regardless of the activities of other genes in the pathway [112–115]. The feminization-1 gene family (*Fem-1*: *Fem-1a*, *Fem-1b*, and *Fem-1c*) is found in human, mouse and zebrafish [116].

36.6.1 Fem Genes in *Macrobrachium nipponense*

In *M. nipponense*, a *fem-1* homolog, (*Mnfem-1*), is expressed only in the ovary of adult prawn; it is highly expressed in both unfertilized eggs

and embryos at the cleavage stage, and thereafter drops to a low level from blastula to zoea, suggesting a maternal origin. In post-larva, it increases by the timing of internal and external sex differentiation [117]. In contrast, *Fem-1* homolog b (*Fem1b*) increases at 10 days after metamorphosis and has the highest expression level in the testis in juvenile and adult prawn [118]. The *Fem-1* genes (*Fem-1a*, *Fem-1b*, and *Fem-1c*) and other sex-determination related genes are annotated in this species [119].

36.6.2 *Fem* Genes in Penaeids

Fem-1 has been mapped in the genome of *P. monodon* [120] and identified in *L. vannamei* [121].

36.6.3 *Fem* genes in Crabs

The three members of the *Fem-1* family have been identified in *E. sinensis*: *EsFem-1a*, *EsFem-1b*, and *EsFem-1c*. These genes are highly expressed in early embryonic development, suggesting a maternal origin [122].

36.7 Concluding Remarks

- 1) Prawn and shrimp have a WZ/ZZ sex determination system (female heterogamety), and male sex differentiation is under the control of the androgenic gland.
- 2) What sex-mechanism precedes the AG-development to elicit maleness, and what precedes and overrules a male-AG sexual

pathway to elicit femaleness, remains unknown.

- 3) RNAi-biotechnology in freshwater prawn has revolutionized sex reversal techniques and the genetic identification of mature neofemales and their all-male-progeny, making control of sex and monosex production a modern aquaculture achievement.
- 4) In crayfish hybrids, inter-specific reciprocal crosses that may yield mostly or all-male progeny by Haldane's rule require further research.
- 5) The induction of triploid shrimp produces partial or total sterility, and it is the only biotechnological approach to skew sex to females, as in the Kuruma and Chinese shrimp.
- 6) The induction of tetraploid shrimp requires new research protocols.
- 7) Genes involved in sex determination in insects and nematodes that have been identified in the genome of a particular crustacean species will gain attention in further gene functional analysis research. This includes those genes that show a high expression in target sex differentiation-related organs, such as ovaries, testes, and AG.

Acknowledgements

This review is dedicated to all pioneer researchers in sex differentiation in Malacostraca, as well as those in monosex prawn and shrimp farming. We thank Consejo Nacional de Ciencia y Tecnología (CONACYT Grants 223157 and 258504).

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Part XI

Sex Determination and Sex Control in Other Fish Species

37

Sex Determination, Differentiation, and Control in Atlantic Cod

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37.1 Introduction

The Atlantic cod (*Gadus morhua* L., family Gadidae) is a cold-water marine species widely distributed throughout the North Atlantic continental shelf. For centuries, stocks of this iconic species supported some of the largest capture fisheries in the world [1], with a peak harvest of 3.9 million metric tons in 1968, but this was followed by a steady 40-year decline in harvests to a low of just 0.8 million metric tons by 2008 (www.fao.org). The collapse of the capture fisheries spurred interest in Atlantic cod aquaculture at the turn of the millennium, and it was soon predicted that farmed production would reach 140–160,000 metric tons by 2010 [2]. However, global aquaculture production peaked at only 23,000 metric tons, in both 2009 and 2010, and has since declined to less than 100 metric tons in 2015, coincident with slowly recovering harvests from capture fisheries (www.fao.org).

Although a number of factors contributed to the loss of interest in Atlantic cod aquaculture, of particular concern were the young age and small size at which fish mature under aquaculture conditions [3, 4]. This led to interest in developing methods for the production of single-sex populations of Atlantic cod, especially for the sake of producing all-female triploid (sterile) populations, which

have the added benefit of ensuring that any fish that escape from farms cannot breed in the wild [5]. The effective production of single-sex populations of fish requires knowledge of the genetic basis of sex determination in the species of interest, as well as the developmental stage at which undifferentiated gonads begin proceeding down the pathway to becoming ovaries or testes, and therefore are amenable to treatments designed to change their functional sex [6].

37.2 Sex Determination

Atlantic cod have a female-homogametic sex-determining system, equivalent to the XX/XY mammalian system (Box 37.1). This was first determined by examining sex ratios in families sired by hermaphrodites that had been obtained by treating larvae with a masculinizing agent (as outlined in section 37.4), and were therefore known to be genetic females [7]. When used to fertilize eggs from untreated females, the milt obtained from each of three such hermaphrodites yielded all-female families, in comparison to the expected 1 : 1 sex ratio from control crosses. Since these crosses were effectively mating genetic females with genetic females, the absence of male offspring provided clear evidence of female being the homogametic sex in Atlantic cod.

Box 37.1 Sex determination

Atlantic cod sex determination is genetic (female homogametic), with no evidence of any environmental component. Although a number of sex-linked loci have been identified, no sex-determining genes have been found as yet.

Further confirmation of a female-homogametic sex-determining system in Atlantic cod comes from studies using uniparental maternal inheritance (i.e., gynogenesis), a tool commonly used to identify the genetic mechanism of sex determination in fish [8, 9]. Three separate groups of researchers have reported the successful production of gynogenetic Atlantic cod populations [10–12], all using the same standard approach. Milt was first collected from mature males, then diluted in a non-activating extender solution, and exposed to UV radiation. The optimum radiation dose does not wholly destroy the ability of spermatozoa to swim and activate embryonic development, but is sufficient to cause conformational changes in DNA structure that prevent mitotic duplication of paternal chromosomes in the developing embryo.

Milt treated in this way was used for *in vitro* activation of embryonic development in eggs, followed by hydrostatic pressure treatment to block the completion of meiosis in the maternal genome and, thereby, retain the haploid second polar body. This protocol results in the production of gynogenetic diploids – that is, fish that have the correct diploid chromosome number, but with their entire chromosome complement inherited from the mother.

Each of the three studies that produced gynogenetic Atlantic cod used a different extender solution for diluting milt prior to irradiation: either Hanks' balanced salt solution and modified turbot extender [10], diluted sea water (2 parts sea water, 1 part fresh water) [11], or Mounib's Medium [12]. One study only used a single dilution

(1 : 80) [10], another tested three dilutions (1 : 20, 1 : 40, and 1 : 160) [11], and, in order to address concerns about variation among males in spermatocrit prior to milt dilution, a third used a modified approach of always first diluting milt to a 15% spermatocrit, and then tested four dilutions (1 : 10, 1 : 20, 1 : 40, and 1 : 80) from this pre-diluted milt, to determine an optimum dilution for irradiation [12].

In all three cases, diluted milt was spread in a thin layer in a glass dish and mixed with a magnetic stir bar during UV exposure. This is necessary because UV radiation has poor penetrating power through water. Diluted milt was kept cold, either by placing the dish on crushed ice [10, 11], or by conducting the entire procedure in a cold room [12]. One study used a standard UV exposure of 60 seconds at 3.689 mW/cm^2 (i.e., 221.3 mJ/cm^2), and did not specify the instrumentation or wavelength used [10]. The two others both used germicidal UV lamps (254 nm) for irradiation, and confirmed actual radiation intensity at the surface of the milt sample using UV meters [11, 12]. Furthermore, they varied exposure times (up to 15 or 20 minutes, respectively), in order to determine the optimum UV dose. On this basis, optimum treatments were deemed to be 1 : 40 dilution and three minutes at 0.5 mW/cm^2 (i.e., 90 mJ/cm^2) [11], and 1 : 10 dilution from 15% spermatocrit and three minutes at 0.63 mW/cm^2 (i.e., 113.4 mJ/cm^2) [12].

Hydrostatic pressure was used in all three cases for retention of the second polar body. One study conducted an experiment to optimize pressure treatment, varying duration and magnitude of the pressure treatment (2–8 minutes at either 34.47 or 55.16 MPa), as well as the time at which pressure was applied (10–35 minutes postfertilization [mpf]), and determined the optimum treatment to be six minutes at 34.47 MPa, beginning 15 mpf [10].

Although they did not state the temperature at which the eggs were held prior to treatment, assuming it was the same as used

for subsequent egg incubation ($5.5 \pm 1^\circ\text{C}$), this would equate to the pressure treatment starting at 82.5 mpf. The two other studies both used a previously standardized treatment [13] of five minutes at 58.6 MPa, beginning at 180 mpf [11, 12].

Uniparental inheritance was not confirmed for presumptive gynogenetic diploids in one study, but can be assumed for most of their fish, based on better survival, absence of characteristic deformities ("haploid syndrome"), and correct chromosome number ($2n=46$), in comparison to gynogenetic haploid controls ($1n=23$) [10]. Both other studies used genotyping with microsatellite DNA markers to confirm the absence of the paternal genome in fish identified as gynogenetic diploids, mostly using the same markers [11, 12].

Although ploidy level was not confirmed for presumptive gynogenetic diploids in one of the studies, it can be assumed for most of their fish, based on the absence of males [11]. The two other studies confirmed diploidy either by determining chromosome number [10], or by flow cytometric measurement of erythrocyte DNA content [12]. The fish produced in one study were not reared past larval stages [10]. Both other studies reared their fish to a size at which sex could be determined by histology, and found that every fish that could be sexed was female [11, 12]. Given the way by which these populations were produced (exclusion of the paternal genome and duplication of the maternal genome), this again provides clear evidence of a female-homogametic sex-determining mechanism in Atlantic cod.

Ovarian development appears to be somewhat affected by gynogenesis in Atlantic cod. One study found oocytes in the ovaries of all gynogenetic diploids examined as 8–9 month old juveniles, but their development was delayed in comparison to sibling diploid controls [12]. In contrast, another study found that 20% of similarly aged gynogenetic diploids still had undifferentiated gonads [11], with this value falling to 15% and 11%,

respectively, in two- and three-year-olds of the same population [14], but neither of these two studies included diploid controls for comparative purposes.

The latter study found that the proportion of fish with oocytes larger than 0.25 mm in diameter (i.e., cortical alveoli or later stages of development and, therefore, indicative of the onset of sexual maturation) was lower than expected for fish of these ages, increasing from just 52% to 77% of the population for two- and three-year-olds, respectively [14]. Furthermore, fecundity estimates for the maturing two-year-old gynogenetic females, as determined by counting the number of late-vitellogenic stage oocytes (>0.43 mm diameter) in an ovarian sample, and then extrapolating to the full ovary size, yielded values approximately half of what would be expected for normal maturing diploids of a comparable size [14].

Only a single study has examined the viability of offspring from gynogenetic Atlantic cod [14], achieved by hand-stripping two- and three-year-old females, and then fertilizing their eggs with milt obtained from normal diploid males. Egg diameter (1.16–1.32 mm) was as would be expected for normal females, and successful fertilization was observed in eggs from most of the fish (71% and 87% for two- and three-year-olds, respectively), but with highly variable fertilization rates (3–82%) and high mortality rates within 24 hours of fertilization. However, surviving larvae were observed to hatch and commence exogenous feeding on rotifers.

Although the fate of these fish was not reported, presumably they would all have been females, thus providing proof-of-concept that gynogenesis can be used to produce all-female populations of Atlantic cod. However, fish produced in this way are expected to be highly inbred, considering that they lack the paternal genome, so diploid gynogenesis, therefore, is not generally seen as an effective approach for the mass production of all-female populations for aquaculture.

37.3 Sexual Differentiation

Precursors to primordial germ cells (PGCs) are identifiable as early as the 32-cell stage of embryogenesis in Atlantic cod, as evidenced by expression of the germ cell markers *vasa* and *nanos3* [15]. Migration of PGCs to the location of the primordial gonad has occurred by 3–8 days posthatch, coincident with the onset of exogenous feeding [15, 16]. Subsequent anatomical and cytological differentiation of the gonads into ovaries and testes has been described in Atlantic cod in three separate histological studies [17–19]. Although all three describe a consistent pattern of sexual differentiation, there are differences among them in the size of the fish at which specific stages of differentiation were observed. The reason for this is not clear but could, conceivably, be a result of using different stocks of fish or different rearing conditions.

Atlantic cod gonads develop as paired organs suspended in the peritoneal cavity below the swim bladder and above the intestine. Undifferentiated gonads, with primordial germ cells, are present in fish as small as approximately 8.2 mm total length (TL) (7.6 mm standard length (SL) [17]. Although early stages of ovarian cavity formation are apparent by approximately 12.4 mm TL (11.5 mm SL) [17], all three studies used the appearance of a fully enclosed ovarian cavity as definitive evidence of anatomical differentiation of the ovaries, with this observed by approximately 14 mm TL (13 mm SL) [17], 18–20 mm TL [18], and 27 mm TL [19] (see Box 37.2).

Box 37.2 Sexual differentiation

Ovarian differentiation in Atlantic cod begins with the formation of an ovarian cavity when fish are 12–14 mm TL, and is completed by 79 mm SL, with the first appearance of primary oocytes. Differentiation of the testes begins when fish are 30 mm TL, and is completed by 65 mm TL.

Fish in the 14–20 mm TL size range can be separated into two groups, based on gonad length and germ cell numbers, with presumptive females having larger gonads, with both a larger number of mitotically dividing germ cells and a larger total number of germ cells, than presumptive males [18]. Cytological differentiation of the ovaries, as evidenced by the first appearance of primary oocytes, is observed much later in development (79 mm TL) [19].

The timing of when anatomical differentiation of the ovaries begins in Atlantic cod is matched by gene expression patterns for the *cyp19a1a* paralog, which encodes ovarian cytochrome P450 aromatase. The principal role of this enzyme is to convert endogenous testosterone substrate to the feminizing hormone 17 β -estradiol (E_2), thus mediating ovarian differentiation of the undifferentiated gonads. Transcription rates for this gene increase steadily from late gastrulation through hatching, yolk absorption, and the onset of exogenous feeding (by 5 mm TL) in Atlantic cod, although with expression mostly in the brain region, rather than the abdomen, and with no bimodal patterns that might suggest earlier differentiation of one sex before the other [20]. However, comparison of expression patterns in all-female populations with that in mixed-sex populations has shown that *cyp19a1a* transcription rates are already much higher in females than in males in fish as small as 12–14 mm TL [18] (i.e., at exactly the same time as the initiation of ovarian cavity formation [17]). Higher *cyp19a1a* transcription rates are maintained in females until fish have reached about 40 mm TL [18].

The gonads of male Atlantic cod remain undifferentiated through these early stages of ovarian differentiation in females. The first evidence of gonadal differentiation of testes comes from gene expression studies, in this case for the transcription of *amh* for the production of anti-Müllerian hormone mRNA. Similar to *cyp19a1a*, expression of *amh* is observed throughout early development (late gastrulation onwards) [20], but sex-specific

differences in transcription rates are not seen until fish exceed 30 mm TL, with higher mRNA levels in males than in females [18]. Presumptive testes are still undifferentiated in fish of this size, but both morphologically and cytological distinct testes are apparent by approximately 65 mm TL (60 mm SL) [17] to 94 mm TL [19] (see Box 37.2).

Other genes-encoding proteins that are associated with sexual differentiation are also expressed in Atlantic cod, preceding and during these developmental stages, including the *sox9a* and *sox9b* paralogs, the *cyp19a1b* paralog, *dax1*, *shp*, and several *dmrt* genes – but none appear to show sex-specific patterns [18, 20, 21].

Taken together, these studies point to the Atlantic cod being a differentiated gonochoristic species (i.e., with undifferentiated gonads developing directly into ovaries or testes, rather than through intermediate stages), with the differentiation of ovaries preceding that of testes.

37.4 Sex Control

The only reported successful production of single-sex populations of Atlantic cod came from a study that had the goal of producing all-female populations by first producing functionally masculinized genetic females (neomales), and then crossing them with normal females [7]. As noted in section 37.2, in species where female is the homogametic sex – as is the case for Atlantic cod – such crosses should yield all-female offspring. Producing neomales is typically done by exposing fish to masculinizing agents at the time of ovarian differentiation [6, 9], and this was the approach used in this one study [7].

Feeds containing the synthetic androgen 17 α -methyltestosterone (MT), at either 5 or 15 mg/kg, were fed to replicate groups of fish during five specific growth intervals: 12–16, 12–21, 12–25, 16–21, and 16–25 mm TL. Although none of these treatments increased the proportion of functional males within experimental groups (42–55%, compared to

47% for controls), the four treatments that spanned the 12–21 and 12–25 mm TL growth intervals resulted in a significantly reduced proportion of females (7–24%, compared with 53% for controls), due to the production of hermaphrodites (24–47%, with none observed in controls). Given the masculinizing effect of MT, and the decline in the proportion of females as the proportion of hermaphrodites increased, it can safely be assumed that these hermaphrodites were genetic females.

As noted in section 37.2, three of these hermaphrodites were subsequently crossed with normal females, and all three gave rise to all-female progeny. Although full functional masculinization was not achieved in this study, the milt obtained from these hermaphrodites was essentially the same as would be obtained from neomales with respect to the ability to produce all-female populations of Atlantic cod. However, obtaining milt necessitated killing the fish, dissecting out their testes, and diluting the milt in an artificial extender solution, prior to fertilizing eggs.

There has been only one other study to investigate hormonal sex reversal in Atlantic cod [17]. Because the genetic mechanism of sex determination was not yet known when this research was undertaken, attempts were made both to functionally masculinize and functionally feminize fish, using the synthetic androgen 17 α -methyl-dihydrotestosterone (MDHT), and the dominant teleost estrogen 17 β -estradiol (E_2), respectively. Steroids were delivered via feed in two separate experiments.

In the first experiment, replicate treatments of MDHT at 0.67, 2, and 6 mg/kg, and of E_2 at 5, 10, and 20 mg/kg, spanning the growth interval of 17–43 mm SL (approximately 18–46 mm TL), had only limited impact on sex ratios. The follow-up experiment, therefore, used higher steroid concentrations (MDHT at 3, 6, 12, and 18 mg/kg, and E_2 at 20, 40, 80, and 100 mg/kg) and started earlier in development, spanning the growth interval of 8–45 mm SL (approximately 9–49 mm TL). In this case, dramatically skewed sex ratios were observed, with a progressive decrease in

Box 37.3 Sex reversal

Although the production of functional sexually mature Atlantic cod neomales has yet to be reported, the optimum treatment for hormonal masculinization is likely to be by feeding MDHT at 12 mg/kg feed through the 9–49 mm TL growth interval.

the proportion of females, with increasing MDHT concentration (to 16, 4, 0, and 0%, respectively) and significant increase in the proportion of females in the two highest E_2 -treated groups (to 59 and 65%, respectively), compared with the control (45% female). Most non-female fish appeared to have normally developing testes, but some “intersex” gonads (containing both ovarian and testis tissue) were observed in all treatment groups in the second experiment, with less than 5% of the populations exhibiting this trait in the MDHT-treated and two lowest E_2 -treated groups, but increasing to 15–20% in the two highest E_2 -treated groups.

Because the experiments were terminated when fish were still juveniles, it was not confirmed whether presumptive neomales would have developed functional testes, or whether the intersex fish would have developed as functional hermaphrodites (Box 37.3).

When using endocrine manipulations to produce neomales in normal, mixed-sex populations, it can be difficult to distinguish these fish from normal males. Sex-specific genetic markers are useful in this regard, but are not available for most species of fish. Potential sex-specific markers were recently found in Atlantic cod, using whole-genome sequence data to identify numerous sex-linked loci [22]. The best of these are associated with a small (55 Kb) region within a linkage group that may contain a gene or genes for sex determination, but sequence data for this region showed no homology to any known sex-determining genes in other species, or for the genes known to code for proteins involved in sexual differentiation in Atlantic cod (see Box 37.1). Nevertheless, the availability of a diagnostic test for genetic sex

will facilitate future research to optimize the production of neomales, and to incorporate them into breeding programs for the production of all-female populations.

37.5 Triploidy

Combining the production of all-female populations with triploidy induction is the best approach currently available to eliminate sexual maturation in farmed fish [5]. Given the female-homogametic sex-determining mechanism in Atlantic cod, the simplest way to do this would be by using milt (either fresh or cryopreserved) from fully functional neomales to fertilize eggs, and then to expose these eggs to treatments designed to retain the haploid second polar body [6].

Although the production of all-female populations of triploid Atlantic cod has yet to be reported, mixed-sex triploid populations have been produced using standard approaches developed for other species. The first study to do so [23] focused on thermal treatments for triploidy induction, comparing cold (two hours at -1.7°C) with heat (20 minutes at 16, 18, 20 or 24°C) for eggs fertilized and held at 6°C prior to treatment. Three experiments used the same treatment start time (120 mpf), but a fourth also varied the time of treatment initiation (i.e., 20°C treatment beginning at 120, 180, or 240 mpf).

Cold treatment yielded very few triploids, but heat treatments were more successful, with the best results obtained from 20 minutes at 20°C , beginning 120 mpf. Other studies with Atlantic cod have used hydrostatic pressure to produce triploids [13, 24–28], always with high success rates, and mostly using a standard treatment of five minutes at 58.6 MPa, beginning at 180 mpf. Two studies that varied conditions for triploidy induction via hydrostatic pressure treatment found little effect of time at which treatment was initiated, i.e., five minutes at 58.6 MPa, beginning at 140, 175, or 210 mpf [27], or magnitude of pressure treatment (i.e., five minutes at 40, 50, or 60 MPa, beginning at 180 mpf [28]).

As is the case for other teleosts, the suppressive effects of triploidy on gonad size and germ cell development are generally more substantial for ovaries than for testes in Atlantic cod. For instance, although relative gonad size (i.e., gonadosomatic index) is lower in triploids than in diploids of both sexes, this reduction is greater in females than in males [13, 26, 27, 29–31]. Triploid males are capable of producing functional spermatozoa, and of competing effectively with diploid males in spawning with diploid females and fertilizing their eggs, although their progeny only survive through early developmental stages [32, 33].

In triploid females, on the other hand, the progression of oocytes through previtellogenic and vitellogenic stages is delayed, compared with diploids [29], and ovulated postvitellogenic oocytes have not been observed in triploids of the same age and size as sexually mature diploids [26, 29]. However, the effects of triploidy on ovarian development in Atlantic cod are not as dramatic as observed in Atlantic salmon (*Salmo salar*) and other salmonids [5], likely due to the much larger number of pre-meiotic oogonia and lesser increase in oocyte size during vitellogenesis in Atlantic cod, compared with salmonids. To some extent, this can be addressed for triploid Atlantic cod by exposing them to continuous artificial lighting, as has already been done to control sexual maturation in diploids [26].

Although triploid Atlantic cod larvae appear to be delayed in making the transition from live prey (*Artemia*) to prepared feeds [24, 25], their growth rate does not differ from that of diploids at either larval or juvenile stages [24–27, 30, 31, 34]. Triploid females do, however, have better growth rates than diploid females through the diploid spawning period, presumably due to their reduced investment in vitellogenesis and lack of spawning, but diploids quickly catch up in size after spawning [26, 30]. As has been reported for other species of fish [5, 35], the incidence of cranial, mandibular, and spinal deformities tends to be higher in triploid Atlantic cod than in diploids (24, 26–28, 30).

37.6 Conclusions

Atlantic cod have been confirmed to have a female-homogametic sex-determining system by demonstrating that:

- 1) milt obtained from genetic females yields all-female offspring when used to fertilize eggs from normal females; and
- 2) gynogenetic diploids are always female.

Furthermore, histological studies have shown that Atlantic cod is a differentiated gonochoristic species, with ovarian differentiation preceding that of the testes. Both gene expression studies and histological observations have shown that ovarian differentiation begins when fish are approximately 12 mm TL, by which time they have completed yolk absorption, and are dependent on exogenous feeds. Feeding juveniles synthetic androgens around this time can be used to produce reproductively functional hermaphrodites, and likely also functional males, and the milt obtained from such fish yields all-female offspring.

Combining this approach with triploidy induction should be an effective way to produce reproductively sterile populations of Atlantic cod for aquaculture, thereby both reducing production losses due to pre-harvest sexual maturation, and eliminating any risk of spawning by farmed cod within cages or subsequent to their escape.

37.7 Future Studies

Due to the current lack of interest in farming Atlantic cod, commercial-scale application of the research results outlined above has yet to be realized. However, if this situation changes, it should be possible to develop programs fairly quickly for the mass production of all-female diploid and triploid populations.

The use of all-female diploids would help alleviate production losses due to early maturation of farmed Atlantic cod, which occurs earlier and more frequently in males than

in females. The characteristics of diploid females derived from all-female populations should be no different from that of females in normal mixed-sex populations. For the sake of commercializing the production of all-female diploid populations of Atlantic cod, future research should focus on optimizing endocrine manipulations for producing functional neomales, capable of spawning naturally in tanks, or that can be stripped non-lethally.

Given that growth and developmental rates are temperature-dependent in all ectotherms, information on the timing of endocrine treatments should always include sufficient information on fish size, age, and rearing temperature to allow for comparisons among studies. Fish size data should be expressed as both standard and total length prior to fixation, and fish age should be expressed relative to temperature (i.e., in acquired thermal units such as °C-days). Scaling up the production of all-female populations would also benefit from the development of simple and reliable PCR-based genetic markers for sex, to distinguish neomales from normal males.

In the presence of wild males, the use of all-female diploid populations would not prevent genetic introgression from farmed populations into wild stocks of Atlantic cod; addressing the risks associated with this requires the use of sterile fish. The commercial-scale production of sterile fish can currently only be achieved through triploidy induction. However, extensive evaluation of triploid Atlantic salmon has shown them to differ in many ways from diploids [5], and future research should, therefore, focus on determining any triploid-specific culture

requirements – for instance, to address the one production problem already recognized for triploid Atlantic cod: cranial, mandibular, and spinal deformities. These same deformities have been associated with triploidy in Atlantic salmon, and have now largely been addressed through diet reformulation [36].

The only relevant research conducted on triploid Atlantic cod to date has focused on muscle structure [25, 34] and gut morphology [31, 37]. The benefits of selecting for triploid performance in breeding programs should also be investigated, given evidence for family effects in Atlantic cod [13, 31]. Future research could also focus on developing alternatives to triploidy for producing reproductively sterile populations, such as interfering with the expression of genes associated with germ cell formation [38–40].

Acknowledgements

My contributions to the study of sex control in Atlantic cod were only possible through teamwork that involved dedicated graduate students and government scientists, and with the generous support of government funding agencies and industry partners. I especially want to thank my former MSc students Jessica (Whitehead) Feindel, Nathaniel Feindel, and Song Lin, and research colleagues Debbie Martin-Robichaud and Ed Trippel (Saint Andrews Biological Station, Fisheries and Oceans Canada) and Mike Reith (Institute for Marine Biosciences, National Research Council of Canada).

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38

Sex Differentiation, Sex Change, and Sex Control in Groupers

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38.1 Introduction

Fish belonging to the Serranidae are distributed in tropical, subtropical, and temperate seas around the world. The Serranidae are subdivided into three subfamilies – Anthiinae, Epinephelinae, and Serraninae – and fish belonging to Epinephelinae are called groupers. A total of 475 species of groupers in 64 genera are known [1], and groupers are commercially valuable as table fish [2]. The catch of groupers in Japan, southeastern Asian countries, and Australia has been gradually declining each year, because of overfishing and habitat deterioration. Aquaculture strives to cultivate larger and more valuable groupers [3], and grouper aquaculture is popular primarily in southeastern Asian countries [4]. Successful seed production for aquaculture of *Epinephelus akaara*, *E. septemfasciatus*, *E. bruneus*, and *E. malabaricus* has been established in Japan [5]. Further basic and physiological studies on sex differentiation, maturation, and sex change in groupers are essential for stable seed production.

Groupers exhibit protogynous sex change during their life history [6–8]. Undifferentiated gonads of all individuals differentiate into ovaries during the fry stage, and mature after they reach adulthood. After functional maturation, the ovary changes into functional testes [6].

Therefore, in general, males of a species are larger than females. Larger males in the wild have decreased remarkably in numbers, owing to overfishing so, although both males and females are essential for offspring production, collecting larger male groupers from the field is very difficult. In addition, substantial amounts of money, labor, and space are necessary for the long-term rearing of large males in captivity, in order to collect sperm.

Our ultimate aim is to carry out female-to-male sex reversal artificially for aquaculture. To achieve successful sex change, analyzing the mechanisms of sex differentiation and sex change in groupers is necessary. Here, the physiological characteristics of sex differentiation, sex change, and artificial control of sex in groupers is discussed. The information presented is a compilation of research results, obtained at the author's laboratories over the past 17 years. Two species of grouper of the genus *Epinephelus* were used: one is the large Malabar grouper, *E. malabaricus* (maximum total length 100 cm) [9], which is cultivated as food in the Okinawa Prefecture, Japan [10], and has been used for a model in research on sex differentiation; the other species is the smaller honeycomb grouper, *E. merra* (maximum total length 25 cm), which predominantly inhabits the coral reefs in Okinawa, and has been

used for model research on sex change because it is easy to catch and rear in captivity.

38.2 Sex Differentiation in Grouper

38.2.1 Histological Characteristics of Sex Differentiation

In many fish, the most effective time to induce artificial sex reversal with exogenous steroid hormones and aromatase inhibitors is the gonadal sex differentiation period [11]. In gonochoristic fish, the commencement of

gonadal sex differentiation is characterized by germ cell differentiation and sex-specific differentiation of somatic cells, such as ovarian cavity formation in the ovary, and efferent duct formation in the testis [12]. However, there is little information on morphological sexual differentiation in groupers. Thus, the morphological characteristics and timing of gonadal sex differentiation in Malabar grouper were examined [13].

Gonads of Malabar grouper from 11–39 dph (0.3–2.3 cm in total length [TL]) were in the undifferentiated stage (Figure 38.1A). The gonads consisted of single germ cells enclosed by a few somatic cells. Morphological sex differentiation starts initially in the gonads of

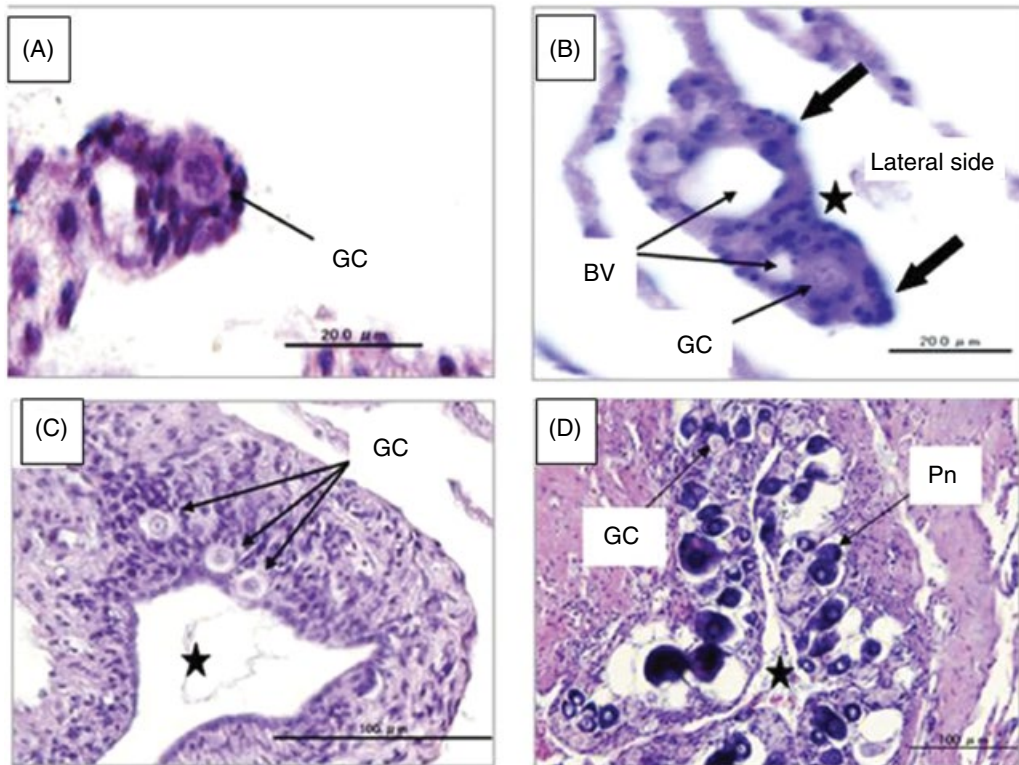


Figure 38.1 Gonadal sex differentiation of Malabar grouper. (See inserts for the color representation of this figure.)

- (A) Undifferentiated gonad at 39 days post-hatch (dph), which consists of individual oogonial germ cells enclosed by a few somatic cells.
 (B) Initial ovarian differentiation at 47 dph. Two elongations of somatic tissues (arrow), indicate initial ovarian cavity formation. Asterisk (*) indicates the side of lateral wall.
 (C) An ovary at 144 dph. Single oogonia are seen in the somatic tissue. Asterisk (*) indicates the ovarian cavity.
 (D) An ovary at 720 dph. Many oocytes at the peri-nucleolus stage (Pn), together with oogonia are seen.
 GC – germ cell. BV – blood vessel.

fish at 47 dph (TL 3.4 cm). Two elongations of somatic cells, indicating initial ovarian cavity formation, were evident in the gonads on the side facing the lateral wall (Figure 38.1B). In contrast with active changes in the somatic cells, the germ cells were not actively dividing, and remained at the oogonial stage. Ovarian cavity formation was observed in all gonads at more than 47–180 dph (Figure 38.1C). Germ cells in the ovaries at 59 and 74 dph (TL 5.3–8.2) remained at the oogonial stage for long after ovarian cavity formation.

The number of germ cells in the ovaries increased gradually through 243 dph (TL 20.8 cm). By this stage, some oocytes had already entered into meiosis (i.e., oogenesis) and had dispersed into the somatic tissue on the side facing the ovarian cavity. Oocytes increased in diameter and in number in the ovaries of fish by 360 dph (TL 24.6 cm). Oocytes at the peri-nucleolus stage appeared and developed in the ovary at 450–1,230 dph (33–60 cm) (Figure 38.1D). Primary males with gonads that begin testicular differentiation directly from the undifferentiated gonad were not seen. Sex change from immature ovary to testis was also not seen during the process of gonadal sex differentiation in Malabar grouper. This fact suggests that monandrous species of sex-changing fish do not possess identified sex-determining genes, as seen in gonochoristic species, or that these genes have become dysfunctional [8].

The results presented herein indicate that, in *E. malabaricus*, the most effective period in which to induce sex reversal from female to male by treatment with androgen may be 30–80 dph.

38.2.2 Endocrine Mechanism of Sex Differentiation

It is well established that gonadal sex differentiation in gonochoristic fish is controlled by the endogenous sex steroid hormones [11, 14]. To clarify the role of endogenous sex steroid hormones for sex differentiation in the Malabar grouper, the expression of three steroidogenic enzymes was examined

immunohistologically in the gonads during and after ovarian differentiation. These were: cytochrome P450 cholesterol-side-chain-cleavage (Cyp11a1), which is an essential key enzyme for steroidogenesis; cytochrome P450 aromatase (Cyp19a1a), which is essential for estrogen (estradiol-17 β : E₂) production; and cytochrome P450 11 β -hydroxylase (Cyp11b), which is important for fish-specific androgen synthesis, such as 11-keto-testosterone (11-KT) [15].

The first appearance of Cyp19a1a was seen in the gonads at the undifferentiated stage (30 dph) and at the initiation of ovarian cavity formation (45 to 57 dph) (Figures 38.2A and 38.2B). However, Cyp19a1a-positive cells were elliptical in shape, like a fibroblast. Cyp11b-positive signals were not detected in any somatic cells prior to ovarian differentiation (Figure 38.2C). Positive immunoreaction against Cyp11b was first seen at the end of ovarian cavity formation, in the cluster of somatic cells near the blood vessels on the dorsal side of the ovary. Later, Cyp11b-positive cells, which were globular in shape, were expressed in the cluster of somatic cells in the ovary tunica near the blood vessels.

Reactions against Cyp11a1 and Cyp19a1a were seen to co-localize in the somatic cells surrounding germ cells on the side facing the ovarian cavity in the ovaries of fish at 130 dph (Figure 38.2D, E and F). Co-localization of Cyp11a1 and Cyp11b was also observed in the cluster of somatic cells in the ovary tunica near the blood vessels. However, Cyp19a1a and Cyp11b did not co-localize in any cells. These results indicated that there were at least two types of steroid-producing cells (SPCs) – estrogen-producing cells, and androgen-producing cells.

Furthermore, these cells have different ultrastructural characteristics [15]. Estrogen-producing cells have a small cytoplasmic volume with few organelles, such as fibroblasts. In contrast, androgen-producing cells have a large cytoplasmic volume with many large mitochondria, a structure typical of SPCs [16–19]. The timing of the first appearance of these cells is also different. These results

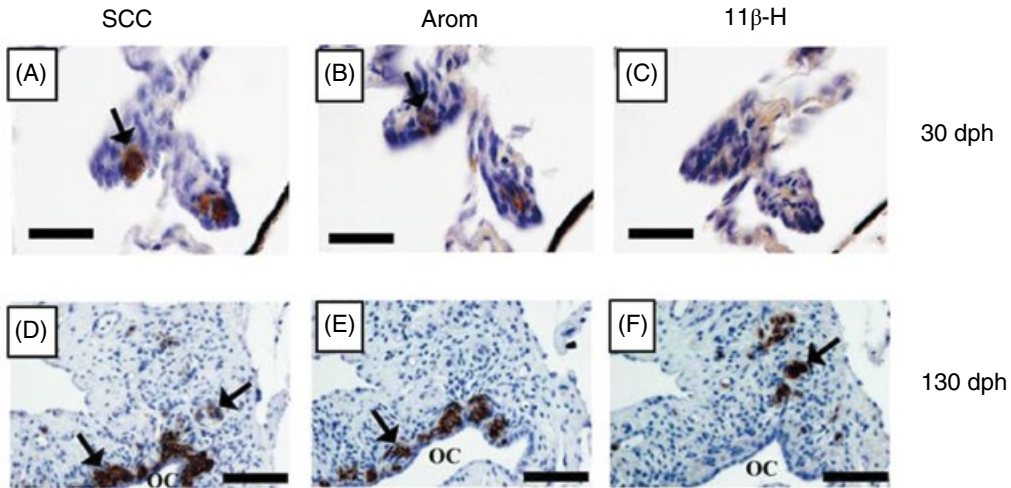


Figure 38.2 Gonads of Malabar grouper during ovarian differentiation. Undifferentiated gonads at 30 dph (A–C). Differentiating ovaries at 130 dph (D–F). (See inserts for the color representation of this figure.) A and D: Immunostaining with anti-Cyp11a1. B and E: immunostaining with anti-Cyp19a1a. C and F: immunostaining with anti-Cyp11b. Arrows indicate positive immunoreactivities. OC – ovarian cavity. Scale bar = 20 μ m (A–C), 50 μ m (D–F).

strongly suggest that two types of SPCs differentiate from somatic cells derived from different origins. Estrogen-producing cells were first in undifferentiated gonads, whereas androgen-producing cells were first detected at the end of ovarian differentiation. Consequently, serum estrogen was detectable during ovarian differentiation, and was present at higher levels than 11-KT during ovarian and ovarian development. This suggests that endogenous estrogen is produced in the gonads at the undifferentiated stage, and may be involved in the initiation of ovarian differentiation.

