THE FISH OOCYTE
From Basic Studies to Biotechnological Applications

The Fish Oocyte

From Basic Studies to Biotechnological Applications

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PREFACE

Fish and their prolific nature serve as symbols in diverse human cultures, religions, and societies. Fish eggs not only serve as food, with caviar as one of the most famous luxuries on our menus, but they also form the basis for aquaculture that produces fish as a plentiful food source. Obtaining "good-quality" eggs is crucial and essential for successful production of fish in captivity. Aquaculture production already accounted for almost 22% of fish produced worldwide in 2002 (http://www.fao.com), and is steadily growing. At the same time, natural fish populations, including some of immense biological, ecological, and commercial importance, are dwindling alarmingly, leading to the need for investigations into ways of replenishing stocks in their natural environment. Research into the formation of fish eggs addresses this issue and strikingly illustrates the statement of William Harvey in 1651: "Omne vivum ex ovo" (All life comes from eggs).

The term "fish" includes hagfishes, lampreys, chondrichthyans (sharks, rays, and chimaeras), actinopterygians (ray-finned fishes), and actinistians (coelacanths and lungfishes). This group is clearly paraphyletic, as there is no character that can exclusively define fishes. Among them, teleost fish represent the largest and most diverse group of vertebrates (about 29,400 species, FishBase, www.fishbase.org, version 09/2006). Fish display an astonishing variety of reproductive modes, i.e. gonochorism (dioecism), protandry, protogyny, true hermaphroditism, and parthenogenesis. The ultimate fate of the developing gonads is governed by a delicate balance of genetic and environmental factors. Ovaries arise as bilateral primordia but, in some species, only one ovary is functional and hermaphrodites may have ovotestis, containing both ovarian and testicular tissue. The ovary is composed of follicles, derived from the germinal epithelium, and contains oogonia that pass through meiosis to become oocytes and, ultimately, ova. While the structure of growing ovarian follicles is remarkably similar in most fishes, their development inside the ovary may be synchronous, group synchronous, or asynchronous, leading to fecundity ranging from millions of eggs per year in some teleosts to a few live-born offspring, e.g. in many sharks. Most fishes are oviparous (egg-laying) with external fertilization, but there are also a number of viviparous (live-bearing) species.

Fish oocyte development attracted specific interest in the last century. Morphological investigations were followed by biochemical, physiological, and endocrinological analyses that extended our knowledge of the dynamic events that take place during oocyte development and egg formation. The development of molecular tools has recently opened new directions and facilitated the discovery of the genes involved in these processes and their evolutionary functional significance. Some fish species, such as zebrafish and medaka, are gaining importance as model organisms for deciphering the functional aspects of specific genes and proteins during oogenesis. We are witnessing a dazzling expansion in the number of publications and scientific meetings concerning fish reproduction. For many years, these focused more on the endocrine regulation pathways aiming at inducing egg production and less on cellular and molecular processes associated with ovarian follicle formation. This book aims to fill this gap by presenting a comprehensive overview of egg production in fish, from the standpoint of the oocyte. Conceptually, we guide the reader through the ontogeny of the oocyte: formation, shaping the envelope, growth by accumulating nutrients (yolk), maturation, ovulation, and fertilization. Genomic and proteomic studies investigate the biological processes occurring within the oocyte. The maternal factors stored within the oocyte and their contribution to embryo development is discussed in another chapter. Several chapters discuss regulation of developmental processes, such as gonadotropin regulation of oocyte development, sex steroid receptors, and oocyte maturation and ovulation. The chapter devoted to oocyte regression and atresia assists in exploring regulatory mechanisms required for proper development. Environmental impact on gonadal development is highlighted in a chapter reviewing current knowledge on the effects of endocrine-disrupting compounds and provides a warning of the long-term effects of man-made contaminants. Novel aspects include analysis of the role of specific molecular water channels (aquaporins) during oocyte hydration, a unique, essential physiological process in marine fish. This vast body of knowledge provides a basis for developing strategies for promoting oocyte development in farmed fish species and preserving the maternal genome.

To summarize, this book provides an insight into the complex mechanisms involved in egg formation in fish, as well as revealing important gaps in our knowledge that should be filled in the near future. We hope that it will also assist students in their choice of future research, as well as bridging the gap between basic studies and technological applications, thus contributing to the growing aquaculture industry. The success of this industry in meeting the nutritional demand of the growing world population may also assist in alleviating overfishing and facilitating the recovery of natural resources.

The Editors

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This book is a tribute to those investigators that were pioneers in the study of reproductive biology and endocrinology of fish species. We are especially thankful to Robin Wallace and Kelly Selman whose studies and ideas inspired a whole generation of researchers in these fields.

The book cover logo was made by Hana Bernard (Israel Oceanographic and Limnological Research, Haifa, Israel) from pictures by Dr. Joan Cerdà (Chapter 12).

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CHAPTER 1

ULTRASTRUCTURAL ASPECTS OF THE ONTOGENY AND DIFFERENTIATION OF RAY-FINNED FISH OVARIAN FOLLICLES

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1. INTRODUCTION

The animal kingdom exhibits two particular diploid cell types: cells forming the organism or somatic cells, which remain diploid; and germinal line cells that generate haploid reproductive cells, or gametes. Diploid cells in the germinal line, known as primordial germ cells (PGC), are singled out very early in embryogenesis and migrate towards the genital crests from which ovaries or testes derive. As soon as the PGC colonize the ovary, they become associated with somatic cells in the stroma, which differentiate at the same time as the germinal cells. Indeed, in Actinopterygii (ray-finned fish), as in other vertebrates, the development of oocytes in the ovaries is inevitably linked with that of the surrounding somatic layers. An oocyte and its layers of specialized somatic cells constitute an ovarian follicle (for detailed reviews of the cellular events in oocyte growth and development in Teleostei see: Wallace and Selman, 1981; Guraya, 1986; Selman and Wallace, 1989; Selman et al., 1993).

2. FROM PGC TO EGG: SHORT REVIEW OF SUCCESSIVE EVENTS

In ray-finned fish species, undifferentiated PGC appear in the ovaries and generate differentiated oogonia. As in other vertebrates, PGC are formed at extragonadal locations and recent studies, mostly performed on zebrafish (*Danio rerio*), identified specific molecular markers and processes implicated in progenitor segregation and their migration to the gonadal anlage (for reviews see: Braat et al., 1999; Raz, 2003, 2004). Gonadal somatic cells then envelop female germ cells destined to become oocytes and form ovarian follicles (Matova and Cooley, 2001). After a number of mitotic divisions, diploid oogonia undergo their first meiotic division, differentiating into primary oocytes. This first meiotic division remains arrested at the end of the prophase in the diplotene stage, for a few days or months in most Teleostei and for years in Chondrostei (e.g. sturgeons). In oviparous vertebrates, tetraploid female germinal cells accumulate the informational components and nutritional reserves needed for embryo development during this arrest, in a process known as vitellogenesis (see Chapter 2), and completes differentiation of its cellular and a-cellular envelopes. At the end of vitellogenesis, primary oocytes resume their progress through the first division of meiosis, and maturational processes occur, leading to primary oocyte nuclear envelope breakdown, i.e. germinal vesicle breakdown (GVBD). This first meiotic division gives rise to two cells differing greatly in size, a very small first polar body, which degenerates, and a large secondary oocyte. Ovulation occurs once maturation is completed. The secondary oocyte separates from its envelopes and drops into the lumen of the ovary, or the abdominal cavity in some species, e.g. salmonids. In ray-finned fish, as in most vertebrates, the second meiotic division of the secondary oocyte proceeds to

metaphase, and pauses until activation during fertilization, which activates the end of the second meiotic division. This leads to the formation of a transient haploid female gamete, also known as an ovum, and a second polar body, which degenerates like the first one. Fertilization occurs immediately by fusion of the haploid ovum nucleus with the haploid spermatozoon nucleus to form a diploid egg. Mitotic divisions of the fertilized egg result in the development of a new individual constituted of genetically alike diploid cells, with the two types of somatic and germinal cells required for reproduction.

3. PGC

Undifferentiated Teleostei gonads consist of a conjunctive stroma containing germinal cells and Chondrostei gonads also contain a large number of adipocytes. PGC (Figure 1A) are present, intermingled with gonia, in all gonads studied in both gonochoristic and hermaphroditic Teleostei. The features are the same in ovaries and testes, and in male and female ovotestis territories. They are bipotential cells, capable of evolving into oogonia or into spermatogonia. It is assumed that in successive hermaphroditic species, oogonia, during the female phase, then spermatogonia, when sexual inversion occurs, originate directly (Bruslé, 1982) or indirectly (Slanchev et al., 2005) from the same stem cells. Their presence in functional gonochoristic gonads indicates that they act as residual cells, ensuring the reconstitution of the germinal stock between two successive cycles (Bruslé, 1982).

PGC are larger than somatic cells in the gonad stroma. Their size is a recognition criterion. They are oval-shaped and easily identifiable on electron micrographs by their irregular outlines, heavy electron density, and high nucleus-to-cytoplasm ratio (Figure 1A, B). In very young females, the pseudopodial structure of the cytoplasm indicates their migratory status in the forming ovary. PGC nuclei are round and eccentrically located. They generally exhibit only one nucleolus in a finely granular chromatin and have few nuclear pores. In the cytoplasm, which contains few organelles, some relatively large mitochondria are located near the nucleus. The mitochondria are intimately linked with dense material called "ciment" (Clérot, 1976), or germinal dense bodies (Hamagushi, 1985). These associations constitute the mitochondrial aggregates first described in stage I oocytes (Clerot, 1976; Wallace and Selman, 1981) and later in PGC (Bruslé, 1980). A dense material of the same kind, known as "nuages", appears scattered through the perinuclear cytoplasm. The electron-dense "ciment" and "nuages" come from the nucleus and are often observed passing through the nuclear pores. They constitute excellent germinal cell markers on electron micrographs (Hogan, 1978). Although the role of these extrusions is not still entirely known, their nuclear nature has been demonstrated (Mazabraud et al., 1975). It has been suggested that "ciment" takes part in mitochondriogenesis (Hogan, 1978; Toury et al., 1979). PGC are associated with a few special somatic cells, which surround them (Figure 1B). These cells have a high electron density and exhibit irregular outlines, mirroring the irregular shape of the PGC. They are linked to each other by desmosomes. The nucleus, generally devoid of nucleoli, is big with a layer of heterochromatin lining the internal nuclear

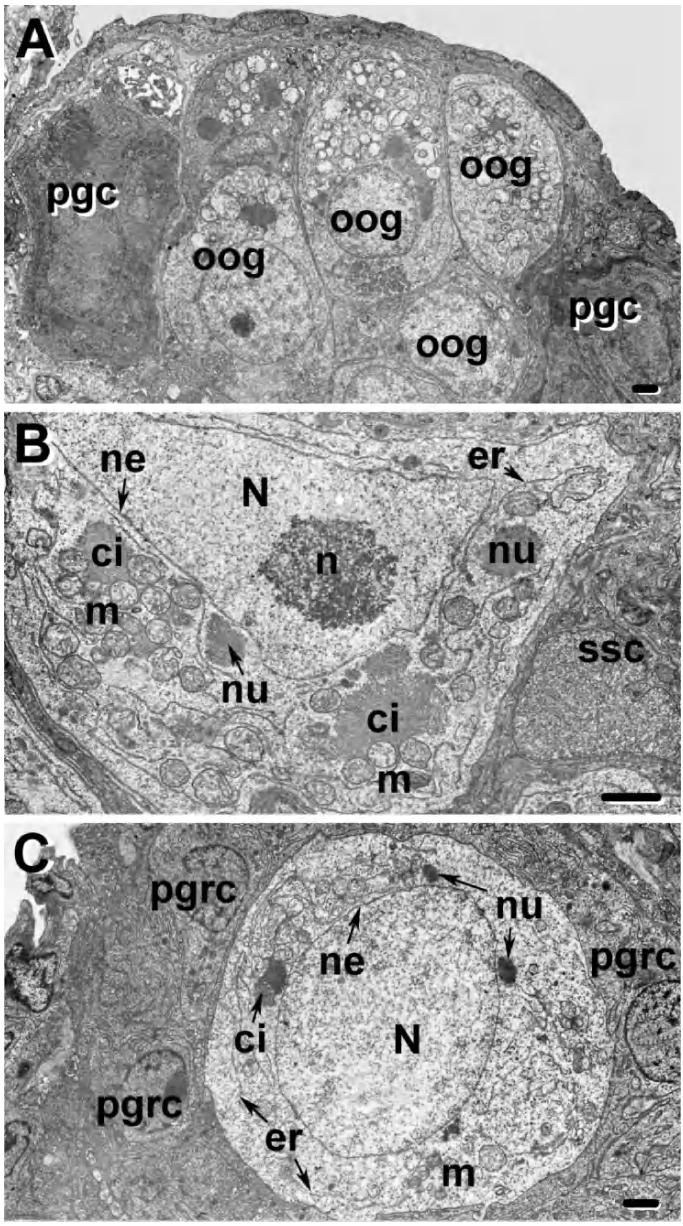

Figure 1. From primordial germ cell to oogonium in *Liza aurata* (Teleostei, Mugilidae). (A) Oogonial nest surrounded by primordial germ cells. (B) Primordial germ cell characterized by its irregular shape and the presence of endoplasmic reticulum. (C) Oogonium characterized by regular shape, round nucleus and high nucleus-to-cytoplasm ratio. *Abbreviations*: ci – "ciment"; er – endoplasmic reticulum; m – mitochondria; N – nucleus; n – nucleolus; ne – nuclear envelope; nu – "nuage"; oog – oogonium; pgc – primordial germ cell; ssc – special somatic cell**.** Scale bar = 1 µm. (Data are from S. Bruslé, University of Perpignan, France.)

membrane, except at nuclear pores. The cytoplasm contains few organelles. The presence in the female gonad of these somatic cells surrounding each PGC indicates the beginning of the ovarian follicle differentiation process.

4. FROM PGC TO OOGONIUM

In several species, the gradual transformation of PGC into oogonia has been observed using electron microscopy (Bruslé and Bruslé, 1978). At first, the electron density of the cell decreases, while cell and nucleus size increases. The outline is irregular. The number of nuclear pores also increases and more organelles are present in the cytoplasm. The cell then continues growing. The granular endoplasmic reticulum is well developed, forming concentric layers and whorls around each mitochondrion. Some Golgi apparatus are present and the mitochondria are more numerous. "Nuages" and "ciments" are greater in size and number. The nucleolus exhibits a central fibrilar structure. Finally, cellular and nuclear outlines become regular and the electron density of the cell even lower. The nucleolus is differentiated and has a fibrilar core (Figure 1C). The oogonium exhibits a large, centrally located ovoid nucleus with a prominent spherical single nucleolus. The cytoplasm contains few ribosomes and has a lower electron density than the PGC on electron micrographs. The perinuclear ooplasm shows some "nuages" near the outer nuclear membrane and a lot of mitochondria aggregates around the "ciment" (Figure 1C). The oogonia multiply rapidly by successive mitosis before the start of meiosis. At one point in time, one oogonium starts a process of successive divisions and the formed oogonia remain linked together with a few surrounding somatic, or pre-granulosa cells. This oogonial nest is separated from the stroma by a basement lamina secreted by the pre-granulosa cells (Figure 2A). The basement lamina is an extracellular matrix secreted by the granulosa epithelium at its base. Isolated oogonia are also observed in the ovarian stroma, each surrounded by a few pre-granulosa cells (Figure 1C). These two types of evolution of oogonia may be observed in the same fish (Bruslé, 1982).

5. FROM OOGONIUM TO PRIMARY OOCYTE AND OVARIAN FOLLICULOGENESIS

Each oogonium is a diploid cell, with a nucleus containing two copies of each chromosome, called homologs, one from the father and one from the mother (Figure 2). During the interphase following the last mitotic division of a diploid oogonium and preceding the first meiotic division of the primary oocyte, each homolog is duplicated by DNA replication, giving rise to a pair of identical chromatids, known as a sister chromatid pair. For each chromosome, a sister chromatid pair from the mother and one from the father are present in the nucleus of the primary oocyte. As a result, the development of primary oocytes occurs in the presence of tetraploid chromatids, i.e. a double amount of DNA for RNA synthesis. This feature is unique to primary oocytes. When the first meiotic division is initiated in an oogonium, the cell becomes a primary oocyte. Oogonia begin meiosis in the

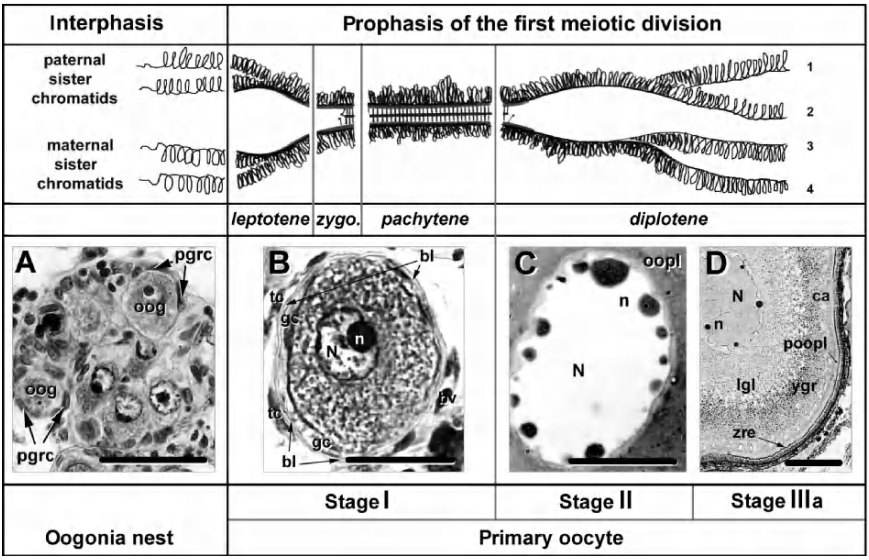

Figure 2. Nuclear stages correlated with primary oocyte development during the prophase of the first meiotic division. Synaptonemal complex structure schemas during nuclear stages are indicated in the upper figure, while corresponding primary follicle development stages are indicated in the lower part. (A) Oogonial nest in *Acipenser baeri* (Chondrostei, Acipenseridae). (B) Ovarian follicle of *A. baeri* stage I oocyte. (C) Nucleus detail of ovarian follicle of *Gobius niger* stage II oocyte (Teleostei, Gobiidae). (D) Ovarian follicle of *Oreochromis niloticus* stage III oocyte (Teleostei, Cichlidae). *Abbreviations*: bl – basement lamina; bv – blood vessel; ca – cortical alveolus; gc – granulosa cell; lgl – lipid globule; N – nucleus; n – nucleolus; oog – oogonium; oopl – ooplasm; poopl – peripheral ooplasm; pgrc – pre-granulosa cell; ygr – yolk granule; zre – zona radiata externa, tc – thecal cell. Scale bars = 30 µm in (A), 50 µm in (B), and 100 µm in (C, D). (Data are from F. Le Menn, University of Bordeaux 1, France in (A–C) and from P. Ndiaye, IFAN, Dakar, Senegal in (D), respectively.)

oogonial nest (Grier, 2000) (Figure 3A) before separating from it. When a young oocyte separates from its oogonial nest, it is surrounded by a monolayer of very flat somatic granulosa cells, which immediately secrete a basement lamina, isolating it from the meso-epithelial environment of the stroma. Outside the basement lamina, a few somatic cells form a monolayer constituting the theca associated with blood vessels. Together with the oocyte, these surrounding granulosa and theca somatic cell layers constitute the ovarian follicle (Figure 2B). Granulosa cells are specialized somatic cells surrounding primary oocytes that secrete the basement lamina and are involved with thecal cells in sexual steroidogenesis during oocyte growth and maturation (Nagahama et al., 1995; Nagahama, 1997) (see Chapter 8). We recommend the use of the term "granulosa cells" as opposed to "follicle cells", as thecal cells and oocytes are also part of the follicle.