38.2.3 Role of Gonadotropin in Sex Differentiation

The control mechanism for the expression of steroidogenic enzymes in the gonads during gonadal sex differentiation in groupers remains to be elucidated. In teleosts, like in other vertebrates, gonadal steroidogenesis is largely controlled by pituitary gonadotropins (GTH), follicle stimulating hormone (FSH), and luteinizing hormone (LH). These GTHs contain a common

glycoprotein hormone α subunit ($GP\alpha$), which forms a heterodimer with unique β -subunits ($Fsh\beta$ and $Lh\beta$) [20].

To clarify the possible role of GTHs in the process of sex differentiation in the grouper, the expression patterns of GTH subunits ($GP\alpha$, $Fsh\beta$, and $Lh\beta$) in the pituitary during gonadal sex differentiation in Malabar grouper was investigated [21]. Both $GP\alpha$ and $Fsh\beta$ were detectable before ovarian differentiation, and significantly increased after ovarian differentiation. In contrast, $Lh\beta$ was not detected before ovarian differentiation, with $Lh\beta$ mRNA expression becoming detectable only after ovarian differentiation. In addition, the $Fsh\beta$ and $Lh\beta$ immuno-positive cells in pituitary during gonadal sex differentiation were examined. As expected, immuno-positive cells were not detected in the pituitary before the gonadal differentiated stage (Figure 38.3 A and B). Both positive signals appeared in the pituitaries after ovarian differentiation. From these results, it appears less likely that pituitary gonadotropins play a major role in the control of gonadal sex differentiation in the grouper.

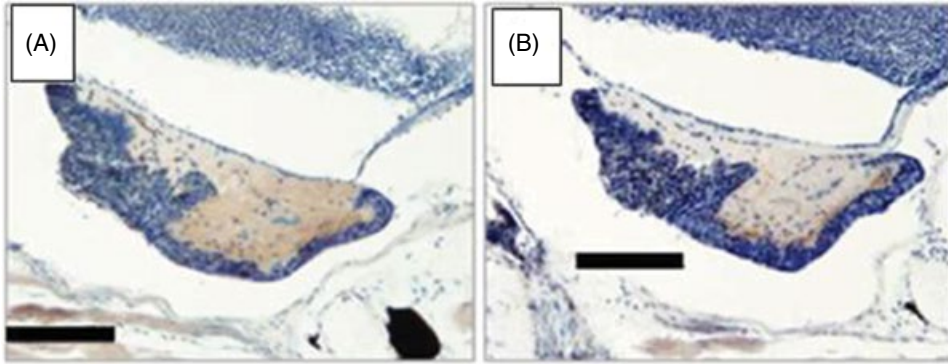


Figure 38.3 Pituitaries of Malabar grouper at sexual differentiated stage. Positive reactions against anti-*Fsh β* (A) and anti-*Lh β* (B) are not detected. Scale bars = 100 μ m. (See inserts for the color representation of this figure.)

38.3 Sex Change of Grouper

As mentioned above, investigation of cultured groupers, such as Malabar grouper, may encounter limitations due to the large size of fish. Therefore, a smaller-size species, honeycomb grouper, the dominant species inhabiting the coral reef in Okinawa, Japan, was chosen as an experimental model for endocrinological and physiological mechanism of sex change. In the next section, some general histological and endocrinological features of sex change in honeycomb grouper are described.

38.3.1 Histological Characteristics of Gonads During Sex Change

To clarify natural sex change of the honeycomb grouper, the sex hormone profile was examined, focusing on the histological characteristics of the gonads during sex change, in addition to recording the season and fish size at sex change [22]. On the basis of histological observation, gonadal stages were divided into four phases, as described below [22] (Figure 38.4).

1) *Female phase* (FP): the gonads in the FP during the non-breeding season had many immature oocytes at the perinucleolus stage in the ovigerous lamellae (Figure 38.4A). An ovarian cavity, in which ovulated eggs are stored, was seen among ovigerous lamella.

- 2) *Early transitional phase* (ET): the gonads in the ET were characterized by degenerating young oocytes and the active proliferation of spermatogonia on the periphery of the ovigerous lamella (Figure 38.4B).
- 3) *Late transitional phase* (LT): active spermatogenic germ cells occupied the ovigerous lamella of LT fishes, with few degenerating oocytes (Figure 38.4C).
- 4) *Male phase* (MP): the gonads of MP fish were filled with mainly spermatogonial germ cells in the non-breeding season. The gonads in the breeding season were occupied by active spermatogenic germ cells. No oocytes were observed in the testes (Figure 38.4D).

The appearance of the ET phase soon after the breeding season, and the LT phase in the pre-breeding season, suggested that sex change is likely to begin soon after spawning, continue through the non-breeding season, and terminate following the spawning season [22]. In another grouper species (*E. diacanthus*), sex change also occurred during the non-reproductive season and the following spawning season [23].

Twenty centimeters of total body length is the threshold for the sex change in honeycomb grouper [22, 24]. It is widely accepted that sex changes in fish are controlled by social factors, such as presence or absence of the dominant female or male in the groups [8]. However, in the case of the grouper, age

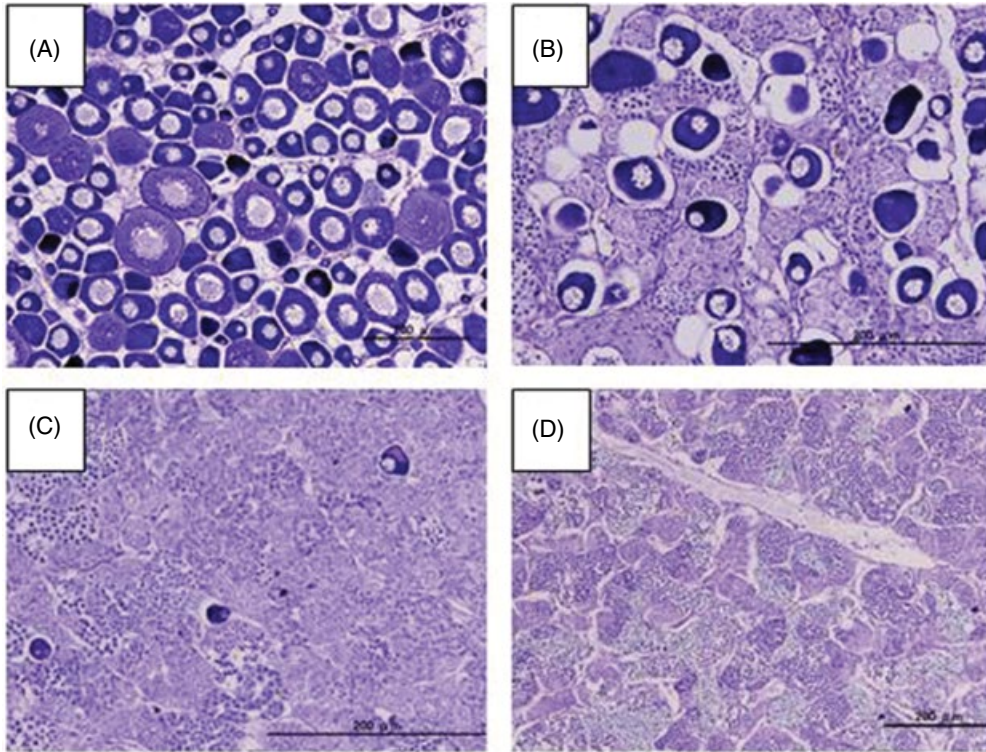


Figure 38.4 Gonadal stages of honeycomb grouper during female-to-male sex change. (See inserts for the color representation of this figure.)

(A) Female phase, containing many immature oocytes at the peri-nucleolus stage.

(B) Early transitional phase, characterized by degenerating young oocytes and the active proliferation of spermatogonia.

(C) Late transitional phase, with active spermatogenic germ cells occupying the ovigerous lamella.

(D) Male phase in the breeding season, with active spermatogenic germ cells.

Scale bar = 200 µm.

and fish size are important factors in the beginning of sex change. No evidence of sex change in *E. merra* and *E. malabaricus* based on social cues could be found.

An ovarian cavity remained in all testes after the sex change in grouper. The presence of the ovarian cavity is one type of evidence of change from ovary to testis, similar to the protogynous saddle-back wrasse (*Thalassoma duperrey*) [17, 25]. However, the ovarian cavity in the testis after sex change does not function. The efferent ducts, which are the storage and transport sites for matured sperm, are newly differentiated during sex change in the wall of an ovarian cavity [24]. Androgen is involved in the differentiation and development of efferent ducts in the gonads.

38.3.2 Endocrine Mechanism of Sex Change

In order to clarify the role of endogenous sex steroid hormones for sex change in the honeycomb grouper, sex steroid hormone profiles during natural sex change were measured [22, 26].

Plasma E_2 levels were high in the FP, and gradually decreased in the males. In contrast, plasma 11-KT levels were low in females, but gradually increased in ET and LT phases, and were significantly high in the males. These results are in good agreement with results obtained with *in vitro* tissue incubations [27]. In other protogynous species, a similar shift in steroidogenesis from estrogen production

to androgen production was observed during sex change [14].

To clarify more details regarding steroidogenesis in gonadal sex change, the immunohistochemical changes of three steroidogenic enzymes (Cyp11a1, Cyp19a1a, and Cyp11b) during sex change were examined [26].

Immuno-positive androgen-producing cells against both Cyp11a1 and Cyp11b antibodies were observed in the tissue near blood vessels in the tunica ovary enclosing the outer periphery of the ovary [28] (Figure 38.5 A and B). The nucleus of androgen-producing cells increased gradually in size accompanying sex change. This phenomenon indicated that androgen-production of these cells became more active in the testis than the ovary.

In addition to these androgen-producing cells, the differentiation of androgen-producing cells derived from the follicle cells enclosing the developed oocytes during sex change was observed. Although neither theca nor granulosa follicle cells enclosing oocytes in the ovary positively reacted against Cyp11b, reactions appeared in the remaining follicle cells enclosing the oocytes in the gonads at the ET, LT, and male phase. Thus, androgens produced in the tunica near blood vessels may provide the stimulus for females to degenerate oocytes and initiate

sex change. In addition, androgens produced both in tunica and the remaining follicle layer of degenerated oocytes possibly play a critical role during testicular differentiation, as well as gonadal restructuring at ET and LT phases during sex change in the grouper.

In addition, the role of sex-related genes, *foxl2* (ovary-specific gene) and *dmrt1* (testis-specific gene), during sex change in the honeycomb grouper, were examined [29]. *foxl2* expression was gradually downregulated from the ET until the completion of sex change. In contrast, *dmrt1* expression was upregulated during the ET until testis spermatogenesis, and continued until the formation of the testis. Therefore, the downregulation of *foxl2* during the ET and LT phases most likely facilitates oocyte degeneration, whereas the upregulation of *dmrt1* promotes the proliferation of gonadal germ cells into spermatogonia, and initiates sex change. Further detailed studies are required to determine if *dmrt1* promotes the proliferation of gonadal germ cells into spermatogonia and initiates sex change.

38.3.3 Role of Gonadotropin in Sex Change

The first stimuli signals for gonadal sex change in fish probably come from the

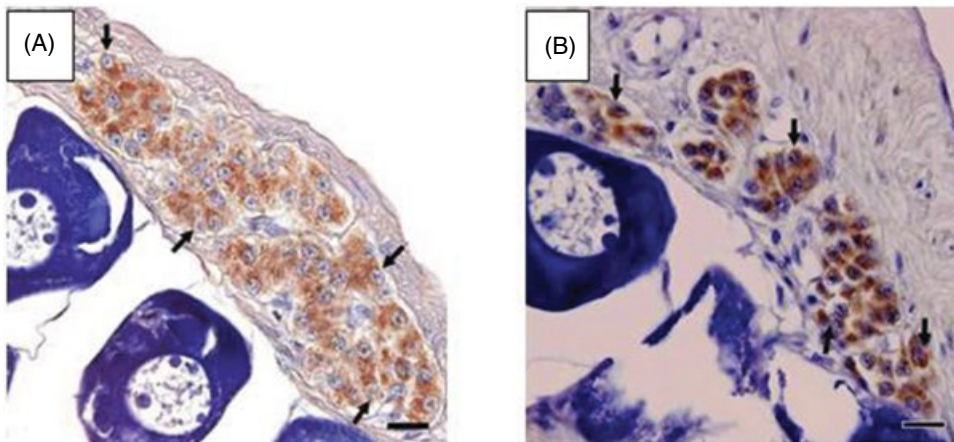


Figure 38.5 Immunopositive reactions against anti-Cyp11a1 (A) and anti-Cyp11b (B) in the tunica ovary of honeycomb grouper. Arrowheads indicate clusters of immunopositive cells. (See inserts for the color representation of this figure.)

Scale bar = 10 μ m.

brain. Therefore, the brain (hypothalamus)-pituitary-gonad (HPG) axis may be involved in the sex change [14]. As described before, two gonadotropic hormones (FSH and LH) produced by the pituitary control gonadal development via sex steroid hormone production in fish, similar to the higher vertebrates [20]. Thus, it is highly possible that endogenous GTHs control the sex change in grouper.

To clarify the role of GTH in sex change, mRNA expression levels of GTH subunits in the pituitaries were quantified during sex change in the honeycomb grouper [30]. The relative mRNA levels of *Gpα* and *Lhβ* were higher during the breeding season than in the non-breeding season. However, there were no significant differences in these levels among different sexual phases. In contrast, the expression pattern of *Fshβ* transcripts showed a marked sexual dimorphism. Although *Fshβ* subunit transcripts were low in the breeding and non-breeding season female phase, significantly increasing *Fshβ* transcripts were observed during natural sex change, especially in ET males.

To confirm the sexually dimorphic expression of *Fshβ* transcripts in the pituitary of honeycomb grouper, the expressions of *Fshβ* and *Lhβ* were immunohistochemically examined, using specific antibodies in the pituitaries of female, ET, LT, and male fish [31]. No *Fshβ* immuno-reactive cells could be observed in the pituitary of FP fish. In contrast, strong *Fshβ* -positive signals were seen in the pituitary of ET and LT males. In females, *Lhβ* immuno-reactive cells were seen in the proximal par distalis and par distalis of the pituitary. The *Lhβ* signals were also seen at the same area of the pituitary of males. These observations are in agreement with the results of mRNA expression of GTH subunits during natural sex change in honeycomb grouper. Similarly, the upregulation of FSH receptor gene (*fshr*) in the gonad is associated with sex change [32]. Interestingly, *fshr* mRNA was localized in the androgen production cells in tunica ovary near the blood vessels. Taken together, these

results suggest that FSH has an important causative role in sex change of honeycomb grouper.

38.4 Artificial Induction of Sex Reversal

As described in the introduction section, protogynous groupers provide a high quality food source and economic value. Thus, several grouper species are cultured in the world, especially in East Asia [2]. However, several problems have been raised for grouper bloodstock management, due to their unique sexuality. For instance, males to be used as brood stock are difficult to catch from the wild, since female-to-male sex change is correlated with body size of fish (Section 38.2.1). In addition, under farming conditions, several years are needed before sex change takes place.

To solve these problems, many attempts have been undertaken to induce the sex reversal in captivity by treatment androgens [33, 34]. The synthetic 17 α -methyltestosterone (MT) has been widely tested for sex reversal induction in various fishes including grouper [14, 33, 35]. However, MT-induced males are transient. After treatment completion, these MT-induced males can spontaneously reverse into females [36, 37]. Obviously, stable male brood stock are very important for grouper aquaculture. In the next section, the methods used in our laboratory to induce sex reversal in juveniles and adult females are described separately.

38.4.1 Artificial Induction of Sex Reversal in Juveniles

As is well accepted, to induce complete sex reversal by treatments with sex steroid hormones, treatments should start when the gonads are still undifferentiated, and continue through completion of sex differentiation [11]. Based on histological observations

of the process of sex differentiation, it was concluded that around 47 dph is the most effective period to treat larvae to induce the sex reversal in Malabar grouper [13].

Over the past years, treatment with aromatase inhibitors (AI), pharmaceutical agents that block Cyp19a1a activity, are very effective to induce female-to-male sex reversal in gonochoristic fishes [38–43]. Thus, the possibility of sex reversal by androgen or AI treatments was examined using various stages of ovaries, after ovarian differentiation, from 0+, 1+, and 3+ year-old fish [15, 44]. The effects of AI on sex change of immature ovaries of grouper were also examined [45]. In total, six experiments were conducted.

In Experiment 1, most fish at 144 dph (0+ old) treated with MT (10 and $50\mu\text{g g}^{-1}$ diet) for six months had testes with active spermatogenic germ cells at various stages of spermatogenesis, including spermatozoa. However, the heads of some spermatozoa were of various sizes.

In Experiment 2, fish at 100 dph (0+ old) were treated with MT (10 and $50\mu\text{g g}^{-1}$ diet) and AI (Aromasin: 10 and $50\mu\text{g g}^{-1}$ diet) for 1–7 months. From 1–6 months after the withdrawal of treatment, gonadal status was examined histologically. In MT-treated fish, spermatogenesis progressed until seven months after the start of the treatment, and 80% of MT-treated fish developed mature testes with active spermatogenesis. However, spermatozoa in the gonads of almost all MT-treated fish disappeared within one month after the withdrawal of MT treatment. Spermatogenic germ cells were not seen in the gonads of AI-treated fish, though efferent duct-like structure was induced.

In Experiment 3, cocoa butter containing MT (4mg kg^{-1} body weight [BW]) and AI (Aromasin: 25 and 100mg kg^{-1} BW) were implanted into the intraperitoneal cavity of fish at 350 dph (0+ old). Spermatogenesis and a few sperm were induced in the gonads of fish at four months after implantation of MT. The gonads of AI implanted fish also showed no spermatogenesis or sperm regardless of dosage.

In Experiment 4, fish at 570 dph (1+ year old) were implanted with cocoa butter containing MT (1 and 4mg per fish) and AI (Aronasin 25 and 50mg kg^{-1} BW) into the body cavity. All the MT-treated fish showed matured testes, which consisted of spermatocytes and many spermatozoa in the efferent duct with regular head size, at two and five months from the start of the experiment, regardless of dosage. Some of the AI implanted fish showed an ovary with an efferent duct-like structure, but no spermatogenesis or sperm were detected.

In Experiment 5, fish at 570 dph (2+ years) were provided a diet containing MT ($50\mu\text{g g}^{-1}$ diet) for 2 and 7 months. All fish were sacrificed at 2 and 7 months after treatment, and had testes with active spermatogenesis. However, all fish had ovaries without spermatogenic germ cells at two months after withdrawal of MT treatment.

In Experiment 6, cocoa butter containing MT (10 mg per fish) was implanted into the body cavity of fish at 1,000 dph (3+ year old). At approximately one month from the start of the experiment, MT-treated fish exhibited nuptial coloration and courtship behavior. All fish treated with MT for five months had matured testis that consisted of spermatocytes and many sperm at five months from the start of the experiment.

From these results, all ovaries of fish from 0+ through 3+ years old had an ability to cause active spermatogenesis in response to the exogenous androgen. In other words, some germ cells in the ovaries of grouper are bipotential, which provides the ability to differentiate into oocytes or spermatozoa (see also Box 38.1).

Precocious sex change from immature ovaries to mature testes can be induced in yearling individuals with MT treatment after ovarian differentiation. However, testes with active spermatogenic germ cells, originating after the transformation from immature ovaries, are highly likely to change back to ovaries after treatment (Experiments 2 and

Box 38.1 Sex differentiation and control in the Malabar grouper

The ability of androgen and AI treatments to induce sex reversal in differentiated but immature ovaries of 0+ (10.0–11.4 cm TL), 1+ (24.6–33.0 cm TL), 2+ (33.0–36.2 cm TL), and 3+ (55.4 cm TL) year old Malabar grouper was examined. Most of the ovaries of fish from 0+ through 2+ years old treated with MT (10–50 µg/g diet, 4 mg/kg BW, 1–4 mg/fish) for 1–7 months, were capable of inducing active spermatogenesis. However, testis with active spermatogenic germ cells that had developed from immature ovaries tended to revert to

ovaries after treatment withdrawal. The 3+ year old fish treated with MT (10 mg/kg BW) exhibited nuptial coloration and courtship behavior. In addition, all MT-treated fish had also mature testes with active spermatogenesis. These results indicated that 3+ year old fish are suitable for obtaining fertilized eggs from artificially sex-changed parental male fish under aquarium conditions. The AI (Aromasin: 2 mg/g diet, 25 and 100 mg/kg BW, 25 and 50 mg/fish) treatments for 1–6 months did not induce spermatogenesis in the immature ovaries.

5) withdrawal, as previously reported for the dusky grouper [37].

The cause of this transient sex change was clarified [45]. The expression of three steroidogenic enzymes (Cyp11a1, Cyp19a1a, and Cyp11b) in the testes of the immature grouper with oral MT treatment were analyzed immunohistochemically at the end of the seven-month treatment. Positive cells against three antibodies were observed in the testes in MT-treated testes. Cyp11a1-positive cells appeared in the somatic cells surrounding germ cells and cysts, the Sertoli cell layer, and somatic tissue in the ovarian tunica area. Cyp19a1a-positive cells appeared only in the somatic cells surrounding germ cells and cysts. Cyp11b-positive cells appeared only in the somatic tissue in the ovarian tunica area, but not in the interstitial tissue of testis. Positive cells against three antibodies for steroidogenic enzyme were seen in the control immature ovary, as described in the previous chapter.

No significant differences in serum E₂ and 11-KT levels were found between control fish, which had immature ovaries, and MT-treated fish, which had mature testes. Thus, in the case of the immature grouper, MT might have little effect on endogenous steroidogenesis in the gonads, suggesting that the precocious sex change from immature ovary to testis by MT treatment in yearling grouper might occur only through direct stimulus of germ cells for spermatogenesis by MT treatment.

38.4.2 Artificial Sex Reversal in Adults

38.4.2.1 Artificial Sex Reversal in Adult by Androgen

Administration of exogenous androgens around the time of sex differentiation is able to induce sex change fish [11, 46]. To elucidate the effect of an androgen on sex change in adult grouper, 11-KT (10 mg kg⁻¹ body weight of fish) was implanted into pre-mature females to induce female-to-male sex change (47). As a result, gonads of fish treated with 11-KT for 75 days had completely transformed to testes (see also Box 38.2). Spermatozoa were present, in addition to germ cells, in advanced stages of spermatogenesis. In contrast, all fish in the initial control had ovaries with immature, vitellogenic oocytes, or ovulated oocytes. The sex-changed males were mated with normal mature female in a tank around the time of a full moon, which is the spawning time of honeycomb grouper [24, 48]. Viable embryos were obtained from these matings, revealing the functionality of the sex-reversed females.

38.4.2.2 Artificial Sex Reversal in Adult by Aromatase Inhibitor (AI)

It has been previously observed that estrogen in female protogynous saddle-back wrasse dropped suddenly just after the onset of sex change [16]. In honeycomb grouper, serum estrogen levels decreased gradually during sex change from female to male [22]. Artificial

Box 38.2 Sex control in the honeycomb grouper

In nature, female-to-male sex change occurs in female honeycomb grouper (total length is less than 20 cm) during the non-breeding season. The onset of sex change is characterized by the degeneration of oocytes and proliferation of spermatogenic cells in the gonad. The artificial induction of female-to-male sex reversal in the honeycomb grouper can be successfully achieved by treatment with aromatase inhibitor (AI: fadrozole) dissolved in cocoa butter and implanted in females with a dose of 1 mg/fish. With this dose,

complete sex reversal takes place within 90 days during the non-breeding season, within 75 days in the early pre-breeding season, and within 42 days in the late pre-breeding season.

Sex reversal in this grouper can also be induced by androgen (11-KT: 10 mg/kg body weight of fish) and gonadotropin (purified bovine FSH: 500 ng/fish). However, the production of females from males (i.e., male-to-female sex change) in this species of grouper has never succeeded in our laboratory.

depletion of estrogen by AI also brought about sex changes in protogynous wrasse [49]. In addition, it was recently demonstrated that estrogen depletion in the protandrous anemone fish *Amphiprion clarkii* by androgen treatment induced testicular differentiation in the mature ovary of females [50].

The effects of artificial induction of estrogen depletion by an aromatase inhibitor were examined for the ovary in grouper [27]. All fish treated with AI (Fadrozole: 10 mg kg⁻¹ BW) had developed testes, similar in structure to those spawners in the wild, and containing spermatogenic germ cells undergoing late stages of spermatogenesis. Artificial sex change was also induced within two full moons by AI (Fadrozole: 1 mg/fish) during the breeding season and spawning in grouper [51]. In contrast, the ovary of AI/E₂-treated fish had either previtellogenic or vitellogenic oocytes. An artificial drop in estrogen by females, from the aromatase inhibitor, induced the female-to-male sex change in grouper (see also Box 38.2). The depletion of estrogen from gonochoristic matured females in Nile tilapia, medaka, and zebrafish was also demonstrated [52, 53].

sex steroid hormones in gonads are usually controlled by the GTHs from the pituitary. These results strongly suggest that FSH is involved in sex change in grouper.

Therefore, the direct effects of GTHs on gonadal sex change of grouper were tested [30]. Purified bovine FSH and LH was implanted into adult females with immature ovaries (Figure 38.6). Most of the ovaries of females that were implanted with FSH began active spermatogenesis within three weeks after implantation (Figure 38.6B). In contrast, LH treatment females maintained immature ovaries, indicating no effects for gonadal sex reversal (Figure 38.6A).

Taken together, these results suggest that FSH may trigger the female-to-male sex change in honeycomb grouper. Plasma androgen level gradually increased during sex change by FSH implantation, though plasma estrogen levels did not change. From these results, we concluded that an unbalanced ratio between estrogen and androgen is related to the trigger of sex change in grouper. In addition, FSH is a potent stimulator of androgen production, which is similar in a number of teleost species [54, 55].

38.4.2.3 Artificial Sex Reversal in Adults by GTH

As mentioned in Section 38.3.3, drastic changes of FSH expression level in pituitaries during natural sex change have been observed in grouper [30]. Biosynthesis and secretion of

38.5 Discussion

Here, we have clarified the characteristics of sex differentiation of the protogynous Malabar grouper. All undifferentiated gonads

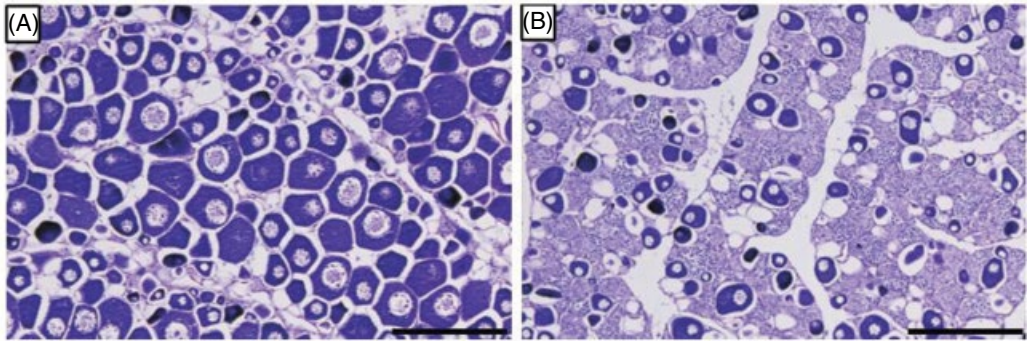


Figure 38.6 Gonadal sections of honeycomb grouper treated with only molten cocoa butter as control (A), or with 500 ng/fish of bovine FSH (B) for three weeks. (See inserts for the color representation of this figure.)

(A) Gonads showing many previtellogenic oocytes.

(B) Primary oocytes and active spermatogonial proliferation were observed in the gonad simultaneously. Thus, we characterized these fishes as sex-changing.

Scale bars = 200 μ m.

of Malabar grouper differentiate into ovaries. There is no testicular differentiation from sexually undifferentiated fish and, thus, all males are the product of sex change (i.e., secondary males), indicating that no primary males exist, as with other groupers [13]. Similarly, all undifferentiated gonads of juveniles of the protandrous anemone fish, *A. clarkii*, first start differentiating into ovaries before fully differentiating and first maturing as males [56]. In the gonochoristic medaka, *dmy*, the male sex-determining gene (SDG), is expressed in the somatic cells surrounding germ cells, and induces testicular differentiation in undifferentiated gonads [57]. Conversely, no expression of *dmy* results in ovarian differentiation.

It is known that androgen treatment can induce genetic XX females to develop as XX males in, for example, tilapia, medaka, and salmonid fish. These XX males, when mated to regular females, produce all-female offspring. Taken together, these observations indicate that monandrous species of groupers do not have a master SDG as such. However, few primary males appear in the sex differentiation of protogynous wrasse (unpublished data). We interpret this as that an SDG in this type of sex-changing fish is evolving out. Further studies are needed to determine the relationships between sex-

determining genes and sex change in hermaphroditic fish.

It is known that endogenous estrogen is involved in the gonadal sex differentiation in gonochoristic species [11, 12]. In general, endogenous estrogen functions as ovarian differentiation, whereas the lack of estrogen acts as testicular differentiation. However, the role of endogenous estrogen in the gonadal sex differentiation of hermaphroditic fish is still unclear. It was observed that *Cyp19a1a*, which is the key enzyme of estrogen production, is expressed immunohistochemically in the undifferentiated gonads of groupers. In addition, serum E_2 levels were high in the trials with undifferentiated gonads during and after sex differentiation. Taken together, it is strongly suggested that endogenous estrogen functions as an ovarian inducer in the gonadal sex differentiation of hermaphroditic grouper.

Aquaculture of groupers has been recently expanding into Central, Southeast, and East Asia [4]. Fishermen in some countries catch large number of fry from fishing grounds, for use as the seed for aquaculture. In addition, natural resources of grouper are decreasing gradually, because of the deterioration of habitat. One issue is the assurance and maintenance of larger males essential for seed production. Larger males in the wild are decreasing rapidly in number, due to overfishing.

The long-term rearing of large fish in captivity costs money and labor. To reduce the economic burden, we expect to establish artificial sex change in smaller females by various methods. We have successfully induced active spermatogenic germ cells, including spermatozoa in the immature females at various ages by androgen treatments. These facts revealed that exogenous androgen induced masculinization of germ cells in the ovary after sex differentiation. However, the amount of sperm obtained from the gonads of juveniles after androgen treatment was severely reduced.

It is difficult to use very small spermatozoon for artificial insemination. It is known that low doses of estrogen and 17α , 20β -dihydroxy-4-pregnen-3-one can stimulate the multiplication of spermatogonia in the testis of eel [58]. These treatments for the immature ovary of fish before the androgen treatment may increase the volume of spermatozoon.

We have established two new methods for artificial induction of sex change in adults, based on FSH and AI administration, in addition

to the androgen method. Although each of the three methods are expected to artificially induce sex change in various species of groupers, the decision regarding which method to use will be made from the viewpoints of economics, safety for fish and man, and reliability for induction of sex change on the basis of the characteristics of the grouper.

Acknowledgments

The present research was supported by grants from the JSPS KAKENHI (Project no. 16H04983, 16H02984 for MN and 16K07873 for YK), and from the Okinawa Churashima Foundation. We express special thanks to all members of our laboratory, Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, for their kind assistance during our study. We also acknowledge Emeritus Professor of the National Institute for Basic Biology, Yoshitaka Nagahama, for the antibodies gift and useful advice.

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39

Artificial Gynogenesis and Sex Control in Large Yellow Croaker

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39.1 Introduction

Large yellow croaker (*Larimichthys crocea*; Perciformes, Sciaenidae) is a sciaenid mainly distributed in the southern part of the Yellow Sea, the East China Sea, and the northern part of the South China Sea, once being one of the most important marine fishery species in China (Figure 39.1) [1]. However, the wild populations of *L. crocea* nearly collapsed due to prolonged over-fishing in the 1960s and 1970s [2]. Research into the hatchery methods of large yellow croaker began in 1985, and a breakthrough was obtained in 1987, which is also the first success of artificial propagation of marine fish species in China.

Since 1995, the industry of culturing large yellow croaker has been developing, and now large yellow croaker is one of the most important commercial cultured fish species in China. To date, the current annual production of this fish species is approximately 140,000 tons, enough to supply the domestic market and even overseas markets, such as those in the United States, Japan, and Korea. In fact, it is one of the most extraordinary export aquatic products of China (more than \$260 million at 2015).

Since the growth rate of large yellow croaker shows sexual dimorphism, the

females growing significantly faster than the males (about 26% for the body weight at the age of 25 months), rearing an all-female population would be helpful to increase the yield. Therefore, techniques and knowledge associated to sex control in large yellow croaker have been developed and accumulated in recent years.

39.2 Sexual Growth Dimorphism

Large yellow croakers exhibit sexual growth dimorphism under both culture and natural conditions, females grow faster than males, especially after 16 months. At the age of 12, 16, and 21 months (March, July, and December 2013), about 500 fish rearing in net cages were randomly chosen by dip net respectively, and their quantitative traits were measured and presented in Table 39.1. The results showed that all the quantitative traits relative to growth were significantly different between sexes, especially for the whole-body weight, visceral weight, and carcass weight.

At the age of eight months (April 2007), 398 fish from a culture population were tagged with PIT tags and transferred to the same net cage in the Fishery Breed



Figure 39.1 The lateral view of large yellow croaker, *L. crocea*.

Table 39.1 Descriptive statistics of five quantitative traits relative growth in *L. crocea* at different ages.

Item	Sex	12 months (♀ 250, ♂ 304)		16 months (♀ 241, ♂ 294)		21 months (♀ 237, ♂ 263)	
		Mean ± S.D.	Difference (%)	Mean ± S.D.	Diff. (%)	Mean ± S.D.	Diff. (%)
Body length (mm)	Female	160.77 ± 17.27	1.78	200.72 ± 21.35	3.17	234.85 ± 29.04	3.37
	Male	157.96 ± 16.12		194.56 ± 18.72		227.19 ± 25.19	
Body height (mm)	Female	42.70 ± 4.72	2.00	54.75 ± 6.19*	6.15	66.61 ± 9.54*	7.38
	Male	41.86 ± 4.18		51.58 ± 5.37		62.03 ± 8.11	
Body width (mm)	Female	23.03 ± 3.01	3.22	27.94 ± 3.51*	6.24	34.53 ± 5.28*	7.83
	Male	22.31 ± 2.67		26.30 ± 3.14		32.02 ± 4.76	
Body weight(g)	Female	71.69 ± 23.74*	7.85	148.77 ± 48.74**	18.79	247.41 ± 99.96**	22.15
	Male	66.47 ± 20.47		125.24 ± 39.22		202.56 ± 77.38	
Visceral weight (g)	Female	—	—	—	—	31.28 ± 18.44**	138.78
	Male	—	—	—	—	13.10 ± 5.72	
Carcass weight (g)	Female	—	—	—	—	214.80 ± 85.80**	13.67
	Male	—	—	—	—	188.96 ± 72.22	

*indicates significant difference between both sexes (t-tests: $P < 0.05$)
**indicates highly significant difference between both sexes (t-tests: $P < 0.01$).

Field of Jimei University in Ninde City, China. The body weights of tagged fish were measured three times: 8 months, 20 months, and 25 months. The gender of each fish was determined by dissection at the last time of measurement. In total, 61 females and 62 males had survived and still held their tag when being dissected. The results showed that the body weight of females was 26.2% higher than that of males at the age of 25 months (Figure 39.2).

39.3 Induction and Genetic Analysis of Artificial Gynogenesis

39.3.1 Meio-Gynogenesis

39.3.1.1 Conditions for Inducing Heterogeneous Gynogenesis

- **Method 1: using large yellow croaker sperm** [3].
Mature females and males were collected from cultured population at Sandu-ao Gulf at Ningde, Fujian province, China.

Figure 39.2 The gender differences of body weight of *L. crocea*.

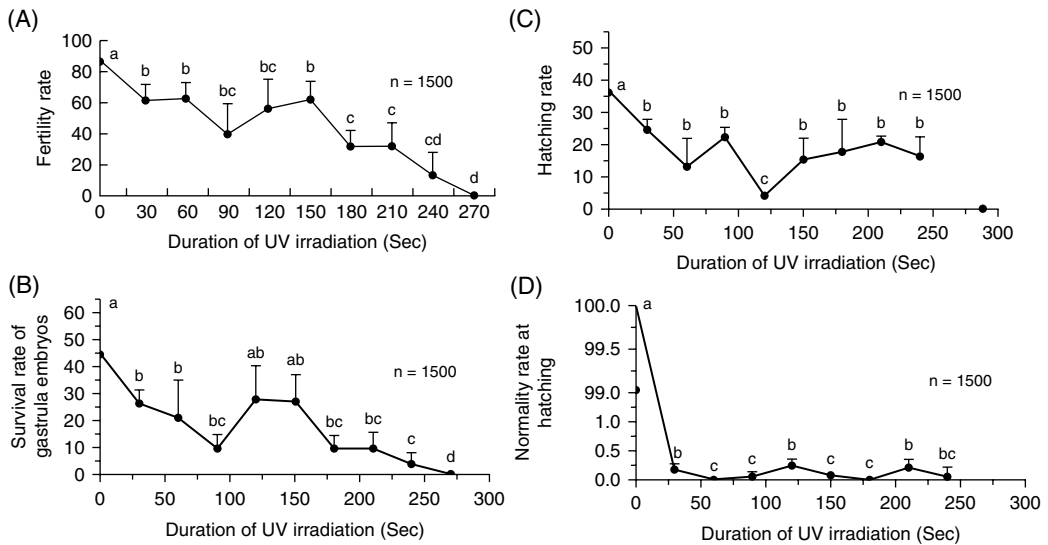
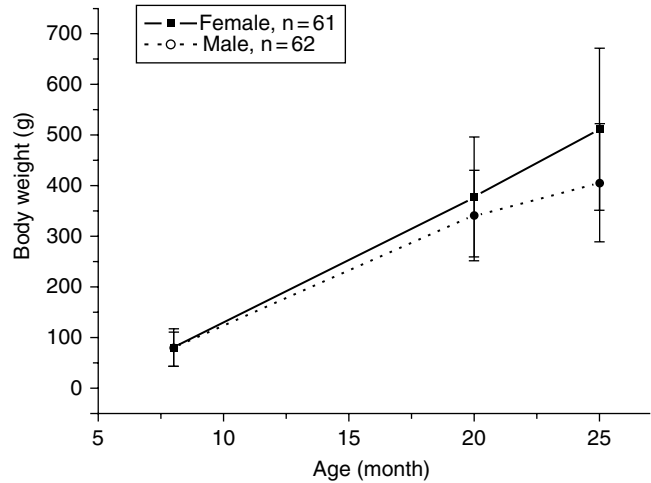


Figure 39.3 Effect of duration of UV irradiation on fertility rate (A), survival rate of gastrula embryos (B), hatching rate (C) and normality rate at hatching (D). $n = 1500$ means the sample size (500 for each treatment, with three replicates); different letters of a, b, c, d mean significant difference in the figure.

Approximately 36 hours after injection with LH-A3 (an animal luteinizing hormone-releasing hormone), semen and eggs were collected by stripping. The conditions for inducing meiotic gynogenesis of large yellow croaker were optimized according to survival and normality of the newly hatched fry (Figure 39.3). The typical Hertwig's effect was observed when the sperm was exposed to UV light, and the effective UV intensity for sperm irradiation covered a wide range,

from $53,800 \mu\text{W cm}^{-2}$ to $406,080 \mu\text{W cm}^{-2}$; and the optimal UV dosage for gynogenetic haploid induction was $355,320 \mu\text{W cm}^{-2}$. Cold shock could inhibit the extrusion of second polar body success, and the induction rates of gynogenetic diploids in the experimental groups were mainly affected by the starting time¹, intensity, and duration

1 The starting time: the time when the cold shock starts, usually expressed as a number of minutes post insemination.

of shocks. The optimal condition of cold shock was at 3°C for 10 minutes, starting at three minutes post-insemination.

- **Method 2: using yellow drum sperm.**

The gynogenesis of large yellow croaker can also be triggered by UV-inactivated sperm of yellow drum (spotted maigre, *Nibea albiflora*), under the same conditions (for sperm inactivation and cold shock) as using large yellow croaker sperm. The surviving fry were all gynogens of large yellow croaker when the eggs of large yellow croaker were inseminated with yellow drum semen, for the true hybrids were proved to be unviable, although the fertilization and hatching rate of the hybridization were as high as the pure cross of large yellow croaker [4]. Therefore, this method can avoid the trouble of subsequent genetic identification by using yellow drum's sperm to trigger the gynogenesis of large yellow croaker. In addition, the spawning season of the yellow drum is similar to that of the large yellow croaker, so it is easy to obtain the brood stock and the sperm of

yellow drum. Thus, inducing gynogenesis of large yellow croaker with UV-inactivated sperm of yellow drum has become a routine way in our laboratory since 2010.

39.3.1.2 Identification of Gynogenesis

The authenticity of gynogenesis needs to be confirmed with fry shape, chromosome count, or molecular markers analysis, for the sperm may not be genetically inactivated well at a very low frequency.

- **Haploid syndrome**

The genome integrity of UV irradiated sperm can be determined according to the putative “haploid syndrome,” such as distorted body with poorly developed tails and small underdeveloped eyes. Figure 39.4 shows the normal and abnormal embryos and newly hatched fry of large yellow croaker. The percentage of embryo and hatched fry exhibiting haploid syndrome were more than 99.9% when the inseminated

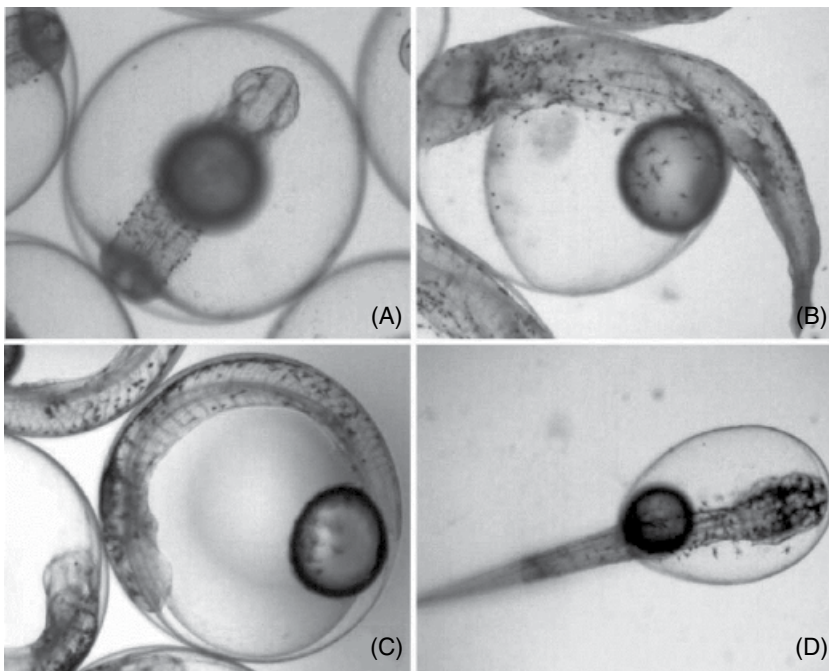


Figure 39.4 Abnormal tail bud embryo (A) and abnormal fry (B), normal tail bud embryo (C) and normal fry (D) of the large yellow croaker.

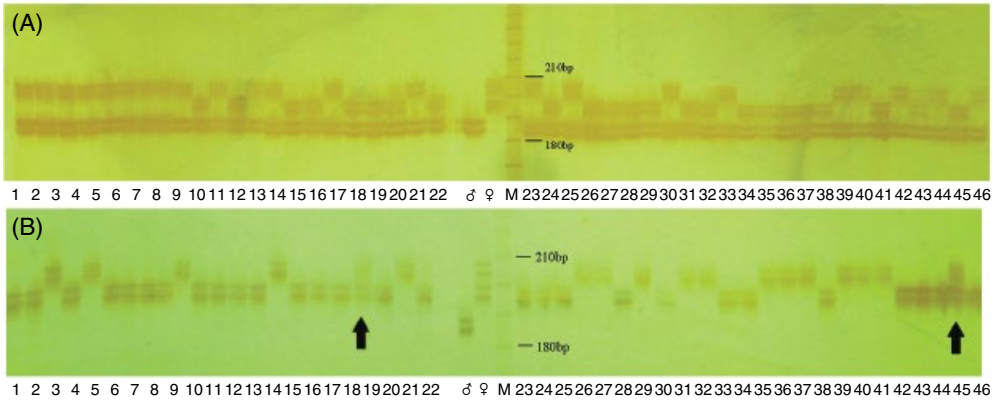


Figure 39.5 Genotypes segregation at microsatellite locus *LYC0022* in control family 1 (A) and Gynogenetic family 1 (B). (See inserts for the color representation of this figure.)

Lane M: DNA ladder; ♂: male parent; ♀: female parent; lane 1–46: samples of progenies; bp: base pair. Arrows indicate the individuals with heterozygous genotype. Adapted from Reference [3].

sperm were genetically inactivated under UV irradiation ($1692 \mu\text{W} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ for 2.5 minutes) [3].

• Chromosome counts

The numbers of metaphase chromosome of embryos in the above experiments were determined to confirm the effect of the manipulation for gynogenesis. The eggs triggered with genetically inactivated sperm (under $1692 \mu\text{W} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ UV irradiation for 2.5 min) developed into embryos with 18–28 chromosomes, but mostly with 24 chromosomes as putative haploid. After cold shock or hydrostatic pressure treatment, most of the surviving embryos had 48 chromosomes as the putative diploid. The results suggested that the above conditions were appropriate for genetic inactivation of sperm with UV irradiation and diploid restoration with cold shock.

• Molecular markers analysis

The gynogenetic families were analyzed with amplified fragment length polymorphism (AFLP) and microsatellite markers [5, 6]. The artificial gynogens lacked paternal specific bands or alleles, while the controls with normal insemination had bands (or alleles) from both parents. The results showed that the percentage of gynogens in the survival fry reached 100% in most

batches (we had bred more than 30 batches with more than 5,000 gynogens in large yellow croaker using UV-irradiated large yellow croaker sperm), and 7.5–12.5% of the survived fry had paternal specific alleles in some batches under the conditions for artificial gynogenesis as described above. Figure 39.5 shows the representative results of microsatellite analysis.

39.3.1.3 Genetic Analysis on Meio-Gynogens of Large Yellow Croaker

• Nucleolus number

The nucleoli in interphase cells of various kinds of samples, including normal diploid, triploid, gynogenetic haploid, and gynogenetic diploid, at different developmental stages, were observed and counted by using a silver staining technique [7]. The modal numbers of the nucleolus were corresponding to the ploidy in normal diploids, normal artificial triploids, and gynogenetic haploids, but the modal numbers of the nucleolus in diploid gynogens and their triploid progenies (derived from diploid gynogens crossed with normal males) were only one and two, respectively (Table 39.2, Figure 39.6). The development stage did not influence

Table 39.2 The results of nucleolus counts in groups with different manipulation (adopted from [7]).

Group	N	No. of cells	Percentage of cells with different nucleolus number					Average	Modal number
			1	2	3	4	5		
2 <i>n</i>	32	3,541	24.94	68.60	4.94	1.52	—	1.83 ± 0.090	2
3 <i>n</i>	30	3,452	9.65	30.56	57.85	1.54	0.12	2.52 ± 0.065	3
G-2 <i>n</i>	30	3,503	65.14	34.48	0.37	—	—	1.35 ± 0.098	1
3 <i>n</i> -G	30	3,210	8.32	47.04	42.62	2.02	—	2.38 ± 0.068	2

Notes: N: number of individuals being tested; 2*n*: normal diploid; 3*n*: artificial triploid; G-2*n*: gynogenetic diploid; 3*n*-G: artificial triploid whose female parents were gynogenetic diploids.

the modal number of the nucleolus in all groups (Table 39.3).

• **Homozygosity and diversity of two successive generation meio-gynogens**

To assess the efficiency to fix genes for artificial meiotic gynogenesis in large yellow croaker, the homozygosity of the meio-gynogenetic populations for meio-G1 (1st generation of meiotic gynogens) and meio-G2 (2nd generation of meiotic gynogens) was studied with microsatellite markers [8]. The results showed that the average homozygosity of the 15 analyzed loci were 0.661 and 0.803 in meio-G1 and meio-G2, respectively, which were much higher than that in the natural mating population (0.376 for the average homozygosity) (Table 39.4). The average similarity coefficient² between individuals within meio-G1 and meio-G2 were 0.5903 and 0.8672, respectively, which were also higher than that in the natural mating population (0.4687 for the average similarity index between individuals). Value of diversity coefficient (*Fst*),

genetic similarity, and genetic distance showed significant genetic differentiation between the populations of meio-G2 and the natural mating population.

Besides this, seven out of 15 analyzed loci (46.7%) were fixed in meio-G2, showing that the homozygosity of most genes can be accelerated by inducing meiotic gynogenesis in large yellow croaker. However, purity is hard to achieve in some loci for their telomeric location. For these loci, homozygosity can be gained by inducing mito-gynogenesis or control cross between individuals having same genotype. The information obtained in the study suggested that artificially induced meiotic gynogenesis is an efficient inbreeding method to accelerate breeding and establish pure-lines of large yellow croaker.

• **Microsatellite-centromere mapping in large yellow croaker by using gynogenetic diploid families**

Inheritance of 22 heterozygous microsatellite loci was examined in normal crossed diploid families and meio-gynogenetic families in large yellow croaker [3]. Two gynogenetic families were produced via inhibition of the second polar body in eggs fertilized with UV irradiated sperm. The ratio of gynogenesis was proven to be 100% and 96.9% in the two families, respectively. Of the 22 examined loci, four showed a segregation distortion in both control and gynogenetic families.

2 Similarity coefficient: genetic similarity between two populations or two individual were evaluated by calculating the similarity coefficient (S_{ij}) according to Lynch(1990), where $S_{ij} = 2 N_{ij} / (N_i + N_j)$, in which N_{ij} is the number of allele shared by the individual *i* and individual *j*, N_i and N_j are number of alleles with individual *i* and *j* respectively.

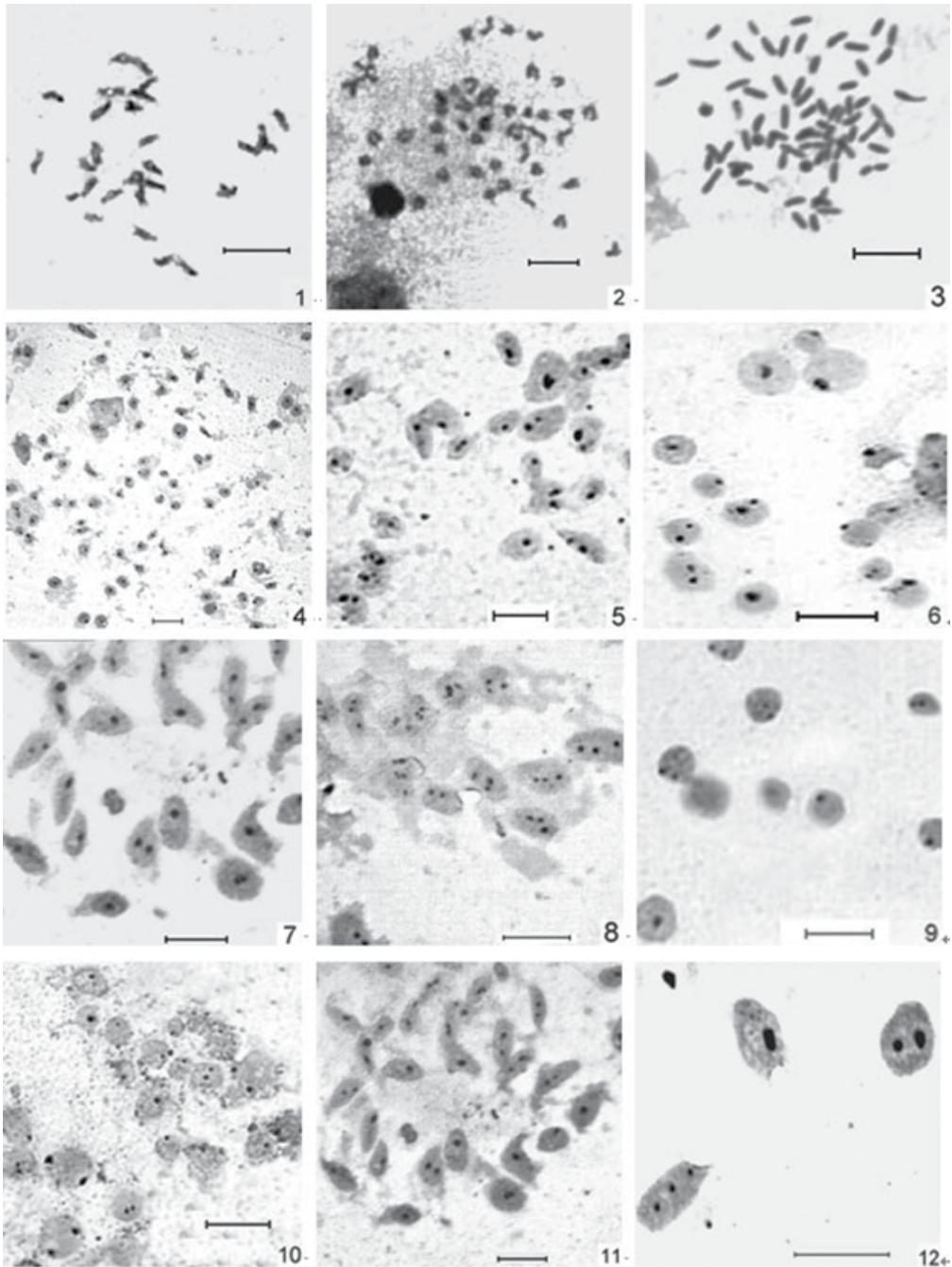


Figure 39.6 Metaphase chromosomes and interphase nucleoli of large yellow croaker.

- 1) & 4) gynogenetic haploid
- 2) & 7) gynogenetic diploid
- 3) & 8) triploid with gynogenetic diploid female parent
- 5) & 9–12) normal diploid
- 6) normal triploid.

Scale bar = 5 μ m (adapted from Reference [7]).

Table 39.3 Comparison of nucleolus number between normal diploid and gynogenetic diploid of large yellow croaker (adapted from [7]).

Group	Stage	Tissue	No. of cells	Percentage of cells with different nucleolus number			Average nucleolus number
				1	2	3	
N-2 <i>n</i>	Embryo	Whole	3,660	28.03	67.85	4.12	1.74 ± 0.061
	Adult	Kidney	3,241	24.94	68.60	6.46	1.83 ± 0.090
		Dorsal fin	3,556	22.34	72.52	5.14	1.92 ± 0.034
		Gill	3,110	23.89	70.13	5.98	1.85 ± 0.055
G-2 <i>n</i>	Fry	Whole	3,503	65.14	34.48	0.37	1.35 ± 0.098
	Adult	Kidney	3,680	55.64	42.03	2.33	1.47 ± 0.087
		Dorsal fin	3,051	57.83	38.04	4.13	1.46 ± 0.095
		Gill	3,184	53.34	45.04	1.62	1.48 ± 0.042

Table 39.4 Genetic diversity and homozygosity for the analyzed microsatellite loci in the control, meio-G1 and meio-G2 (adopted from [8]).

Genetic parameters	Control	Meio-G ₁	Meio-G ₂
Mean number of alleles	5.3	3.9	2.1
Mean number of genotypes*	10.1	4.5	2.9
Mean observed heterozygosity	0.624	0.339	0.197
Mean expected heterozygosity	0.672	0.542	0.219
Mean PIC	0.616	0.455	0.176
Homozygosity	0.376	0.661	0.803

*Number of genotypes: number of genotypes for each loci. All the parameters in Table 39.4 were calculated by the software Cervus 3.0.

Microsatellite-centromere (M-C) map distances were examined using 18 loci with normal Mendelian segregation (Table 39.5). Estimated recombination rates ranged between 0 and 1.0 under the assumption of complete interference. The average recombination frequency was 0.586. Ten loci showed high M-C recombination with frequency greater than 0.67. Thus, high recombinant frequencies between heterozygous markers and the centromere were found in large yellow croaker, as in other teleosts. M-C distances provide useful information for gene mapping in large yellow croaker.

39.3.2 Mito-Gynogenesis

Diploidization of the maternal chromosome set can be obtained by blocking the first cleavage, which is termed mito-gynogenesis (or endomitosis). Double haploids (DH), homozygous at all loci, can be induced by suppressing the first cleavage of gynogenetically developed embryos. Gynogenetic double haploids (GDHs) have potential applications in genetic improvement in aquaculture, such as rapid establishment of clone lines, accelerated elimination of recessive deleterious genes from aquaculture population, and genetic mapping.

Table 39.5 Microsatellite-centromere recombination rate (second meiosis segregation frequency = y) and map distance (x) of 18 microsatellite loci examined in two gynogenetic families of large yellow croaker (adopted from [3]).

Loci	Family	No. of genotyped samples	Genotypes of gynogens			y	x (cM)	χ^2 A/A : B/B = 1 : 1
			A/A	A/B	B/B			
LYC0002	F ₁	89	2	86	1	0.966	48.9	0.34
	F ₂	90	0	89	1	0.989		1.00
LYC0008	F ₁	93	33	24	36	0.258	12.9	0.13
LYC0011	F ₁	90	41	8	41	0.089	4.4	0.00
LYC0013	F ₁	92	4	82	6	0.891	45.8	0.40
	F ₂	86	3	81	2	0.942		0.20
LYC0014	F ₁	92	2	90	0	0.978	49.2	2.00
	F ₂	90	0	89	1	0.989		1.00
LYC0015	F ₂	90	1	87	2	0.967	48.3	0.33
LYC0017	F ₁	94	9	79	6	0.840	42	0.60
LYC0021	F ₂	88	47	2	39	0.023	1.15	0.74
LYC0022	F ₁	92	47	5	40	0.054	1.6	0.56
	F ₂	90	36	1	53	0.011		3.25
LYC0025	F ₁	89	21	0	68	0.000	0	24.81 **
	F ₂	88	46	0	42	0.000		0.18
LYC0026	F ₁	94	22	46	26	0.489	17.5	0.33
	F ₂	90	39	19	32	0.211		0.69
LYC0027	F ₁	94	50	0	44	0.000	0	0.38
LYC0036	F ₁	94	7	22	65	0.234	13.6	46.72**
	F ₂	90	36	28	26	0.311		1.61
KPC43	F ₁	93	6	83	6	0.892	44.6	0.00
KPC45	F ₁	92	0	90	2	0.978	48.9	2.00
	F ₂	94	2	92	0	0.979		2.00
KPC49	F ₂	94	0	94	0	1.000	50.0	0.00
KPC10	F ₂	94	3	81	10	0.862	43.1	3.77

Notes: * $p < 0.05$, ** $p < 0.005$, Data were eliminated for their significant difference with the frequencies of the two homozygous classes (PG0.05).

39.3.2.1 Conditions for Inducing Mito-Gynogenesis in Large Yellow Croaker

• Mitotic interval at different temperatures

The starting times of 1st cleavage (τ_1) and mitotic interval (τ_0) at different temperatures were determined in large yellow

croaker [9]. The higher the temperature, the faster was the first cleavage, about 3.1 minutes faster every 1°C. τ_0 was shorter when the temperature rose. The relations between τ_0 and temperature was fit as $\tau_0 = 1304.22225e^{T/(-4.50006)} + 9.7338$ ($R^2 = 0.99999$, $P = 0.00171$). The ratio of

$\tau I/\tau 0$ ranged from 2.24 to 2.91, which increased with the temperature. The results would provide a foundation to improve the technique for chromosome manipulation in large yellow croaker.

- **Start timing of hydrostatic pressure shock**
Figure 39.7 shows the effect of the start timing of hydrostatic pressure shock on the survival of embryos at the tail bud stage, the hatching rate, and the production of normal fry. All three curves were bell-shaped. In the haploid control groups, the production of total fry was relatively high

Table 39.6 Time of egg cleavages under different temperature in large yellow croaker (adopted from [9]).

Temperature (°C)	Time of egg cleavages (minutes post-insemination)				
	τI	τII	τIII	τIV	τV
19.1 ± 0.4	63.9	100.2	127.7	152.8	177.7
22.4 ± 0.4	50.7	68.0	87.8	106.2	125.7
25.0 ± 0.5	43.0	52.5	69.0	85.8	102.3
28.2 ± 0.3	35.3	47.2	61.3	70.9	84.1

τI – τV : the 1st to the 5th cleavage.

(20.82% ± 5.34%), but all the embryos and fry were abnormal, showing typical haploid syndrome. Normal fry appeared when pressure shocks were applied to the eggs from 35.4 minutes post-insemination (p.i.) to 39.4 minutes p.i. Maximum production of normal fry (9.36% ± 2.97%) was recorded at 38.1 minutes p.i. However, there was no significant difference in the production of normal fry among four start times: 36.1 minutes, 36.7 minutes, 37.4 minutes, and 38.1 minutes ($P > 0.05$). The normal fry from the 38.1 minutes group were verified as diploids by ploidy analysis.

- **Intensity of pressure shock**
In two trials, all fry in the haploid control group showed obvious haploid syndrome. Haploid-syndrome fry could be easily distinguished from normal fry by their curved spine and vague sarcomere, while the normal fry had straight spines and clear sarcomeres (Figure 39.4). The fry with haploid-syndrome were verified as containing half the DNA content of a normal diploid by ploidy analysis. Figure 39.8 shows the effect of hydrostatic pressure shock intensity on the hatching rate and production of normal fry. The hatching rate decreased as the

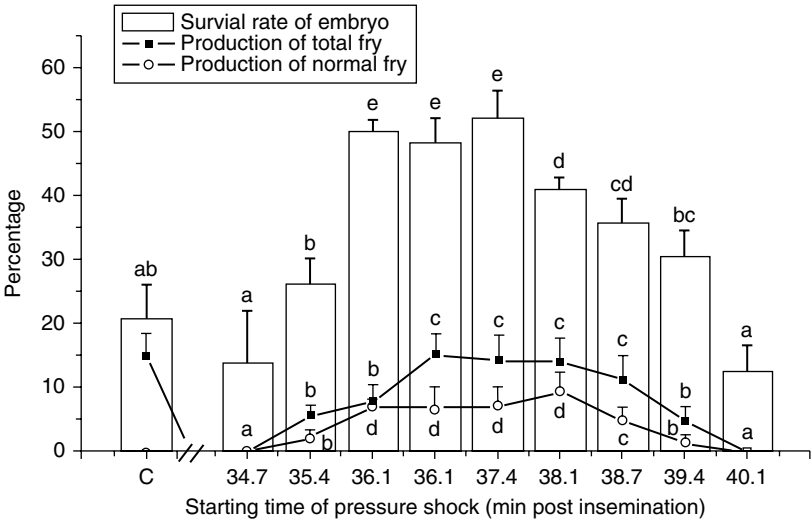


Figure 39.7 The effect of the starting time of hydrostatic pressure shock on the survival rate of embryos at the tail bud stage, the hatching rates, and the production of normal fry at four hours after hatching. ‘C’ on abscissa indicates the haploid control group without pressure shock. Different letters of a, b, c d in the figure mean significant difference.

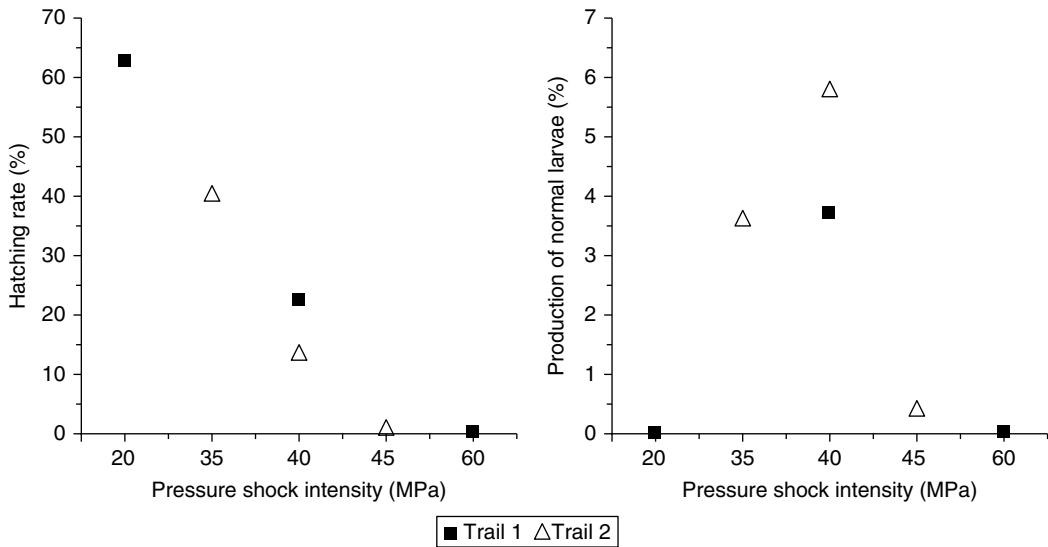


Figure 39.8 The effect of the intensity of the hydrostatic pressure shock on the hatching rate and the production of normal fry. The inseminated eggs of the large yellow croaker were shocked for three minutes, beginning at the starting time of the first cleavage.

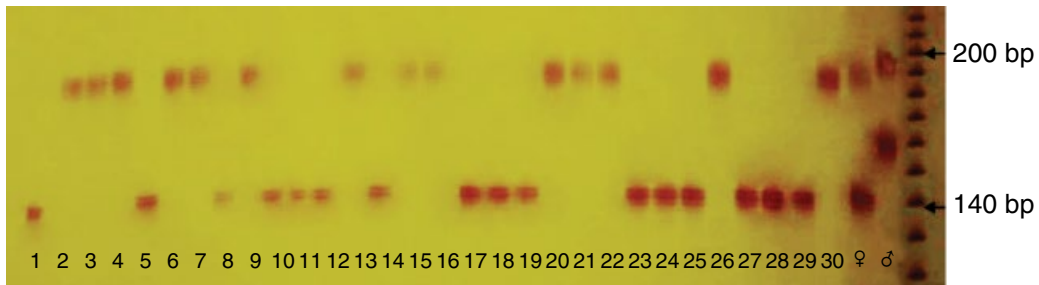


Figure 39.9 The electrophoresis patterns of family GF1 at LYC0026 microsatellite locus (adapted from [11]). (See inserts for the color representation of this figure.)

pressure increased, while the production of normal fry rose at first, reaching a maximum at 40 Mpa in both trials, and then declined. The maximum productions of normal fry were 5.76% and 3.70% of treated eggs in the two trials, respectively. The normal fry collected from 40 Mpa groups were verified as diploids by ploidy analysis.

39.3.2.2 Genetic Analysis on Mito-Gynogens in Large Yellow Croaker

• Microsatellite analysis

Two mitotic gynogenetic families, GF1 and GF2, were produced. All of the fry in the control families were abnormal, while normal fry

reappeared in GF1 and GF2 after hydrostatic pressure shock, with normality rate of 40.0% and 17.1% respectively (Figure 39.7). The inheritance and segregation of 10 microsatellite loci in putative gynogenetic doubled haploids (GDH) was investigated [11]. In GF1, 20 genotypes were observed in 30 assayed progenies. All samples of GF1 were demonstrated as GDH for exclusive maternal inheritance and homozygous at each locus (i.e., LYC0026 in Figure 39.9).

In 30 tested offspring in GF2, 27 fry were demonstrated as GDH, two contained male parent specific band, and one fry remained undefined. These results suggested that the homozygous gynogenesis

Table 39.7 Segregation and χ^2 test of microsatellite markers in two mito-gynogenetic families (adapted from [12]).

Locus	Family	Males	Females	Offspring*		χ^2 (df=1)	P value
				F/F	S/S	F/F : S/S = 1 : 1	
LYC0002	GF1	B/C	A/D	A/A (17)	D/D (13)	0.53	0.467
	GF2	C/C	A/B	A/A (18)	B/B (9)	3.00	0.083
LYC0004	GF1	A/A	A/B	A/A (16)	B/B (14)	0.13	0.718
LYC0011	GF1	B/B	A/B	A/A (10)	B/B (20)	3.33	0.068
LYC0012	GF2	C/C	A/B	A/A (9)	B/B (18)	3.00	0.083
LYC0013	GF1	A/B	B/C	B/B (14)	C/C (16)	0.13	0.718
LYC0014	GF1	B/C	A/D	A/A (17)	D/D (13)	0.53	0.467
LYC0026	GF1	B/D	A/C	A/A (16)	C/C (14)	0.13	0.718
	GF2	C/D	A/B	A/A (8)	B/B (19)	4.48	0.034
LYC0053	GF2	C/C	A/B	A/A (7)	B/B (20)	6.26	0.012
LYC0109	GF1	A/B	C/D	C/C (18)	D/D (12)	1.2	0.273
LYC0114	GF1	A/B	A/B	A/A (13)	B/B (17)	0.53	0.467

Notes: in parentheses are given the numbers of individuals observed for each genotype; F and S were the fast and slow allele in the electrophoresis patterns, respectively.

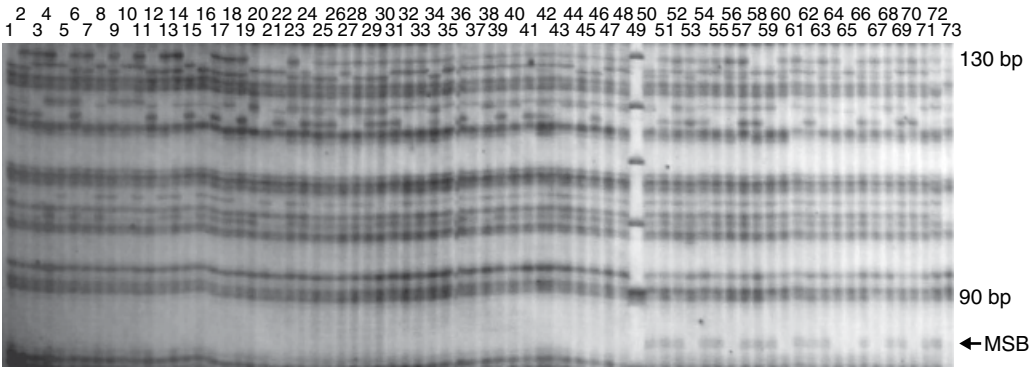


Figure 39.10 AFLP fingerprint pattern of control family (Lane 50–71), meio-gynogenetic family (Lane 25–48), and mito-gynogenetic family (1–24), using E-AAC/M-CAA primer pair.
Lane 49: DNA Ladder; lane 72: male parent; lane 73: female parent; MSB: male parental specific band; bp: base pair.

could be induced with the method reported as described above. In addition, the segregation of microsatellite markers in GDHs was consistent with the expected ratio according to Mendel’s law at all the loci except LYC0026 and LYC0053 (Table 39.7). We also found that the segregation mode of GDH was completely identical between LYC0002 and LYC0014.

- **AFLP analysis**
Segregation patterns of amplified products of five pairs of AFLP primers and seven pairs of microsatellite primers were investigated in a meiotic gynogenetic family (MeGF), a mitotic gynogenetic family (MiGF), and a normal family as control (CF) (Figure 39.10), using the same female and male fish as the parents [10].

No male parent specific alleles were detected in all offspring in either MeGF or MiGF, indicating 100% success of gynogenesis for the two families. The results showed that 40.8% heterozygous gene could be fixed after one generation of meiotic gynogenesis, but it was very difficult to achieve homozygote by meiotic gynogenesis for a large number of loci near the telomere.

Among the segregated AFLP loci in the MiGF, only four AFLP loci segregated deviated from the expected 1 : 1 ratio, compared with that in the CF, suggesting that the selection had no obvious effect on the mito-gynogens in most of the detected loci at the hatching-out stage (Table 39.8). Two recessive lethal linked AFLP markers – aagcag70 and aaccag255 – were screened out for unusual phenotype frequencies as all or none in MiGF.

39.4 Sex Determination in Large Yellow Croaker

A wide variety of techniques have been used to analyze sex determination in large yellow croaker, such as induced gynogenesis, karyotype analysis, molecular, and genomic methods.

39.4.1 Elucidation of sex-Determining Systems Without the use of Markers

Analyzing phenotypic sex ratios among fish produced by methods such as hybridization and chromosome set manipulations (gynogenesis, androgenesis), and progeny testing of sex-reversed individuals, has allowed identification of simple genetic sex-determining systems (e.g., XX/XY, WZ/ZZ). Many species of importance to aquaculture have been analyzed using such techniques and, in several cases, significant commercial monosex production programs have been developed. In large yellow croaker, the induced meiotic gynogens present a high proportion of females (average 99.3%) in 47 examined batches.

Table 39.8 Inheritance and segregation of female parent specific AFLP loci in normal family, meio-gynogenetic family and mito-gynogenetic family.

Locus name	CF (n=22) Present : absent	MeGF (n=24) Present : absent	MiGF (n=24) Present : absent
aaccaa350	10 : 12	20 : 4*	14 : 10
aaccaa260	13 : 9	18 : 6*	11 : 13
aaccaa225	16 : 6	23 : 1**	12 : 12
aaccaa173	6 : 16	24 : 0**	11 : 13
aaccaa138	11 : 11	10 : 14	14 : 10
aaccaa129	6 : 16	14 : 10	17 : 7
aaccaa127	11 : 11	12 : 12	10 : 14
aggcag165	12 : 10	23 : 1**	7 : 17
aggcag139	14 : 8	24 : 0**	14 : 10
aggcag70	12 : 10	24 : 0**	11 : 13
aagcag278	12 : 10	24 : 0**	12 : 12
aagcag272	10 : 12	10 : 14	12 : 12
aagcag255	12 : 10	12 : 12	17 : 7
aagcag170	10 : 12	24 : 0**	11 : 13
aagcag154	13 : 9	16 : 8	13 : 11
aagcag138	8 : 14	21 : 3**	11 : 13
aagcag129	10 : 12	13 : 11	12 : 12
aagcag86	11 : 11	23 : 1**	13 : 11
aagcag70	12 : 10	24 : 0**	0 : 24**
accag350	17 : 5*	24 : 0**	16 : 8
accag340	10 : 12	23 : 1**	12 : 12
accag335	22 : 0**	24 : 0**	24 : 0**
accag323	12 : 10	16 : 8	9 : 15
accag235	22 : 0**	24 : 0**	24 : 0**
accag138	11 : 11	11 : 13	11 : 13
accag115	11 : 11	20 : 4*	13 : 11
accag103	12 : 10	24 : 0**	6 : 18
accag76	13 : 9	16 : 8	10 : 14
accag-67	17 : 5*	8 : 16	11 : 13
aaccag350	16 : 6	17 : 7	11 : 13
aaccag255	13 : 9	24 : 0**	24 : 0**
aaccag105	12 : 10	13 : 11	16 : 8
aaccag340	22 : 0**	24 : 0**	24 : 0**

*Significantly different from 1 : 1 in inheritance ratio (0.001 < P < 0.05).

**Highly significantly different from 1 : 1 in inheritance ratio (P < 0.001).

Box 39.1 Type of sex determination

Type: simple genetic sex-determining systems (XX-XY).

Sex-determining gene (candidate): *dmrt1*

Sex markers:

- 1) MFS [a co-dominant marker, two bands for the genetic males (XY) and one band for the genetic females (XX). Primer sequences: F: 5'-TGGCTCTGTGAGGCGTCT-3', R: 5'-ATACAATGATGACATCAATCCTGAT-3'];
- 2) MS [an Y-specific dominant marker, one band for the genetic males (XY) and no band for the genetic females (XX). Primer sequences: F: 5'-GGCTCTGTGAGGCGTCTT-3', R: 5'-CTTACAGTTATCTGCAATTTGTATG-3']

When the gynogenetic diploids were administered orally with 17 α -methyltestosterone at the concentration of 20 ppm (μ g/g diet) from 20–55 mm in total length, 53.3–76.6% of treated gynogenetic diploids became males. More than 96% of progenies produced from the cross between the sex-reversed gynogenetic females (phenotypically males) and normal females were females. Conversely, the sex ratios were almost 1 : 1 in the control cross between normal females and males. These facts strongly suggest that the genetic sex determination mechanism in large yellow croaker is basically XX-XY type (Box 39.1).

39.4.2 Karyotypical Analysis

The chromosome characteristics of large yellow croaker, *L. crocea*, were examined with several cytogenetic methods. The karyotype of large yellow croaker comprised 48 chromosomes ($2n = 2sm + 4st + 42t$; Figure 39.11) [12]. Although the preliminary results showed the short arm of one of chromosome 10 was longer slightly than that of its homologous chromosome (Figure 39.12), it is still difficult for this to be used as a cytogenetic marker of sex, as a large variation existed in the length of the chromosome. Furthermore, whether this pair of

chromosomes are the sex chromosomes also needs more evidence.

39.4.3 DNA Markers for Sex

The advent of DNA analysis techniques has facilitated the search for sex-linked and sex-specific sequences. In large yellow croaker, we have attempted to screen sex DNA markers through several methods, including comparison of male and female DNA using AFLP fingerprinting, linkage mapping, and genome-wide association study (GWAS), and in-depth comparative analysis using multiple sets of male and female whole genome re-sequencing data.

39.4.3.1 AFLP Fingerprinting

The phenotypical male and female large yellow croaker were screened for amplified fragment length polymorphisms (AFLPs) to search for sex-linked or sex-specific markers with bulked segregant analysis (BSA). In total, two male-specific markers (M2-P8-359 and T3-E4-350) were indentified from 23,809 AFLP bands, produced with 256 selective amplification primer combinations of four sets of restricted enzymes (*EcoR* I + *Mse* I, *EcoR* I + *Taq*I, *Pst*I + *Mse*I, and *Pst*I + *Taq*I). Consistent results were obtained in unrelated individuals with both markers. However, it failed to transfer the AFLP sex markers to the sequence characterized amplified region (SCAR) markers. The results suggested that the divergence of genomic DNA between two sexes in large yellow croaker is very low [13].

39.4.3.2 Linkage Map, GWAS, and in-Depth Comparative Analysis of Male and Female Whole Genome Re-sequencing Data

Sex determination regions of large yellow croaker were identified with sex-linked QTL mapping and association analysis, with a croaker family based on a high-density genetic map consisting of 3,448 single-nucleotide polymorphism (SNP) markers [14, 15]. The markers associated with the sex significantly were detected with composite interval mapping and multiple QTL model (MQM).

Figure 39.11 DAPI-staining of large yellow croaker (*L. crocea*).
1, 2: female;
3, 4: male.
Scale bar=5 μ m (adapted from [12]).