In all species studied, the development of the primary ovarian follicle is divided into four stages, which reflect the four characteristic physiological steps in the development of primary oocyte associated with the development of the surrounding cell layers (Figure 2; Table 1). Stage I oocytes are characterized by the formation of the

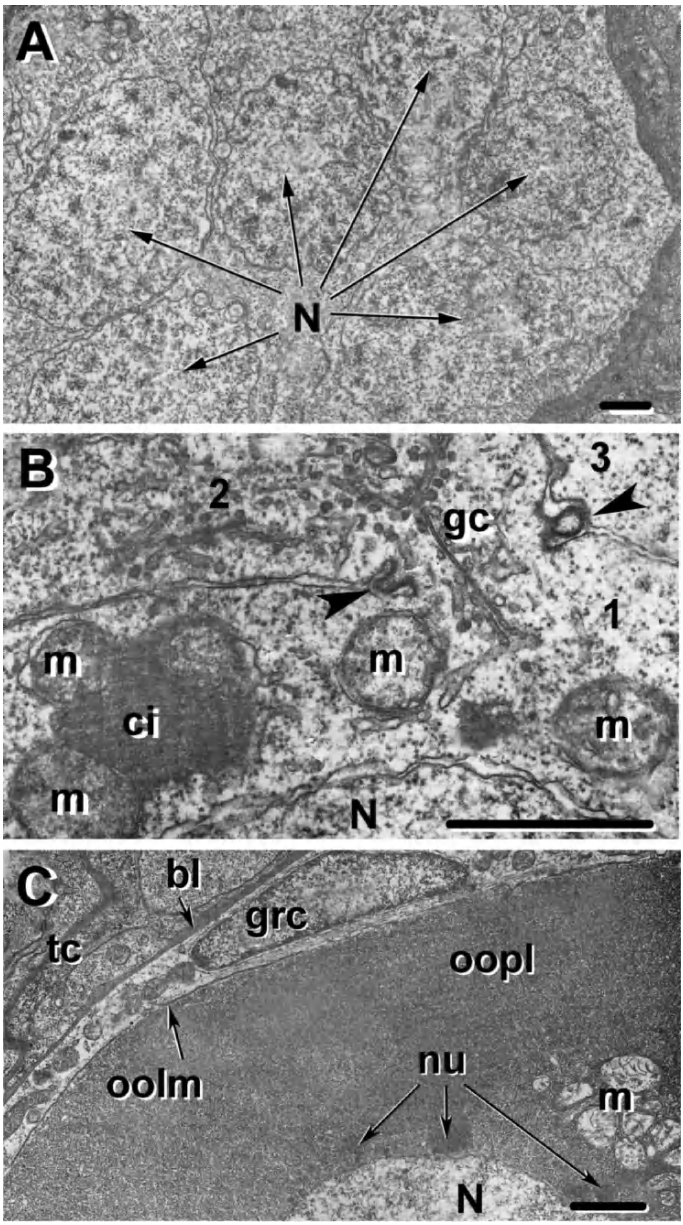

Figure 3. Ovarian follicle of stage I, *Liza aurata* oocyte (Teleostei, Mugilidae). (A) Nest of oocytes initiating their first meiotic division in a synchronic manner. They are not yet associated with follicle surrounding layers. (B) Ooplasmic communication between two oocytes (labelled 1 and 2) still associated in a nest with a third one (labelled 3). The black arrowheads indicate the margins of a common cytoplasmic bridge between these two oocytes. A Golgi apparatus complex extends over the bridge. (C) Ovarian follicle of stage I oocyte. *Abbreviations*: bl – basement lamina; ci – "ciment"; gc – Golgi apparatus complex; grc – granulosa cell; m – mitochondria; N – nucleus; nu – "nuage"; oolm – oolemma; oopl – ooplasm; tc – thecal cell. Scale bar = 1 μm. (Data are from S. Bruslé, University of Perpignan, France.)

Table 1. Ultrastructural aspects of oocyte growth in ray-finned fish

	Follicle layers	Oocyte nucleus	Nucleoli	Oolemma	Ooplasm organelles	Cortical alveoli	Endocytosis and yolk deposition
Primary Oocyte							
Stage I	Follicle formed: oocyte surrounded successively by granulosa, basement lamina, and theca including blood vessels	Leptotene, zygotene, and pachytene of prophase I with formation of synaptonemal complexes	One or several	Smooth, adherent to granulosa apical membrane	Mitochondria aggregates around "ciment". "Nuages"	None	None
Stage II	Multiplication of granulosa cells in mono layer, increase of blood vessels in theca	Arrested in diplotene of prophase I with disassembly of synaptonemal complexes and intense mRNAs transcription, lampbrush chromosomes	Numerous nucleoli located in the concavities of the nuclear envelope	Synthesis of extraoocyte matrix and formation of oocyte microvilli towards granulosa	Mitochondria migration towards peripheral ooplasm "Nuages"	None	None
Stage IIIa	Zona radiata externa deposition, secondary envelope secretion	Arrested in diplotene of prophase I	Fewer than stage II	Microvilli elongation in parallel to the increase in thickness of the zona radiata, setting up of gap-junctions with granulosa microvilli, and granulosa basal membrane	Numerous Golgi apparatus, multivesicular bodies	Synthesized in oocyte of Teleostei	Endocytosis becomes apparent Lipids > vitellogenin. *Type I vitellogenesis.* Yolk granules, few yolk globules, numerous lipid globules

Stage IIIb	Zona radiata interna deposition, full secretion of the secondary envelope by granulosa cells	Arrested in diplotene of prophase I	Fewer than stage II	Same as stage IIIa	Numerous Golgi apparatus, multivesicular bodies	Synthesized in oocyte of Chondrostei (Acipenseridae)	Maximal endocytosis Vitellogenin > lipids. *Type II vitellogenesis.* Yolk granules, lipid globules, numerous yolk globules/ platelets
Stage IV	Granulosa microvilli retraction from zona radiata channels	Diacinesis leading to germinal vesicle break down	Further reduction in number	Oocyte microvilli retraction from zona radiata channels	Reduced	Located in a mono layer under the oolemma	Reduced to stopped endocytosis, Yolk liquefaction that could be associated with platelets dissolution, lipid moiety separated
Secondary Oocyte							
Stage V	Zona radiata transformed in chorion	Arrested in metaphase II of the meiosis	None	Smooth	Few, and concentrated at the animal pole	Located under the oolemma	Minimal endocytosis

ovarian follicle and nuclear genetic recombination of chromosomal chromatids at synaptonemal complexes. During stage II, or pre-vitellogenesis, organelles, and molecules used during the later stages are synthesized. Stage III, or vitellogenesis, and its sub-stages IIIa and IIIb according to the ratio between lipid and yolk globules, is characterized by yolk deposition and the formation of the zona radiata (ZR), a protective structure surrounding the future egg. Stage IV is characterized by the maturation processes preceding the end of the first meiotic division.

6. OVARIAN FOLLICLE DEVELOPMENT BEFORE VITELLOGENESIS

During the prophase of the first meiotic division, the nucleus passes through five successive stages: leptotene, zygotene, pachytene, diplotene, and diacinesis (Figure 2). The first three nucleus stages occur during stage I of primary oocyte development.

6.1. Stage I

6.1.1. Oocyte events

Young primary oocytes (Figure 2B) look like oogonia, except for the fine structure of their nuclei. There is generally only one large nucleolus. The nuclear envelope is almost smooth and nuclear pores are regularly spaced. As mentioned above, a sister chromatid pair from the mother and one from the father for each chromosome are present in the nucleus of the primary oocyte. In the leptotene nucleus phase, both maternal and paternal sister chromatid pairs attach each of their ends to the inner membrane of the nuclear envelope and, recognizing each other from a distance, they move closer together. In the following zygotene stage, the maternal and paternal homologous sister chromatid pairs join together. A like-with-like recognition is mediated by a long central proteinous ladder-like core that gradually forms a synaptonemal complex, with a maternal sister chromatid pair on one side and a paternal sister chromatid pair on the other (Figure 2). This tetrad chromatid structure or bivalent is easily observable by electron microscopy (Figure 4A, B). During the pachytene stage, chromosomal exchanges occur between opposite non-sister chromatids with the help of recombination modules located along the central protein core of the synaptonemal complex. The cytoplasm, or ooplasm, still contains few organelles. Numerous mitochondrial aggregates, intimately associated with "ciment", are distributed in the

in *Gobius niger* stage I oocyte. (E) Tangential section of the nucleus in *Gobius niger* stage I oocyte exhibiting "nuages" protruding from nuclear pores and patches of "ciment". *Abbreviations*: ci – ciment; m – mitochondria; ma – mitochondrial aggregate; N – nucleus; n – nucleolus; ne – nuclear envelope; nu – nuage; np – nuclear pore; oopl – ooplasm; sc – synaptonemal complex (black arrowheads). Scale bar = 1 μm. (Data are from S. Bruslé, University of Perpignan, France for *Liza aurata* and from F. Le Menn, University of Bordeaux 1, France for *Gobius niger*, respectively.)

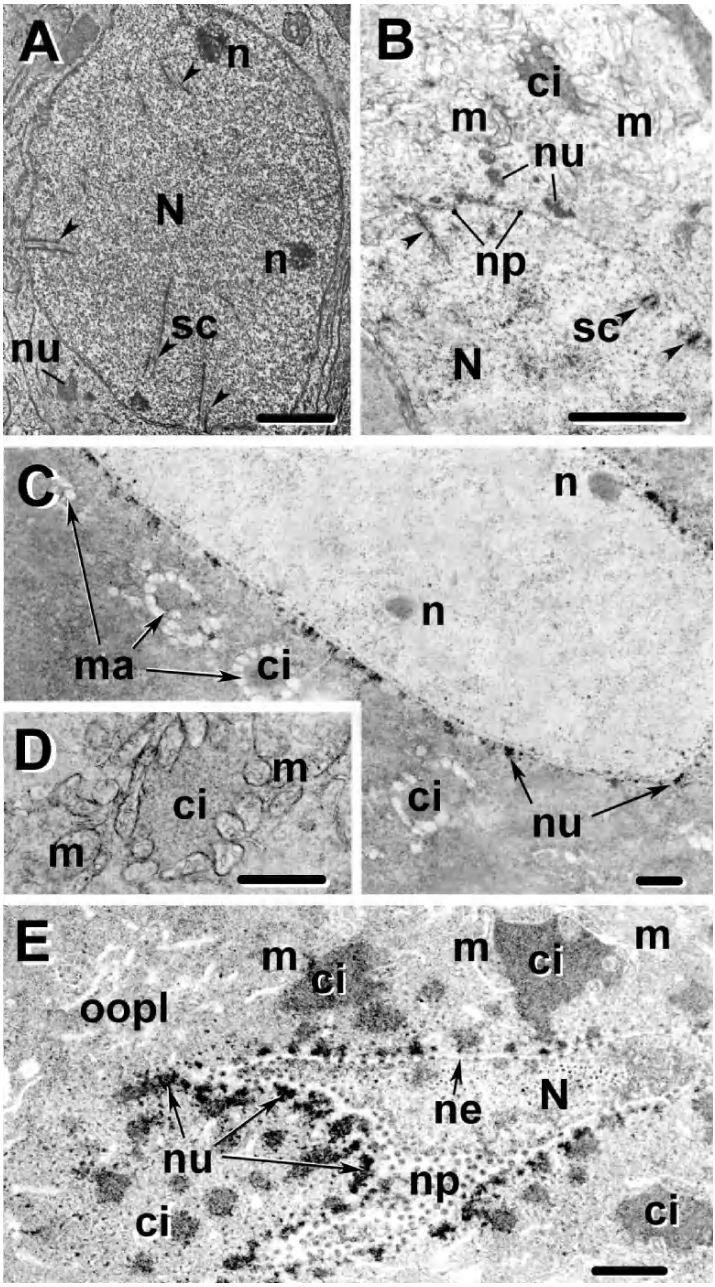

Figure 4. Nuclear events of *Liza aurata* (Teleostei, Mugilidae) and *Gobius niger* stage I oocytes (Teleostei, Gobiidae). Synaptonemal complexes during nuclear pachytene stage in (A) *Liza aurata* and (B) *Gobius niger*. (C) Detail of the perinuclear ooplasm of *Gobius niger* stage I oocyte showing "nuages" and mitochondrial aggregates around "ciments". (D) Detail of a mitochondrial aggregate

perinuclear ooplasm (Figures 3C; 4C, D). These structures correspond to an intense multiplication of mitochondria (Clérot and Wegnez, 1977). In the vicinity of the outer nuclear membrane, a crown of spotted, highly electron-dense "nuages" seems to emerge from the nuclear pores (Figure 4C, E). The oocyte membrane, or oolemma, is smooth. When stage I oocytes develop in the oogonial nest, the initiation of the first meiotic division is generally synchronized, with all nuclei in the same stage (for example: pachytene stage in Figure 3A). Ooplasmic communication is then often observed between adjacent stage I oocytes (Figure 3B).

6.1.2. Surrounding events

The somatic granulosa cells, associated with germinal cell from PGC stage, multiply, forming a regular epithelium of flattened cells. In numerous ray-finned fish species, their prominent nuclei lead to a crenulation of the oolemma closely adhering to the apex membrane. They complete the secretion of the basement lamina from their basal membrane. Outside the basement lamina, multilayers of unorganized meso-epithelial cells constitute the thecal layer, or theca, irrigated by numerous blood vessels (Figure 3C).

6.2. Stage II

6.2.1. Oocyte events

This is the pre-vitellogenesis stage, i.e. preceding the entry of yolk precursors into the oocyte (Le Menn and Burzawa-Gerard, 1985). In stage II, the nucleus exhibits a new feature, characteristic of the diplotene stage of the prophase of the first meiotic division. Two kinds of processes occur. The first is the de-structuring of the synaptonemal organization by disassembling the lateral protein axes of the ladder-like central core. Each sister chromatid pair is highly active in RNA synthesis, due to the double amount of DNA, compared to somatic cells. The newly transcribed RNAs, packed into dense RNA/protein complexes, are clearly visible on electron micrographs on large chromatin loops, emanating from the linear chromatid axis. At this pre-vitellogenic stage, the so-called lampbrush chromosome structure appears and persists throughout the diplotene phase, i.e. during vitellogenic stages IIIa and IIIb (Figure 2). The second is an increase in nucleoli due to a huge amplification of the nucleolar organizer genes. The nucleus is then generally characterized by a large central nucleolus and numerous smaller nucleoli. Nucleoli are localized in the vicinity of the crenulated nuclear envelope, in the crests protruding out into the ooplasm (Figure 2C). At the same time, both ribosomal RNA and mRNAs are transported to the ooplasm, appearing as an electron-dense feature on electron–electron micrographs. For example, large quantities of vitellogenin receptor mRNA are accumulated in the ooplasm during stage II (Davail et al., 1998; Perazzolo et al., 1999; Agulleiro et al., 2007).

The size of the oocyte increases as these nuclear events progress. The ooplasm is very electron-dense, due to numerous tightly packed ribosomes. It exhibits a scattered smooth endoplasmic reticulum and mitochondria begin to migrate towards the peripheral ooplasm, where a few Golgi apparatus can be observed. In numerous Teleostei, a roughly spherical cluster of organelles can be observed halfway between the nucleus and the oolemma, using photonic histology. This cluster is the strongly stained Balbiani body, sometimes improperly called yolk nucleus (Kobayashi and Iwamatsu, 2000). Electron micrographs show that it may be an artefact aggregation of numerous mitochondria, endoplasmic reticulum, and Golgi apparatus elements, due to fixation shock (F. Le Menn, personal observations). However they have been observed in living oocytes after staining (Begovac and Wallace, 1988). They may be involved in the extensive organelle formation during this pre-vitellogenic stage (Guraya, 1979).

The oolemma separates from some areas of the apical granulosa cell surface. The intervening space is filled with a flocculent, electron-clear material, corresponding to an extra-oocyte matrix (Figure 5A). In this matrix, oocyte microvilli protrude from the oocyte surface towards the granulosa epithelium. In numerous species, microvilli are grouped together in batches (Figure 5B). In sturgeon, circular oocyte microvilli batches lay under the umbrellas formed by the apex of the granulosa epithelium. This apical granulosa membrane adheres to the oolemma around each batch of microvilli (Figure 5B, C). These residual junctions between the oolemma and the follicular epithelium are classic examples of gap junctions (Figure 5C). As soon as the junctions between the oolemma and the apical membrane of the granulosa epithelium have disappeared, oocyte microvilli appear in the extra-oocyte matrix in a radial position towards the granulosa cells, as observed on cryofracture preparations (F. Le Menn, personal observations).

6.2.2. Surrounding events

Granulosa cells multiply to form an epithelial coating of cubic cells, joined together by gap junctions (Figure 10A, B). In species such as sturgeon, the basement lamina may be exceptionally thick, up to five times as thick as the granulosa epithelium (Figure 5D). Depending on the species, the thecal layer may consist of just a few cells located close to blood vessels (Figure 5D), or so many cells that they form a continuous two- or three-ply layer, largely irrigated by blood vessels and linked by numerous desmosomes (Figure 10C). At the end of pre-vitellogenic stage II, the oocytes contain all the molecules and organelles necessary for its subsequent endocytic and exocytic activities during oocyte vitellogenesis. It is surrounded by cellular and a-cellular layers, i.e. the theca, basement lamina, and granulosa, organized in a centripetal pattern. The oocyte has started forming microvilli, which will become the indispensable means for managing exchanges with the general blood flow via its surrounding cellular layers in the following stages.