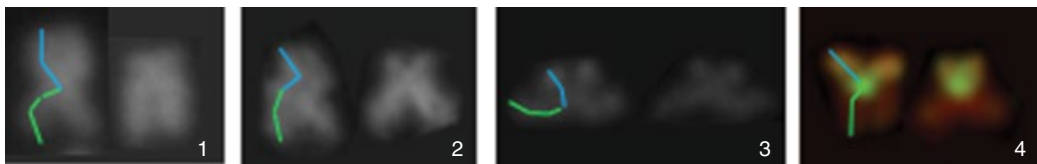
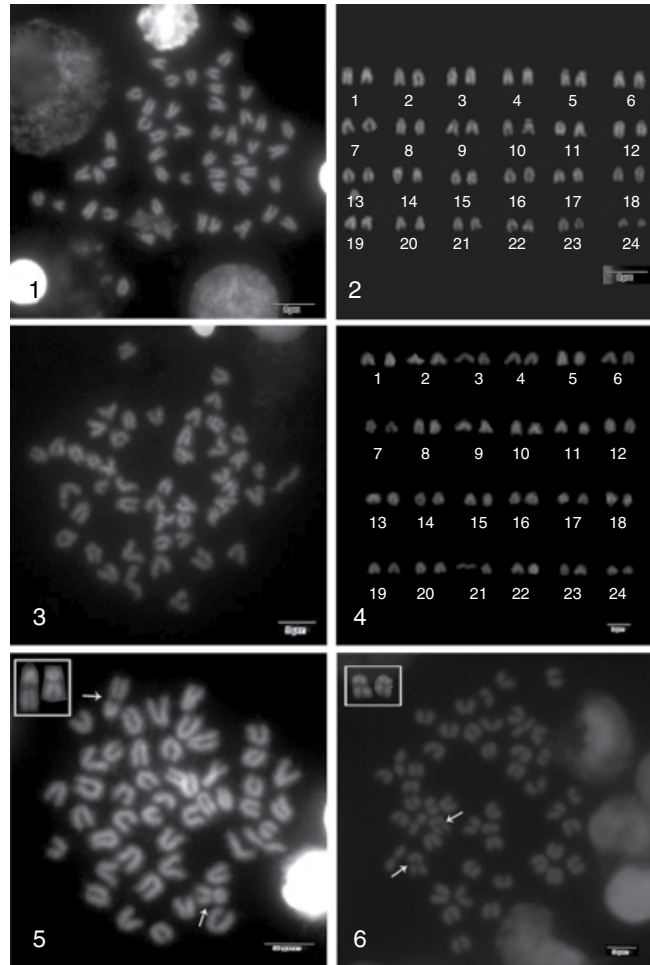


Figure 39.12 Chromosome 10 of *L. crocea* male 1: PI-staining; 2: DAPI-staining; 3: DAPI-staining; 4: chromosomal localization of H-P3K by FISH. Blue lines indicate the midrib line of the short arms, green lines indicate the midrib line of the long arms. Adapted from [12]. (See inserts for the color representation of this figure.)

With markers being of LOD over a threshold of 30, a unique pseudoautosomal locus linking to sex determination closely was found at 30–32 cM in the linkage group (LG) 9 by using MapQTL47 (Figure 39.13).

The differences in the DNA sequences between the two sexes in the regions were

screened out by comparing six re-sequencing databases (two males, two females, one male pool containing 50 male fish, and one female pool containing 50 female fish; data not shown). Consequently, a male-specific 15-bp-deletion was found. Sex DNA markers were developed on the basis of the deletion.

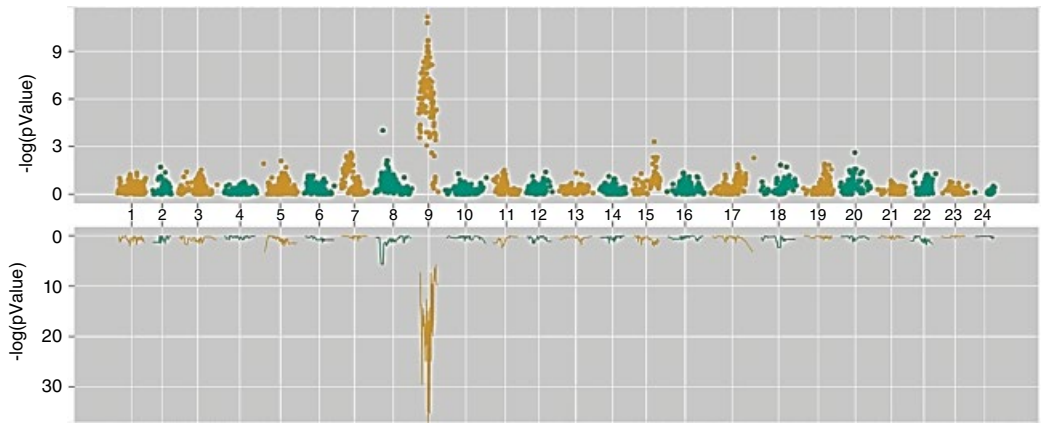


Figure 39.13 QTL (below) and GWAS (above) of sex in large yellow croaker.

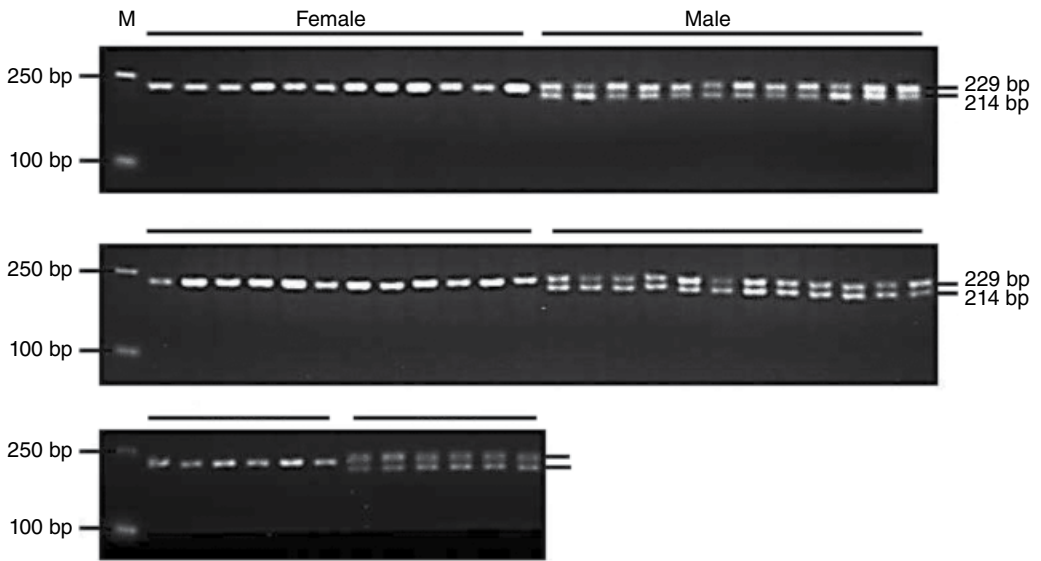


Figure 39.14 PCR amplification results using the MFS primer pair in 30 females and 30 males. The 229 bp long band shared by males and females, and the shorter 214 bp male-specific band, are each indicated by arrows on the right. The DL 2000 DNA marker sizes are shown on the left. Adapted from [16].

Two sets of PCR primers were designed to amplify the sex differentiated locus containing the deletion:

- 1) MFS-F (5'-TGGCTCTGTGAGGCGTCT-3') and the reverse primer MFS-R (5'-ATACAATGATGACATCAATCCTGAT-3'), which can amplify two bands

- in genetic males (XY) and one band in genetic females (XX) (Figure 39.14);
- 2) MS-F: 5'-GGCTCTGTGAGGCGTCTT-3', and the reverse primer MS-R: 5'-CTTACAGTTATCTGCAATTTGTATG-3', which can amplify a band specifically in genetic males (XY) and no band in genetic females (XX) (Figure 39.15) [16].

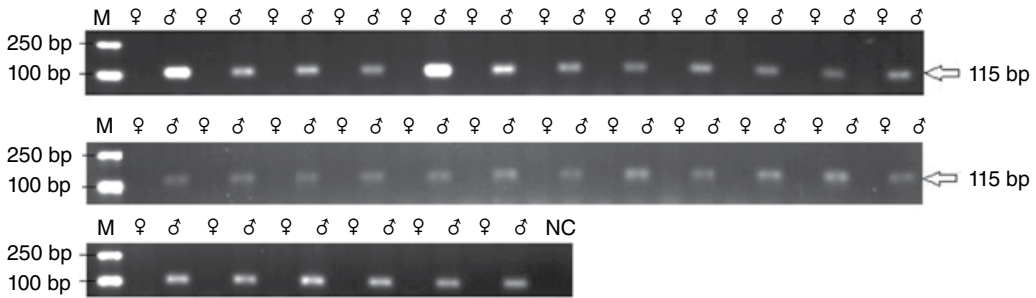


Figure 39.15 Agarose gel separation of PCR products of 30 females (♀) and 30 males (♂) using Y-specific marker MS confirmed the XY-type sex determination in large yellow croaker. “NC” denotes “Negative Control.” The 115 bp male-specific fragments are indicated by arrows on the right, and the DL 2000 DNA marker sizes are shown on the left. Adapted from [16].

These two markers had been confirmed with more than 900 fish from different groups. The success of developing male-specific DNA markers in large yellow croaker confirmed that this species has a monofactorial genotypic sex determination, as XX-XY type.

In addition, we applied an association study with the simple linear regression model, using PLINK package to identify the sex associated genes, and found *dmrt1* might be the candidate (male) sex determination gene for large yellow croaker (Box 39.1).

39.5 Histological Observation on Gonadal Sex Differentiation

Reproduction is the most important node of a life cycle, and it depends on normal differentiation of gonads at the early stage of life. Gonadal sex differentiation includes a series of changes in cells and tissues. Identification of these changes will lay the foundation for further studies of fish sex differentiation mechanism, and will provide the basis for determining the appropriate time for sex control, as well as background for the investigations on the effect of climate change and pollution on reproductive biology. Therefore, histological and cytological characteristics of important events occurring in gonadal differentiation in large yellow croaker were investigated, to

provide a theoretical basis for sex control in large yellow croaker.

The process of gonadal development and differentiation of large yellow croaker was investigated using histological methods [17]. The sampled fries hatched on September 22 in 2009, at 26 °C. The temperature for nursery and culture were 22.0–25.8 °C and 11.5–25.6 °C, respectively. At the age of 20 days post-hatch (dph), when the fry gained 17.6–19.2 mm in body length (BL), a pair of primordial gonads was present in the abdominal cavity. Ovarian differentiation occurred at 55 dph (BL 27.5–37.0 mm), and was characterized by the presence of clusters of oogonia. The formation of ovarian cavity and meiosis of germ cells began simultaneously at the age of 60 dph (BL 28.0–37.2 mm). The presence of primary oocytes occurred at the age of 120 dph (BL 39.2–51.0 mm). The differentiation of testis began at the age of 95 dph (BL 38.0–48.0 mm), and was characterized by the presence of efferent duct and the scattering of somatic cells throughout the gonad. Cyst of spermatocytes could be seen at 215 dph (BL 44.0–59.2 mm), and testis lobules started to form at 230 dph (BL 56.2–72.8 mm). These results suggested that large yellow croaker is a differentiated gonochorist, and that differentiation of the ovary occurs earlier than that of testis (Table 39.9, Figures 39.16 and 39.17).

Table 39.9 Summary of histological observations on the gonadal differentiation of large yellow croaker (adapted from [17]).

Age (dph)	Body length (mm)	Sex			Degree of histological differentiation of gonads
		U	F	M	
20	17.6–19.2	10	0	0	A pair of primordial gonads hanging from the abdominal epithelium Round-to-ovoid nuclei in pgcs
30	16.0–24.5	10	0	0	Enlargement of gonads mainly by proliferation of somatic cells Formation of blood vessels in all individuals
50	25.5–34.6	0	6	4	Active germ cells mitosis in half of the individuals (♀)
55	27.5–37.0	0	4	6	Increases in the number of germ cells and somatic cells; cluster of oogonia (♀)
60	28.0–37.2	0	4	6	Meiosis; small protuberance of gonads adjacent to blood vessels in presumptive ovary (♀)
65	27.1–41.0	0	5	5	Active germ cells meiosis; lateral gonadal tissue adjacent to blood vessels grew towards the dorsal side of the gonad (♀)
95	38.0–48.0	0	6	4	Active germ cells mitosis; the formation of efferent duct (♂)
120	39.2–51.0	0	6	4	Primary oocyte and the gathering somatic cells in the stalk-like dorsum of gonads (♀)
215	44.0–59.2	0	6	4	Enlargement of efferent duct and the presence of cyst of spermatocytes (♂) Closure of ovarian cavity by fusion of dorsal somatic cells outgrowth with lateral gonadal tissue (♀)
230	56.2–72.8	0	4	6	Massive primary oocytes (♀) Formation of testis lobules (♂)
235	61.0–76.2	0	3	7	Production of spermatids (♂)

39.6 Effects of Exogenous Hormone and Temperature on Sex Differentiation of Large Yellow Croaker

The effects of methyl testosterone, aromatase inhibitor, and rising temperature on sex differentiation of larvae and juveniles of large yellow croaker were preliminarily studied [18]. After treatments with methyl testosterone, Letrozole, and high temperature, the sex of each individual was determined by observing its gonadal tissue slices, and then the sex ratio of males was calculated, respectively. Larvae and juveniles of large yellow

croaker were fed with oral feed soaking in different concentrations of methyl testosterone (25, 50, 100, 200, /L and 400 µg/L) with three different treatment periods (one, two, and three months). The results showed that the male sex ratios were 30.95–67.65% with treatment of one or two months, but they increased significantly with treatment of three months with all dosages (80.00–95.65%; Figure 39.18). The sex ratios in all groups treated with oral Letrozole-soaked feed (concentration: 25, 50, 100, 200, and 400 µg/L; periods: one, two, and three months) and high temperature (Table 39.10) were not significantly different from those in the control groups.

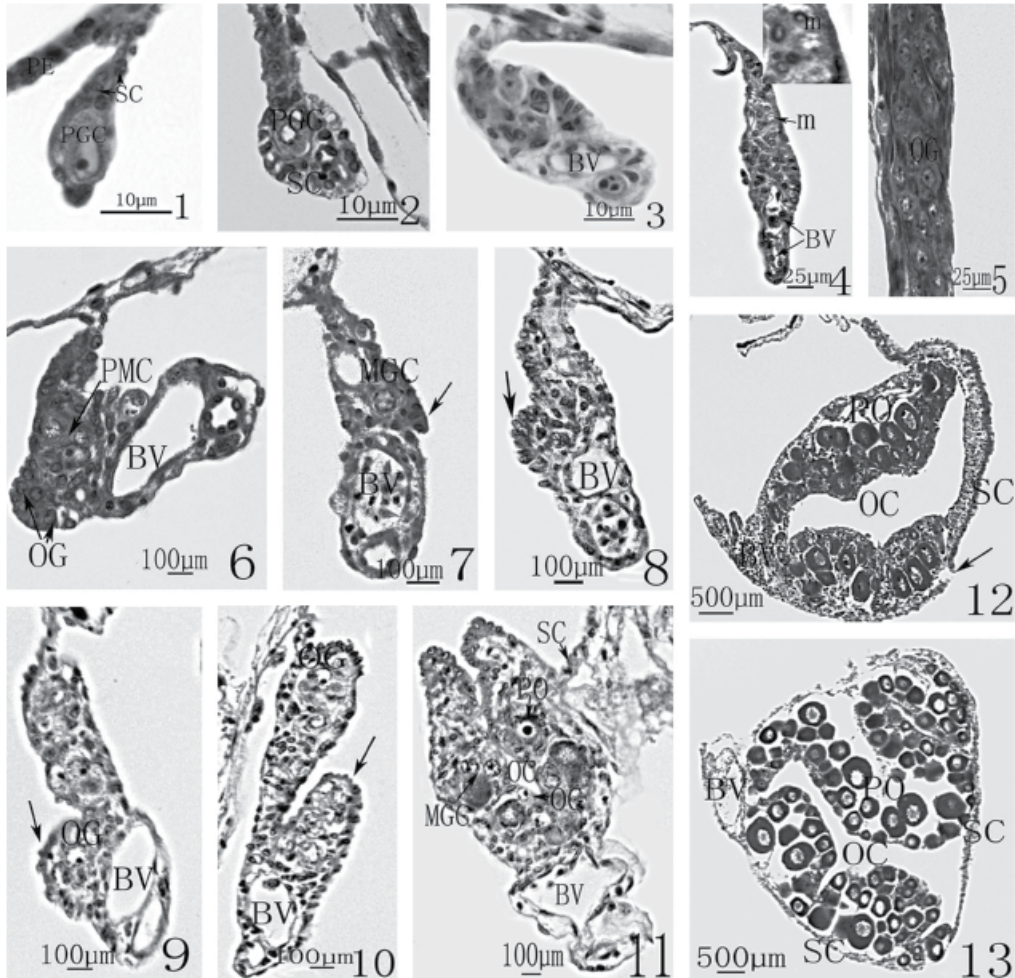


Figure 39.16 Development and differentiation of ovary in large yellow croaker.

- 1: Primordial gonad at 20 dph, showing the PGCs;
 - 2: Undifferentiated gonad at 25 dph, showing the increases in the number of somatic cells;
 - 3: Undifferentiated gonad at 30 dph, showing the formation of blood vessels in the ventral aspect of gonad;
 - 4: Presumptive ovary at 50 dph, showing germ cells undergoing mitosis;
 - 5: Presumptive ovary at 55 dph, showing the appearance of clusters of oogonia;
 - 6: Presumptive ovary at 60 dph, showing cysts of pre-meiotic germ cells;
 - 7: Presumptive ovary at 60 dph showing germ cells undergoing meiosis and small protuberance of gonads adjacent to blood vessels(arrow);
 - 8, 9, 10: Presumptive ovary at 60–110 dph, showing the growth of lateral gonadal tissue (arrow);
 - 11: Presumptive ovary at 120 dph, showing the primary oocytes and the somatic cells in the stalk-like dorsum of gonad;
 - 12: Presumptive ovary at 205 dph, showing the fusion of dorsal somatic cells outgrowth with lateral gonadal tissue (arrow);
 - 13: Presumptive ovary at 215 dph, showing massive primary oocytes.
- AC – abdominal cavity; PE – peritoneal epithelium; PG – primordial gonad; PGC – primordial germ cell; BV – blood vessel; SC – somatic cell; m – mitosis; OG – oogonium; MGC – meiotic germ cell; PMC – Pre-meiotic germ cells; PO – primary oocyte; OC – ovarian cavity (Adapted from [17]).

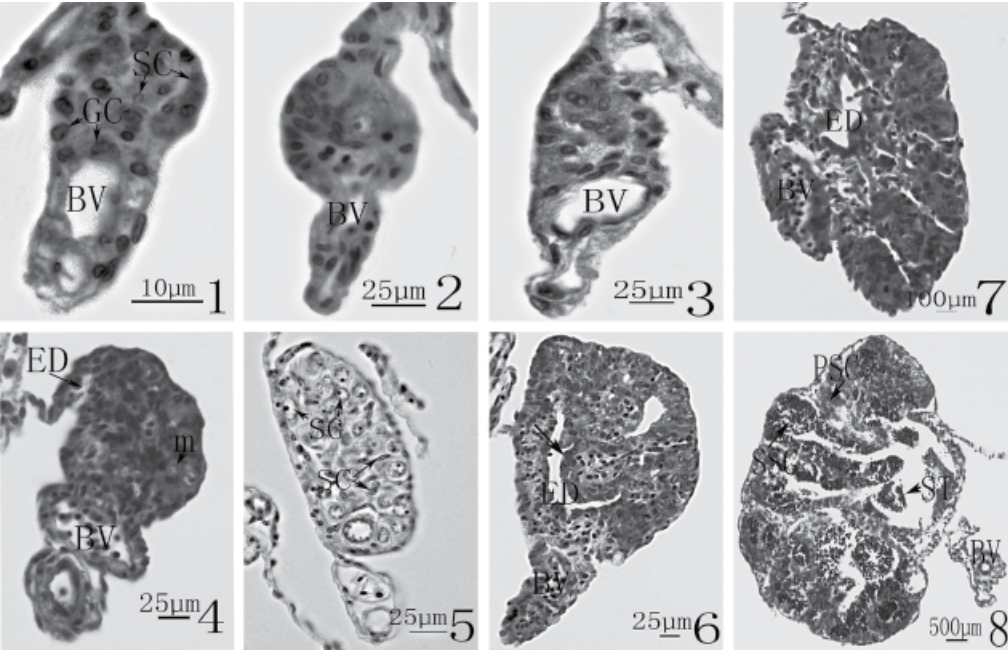


Figure 39.17 Development and differentiation of testis in large yellow croaker.
1, 2, 3: Presumptive testis at 50–75 dph;
4: Presumptive testis at 95 dph, showing germ cells undergoing mitosis and the efferent duct;
5: Presumptive testis at 95 dph, showing spermatogonia and somatic cells scattering throughout the gonad;
6: Testis at 215 dph showing cysts of spermatocytes (arrow);
7: Testis at 230 dph showing the formation of testis lobules;
8: Testis at 230 dph showing the production of spermatids.
ED – efferent duct; SG – spermatogonium; SC – somatic cell; PSC – primary spermatocyte; SSC – secondary spermatocyte; ST – spermatids (adapted from [17]).

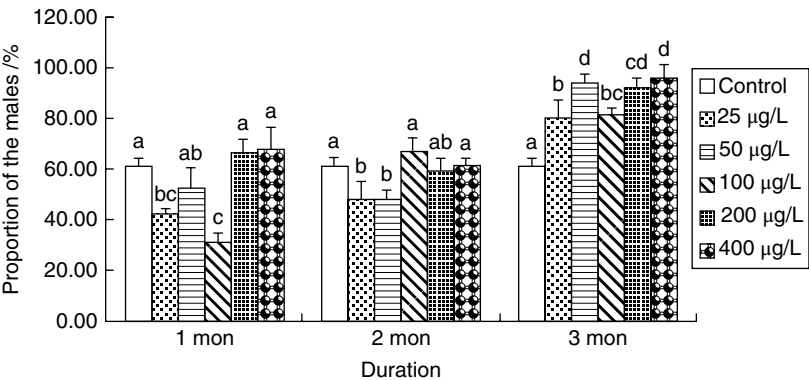


Figure 39.18 The proportion of males with treatment of methyl testosterone. Adapted from [18].

In conclusion, the proportion of males can be improved by treatment with methyl testosterone (25–400 µg/L, at 22 ± 1 °C) from 1–3 months of age, and the liable

period of the treatment is from 2–3 months of age, which is roughly consistent with the first round of germ cells mitosis in gonad (Box 39.2).

Table 39.10 Experiment designation of temperature treatment.

Initial body length (cm)	Temperature (°C)				Period (day)
	26 ± 0.5	26 ± 0.5	26 ± 0.5	22 ± 1	
1.5	A1	A2	A3	Control-A	63 days
2.5	B1	B2	B3	Control-B	55 days
3.5	C1	C2	C3	Control-C	21 days

Box 39.2 Best treatment for sex control

Type of steroids: methyl testosterone.
 Dosage: 50–400 µg/L.
 Timing: 2–3 months age.
 Expected sex ratio: 80%.

39.7 Conclusions and Perspectives

After years of efforts, we have established the procedures for inducing meio- and mitogynogenesis in large yellow croaker, and produced more than 20 batches of meio-gynogenetic adults including of two successive generations of gynogens, which have been applied: to elucidate the sex determination system; to estimate the microsatellite-centromere distances; and also to produce highly homologous material to gain the reference genome sequence. The genetically female gynogens have been reversed to phenotypically males (pseudo-males) with oral treatment of methyl testosterone. Eventually, batches of all-female populations have been produced by mating the neomales and the normal females. However, there are still several problems to be solved to upgrade the platform of sex control, and expand its application in the culture industry of large yellow croaker.

39.7.1 Culture Platforms and Technology

The infectious diseases of large yellow croaker become more and more severe as industry expands, which has not only hampered the progress of culture industry of

large yellow croaker, but has also affected the development of sex control techniques in this species. In addition, the fitnesses of the induced gynogens and pseudo-males are even lower for homozygosity of recessive deleterious genes. In fact, multiple batches of gynogens and pseudo-male produced in our laboratory have died out due to white-spots disease, severely hampering the progress of the study. Therefore, it is essential to develop special fine culture technology and a platform for the products of induced gynogenesis and sex control in large yellow croaker.

39.7.2 Parameters of Sex Control

It is possible to achieve 100% sex reversal by means of oral or soaking treatment of sex hormones, as suggested by the experiences in other fishes. The effect of sex reversal manipulation is determined by the starting time, duration and intensity of the treatment, and is also influenced by the feeding regime, temperature, and so on. We have learned that the liable period of the treatment of oral methyl testosterone (25–400 µg/L) in large yellow croaker is from 2–3 months of age, but the percentage of pseudo-males is still low (50–70%) after the gynogens were treated with androgen. Therefore, it is still necessary to optimize the parameters in the procedure step by step to improve the efficiency of sex reversal.

39.7.3 Mechanism of Sex Determination and Differentiation

New technologies, including RNA interference, gene editing, and gene knockout, have been applied in sex control of aquatic

animals ([e.g., in *Macrobrachium rosenbergii* Hayakijkosol and Owens, 2012] and in *Cynoglossus semilaevis* [Songlin Chen, personal communication]). In large yellow croaker, the evidence obtained in our laboratory supported that the sex of large yellow croaker was determined by a single key gene, and *drmt1* was the strong candidate. However, the mechanisms of sex determination and sex differentiation need further studies, to support the development of new methods such as gene editing for sex control in large yellow croaker.

39.7.4 Relations Between Growth and Gonad Development

The weight of two year old treated individuals with methyl testosterone as well as high water temperature was only 100–150 g, much lower than that of the controls (500 g or so), suggesting that the treatment for inducing genotypical females to reverse to phenotypical males would inhibit the growth of the

treated fish. The small size of the pseudo-males produced only a little semen, which would affect the scale of production of all-female fry. Therefore, the relationship between growth and gonadal development in large yellow croaker needs to be studied, to answer why the treatments to induce androgenic inhibit the growth.

Acknowledgements

The studies in this chapter were supported by the National Natural Science Foundation of China (U1205122 & 31272653), the Science and Technology Key Project of Fujian Province (2001Z009), the Special Fund for Agro-Scientific Research in the Public Interest (200903046-5), and partially supported by the National High Technology Research and Development Program of China (863 Program) (2006AA10A405 & 2012AA10A403).

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40

Sex Determination and Control in Eels

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40.1 Introduction

The rice field eel (*Monopterus albus*) or the Asian swamp eel, belonging to the Synbranchidae family of the order Synbranchiformes [1, 2], is a freshwater fish with an eel-like body shape. It is native to East and Southeast Asia, and is especially widespread in China. It is also found in Northern Australia and the Southeastern United States, and is identified as an invasive species in the North American Everglades [3]. This fish is emerging as a specific model organism for vertebrate sexual development studies, because of its small genome size and natural sex reversal characteristic [4].

Unlike the rice field eel, the other eels belong to the Anguillidae family, of the order Anguilliformes, which contains four suborders and only one genus named *Anguilla*, with 19 species [5, 6]. The eels are elongated fishes, having a snake-like body, without scales. The dorsal and anal fins are fused with the caudal fin [7]. As catadromous fishes, they spawn in the sea, but spend the rest of their life in fresh water.

Anguillid eels are widely distributed in the world's oceans, with three main temperate species in the Northern Hemisphere: the European eel (*Anguilla anguilla*), the Japanese eel (*Anguilla japonica*) and the American eel (*Anguilla rostrata*) [8].

All three of these eel species are studied and extensively cultured. The Atlantic Ocean-originated European and American eels spawn in the Sargasso Sea within a limited area, but their leptocephali drift back to different continental areas of Europe and North America for recruitment, and become widely distributed in freshwater habitats [9]. The spawning area of the Japanese eel is west of the Mariana Islands in the Pacific Ocean, and leptocephali are transported via the North Equatorial Current and the Kuroshio Current to their growth habitats in China, Japan, and Korea [10].

In this chapter, research progress on sex differentiation and sex control of the rice field eel and the other three eel species is reviewed. This review can lay the foundation for a deeper understanding of molecular mechanisms controlling the gonad development in the rice field eel, and can help sex control practices in eel culture.

40.2 Biology of the Rice Field Eel

40.2.1 Basic Biology

The rice field eel is one of the popular freshwater fishes in China. This scaleless, snake-like slender-bodied fish typically is

sold at sizes of 25–60 cm (the longest is over 80 cm) in marketplaces. They have a blunt snout and tapering tail. They lack pectoral and pelvic fins, and their dorsal, anal, and caudal fins are rudimentary. Their mouths are large and protractile, with tiny teeth on upper and lower jaws. Their body and head have a yellowish-brown color, covered with irregular dark spots. Dorsal and ventral sides of their body are gray-colored. The rice field eel lives in freshwater fields, ditches, shallow pools, and stagnant waters that store rich organic swamp soil. The fish breathes air, partially by skin respiration. The rice field eel eats other small fishes, worms, crustaceans, and aquatic animals at night [11, 12].

The rice field eel is an egg-laying freshwater fish. Their prolonged spawning season may extend from May to September, but it is shorter (mainly from June to August) in China. Spawning occurs near the caves where they live and build a foam nest. Eel parents defend their nests, and provide protection for their offspring until yolk sac absorption [11, 13]. However, parents might eat their eggs at hatching if they sense danger or are frightened [13]. Before two years of age (total length <40 cm), all the rice field eels are always female. After the second year, females gradually transform to males via an intersex period lasting about two years (between total lengths of 40–60 cm). After this prolonged period of intersexuality, they become male, which are always the largest (total length >60 cm) [12].

40.2.2 Genome and Karyotype

Compared to other model fish, the genome size of the rice field eel (600 Mb) is smaller than that of zebrafish (1700 Mb). Because of its small genome size, the rice field eel is a specific model species for studies of fish, and even vertebrate sex differentiation and sexual development.

The karyotype of the rice field eel has several characteristics that differ from those of other fishes. First, they have a low number of chromosomes ($2n = 24$), with a limited number of arms (N.F. = 24). Their chromosomes

are small, with average relative length of an individual chromosome in the chromosome complements ranging between $11.21 \pm 0.46\%$ to $5.41 \pm 0.46\%$ [12, 14]. Additionally, all their chromosomes are metacentric, and they have no heteromorphic sex chromosomes.

40.2.3 Life Cycle

The rice field eel is a protogynous hermaphrodite fish, with a natural female-to-male sex reversal phenomenon in its lifecycle [15, 16]. Anatomical evaluations of gonadal development show that ovaries transform to testis, with an intersexual phase in between, as a natural process in their life cycle. The ovarian epithelium develops rapidly and is replaced by testicular tissue during this transformation. When the gonad completes its transformation to testis, they finally become real males [16, 17]. Histological sections of gonadal tissue from individual fish at different developmental stages could provide further direct and important evidences for natural sex reversal. This natural sex reversal phenomenon within a life cycle is a hot research spot in the field of sex differentiation in fish.

40.2.4 Histology of Gonadal Development

Usually, all the rice field eel individuals are females, from the embryonic stage to first sexual maturity. After the first sexual maturity, the ovarian follicle and tissue degenerate gradually, while the spermatogonia on the germinal fold begin to multiply and form spermatocysts. During this intersex phase in adulthood, the degenerating female and developing male germ cells settle in the same gonad. Furthermore, this female-to-male sex reversal is unipotent [17, 18]. In a recent study, He (2014) evaluated the serial sections of gonads of rice field eels from 0–60 days old. He observed the formation of undifferentiated gonads from gonadal primordium and cystovarian differentiation of ovaries in juvenile fish. His study also showed that the rice

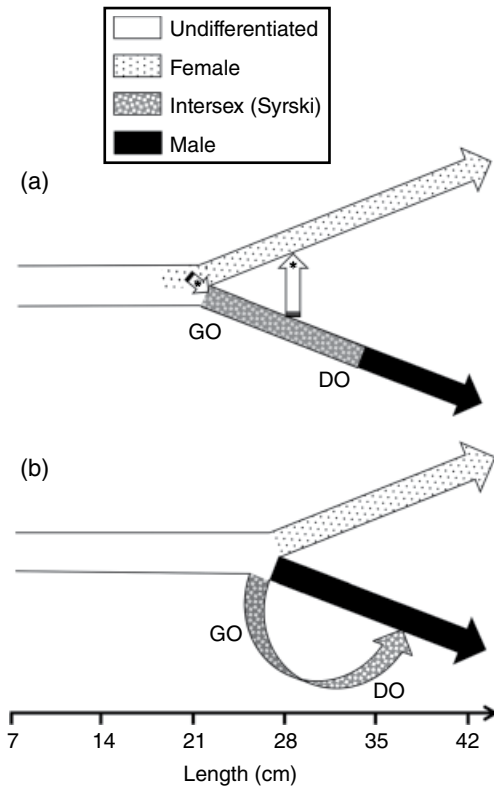


Figure 40.1 Schematic representation of gonad development in eels (according to Geffroy, 2016).

(a) All males differentiate through a Syrski organ (modified from Colombo and Grandi, 1996), “quite unlikely in experimental conditions.”

(b) Males could differentiate directly or through a Syrski organ (according to Geffroy *et al.*, 2013).

Do – degenerating oocytes; Go – growing oocytes.

field eel possess a single elongated gonad in their abdominal cavity [19].

In two Anguillid eels, the research of Satoh (1962), Colombo (1996), and Grandi (1997) described the Japanese eel and the European eel gonadal development, respectively. Satoh’s results showed that the primordial germ cells steadily increase at 18 cm in total length of the body in Japanese eels, and the sex may differentiate at this stage, while ovaries and testes can hardly be distinguished until the Japanese eel grows to about 30 cm in total length [20]. The results of Colombo (1996) and Grandi (1997) reported the development and sex differentiation of the gonad in the

European eel, including the appearance of primordial germ cells, the formation of gonadal primordia, the presence of oogonial clones and spermatogonium B clones, and differentiation of ovary or testis after undergoing a juvenile ambisexual stage, respectively [21, 22]. Furthermore, Colombo’s (1996) data also suggested that the undifferentiated gonad might develop directly into an ovary or a Syrski organ, which would then develop into an ovary or a testis. Geffroy (2013, 2016) proposed that testes of the European eel would develop either directly from an undifferentiated gonad tissue, or from an intersexual organ containing oocytes, so that later degenerate ovaries would develop directly from an undifferentiated gonad [23, 24] (Figure 40.1).

40.3 Sex Determination and Differentiation in the Rice Field Eel

The phenomenon of natural sex change in the rice field eel was first observed by Liu (1944). Afterward, lots of histological, hormone regulation and molecular studies have been conducted to investigate the sex determination and differentiation mechanisms in the rice field eel. Histological analyses provided evidence that hormones can partially induce sex reversal in the rice field eel [25]. Although genetic sex determination has been proposed as the key mechanism in this species [12], the molecular mechanisms of the sex change are poorly understood in the rice field eel.

40.3.1 Roles of Certain Key Genes in Sex Determination and Differentiation

Up to now, several genes have been implicated in the processes of gonadal sex determination and differentiation in vertebrates [26, 27]. The most extensively studied genes are: *SRY* (Sex-determining region of the Y chromosome); *dmy* (DM-domain on the Y chromosome) [28]; *gsdf* (gonadal

somatic cell derived factor); *SOX9* (SRY-related HMG box-9); *DMRT1* (Double sex and mab-3 related gene-1); *AMH* (Anti-Müllerian hormone); *DAX1* (X-linked Dosage-sensitive sex reversal, congenital adrenal hypoplasia); *FOXL2* (Forkhead transcription factor-2) [29, 30], and so on.

In recent years, much effort has been spent by different laboratories to search for key gene(s) involved in sex determination and differentiation of the rice field eel. Female sexual differentiation gene *foxl2* [31], male sexual differentiation genes: *sox* family [32–35], *dmrt* [36–38], and other important genes [39, 40] have been suggested to play important roles in the rice field eel sex differentiation.

40.3.1.1 *Foxl2*

FOXL2/foxl2 is a member of the fork-head family of transcription factors [41]. It participates in many biological processes, including cellular proliferation, tissue development, and development of muscles in the eyelids of vertebrates [42, 43]. In mammals, *FOXL2* is also female-specific, and is detected during early phases of gonadal development [44]. Earlier studies with fish showed that *foxl2* is involved in early development of female gonadal tissue, maintenance of adult ovarian function and regulation of *cyp19a1* gene, and so on [45–48].

In the rice field eel, the complete cDNA of the *foxl2* gene has been isolated from ovaries [31]. RT-PCR results demonstrate that this gene is expressed in the brain and eye, but mainly in gonads. The expression level of *Foxl2* is high in the ovary before sex change, and there is no obvious expression difference between ovotestis and testis. Immuno-histochemical staining also shows that *foxl2* is strongly expressed in the granulosa cell layer of the ovarian follicles surrounding immature oocytes, and in the interstitial cells of ovotestis and testis, but not the mature oocytes. The results of this study allow us to conclude that *foxl2* expression level in the gonadal tissue of the rice field eel is in line with the process of sex development and

maintenance of ovarian function. The experimental results additionally indicate that *foxl2* may play an essential role during the natural sex reversal phenomenon in the rice field eel [31].

40.3.1.2 *Sox* family

SOX genes belong to the HMG-box domain family, which is homologous to *SRY* (key sex-determining gene) in mammals [49, 50]. *SOX/sox* genes encode some transcription factors functioning in the testicular development. A few of the *SOX/sox* genes are involved in sex determination and differentiation.

Five types of *sox* genes have been reported in the rice field eel, including *sox1*, *sox4*, *sox14*, *sox9*, and *sox17*. The first three genes do not have the HMG-box, and are not evolutionarily conserved [32]. The latter two genes, *SOX9* and *SOX17/sox9* and *sox17*, have the HMG-box and are very important in mammalian and medaka (*Oryzias latipes*) sex differentiation [51, 52].

SOX9/sox9 is one of the main genes related with sexual differentiation in vertebrates [53]. This gene is also critical for testis determination and chondrogenesis in vertebrates [54, 55]. In fish, the *sox9* gene has two duplicated orthologs during the evolution of some fish lineages, such as *sox9a* and *sox9b* in zebrafish. *sox9a* has been detected in testis and other organs (brain, kidney, muscle), and *sox9b* only in ovary [56]. On the other hand, two forms of *sox9a* genes – *sox9a1* and *sox9a2* – have been identified in rainbow trout (*Oncorhynchus mykiss*). *Sox9a1* shows a sexually dimorphic pattern of expression, and higher expression levels are found in males before sexual differentiation [57].

The *sox9* gene was cloned in the genome of the rice field eel in 2002 by Zhou. In further research, the duplicated copies of this gene, named *sox9a1* and *sox9a2*, were cloned in the gonadal tissues of the rice field eel. The expression patterns of two *sox9a* genes in the rice field eel are similar to each other, but different from the *sox9a* gene of zebrafish. By RT-PCR analysis, the expression of *sox9a1* and *sox9a2* has been detected

in testis, ovary, and ovotestis of the rice field eel. By *in situ* hybridization, both *sox9a1* and *sox9a2* are expressed in the outer layer (mainly gonocytes) of gonadal epithelium of male, intersex, and female fish. These results indicate that *sox9a* genes may play an important role in gonadal differentiation from female to ovotestis and testis during natural sex reversal. The presence of double copies of *sox9a* genes in the rice field eel suggest that the two copies serve as threshold for the transformation of gonadal tissue from female to male [35, 54].

The *sox17* gene also belongs to the large family of HMG-box domain genes [32]. The rice field eel *sox17* gene is located on chromosome 5 [58], and includes two exons that encode a 399 amino acids-long protein with a conserved HMG box. The expression of *sox17* is detected in testis, ovary, and ovotestis. The results of *in situ* hybridization studies have suggested that *sox17* gene is expressed in the germinal lamellae of ovary, ovotestis, and testis, as well as the developing germ cells of immature gonads. It seems that the expression level of *sox17* accompanies gonadal development. This pattern of expression indicates that *sox17* has significant function during gonadal differentiation [59].