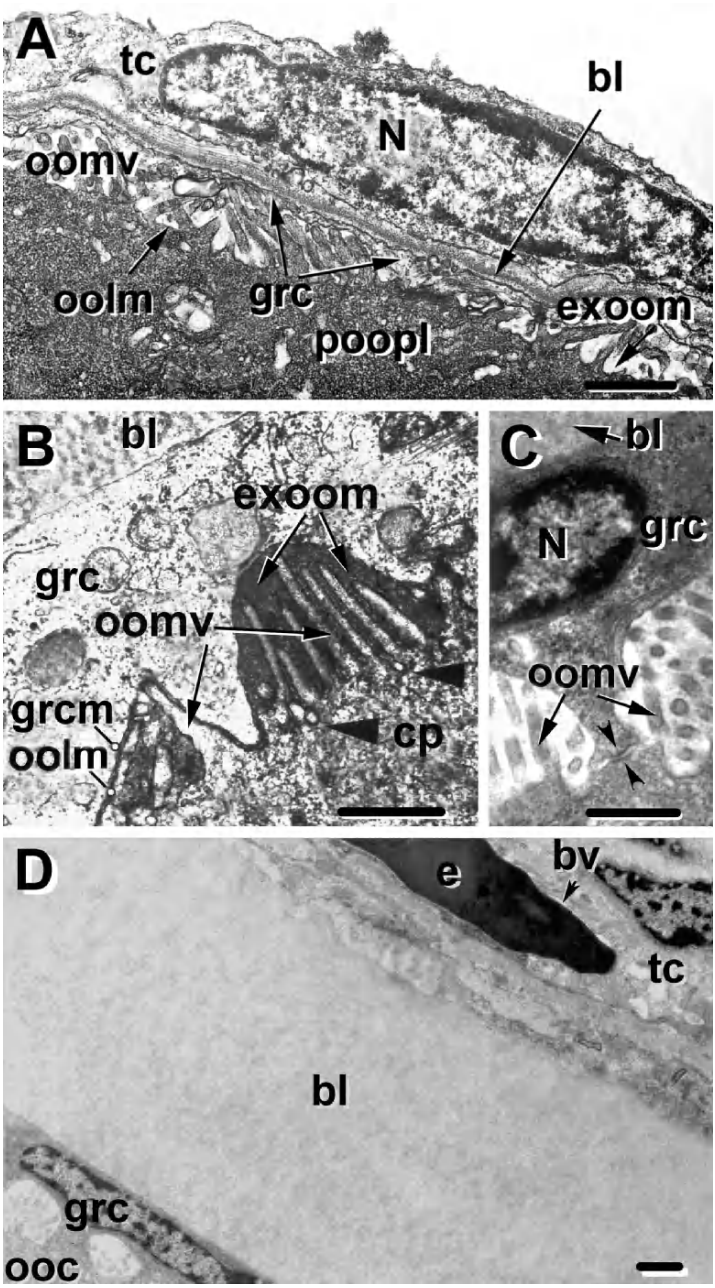

Figure 5. Ovarian follicle of *Liza aurata* (Teleostei, Mugilidae) and *Acipenser baeri* (Chondrostei, Acipenseridae) stage II oocytes. (A) Peripheral ooplasm and oocyte surrounding layers in *Liza aurata*. (B) Oolemma events in *Acipenser baeri* early stage II oocyte. (C) Detail of oocyte microvilli in *Acipenser baeri* late stage II oocyte. (D) Detail of the basement lamina in *Acipenser baeri* late

7. OVARIAN FOLLICLE DEVELOPMENT DURING VITELLOGENESIS

Ovarian follicle enlargement occurs in stage III, while the nucleus remains in the diplotene stage (Figure 2). During this stage, the oocyte accumulates the yolk containing nutritional reserves from the blood stream needed for embryo development, and completes differentiation of its cellular and a-cellular envelopes. In ray-finned fish, as in other oviparous vertebrates, the sexual cycle and ovarian follicle development are controlled by environmental factors, e.g. photoperiod and temperature. In response to these factors, the central nervous system induces a cascade of neurohormones leading to secretion of gonadotropin-releasing hormone (GnRH) by specialized neurons. The GnRH acts on pituitary cells, which secrete the follicle-stimulating hormone (FSH). The FSH signal, mediated by specific receptors located on thecal and granulosa cells, leads to the synthesis of sexual steroid hormones, such as 17β-estradiol (E2) (see Chapters 7 and 8). E2 is secreted into the theca blood vessels to reach the blood stream. In response to E2, specific receptors in hepatocytes mediate the synthesis and release into the blood of vitellogenins (Vtgs), the main yolk precursors in plasma. These are specifically incorporated by the oocytes via receptor mediated endocytosis. (Stifani et al., 1990; Chan et al., 1991; Davail et al., 1998; Hiramatsu et al., 2004) (see Chapter 2). The term vitellogenesis has a double meaning. Strictly speaking, it is the hepatic synthesis of Vtg but, in fact, the term is generally used to describe the incorporation of Vtgs by the oocyte and further processing into yolk proteins.

7.1. Stage IIIa

This sub-stage (Figures 2 and 6) is characterized by the first, discrete entry of Vtg into the oocyte ooplasm, which is only detectable using electron microscopy, as well as, in many species, a huge accumulation of lipid globules. This has previously been described as type I vitellogenesis (Breton et al., 1983). In Teleostei, cortical alveoli are synthesized during this sub-stage.

7.1.1. Oocyte events

The Vtg reaches the oocyte surface by passing first between thecal cells, through the basement lamina, between the granulosa cells, and, finally, along the oocyte microvilli (Selman and Wallace, 1982; Abraham et al., 1984). It is now well established that Vtg is selectively sequestered by growing ovarian follicles via specific receptors located in the oolemma, which become effective at this early stage in vitellogenesis. Numerous endocytic clathrin-coated pits

stage II oocyte. *Abbreviations*: bl – basement lamina; bv – blood vessel; cp – coated pit (black arrow); e – erythrocyte; exoom – extraoocyte matrix; grc – granulosa cell; grcm – granulosa cell membrane; N – nucleus; ooc – oocyte; oolm – oolemma; oomv – oocyte microvilli; poopl – peripheral ooplasm; tc – thecal cell. Scale bar = 1 μm. (Data are from S. Bruslé, University of Perpignan, France for *Liza aurata* and F. Le Menn, University of Bordeaux 1, France for *Acipenser baeri*.)

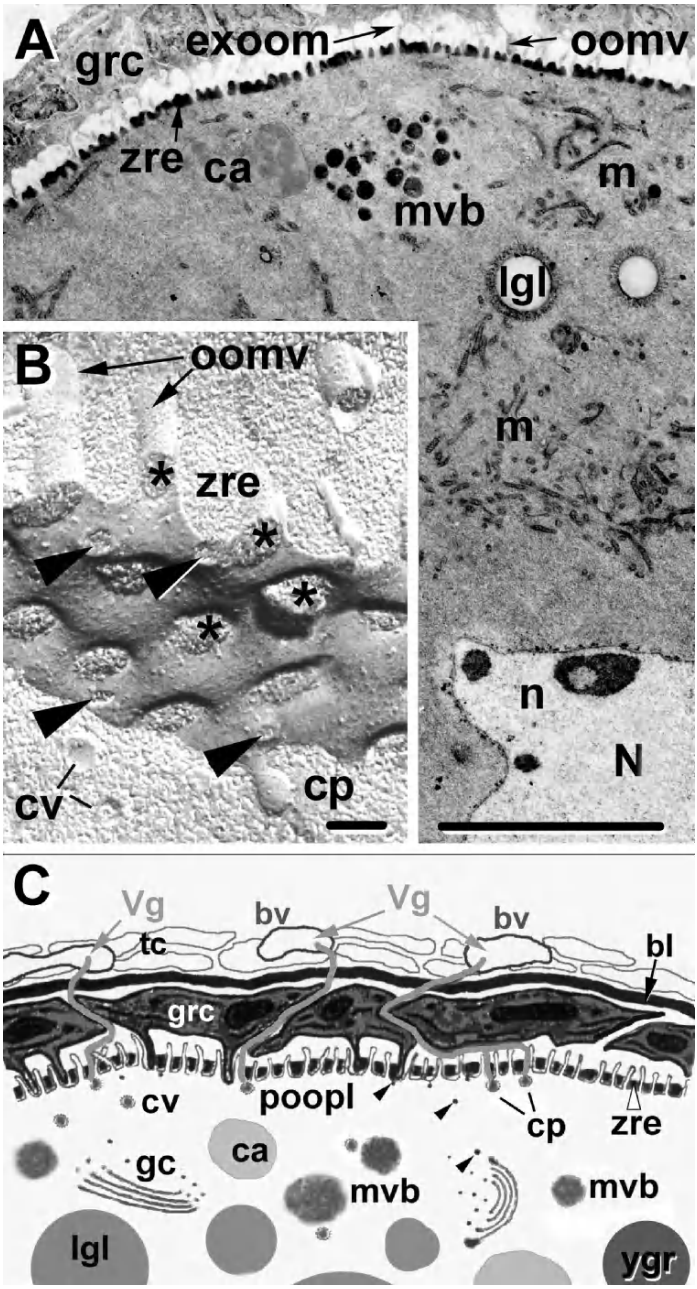

Figure 6. Ovarian follicle of *Gobius niger* (Teleostei, Gobiidae) stage IIIa oocyte (type I vitellogenesis). (A) Transmission electron micrograph of the oocyte. (B) Detail of the endocytosis process observed on cryofracture at oolemma level. View from the peripheral ooplasm towards the inside of the oolemma.

(Pearse, 1976) develop in the oolemma, leading to the formation of coated vesicles that move into the peripheral ooplasm (Figure 6B). These Vtg-containing coated vesicles fuse with lysosomes, known as multivesicular bodies (MVB), originating from the Golgi apparatus (Figures 6A, 7A, B) (Busson-Mabillot, 1984; Wallace and Selman, 1990). The MVB contain lysosomal enzymes, including cathepsin D, that possibly cleave the Vtg into smaller yolk proteins (Sire et al., 1994; Carnaveli et al., 1999). The MVB increase in size and are gradually transformed into small yolk granules, then into large yolk globules (Figures 6C, 7C). In some Teleostei, such as rainbow trout (*Oncorhynchus mykiss*), sub-stage IIIa is characterized by an enormous accumulation of MVB and their transformation into yolk granules and globules after initiation of Vtg endocytosis. Cortical alveoli observed in the vicinity of numerous Golgi apparatus result from the biosynthesis activity of the oocyte. This occurs during sub-stage IIIa in Teleostei and sub-stage IIIb in Chondrostei. These alveoli originate in the ooplasm in the vicinity of smooth endoplasmic reticulum vesicles and are surrounded by one or more layers of rough endoplasmic reticulum, depending on the species. In killifish (*Fundulus heteroclitus*), nascent cortical alveoli contain cathepsin F, a cysteine protease which may be involved in processing the content of the alveoli (Raldúa et al., 2006). A direct relationship has been established between cortical alveoli and Golgi apparatus (Ulrich, 1969; Selman and Wallace, 1989; Le Menn and Pelissero, 1991). These organelles are not used by the embryo and cannot be considered part of the yolk. At the end of vitellogenesis, they are distributed in a single layer underlying the oolemma. At fertilization, they fuse with the oolemma and discharge their glycoprotein content at the oocyte surface during the cortical reaction of the female gamete by exocytosis (Kobayashi, 1985; Selman and Wallace, 1989). This release leads to a restructuring and hardening of the ZR to become a chorion (Shibata et al., 2000). This hardened structure prevents the fusion of other spermatozoa with the ovule and protects the early embryo.

In rainbow trout, as in some other ray-finned fish species where the full-size primary oocyte becomes particularly large, a huge amount of cortical alveoli are deposited just under the oolemma. Consequently, this period has often been incorrectly considered a specific oocyte stage. Synthesis of the cortical alveoli occurs at the beginning of yolk accumulation, when the internalization of Vtg is so discrete that it can only be perceived using meticulous electron microscopy

(C) Schematic draft of type I vitellogenesis of stage IIIa oocyte. *Abbreviations*: bl – basement lamina; bv – blood vessel; ca – cortical alveoli; cp – coated pit; cv – coated vesicle; exoom – extraoocyte matrix; gc – Golgi apparatus complex; grc – granulosa cell; lgl – lipid globule; m – mitochondria; mvb – multivesicular body; N – nucleus; n – nucleolus; oomv – oocyte microvilli; poopl – peripheral ooplasm; tc – thecal cell; Vg – vitellogenin; ygr – yolk granule; zre – zona radiata externa. The asterisks indicate the oocyte microvillus section showing the internal actin skeleton. Black arrowheads in (B) show coated pit neck section. Scale bar = 1 µm (A), 0.1 µm (B). (Data are from F. Le Menn, University of Bordeaux 1, France.)

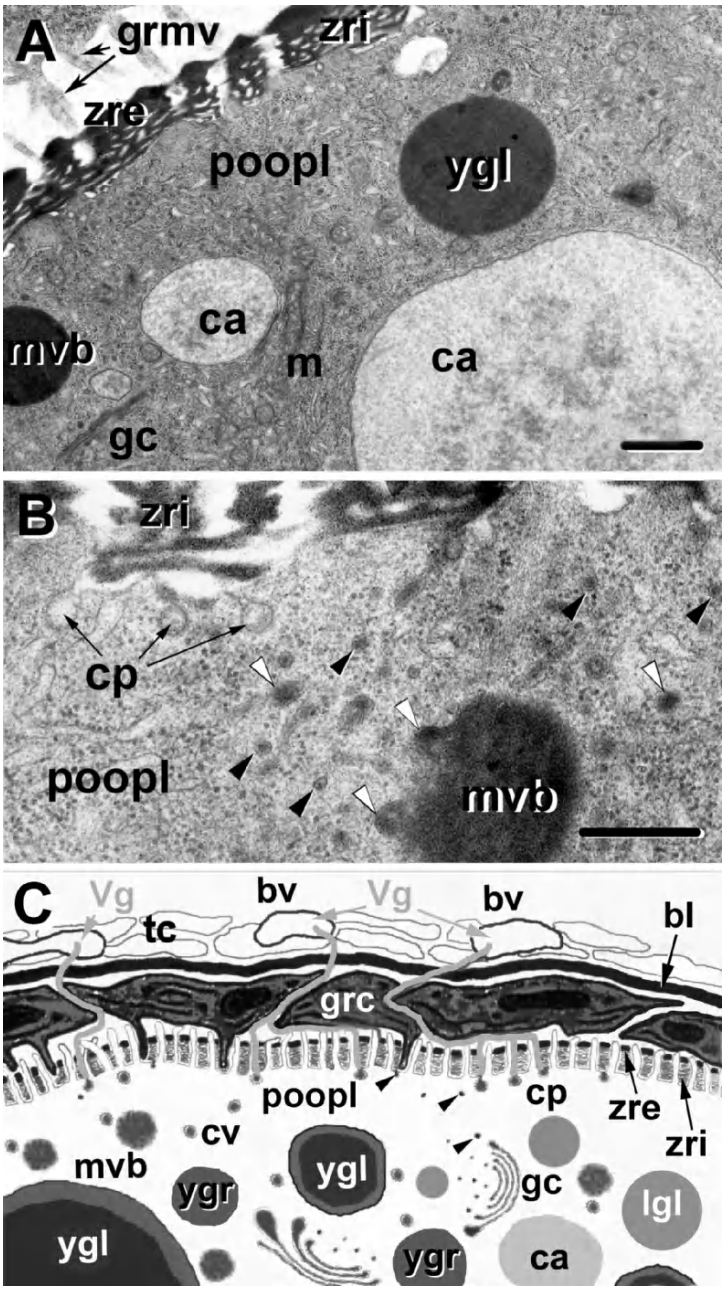

Figure 7. Ovarian follicle of *Gobius niger* (Teleostei, Gobiidae) stage IIIb oocyte (type II vitellogenesis). (A) Surrounding layers and peripheral ooplasm. (B) Detail of endocytic and exocytic events in the peripheral ooplasm. (C) Schematic draft of type II vitellogenesis in stage IIIb oocyte.

(Perazzolo et al., 1999). At the same time, lipid globules, the other yolk moiety, appear in the ooplasm, intermingled with cortical alveoli. Little is known about this deposition and storage. Physiological observations and knowledge of the lipid metabolic behaviour of other kinds of tissue lead to the hypothesis of a sequestration of lipids from plasma very low-density lipoproteins (VLDL), which exhibit a significant surge in sub-stage IIIa (Wallaert and Babin, 1994). This sequestration may occur by binding apolipoprotein B in VLDL (Babin, 1987) with O-linked sugar-specific VLDL/Vtg receptors (Bujo et al., 1995) anchored in the plasma membrane of the oocyte (see Chapter 2) or/and action of an endothelial-attached lipoprotein lipase (Black and Skinner, 1987; Ibanez et al., 2003). Lipolysis of VLDL triacylglycerols and esterification of their subsequent fatty acids inside the oocyte cytoplasm may result in neutral lipid storage in the form of lipid globules. In sub-stage IIIa, yolk and lipid globules are present in the ooplasm, but lipid accumulation is largely predominant over Vtg endocytosis and yolk globule deposition. In numerous Teleostei, cortical alveoli and lipid and yolk globules appear on histology slices arranged in concentric rings. The cortical alveoli are located in the peripheral ooplasm in all cases (Figures 2D; 6A). However, some species, such as zebrafish, do not have lipid globules in their ooplasm. The lipids, mostly phospholipids, necessary for embryo development are associated with Vtg derivatives in the yolk globules (Selman et al., 1993).

In sub-stage IIIa, spots of electron-dense "nuages" are still located near the nuclear pores, but the patches of "ciment" have disappeared. Mitochondria are now scattered throughout the ooplasm. In many species, they are elongated and distorted. The ooplasm contains an abundant smooth endoplasmic reticulum. In the nucleus, nucleoli nested in the crenulated nuclear envelope concavities, exhibit a granulated feature at the periphery (Figure 6A).

Exocytosis events occur in the peripheral ooplasm and oolemma, associated with the first deposition of a protective layer between oocyte microvilli that have received different denominations, including ZR, zona pellucida (ZP), vitelline membrane, vitelline envelope, or chorion, according to various authors. The term "ZR" seems to suit the structure of this protective layer perfectly, as it describes the radial channels formed by the deposition of the ZR around the microvilli, arranged radially in relation to the oocyte centre, which lengthen as the thickness of the ZR increases. Finally it reflects the fact that the microvilli are indispensable for contact between the oocyte and surround-

Abbreviations: bl – basement lamina; bv – blood vessel; ca, cortical alveolus; cp, coated pit; cv, coated vesicle; gc – Golgi apparatus complex; grc – granulosa cell; grmv – granulosa microvilli; lgl – lipid globule; m – mitochondria; mvb – multivesicular body; poopl – peripheral ooplasm; tc – thecal cell; Vg – vitellogenin; ygl – yolk globule; ygr – yolk granule; zre – zona radiata externa; zri – zona radiata interna. Black arrowheads show dense-cored vesicles of the Golgi apparatus and white arrows show coated vesicles and their fusion with multivesicular bodies. Scale bar = 1 μm (A), 0.5 μm (B). (Data are from F. Le Menn, University of Bordeaux 1, France.)

ing granulosa. In this elegant manner, the follicle builds its envelope while accumulating yolk and receiving information from the blood circulation via its cellular layers. Numerous Golgi apparatus in the peripheral ooplasm secrete smooth dense-cored vesicles, which fuse with the oolemma and release their contents into the extraoocyte matrix between the oocyte microvilli to form the ZR externa. In numerous species, this release appears to be the final step in cooperation between the liver and oocytes to produce the ZR. The major proteins involved in ZR synthesis are evolutionarily related to mammalian ZP proteins (see Chapter 5). These ZP proteins are synthesized by the liver under estrogenic induction at the same time or almost the same time as Vtg (Celius and Walther, 1998) (Chapter 5). ZP proteins probably enter the oocyte via endocytic-coated pits and are reshuffled with oocyte glycoproteins in Golgi apparatus before being deposited by exocytosis of dense-cored vesicles on the oocyte surface in the extra-oocyte matrix (Tesoriero, 1977a, b; 1978). However, recent data using molecular biology approaches, have demonstrated that, in some Teleostei, ZP proteins originate from the oocyte itself (Chang et al., 1996; Chang et al., 1997).