Overall, the *sox9* and *sox17* gene families are expressed in both males and females, or intersex fish, with no apparent sex specificity. Their expression levels keep along with the gonadal transformation from ovary to testis. These genes may be associated with sex reversal and gonadal differentiation in the rice field eel.

40.3.1.3 *Dmrt* Family

The *DMRT/dmrt* gene family has a cluster of Doublesex and Mab-3 (DM) domains, sharing a common DM domain that codes for a conserved transcription factor in vertebrates and invertebrates [60]. For instance, *Dmrt1* is involved in human sex reversal [61]. Deletion of *DMRT1* in mice leads to abnormal testicular differentiation after sex determination [62]. In fish, the *dmrt1* gene is expressed in the gonads of several species and is related to

sex determination and differentiation [63]. Moreover, it has been proved that a duplicated copy of *dmrt1* on the Y chromosome of medaka, called *dmy/dmrt1Y*, is necessary for the male sex determination in this fish [26, 64]. In addition to the above function, the *DMRT/dmrt* gene family also has a role in non-gonadal tissues [65], like *dmrt3*, which is involved in olfactory placode development [64]. This gene family is highly conserved and plays an important role in the organ development process.

In the rice field eel, the homology of the *dmrt* gene was reported in 2002 [36]. Four alternative spliced types of *dmrt1* gene cDNA were then observed in the rice field eel gonad (*dmrt1a*, *dmrt1b*, *dmrt1c*, and *dmrt1d*). RT-PCR analysis showed that the expressions of four types of *dmrt1* were detected in ovary, ovotestis, and testis. The expression level becomes upregulated with the gonadal transformation from ovary to testis. The expression pattern of *dmrt1a* shows the same trend with *dmrt1b*, but its expression level is higher. However, the expression of *dmrt1d* does not change during natural sex reversal. The results of *in situ* hybridization suggested that the location of *dmrt1* expression is the outer layer of gonadal epithelium, containing primarily somatic cells and undifferentiated germ cells [37].

To further understand the function of the DM domain genes in the rice field eel, and their expression during sex reversal, cDNA of the other five *dmrt* genes (*dmrt2*, *dmrt2b*, *dmrt3*, *dmrt4*, and *dmrt5*) have been cloned and characterized in this species. The sequence analysis showed that the *dmrt* gene of the rice field eel has a high level of sequence homology with the other vertebrates, especially in the conserved DM domains. The results of RT-PCR showed that the *dmrt* genes are expressed in different periods of the gonadal development. The expression levels of *dmrt* genes are upregulated during the transformation of gonad from ovary to testis. In addition, the expression levels of *dmrt2* and *dmrt2b* show

a similar pattern. Both *dmrt3* and *dmrt5* are expressed at the same level in all gonads.

In situ hybridization demonstrates that all five *dmrt* genes are expressed in the ovary, ovotestis, and testis. In ovary, *dmrt* genes are mostly expressed in developing and mature follicles. In ovotestis, expressions of *dmrt* genes have been observed in both degraded follicles and in the outer layer of developing testicular epithelium. In testis, *dmrt* genes are expressed in developing sperm cells [38]. Upregulation during gonadal development and differentiation shows that *dmrt* genes play a role in sexual development of the rice field eel [67].

40.3.1.4 *Gsdf*

Gsdf was first identified from rainbow trout and belongs to the transforming growth factor- β (TGF- β) superfamily [68]. It has been identified as the candidate gene for sex determination in medaka, and it stimulates testis differentiation [68, 69]. In the rice field eel, the *gsdf* gene has been cloned and identified.

Expression of this gene is only located in gonads. Although its expression is strong in testicular somatic cells of testis and ovotestis, it weakens in granulosa cells surrounding oogonia and primary oocytes [39]. These variations in expression levels reveal that *gsdf* functions in testicular differentiation and sex transformation from female to male in the rice field eel.

40.3.1.5 Other genes

Several other genes are suggested to be important in the rice field eel sex determination and differentiation, such as aromatase (*cyp19a*) [70, 71], *amh* [72], and other candidate genes selected by our laboratory.

Aromatase (P450arom) is a key steroidogenic enzyme encoded by the *cyp19a* gene. This gene is required for estradiol-17 β (E_2) synthesis. Aromatase regulates the amount of estrogens (mainly E_2) and keeps its levels within adequate ranges for ovarian differentiation [73]. In fish, two genes code for aromatase enzymes: *cyp19a1* and *cyp19a2* [74]. Gene *cyp19a1* expression is restricted to the gonads, and related to sexual differentiation of gonads, while *cyp19a2* is mainly expressed

in the brain, and is indirectly involved in sexual differentiation [75].

The expression of gene *cyp19a1* is regulated by several factors, such as *foxl2* and so on [19, 48, 76]. In the rice field eel, the analysis of gonadal expression shows that aromatase is predominantly expressed in ovaries, and its expression decreases greatly in ovotestis and is barely detected in testis [71]. These results show that the *foxl2 cyp19a1* genes may be linked in a conserved gene cluster, and may play a necessary role in female-to-male sex reversal in the rice field eel [31, 71].

AMH, known as Müllerian Inhibiting Substance, is one of the glycoprotein members of TGF- β superfamily [77, 78]. In mammals, *AMH* is expressed strongly in sertoli cells and causes the regression of Müllerian, leading to suppression of female organogenesis during testicular differentiation [79, 80]. In the rice field eel, cDNA of the *amh* gene was cloned by Hu (2015). *In situ* hybridization has shown that *amh* is located in follicular and, mostly, in granulosa cells of the ovary. In ovotestis, this gene is detected in degenerated follicle cells of ovary and early sertoli cells of testis. In testis, a high level of *amh* expression can be detected in sertoli cells [72]. Although expression of this gene has been detected in ovary, ovotestis, and testis, no obvious signal has been obtained from the other tissues, suggesting that *amh* is a gonad-related gene, mainly expressed in gonads. Hence, the high expression level of *amh* is necessary for the regular maintenance of testis function and differentiation [72, 81].

So far, many homologous candidate genes involved in sex determination and differentiation in the other vertebrates have been found in the rice field eel. However, the genetic mechanisms behind the natural sex reversal phenomenon are still poorly understood. In our laboratory, suppression subtractive hybridization (SSH) libraries were constructed by using mRNA from the stage IV ovaries and ovotestis. High-quality SSH cDNA libraries and 90 ESTs were obtained. Four candidate genes: *F11*, *F63*, *R11*, and *R47*, which were not gonadal tissue specific, were identified. The expression analysis results

showed the transcription level of *F11* and *F63* genes was significantly increased, while that of *R11* and *R47* genes was significantly decreased in ovaries from stage IV or V [82].

In a follow-up study, 14 differentially expressed genes were characterized within the transcripts isolated from stage IV ovaries and ovotestis, by the annealing control primer-based differential display reverse transcription PCR method. One of these 14 genes, *G2*, showed a higher transcription level in the ovotestis than ovaries, and its expression increased significantly in parallel to gonadal development [83]. These differentially expressed genes may play an important role in sex reversal and testis development in the rice field eel. Nevertheless, further studies are still required in the future to understand the function of these genes.

40.3.2 Summary and Perspectives

Since first reported by Liu in 1944, the natural sex reversal of the rice field eel has been a research hot spot in sex differentiation studies. Some important sexual development related genes reported in fish are verified, and some still remain to be verified in the rice field eel. Together with this, the key molecular mechanisms controlling the sex determination and differentiation in the rice field eel have not been ascertained yet.

A brief review of some key genes involved in gonadal development of the rice field eel shows this species may not have a sex determination gene, but only have sex differentiation genes to regulate the female differentiation and male sex reversal. Based on the previous studies of important candidate genes, which are involved in sexual differentiation of the rice field eel, we could draw a conclusion that the pathway of sex differentiation is regulated by a complex genetic network. Even though the complete function of the key sex reversal gene has not been identified, the combination of research results from all vertebrates suggest that the downregulation of *foxl2* and upregulation of *dmrt1* genes function

together to initiate the ovarian tissue degradation and testicular tissue development during the natural sex reversal process in the rice field eel.

It could be speculated that the *foxl2-dmrt1* genes' interaction is linked into a gene cluster in the gene expression pathway during gonadal development and sex differentiation. As a model species of natural sex reversal, gene expression patterns related to sex differentiation in the rice field eel will help us to further understand the genetic regulatory mechanism of sex differentiation in vertebrates. Together with this, other methods, such as gene knockout, could also provide important opportunities in elucidating the genetic regulatory mechanism of sex differentiation in the rice field eel.

Key information on sex differentiation of rice field eel is summarized in Box 40.1.

40.4 Sex Control

In general, sex control means altering the direction of gonadal differentiation to the desired sex. This is an important biotechnology used in the culture of some commercially important fish species, or in research. With this technology, normal sexual development processes of animals are interrupted via human intervention. There are some basic methods used for sex control in fish, such as:

Box 40.1 Key information on sex differentiation of rice field eel

The pathway of sex differentiation is regulated by a complex genetic network.

The downregulation of *foxl2* and upregulation of *dmrt1* genes function together to initiate the ovarian tissue degradation and testicular tissue development during the natural sex reversal process in the rice field eel.

The interaction between the *foxl2-dmrt1* genes seems the most probable drive for the rice field eel sex change.

hormonal sex reversal; interspecific hybridization; and manipulation of environmental temperatures, photoperiod, rearing density, and so on.

Hormonal sex reversal method can be applied directly or indirectly [84]. The direct method involves the administration of androgen or estrogen hormones during the labile period of gonadal differentiation. Production of populations containing only the desired sex could easily be achieved in one generation, although the sex steroid-treated fish might cause public disapproval. The indirect method combines hormonal sex reversal and back-crossing(s) of treated fish with untreated fish. Back-crossings might require certain and sometimes complicated breeding steps and, of course, knowledge of the genetic sex determination mechanism [84]. Generally, estrogen treatments cause feminization in genetic males, and androgen treatments cause masculinization in genetic females [84, 85].

On the other hand, the research concerning the role of environmental factors in fish sex differentiation is scarce, and deserves further study. Research in this area could eventually lead to sex control in fish without the use of sex steroids. Since this method is also complicated, more research studies are needed to test the main factors influencing the direction of gonadal differentiation in different species.

With the rapid development of cell biology, molecular biology, and immunology, the future method of sex control will be introduced at the molecular level. For example, control will be performed at the genetic level by using gene targeting technology to knock out or insert the sex-determining gene, and to regulate gene replication, transcription, and translation. Right now, these methods are not well developed, and can only be tested at the individual operation level. Direct and indirect hormonal sex reversal methods have been used to control sex in many fish species, including medaka [86, 87], rainbow trout [88], and goldfish (*Carrassius auratus*) [89].

40.4.1 Sex Control in the Rice Field Eel

Aquaculture of the rice field eel in China is still at the elementary stage. Farming activities rely on capturing a high number of wild juveniles, which is not an easy task to achieve, due to their protogynous hermaphroditic nature and the low fecundity of small-sized females [90]. Additionally, no effective technique for large-scale artificial breeding has yet been developed.

Under this pressure, researchers try to use exogenous hormones to induce male-to-female sex change in the rice field eel. So far, however, none of these attempts have yielded complete success [17, 91–93]. In the post-spawning stage, ovine-luteinizing hormone can facilitate sex reversal to male in the rice field eel [94]. 17α -Methyltestosterone (MT) treatments of post-embryonic rice field eels can induce sex reversal to the male direction and accelerate the timing of male maturation [95]. Diethylstilbestrol (DES) treatments can delay the timing of female-to-male transformation after the first spawning, and increase body weights and lengths [90].

Until now, no exogenous hormone treatments have induced complete sex reversal in this species. Hence, further research on its sex differentiation at the labile period are needed [84]. Research concerning the effects of environmental factors such as temperature, rearing density, and photoperiod on their sexuality is rarely reported. Yuan's (2011) results showed that ovotestis and male ratio increase with culture density increase in rice field eel [93].

40.4.2 Sex Control in the Other Eels

Catadromous anguillid eels enter fresh water as sexually undifferentiated glass eels, and then develop into males and females. Females develop ovaries directly from the ambiguous primordial gonads, whereas males pass through a transitional intersexual stage before developing testes [21]. Sexual development in the *Anguilla* species is not univocally determined by a gene or genes, but is

presumably influenced by environmental and social factors [24, 96–98]. However, heteromorphic sex chromosomes have been found in the European, Japanese, and American eels [24, 99–101]. These anguillid eels show clear sexual dimorphism [98, 102], with females growing faster and attaining greater body sizes than males [103, 104], despite the fact that males may grow faster than females prior to sexual differentiation [103, 104]. The following sections will mainly deal with sex control in the Japanese, European, and American eels, through exogenous hormones and environmental manipulations.

40.4.2.1 Sex control in the Japanese Eel

The Japanese eel is widely cultured in China, Japan, and the other countries in Asia. Until now, the sex determination mechanism in the Japanese eel has not been clearly illuminated. Japanese researchers have shown that cultured adult females are mostly three times as large as males, but almost all cultured eels are males, and the ratio of female eels in culture is only 3.5% [103, 105, 106]. This situation demands the development of sex control techniques in their culture, to obtain all or almost all female populations [103, 106].

In the earliest study, Satoh (1992) reported that oral sodium diethylstilbestrol (DES-Na) treatments at doses of 0.5, 0.75, and 1.0 ppm affected sex differentiation and sex ratio when administered to the Japanese eels of the same age. DES-Na treatments decreased the male ratios and increased the female ratios in a dose-dependent manner. The male ratio was 64.1% in the control, and decreased to 32.0–40.4% in the DES-Na treated groups. Correspondingly, female ratios increased with the increasing dosage of DES-Na treatment. The total lengths and body weights measured at the end of the experiments showed that females were the longest and heaviest of all fish. This experiment provided the possibility of sex control in the Japanese eel by oral administration of DES-Na.

In another study conducted by Chiba (1993), juveniles of the Japanese eel were fed diets supplemented with E_2 at doses of 25, 50,

and 75 mg/kg feed. The growth rates, sex ratios, and body lengths were measured at the end of the experiments. The results showed all or almost all (95–100%) of the E_2 -treated groups consisted of females. The growth rate of fish treated with 25 and 50 mg E_2 /kg diet, starting from the early juvenile stage, was significantly higher, and the body length increased from 5–9 cm to 18–22 cm at the end. The growth rate of fish treated with 75 mg E_2 /kg diet starting from the early juvenile stage was higher than the control, but had no significant change. A lower dose (25 and 50 mg) of E_2 positively affected the growth and induced feminization in the Japanese eel [103].

Based on the above two experiments, a conclusion could be drawn that E_2 has higher feminization potency on the Japanese eel than DES-Na. The naturally higher growth potential of female eels encourages us to extend the studies on sex control in the Japanese eel culture, such as effective dosage, initial timing, and duration of the estrogen treatments. According to the above studies and the timing of gonadal sex differentiation in the Japanese eel, effective E_2 treatments could be started at the glass eel (post-larvae or early juvenile) stage, while lower doses (10–20 mg/kg diet) could be used for shorter durations of 30–60 days. Optimization of the effective dosage and duration of E_2 treatments requires further research. There have not, however, been enough investigations concerning the effects of androgen treatments, or environmental factors such as temperature, population density, and other factors, on sex ratios and gonadal differentiation of the Japanese eel.

40.4.2.2 Sex control in the European Eel

The European eel is reported as an undifferentiated gonochoristic species [107, 108]. The mechanism of its sex determination is also not clear [21, 24]. The present data show that population density and environmental temperature have important effects on their sex ratios, and that both high densities and temperatures promote male-biased sex

ratios [96, 109, 110]. However, the experiments conducted in Sweden produced a controversial outcome of significant increases in the number of females with increasing temperatures [111, 112]. Hence, the effect of temperature on gonadal differentiation has still not been clarified in this species [111].

Wiberg (1983) [109] hypothesized that sex determination may be metagametic and sex inversion may occur in the European eel. Under high density rearing conditions, a very large percentage of the European eel develops as male in aquaculture [108, 113]. Researchers also investigated the effects of sex steroids on gonadal differentiation of this species [24]. Experimental administration of E_2 to the limited number of elvers and juveniles via injections resulted in feminization, but the same treatment regimen with testosterone (T) showed no masculinization effect [114, 115].

David and Degani (1992) [104] examined the effects of dietary E_2 (0, 30, and 60 mg/kg) treatments on sex ratios of the European eel. They observed no differences between the gonadal developments of treated and control groups in the first year but, in the second year, they identified 70%, 32%, and 26% of eels as female in 60 and 30 mg/kg E_2 fed groups and the control group, respectively.

In another experiment, conducted by Colombo and Grandi (1995) [108], two kinds of hormones were used – MT, and 17 α -ethynylestradiol (EE_2). The hormones were added

into the diet at 0.1 and 1 mg MT/kg diet, or 1 and 10 mg EE_2 /kg diet doses, and they were administered starting from different developmental stages (6–8 cm elvers, 15–18 cm eels, and 22–25 cm eels). No masculinizing effect of MT could be demonstrated, but MT treatment at 1 mg/kg dose accelerated testis differentiation when it was initiated with 22–25 cm long eels. The EE_2 treatment at 10 mg/kg dose induced about 90 and 66% feminization when it was initiated with elvers and 15–18 cm long eels, respectively. The proportion of females in the control group was only 2%, and the EE_2 treatments had no significant feminization effect when they were initiated with 22–25 cm long eels. Additionally, 10 mg/kg EE_2 treatments improved the growth rate of elvers and 15–18 cm eels, but suppressed the growth rate of 22–25 cm eels (Table 40.1).

On the basis of their results, researchers suggested that the best developmental stage to induce feminization in the European eel is around 12–13 cm. They also proposed that a high dose of EE_2 (10 mg/kg diet) and a longer treatment duration (83 days or longer) are necessary for a high level of feminization.

In a more recent study, Tzchori (2004) [116] fed the European eel juveniles with undifferentiated gonads E_2 or phytoestrogens containing diets for 100–150 days. The results showed that feeding E_2 increased the body weight, compared with controls. Both E_2 and phytoestrogens significantly increased

Table 40.1 Body length in eel samples (from Colombo, 1995).
The experimental groups at successive days from the beginning of the treatments in experiments on 6–8, 15–18 and 22–25 cm eels.

Experimental groups (cm)	Length (cm)	Control	EE_2 (10 mg kg ⁻¹)
6–8	X \pm S.D.	30.81 \pm 8.80	39.69 \pm 7.88
	Range	15.2–49.3	14.3–54.0
15–18	X \pm S.D.	26.17 \pm 6.14	26.09 \pm 6.38
	Range	16.2–38.0	16.4–48.0
22–25	X \pm S.D.	35.79 \pm 2.18	29.03–2.12
	Range	31.6–40.0	35.0–33.6

the percentage of females in the experimental groups. The highest feminization rate of 70% was obtained by feeding undifferentiated juveniles with 20 mg E_2 /kg diet for 100 days. Finally, Fazio (2008) [117] induced significantly male-biased sex ratios in this species by means of 11-ketotestosterone injections.

From the above research, it could be speculated that sex steroid treatments on the European eel juveniles can induce feminization or masculinization. Studies conducted with this species so far suggest that sex steroids are effective at the earliest stage of gonadal differentiation, and the direction of gonadal differentiation determines the growth potential in this species because of sexually dimorphic growth [108, 118, 119]. Moreover, environmental factors (temperature and population density) could influence the direction of gonadal differentiation in the European eel. As these factors are interrelated, it is, however, difficult to verify the exact role of a single factor on sex differentiation without further studies.

40.4.2.3 Sex control in the American eel

The mechanism of sex determination in the American eel is also not clear [120]. Numerous environmental factors, such as temperature, density, salinity, latitude, and habitat, have been reported to have an effect on the direction of gonadal differentiation in the American eel [98, 121–125]. However, among all these environmental factors, temperature was the only one with a consistent effect on gender of this species, so the other factors were disputed [98, 125].

Oliveira [124] reported that eels migrating from lacustrine habitats within a river were predominately female, while eels migrating from fluvial habitats were predominately male in the American eel. Vladikov [126] reported that males predominate the natural populations at the yellow stage in a New Brunswick lake. Krueger and Oliveira [127] reported that both sexes of the American eel are more widely distributed, and that males predominate in the northern subpopulation.

Krueger and Oliveira [97] concluded that high population densities cause the higher male ratios, whereas low population densities resulted in the predominance of females.

Meanwhile, the data collected for two decades from the Annaquatucket River, Rhode Island, United States, showed that the number of males were three times higher than females among the migrating silver eels. The researchers argued that population density may be the main factor influencing the sex ratios, and not the genotypic sex determination mechanism, but that environmental sex determination mechanism plays the main role in the gonadal differentiation of this species [97].

The skewed sex ratios of wild populations observed in the above studies might imply environmental control of sexual differentiation in the American eel. Nevertheless, further investigations are necessary to clarify the exact influence of these environmental factors. Additionally, there is not sufficient research on the effects of exogenous hormone treatments in this species so far. Hence, research studies on effective hormone treatment strategy in the American eel at post-larvae or juvenile stage are needed.

40.4.3 Summary and Perspectives

The sex determination mechanisms of the rice field eel and the three anguillid eels are not clear. There are no heteromorphic sex chromosomes in the rice field eel, but heteromorphic ZW chromosomes are present in the anguillid eels. Wiberg concluded that this heteromorphism was not sex-specific, and that sex determination is metagamic in anguillid eels [109].

Together with this, environmental factors, especially population density and temperature, have been proposed to influence the direction of gonadal differentiation in the anguillid eels. However, the exact mechanisms of how these factors take over the control of sexual differentiation are not clear, and the extent of their impact requires further studies. None of the limited numbers

Box 40.2 Key information

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| <ol style="list-style-type: none"> 1) Further research on sex reversal of the rice field eel during the labile period is needed. 2) The optimal dosage (10 or 20 mg/kg diet E₂) and treatment duration (30 or 60 days) requires further research in the Japanese eel and the European eel at the glass eel stage. | <ol style="list-style-type: none"> 3) In the three anguillid eels, sex differentiation is governed mostly by environmental factors. The density of individuals is the main factor affecting sex differentiation. 4) Environmental factors influence the direction of gonadal differentiation in the anguillid eels, and this requires further studies. |
|--|--|

of exogenous hormone treatments induced full sex-reversal in the rice field eel, but exogenous estrogen treatments were effective to induce feminization in the Japanese and European eels, and no hormonal sex-reversal study has been conducted on the

American eel so far. Further research is needed to develop effective hormone treatment strategies in the rice field eel and the American eel. The future perspectives and related key information are summarized in Box 40.2.

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41

Sex Control and Chromosome Manipulation in Cyprinidae: Common Carp and Grass Carp

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41.1 Introduction

Fishes in the family Cyprinidae are the most widely cultured food fish, and with the longest history as a farmed fish. Culture of common carp, *Cyprinus carpio*, began in Europe, and the Chinese carps were historically major culture fishes in Southeast Asia. Common carp is a traditional species for studies on sex control and chromosome manipulation methods. The grass carp, *Ctenopharyngodon idella*, has been distributed worldwide in both hemispheres, mainly as a biological control for nuisance aquatic vegetation. Environmental concerns over using exotic fishes have been addressed by management of reproduction through monosexing or triploidization; these can be applied individually, or as a part of an integrated breeding program. Sterile triploid grass carp are now commercially produced in the United States.

41.2 Management of Reproduction – Artificial Propagation and Sex Manipulation

Reproduction in fishes is the most diverse assemblage of modalities and strategies among vertebrates. The control of fish reproduction

through artificial propagation has provided tremendous opportunities in culture and management. Early culture of lotic-spawning cyprinids was achieved by capture of eggs and larvae. However, developments in artificial propagation have revolutionized the culture of both river spawners and lentic-spawning species, opening potential for more sophisticated reproductive manipulations of the phenotype and genotype.

Management of reproduction has been central to the development of contemporary aquaculture, as seedstock availability is commonly an impediment to commercial fish farming. The capability to spawn fishes under controlled conditions assures an adequate supply of young, and removes the constraints of limiting culture to the geographic proximity of their native range [1–3]. Artificial propagation has facilitated the capability to move fishes to new areas, maintain them, and establish culture for these species far outside their natural range [4]. Manipulation of the reproductive system also can provide the capacity to control unwanted spawning, and offer new tools in utilizing non-native organisms in an ecologically responsible manner. Thus, management of fish reproduction can be considered from these two perspectives: one involves the production of seedstock under controlled conditions; while the other limits unwanted reproduction [5]. Both are

valuable for aquaculture, either as a tool in culture, or as a “prophylactic” measure to avoid naturalization.

Selective breeding in fishes is facilitated by external fertilization, high fecundity, and potential for hybridization. Domestication of an organism involves its adaptation to, and modification for, the new environment. Common carp has been the most intensively domesticated warm water species. Artificial propagation can range from simple environmental manipulation to more sophisticated physiological control [1, 6].

Hormonal therapy was only started in the 1930s and, initially, it was restricted to the injection of homologous or heterologous pituitary glands. The simple dried glands and pituitary extracts are currently still in wide use. Hypophysation has been used to overcome impediments to reproduction under conditions where one or more critical natural spawning stimuli are absent. This central endocrine gland is the source of Gonadotropic Hormone (GtH) synthesis, and is the link between environmental factors and physiological control over gametogenesis, thus orchestrating natural reproduction so as to produce offspring when food and conditions are optimal for their survival.

Gonadotropin-Releasing Hormone (GnRH – aka LH-RH), is produced in the hypothalamus and regulates GtH production and release from the pituitary gland. Synthetic super-active analogues (GnRHa or LH-RHa) have now been developed, and are increasingly being used [2, 6, 7]. Today, salmon and carp GtH have been extracted, purified, and packaged for more convenient use, and are also available in calibrated and efficacy bioassayed form (CCPE – Calibrated Carp Pituitary Extract). The period between hormone injection and ovulation, the latency period, is affected by temperature as well as by the action of the inducing agent target (pituitary or gonad). The post-injection latency period for GnRH is longer than pituitary preparations that act directly on the gonad [8]. The capability to regulate and predict the availability of fresh gametes is critical to reproductive manipulation.

Artificial propagation techniques for cyprinids are well established and relatively routine. Currently, tutorial videos on artificial breeding of common carp can be found on the internet (<https://www.youtube.com/watch?v=4JqKNdRr8xc>). Breeding induction procedures and nursing for carps have been reviewed [7–9]. Post-ovulatory gamete treatment among the carp species is different, relative to natural spawning. Chinese carps are stimulated by flooding rivers, and their buoyant eggs can be conveniently incubated under hatchery conditions in flow-through or upwelling incubators, while common carp spawn in lentic conditions and their adhesive, demersal eggs must be treated before they can be incubated in flow-through zuger-type jars. Traditionally, clay or milk are used to coat the eggs, and some chemical treatments have also been developed [9, 10]. Incubation period to hatching is inversely proportional to temperature; the duration for Chinese carps is much shorter than the time to hatching for common carp. The shorter incubation period for lotic-spawning species, compared with lentic spawners, is presumably an adaptation to the more variable riverine aquatic environment.

Developments in the management of fish reproduction have had major impacts on the growth of aquaculture [2]. Various manipulation tools can be used in the contemporary management of fish reproduction, including sex reversal and chromosome manipulation. The efficiency of each of these management approaches is interdependent on the optimization of physiologically based induction parameters [11]. Sex-reversal protocol is different for each species, because of phylogenetic differences in phenotypic sex development (gonadal differentiation), and the efficacy of ploidy manipulation depends on effective induction characteristics in relation to the timing of species-specific meiosis or initial zygotic mitosis. Optimized induction variables for gynogenesis can be directly evaluated by simple enumeration of viable diploid progeny, particularly if a phenotypic marker is used. Further, optimized gynogenetic protocol can be

used to facilitate the design of treatment for polyploid induction: 1) type of shock (pressure, heat, cold), intensity, and 2) post-activation time of application and duration.

Direct monosex induction treatment can be developed rapidly, but must be applied to each individual [11]. The various induction options can be used independently or

combined in a programmatic chronology. For example, induction of gynogenesis for homogametic species can be used in combination with steroid-induced sex reversal as a means of developing neomale (genetic female) brood stock to be used in a monosex breeding program for species with homogametic females (Figure 41.1).

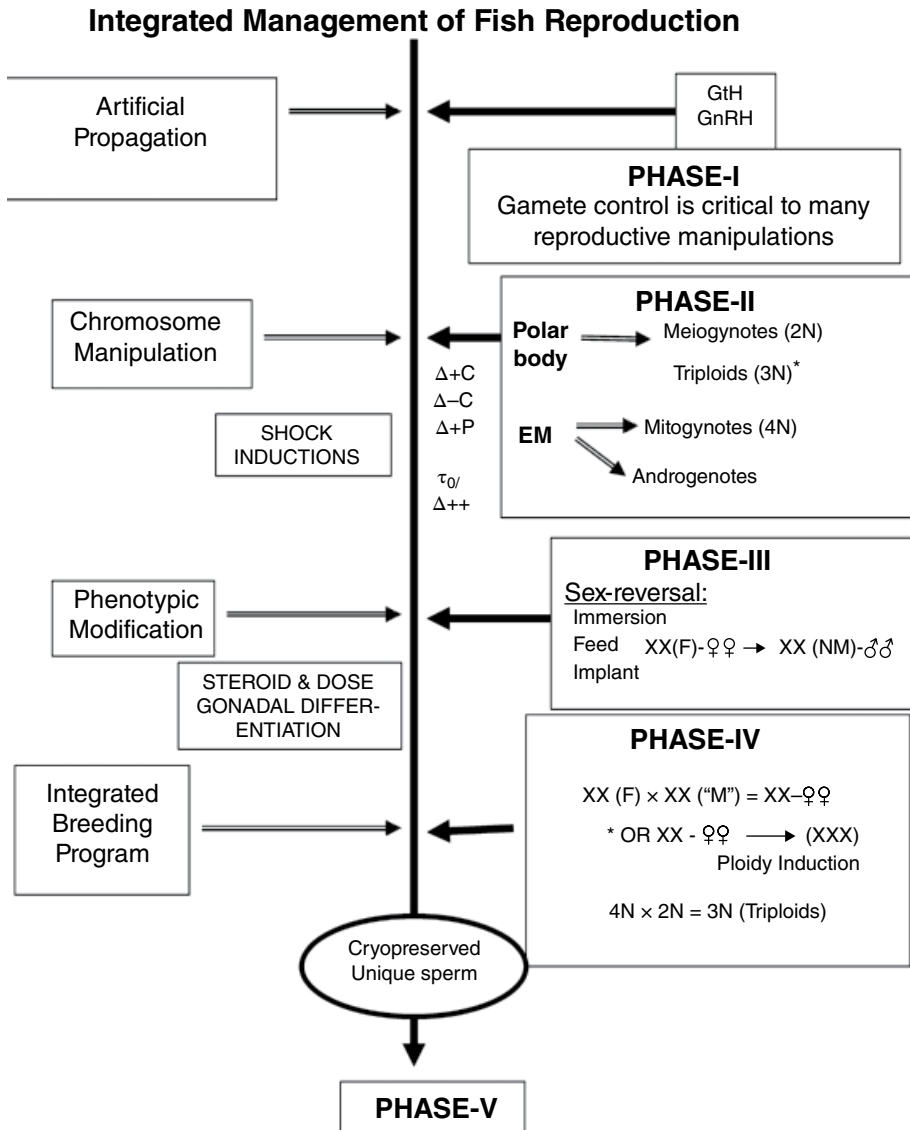


Figure 41.1 Program for reproductive management of fishes with homogametic female sex determination. Phase chronology can be altered or can be used independently.

Terminology and symbols: Shock induction – $\Delta+$ = heat shock or pressure shock; τ_0 – mitotic interval used to adjust shock time for different pre-shock incubation temperature; meiogynote and mitogynote – diploid progeny from early or late shock, respectively.

41.3 Common Carp

41.3.1 Genetic Sex Determination

While our understanding of sex determination is far from complete, we can consider certain fundamentals. Sex determination systems in fishes and how these are transcribed into functional phenotypic sex during gonadal differentiation have been well reviewed [12]. The genetic basis for phenotypic sex development is initiated at fertilization, usually translating gonadal sex with fidelity but, because of the sexual bipotentiality of premeiotic germ cells, they can be exogenously influenced [1, 13]. Sex determination in vertebrates is generally characterized by genetic models of either female homogamety (XX) and male heterogamety (XY), or male homogamety (ZZ) and female heterogamety (WZ). Both systems are present in fishes [14].

Common carp is characterized by male heterogamety (XY) and, correspondingly, female homogamety (XX). Initially, this type of sex determination in common carp was revealed by the presence of females only in meiotic gynogenetic progenies [15–17]. Later, male heterogamety in common carp was confirmed by results of crosses of sex-reversed males (XX-neomales) which, when crossed with normal XX females, produced all-female progenies [18, 19].

41.3.2 Sex Differentiation

Earlier maturing fish species have earlier gonadal differentiation. For example, cichlids differentiate within a few weeks post-hatching, and sexual maturity occurs early [20]. Common carp can mature within the first year of age, and gonadal differentiation occurs at about two months post-hatching [21] while, in the Chinese carps, sexual maturation does not occur for several years, with gonadal differentiation occurring within the first year or two, depending on the species [22]. In cyprinid fish, the first sign of sex differentiation is the development of a difference in anatomic structure between ovaries and testes [22–26]. The developing ovary becomes attached to the dorsal peritoneum at two points, forming an ovarian cavity between the gonad and body wall. The sac-like testis develops as a longitudinal anlagen narrowly attached to the dorsal body wall. After anatomical differentiation, the cytological sex differentiation begins by formation of gonia and the processes of oogenesis and spermatogenesis in females and males, respectively.

Table 41.1 summarizes data on age and size of common carp at anatomical and cytological differentiation of female gonads. As will be discussed, most studies on hormonal sex-reversal in common carp have been to induce sex reversal of genotypic females into functional males. Therefore, these data are

Table 41.1 Common carp age (days post-hatching, dph) and size at anatomical and cytological differentiation of gonads to female direction.

Anatomical sex differentiation		Cytological sex differentiation		Reference
Fish age, dph	Fish size, cm	Fish age, dph	Fish size, cm	
60–70	–	–	–	[24]
65–90	3.9	90–123	–	[25]
70–112	3.2–4.7	119–140	5.2–8.2	[26]
50–112	3.3–5.4	91–140	4.2–6.0	[21]
70–90	–	80–110	–	[27]

important to identify the critical period for hormonal treatment to successfully sex-reverse genotypic females. The appearance of an ovarian cavity (anatomical differentiation) and gametic-cytological differentiation (appearance of meiotic oocytes) in genotypic females at the ages of about two and three months, respectively, signals the process of sexual or gonadal differentiation.

41.3.3 Inducement of Sex Reversal by Androgens and Aromatase Inhibitors

Steroid-induced sex reversal has been practiced the longest in tilapias, in order to manage early sexual maturity and unwanted reproduction and recruitment, as well as producing all-male populations to take advantage of the greater growth potential of males [1, 6, 20, 28]. Studies of sex reversal for tilapias were initiated in the mid-1960s, underwent rapid experimental development in the 1970s, and attained commercialized application during the 1980s [29]. While procedures for various fish species will differ in detail, certain fundamentals from these early studies can be applied to cyprinids.

The basic assumptions are that:

- 1) treatment must proceed during a critical period of gonadal differentiation;
- 2) steroids (androgens/estrogens) mimic natural induction by genetic sex-determining factors, so as to alter development of the phenotypic or gonadal sex;
- 3) the exogenous steroid must be efficacious, adequately concentrated, and efficiently delivered during gonadal differentiation, so as to provide a physiologically or pharmacologically effective dose (PED) [11];
- 4) steroid-induced development of gonadal sex does not spontaneously revert; and
- 5) genotypic sex is not affected by the phenotypic alteration.

Efficacious steroid-induced sex reversal depends on several factors in addition to the induction treatment relative to gonadal differentiation. A hormone with androgenic- or estrogenic-inducing effectiveness, at a

physiologically or pharmacologically effective dose or concentration, must be delivered to encompass the period of gonadal differentiation when gonial cells are labile. Adding steroids to feed is the most convenient means of delivery; treated feed is easily prepared, and oral delivery is convenient, since the proportional dose rate is maintained as fish grow and consume greater amounts of feed.

Absorption and digestive breakdown must be considered in the design of an effective protocol. Synthetic steroids are more effective than naturally occurring ones. Androgen treatments for tilapias through oral delivery are usually 95–100% effective, but other means of hormone administration are required for some species. Characterizing steroid concentration in feed is the usual way of reporting dosage, although the steroid concentration plus the amount of treated feed eaten is a more accurate consideration. Restricted feeding versus *ad libitum* intake will affect internal hormone levels.

The product of feeding rate (percentage body weight/day, or per feeding) and steroid concentration (mg or μg /weight of feed) equals ($\mu\text{g/g}$ body weight/day); this approximates the Pharmacologically Effective Dosage concept suggested by Shelton [11]. Steroid-PED levels for sex reversal of tilapias have been in the range of 1.5–3 $\mu\text{g/g}$ of body weight/day. However, some fishes are less prone to accept non-living food or, as with grass carp, have limitations relative to absorbing the hormone (discussed later). Thus, it was necessary to develop another means of hormone delivery for the Chinese carps; implant delivery will be discussed relative to sex reversal of Chinese carps.

Functional sex reversal can be a direct means of producing monosex fish, or can be a component of a breeding program (Figure 41.1). The genetic basis for phenotypic sex development is determined at fertilization, and usually directs the formation of the gonadal sex, but genetic regulation is complicated by a labile period, where environmental factors can affect gonadal type. For steroid induction, control factors

include the selection of steroid, concentration, mode of delivery, age/or size of treatment initiation, duration, and treatment conditions [20]. The appropriate concentration of an efficacious steroid must be delivered throughout the period of gonadal differentiation. The labile period of gonadal differentiation can be identified through histological means, as has been done for the carps [21, 22, 26].

As mentioned above, common carp have male heterogamety (XY – males, XX – females). Also, females in common carp grow larger than males [30, 31]. Therefore, studies on hormonal sex reversal in this species have been aimed at production of sex-reversed XX-males (neomales) by treatment with androgens (or aromatase inhibitors), then crossing them with normal females (XX) for production of all-female progenies (Figure 41.1).

41.3.3.1 Review of Literature Data

The first report of successful sex reversal of genotypic female common carp by androgen treatment was made by Nagy *et al.* [18]. Treatment with 17 α -methyltestosterone (MT) was applied to all-female progenies obtained by meiotic gynogenesis, and MT was added to a prepared diet at a dose of 100 mg/kg and administrated at five different 36-day periods beginning from 8–80 days after hatching. These androgen treatments were tested at water temperatures of 20°C and 25°C. Fish weights at the beginning of androgen treatment varied in different experimental groups, from 0.003–6.0 g. The experimental groups of fish were raised in 160 L aquaria with filtered and aerated water.

There were significant differences in androgen treatment effectiveness at different water temperatures. The percentages of males in experimental groups raised at 20°C ranged from 15.4% in the group receiving androgen from 26–62 days, to 70% in the group treated in the period 80–116 days. At a water temperature of 25°C, the percentages of males in groups where hormonal treatment started from 8–62 days was relatively high, and varied from 71.4% to 88.9% (Table 41.2). In the groups where androgen

feed was started at 80 days, the percentage of males was only 20%.

Hormonal sex reversal was achieved in two consecutive generations of common carp with a dosage of 100 mg/kg MT in feed [19]. In experiments performed in 1982, sex reversal was induced in all-female progeny obtained by meiotic gynogenesis. After initial nursing in an earthen pond, two-month-old fry were transferred to aquaria in a recirculating system. In some aquaria, fish received MT (100 mg/kg) with a diet for 36 days from day 62 to 98, while fish in other aquaria in the same system were fed with an androgen-free diet. Water temperature in the recirculating system was kept at 25°C. Mean weight of fish at the beginning of hormonal treatment was 9 g.

In the group receiving MT-feed, the percentage of males was 83.3%, while 87.5% of the fish fed with MT-free feed, but kept in the same recirculating system, were males. Since female gynogenotes were used in this experiment, neomales must have been induced by androgen residue in the recirculating water. Apparently, MT (or its metabolites) leached from treated feed and excrements (from aquaria where fish received MT-feed) into the water of the recirculating system.

For sex reversal experiments performed in 1983, all-female progeny were obtained by crossing sex-reversed males from the 1982 generation with normal common carp females. As in the 1982 experiment, larvae were stocked in earthen ponds for nursing. In 1983 experiments, fish were kept in flow-through tanks at 25°C and fed artificial diet containing 100 mg/kg of MT for 40 days (from 78–118 days after transition to active feeding); fish mean weight at the beginning of androgen treatment was 12 g. Only females were present in the control group, while 51% were males in the group receiving MT-treated feed.

A similar experiment on sex reversal was performed in 1984, where all-female progeny from neomales produced in 1983 were used (Gomelsky, unpublished). Androgen-treated feed (100 mg/kg) was given to fish for 40 days,

from 60–100 days after transition to active feeding; mean fish weight at the beginning of androgen treatment was 5.8 g. In a control group fed an androgen-free diet, only females were present, while 73.4% of the fish in the group receiving MT with food were males (Table 41.2).

Gomelsky [19] performed histological investigation of the process of sex reversal in genotypic females under influence of androgen treatment in an experiment performed in 1983. Fish of 78 days old after transition of active feeding (at the beginning of MT treatment) had presumptive ovaries at the anatomically differentiated stage, which were attached to the dorsal body wall at two points, forming an ovarian cavity. The androgen treatment induced cytologically differentiated male gonidia in genotypic females, and advanced spermatogenesis and normal male reproductive morphology. The ovarian cavity in testes gradually diminished and finally disappeared.

Komen *et al.* [32] investigated the effects of a five-week oral administration of MT on sex ratios in normal mixed-sex progeny of common carp. Androgen treatment in experimental groups was started at fish ages of three, six, and 10 weeks after hatching. Doses of MT were 50 and 100 mg/kg for all variants, while the dose 150 mg/kg was only tested in groups receiving androgen from six weeks after hatching. Fish were kept in aquariums of a recirculation system, and the water from aquaria with fish receiving hormone with a diet was not recirculated during the periods of treatment. The water temperature was kept at 25°C. Initial mean weight of fish at start of MT treatment was 0.02 g, 0.9–1.0 g and about 12.0 g for experimental groups, which received hormone with food from three, six, and 10 weeks after hatching, respectively. In control groups, the percentage of males was 64.4%. The highest percentage of males (92.7%) was in the group that received MT at dosage 50 mg/kg in period 6–11 weeks after hatching; in groups that received doses 100 and 150 mg/kg of MT during the same period, the percentages of

males were 80.2 and 76.3%, respectively (Table 41.2). In groups of fish that were MT-treated 10–15 weeks after hatching, the percentages of males were 74.7 and 80.4% for doses of MT 50 and 100 mg/kg, respectively.

The androgen treatment during the 3–8 weeks after hatching had a predominately sterilizing effect. It was noted [32] that, for a more precise description of the hormonal treatment procedure, the amount of MT per unit of body weight, or per unit of body-weight gain should be determined.

Gomelsky *et al.* [33] reported results of several MT-treatment experiments on sex reversal in all-female gynogenetic progenies of common carp. The design of experiments was similar to those described earlier [19]. After nursing in earthen ponds, fish were stocked in recirculating water systems and tanks with running water for 40-day MT administration with diet (100 mg/kg). When 27-day-old fish were treated from 27–67 days after hatching (mean weight at initiation of MT treatment – 2.8 g), the group receiving MT-feed, and the group exposed to hormone only through water of a common recirculating system, contained 82.4% and 96.6% males, respectively (Table 41.2).

When fish from the same progeny received MT from 40–80 days after hatching (mean weight at initiation of treatment – 9.2 g), the percentages of males in corresponding groups in recirculating system were 66.7 and 68.6%, respectively. The percentage of males was only 43.8% in fish kept in flow-through tanks and fed MT-containing feed in the same period (from 40–80 days). In a similar experiment with other gynogenotes, fish with initial weight of 8.7 g received MT in the same period (from 40–80 days), and the resulting sex ratios were 75.0 and 88.1% in groups from the recirculating system (Table 41.2) and 25.6% in a group of fish treated in the flow-through tank. Fish at the beginning of less successful MT treatment were 34 days post-hatching, with a mean weight 14.5 g. In this experiment, 46.7% and 53.8% of males were found among fish in the

Table 41.2 Description of most successful experiments on inducement of sex reversal in common carp by androgens and aromatase inhibitors.

Treated progeny (sex composition and origin)	Conditions of fish rearing before treatment	Fish mean weight at onset of treatment (g)	Dose of hormone in diet (mg/kg)	Duration of treatment and age of fish at start and finish of treatment (days)	Conditions of fish rearing during treatment (system and water temperature)	Percentage of males	Reference
Methyltestosterone							
All-female, gynogenetic	Aquariums, 25 °C	0.003–1.3	100	36 days from 8, 26, 44 or 62 days	Aquariums, 25 °C	71.4–88.9	[18]
All-female, gynogenetic	Earthen pond	9.0	100	36, 62–98	Tanks of recirculating water system, 25 °C	83.3–87.5	[19]
All-female, from sex-reversed males	Earthen pond	5.8	100	40, 60–100	Tanks with running water, 25 °C	73.4	Gomelsky, unpublished
Normal, mix-sex	Aquariums, 25 °C	0.9	50, 100	35, 42–77	Aquariums, 25 °C	92.7, 80.2	[32]
All-female, gynogenetic	Earthen pond	2.8	100	40, 27–67	Tanks of recirculating water system, 25 °C	82.4–96.6	[33]
All-female, gynogenetic	Earthen pond	8.7	100	40, 40–80	Tanks of recirculating water system, 25 °C	75.0–88.1	[33]
All-female, gynogenetic	Earthen pond	2.4–3.9	100	45 or 50 days, starting at 50 days	Tanks of recirculating water system, 25–26 °C	93.1–100	[36]
Methyldihydrotestosterone							
Normal, mix-sex	Concrete pond	0.05	50, 100	50, 51–100	Tanks with water exchange	100	[37]
Fadrozole (aromatase inhibitor)							
All-female, from sex-reversed males	Earthen pond	3.5	100, 200, 400	36 or 50 days starting at 30 days	Tanks with recirculating water system, 26–28.5 °C	86–97	[35]

recirculation system and, among the fish fed with MT-containing food in tank with running water, only 20.0% were males.

Gomelsky *et al.* [33] noted that the experimental results agreed with observations made in a previous study [19], where sex reversal was induced in fish not receiving androgen in their diet, but exposed to it through shared water in the recirculation system. It was suggested [34] that MT consumed by fish might be transformed in the liver into soluble, active metabolite(s) and excreted with the bile. The data from several experiments showed that induction of sex reversal was more successful in fish kept in a recirculating water system, than in tanks with flow-through water. It was noted [33] that, for routine application of sex reversal, it is important to choose a practical indicator of the onset of optimal period for androgen treatment.

The highest percentage of reversed males (up to 96.6%) was achieved by treating the youngest fish (from 27 days after hatching) with the lowest weight (2.8 g), while the least successful treatments were those with fish of similar age (from 34 days after hatching) but with a larger mean weight (14.5 g) at the start of androgen treatment. Gomelsky *et al.* [33] compared hormonal sex reversal in a series of experiments conducted under different climatic conditions – one in a moderate, continental climate (Moscow region, Russia) and the other in a Mediterranean climate (Israel). The mean weight of fingerlings at the beginning of androgen treatment was similar in the most successful experiments (up to 82–96% of males in gynogenetic progenies): 2.7–9.2 g and 5.8–9.0 g in Israeli and Russian climatic conditions, respectively. However, the age of the fish at the time these weights were attained was quite different: 27–40 and 63–65 days after hatching, respectively. Based on these data, it was suggested [33] that the weight of the fish (rather than their age) should be used as a practical criterion for determining the appropriate time to begin androgen treatment. Body size can be

regulated for common carp fry through differential stocking rate during pre-treatment nursing [21].

Tzchori *et al.* [35] demonstrated the efficacy of an aromatase inhibitor fadrozole to induce sex reversal in genotypic females of common carp. All-female progenies obtained from sex-reversed males were used in experiments. Two experiments were performed; in both experiments, larvae were stocked for nursing in earthen ponds and later transferred to tanks of recirculating systems. In the first experiment, 22-day-old fish with mean initial weight 4.0 g were fed with a diet containing 200 mg/kg of fadrozole for 40 days; the resulting percentage of reversed males in treated group was 58.6%. In second experiment, 30-day-old fry with an initial fish weight of 3.5 g were fed with diet containing fadrozole at doses 100, 200, and 400 mg/kg feed for 36 or 50 days. At dose 100 mg/kg, the percentage of males was 86%, while at doses 200 and 400 mg/kg, it was increased up to 97% (Table 41.2).

Hulak *et al.* [36] investigated the effects of water with MT on masculinization of common carp kept in recirculating water systems. The experimental design in this study was similar to that previously used [19, 33]: Fish were fed with MT-containing diet in some tanks of recirculation systems while, in other tanks of the same system, fish received MT-free diet. Androgen treatment was applied to all-female progeny obtained by meiotic gynogenesis. After transition to active feeding and before hormone treatment, the larvae were stocked into earthen ponds for a 50-day nursing period, and groups were then transferred to recirculating systems for androgen feeding. Three periods were examined (40, 45, and 50 days after the 50-day pond nursing) when fish were fed MT-containing diet (100 mg/kg); the mean fish weight at the initiation of androgen treatment varied from 2.1 to 3.9 g. Histological analysis showed that, at the beginning of androgen treatment, fish were sexually undifferentiated. Water temperature in recirculating systems was 25–26 °C.

The results of this study confirmed previous observations that feeding fish with MT-containing feed causes sex reversal in fish that are kept in other tanks of the same system and that eat an androgen-free diet. In recirculation systems with a duration of androgen treatment of 40 days, 61.5% were males, among fish fed with MT-containing diet, while 81.2% were males in groups fed with MT-free diet and were exposed to hormone only through water of the recirculating system.

Longer durations of treatment yielded higher percentages of males; the 45- and 50-day-long feeding with MT gave 93.1% and 94.5% of males, respectively, in groups fed with MT-containing feed, and 100% were males (in both duration variants) in groups influenced only by the hormonally-polluted water (Table 41.2). The control groups reared in separate tanks with no androgen exposure consisted of females only. Chemical analyses of the water detected 0.33–2.68 µg/L of MT from the recirculation systems that housed MT-fed groups of fish [36].

Basavaraju *et al.* [37] used two androgens (MT and 17 α -methylidihydrotestosterone, MDHT) to induce sex reversal of genotypic females in normal mixed-sex progenies of common carp. Before the experiment, fry were nursed in concrete ponds. Among variants treated with MT, a maximum of 77.1% were males in group of 60-day-old fish fed a diet containing 100 mg/kg MT for 40 days; the mean weight of fish at initiation of the treatment was 0.10 g. When MDHT (50 or 100 mg/kg) was given to 50-day-old fish with a mean initial weight of 0.05 g for 50 days, only males were found in the treated groups (Table 41.2). When a similar treatment was applied to fish of the same age, but with larger initial mean weight (0.24 g), the percentage of males was significantly lower (approximately 70–80%). Based on these data, the authors suggested that not only the age, but also the weight, of the fish is equally important in determining the appropriate period of androgen treatment [37].

41.3.3.2 Analysis of Literature Data and Determination of Optimal Sex Reversal Parameters

Guidelines for sex reversal can be concluded from information in Table 41.2, which describes the most successful experiments. In most experiments, all-female groups were used, either gynogenotes, or progeny from neomales. If all-female progenies are used in sex-reversal experiments instead of normal mix-sex progenies, efficacy determination is more precise. Also, there is no need to progeny-test to identify functionally sex-reversed XX-males. In most studies, larvae were stocked for nursing in earthen ponds (Table 41.2) before being used in experiments on sex reversal; nursing in ponds is technically simple and, during pond rearing, fish are able to consume some natural food.

Sex reversal was induced in only one study [18], where hormonal treatment started soon after transition to active feeding (from eight days after hatching). Other successful treatments started with older fry of approximately 30–60 days of age (Table 41.2). Anatomical sex differentiation occurs in common carp at about two months old, thus, verifying the criteria that successful steroid-induced sex reversal in fish should start before gonad sex differentiation is complete [38].

The important issue is determining which parameter – fish age or size (weight) – should be used as a practical indicator for the onset of hormonal treatment. Fish weight at onset of hormonal treatment varied from 1–9 g, and it was recommended [33] that fish weight (rather than their age) should be used as a primary indicator of the best time to start androgen treatment, since rapidly growing fish of appropriate age could be too large for successful sex reversal. This conclusion agrees with reports on density dependent effects on gonadal differentiation in common carp [21]. It can be recommended that the weight of fish should be monitored during nursing and, when they reach about 3–5 g, they should be collected for hormonal treatment (Box 41.1).

Box 41.1 Female monosex production of common carp

Genetic mechanism of sex determination: male heterogamety (XY/XX system).

Gonochoristic species: differentiated (straight) type of gonadal sex differentiation.

Raising of all-female progenies is attractive because of sexual dimorphism (females are larger than males), and the possibility of preventing uncontrolled reproduction. All-female progenies can be obtained by crossing sex-reversed males (XX-neomales) with normal XX females.

Sex reversal in genotypic females to produce neomales can be induced by feeding fish with food containing androgen 17 α -methyl-testosterone at dose 100 mg per kilogram of food, for 36–40 days. Recommended weight of fish at the beginning of androgen treatment is 3–5 g; recommended fish age is 1–2 months.

During androgen treatment, water temperature should be kept at 25 °C. To increase the effectiveness of MT-treatment, it is recommended to keep fish in closed recirculating water systems.

As mentioned above, it was reported [19] that successful MT-sex reversal in genotypic female common carp was achieved when treatment started after anatomical differentiation of presumptive ovaries. The androgen treatment caused subsequent cytological differentiation into male gonads. A similar process was described by Jensen *et al.* [39], in studies on sex reversal in grass carp using intraperitoneal implants (see Section 41.4). The gonads were not cytologically differentiated at the start of MT treatment, but presumptive ovaries were anatomically differentiated. Based on these data, it can be concluded that the critical androgen-labile developmental stage in cyprinids occurs after anatomical gonad differentiation, but prior to cytological sex differentiation.

The androgen most commonly used was MT, at dietary dosage 100 mg/kg. Also, methyl-dihydroxy-methyltestosterone, and the aromatase inhibitor fadrozole, were effective. Treatment was most effective in recirculating water systems. Carp fed with MT-containing feed were sex-reversed, but sex reversal was also observed in fish fed a hormone-free diet and exposed to androgen in recirculating water. Similar implications of hormone treatment in closed water systems have been described in experiments with Nile tilapia [40, 41]. As mentioned above, Hulak *et al.* [36] showed that water of recirculating systems was polluted with MT when fish are fed with MT in some tanks. Also,

additional studies should be considered relative to the role of liver metabolites as inducers of sex transformation in recirculating systems [34].

41.3.4 Genetic Sex Regulation and Advantage of Raising All-Female Progenies

Production of all-female progenies by crossing of sex-reversed males (neomales-XX) with normal females (XX) was performed in many studies [18, 19, 31, 37]. Common carp neomales have normal sperm ducts, in contrast to anomalies reported in sex-reversed male rainbow trout [42]. Normal development of sperm ducts permits stripping of sperm from neomale common carp for use in breeding for all-female progenies.

Raising all-females can increase yield in practical common carp aquaculture. Female cyprinids grow larger than males of the same age, and can increase yield. Also, males reach sexual maturity at an earlier age than females and, when they mature, their growth rate decreases. Fish reach maturity sooner at higher temperatures, so raising all-female common carp in warm climates should be advantageous, especially when they reach maturity before attaining market size.

In the Israeli climate, most 14-month-old carp of market-size (about 1 kg) are mature. Cherfas *et al.* [31] compared growth of all-female and normal mix-sex progenies, and

found that yield of all-female groups was 7–8% higher, and females were 15% heavier than males. Further, unwanted spawning was prevented. Under moderate Central European climatic conditions, growth of all-females was 6–8% higher than mixed-sex progenies, reaching 1.5–1.8kg after three years [43]; all males, and half of females, were mature. However, these authors concluded that the economic benefit from rearing all-female carp populations under these conditions must consider the additional expense of establishing and renewing brood stock of neomales.

41.3.5 Induced Gynogenesis

Gynogenesis is defined as embryonic development without a paternal genetic contribution; insemination is usually induced by genetically inactivated sperm, which results in only maternal heredity. Spontaneous gynogenesis occurs, but diploidization frequency can be increased by a physical shock (cold, heat or pressure) to block second polar body formation (Meiotic, or Early Shock), or by blocking of the first mitotic division in haploid embryos (Endomitotic or Late Shock). Optimized induction (shock) variables of intensity, magnitude, and duration must be applied at the most effective time after gamete activation [44–46].

Optimization of induction parameters must be determined empirically and, because of variation in the cytological sequence among a population of gametes, even at a single temperature, only a percentage of the cells will be within the optimum induction window and therefore be affected by the shock. This pattern was well illustrated for

tilapia by Shirak et al. [47]. Usually, heterologous male chromosomes are inactivated by sperm irradiation with ultraviolet light (UV). Gynogenetic origin of fish produced in experiments can also be confirmed by application of genetic markers. In common carp, mutations of scale cover types and color mutations are traditionally applied for this purpose.

Presently, microsatellite DNA markers are widely used for confirmation of gynogenetic origin of fish. Treatment standardization is important, particularly with reference to pre-shock incubation temperature. The species-specific developmental duration unit (τ_0), or mitotic interval index [48, 49], is a useful tool to standardize different pre-shock temperatures in terms of τ_s/τ_0 (where τ_s absolute shock time in minutes). Values of one τ_0 in common carp and grass carp at different temperatures are presented in Table 41.3.

Gynogenesis induction protocol has been developed for common carp [52–56], in the Chinese carps with no shock (spontaneous Pb retention) [57, 58], and with heat, cold, or pressure shock [59–63]. Optimized gynogenetic induction protocol (Pb = 2nd polar body, or Em = endomitotic) will provide the best first estimate for polyploid induction (triploidy – early shock, or tetraploidy – late shock), requiring only the substitution of normal homologous non-irradiated sperm at insemination. Haploids may survive to hatching, but they die before swim-up; therefore, only a direct count of viable larvae indicates the best induction protocol.

Gomelsky [56] reviewed literature on induced gynogenesis in common carp, and described practical instructions for production of gynogenetic progenies, based on the

Table 41.3 Values of one τ_0 (in minutes) in common carp and grass carp at different temperatures.

Temp. (°C)	16	17	18	19	20	21	22	23	24	25	26	27	28	Ref.
Common carp	53	45	38	32	28	24.5	22	20	19	19	–	–	–	[50]
	53	45	39	34	30	26	24	21	19	17	16	14	13	[51]
Grass carp	–	–	–	–	26	23	20	18	16	14	13	11	10	[51]

results of recent studies [64]. Sperm was UV-irradiated using a FisherBiotech Crosslinker. The dosage of irradiation of common carp sperm was 3,000 or 4,000 J/m². For UV irradiation, sperm was diluted with saline solution (1 ml of sperm per 9 ml of 0.85% NaCl solution); 2 ml of diluted sperm was placed in a 6 cm glass Petri dish with approximate 0.07 cm thickness, and six Petri dishes were placed in the Crosslinker simultaneously. Uniform irradiation of spermatozoa was achieved by placing Crosslinker on a rotating shaker table. Suppression of the second meiotic division (meiotic gynogenesis) or first mitotic division (mitotic gynogenesis) in eggs was induced by heat shock. For meiotic gynogenesis, a two-minute heat shock (39°C) was initiated 0.2 τ_0 after insemination (5–6 minutes at water temperature 20°C); for mitotic gynogenesis, a two-minute heat shock (39.5–40°C) was initiated 1.5–1.6 τ_0 (42–45 minutes at water temperature 20°C) after insemination.

Meiotic gynogenetic progenies consisted of females only [15–17]. Usually, mitotic gynogenetic progenies are also all-female; however, a recessive mutation of a sex-determining gene results in the appearance of males in some gynogenetic progenies [65]. Gynogenesis increases the homozygosity of the genome [56], with the rate of increase depending on the type of gynogenesis. For meiotic gynogenesis, heterozygosity results from crossing over between gene and centromere, and may differ to a great extent for different genes.

Based on analysis of recombination rate for many genes and DNA markers, one generation of meiotic gynogenesis in common carp results in higher increase of homozygosity than self-fertilization, with coefficients of inbreeding (F) of 0.60 and 0.50, respectively. Mitotic gynogenesis results in homozygosity for all genes ($F=1.0$), since homologous chromosomes are replicated by simple mitosis in haploid embryos. Induced gynogenesis is not used for direct sex control, because of possible inbreeding depression, and the induction procedures are relatively complex,

and diploid yield is lower, than with normal fertilization. However, all-female meiotic gynogenotes are commonly used prior to hormonal sex reversal (Figure 41.1). Neomales (XX) produced by androgen-treating all-female gynogenote progeny can be used as male brood stock to breed for all-female progeny. Further, replacement neomale brood stock can be produced by sex reversing progeny from such crosses, without the need for further gynogenesis (See Figure 41.2 and grass carp discussion – Section 41.4.7).

41.3.6 Induced Triploidy

Induced polyploidy in aquaculture and fisheries can provide triploid fish (i.e., fish whose karyotypes contain three haploid chromosome sets). As a rule, triploid fish are genetically sterile, because of complications in pairing of homologous chromosomes during meiosis. Triploid fish have complete or partial reduction of gonads, and this usually differentially affects ovarian versus testicular formation. Triploid fish can be produced by suppression of the second meiotic division in eggs after insemination by intact spermatozoa. This method uses the same shock protocol, optimized to induce diploid meiotic gynogenesis. Application of shock should provide a high frequency of triploids but, at the same time, does not reduce embryo survival significantly.

Gomelsky [56] provided literature review on production and properties of triploids in common carp, and described practical instructions for production of mass triploid progenies in this species, based on previous studies [66, 67]. The following parameters of heat shock are recommended as optimal: 40°C for two minutes, or 41°C for 1.5 minutes, starting at 0.2 τ_0 after insemination (5–6 minutes at pre-shock water temperature 20°C). Effectiveness of this method was verified in further studies [68, 69].

Somatic growth can slow after gonadal maturation in normal diploid fish. This has led to speculation that triploids might have improved somatic growth through energy

saving not invested in gonadal development. Growth of triploid common carp was compared with diploids in two studies [70, 71]. Gonads, especially ovaries, were reduced in the triploids, although some triploid females had well-developed ovaries [70]. However, triploid fish grew slower than diploid fish in almost all comparative trials [70, 71]. This growth differential between fish with the two ploidy levels also has been reported for grass carp, and this slower growth of the $3n$ fish is used in commercial triploid production (see Section 41.4.8.2).

Recently, it has been reported [72, 73] that some triploid ornamental koi carp females developed unexpectedly well-developed ovaries that were filled with fully grown oocytes. Crosses of triploid koi females with normal diploid koi males yielded mass aneuploid progenies, having very low viability. Most of aneuploids had ploidy ranging from $2.14n$ to $3.0n$, with mean ploidy level around $2.5n$. Since aneuploid fish have, in their genomes, one haploid set from parental males, the obtained results suggest that triploid koi females produced aneuploid eggs with ploidy range from haploid to diploid level, and a modal ploidy level around $1.5n$. Earlier, similar range of ploidy was observed in aneuploid spermatozoa that were produced by triploid males of different fish species [74].

41.4 Grass Carp

41.4.1 Artificial Propagation and Sex Manipulation

Traditional culture of the Chinese carps was based on capturing wild-spawned seedstock within their natural range, until techniques for induced spawning of these species were developed. Artificial propagation through controlled final maturation, ovulation, and spermiation permit genetic selection to improve stocks for various desirable traits, even for those species that will reproduce under most culture conditions, such as common carp. Induced ovulation has permitted

more efficient management, and has provided greatly enhanced capacity to conduct breeding programs and perform manipulations [7, 28, 44, 46, 56, 75].

Grass carp have been introduced into numerous countries, as a biological control of aquatic vegetation [3, 76]. The US program was initiated in 1963, when about 70 fingerlings were imported from Malaysia by the US Fish and Wildlife Service Laboratory at Stuttgart, Arkansas (FWS), and Auburn University, Alabama (AU) obtained 13 fingerlings from Taiwan. These fish matured and were spawned at both facilities in 1966. Concerns over using exotic fishes in the US stimulated research on reproductive control methods, first through monosexing, and later triploidy, after chromosome induction techniques were developed. Protocols for grass carp monosexing were independently studied at two laboratories – FWS from 1972–1976 and AU from 1972–1982. The objectives of these investigations were to sex-reverse XX-females and use homogametic neomale brood stock (XX-♂♂) to fertilize eggs from normal females, thereby producing all-female progeny [57, 77–80]. A breeding program for monosex grass carp was developed at AU between 1973 and 1984, and then re-tested in Israel in 1994–2000 [58, 63].

All-female progeny produced by gynogenesis to develop reproductively limited fish was one of the first applications of chromosome manipulations in warm water species [57, 78]; however, at the inception of these grass carp studies, key elements were not yet available. Neither ploidy manipulation induction methodology, nor sex-reversal techniques for grass carp, had been studied. Gynogenesis can be a means to directly produce monosex progeny, but it is inefficient without using shock methods to increase second polar body retention. However, the combination of gynogenesis and sex reversal as key elements in a more comprehensive integrated monosex breeding program is of greater significance (Figure 41.1).

These early studies on gynogenesis used UV-treated common carp spermatozoa to

activate grass carp eggs, but diploid progeny were produced only by spontaneous retention of the second meiotic polar body (ca. 0.2–0.5%). About 45,000 diploid gynogenotes were produced from over 58 million eggs (FWS) for a large-scale study in Lake Conway, Florida [80, 81], and about 850 diploid gynogenotes were produced through spontaneous Pb retention at AU in the initial developmental of a monosex breeding program [82–84] (Figure 41.2).

Subsequent developments in ploidy manipulation have facilitated additional options in sex control, including commercial-scale triploid grass carp production. Reproductively limited fishes now can be developed by various techniques, singly or in combination. These include induced gynogenesis, androgenesis, and polyploidy

manipulation, and provide numerous options for genetic selection, sterility, and sex control [1, 11, 29, 44, 46, 85, 86].

A monosex breeding program for homogametic female species involves the initial progeny development through gynogenesis, then neomale brood stock production through functional sex reversal of gynogenote females (Figure 41.2). Long-term program continuity is perpetuated through sex reversal of female progeny (F_2) from neomales and normal females, without the further need to use gynogenesis. The initial development of components of this multi-tiered reproductive management program for grass carp was conducted at AU over a 10-year period (1973–1984), and verified in a collaborative study in Israel (1994–2000), using an albino grass carp (AGC) model

Figure 41.2 Phases of grass carp broodstock development and breeding chronology for all-female production.

a. Brood stock development.
b. Breeding (see Figure 41.1).

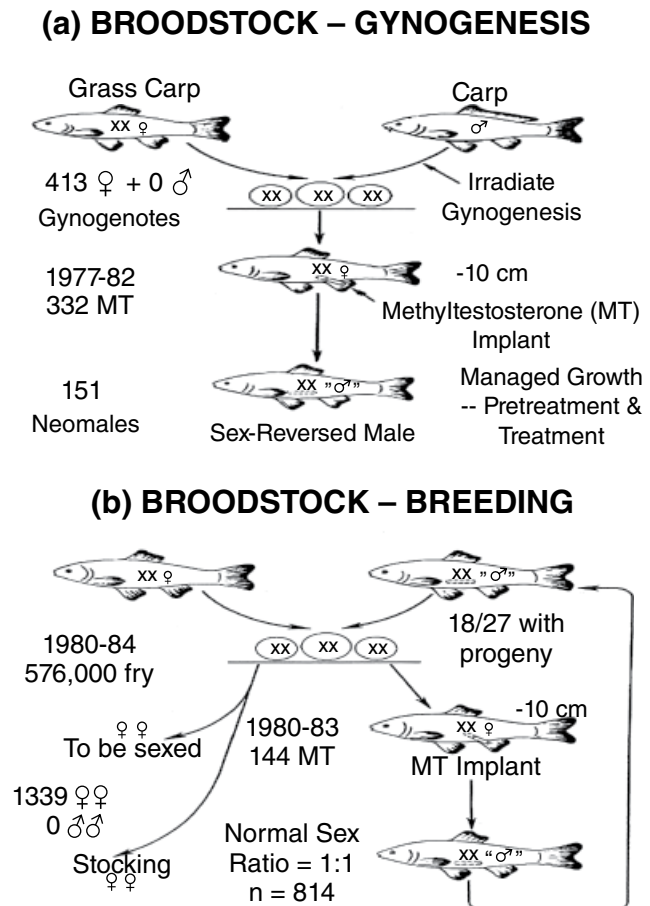


Table 41.4 Grass carp monosex breeding program milestones – normal grass carp and albino broodstock (updated Shelton 1986 [58] and Rothbard *et al.* 2000 [63] – Reference Figures 41.1 & 41.2).
Spontaneous Pb retention occurs infrequently but shock increases incidence; @ = age or size;
MT = methyltestosterone; Δ + P = increased pressure shock, Δ + C = heat shock, Δ – C = cold shock.

	Development (1973–1984)	Verification –AGC model (1994–2000)
	Phases I & II	
	<u>1977–1979</u>	<u>1994</u>
Gynogenote	413 (all ♀♀ : 0 ♂♂) (Spontaneous Pb)	850 AGC 2N gynogenotes Shock: Δ + P 7000/1.5 min; τ ₀ = 0.2–0.3 Δ + C 40 °C/2 min; τ ₀ = 0.15–0.20
Normal Fertilization	814 (1♀ : 1♂)	Δ – C 10 °C/10 min; τ ₀ = 0.11–0.17
	Phase III	
	<u>1977–1982</u>	<u>1994–1995</u>
Methyltestosterone implanted (5 mg)	332 gynogenote ♀♀ 55–75 days-old @ 65–135 mm 151 sex-reversed of 212 MT (71%)	33 gynogenote ♀♀ @ 10 mos, 58–84 mm (73% rejected implants) matured in 1997
	Phase IV	
	<u>1980–1984</u>	<u>1997</u>
Neomales (“♂♂”) Spawned	27 neomales spawned of 79 (18 with progeny); F ₂ progeny (♀♀) = 576,000 fry; 92,000 juveniles	8 of 12 MT-treated gynogenote spermiating = neomales (67% sex-reversed) Two neomales spawned 20,000 all-♀♀ fry
F₂ Progeny Sexed	1,347 – all females	none sexed
	<u>1980–1983</u>	<u>1998</u>
Neomale Progeny MT-treated	144 F ₂ (♀♀) MT-implanted	31 F ₂ (♀♀) implanted (81–105 mm)
GYNOGENESIS NO LONGER NECESSARY TO CONTINUE MONOSEX BREEDING PROGRAM		
		<u>1998</u>
Monosex Triploid	—	326 putative 3N ♀♀ fry
(AGC X AGC) — see gynogenetic induction protocol		

[58, 63] (Table 41.4). The total number of monosex fish needed for the Lake Conway study [80] were produced 10 times over by using a single XX-neomale to fertilize eggs from only one female.

41.4.2 Sex Determination

Cyprinids in general, and common carp and grass carp in particular, have female homogamety [55, 58, 78, 87]. The basis for sex determination is pertinent to reproductive manipulations, and particularly relative to

breeding of phenotypically altered individuals such as sex-reversed neomales, as well as for the sex of ploidy-manipulated fishes (gynogenotes and triploids) (Figure 41.1). In the direct induction of female monosex (XX) or sterile triploids (XXX or XXY), the treatment protocol can be developed rapidly but, because of individual biological variation, absolute efficacy is rarely achieved. However, brood stock can be developed using gynogenesis and steroid-induced sex reversal sequentially, providing the possibility to mass-produce monosex offspring, thereby

using the full reproductive potential of the species and, further, all-female triploids would open new options [1, 83, 84].

41.4.3 Sex Differentiation

Gonadal or sex differentiation is a relatively early ontogenetic process, while initial sexual maturation develops later. The time of gonadal differentiation is somewhat proportional to the age of sexual maturity, but with a species-specific relationship between the relative timing of these two events. For example, the gonads of tilapia differentiate at a small size within a few weeks post-hatching, and sexual maturation occurs within a few months, while the gonads of black carp *Mylopharyngodon piceus* differentiate only after a year or more of age, and sexual maturity is not reached for several years [61]. Therefore, specific timing of critical developmental events must be considered within a reproductive management program, in order to apply an effective treatment for various manipulations. The effective induction of phenotypic sex reversal must correlate with the labile period during gonadal differentiation, and efficient induction of ploidy manipulation must consider the timing of nuclear division [11].

Gonadal differentiation of phenotypic sex can be identified histologically by morphological differences prior to the initiation of gametogenesis, and this has been documented for common carp [21, 25, 26], and for Chinese carps [22, 60]. Premeiotic germ cells (oogonia or spermatogonia) develop from primordial germ cells (PGC) and proliferate mitotically in the presumptive gonad until they are transformed into gonocytes at the initiation of the first meiotic prophase; the natal gonad has sexual bipotentiality during this phase of development.

Cytological differentiation is most clearly observed in females, and is characterized by the transformation of gonial cells (oogonia) into oocytes [88, 89]. Meiosis is initiated at this time, but further progress is suspended until ovulation, when meiosis resumes and

the first polar body is formed. The second meiotic polar body is ejected when the ovum is activated by a spermatozoon at spawning, and diploidy is restored during normal fertilization.

Prior to cytological differentiation, sexual phenotype can be modified by steroid exposure. The anatomically differentiated gonad is considered labile and subject to exogenous influence, while the phenotypic sex is thought to be genetically fixed at cytological differentiation. The pattern of gonadal differentiation is a primary consideration for effective sex-reversal treatment, and varies in different cyprinids, somewhat proportional to age and size at first maturity. Gonadal differentiation is also affected by growth rate relative to chronological age.

41.4.4 Age-Size Effects on Gonadal Differentiation

The chronology of ontogenetic processes, such as gonadal differentiation and sexual maturity, are affected by environmental factors altering rates of development so, consequently, any induced manipulations must take appropriate trajectories into consideration [1]. The physiological processes that are involved in gonadal differentiation or meiosis/mitosis must be considered in developing protocols that attempt to alter a functional phenotype, or to induce chromosomal manipulations, respectively [11]. Examples include the effect of growth rate on sex reversal treatment and the temperature effect on developmental rate relative to timing of ploidy manipulations.

The growth rate of fish is controlled within genetic constraints by various environmental factors. Temperature is one of the most important abiotic components influencing growth rate, and food is the dominant biotic factor. Growth is further influenced by various density-dependent factors. Individual growth within a population is altered by density-dependent factors, such as biomass and carrying capacity [90, 91]. Growth is expected to be inversely related to population densities.

Growth patterns of individual common carp vary during early post-hatching. Moav and Wohlfarth [92] and Hulata *et al.* [93, 94] studied growth patterns within carp populations. They examined interactions between genetic and environmental effects which lead to growth variation, deviation and depensation, or the Tobi-Koi “jumper-laggard” phenomenon [95]. Growth depensation affects the physiological interrelationships of chronological age and respective size. The overall pattern of density-dependent growth can be documented by comparing the final sizes in pond-cultured fish at various population densities.

A conceptual model presented for carps [21, 58, 77] characterizes a changing size/age relationship, during which gonadal differentiation progresses under genetic control, but is physiologically labile and can be influenced by exogenous factors. Under conditions of variable growth rates, the ontogeny of some organ systems is differentially affected by size and age. The interface of the genetic/environmental interaction and gonadal ontogeny is related to size as well as age, but not necessarily in a fixed proportion. Temperature and population density affect growth and, therefore, alter this ontogenetic process. Different growth rates will affect the size/age relationships of gonadal ontogeny. The longer the growth period to these physiological stanzas, the greater will be the effects of population density on growth differential relative to age. Using size or age independently to characterize expected progression of gonadal development will not accurately reflect the phase. Characterization of this growth-rate influenced realm of gonadal development has been critical in the development of an effective sex reversal protocol for grass carp.

Jensen and Shelton [22] documented that gonads were undifferentiated in grass carp younger than 40–50 days, but that anatomical differentiation occurred over the next 25 days between 47 and 50 mm SL; cytological differentiation in females occurred between 180–232 days at 112–130 mm, but was delayed in slower growing individuals.

Clusters of oogonia were documented in 150-day old fish (76 mm SL), but were not apparent in smaller, slower-growing fish of 170–225 days (56 mm).

41.4.5 Density-Dependent Growth Management

Growth management for grass carp has been practiced in Southeast Asia relative to market size. Grass carp fry are stocked in China at about one million per hectare, to produce fish 40–100 mm in length. Shelton *et al.* [96] applied this practice to first-year juvenile grass carp; growth control of grass carp was tested by stocking rates of young-of-the-year in a series of mud-bottom ponds and concrete tanks at population densities between 14,000 and 470,000/ha. Different density-affected size distributions developed during the first growing season in a clear density dependent-size relationship (Figure 41.3). Modal length at the lowest population density was about 17 cm, while that for the highest was about 7.5 cm.

Growth trajectories for grass carp in ponds and tanks at various population densities were also density-dependent; growth differential was well established within the first month of nursing, and these patterns continued throughout the growth period. Growth curves plateau at generally expected sizes during the first season of growth (Figure 41.4). Thus, the size of carps can be somewhat predictably managed by variable stocking densities, and growth management is essential in steroid-induced sex reversal of grass carp using MT implants. Growth management is also being practiced for triploid grass carp production, which will be described subsequently.

41.4.6 Grass Carp Sex Manipulation – Initial Development (1973–1984)

Factors that affect growth can affect the efficiency of sex reversal, since physiological processes such as gonadal differentiation are

Figure 41.3 Size distribution of grass carp fingerlings stocked at 50–60 days post-hatching after one growing season in ponds. Adapted from [96].