7.1.2. Surrounding events

The most visible event is the deposition of the ZR as homogeneous ZR externa between oocyte microvilli parallel to the granulosa cells (Figure 6A–C). In the tangential section, this ZR appears to be perforated by channels, through which the oocyte's microvilli pass (Figure 8A). In Chondrostei and Teleostei, the ZR externa is formed by the deposition of two distinct layers, which exhibit amorphous structures of different electron densities (Riehl and Schulte, 1977; Hosokawa, 1985; Fausto et al., 2004). In all species studied, the ZR externa was visible using high-magnification light microscopy by sub-stage IIIa (Figure 2D). It appears as a fine, neutral glycoprotein, periodic acid-Schiff reagent-positive line located at the oolemma surface. This deposition is correlated with the first entry of Vtg into the oocyte and indicates the beginning of type I vitellogenesis (Figures 2D and 6) (Le Menn et al., 1999). In some Teleostei, granulosa cells secrete the materials constituting the secondary envelope between their lateral plasma membranes (Wourms and Sheldon, 1976; Busson-Mabillot, 1977; Dumont and Brummet, 1980; Selman and Wallace, 1982; Thiaw and Mattei, 1991; Andrade et al., 2001; Francolini et al., 2003). The formation of a secondary envelope plays a role in the protection, resilience, adhesiveness, or buoyancy of the egg, depending on the species (Stehr and Hawkes, 1983).

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structure. (C) Endocytosis and exocytosis traffic in the peripheral ooplasm of *Solea solea* oocyte. *Abbreviations*: af – actin filaments anchoring microvilli in the terminal web; cp – coated pit; cv – coated vesicle; gc – Golgi apparatus complex; grc – granulosa cell; m – mitochondria; mvb – multivesicular body; oomv – oocyte microvilli; oopl – ooplasm; zre – zona radiata externa; zri – zona radiata interna. Black arrowheads are dense-cored vesicles of the Golgi apparatus. Scale bar = 1 μm. (Data are from S. Bruslé, University of Perpignan, France for *Liza aurata* and J. Nuñez, University of Bordeaux 1, France for *Solea solea*.)

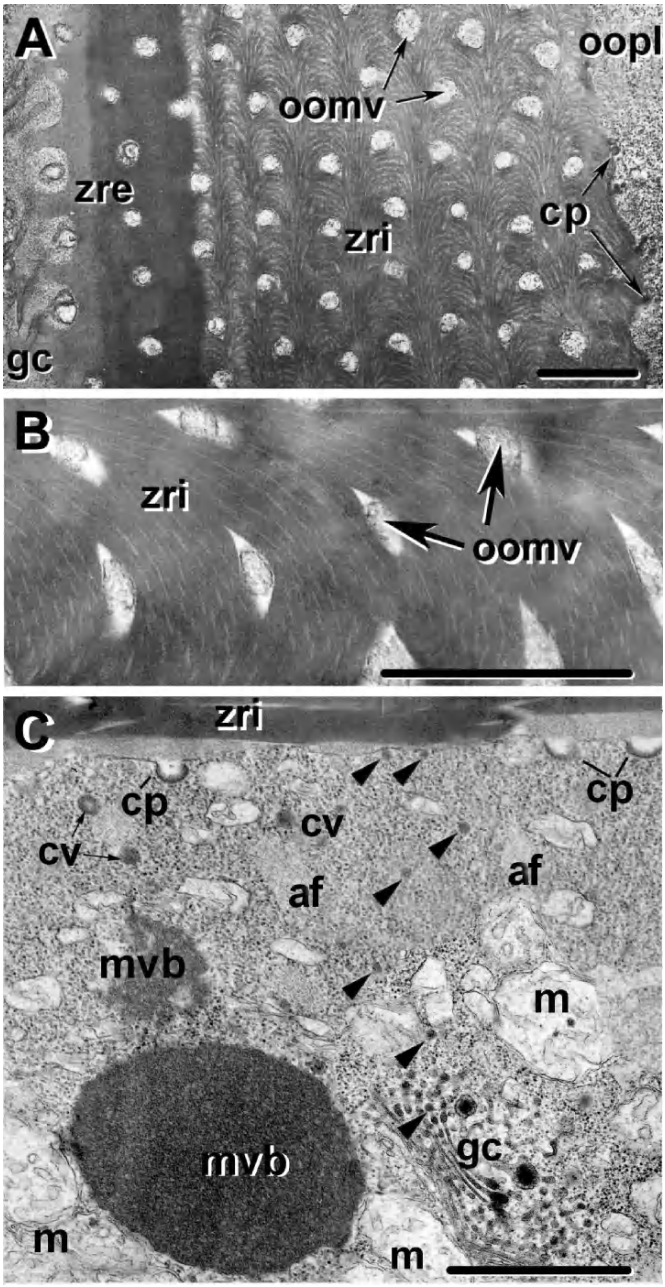

Figure 8. Ovarian follicle of *Liza aurata* (Teleostei, Mugilidae) and *Solea solea* (Teleostei, Soleidae) stage IIIb oocytes (type II vitellogenesis). (A) Transverse section of the *Liza aurata* zona radiata. (B) Transverse section of *Solea solea* zona radiata interna, exhibiting its cholesteric

In the thecal layer, some cells have irregularly shaped nuclei, well-developed smooth endoplasmic reticula, and characteristic crested mitochondria. These steroidogenic cells are involved in androgen synthesis induced by FSH. The androgens are converted into estrogens in the underlying granulosa cells and E2 passing through basement lamina and thecal cells is discharged into the blood stream via thecal vessels (Kagawa et al., 1981; Kagawa, 1985; Nakamura and Nagahama, 1985; Nakamura et al., 1993). However, steroidogenesis does not necessary require the involvement of the both somatic cell types, as shown in killifish (Petrino et al., 1989).

7.2. Stage IIIb

At this sub-stage, the acting molecules and organelles are exactly the same as in sub-stage IIIa. However, the proportion of lipid and yolk globules deposited is inverted (Table 1). This stage was previously described as type II vitellogenesis (Breton et al., 1983).

7.2.1. Oocyte events

The Vtg endocytosis is considerable at this stage, inducing a rapid growth of the follicle. All yolk and lipid globules are intermingled in the ooplasm. However, the peripheral ooplasm, a thin layer of cytoplasm underlying the oolemma, is devoid of globules and organelles. In this peripheral ooplasm, a huge amount of endocytic vesicles transfer Vtg towards the MVB centripetally (Figure 7B, C), whereas numerous dense-cored vesicles originating from the Golgi apparatus reach the oolemma centrifugally to deposit the ZR interna at the oocyte surface by exocytosis (Figures 7B, C; 8C). These transfers occur in, and are probably helped by, the presence of a cytoskeleton network underlying the oolemma. This network, known as the terminal web, is formed by actin filaments, where the internal actin skeleton of each oocyte microvillus is anchored (Figure 8C). Extremely large numbers of endocytic vesicles bud off from the oolemma near the bases of the oocyte microvilli, in correlation with the enormous amount of yolk stored in the oocyte. These coated pits have never been observed in the oolemma covering the microvilli, probably due to the presence of axial actin filaments that run in a parallel bundle, forming an internal skeleton that prevents any distorsion of the oocyte plasma membrane (Figure 6B). In *Xenopus laevis*, a small toad with the same oocyte yolk deposition process, it has been calculated that several million endocytic vesicles are required to internalize Vtg and produce a single large yolk globule (Wallace et al., 1983). In stage IV, oocytes are over five orders of magnitude larger than in stage I. For example, oocyte diameter increases from around 50 μm to around 2,800 μm in sturgeon (*Acispenser baeri*) or around 4,000 μm in rainbow trout.

In numerous species, the formation of crystals within platelets seems to enhance the capacity of the oocyte to store yolk (review in Wallace and Selman, 1981). Yolk platelets appear as regularly or irregularly shaped crystalline cores

distributed in an apparently amorphous matrix (Karasaki, 1967). At higher magnification, the crystalline part shows an intersecting band pattern, with very similar architecture in the various species studied. This is probably physiologically significant (Lange et al., 1983). A close association between the lipovitellin-phosvitin crystal cores may have a cation-trapping property. By sub-stage IIIb, exocytosis events persist in the peripheral ooplasm and at oolemma level, constituting the ZR interna. Numerous dense-cored vesicles are observed between Golgi apparatus and the oolemma (Figure 8C). In Chondrostei, cortical alveoli are synthesized from Golgi apparatus located peripheral to the oopolasm during this late vitellogenesis sub-stage (Figure 9A). They exhibit a fibrillar structure containing a few accumulations of electron-dense material (Figure 9B). At the end of sub-stage IIIb, these alveoli are located under the oolemma (Figure 9C).

7.2.2. Surrounding events

The ZR interna is deposited in the extra-oocyte matrix, gradually displacing the ZR externa towards the granulosa cells. Unlike the amorphous structure of the ZR externa, the ZR interna has reticulated deposits with twisted arrangements, giving it the arched appearance of a polymerized fibrillar secretion deposited in a matrix. The electron density of this ZR interna matrix hides the fibrillate texture to a greater or lesser extent in different species (Flügel, 1967). The parabolic lamellar pattern appears clearly on electron oblique sections (Figure 8A, B). The ZR interna structure is common to all oviparous Teleostei and Chondrostei fish species studied. Many biological polymers produce these twisted fibrous arrangements by deposition of microfibrils in regular helicoidal arrays, providing pliant structures, which generally have a protective function (Bouligand, 1972; Giraud-Guille, 1996). These formations show a similar pattern to the well-defined molecular state known as the cholesteric phase, as they were first described in microscopic preparations of cholesterol derivatives (Friedel, 1922). They are obtainable *in vitro* with aqueous suspensions of cellulose crystallites (Revol et al., 1992), chitin crystallites (Revol and Marchessault, 1993), and, in general, all fibrous biological composites (Martin et al., 2003; De Luca and Rey, 2004). These supra-molecular arrangements appear in a viscous state with some fluidity, as molecules are free to move with respect to each other (Besseau and Giraud-Guille, 1995). In ray-finned fish, this particular structure may be gradually stretched, making enormous enlargement of oocyte volume possible during its rapid growth phase in sub-stage IIIb.

At least four ZP proteins participate in assembling the ZR (see Chapter 5). They all have a ZP domain and are members of extracellular matrix proteins (Sutton et al., 2002). A comparison between fish ZR interna formation and models of the mouse-egg matrix (Jovine et al., 2002) shows that (i) ZP domains are responsible for polymerization of these ZP proteins into supra-molecular structures, and (ii) the formation of the biologically functional ZR interna requires all the ZP proteins, as each ZP domain contains distinct information about the structure being assembled (Sutton et al., 2002). Future investigations may focus on the relationship between the polymerization mechanisms of ZR interna proteins, as well as the role of ZP domains in the formation of the cholesteric architecture.

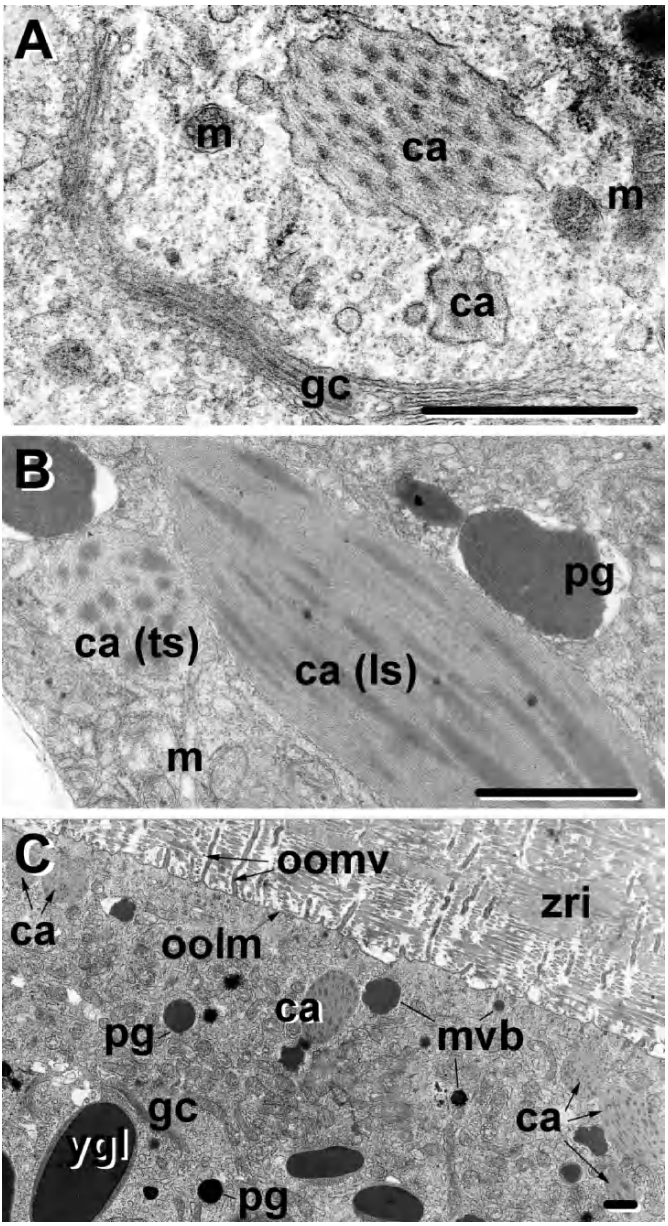

Figure 9. Synthesis of cortical alveoli in the ovarian follicle in *Acipenser baeri* (Chondrostei, Acipenseridae) stage IIIb oocyte. (A) Synthesis of cortical alveolus by the Golgi apparatus complex. (B) Longitudinal (ls) and transverse (ts) sections of cortical alveoli. (C) Location of cortical alveoli in peripheral ooplasm under the oolemma. *Abbreviations*: ca – cortical alveolus; gc – Golgi apparatus complex; m – mitochondria; ls – longitudinal section; mvb – multivesicular bodies; oolm – oolemma; oomv – oocyte microvilli; pg – pigment granule; ts – transverse section; ygl – yolk globule; zri – zona radiata interna. Scale bar = 1 μm. (Data from F. Le Menn, University of Bordeaux 1, France.)

In each transversal channel, one or two (depending on species) granulosa microvilli run parallel to the oocyte microvillus. These paired microvilli are often particularly visible in the extra-oocyte matrix (Figures 7A; 10D–F). Oocyte microvilli establish electron-dense membranous contacts with the apical granulosa surface (turned towards the oocyte) and granulosa microvilli (Figure 10E, F). The micropyle is fully formed during sub-stage IIIb. The micropylar cell (or cells, depending on the species), which originate(s) from the granulosa epithelium invade(s) the ZR to the oolemma, maintaining contact throughout choriogenesis (Wallace and Selman, 1981). They induce reorganization of the ZR into one or more funnels, depending on the species, which constitute the only entrance for the spermatozoon (Dumont and Brummett, 1980; Cherr and Clark, 1982; Amanze and Lyengar, 1990). The location of the micropyle on the oocyte surface delineates the future animal pole. ZR externa glycoproteins apparently have an affinity for spermatozoa and guides sperm into the micropyle (Amanze and Lyengar, 1990; Iwamatsu et al., 1997).

8. CELL–CELL INTERACTION IN OVARIAN FOLLICLES

Germ cells interact with somatic cells not only during migration but also after settlement in the gonadal primordium. The completion of the oocyte differentiation programme within the follicle requires support from the gonadal somatic cells, with which continuous two-way communication is established via various cell–cell adhesion and coupling structures and secreted factors (Matzuk et al., 2002).

8.1. Cell Adhesion and Junction Complexes

During early oogenesis in Teleostei, a single layer of somatic pre-granulosa cells initially surrounds the entire nest of early meiotic oocytes (Selman et al., 1993; Grier, 2000; Higashino et al., 2002). As the cells in the nest cellularize, the cyst structure of the oocyte is gradually broken by the invagination of somatic cells and, finally, the oocyte becomes a single cell surrounded by a sheath of squamous granulosa cells. Later, the granulosa cells gradually increase in height to become more cuboidal and separate from each other to form intercellular spaces. During the ovarian follicle formation, cytoplasmic projections from the granulosa cells and oocyte, presumably microvilli in *statu nascendi*, make focal contact and these connections are maintained throughout oocyte growth (Figure 10A). During the growth period, the microvilli elongate as the ZR is deposited around them and appear to be located in "pore canals" in the developing oocyte envelope. Some of them emerge from the ZR and penetrate deep into the spaces between adjacent follicle cells. In contrast, there are usually fewer microvilli projecting from the granulosa cells and they frequently do not reach the oocyte surface (Kessel et al., 1988; Cerdà et al., 1999; Ravaglia and Maggese, 2003). During folliculogenesis, a number of typical junction complexes, such as desmosomes and tight (occluding) junctions, are established between granulosa

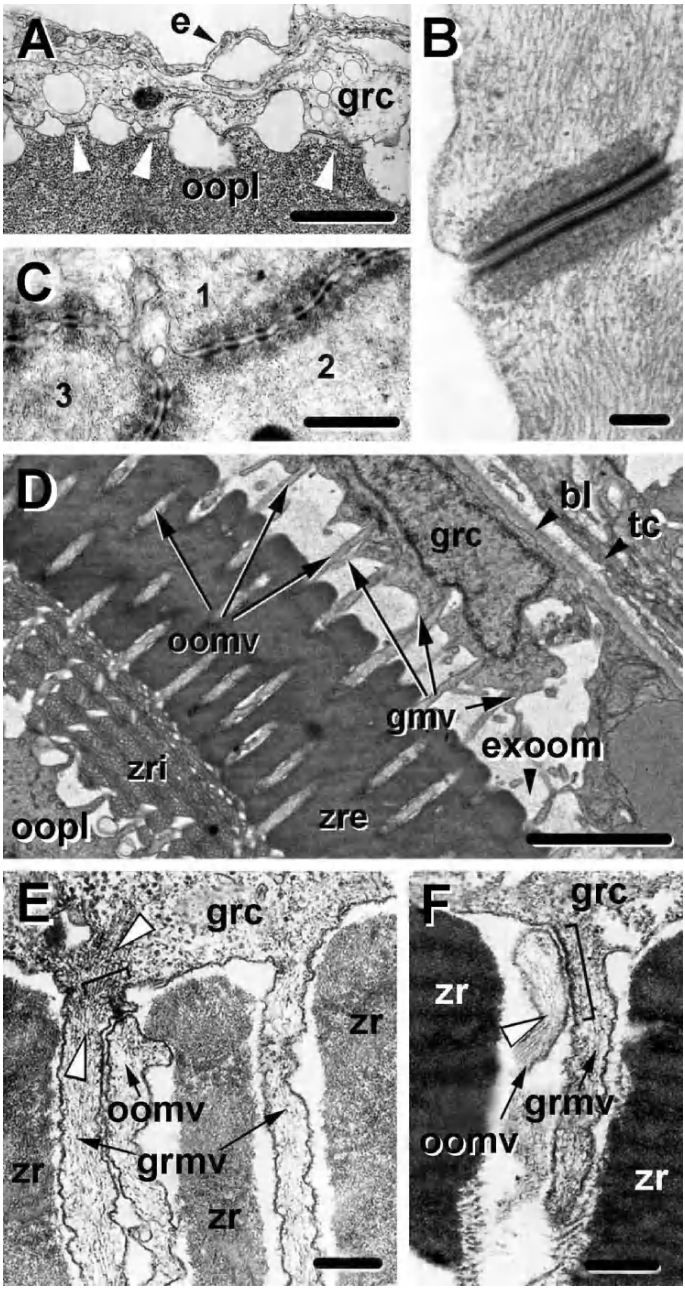

Figure 10. Ultrastructure of junction complexes in Teleostei ovarian follicles. (A) *Danio rerio* (Teleostei, Cyprinidae) early follicles in which the zona radiata is not yet formed and the oocyte has started to make "focal-type" contacts with differentiating granulosa cells. The white arrowheads

cells (Toshimori and Yasuzumi, 1979a, b; Kobayashi, 1985; Kessel et al., 1988). More remarkably, different cell contact structures are formed between microvilli and granulosa cell surfaces (Flegler, 1977; Abraham et al., 1984; Kobayashi, 1985; Cerdà et al., 1999) (Figure 10B–F). However, the nature and molecular composition of these adhesion structures have still not been clearly defined.

In mammalian models, it is well established that cell–cell adhesion within a tissue is mediated by different cell junctions, including such prominent structures as adherens junctions and desmosomes, which form semi-stable intercellular contacts (reviewed by Schmidt et al., 1994). Characteristically, all these adhering junctions consist of transmembrane glycoproteins in the cadherin family and cytoplasmic plaque proteins that anchor the cytoskeleton to the cadherins. The adherens junctions are formed by "standard" cadherins (e.g. E-, N-, and P-cadherin) that associate with specific plaque proteins, such as α- and β-catenins, plakoglobin, vinculin, α-actinin. Desmosomes are clusters of specific cadherins (desmogleins and desmocollins) that assemble with several desmosome-specific proteins, such as desmoplakins I and II and plakophilins, in addition to the common plaque protein, plakoglobin (reviewed by Borrmann et al., 2000; Getsios et al., 2004). In Teleostei, at least some junction complexes formed between granulosa cells and oocytes strongly resemble mammalian adhering junctions on electron micrographs. In zebrafish, Cerdà et al. (1999) recently obtained immunological evidence for the enrichment of α- and β-catenin and E-cadherin-like molecules in the oocyte cortex, notably at oocyte-granulosa cell contact sites, suggesting the presence of *hitherto* unknown heterotypic adherens junctions between these cells (Figure 11). In contrast, plakoglobin and N-cadherin are restricted to cell–cell contacts between granulosa cells. During oocyte maturation, mRNAs coding for αE- and β-catenin and plakoglobin accumulate in the granulosa and all three plaque-forming proteins are stored in unfertilized eggs, indicating that these junction proteins may play a role in early embryogenesis. These findings suggest that at least some heterotypic adhesion contacts between zebrafish granulosa cells and oocytes may be mediated by E-cadherin and catenin molecules.

In recent years, it has been shown that some junction plaque molecules, such as β-catenin and plakoglobin, have important transcriptional functions during

indicate cell contact points. (B) Typical desmosome between two granulosa cells in *Gobius niger* (Teleostei, Gobiidae). (C) Series of desmosomes between three thecal cells (numbered 1–3) in *Gobius niger*. (D) The zona radiata of *Sparus aurata* (Teleostei, Sparidae) fully grown ovarian follicle. The somatic cell layers surrounding the oocyte are differentiated. (E) and (F) Contact points between the oocyte and granulosa cell processes appear associated with electron-dense material (brackets) and microfilaments (white arrowheads). *Abbreviations*: bl – basement lamina; e – epithelium; exoom – extraoocyte matrix; grc – granulosa cell; grmv – granulosa microvilli; oomv – oocyte microvilli; oopl – ooplasm; zr – zona radiata; zre – zona radiata externa; zri – zona radiata interna; tc – thecal cell. Scale bar = 0.5 μm in (A, E, F), 0.1 μm in (B), and 1 μm in (C, D). (Data are modified from Cerdà et al. (1999) in (A, D–F) and F. Le Menn, University of Bordeaux 1, France in (B, C).)

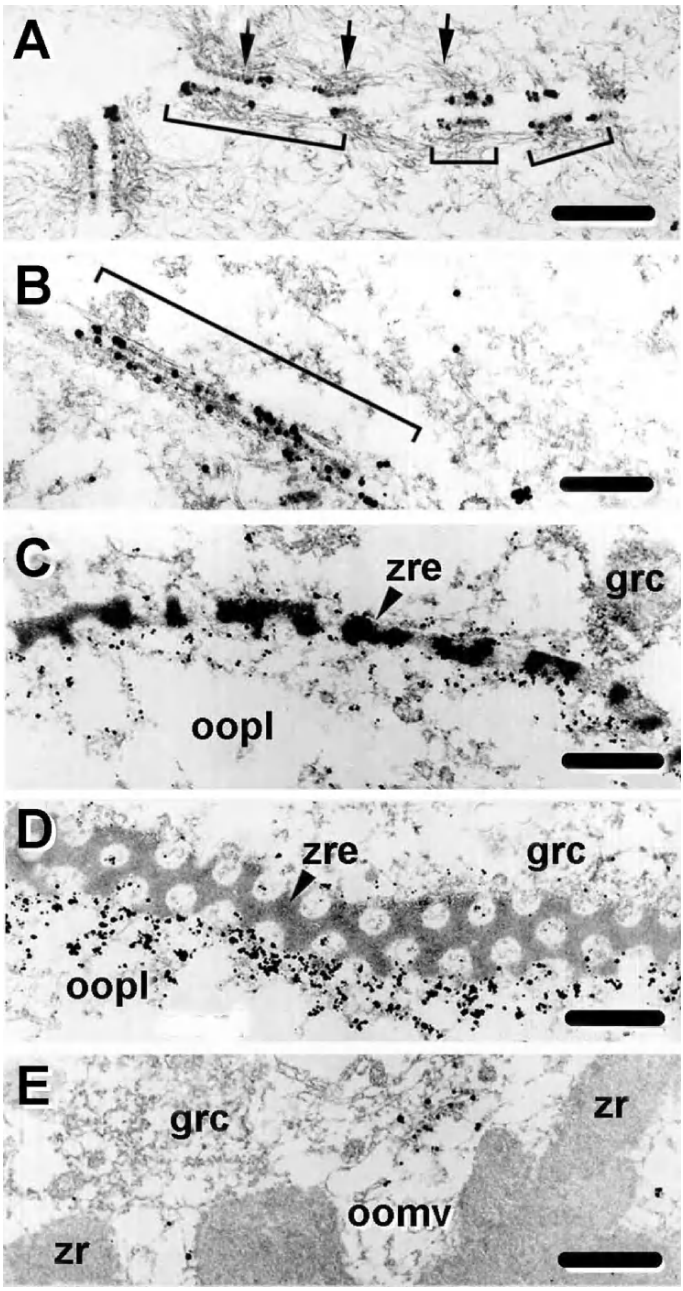

Figure 11. Immunogold localization of cell adhesion-associated proteins in *Danio rerio* ovarian follicle. Immunogold localization of plakoglobin (A, B), E-cadherin (C), α-catenin (D), and β-catenin (E). Plakoglobin immunodetection in both (A) desmosome-associated bundles of intermediate

development in addition to their structural role mediating cell–cell adhesion. Observations in non-vertebrate and vertebrate species, including zebrafish, suggest that this mechanism is highly conserved during evolution (reviewed by Bienz, 2005). The action of β-catenin on gene transcription is regulated by the Wnt-signalling pathway, which controls the degradation of β-catenin during embryo development and cancer progression. Plakoglobin is also involved in the Wnt pathway, but recent evidence suggests that this protein plays a unique role in Wnt signalling, different from that of β-catenin (Zhurinsky et al. 2000). Wnt-secreted factors are also essential for follicular growth and ovulation in mammalian ovaries, and are also involved in male and female sexual determination pathways (Richards et al., 2002). In Teleostei ovaries, however, the potential physiological roles of β-catenin and/or plakoglobin and Wnt factors during oogenesis and folliculogenesis are completely unknown.

8.2. Intercellular Communication Through Gap Junctions

During oocyte growth, granulosa cells and oocytes communicate via heterologous gap junctions. These cellular structures provide a mechanism for the transfer of small molecules (Mr < 1,200) from one cell to another, thus coupling the growing oocyte and its surrounding granulosa cells in a functional syncytium. Gap-junction channels consist of connexins, a homologous family of over 20 proteins that assemble into a connexon. Each connexon is a hexamer of connexins (Cxs) and the end-to-end docking of connexons from two adjacent cells creates the intercellular channel (reviewed by Bruzzone et al., 1996). The occurrence of functional gap junctions between mammalian oocytes and the surrounding granulosa cells has been well established by fluorescent dye transfer and metabolic coupling assays, as has the importance of the signalling through these channels during folliculogenesis (reviewed by Kidder and Mhawi, 2002).

In Teleostei ovarian follicles, heterologous gap junctions between oocytes and overlying granulosa cells have been well documented (Toshimori and Yasuzumi, 1979b; Kessel et al., 1985; Kobayashi, 1985; Iwamatsu et al., 1988; Iwamatsu and Ohta, 1989; York et al., 1993; Patiño and Kagawa, 1999). Particularly clear evidence for the existence of heterologous gap junctions has been provided by transmission electron microscopy in the presence of lanthanum and freeze-fracture replicas of the granulosa, as, for instance, in *Plecoglossus altivelis* (Toshimori and Yasuzumi, 1979b) and zebrafish (Kessel et al., 1985).

filaments (black arrows) and (B) adherens junctions (brackets) in follicle cells. (C) Localization of E-cadherin-like protein at the cortex of stage III oocytes and follicle cells. (D) Stage III oocyte showing a strong signal for α-catenin at the oocyte cortex adjacent to the zona radiata externa. Note that the signal in the overlying follicle cells is not as strong as in the oocyte. (E) Immunogold localization of β-catenin at contact points between granulosa cells and oocyte microvilli. *Abbreviations*: grc – granulosa cell; oomv – oocyte microvilli; oopl – ooplasm; zr – zona radiata; zre, – zona radiata externa. (Data are modified from Cerdà et al. (1999).)

Interestingly, gap junctions are formed mostly between oocyte microvilli and granulosa cells rather than granulosa microvilli and oocytes (Toshimori and Yasuzumi, 1979b; Kobayashi, 1985; Cerdà et al., 1999). Recently, fluorescent dyes, such as lucifer yellow, have shown that oocytes and granulosa cells in different species are functionally coupled through gap junctions during oocyte growth (Cerdà et al., 1993; Yoshizaki et al., 2001). Communication is resumed when microvilli retract from pore canals in the ZR during oocyte maturation prior to ovulation and, accordingly, the number of both heterologous and homologous (between granulosa cells) gap junctions appears to decline as maturation proceeds (Bolamba et al., 2003). During maturation, cell adhesion structures also become disorganized and E-cadherin, as well as α- and β-catenin, disappear from the oocyte-granulosa cell contact points (Cerdà et al., 1999).

Nevertheless, the specific physiological roles of gap junctions during ray-finned fish ovarian follicle development are not well known. Iwamatsu and Ohta (1981) provided some evidence that the maintenance of junction associations between granulosa cells and oocytes is necessary for medaka (*Oryzias latipes*) oocytes to acquire normal developmental capacity. In killifish, functional heterologous gap junctions are required for steroid-induced oocyte hydration during the resumption of meiosis (see Chapter 12) and there is evidence that this communication is necessary to maintain oocyte meiotic arrest (Greeley et al., 1987; Cerdà et al., 1993). In other fish species, such as Atlantic croaker (*Micropogonias undulatus*) and red sea bream (*Pagrus major*), gap junctions and mRNAs encoding Cx32.2, Cx31.5, and Cx32.3 increase during gonadotropin-induced maturational competence (OMC) of oocytes and during steroid-induced oocyte maturation (Yoshizaki et al., 1994; Patiño and Kagawa, 1999; Choi and Takashima, 2000; Choi and Takemura, 2001). In croakers, Cx32.2 and Cx32.7 RNAs are highly expressed in somatic cells, while low levels of both Cx RNAs are found in ovulated eggs (Chang et al., 2000). The stimulation of OMC and Cx32.2 mRNA levels by gonadotropin is apparently mediated by protein kinaseA-dependent pathways and antagonized by protein kinase C-dependent mechanisms (Chang et al., 1999; Yoshizaki et al., 2000). These findings have led to the suggestion that gap junction communication is necessary to transfer signals from granulosa cells into oocytes to induce or modulate OMC (Patiño et al., 2001). However, direct experimental evidence supporting this hypothesis is lacking and, thus, the physiological significance of the increase in gap junctions during OMC, as well their disappearance before ovulation, remain unknown.

9. FROM PRIMARY OOCYTE UNDERGOING VITELLOGENESIS TO SECONDARY OOCYTE: STAGE IV AND MATURATIONAL PROCESSES

During maturation, or stage IV, the primary oocyte leaves the diplotene stage and restarts the first meiotic division. Unfortunately, the term maturation is sometime improperly used to describe primary oocyte size enlargement during arrest

in the prophase of the first meiotic division, leading to confusion between researchers and fish farmers. Maturation processes are induced by gonadotropic hormones and occur in the ooplasm and nucleus of primary oocytes. The maturation signal consists of a new pulse of gonadotropic hormones, which stimulate ovarian somatic follicle cells, triggering synthesis of the maturation-inducing steroid (MIS) (see Chapter 11). MIS acts on specific receptors located on the oolemma, which, in turn, trigger the synthesis and activation of maturation-promoting factor (MPF) in the ooplasm (Yamashita et al., 1998; Senthilkumaran et al., 2004) (see Chapter 11).

The morphological events during maturation generally follow a similar sequence in all ray-finned fish species. The oocyte nucleus migrates towards the animal pole, i.e. the end of the oocyte-containing ooplasm and organelles, associated with the micropyle in the granulosa cell layer. In some species, such as sturgeon, this displacement may take several months. Yolk globules can fuse with lipid globules to form a liquid yolk mass located at the vegetal pole of the oocyte. In pelagophil fish species, which produce buoyant eggs, lipid globules fuse independently to form one or larger lipid droplet(s), which contribute to the buoyancy of the eggs and early embryonic stages (see Chapter 12). In some ray-finned fish species, a new, thin, irregular external layer of homogenous material, probably secreted by the granulosa epithelium, is deposited on the surface of the ZR externa (Le Menn and Pelissero, 1991). In zebrafish, a light flocculent material covers the outer surface of the ZR externa, and is periodically interrupted by masses of electron-dense material (Selman et al., 1993). Hydration of the oocyte is indispensable for normal embryo development and exists in pelagophil as well as benthophil species. However the buoyant eggs of pelagophil species are considerably more hydrated than those of benthophil species (see Chapter 12). This process involves the breakdown of part of the yolk protein stored in the oocyte, releasing free amino acids and small peptides, which act as osmotic effectors for water influx. Oocyte microvilli then retract from ZR channels. MPF acts on the lamina covering the inside of the internal nuclear membrane, phosphorylating lamina serines and leading to de-structuring of the nuclear envelope and GVBD (see Chapter 11). Metaphase of the first meiotic division occurs, producing two cells, a large secondary oocyte, and a small first polar body, which degenerates. The secondary oocyte undergoes the second meiotic division, which is arrested in the metaphase, as in other vertebrates. The secondary oocyte separates from its somatic surrounding layers during ovulation. In the wild, spawning occurs spontaneously. In farmed fish, spawning may require manual stripping, i.e. a gentle pressure on the female's abdomen. In all cases, the spawn cells are matured secondary oocytes or ova. Fusion with a spermatozoon during fertilization produces a fertilized egg, i.e. the starting point of embryo development.

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CHAPTER 2

MOLECULAR ASPECTS OF OOCYTE VITELLOGENESIS IN FISH

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1. INTRODUCTION

The ability of an oocyte to develop into a viable embryo depends on the accumulation of specific maternal information and molecules. Oocyte growth, particularly in oviparous species, is characterized by the intense deposition of products; e.g. RNAs, proteins (including growth and transcription factors), lipids, vitamins, and hormones. The deposit and storage occur in teleost fish species in a physiologically arrested cell at the G2/M border in first meiotic prophase (see Chapter 1). Whereas the nucleus remains in the diplotene stage, maternal RNAs are produced endogenously by the oocyte (see Chapters 3 and 6). As in other oviparous vertebrates, oocyte growth occurs by the uptake of plasma egg yolk precursor proteins (EYPP), predominantly vitellogenins (Vtgs) and putatively very low-density lipoprotein (VLDL) constituents during the vitellogenesis phase of oogenesis (for reviews see: Wallace, 1985; Mommsen and Walsh, 1988; Wallaert and Babin, 1994; Tyler and Sumpter, 1996; Wiegand, 1996). In females, these lipoproteins are synthesized in the liver, mainly under 17β–estradiol (E2) control (Wallaert and Babin, 1992), and

are massively transported to the oocyte, leading to full-grown gonads that may account for about 20% of the total weight of the fish at the end of vitellogenesis. The many molecular events involved in the overall process require tight, coordinated regulation under strict endocrine control (see Chapters 7 and 8), and this hormonal regulation may be disrupted by environmental estrogenic compounds (for reviews see: Arukwe and Goksøyr, 2003 and Chapter 10).

2. THE VITELLOGENIN GENE FAMILY AND THE STRUCTURE, EVOLUTION, AND EXPRESSION OF PRECURSOR PROTEINS

2.1. The Large Lipid Transfer Protein (LLTP) Superfamily

In animals, the extracellular transport of hydrophobic lipids is mediated by lipoproteins, and to a lesser extent by proteins able to bind non-esterified fatty acids, e.g. serum albumin. The term "vitellogenin" was proposed over 35 years ago (Pan et al., 1969) to describe female-specific insect hemolymph protein precursors of egg yolk, regardless of their amino acid sequences or structures. This term, based on a functional criterion, was later adopted with regard to other egg-laying animals and is widely used in the scientific community and sequence databases. Vtg is the main EYPP in most oviparous species, and its molecular characterization has revealed that this high-molecular weight glycolipoprotein is conserved among species, suggesting derivation from a common ancestor (Wahli, 1988; Byrne et al., 1989; Chen et al., 1997; Sappington and Raikhel, 1998a). However, molecular data from some species has revealed that the main EYPP are unrelated to the Vtg protein family. For example, major EYPP are related to transferrin in sea urchins (Brooks and Wessel, 2002) and lipase in higher *Diptera* (Bownes and Pathirana, 2002). A unique highly polymerized glycosylated yolk protein was reported from cochineal insect *Dactylopius confusus* (Ziegler et al., 1996). Multiple alignments of vertebrate and non-vertebrate Vtg sequences revealed five relatively well-conserved regions (Chen et al., 1997; Lim et al., 2001). Regions I to III, located in the N-terminal part, correspond to the lipovitellin 1 subunit of vertebrate Vtg, while regions IV and V, located in the C-terminal part, correspond to the lipovitellin 2 subunit. The sequence and deduced structural homologies indicated an evolutionary relationship of Vtg with three mammalian proteins: apolipoprotein B-100 (apoB), the large subunit of microsomal triglyceride transfer protein (MTP), and the von Willebrand factor (VWF) (Baker, 1988a, b). The identification of conserved amino acid sequence motifs and ancestral exon boundaries in apoB, MTP, non-vertebrate and vertebrate Vtg, insect apolipophorin II/I (apoLp-II/I), and decapod crustacean apolipocrustacein (apoCr), indicated that large lipid transfer proteins (LLTP) are members of the same multigene superfamily and have emerged from a common ancestral molecule. This molecule is thought to have played a pivotal role in the intracellular and extracellular transfer of lipids

and liposoluble substances (Babin et al., 1999). A recent phylogenetic analysis conducted using maximum likelihood and Bayesian inference methods strongly suggested that the extracellular LLTP members diverged after an ancient duplication event leading to the separation of an APO paralogous family group (apoCr, apoLp-II/I, apoB) from Vtg/clotting protein (CP) sequences (Avarre et al., 2007). This second group of sequences, named the Vtg/CP family, grouped with confidence in separate clusters: vertebrate, mollusk, nematode, and insect Vtgs, as well as crustacean CP (Avarre et al., 2007).