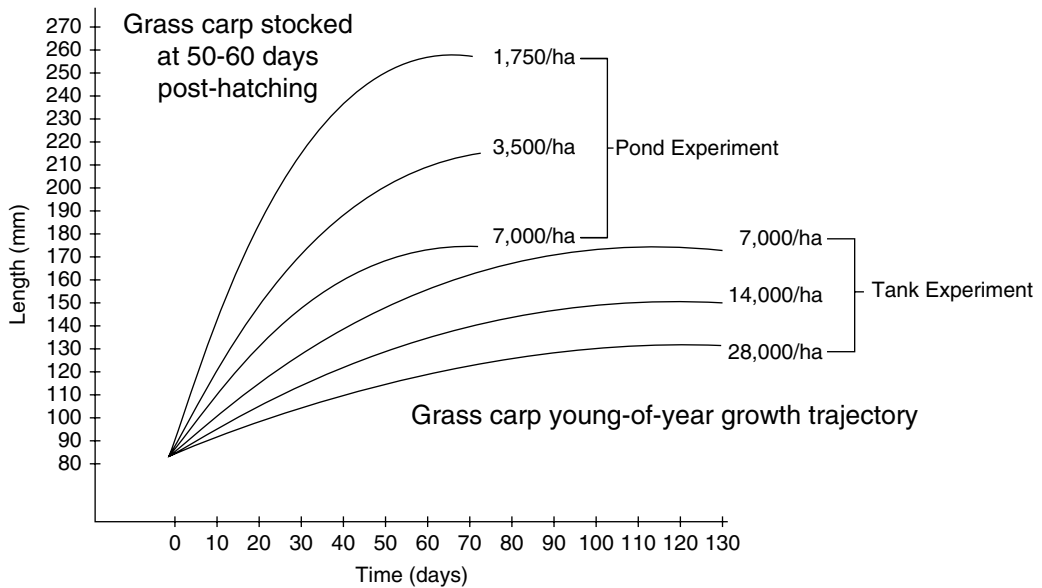
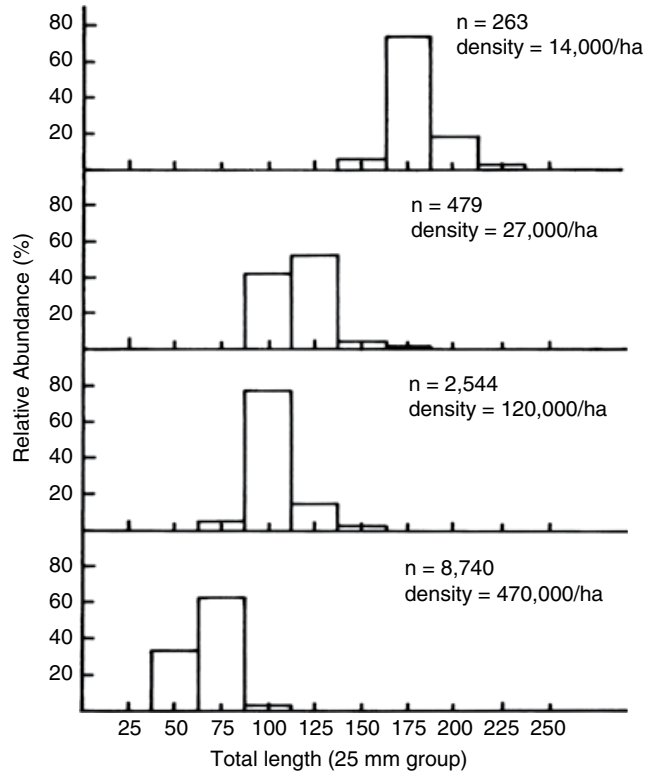


Figure 41.4 Growth curves for young-of-the-year grass carp at different population harvest densities after one growing season.

altered by a balance between chronological age (time) and growth (size) [21]. Therefore, the “period” of treatment must consider age and size. This “window” of opportunity for treatment optimization was conceptualized for tilapias [20] and applied to common carp [21, 32, 87] and grass carp [39, 97]. However, the traditional application of steroids to sex reverse fishes was not successful for grass carp; oral delivery of steroids to sex reverse grass carp was independently tested in two studies [77, 98], but both failed.

Stanley and Thomas [98] applied the accepted practice for oral delivery of methyltestosterone (MT) as a means of sex-reversing grass carp, but failed to develop the requisite protocol. Shelton and Jenson [77] also fed MT diets to gonadally undifferentiated grass carp [22] but, again, failed to induce sex reversal. Thus, these studies demonstrated that oral delivery of sex-reversing steroids is not feasible for grass carp; some species have specialized feeding habits and will not accept, nor do well, on an artificial diet. Jensen *et al.* [99] reported progress on developing a prototype MT-intraperitoneal implant and, subsequently, grass carp were sex reversed [39, 97]. However, hormone delivery by implant requires management of growth, so as to ensure efficacious dosage during the physiologically labile period of gonadal development, and also because of peculiarities of the steroid release.

41.4.6.1 Steroid Delivery from Intraperitoneal Implants

Sex reversal of grass carp failed when given MT-treated feed so, by necessity, an alternative delivery system was developed [39, 97]. The prototype implant contained 10 mg of MT but, subsequently, an implant containing 5 mg of MT was used [58]. For species such as the Chinese carps, in which gonadal differentiation occurs at a relatively large size, and for which feeding hormone-treated diet is not an option, a less controlled, but effective, steroid delivery can be achieved through intra-peritoneal implants [100].

The implant that was developed to sex reverse grass carp was made from sections of Silastic Medical-Grade Tubing (12 mm lengths, 1.02 mm [ID], 2.16 mm [OD]), which were hand-packed with 5 mg of MT and inserted intraperitoneally through a small abdominal incision in juveniles. Gynogenote females were sex-reversed into functional males under appropriate growth conditions [39, 58, 97]. The implant also was used to sex reverse silver carp gynogenotes that contained only females; the MT-treatment protocol mirrored that of grass carp [60].

41.4.6.2 Sex Reversal – Implant and Growth Management

Sex reversing grass carp is based on a time release of MT implant; hormone delivery from an intraperitoneal implant and growth management provide the mechanism to regulate hormone dose [77, 82, 83]. Sex reversal for grass carp is based on size at treatment, and taking into account implanting at the appropriate size and diffusion rate of MT from the steroid implants (Box 41.2).

To induce phenotypic sex reversal of grass carp using an implant delivery requires a balance between growth rate (increasing biomass) and the inherent characteristic of diminishing steroid diffusion. Diffusion is affected by temperature, and is also higher initially [97]. Growth management regulates the delivery of an effective dose, since diffusion from the implant is not controlled [58] (Figure 41.5). Stocking density is used to effect the relative steroid dose level relative to release from the implant. Fish at lower stocking densities initially grow more rapidly than those at higher densities, and the rate becomes asymptotic sooner.

The manipulation of fish growth by controlled stocking density affects the temporal pattern of gonadal differentiation and, thus, provides the means of affecting the MT delivery from implants [39]. Thus, this grass carp sex-reversal model is an amalgamation of density-dependent growth management relative to gonadal differentiation, and the changing *in vivo* implant diffusion.

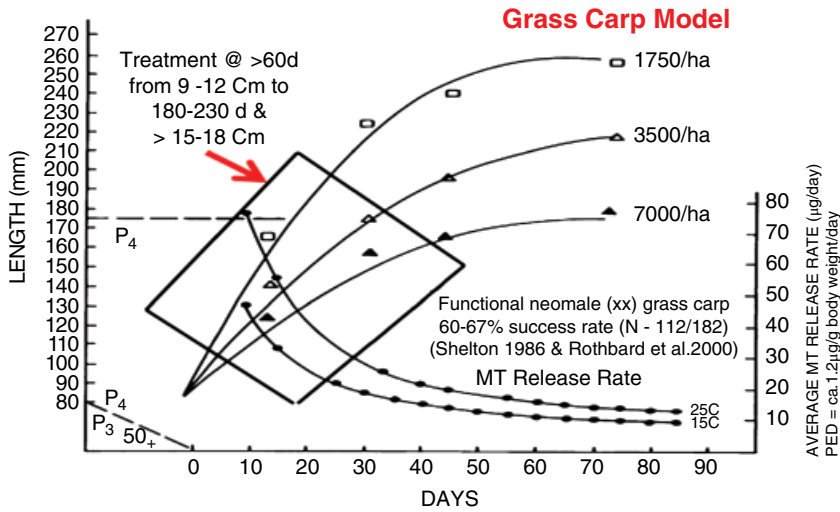


Figure 41.5 Grass carp sex reversal model, relating density-dependent growth curves and exposure to MT-release pattern relative to gonadal differentiation period; P3 = morphological differentiation; P4 = cytological differentiation. Modified from [11].

Box 41.2 Grass carp broodstock development protocol for all-female breeding program

- **Sex determination:** Female homogamety (XX).
- **Gonadal differentiation:** Undifferentiated < 40–50 dph (days post-hatch) and < 50 mm TL.
- **Morphological to cytological differentiation:** between 50 and 150–230 dph at 10–12 cm (population density-dependent).
- **Gynogenetic Induction:** Donor male – common carp sperm UV treated (1,000–4,000 J/m², depending on spermatozoa concentration and saline dilution).
- **Post-activation incubation at 21°C until shock:** Optimum time = $0.2\tau_0$ (0.1–0.3) = 4 minutes.
 - Heat: 40°C (38–42) for 2 minutes (1–3 minutes).
 - Cold: 10°C (or 5–8) for 10 minutes (or for 3–10 minutes).
 - Pressure: 7,000–8,000 psi for 1.5 minutes (1–2 minutes)
- **Gynogenote nursing:** Stock swim-up fry in fertilized pond at ≈ 500,000/ha, 50–60 days.
- **Fingerling nursing:** Restock 5 cm young at ≈ 10,000/ha for 45 days, harvest size = 8.5–10.5 cm.
- **MT-treatment:** Anesthetize, insert 5 mg MT implant through mid-abdominal incision (< 5 mm).
 - Restock implanted XX-female fingerlings (8.5–10.5 cm) @ < 10,000/ha for 30–60 days or more, expected size 18–20 cm (implant will release MT for about one year).
- **Neomale maturation:** Sex-reversed males (XX) will mature and begin spermiating after two years.
- **Breeding:** Neomale × Normal Female = only XX-female progeny (Note: progeny from second generation neomales can be MT-implanted; thus, no further gynogenesis is required).
- **Monosex triploid production:** Shock treat progeny from neomale × Female = sterile female triploids (Note: will require culling residual diploid females as described using growth management and Coulter-counter examination).

Dose rate from steroid implants depends on two contrasting factors, one physical and the other biological:

- 1) diffusion rate from the capsule diminishes over time; and
- 2) fish grow, diminishing the physiological dose rate (PED) even during the stabilized release period as body weight increases.

Therefore, effective MT exposure must balance a declining physiological steroid level during the period of gonadal differentiation, but management of growth rate somewhat ameliorates this disparity. Diffusion of the steroid from the implant is affected by the molecular size of the material in the capsule, its solubility, and the relative pore size of the silastic and temperature [97]. Further, the diffusion rate during the initial 10–30 days is about 30–80 $\mu\text{g}/\text{d}$, but approaches asymptotic (10–20 $\mu\text{g}/\text{d}$) after about one month.

Grass carp sex reversal is based on size at treatment, and average steroid diffusion rate for the entire treatment of about 1.2 $\mu\text{g}/\text{g}$ body weight/day; a 5 mg implant will release MT for about one year. The recommended treatment protocol for grass carp relative to implant diffusion and growth management is summarized in Box 41.2. Grass carp between 75–100 mm TL can accommodate a 12 mm long implant, but rejection increases in fish smaller than 75 mm [58, 63]. The initial fish size recommended for treatment of grass carp is between 10–12 cm TL (5–10 g). Gonadal differentiation for grass carp relative to sex-reversal treatment occurs between ages of about three and six months before reaching about 13–18 cm. The complete program was first presented by Shelton [58], and later was updated, with additional data, at the various milestones and verifications (Table 41.4).

41.4.7 Integrated Monosex Breeding Program Verification: Albino Grass Carp Model (1994–2000)

Collaborative studies using a unique Albino Grass Carp (AGC) model were conducted in

Israel between 1994 and 2000. The overall goal was to verify the sex reversal protocol, and to incorporate ploidy induction techniques, so as to test the entire integrated management concept. Albino female grass carp provided a recessive phenotypic marker to facilitate the identification gynogenote progeny [101]. Chromosome-set manipulation and hormonal sex reversal were integrated, so as to produce monosex diploid females or sterile all-female triploid grass carp.

41.4.7.1 Gynogenesis

Gynogenesis induction techniques were tested using albino grass carp (AGC) eggs activated with sperm from various species [heterologous donors (common carp, wild-type color or ornamental koi carp, or golden tench) or homologous donor (normal-color grass carp)]. Compatibility of gametes is of relevance to ploidy manipulation. The recommended protocols for induced gynogenesis include using a heterologous sperm donor to activate egg development and, as further assurance that the gynogenote offspring carries only the maternal genome, the spermatozoa are usually UV treated at about 1,000 J/m², to neutralize the male DNA. Dosage varies with the density of milt and saline dilution [63].

Diploidy of the zygotes was restored by retention of the second polar body (2Pb) using thermal or pressure shocks. Similarly, AGC females were used in triploid-induction trials with AGC male sperm (Table 41.5). Other chromosome manipulation studies were also done, but only early shock manipulation (gynogenesis and 3N) are discussed here. The three common types of shock used in grass carp ploidy manipulation are: pressure shock – usually 7,000–8,000 psi; thermal (cold) \approx 5–10 °C; or thermal (heat) \approx 39–42 °C. The precision of the time of shock application differs somewhat for the types of shock, which might affect the induction efficiency. The effectiveness of the shock type on the induction efficiency may vary with species, and whether 2Pb or Mt shock is applied.

Activated AGC-eggs were exposed to early shock (at 0.15–0.2 τ_0) in order to retain the

second polar body. Ploidy induction for AGC grass carp has been most effective for meiotic Pb-shock, at $0.24\text{--}0.28 \tau_0$ or $1.6\text{--}1.9 \tau_0$ for late shock. Survival of hatched larvae for cold shock (10°C) was inversely proportional to the duration of shock: 11.1, 1.3, and 0.1%, at 10, 20, and 30 minutes, respectively.

Grass carp eggs are sensitive to lower temperatures [102]. Survival of 8–10 hours old embryos from heat- or pressure-shocked treatments was 89.0%, but decreased for swim-up larvae. These experiments demonstrated that large-scale production of all-female AGC could be developed relatively quickly. Further, all-female sterile triploids can be produced using this approach, with only minor modifications to the protocol (Tables 41.4 and 41.5).

41.4.7.2 Steroid-Induced Sex Reversal of AGC Gynogenotes

Homozygous recessive phenotypic markers in females are useful in ploidy manipulation to verify that progeny are gynogenotes and not hybrids. In 1994, 850 AGC gynogenotes

were produced in the verification study, then in 1995, 5-mg MT implants were introduced into 33 of these gynogenotes (Size = 58–84 mm) in order to develop neomale AGC (female genotype) (Table 41.4). These were retained in aquaria for observation, and within 14 days, 21 implants out of 33 were rejected; however, all treated fish were stocked into grow-out ponds. In 1997, 12 of these fish were recovered and eight were spermiating. Two were used to fertilize eggs from AGC females to produce an F_2 generation. Thirty-one female AGC (81–105 mm) were implanted, but these were not gynogenotes, instead they were progeny from sex-reversed neomales implanted in 1995. These fish were larger than the 1995-treatment fish and only six rejected the implant. About 20,000 female monosex fry were produced from the neomale AGC and AGC females.

41.4.7.3 Monosex Triploid AGC

All-female progeny can be produced through gynogenesis, but optimized protocol also can be an efficient means of estimating optimum

Table 41.5 Optimized induction of gynogenesis and triploidy for grass carp.

Induction treatment	Parameters: psi or $^\circ\text{C}$ /duration in minutes (best % yield)	Application time τ_0 (pre-shock $^\circ\text{C}$)	Ref.
Heat ($^\circ\text{C}$)	$40^\circ\text{C}/1 \text{ min}$ (8%)	$0.3 \tau_0$ (+4 min @ 25.5)	119
	$42^\circ\text{C}/1 \text{ min}$ (100%)	$0.3 \tau_0$ (+4 min @ 25.5)	112
	$40^\circ\text{C}/1 \text{ min}$ (50%)	$0.3 \tau_0$ (+4 min @ 25.5)	112
	$38^\circ\text{C}/3.0\text{--}3.5 \text{ min}$ (80%)	$0.08 \tau_0$ (+1 min @ 26.0)	113
	$40^\circ\text{C}/2 \text{ min}$ (600 gyno.)	$0.15\text{--}0.25 \tau_0$ (+4 min @ 21.0)	63
Cold ($^\circ\text{C}$)	$5\text{--}7^\circ\text{C}/25 \text{ min}^*$ (50–100%)	$0.11\text{--}0.3 \tau_0$ (+2.0–4.5 min @ 25.5)	119
	$4^\circ\text{C}/3 \text{ min}$ (40%)	$0.2 \tau_0$ (+2.5 min @ 26)	113
	$4\text{--}6^\circ\text{C}/10\text{--}12 \text{ min}^{**}$	$0.11\text{--}0.17 \tau_0$ (+2–3 min @ 23)	120
	$10^\circ\text{C}/10 \text{ min}$ (11%)	$0.15\text{--}0.25 \tau_0$ (+4 min @ 21)	63
	$10^\circ\text{C}/20 \text{ min}$ (1%)	$0.15\text{--}0.25 \tau_0$ (+4 min @ 21)	63
Pressure	8,000/1.5 min (67%)	$0.3 \tau_0$ (+4 min @ 25.5)	112
	7,000–8,000/1–2 min (78%)	$0.15\text{--}0.20 \tau_0$ (+3.4–4.5 min @ 21)	63

*Temperatures of less than $5\text{--}7^\circ\text{C}$ for less than 25 minutes did not induce 3N, and durations of longer than 30 minutes were lethal.

**Temperatures of less than 4°C or $\Delta\text{--}^\circ\text{C}$ longer than 12 minutes increased mortality; 18,170 gynogenotes produced in five years.

treatment protocol for polyploidization [54]. Early shock gynogenesis optimization identifies the treatment parameters for best triploid production; monosex triploids were produced in the verification study. Evaluation of optimal treatment relationships through gynogenesis is further facilitated if a visual phenotypic marker is used.

In triploidization procedure for grass carp, fertilized eggs are early-shocked in order to retain the 2PB, which comprises the third set of chromosomes in the triploid fish. In general, triploid fish have poorly developed gonads when compared to diploids. Triploids generated by such a method possess two possible sex genotypes – either XXY (male) or XXX (female) triploid. The ovaries of XXX-triploids are totally undeveloped, with the exception of occasional ova [103]. Males (XXY) may have nearly normal-size testes but with limited spermatogenesis; only few aneuploid germ cells appear, and the production of mature gametes does not recover with age [104]. Cytological studies have demonstrated only about 60 viable spermatids for every billion cells, and that even with artificial insemination, using normal eggs from diploid females, no viable larvae were produced [103, 105, 106]. In 1984, the US Fish and Wildlife Service issued a biological opinion that female triploid grass carp are functionally sterile, and that gametes are probably non-functional [107].

Eggs collected from AGC females fertilized with AGC-sperm were used in experiments to induce triploidy [63] (Table 41.5). Pressure- and thermal (cold and heat)-shock induction were compared with fertilized eggs from each female; one batch was pressure-shocked (7,500 psi/70 seconds), while the other cold-shocked ($10 \pm 1^\circ\text{C}/10$ minutes). Fertilized AGC eggs of other females were exposed at $0.2 \tau_0$ to pressure-shock (7,000–8,000 psi/1–2 minutes) or heat-shock ($40 \pm 1^\circ\text{C}/2$ minutes). The survival rates of 8–10 hour old embryos were examined. Induction of triploidy was successful in most of the trials conducted. The yield of triploids out of total fish showed

either low rates (10–20%), or very high rates (90–100%). The results establish the ability to produce large numbers of monosex triploids.

Since triploid induction using normal grass carp brood stock will produce both males (XXY) and females (XXX), monosex triploid grass carp might offer a safer stocking option. Production of exclusive female-triploid populations would add an increment of security against unwanted reproduction. All-female triploid grass carp have lower reproductive potential than triploid males, based on gonadal development differential. Females are totally sterile, unlike the triploid males (XXY), which do possess some testicular fragments and, occasionally, produce a very low number of viable spermatozoa.

All-female triploid grass carp (XXX) were produced by early-shock (Pb) of AGC eggs fertilized with sperm of neomales (sex-reversed gynogenotes). The use of neomales provides a mechanism for commercial-scale production of sterile all-female AGC. In 1997, two AGC neomales were available after only two years from the initiation of the study; one was used to fertilize eggs from an AGC female, yielding about 20,000 offspring.

41.4.8 Commercial Triploid Production in the United States

41.4.8.1 History of Triploid Grass Carp Production in the United States

The application of reproductively limited fish to aquaculture was a logical precursor to considering exotic fish introductions, but the control measures that are now available were developed only after the earlier stocking of mixed sex fish had occurred [58]. Sterile triploidy was first developed in grass carp in a serendipitous hybridization study [108]. The induction of polyploidy is analogous to gynogenesis, except that non-irradiated sperm from conspecifics is used for fertilization, and the male genome is incorporated [109].

The triploid-hybrid grass carp (grass carp female X bighead carp male) had a particular

significance to triploid grass carp production in the United States. Through personal communication with Hungarian scientists, a US fish farmer learned of this cross. Diploid hybrids from grass carp and bighead carp are generally inviable but, when the second meiotic polar body is retained, only viable 3N-hybrids survive. This phenomenon was used by a private US fish farmer to market sterile-hybrid grass carp for aquatic weed control during the early 1980s [76, 110, 111]. The commercial success of the triploid-hybrid grass carp stimulated research into direct triploidization of grass carp [112, 113]. The commercial production of triploid grass carp has been practiced in the United States since about 1985. However, since some diploids are also produced during direct induction, ploidy examination is required for each fish, so as to cull diploids.

41.4.8.2 Grass Carp Triploidy Protocol in the United States

Individual verification of triploidy is necessary by one of several techniques: karyotyping, red-blood-cell nuclear analysis (microscopic or coulter counter), or quantitative DNA determination (flow cytometry) [114, 115]. Machine techniques for ploidy verification have facilitated the process [106], and have provided a means of commercialization of 3N-grass carp production in the United States. [4]. In practice, the fish are tested several times by the producer, then rechecked before sale to assure that the group is free of diploids. Each individual fish is tested by the producer one or more times during grow-out, and then to certify that the group is free of diploids, a sub-sample is independently examined before shipment to the client and, finally, another sub-sample is usually checked at the destination. The US Fish and Wildlife Service has facilitated verification through the National Triploid Grass Carp Inspection and Certification Program (<https://www.fws.gov/warmsprings/FishHealth/frgrscrp.html>).

To meet the demands for biological control of nuisance aquatic plants, the production of

triploid grass carp in the United States has greatly expanded in the last few decades, and quality control has been enhanced by operational techniques in growth management. Diploid fingerlings grow more rapidly than triploids under the same conditions [116], and this characteristic is used to progressively cull a high percentage of diploids. The basis of ploidy differentiation is that red blood cell nuclei of triploids is larger than diploids, and also contain more DNA.

The coulter counter is most commonly used in the industry for triploid testing. The equipment is relatively inexpensive, and a regimen of workers can test a reasonable number of fish in a day. A three-person team collects a 1 μ L blood sample from each fish and, using a coulter counter, can evaluate around 2,000 small fingerlings in an eight hour day [117]. This initial sampling is preceded by a regimented nursing protocol [113]. Four-day swim-up fry are stocked at about 250,000/ha for 28 days, then harvested and graded. Bar-graders, with 7.9, 12.5, and 15 mm spacing, are used to separate the fish into size groups. Blood from fish in each of these four size groups is tested on a coulter counter. Because of the faster growth of 2N fish, only fish in the size groups with greater than 75% 3N are re-stocked [116].

The second stocking of predominantly 3N-fish is at about 25,000/ha for a growth period of four months. A second harvest repeats the analysis, and only verified 3N fish are restocked to grow to market size of greater than 30 cm TL, which is recommended for fishery management; size at stocking into non-culture ponds provides minimal likelihood of predation by large-mouth [118].

Acknowledgements

The authors thank Alexander Recoubratsky for providing some articles, and sincerely appreciate the extensive contributions of students and long-time collaborators.

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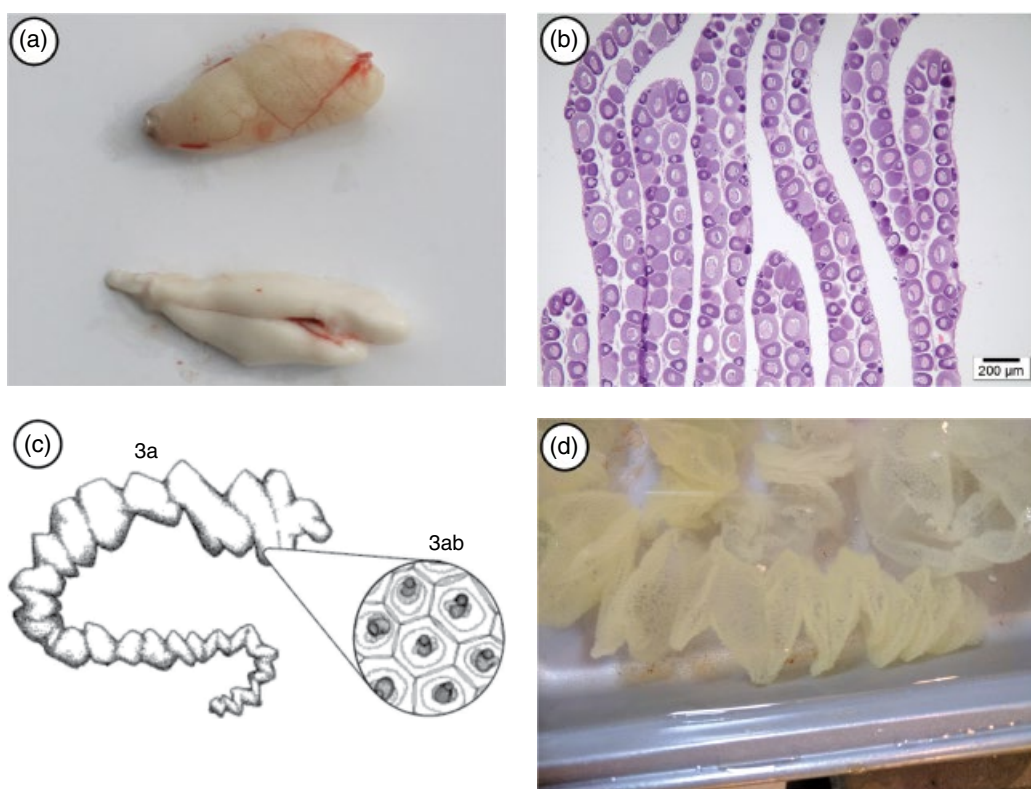


Figure 20.1 Unique ovary morphological structure of yellow perch *Perca flavescens*.

a) single ovary vs. paired testis.

b) ovary histology displays advanced internal organization of oocytes.

c) egg ribbon and magnified eggs (credited to J. M. Hinshaw, North Carolina State University).

d) egg ribbon right after fertilization.



Figure 20.2 Ovatestis in the 54–78 dph group with 20 mg/kg treatment.

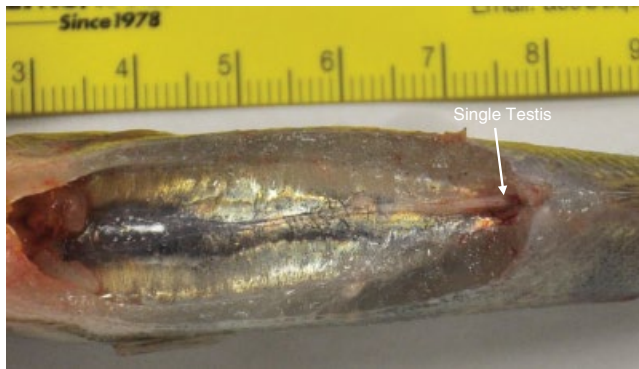


Figure 20.3 Sex-reversed neomales with a single testis in the 54–78 dph group with the 50 mg/kg treatment.

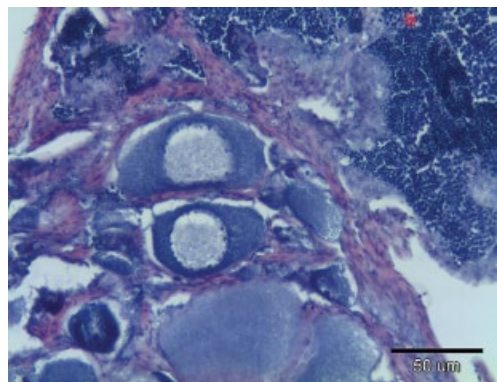
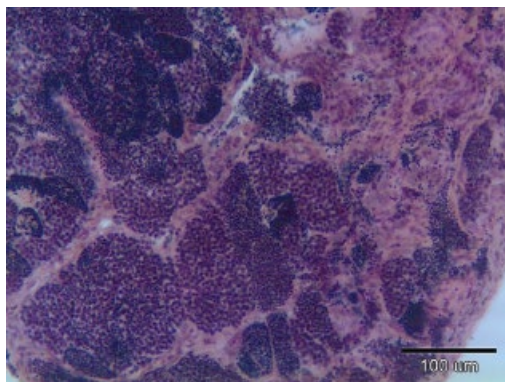
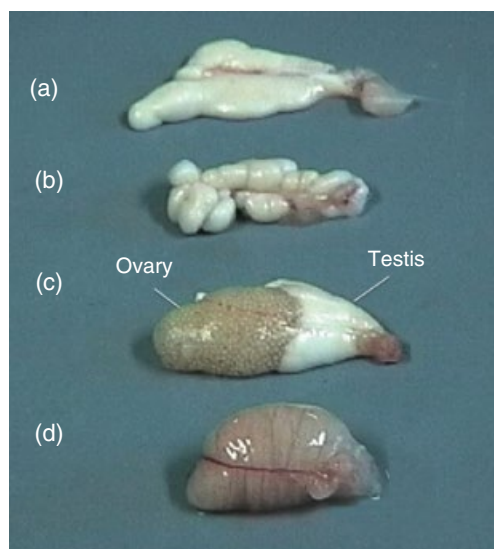


Figure 20.4 Intersex tissue in ovatestis in the 54–78 dph group with 20 mg/kg treatment.

Figure 21.5 Gross morphology of Eurasian perch gonads.

- a) Double testis of normal XY male;
- b) single twisted testis with nodules of hormonally (MT) sex-reversed XX males;
- c) ovotestis with ovarian and testicular tissues of partially hormonally (MT) sex-reversed XX males;
- d) normal single ovary of normal XX female (from Rougeot *et al.* [19]).



(a)



(b)

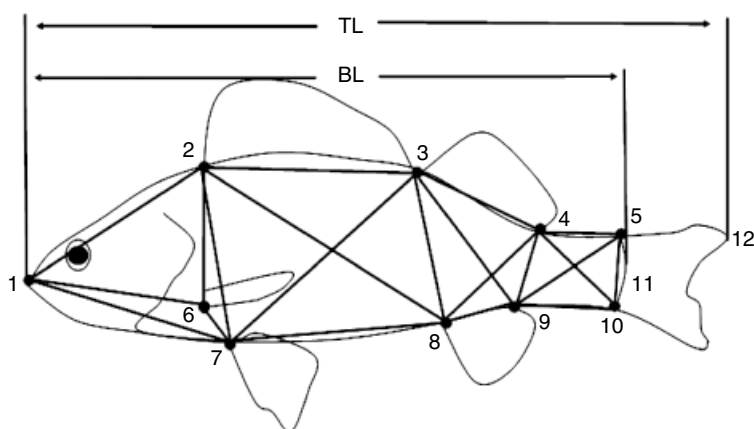


Figure 22.1 The truss network, based on 12 landmarks, was used for morphological measurement on yellow perch. The morphological characters described in this study were shown in Table 22.2.

- a) One specimen of *Perca flavescens* with 12 landmarks (red stars refer to the locations of landmarks).
- b) One pattern of morphometric measurements that were measured between the landmarks as lines. 1: anterior tip of snout; 2: anterior insertion of first dorsal fin; 3: posterior insertion of first dorsal fin; 4: posterior insertion of second dorsal fin; 5: dorsal origin of caudal fin; 6: bottom of pectoral fin; 7: origin of pelvic fin; 8: origin of anal fin; 9: terminal of anal fin; 10: ventral origin of caudal fin; 11: anterior margin of the caudal fin; 12: upper terminal of caudal fin.

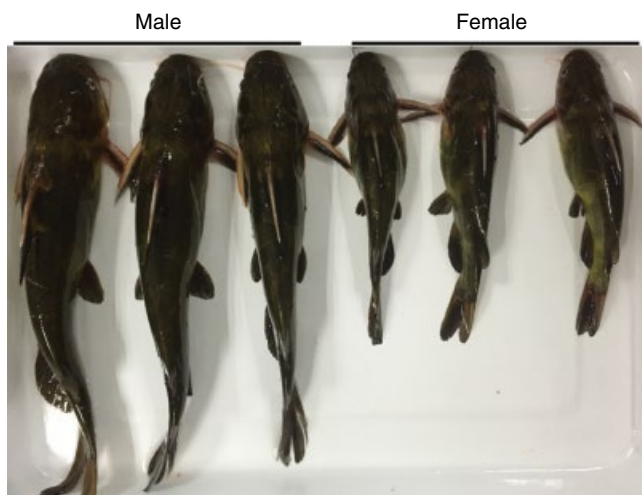


Figure 24.2 The body size difference between male and female yellow catfish.

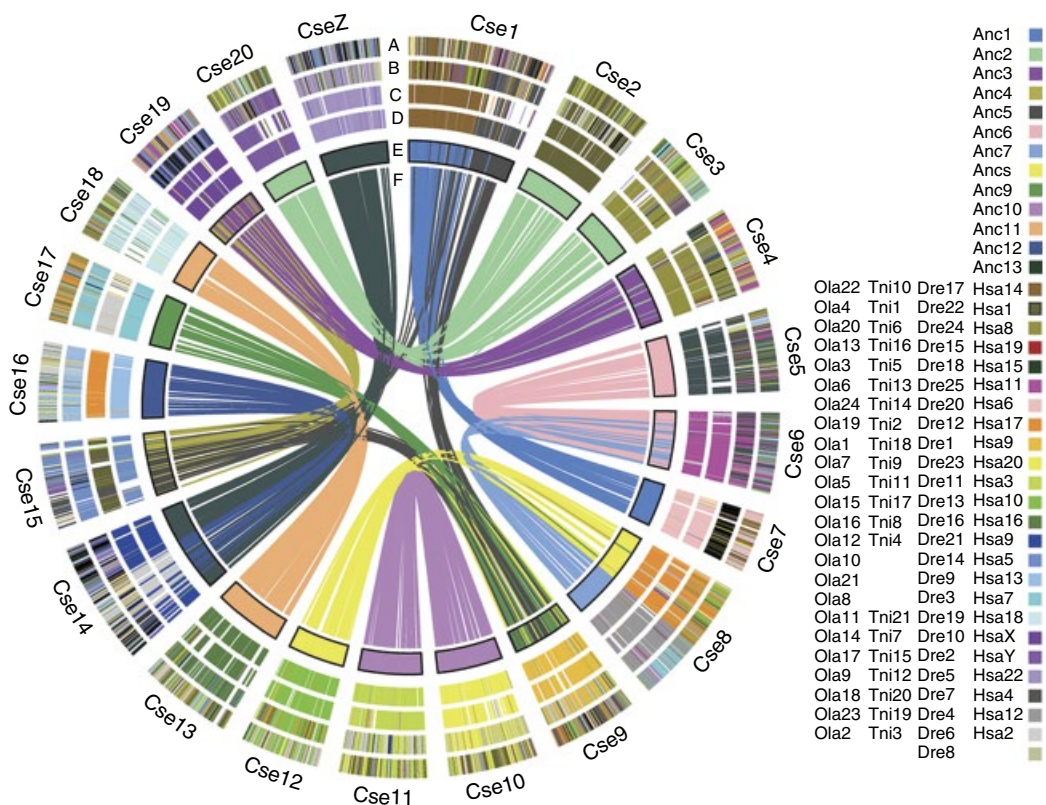


Figure 26.3 Teleost genome evolution. WGD in the tongue sole and orthology in the medaka, *T. nigroviridis*, zebrafish and human genomes. The arcs of concentric circles represent each tongue sole chromosome (Cse1–Cse21 and Z). A–D represent tongue sole chromosomes painted with different colors according to the location of the orthologs in the human (Hsa), zebrafish (Dre), *T. nigroviridis* (Tni), and medaka (Ola) genomes. A 100 kb region around a gene is painted in the same color. E represents tongue sole chromosomes painted by the corresponding ancestral chromosomes (Anc1–Anc13). In F, each line joins duplicated genes at their respective positions (Cited from [6]).

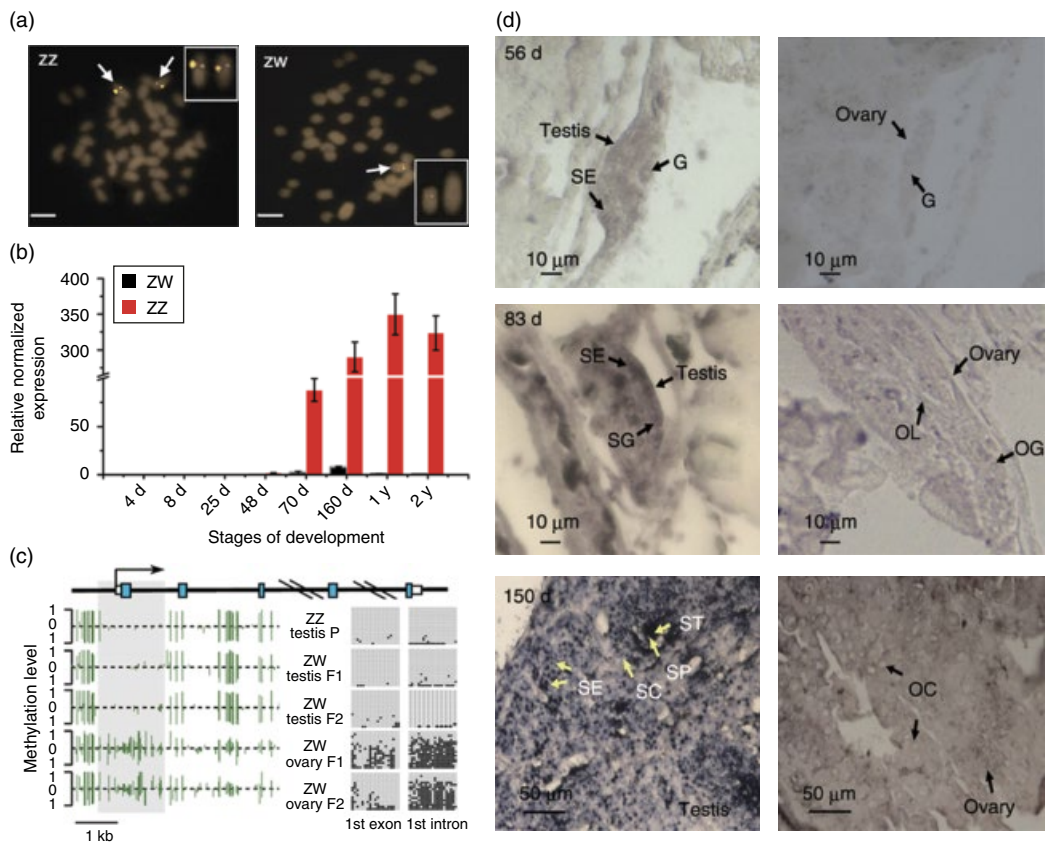
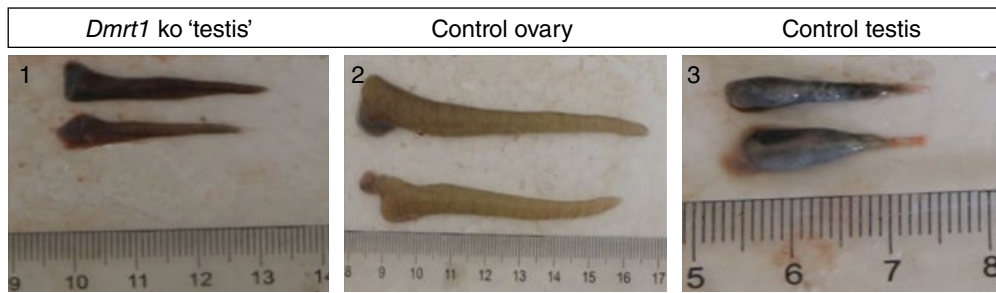


Figure 26.4 Characterization of *dmrt1* in tongue sole.