2.2. Domain Architecture and Conserved Sequence Motifs of Vertebrate VTG

Vertebrate Vtgs are extracellular and shorter (~1,600–1,800 amino acids) LLTP members, and their amino acid sequence include three conserved structural domains (Figure 1). A lipoprotein N-terminal domain also known as vitellogenin_N (Pfam http://www.sanger.ac.uk/Software/Pfam/index.shtml accession number PF01347) and enclosed in the large lipid transfer (LLT) module (Babin et al., 1999) was identified in the N-terminal part of LLTP members, and a VWF type-D domain (VWD) (Pfam accession number PF00094) at the C-terminal end of both apoCr/apoLpII/I subfamilies and Vtg/CP family members (Avarre et al., 2007). While the VWD domain, initially identified in the human VWF, is distributed over a wide range of proteins, the LLT module/LPD-N domain, part of the lipovitellin 1 subunit of vertebrate Vtg (Chen et al., 1997; Lim et al., 2001), contains 22 N-terminal-conserved amino acid sequence motifs (N1–N22) as a common denominator of LLTP (Babin et al., 1999). The Vtgs of vertebrate and some non-vertebrate species also contain polyserine tracks that correspond to the phosvitin domain inserted in the middle part of the protein. However, this domain can also be absent, as demonstrated with zebrafish Vtg3 (Wang et al., 2000), in *Gambusia afinis* (Sawaguchi et al., 2005a, b) and *Pagrus major* (Sawaguchi et al., 2006). It should be noted that an additional DUF1081 domain (Pfam accession number PF06448) of unknown function was identified in the central region of APO family members (Avarre et al., 2007).

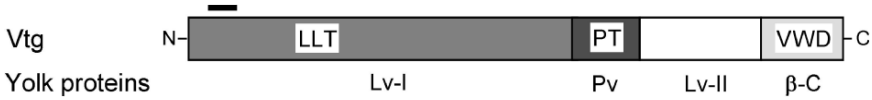

Figure 1. Domain architecture of teleost fish Vtg. Large lipid transfer (LLT) module also referred to as Vitellogenin_N and LPD-N domain, polyserine track (PT) domain, and von Willebrand-factor type-D domain (VWD) are indicated from the N-terminal to the C-terminal ends of the plasma precursor protein. The horizontal line indicates the receptor-binding region to VtgR. Lipovitellin I (Lv-I) and II (Lv-II), phosvitin (Pv), and β-component (β-C) are the yolk proteins generated by the enzymatic cleavage of Vtg inside the oocyte.

2.3. VTG Gene Clusters and Their Expression in Fish

2.3.1. Vtg genes and their expression sites

The presence of multiple Vtg genes (*vtgs*) has been demonstrated in the genome of some teleost fish species (Buisine et al., 2002; Wang et al., 2005). Comparative genomics analyses suggested two whole genome duplication events occurring, one before and the other after the divergence of ray-finned and lobe-finned fishes, prior to teleost radiation (Amores et al., 1998; Postlethwait et al., 1998; Jaillon et al., 2004; Brunet et al., 2006). The additional number of genes resulting from these events may have facilitated the evolutionary radiation and phenotypic diversification in teleost fish. Alternatively, the loss of different duplicates in various populations may have promoted speciation in ancient teleost populations (Hoegg et al., 2004). A model for *vtgs* amplification for teleost fish was proposed from Salmonidae, where ancestral *vtg* duplications gave rise to two paralogous gene clusters, VtgA and VtgB (Buisine et al., 2002). Archaic salmonids harboring low *vtg* copy numbers reflected an ancestral state, while species harboring high *vtg* copy numbers reflected the most recent state of differentiation. The ultimate state of differentiation appears in *Oncorhynchus* species having lost VtgB-related genes in the course of evolution, and exhibiting a unique VtgA locus containing a set of 20 highly similar tandemly arranged genes. The *vtgs* of *Oncorhynchus mykiss* differ from each other because of insertion, deletion, and rearrangement events during evolution, but exhibit a high degree of similarity at sequence level (Trichet et al., 2000). The zebrafish (*Danio rerio*) genome contains at least seven *vtgs* that encode homologous Vtgs with three distinct types mapped onto two different chromosomes (Wang et al., 2005). One *vtg* transcript has been characterized in the silver lamprey, *Ichthyomyzon unicuspies* (Sharrock et al., 1992) and the white sturgeon, *Acipenser trasmontanus* (Bidwell and Carlson, 1995); at least two have been cloned in mummichog (*Fundulus heteroclitus*) (LaFleur et al., 1995, 2005), Japanese medaka (*Oryzias latipes*) (GenBank http://www.ncbi.nlm.nih.gov/Genbank/index.html accession numbers: AB064320 and AB074891; Tong et al., 2004), Japanese goby, *Acanthogobius flavimanus* (Ohkubo et al., 2004), tilapia (*Oreochromis aureus*) (Lee et al., 1994; Lim et al., 2001), and haddock (*Melanogrammus aeglefinus*) (Reith et al., 2001); and multiple transcripts were demonstrated in mosquitofish (*Gambusia affinis*) (Sawaguchi et al., 2005a, b). A scheme has been proposed for the classification of vertebrate Vtg genes based on three rounds of whole genome duplications, and lineage-specific gene duplications (Finn and Kristoffersen, 2007).

It has been shown that vitellogenesis of Chondrichthyes is similar to that of other vertebrates in both synthesis and storage mechanisms of oviparous dogfish, *Scyliorhinus canicula* (Craik, 1978a–d, 1979) and in skate, *Raja erinacea* (Perez and Callard, 1992). However, in *Torpedo marmorata*, oocyte growth is not only due to the uptake of extraovarian Vtg, but also to the contribution of somatic cells of the ovarian follicle (Prisco et al., 2002, 2004). Northern blot results performed in zebrafish showed that all seven *vtgs* were expressed predominantly in the liver and adipose tissue of female fish or E2-induced males, and that *vtg1* is the most highly

expressed (Wang et al., 2000; Tong et al., 2004; Wang et al., 2005). *In situ* hybridization experiments confirmed an extrahepatic expression of zebrafish *vtg* genes in adipocytes associated with several organs such as the ovary (Wang et al., 2005).

2.3.2. Hormonal regulation of Vtg synthesis

Vitellogenin is present in the blood of females of all oviparous vertebrates species undergoing vitellogenesis and is produced mainly in the liver under multihormonal control. From a hormonal point of view, vitellogenesis may be defined as the E2-induced hepatic synthesis of EYPP, their secretion and transport via the bloodstream to the ovary, and their uptake into developing oocytes. The brain modulates the release of gonadotropins from the pituitary by the stimulatory effect of the hypothalamic gonadotropin-releasing hormone and by the inhibitory effect of dopamine. These neurohormones control the release of gonadotropins from the anterior pituitary (see Chapter 7). The follicular-stimulating hormone predominates during oocyte growth and is involved in eliciting steroid release from the follicle wall, thereby controlling a variety of processes, including vitellogenesis. It is well known that plasma E2 levels in females increase during vitellogenesis and decline during maturation. In a great number of teleost species, the hepatic synthesis of Vtg is induced by ovarian estrogens (for a review see Polzonetti-Magni et al., 2004). Many estrogen actions are mediated by classical nuclear estrogen receptors (ER) belonging to a steroid/nuclear receptor superfamily of enhancer proteins. These are important ligand-activated transcription factors (for a review, see Klinge et al., 2004). Following their specific interaction, dimeric ligand/receptor complexes recognize and bind the DNA motif, the "estrogen responsive element" (ERE). Vertebrate genomes contain at least two ER subtypes, ERα (*esr*1) and ERβ (*esr*2), which have now been found in species ranging from teleosts to mammals. ERα and ERβ have been cloned and characterized from different teleost fish species as illustrated by data on the hormonal specificity, binding characteristics, and transactivating capacities (for reviews, see Menuet et al., 2002; Tingaud-Sequeira et al., 2004). The hormone/receptor complex binds tightly in the nucleus at EREs located upstream of, or within the estrogen-responsive genes in DNA. This results in the activation or enhanced transcription of *vtgs* and subsequent increase and stabilization of *vtg* messenger RNAs as fully demonstrated with amphibian (*Xenopus laevis*) *vtgs* (Cooper et al., 1987; Shapiro et al., 1989). In addition, *vtgs* may be committed to tissue-specific expression early in development, and a single injection of E2 at 15 days of chick embryogenesis can retain memory for up to 6 months after hatching (Edinger et al., 1997). The persistence of memory through several rounds of cell division is due to estrogen-dependent alterations in the chromatin structure of these genes. The memory effect is obvious when one compares the primary and secondary responses; during the latter, the response has a shorter lag, and reaches a higher magnitude (Beuving and Gruber, 1971).

E2 is considered the most potent steroid in inducing hepatic Vtg synthesis. The administration of this hormone to juvenile and male teleost fish induced Vtg accumulation in their bloodstream (Mommsen and Walsh, 1988). This hormonal induction may be also performed in the other so-called fishes of the Craniata lineage, e.g. the Mixiniformes *Lampetra fluviatilis* (Mewes et al., 2002). A different sensibility of *vtgs* to estrogens within the same genome, was observed, e.g. in Japanese goby (Ohkubo et al., 2004) and tilapia (Takemura and Kim, 2001). The level of circulating Vtg reflects the level of estrogen in the plasma, as observed, for example, in *Sparus aurata*, where high levels of both Vtg and E2 were found concomitantly in pre-spawning and spawning females. In this model, the plasma Vtg concentration reflects a balance between the protein produced by the liver and the one sequestered by growing oocytes (Mosconi et al., 1998). Vtg assessment is currently considered a useful approach to evaluating female vitellogenesis related to peripheral gonadal steroid changes and to assessing sex ratio. Vtg quantitative assays have been optimized in different teleost species (e.g. Specker and Sullivan, 1994; Mosconi, et al., 1998; Ndiaye et al., 2006). Beside E2, cortisol induced a rapid and transient transcription of *vtg* mRNA in the tilapia, *Oreochromis aurens* (Ding et al., 1994). A combined treatment of E2 plus progesterone or cortisol enforced *vtg* mRNA expression in the hepatocyte culture of immature rainbow trout, *Oncorynchus mykiss*, although injections of cortisol alone failed to induce this messenger expression (Mori et al., 1998). Moreover, the involvement of androgens in Vtg synthesis has also been reported in certain teleost fish (Hori et al., 1979; Le Menn et al., 1980; Peyon et al., 1997; Kim et al., 2003). *In vivo* experiments showed that Vtg was induced in the bloodstream after 17α-methyltestosterone and 5α-dihydrotestosterone treatment in goldfish *Carassius auratus* (Hori et al., 1979) and in *Gobius niger* (Le Menn et al., 1980). While rainbow trout androgens or testosterone (androsterone and 17α-methyltestosterone) induced the *in vitro* expression of *vtg* transcript (Mori et al., 1998), their oral administration in *Oreochromis niloticus* inhibited both the hepatic messenger expression and the appearance of Vtg in the bloodstream (Lazier et al., 1996).

A large number of publications are known on the involvement of pituitary hormones in Vtg synthesis. In teleost fish, even if hepatic Vtg synthesis has mainly been found due to the direct action of E2, *in vitro* experiments performed on immature eel hepatocytes revealed that when physiological doses of E2 were added to the medium, growth hormones (GH) or prolactin were required for its induction (Kwon and Mugiya, 1994; Peyon et al., 1996). In addition, the synthesis and secretion of hepatic Vtg under the effect of estrogenic compounds and GH is probably related to different reproductive phases (Polzonetti-Magni et al., 2004). Recombinant sea bream parathyroid hormone related protein (PTHrP) has a potentiating effect on E2 stimulation of Vtg production by sea bream hepatocytes (Bevelander et al., 2006). It should be pointed out that Vtg may be able to regulate its own synthesis and incorporation (Reis-Henriques et al., 2000; Nath and Maitra, 2001). Many studies confirmed the hormonal

control of the onset of vitellogenesis in fish (Specker and Sullivan, 1994; Ohkubo et al., 2004), although little or no studies exist on vitellogenesis termination. During the quiescent period, the termination of Vtg synthesis occurs when relatively high peripheral E2 levels are still present.

Vtg is considered a useful biomarker of exposure to endocrine disrupting compounds (EDCs) (Kime et al., 1999; and Chapter 10). Measuring Vtg in oviparous vertebrates is generally agreed to be a good indicator for agonist or antagonistic effects, and it is proposed as one of several endpoints in fish assay for endocrine disruptor screening (Kime et al., 1999). Many toxic chemicals are known to disseminate regularly into the environment, and these endocrine disrupters are able to interact with the endocrine system, mimicking the action of E2 and thus impairing the reproduction of aquatic animals. EDCs can have organizational effects on sex differentiation and on expression of secondary sex characteristics in fish (Kime 1996, 1998; Sumpter, 1998; Metcalfe et al., 2000; Edmunds et al., 2000; Cardinali et al., 2004). Xenoestrogens can trigger agonist or antagonistic responses by binding to ER. These compounds have been largely studied due to their ability to induce morphological and functional alterations of the reproductive tract, primarily in males. This is especially true of alkylphenolic compounds from a group of industrial chemicals that has been shown to have estrogenic properties both *in vivo* (Jobling et al., 1996; Schwaiger et al., 2000; Goksøyr et al., 2003; Maradonna et al., 2004) and *in vitro* (Pelissero et al., 1993; Sumpter and Jobling, 1995; Celius et al., 1999). Vtg can also act as a biomarker of female reproductive dysfunctions; abnormally low Vtg levels inhibit ovarian growth. Much is known about the negative interaction of some environmentally released toxicants with gonadal steroidogenesis, either directly on the gonad's steroidogenic enzymes, or by decreasing pituitary gonadotropin production, which in turn decreases steroid levels and the gonadosomatic index (Kime 1996, 1998; Chapter 10).

2.4. VTG Posttranslational Modifications and the Ligand-Binding Domain

After synthesis of the protein backbone, substantial posttranslational modifications occur to Vtg, prior to its secretion into the circulatory system. Vtg is specifically devoted to the massive deposit of yolk reserves and constituents inside the oocyte. The Vtg molecule is phosphorylated, lipidated, and glycosylated, enabling the release of a phospholipoglycoprotein that also serves as a major supply of minerals such as calcium, magnesium, zinc, and iron to the oocyte, and therefore to the developing embryo. The increase in plasma protein, calcium, magnesium, and phosphoprotein content can be used as indicators of plasma Vtg levels (Mommsen and Walsh, 1988; Silversand and Haux, 1995). Like other plasma lipoprotein classes, Vtg is also able to bind and transport lipophilic hormones such as thyroid hormones (Babin, 1992) and retinals (see below). Through their LLT module/LPD-N domain, Vtg retained the capacity to bind and transport lipids, a function that is probably the ancestral function

of LLTP (Babin et al., 1999, Shelness et al., 2005). It was suggested that this domain may form a lipovitellin-like "proteolipid" intermediate containing a pocket-enabling lipid loading of Vtg (Banaszak et al., 1991; Sharrock et al., 1992; Anderson et al., 1998; Segrest et al., 1999) that requires MTP for assembly (Sellers et al., 2005). *Drosophila* MTP promotes the assembly and secretion of human apoB-41 (Sellers et al., 2003), and human MTP enhances the secretion of *X. laevis* Vtg (Sellers et al., 2005). A mutant form of human MTP was still able to promote *Xenopus* Vtg synthesis, whereas secretion of human apoB was abolished, suggesting that the requirement of apoB for interacting with MTP is more stringent than that of Vtg. The circulating native Vtg is usually a homodimer (e.g. Babin et al., 1987a; Matsubara et al., 1999), and each polypeptide chain contains a VWD domain at their C-terminal end that may be implicated in the clotting cascade and multimerization, as demonstrated with human VWF (Jorieux et al., 2000). The functional significance of the VWD domain in Vtg calls for additional elucidation. Recent data demonstrated that teleost fish Vtg exhibits an agglutinin activity, and may be involved in defensive reactions (Zhang et al., 2005; Shi et al., 2006).

As demonstrated in *O. aureus*, the amino-terminal region of the lipovitellin I domain of Vtg interacts with the ligand-binding domain of LR8/Vtg receptor (VtgR). Computational analysis of the binding sequence indicates that tilapia Vtg has a receptor-binding region similar to human apoE and apoB (Li et al., 2003). This is consistent with earlier studies demonstrating that, as in *Xenopus* (Stifani et al., 1990b), the binding of Vtg to fish oocyte receptors was mediated by the lipovitellin I domain of Vtg (Stifani et al., 1990a; Hiramatsu et al., 2002a).

3. THE LOW-DENSITY LIPOPROTEIN RECEPTOR GENE FAMILY: EVOLUTION IN MAMMALS AND OVIPAROUS SPECIES

The evolution of Vtg and receptors for Vtg uptake into oocytes is a prime model for the emergence of ligand/receptor systems designed to sustain the reproductive effort of oviparous species. This evolutionary path is paralleled by one that guided the emergence of related ligand/receptor systems that are important for normal physiology of animals not relying on oviposition for reproduction. As will become apparent below, VtgR genes belong to the supergene family of low-density lipoprotein receptor (LDLR) related proteins (LRPs) that have coevolved in egg-laying and viviparous animals. These receptors, at least in mammals, have in addition to their function in ligand transport acquired functions in cellular signal transduction pathways. We are not aware of such function(s) in fishes, but this aspect is without doubt one of the most interesting open issues in LR biology of piscine species.

In this section, we will provide a synopsis of our knowledge of the receptors for Vtg in the context of the LR supergene family. Starting from the first identified representative of a group of about a dozen LRs (to date mainly, but not exclusively, identified in man, rodents, and chicken), the human LDL receptor,

subsequently characterized LRs will be described in detail in section 4, in order to gain an appreciation of the relation of these receptors to VtgRs in fish. The review of information on VtgRs will emphasize molecular biological studies, but will also deal with biochemical results.

3.1. The Supergene Family of LDL Receptor Relatives (LRs)

The LDLR family comprises a large and growing number of genes, whose products contain a characteristic set of structural domains. As stated above, attempts to delineate the evolutionary history of this gene family must take into consideration newly discovered functions, including binding a still expanding list of ligands by the extracellular domains, ligand uptake through clathrin-mediated mechanisms, and signal transduction via the cytoplasmic regions of the receptors. As will become apparent from the summary of our knowledge about this gene family in the next few sections, we are only at the beginning of a long road towards gaining major new insights into their piscine relatives, both from a structural, but more importantly, from a functional point of view.

3.1.1. The prototype LR: The human low-density lipoprotein receptor and relationships between structure and function of the LDL receptor

The LDLR is an important component in the feed-back regulated maintenance of cholesterol homeostasis, a well-understood mechanism in mammals (Goldstein et al., 1985). Following LDLR-mediated endocytosis, LDL-derived cholesterol and its intracellularly generated oxidated derivatives mediate a complex series of feedback control mechanisms, termed LDL receptor pathway, that protect the cell from overaccumulation of cholesterol. The benefits of this LDLR-mediated regulatory system are the coordination of the utilization of intra- and extracellular sources of cholesterol. Most cells are able to subsist in the absence of exogenous sources of cholesterol, because they can synthesize it from acetyl-CoA. When LDL is available, the cells primarily use the LDLR to import LDL cholesterol and keep their own synthesis suppressed. Thus, a constant level of cholesterol is maintained within the cell, while the external supply in the form of lipoproteins can undergo large fluctuations. *In vivo*, LDLRs have two important functions: first, to supply cells with cholesterol; and second, to remove cholesterol-rich lipoprotein particles from the bloodstream in order to prevent their accumulation in the circulation. These concepts have been elucidated in cultured fibroblasts from normal subjects and from patients with the disease, familial hypercholesterolemia (FH). Deranged cholesterol homeostasis in FH fibroblasts led to the conclusion, and subsequently to the direct demonstration, that the abnormal phenotype is caused by lack of LDLR function, i.e. a disrupted LDL receptor pathway. As a consequence of the disturbed balance between extra- and intracellular cholesterol pools in FH, pathological hypercholesterolemia with accelerated development of atherosclerosis and its complications ensues. Comprehensive information about *LDLR* mutations in FH patients can be found at http://www.ucl.ac.uk/fh/.

Studies at the levels of protein chemistry, molecular biology, and cell biology have led to a detailed understanding of the biology of the LDL receptor. The mature receptor is a highly conserved integral membrane glycoprotein consisting of five domains (Figure 2). In order of appearance from the amino terminus these domains are: (1) the ligand-binding domain; (2) a domain that has a high degree of homology with the epidermal growth factor precursor (EGFP); (3) a domain that contains a cluster of O-linked carbohydrate chains; (4) a transmembrane domain; and (5) a short cytoplasmic region. Until direct information on the three-dimensional structure of the complete 839-residue receptor becomes available, an arrangement of these domains as presented in Figure 2 may serve as a useful model.

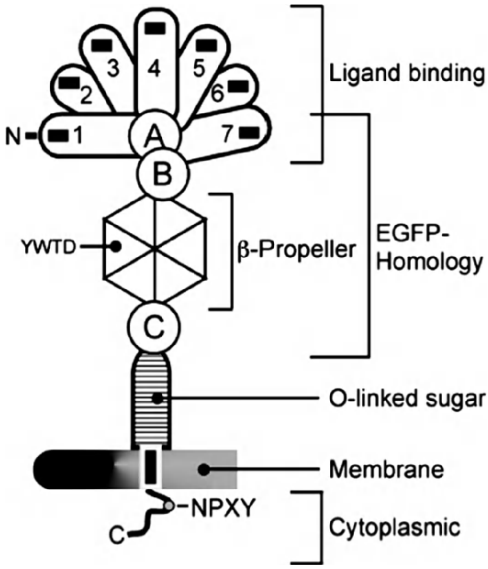

Figure 2. Domain model of the LDL receptor. The five domains of the mature protein, from the N-terminus (bold N) to the carboxyterminus (bold C) are: (1) the ligand-binding domain, characterized by 7 cysteine-rich repeats, each with a cluster of negatively charged amino acids whose core consists of Ser-Asp-Glu ("SDE"); (2) the EGF-precursor (EGFP) homology region, consisting of 400 amino acid residues (central hexagon); adjacent to the ligand binding domain and at the carboxy terminus of this region, respectively, are located 3 repeats with high homology to repeat motifs found in the precursor to epidermal growth factor (encircled letters A, B, and C, respectively). The remaining portion of this domain consists of 6 internally homologous stretches of approximately 50 amino acid residues each of which contains the sequence Tyr-Trp-Thr-Asp (YWTD) called beta-propeller; (3) the O-linked sugar region, consisting of 58 amino acids with 18 serine and threonine residues containing O-linked carbohydrate chains; (4) a single membrane-spanning domain; and (5) the cytoplasmic tail with 50 amino acid residues containing the internalization sequence Asn-Pro-Val-Tyr (NPVY; the Val (V) is not absolutely conserved in all species).

The Ligand-Binding Domain. This domain mediates the interaction between the receptor and lipoproteins containing apo B-100 and/or apoE (Esser et al., 1988). The function is localized to a region at the amino terminus of the receptor, comprised of seven repeats of approximately 40 residues each (called type A ligand-binding repeats, or LA repeats). These seven repeats have 6 cysteines each, which presumably mediate the folding of the domain into a rigid structure with clusters of negatively charged residues on its surface (with the signature tripeptide Ser-Asp-Glu, SDE). These LA clusters are thought to participate in the binding of lipoprotein(s) via positively charged residues on apo B-100 (apoB) or apo E (Jeon and Blacklow, 2005).

The Epidermal Growth Factor Precursor Homology Domain. This region of the LDL receptor lies adjacent to the ligand-binding site and is comprised of approximately 400 amino acids; the outstanding feature is the sequence similarity of this region to parts of the EGF precursor, i.e. three regions termed "growth factor repeats" (also called type B repeats). Two of these repeats are located in tandem at the amino-terminus, and the third is at the carboxyterminus of the precursor homology region of the LDL receptor. The remainder consists of modules of about 40 residues with a consensus tetrapeptide, Tyr-Trp-Thr-Asp (YWTD); six of these modules, tandemly arranged, form a so-called six-bladed beta-propeller. In turn, the beta-propeller and the type B repeats constitute the epidermal growth factor (EGF) precursor homology domain of LRs.

The O-linked Sugar Domain of the human LDL receptor is a 58 amino acid stretch enriched in serine and threonine residues, located just outside the plasma membrane. Most, if not all, of the 18 hydroxylated amino acid side chains are glycosylated. The O-linked oligosaccharides undergo elongation in the course of receptor synthesis and maturation: when leaving the endoplasmic reticulum, N-acetylgalactosamine is the sole O-linked sugar present, and upon processing in the Golgi, galactosyl and sialyl residues are added. Despite the detailed knowledge about the structure of this region, its functional importance remains unclear (Davis et al., 1986).

The Membrane Anchoring Domain lies carboxyterminally to the O-linked carbohydrate cluster. It consists of some 20 hydrophobic amino acids; as expected, the deletion of this domain in certain naturally occurring mutations, or by site-directed mutagenesis, leads to secretion of truncated receptors from the cells.

The Cytoplasmic Tail of the LDL receptor constitutes a short stretch of 50 amino acid residues involved in the targeting of LDL receptors to coated pits. Naturally occuring mutations and site-specific mutagenesis (Lehrman et al., 1987) have identified an "internalization signal," Asn-Pro-Xxx-Tyr (NPXY in Figure 2, where Xxx denotes any amino acid). Recently, the cytoplasmic domains of the LDL receptor and structural relatives have come into new focus, since they hold the key to the involvement of these receptors in signal transduction. For further details on these aspects, see May et al., 2005.

3.1.2. An evolving family

Although the human LDLR and subsequently LDLRs from other species were the first vertebrate LRs described, this multifunctional receptor is likely a recent result of evolution. Following the groundbreaking studies on the LDLR, many membrane proteins with a high degree of structural similarity to the LDLR have been discovered. Here, these proteins are referred to as LDLR relatives or LRs; several of them are still better known under their originally proposed names, and where appropriate, these will also be mentioned.

LRs comprise composite membrane proteins, which among performing other tasks are engaged in receptor-mediated endocytosis of a broad variety of ligands, a list of which now reaches far beyond lipoproteins. The most prominent members of the family are, listed in the order of their discovery, LDLR; LDL receptor related protein (LRP1); megalin (also called LRP2, and originally named gp330); VLDL receptor (VLDLR/VtgR; in chicken termed LR8); LR11 (also named sorLA); apolipoprotein E (apoE) receptor type 2 (apoER2), also known as LR7/8B; LRPs 3, 4, 5, and 6; and LRP1B (also termed LRP-DIT).

Common features of these proteins are the structurally and functionally defined domains described above for the human LDLR (i.e. the ligand-binding domain comprising LA repeat clusters; the EGF-precursor domain consisting of type B repeats, YWTD-motifs, and the beta-propeller domain; a facultatively present O-linked sugar domain; and the short cytoplasmic domain) and frequently specified by distinct exons in the corresponding genes.

3.1.3. The largest family members

LRP1. The extracellular part of LRP1, one of the largest members of the family known, is highly complex and contains 31 LA repeats organized in four clusters of 2, 8, 10, and 11 of these repeats, respectively, separated from each other by EGF precursor domains. The mature receptor is a heterodimer of a short membrane-spanning and a long extracellular subunit, respectively, which arise through furin cleavage of a 600 kDa precursor; this processing is required for efficient surface expression. LRP1 is a multiligand receptor involved in apparently independent processes, for example binding and/or metabolism of lipoproteins, extracellular proteinases and proteinase/inhibitor complexes, glycosphingolipids, and connective tissue growth factor, and may act as signaling co-receptor for the PDGF receptor. Of evolutionary significance is the identification of a gene for LRP in the nematode, *Caenorhabditis elegans*; there are no published reports yet about any piscine LRP1 (Roudier et al., 2005).

LRP2/Megalin. LRP2 (megalin) is another large member of the LR gene family containing four clusters of LA repeats. Although many proteins which bind to LRP1 are also ligands for megalin, its expression pattern and specificity for certain ligands account for physiological roles distinct from those of LRP1. For instance, a chicken oocyte-specific 380 kDa LR was found to bind clusterin (Mahon et al., 1999), known to be a ligand of megalin, suggesting that oviparous species also express megalin or a highly homologous LR. In mammals, megalin is

essential for development of the forebrain via uptake of apoB-containing lipoproteins into the embryonic neuroepithelium (reviewed in: Willnow, 1999). Another important function is its involvement in the metabolism of certain lipophilic vitamins. Dietary vitamin B12 in complex with intrinsic factor is taken up by binding to cubilin, which in turn is internalized by ileal epithelial cells via megalin. In the kidney, on the other hand, vitamin B12/transcobalamin complexes are recaptured from the ultrafiltrate directly by binding to megalin on proximal tubule cells. Furthermore, megalin mediates the reabsorbtion of 25-(OH) vitamin D3/vitamin-D-binding protein complexes, a key step in converting the precursor into active vitamin D3 in the kidney.

LRP1B (LRP-DIT). This 4599 residue type I membrane protein contains 32 LA repeats in its extracellular portion. Among all of its relatives, LRP1B shows the highest homology to LRP1, containing one additional ligand binding repeat and an insertion of 33 amino acids in the cytoplasmic domain compared to LRP1. Homology searches revealed that LRP1B is identical to the product of a candidate tumor suppressor gene, *lrp1b* (Liu et al., 2001). The human gene locus was mapped to chromosome 2q21 by fluorescence *in situ* hybridization. A total of 91 exons, varying in size from 77 bases (exon 87) to 1899 bases (exon 91), were identified in the more than 500 kb spanning gene. Comparative analysis of the genomic structures of LRP1B and the homologous LRP1 gene revealed a striking similarity of the location and sizes of their exons. LRP1B's biochemical properties are similar to those of LRP1 (with the exception of low endocytic activity of LRP1B), but only future studies will reveal the true physiological role(s) of this close relative of LRP1.

3.1.4. The so-called VLDL receptors

This group of small LRs has been discovered originally in the rabbit (Takahashi et al., 1992), about 15 years after the first characterization of the LDL receptor. The hallmark structural difference to the LDLR is the presence of 8, rather than 7, LA repeats in the ligand-binding domain. This structural feature appears to be important for the subtle differences in ligand recognition between the two receptor classes. Important biological characteristics of the VLDLR include the tissue distribution and resistance to regulation by intra- and extracellular sterol levels. Much of the understanding of this group of proteins has been gained from studies that unraveled the function of the homologue in the laying chicken, as described below. Before introduction of the subject in avians, the characteristic features of mammalian VLDLRs will be outlined.

Mammalian VLDLR. As stated above, the overall modular structure of the VLDLR is virtually superimposable with that of the LDLR, except that its ligand-binding domain contains 8, rather than 7, LA repeats. However, both receptors contain a so-called 8–10 residue linker region, in the LDLR between repeats 4 and 5, and in the VLDLR between repeats 5 and 6; furthermore, repeats 3–5 in the LDLR and 4–6 in the VLDLR are encoded by single exons, suggesting that repeat 1 represents the "extra" repeat in the VLDLR. The VLDLR shows

an amazing degree of conservation among different species, e.g. 95% identity within mammals. In addition, even the proteins of more distant species such as the chicken and *X. laevis* share about 80% identical residues with the human VLDLR. The mammalian VLDLR exists in variant forms arising from differential splicing. The longer and predominant splice form contains an additional exon specifying an O-linked sugar domain similar to that always present in LDLRs (28–30 amino acids long). The O-linked sugars are suggested to hinder access to extracellular proteolytic enzymes, which have been reported to release from the cell surface a soluble form of the receptor if it lacks the O-linked sugar domain. In the following section and in 4.3., the specific aspects of the relationships between receptors for VLDL and Vtg, and between the VLDLR and oocyte receptors for Vtg, respectively, will be considered.

The Avian VLDLR also is a Receptor for Vtg. A particularly interesting VLDLR homologue is that of the chicken (termed LDL receptor relative with 8 binding repeats, or LR8), as its functions are documented by both biochemical and genetic evidence: it mediates a key step in the reproductive effort of the hen, i.e. oocyte growth via deposition of yolk lipoproteins (Bujo et al., 1994). This conclusion is based on studies of a non-laying chicken strain carrying a single mutation at the *lr8* locus that disrupts LR8 function (the "restricted ovulator," R/O, strain) (Bujo et al., 1995b). The mutant hens fail to deposit into their oocytes VLDL and – very importantly – the lipophosphoglycoprotein Vtg, which are produced at normal levels in the liver. Consequently, the mutant females fail to lay eggs, and develop severe hyperlipidemia and features of atherosclerosis. The phenotypic manifestations of the single-gene mutation in R/O hens revealed the extraordinary multifunctionality of LR8, i.e. that the receptor recognizes in essence over 98% of all the yolk precursors that eventually constitute the mass of the fully grown oocyte. Obviously, R/O hens, which in fact represent a unique animal model for an oocyte-specific receptor defect leading to a disease akin to human familial hypercholesterolemia, are sterile due to non-laying.

Those tissues which express the VLDL receptor in mammals, i.e. heart, skeletal muscle, brain, and adipose tissue, but not the liver, also express this receptor in chicken, although at very low levels compared to the oocytes (Bujo et al., 1995a; Lindstedt et al., 1997). One difference in the structures of the major VLDL receptors in mammals and chicken LR8 is the presence (in mammalian tissues) and absence (in chicken oocytes) of the O-linked sugar domain, respectively. As described above, in mammalian VLDL receptors, the absence or presence of this domain arises by differential splicing of the mRNA; for simplicity, the longer form is designated LR8+, and the shorter one, LR8–. It was found that in chicken, the somatic cells and tissues, in particular the granulosa cells surrounding the oocytes, heart, and skeletal muscle express predominantly LR8+, while the oocyte is by far the major site of LR8– expression (Bujo et al., 1995a). Section 4.4. will describe findings relating to these findings in fish species. In the testes of roosters, the same expression dichotomy exists in that somatic cells (mainly the Sertoli cells) express the larger, and spermatocytes the shorter, form of LR8 (Lindstedt et al., 1997).

The properties of LR8 strengthen the hypothesis that the avian receptor is the product of an ancient gene with the ability to interact with many, if not all, ligands of younger relatives of the LDLR gene family. In this context, Vtg, absent from mammals, and apoE, not found in birds, possess certain common biochemical properties and regions of sequence similarities, and have been suggested to be functional analogues (see section 4.4.4). Even high-density lipophorin, an abundant lipoprotein in the circulatory compartment of insects, is likely endocytosed in a variety of tissues via an LR8 homologue with very high similarity to the VLDLR/LR8/VtgR group (Dantuma et al., 1999). Presumably, binding of lipophorin to this receptor is mediated by apoLpII/I, which share sequence homology with apoB and Vtg (Babin et al., 1999).

In any case, the requirement for LR8 in oocyte development, and thus reproduction, suggests that the VLDLR/LR8/VtgR family branch is among the oldest in the evolutionary line. Furthermore, studies in the chicken, as well as in fish, have revealed that members of the LDLR gene family from different animal kingdoms have common structures, and share a growing list of physiological roles, including the most recently discovered function(s) in signal transduction (May et al., 2005). One prime example for such a role is described in the following section. The involvement of the VLDLR together with the so-called apoE receptor-type 2 (ApoER2) in neuronal migration is based on the receptors' recognition of the extracellular matrix protein, reelin.

3.1.5. LRs mainly expressed in the brain

ApoER2. Differential splicing of LR transcripts is likely to be frequently due to the presence of repeated sequences, e.g. in the regions coding for clusters of LA repeats. Yet, so far only two LRs have been found to exist in splice variant forms, i.e. the VLDLR (see above) and ApoER2. The products of ApoER2 splice variants are receptors (i) with variant numbers of LA repeats; (ii) containing or lacking the so-called O-linked sugar domain; (iii) containing or lacking a potential furin-cleavage site downstream of the LA repeats; or (iv) differing by a short insertion in their cytoplasmic tail. The structure of the apoER2 gene is reminiscent of that of the smaller members of the family, LDLR and VLDLR, but the produced proteins are now known to harbor a cluster of either 3, 4, 5, 7, or 8 LA repeats (Brandes et al., 2001). Differential splicing affecting the cytoplasmic domain of the protein can generate a shorter version lacking a 59-residue insertion near the carboxyl terminus. This insertion is encoded by a single exon in man and mouse, but is absent from the corresponding chicken gene.