- a) *dmrt1* BAC FISH analysis of tongue sole chromosomes, showing a double signal in males and a single signal in females. BAC clone Hind012D10-3J, which contains the full-length *dmrt1* gene, was labeled and used to probe male (ZZ) and female (ZW) chromosome spreads. Scale bars: 5 μ m.
- b) RT-PCR analysis of *dmrt1* during developmental stages in female (black bar) and male (red bar) tongue sole. The data are shown as the mean \pm s.e.m. ($n=3$).
- c) Methylation status across the differentially methylated region (DMR) of *dmrt1* in the gonads of an adult WZ female, a ZZ male and a WZ female, compared with male sex-reversed fish. The schematic diagram at the top shows the genomic structure of *dmrt1* in tongue sole. Exons are depicted as blue boxes, and the 3' and 5' UTR regions are indicated by white boxes. The black arrow indicates the direction of the *dmrt1* gene from transcriptional start site. Also shown is the methylation level of each cytosine, indicated by a green line, identified on both DNA strands throughout the *dmrt1* gene in female and male fish. The gray shadow indicates the DMR. Open and filled circles represent unmethylated and methylated cytosines, respectively, validated by TA clone and Sanger sequencing. ZZ testis P – testis of the male parent; ZW testis F1 – testis of a pseudomale in the first generation (temperature induced); ZW testis F2 – testis of a pseudomale in the second generation (untreated); ZW ovary F1 – ovary in the first-generation female; ZW ovary F2 – ovary in the female offspring of a pseudomale.
- d) Specific expression of *dmrt1* in testis. Gonad *in situ* hybridization using the antisense RNA probe of *dmrt1* performed in tongue sole larvae at 56, 83 and 150 days during the gonad-development stage. G – gonium; OG – oogonium; OL – ovarian lamellae; OC – oocyte; SG – spermatogonia; SC – spermatocyte; SE – sertoli cell; SP – spermatid; ST – spermatozoa.
- (Cited from [6]).

(a)



(b)

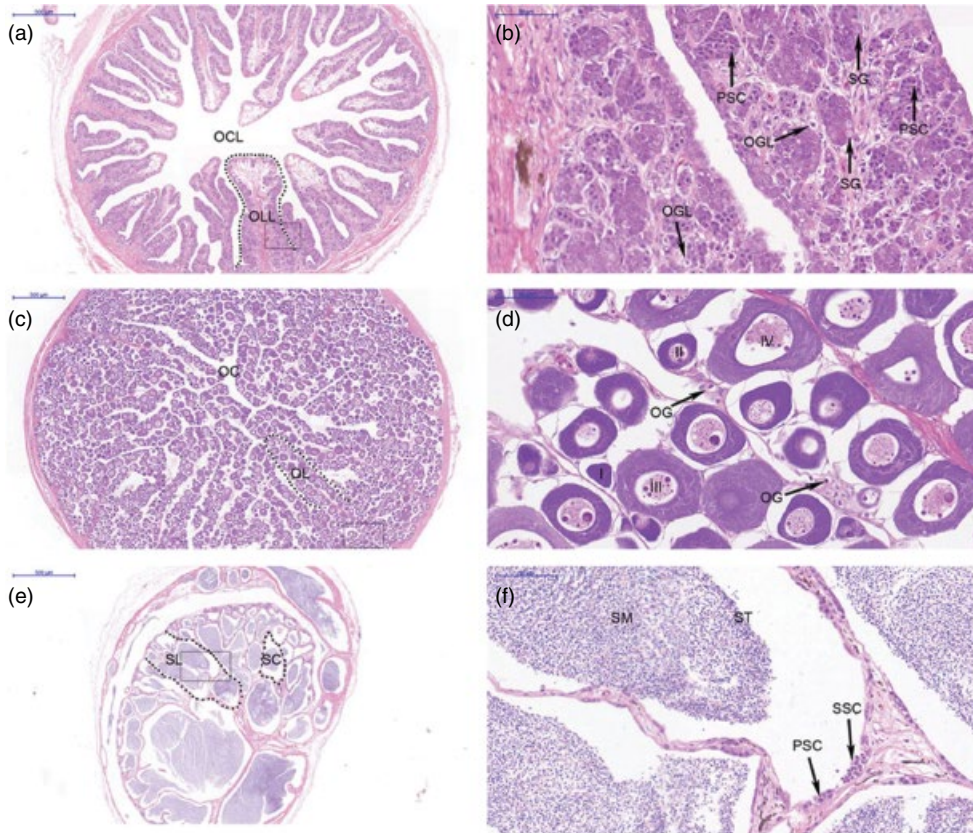


Figure 26.5 Effects of *dmrt1* disruption on gonad phenotype, sex differentiation.

a) Gross morphology of gonads from approximately one year old fish: (1) *dmrt1*-deficient "testes"; (2) wild-type ovaries; (3) wild-type testes.

b) histology of gonads from approximately one year old fish: (a) *dmrt1*-deficient testis. The development of testis is ceased. The shape of the *dmrt1*-deficient testes in transverse sections is similar to control ovaries, and there are structures resembling ovarian cavity and ovarian lamella in the gonad of the mutant male fish. Ovarian cavity-like (OCL); ovarian lamella-like (OLL); (b) large magnification of frame area in (a). No secondary spermatocytes, spermatids and sperm are observed. Oogonia-like (OGL); spermatogonia (SG); and primary spermatocytes (PSC). (c) Ovary of control female, including ovarian cavity (OC), ovarian lamella (OL); (d) large magnification of frame area in (c). Four stages of oocytes: stage I-IV and oogonia (OG). (e) Testis of control male. seminiferous lobuli (SL), seminiferous cyst (SC); (f) larger magnification of frame area in (e). Secondary spermatocytes (SSC), spermatids (ST) and sperm (SM). Scale bar is shown in the figures.

(Cited from [13].

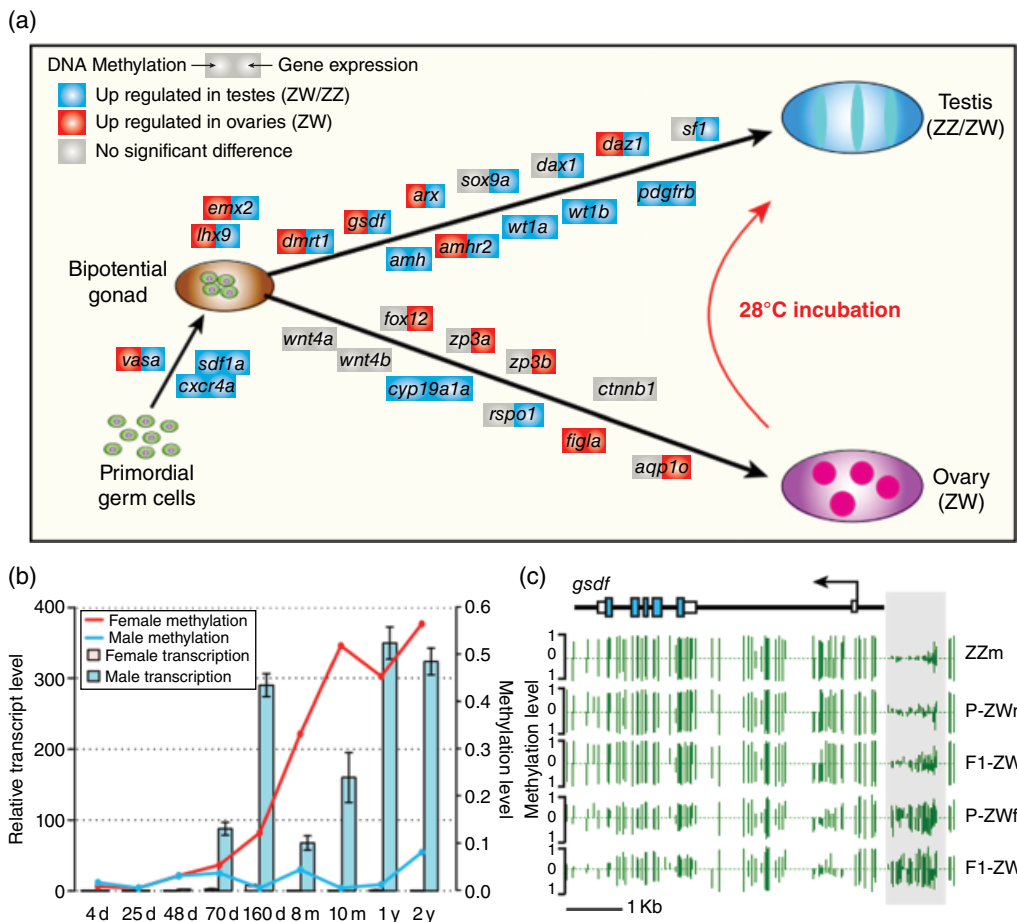


Figure 26.6 Differential methylation and sex determination.

- a) Differentially methylated and differentially expressed genes in the putative sex determination pathway of tongue sole. For each gene presented in the pathway, the methylation (left square) or expression (right square) changes when comparing testes with ovaries are shown by different colors.
- b) DNA methylation and transcription of *dmrt1* in different developmental stages after hatching. The methylation levels of different stages were estimated using bisulfite-PCR, followed by TA-cloning with a pair of primers targeting the first exon, always using at least 10 randomly selected clones for sequencing for each stage.
- c) DNA methylation profiles of *gsdf* in the five gonadal samples. Green vertical lines indicate the methylation level of cytosines. The light gray box indicates the DMR upstream of *gsdf*. (Cited from [14]).

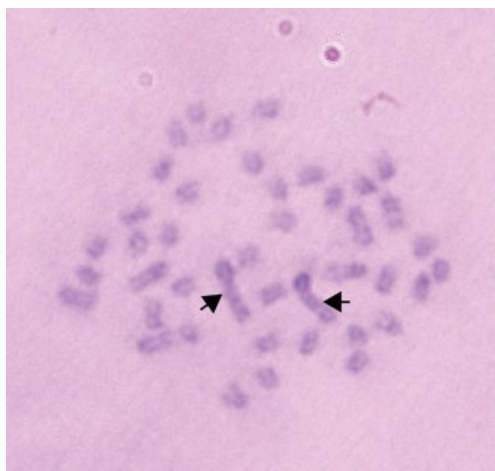


Figure 27.8 Karyotype of gynogenetic WW embryo; two huge WW chromosomes are indicated by arrows.

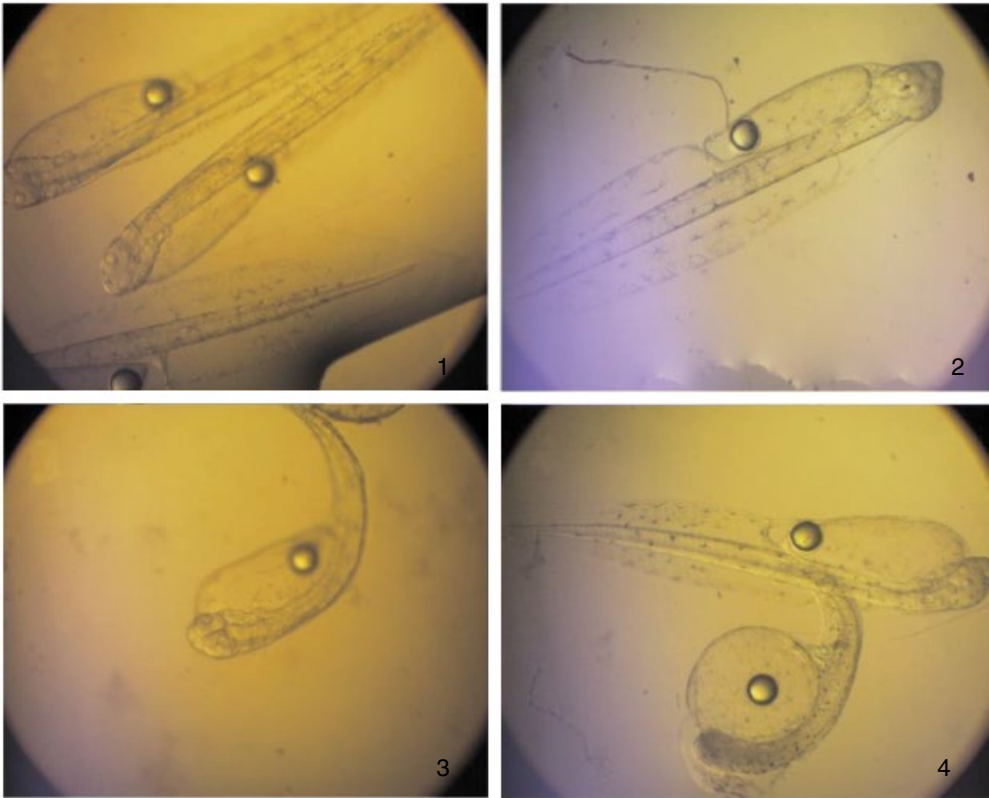


Figure 29.4 Larvae morphology of gynogenetic haploid and diploid in summer flounder. 1: normal diploid; 2: gynogenetic diploid induced with homologous sperm; 3: gynogenetic haploid induced with heterologous sperm; 4: gynogenetic diploid (upper) and haploid (lower) induced with heterologous sperm. Figure reproduced from that published by Yang [37].

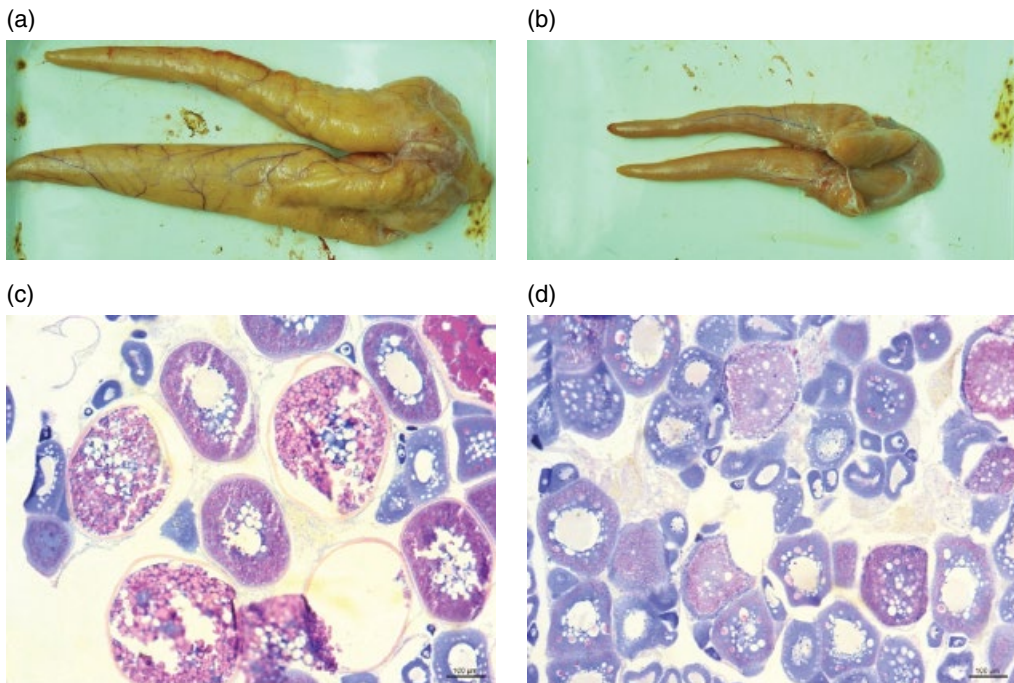


Figure 30.2 The shape and histological sections of fertile and sterile gonads of doubled haploid Japanese flounder, *Paralichthys olivaceus*.

A: Shape of fertile gonad; B: Shape of sterile gonad; C: Histological section of fertile gonad; D: Histological section of sterile gonad.

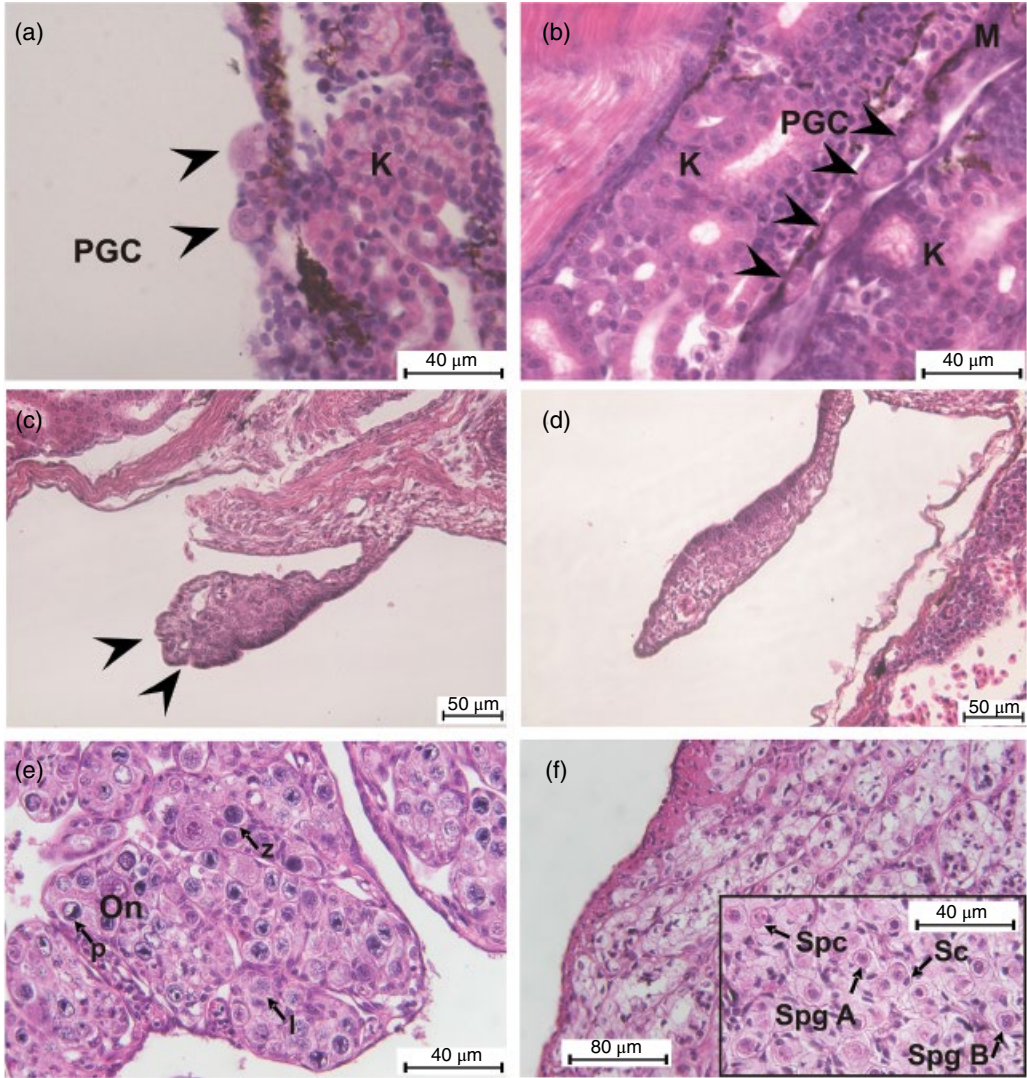


Figure 33.1 Early cytotological germ cell and anatomical gonad differentiation in sterlet *Acipenser ruthenus*, from germ cell migration, genital ridge formation to anatomically distinct gonads:

- PGCs (arrowhead) with a high nucleus/cytoplasm ration (25–30 μm) migrating within the genital ridge of the embryo 5 dpf (sagittal longitudinal section);
- PGCs observed along the dorsal mesentery (M) between the right and left kidney (K) in 28 dpf larvae (coronal longitudinal section);
- anatomical differentiation of the ovary 82 dpf indicated by notches/folds (arrowheads) of the columnar epithelium compared to a;
- "smooth" epithelium without notches, indicative of a male gonad (juveniles were 11.8 cm and 9.8 cm);
- ovary of a 9-month-old female with nests of oocytes (On) at different meiotic stages (l-leptotene, p-pachytene, z-zygotene respectively);
- testis of a 10-month-old male with spermatogonia A and B (Spg A, Spg B), spermatocysts (Spc) and sertoli cells (Sc). HE staining

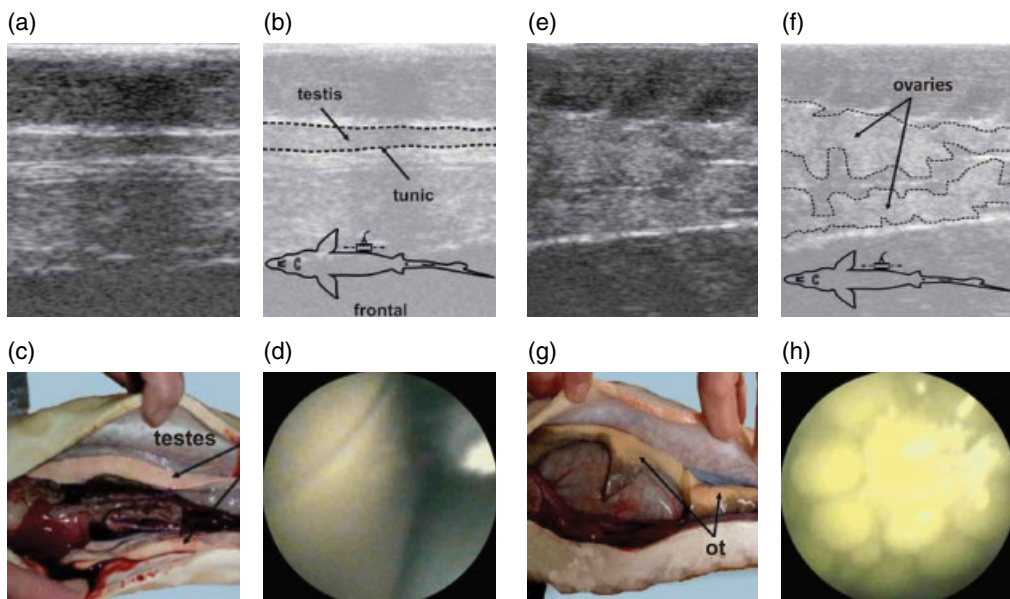


Figure 33.3 Current sexing methods in sturgeon aquaculture, illustrated in a male (a–d) and a female (e–h) Russian sturgeon. The testes (a, b) appear as a homogenous tissue strand with smooth margins, compared to the irregular form of the fine grained ovarian tissue (e, f) using sonography. The irregular form of the ovigerous lamellae can be observed macroscopically (g), whereas the margin of the testes appears rather smooth and continuous (c). By endoscopy, small oocytes can be observed (h). ot- ovarian tissue From: Chebanov, M.S. and Galich, E.V. (2010). *Ultrasound diagnostics of sturgeons*. FSGTSR, Krasnodar. Izdatel' stvo Prosveshchenie-Yug. 135 pp.

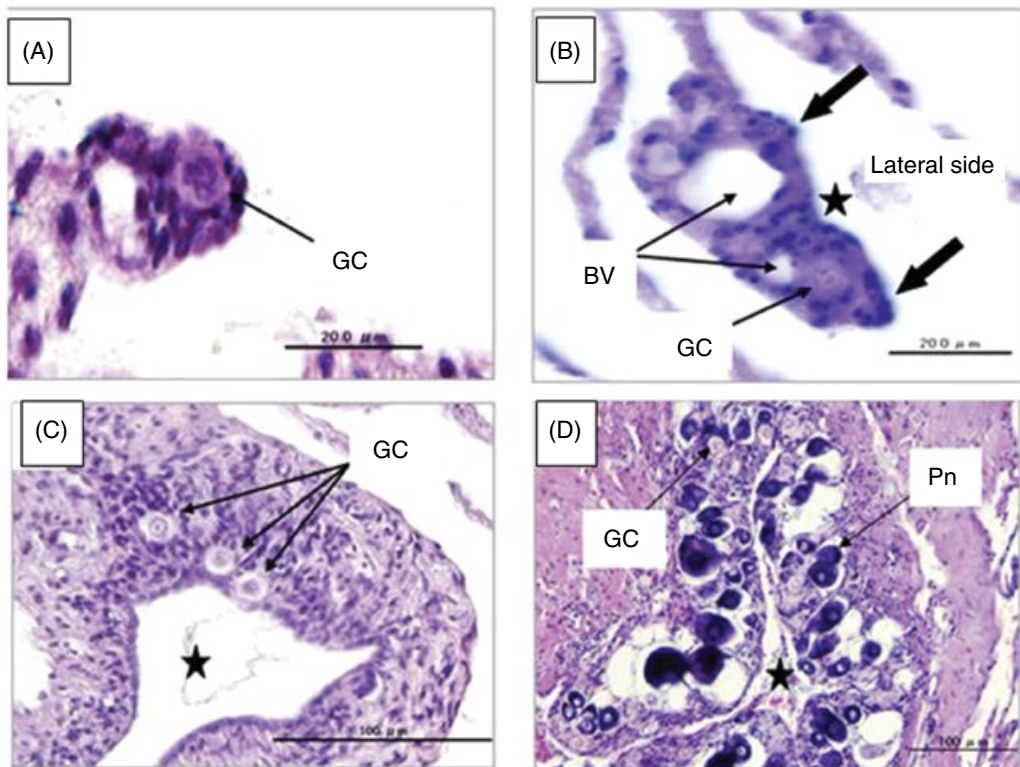


Figure 38.1 Gonadal sex differentiation of Malabar grouper.

(A) Undifferentiated gonad at 39 days post-hatch (dph), which consists of individual oogonial germ cells enclosed by a few somatic cells.
 (B) Initial ovarian differentiation at 47 dph. Two elongations of somatic tissues (arrow), indicate initial ovarian cavity formation. Asterisk (*) indicates the side of lateral wall.
 (C) An ovary at 144 dph. Single oogonia are seen in the somatic tissue. Asterisk (*) indicates the ovarian cavity.
 (D) An ovary at 720 dph. Many oocytes at the peri-nucleolus stage (Pn), together with oogonia are seen.
 GC – germ cell. BV – blood vessel.

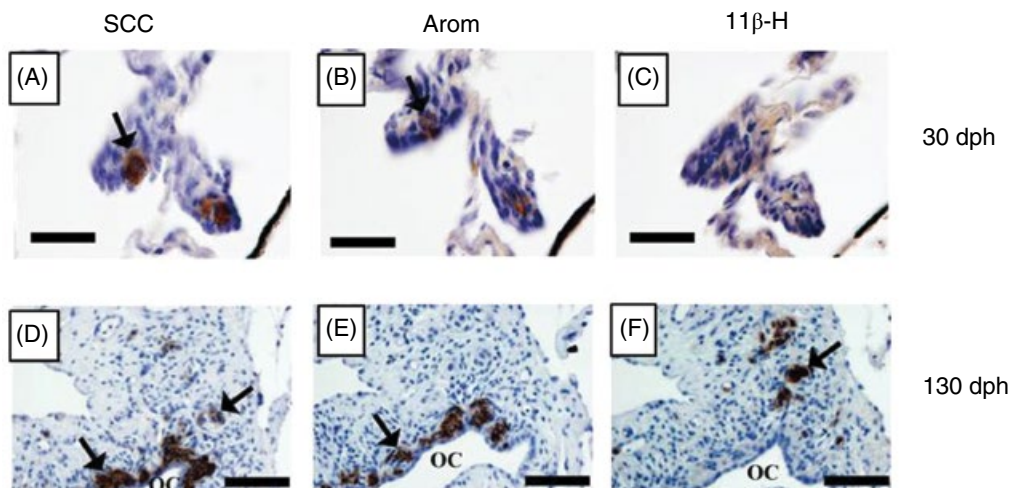


Figure 38.2 Gonads of Malabar grouper during ovarian differentiation. Undifferentiated gonads at 30 dph (A-C). Differentiating ovaries at 130 dph (D-F). A and D: Immunostaining with anti-Cyp11a1. B and E: immunostaining with anti-Cyp19a1a. C and F: immunostaining with anti-Cyp11b. Arrows indicate positive immunoreactivities. OC – ovarian cavity. Scale bar = 20 μ m (A–C), 50 μ m (D–F).

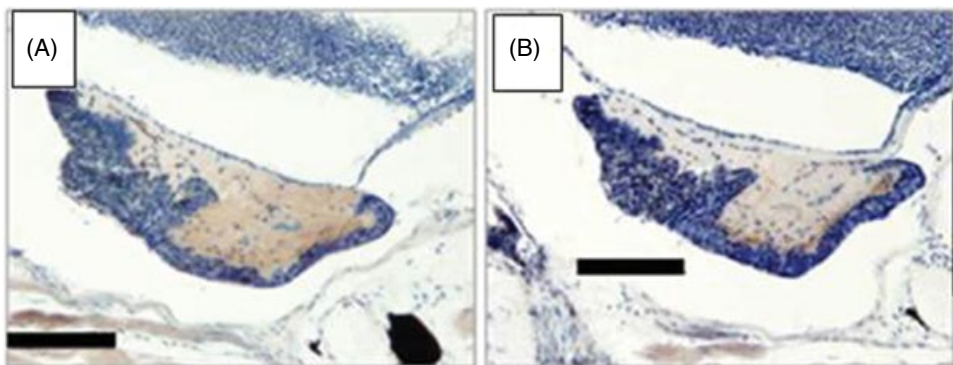


Figure 38.3 Pituitaries of Malabar grouper at sexual differentiated stage. Positive reactions against anti-*Fshβ* (A) and anti-*Lhβ* (B) are not detected. Scale bars = 100 μ m.

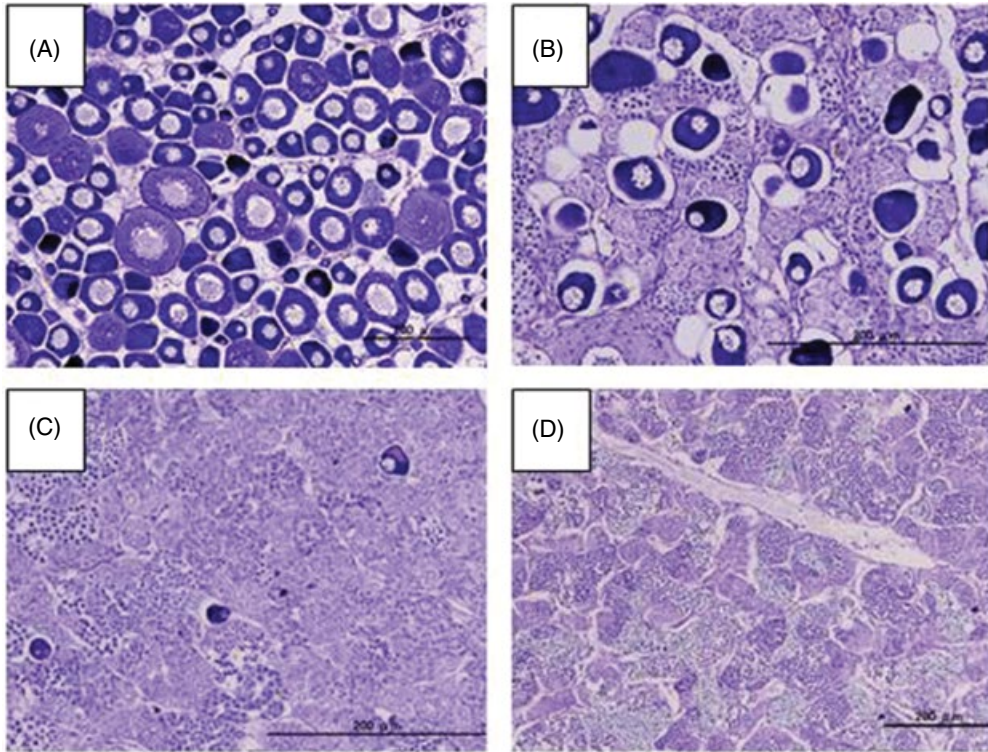


Figure 38.4 Gonadal stages of honeycomb grouper during female-to-male sex change.
 (A) Female phase, containing many immature oocytes at the peri-nucleolus stage.
 (B) Early transitional phase, characterized by degenerating young oocytes and the active proliferation of spermatogonia.
 (C) Late transitional phase, with active spermatogenic germ cells occupying the ovigerous lamella.
 (D) Male phase in the breeding season, with active spermatogenic germ cells.
 Scale bar = 200 µm.

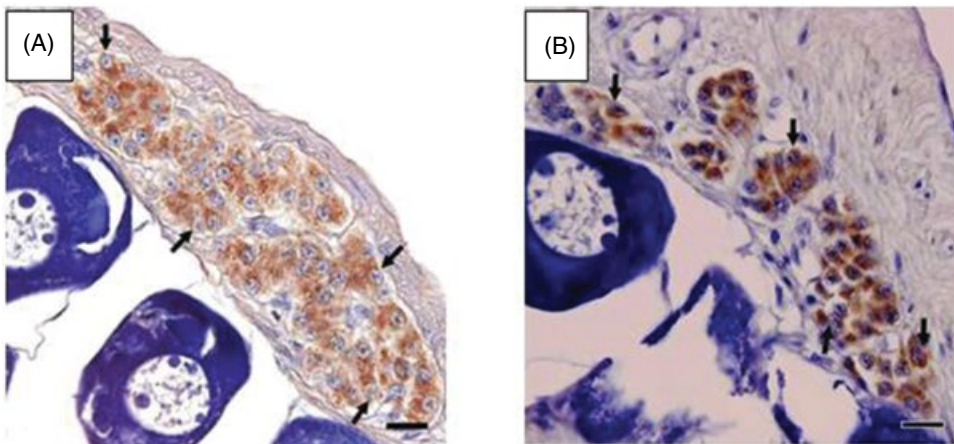


Figure 38.5 Immunopositive reactions against anti-Cyp11a1 (A) and anti-Cyp11b (B) in the tunica ovary of honeycomb grouper. Arrowheads indicate clusters of immunopositive cells.
 Scale bar = 10 µm.

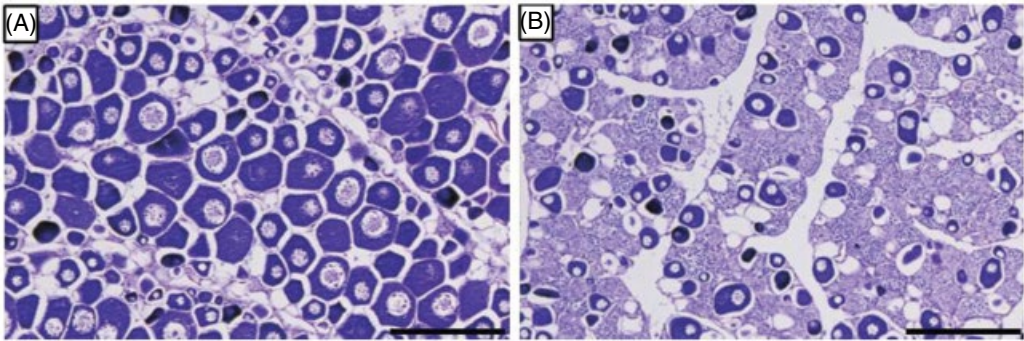


Figure 38.6 Gonadal sections of honeycomb grouper treated with only molten cocoa butter as control (A), or with 500 ng/fish of bovine FSH (B) for three weeks.
 (A) Gonads showing many previtellogenic oocytes.
 (B) Primary oocytes and active spermatogonial proliferation were observed in the gonad simultaneously. Thus, we characterized these fishes as sex-changing.
 Scale bars = 200 μ m.

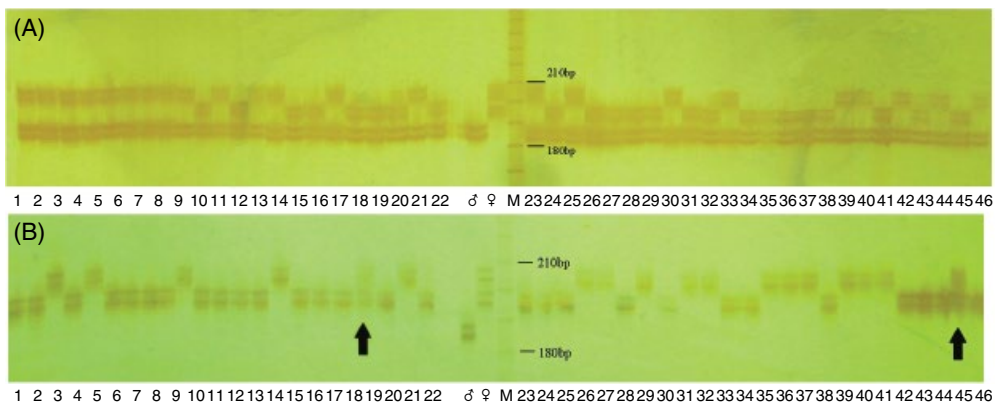


Figure 39.5 Genotypes segregation at microsatellite locus *LYC0022* in control family 1 (A) and Gynogenetic family 1 (B). Lane M: DNA ladder; ♂: male parent; ♀: female parent; lane 1–46: samples of progenies; bp: base pair. Arrows indicate the individuals with heterozygous genotype. Adapted from Reference [3].

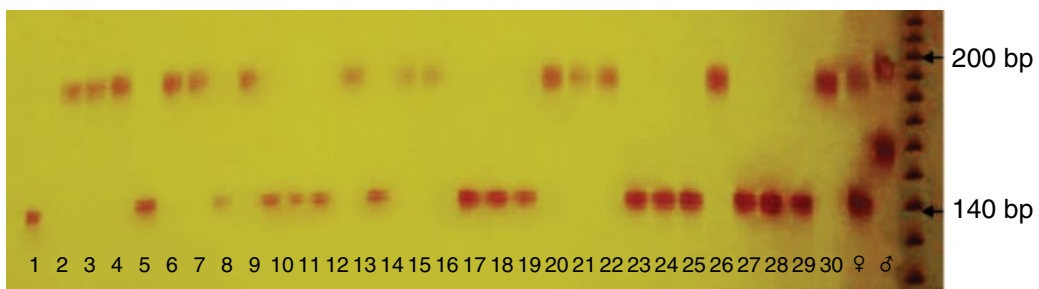


Figure 39.9 The electrophoresis patterns of family GF1 at LYC0026 microsatellite locus (adapted from [11]).

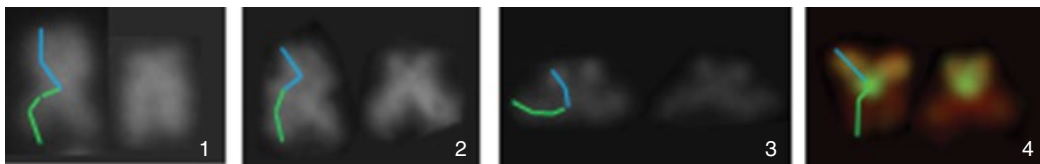


Figure 39.12 Chromosome 10 of *L. crocea* male 1: PI- staining; 2: DAPI- staining; 3: DPI- staining; 4: chromosomal localization of H-P3K by FISH. Blue lines indicate the midrib line of the short arms, green lines indicate the midrib line of the long arms. Adapted from [12].