As for functional details, investigations continue to focus on the variations in the LA repeat cluster and, more recently, on the cytoplasmic domains, which are involved in important signal transduction pathways, likely independent of ligand internalization. Double-knockout mice (*VLDLR*$^{-/-}$, *apoER2*$^{-/-}$) display a dramatic phenotype essentially identical to that of mice lacking the extracellular matrix glycoprotein reelin; single-knockout mice of either receptor gene show only very subtle phenotypes. The reason for the abnormal phenotype of the

double-knockout mice, i.e. disturbed foliation of the cortical layers, is that reelin normally interacts with the extracellular domains of both the VLDL receptor and apoER2, but the subsequent signal cascade remains inactivated when both receptors are missing. Reelin binding to the VLDL receptor and to apoER2 leads to phosphorylation of the cytoplasmic adapter protein Disabled-1 (mDab-1), which is associated with the NPxY-motifs present in the receptors' tails. Reelin-triggered tyrosine-phosphorylation of mDab-1 initiates kinase cascade(s) controlling cellular motility and shape by acting on the neuronal cytoskeleton. The specificity of the reelin signaling via apoER2 and VLDL receptor seems to be achieved by selective binding of reelin to these receptors and not to other LRs. This is an important aspect, since mDab-1 not only associates with VLDL receptor and apoER2, but also with LRP1 and the LDL receptor.

LR11/sorLA and Relatives. LR11 (also termed sorLA) (Yamazaki et al., 1996), an unusually complex member of the LDLR gene family, has been characterized in man, rabbit, mouse, and chicken. The 250 kDa gene product is made up of seven distinct domains. One extracellular domain consists of a cluster of 11 LA repeats, hence its designation; it also contains 5 YWTD repeats and one 8-cysteine module. Additional features are a large domain (~400 residues, 12 cysteines) highly homologous to a yeast receptor for vacuolar protein sorting, VPS10; for this reason, LR11 has also been called sorLA (sorting protein-related receptor-containing LA repeats); and modules found in cellular adhesion molecules (6 fibronectin type III repeats). The membrane-spanning and cytoplasmic domains are conserved up to 100%.

Based on the presence of the VPS10 domain, an LR subfamily can be defined that comprises four smaller proteins in addition to LR11, termed sortilin and SorCS1–3 which lack LA repeats. Analysis of large-scale sequencing data revealed that the three *SorCS* genes are among the largest known human genes, and map to chromosomal localizations of known genetic diseases (Hampe et al., 2001). As stated, the greatest similarities between the SorCSs, sortilin, and LR11 are found in their VPS10 domains; such domains are found from *Dictyostelium* to man. Whereas in yeast, the VPS10 domain is known to function in vacuolar hydrolase sorting, an analogous role in vertebrates has not (yet) been demonstrated. Recent reports (Andersen et al., 2005; Andersen et al., 2006) point to a role of LR11 in the trafficking of amyloid precursor protein in neuronal cells, i.e. a possible involvement in the etiology of Alzheimer's disease.

3.1.6. LRP 3, 4, 5, and 6

Human LRP3 is a 770 residue membrane protein with clusters of two and three LA repeats, respectively. LRP4 contains two clusters of LA repeats with 3 and 5 modules, respectively. It is not known whether these proteins are capable of binding ligands that have been shown to interact with other LRs, whether they are endocytically competent, and of course, what their physiologic functions are.

LRP5 and LRP6 are closely related type I membrane proteins, approximately 1600 residues long (about twice as large as the LDLR), and their extracellular

domains are organized exactly as a portion of LRP1. The cytoplasmic domains of LRP5 and LRP6 contain motifs (dileucine; and aromatic-X-X-aromatic/large hydrophobic) similar to those known to be functional in endocytosis of other receptors. Importantly, they harbor serine- and proline-rich stretches that may serve as ligands for Src homology 3 (SH3) and WW (a variant of SH3) domains, properties that relate these receptors to signal transduction pathways different from those of apoER2 and the VLDL receptor described above. Indeed, LRP6 has been shown to be a co-receptor for proteins of the Wnt family, which trigger signaling pathways important for correct development of anterior structures (Mao et al., 2001). Recently, LRP6 has been demonstrated to interact with proteins called Dickkopf and Axin, respectively. Dickkopf inhibits Wnt signaling by releasing receptor-bound Wnt. Axin is one of the components in the cascade that regulates the activation of gene expression in the nucleus of target cells. The interplay of Wnt, Dickkopf- and Axin-binding to LRP5/6 appears to hold the key to important developmental signals, similar to the role of VLDL receptor and apoER2 in neuronal migration.

3.2. Analyses Aimed at Evolutionary Insights

Attempts at gaining insights into evolutionary aspects of the LRs have largely consisted of systematic analyses of their LA repeats by multiple sequence alignment. One such analysis has been presented (Sappington and Raikhel, 1998b), in which each LA module was "fingerprinted" by identifying amino acids that are both relatively conserved and unique within a given cluster of LA repeats. Four cohorts (A, B, C, and D) of modules refelecting shared recent ancestry were distinguished, which satisfied the criteria for all but two of the 57 modules examined. It appears that all clusters are derived from a single primordial hepta-cluster with a consensus arrangement of CDCADBC. LA repeat clusters are generally not directly homologous to each other and smaller one-cluster and two-cluster LRs likely have not arisen by breakup of larger 2- or 4-cluster LRs. Multicluster LRs are proposed to have evolved independently, but possibly from the same single-cluster receptor. Such analyses might become more predictive in terms of evolutionary insight when applied once all members of this family will have been identified. Furthermore, and if only on functional grounds, sequence analyses must extend to regions with less similarity than among the LA repeats, such as the cytoplasmic domains.

4. RECEPTOR-MEDIATED ENDOCYTOSIS IS THE MAJOR MECHANISM FOR VITELLOGENIN UPTAKE BY OOCYTES

4.1. Receptor-Mediated Endocytosis of LDL by Vertebrate Somatic Cells

This multistep process, originally defined as a distinct mechanism for the high-affinity cellular uptake of macromolecules, emerged from studies by M.S. Brown, J.L. Goldstein and their colleagues which they performed in the mid-1970s

in order to elucidate the normal function of LDL. The salient features of the itinerary of an LDL particle (mean diameter, ~22 nm) from the plasma into a normal human fibroblast are as follows. First, the lipoprotein particle binds to one of the approximately 15,000 LDL receptors on the surface of the cell. LDL receptors are not evenly distributed on the cell surface; rather, up to 80% are localized to specialized regions of the plasma membrane comprising only 2% of the surface area. These regions form pits and are lined on their cytoplasmic side with material that in electron micrographs has the appearance of a fuzzy coat. Each of these "coated pits" contains several kinds of endocytic receptors in addition to LDL receptors, but LDL particles bind only to "their" receptors, due to their extremely high affinity and specificity.

Next, the receptor/LDL complex undergoes rapid invagination of the coated pit, which eventually culminates in pinching-off of the coated pit into the interior of the cell. At this point, the coated pit has been transformed into an endocytic "coated vesicle," a membrane-enclosed organelle that is coated on its exterior (cytoplasmic) surface with a polygonal network of fibrous protein(s), and the main structural component of which is a fascinating protein called clathrin (Goldstein et al., 1985). Subsequently, the coat is rapidly removed, in concert with acidification of the vesicles' interior and fusion with other uncoated endocytic vesicles. Transiently, LDL and the receptor are found in smooth vesicles in which the lipoprotein particle dissociates from the receptor due to the acidic environment. LDL is then delivered to lysosomes, where it is degraded, while the receptor escapes this fate and recycles back to the cell surface, homes in on a coated pit and is ready to bind and internalize new ligand molecules (Goldstein et al., 1985).

There are variations to the theme, as not in all systems of receptor-mediated endocytosis are ligand degradation and receptor recycling coupled; however, all have in common the initial steps leading to the formation of endosomes. Then, the receptors are either degraded, recycled back to the cell surface, or are transported (for example, across polarized cells); their respective ligands can follow the same or divergent routes (Goldstein et al., 1985). The reutilization of the LDLR via recycling constitutes an economical way to ensure efficient removal of LDL from the extracellular space.

4.2. Receptor-Mediated Endocytosis of Vitellogenin by Oocytes

This uptake in principle follows the general pathway outlined above for LDL in somatic cells; only specific aspects will be considered in the following section. When compared with other plasma proteins, such as albumin, the rate of uptake of Vtg by growing oocytes is 20–25 times more rapid, i.e. is specific, and is saturable (Opresko and Wiley, 1987b, c).

In oocytes, which in fact are the cells where coated pits and vesicles actually have been first observed and morphologically described (Roth and Porter, 1964), we encounter a slight variation in the final step described above for the LDLR

in fibroblasts: there are apparently no true lysosomes in oocytes, and thus the final target for the ligands is the endosomal compartment. These endosomes, membrane-enclosed organelles with a proteolytic capacity, which is very low in comparison to true lysosomes (Retzek et al., 1992) form the bulk of yolk. Nevertheless, given the enormous receptor-mediated endocytotic activity of a rapidly growing oocyte, receptors must be reutilized via recycling, in this case out of endosomes. It has been suggested that in frog oocytes, the recyling rate could be influenced by ligand concentration and/or hormones in contrast to the LDLR system. It can be assumed that the specific intracellular processing of Vtg in the oocyte (see section 5) is tightly coupled to the event of receptor-mediated endocytosis. Such coupling has been clearly demonstrated for the lysosomal degradation of LDL in human fibroblasts, where the key player is the LDLR, the prototype of the VLDLR/VtgR family.

4.3. Vitellogenin Receptors are Members of the LR Gene Family

As outlined above, a well-understood system of Vtg uptake into oocytes via specific receptors is represented by the laying hen of the chicken. Interestingly, and of increasing significance for lipoprotein receptor studies in fish (see below) is the fact that LRs come at least in pairs, and sometimes in groups. They are products of genes expressed in mutually exclusive fashion in somatic cells and germ cells, respectively. One biological explanation for this is that oocyte growth must not be feedback-inhibited by intracellular accumulation of ligands, as is the case for cholesterol in somatic cells (Hayashi et al., 1989). Thus, the regulation of the two receptor gene groups must be different. The functional counterpart of LR8 (see section 3.1.4) in somatic cells is a 130 kDa sterol-regulated LDLR (Hummel et al., 2003). The chicken LDL (apoB) receptor cannot bind apoE nor Vtg, indicating the important physiological differences between ligands of mammalian and avian LRs, respectively. The chicken LDLR is thus the only known LDLR that interacts with apoB, but not apoE. It will be interesting to determine the structural element(s) responsible for this unique property.

Most of the biochemical experiments on the characterization of VtgRs have been, and still are, based on two assumptions; fortunately, these have become increasingly validated through detailed studies. First, these receptors are presumed to share structural and immunological properties with unambiguously identified VtgRs, such as the avian LR8, and second, they can be visualized by the technique of ligand blotting. This is a technique originally developed for the visualization of the human LDL receptor (Daniel et al., 1983). It relies on the ability of receptors with a cysteine-rich ligand-binding domain to interact with their ligands following SDS-polyacrylamide gel electrophoresis under nonreducing conditions. The ligands are labeled with radioiodine (Barber et al., 1991) or by biotinylation (Elkin and Schneider, 1994), and used in incubations of nitrocellulose sheets that contain SDS–PAGE-separated oocyte membrane proteins following electrophoretic transfer. Unbound ligand is removed by careful

washing, and proteins specifically interacting with the ligands visualized based on their label. Thus, for instance *X. laevis* Vtg bound to a single 95 kDa membrane protein in chicken oocytes (Stifani et al., 1990b); this protein corresponds to chicken LR8. Similarly, only one membrane protein from *Xenopus* oocytes, having an Mr of approximately 115 kDa, interacted with radiolabeled *Xenopus* Vtg. Importantly, the binding of Vtg to both chicken and *Xenopus* oocyte membrane proteins was totally abolished when membrane proteins had been exposed to disulphide-bond reducing agents during gel electrophoresis, in agreement with the fact that intrachain disulphide bonds within the receptor molecule are necessary for retention of its biological activity (George et al., 1987; Daniel et al., 1983). Based on these results, the VtgR in *Xenopus* oocytes has an apparent Mr of 115,000, and recognizes avian and autologous vitellogenins.

In immunological studies, an antibody raised against the pure bovine LDLR and shown to cross-react with chicken LR8, as well as an antibody raised against LR8, recognized the same bands as those identified in *Xenopus* oocytes by ligand blotting. Importantly, these results were the first to suggest that oocyte VtgRs are not only related among oviparous species, but also share structural features with LRs in mammalian species, a contention now strongly confirmed by the cloning efforts described below.

4.4. Fish Oocyte Vitellogenin Receptors

4.4.1. Oocyte vitellogenin receptor from coho salmon (Oncorhynchus kisutch)

The Vtg oocyte receptor of coho salmon (*Oncorhynchus kisutch*) was the first VtgR from piscine species to be characterized (Stifani et al., 1990a). It was identified as a 100 kDa protein in oocyte membrane extracts by ligand blotting (see above). Underscoring the close relationship of all VtgRs studied so far, this receptor showed cross-reactivity with the chicken and frog systems both in terms of ligand recognition and immunoreactivity. Binding of isolated chicken lipovitellin to chicken LR8 and to the fish receptor confirmed the results obtained with reverse ligand blotting performed with the *Xenopus* receptor (Stifani et al., 1990a, b), namely that the lipovitellin I portion of Vtg harbors the receptor-binding domain of the ligand (see sections 2.2 and 2.4).

In these experiments, suramin, a polysulfated polycyclic hydrocarbon (also known as Germanin or Bayer 205), originally described as antitrypanosomal agent, was shown to abolish Vtg binding to the fish receptor, in agreement with previous results on the human LDLR (Schneider et al., 1982) and chicken LR8 (Stifani et al., 1988), as well as the VtgR from mosquito (Cheon et al., 2001; see below), and the putative locust VtgR (Roehrkasten and Ferenz, 1992). Inhibition by suramin is thought to be caused by the high negative-charge-density of the compound, likely similar to that found on the surface of the binding domains of receptors of the LR superfamily. Suramin can be added to the list of useful tools in ligand blot and solid phase-binding analysis (not considered here) of VtgRs.

It is of interest that Tateno et al. (1998) reported the characterization of rhamnose-binding lectins from *O. mykiss* (steelhead and rainbow trout) eggs, STL1 and STL2 that contain domains homologous to LA repeats of LRs. Although the homology with LA repeats is less than 30%, and is particularly low in signature sequence motifs (e.g. in the clusters of negatively charged residues, SDE) and cysteine conservation, it may be significant that the lectins interact with lipovitellin and a fragment thereof, as determined by surface plasmon resonance measurements, possibly via the LA-like domain.

4.4.2. Vitellogenin receptor of rainbow trout (Oncorhynchus mykiss)

The first attempt to molecularly characterize the ovarian VtgR of the rainbow trout led to the isolation of partial cDNAs encoding two proteins with high homology to LRs (Prat et al., 1998). The proteins are made up of all of the 5 domains that are the structural hallmarks of LRs. The sequences of the two cDNAs differed by a stretch of 105 nucleotides encoding an O-linked sugar domain. However, the aminoterminal portion of the specified proteins could not be obtained in its entirety, as it contained only 2, rather than the 8 LA repeats that are usually present in VLDL/VtgR in other species. In concordance with findings in the chicken, the authors reported the expression of the shorter form of the protein in the ovary, and of the longer protein in addition to the ovary, in muscle, liver, spleen, heart, and intestine, indicating that the shorter form represents the oocyte receptor for lipoproteins such as Vtg, as was found in the chicken.

The complete molecular cloning and characterization of the rainbow trout oocyte receptor for Vtg was eventually achieved by Davail et al. (1998). The 97 kDa protein contains a ligand-binding domain with 8 LA repeats, lacks an O-linked sugar domain, and is endocytically active via an FDNPVY motif in the cytoplasmic domain. Furthermore, the authors also showed that the timing of transcription of the VtgR gene, which predominates during previtellogenesis, suggests that the VtgR is recycled to the oocyte surface during the vitellogenic growth phase (Perazzolo et al., 1999). This is consistent with the demonstration that ovarian level of the transcript encoding this receptor in *Solea senegalensis* has a significant positive correlation with the percentage of follicles in previtellogenesis and may be used as a precocious functional marker to quantify the number of oocytes recruited for vitellogenesis (Agulleiro et al., 2007).

4.4.3. Vitellogenin receptor from white perch (Morone americana)

Sullivan and colleagues reported two studies (Tao et al., 1996; Hiramatsu et al., 2004) on the characterization of a VtgR from the teleost white perch, a species which, in contrast to the trout, produces fatty pelagic eggs. In 1996, the receptor was reported to be a 157 kDa protein that was identified by ligand blotting with autologous Vtg labeled *in vivo* with [3H]-leucine or *in vitro* with ^{125}I using extracts of ovarian membranes (Tao et al., 1996). There was also a 106 kDa cross-reactive protein, considered to be a breakdown product of the larger protein. The Vtg was purified from the plasma of estrogen-treated perch by DEAE-agarose chromatography, and also applied as ligand in binding assays with isolated

membranes. It was shown that this binding was saturable, displaceable by chicken egg yolk VLDL, and that the binding sites showed several biochemical characteristics typical of LRs, such as sensitivity to the polyanionic drug suramin. Furthermore, binding to liver and ovary membranes displayed similar affinity for Vtg, which in turn was fourfold higher than that in muscle tissue. In the context of the two forms of LR8 in chicken (see above), it was assumed that the ovarian-binding site represents the form lacking an O-linked sugar domain, whereas the low-affinity muscle-binding site is the longer isoform of the white perch LR8 homologue (Tao et al., 1996).

More light on this notion was shed by the subsequent molecular characterization of the ovarian cDNA for the VtgR of the white perch (Hiramatsu et al., 2004). The receptor is an 844 amino acid residue protein with high homology to other LR members with 8 LA repeats, and lacks an O-linked sugar domain. It was noted that a splice variant containing an O-linked sugar domain was not discovered; however, the authors only tested liver (which does not express LR8+ in chicken) for expression of the longer transcript, but not muscle, the main source of LR8+ in trout (Prat et al., 1998), chicken, and mammals. Thus, the question of splice variant expression in terms of the O-linked sugar domain and of the nature of the hepatic-binding site described previously by the authors (Tao et al., 1996) remains unanswered in the case of the white perch.

4.4.4. Vitellogenin receptor from tilapia (Oreochromis aureus)

Li et al. (2003) characterized the cDNA for an 8 LA repeat-containing LR by PCR with consensus sequence primers and RACE-PCRs from previtellogenic ovary mRNA of the tilapia species, *O. aureus*. Again, the tilapia VtgR shows a very high degree of similarity to other VtgRs. In addition, PCR-based evidence for the expression in several tissues of splice variants lacking or containing 20 amino acid residues in the region normally representing an O-linked sugar domain was obtained. By Northern blotting, the ovarian transcript was the only one sufficiently abundant to be detected. The authors suggested that the low levels of the splice variant(s) in extraovarian tissues might contribute to the clearance of VLDL or VLDL-like lipoproteins.

Thus, in an effort to understand the multiligand-binding properties, the authors also provide a detailed analysis of the ligand-binding properties of the LA repeats of the receptor. By expression of 25 different fragments of the VtgR-binding domain, the first three LA repeats were found to be essential for Vtg binding, a property which was tested in a semiquantitative yeast-two-hybrid protein–protein interaction system. The crucial results were confirmed by direct-binding assays based on pull-down methodology. Secondly, the key functional features of Vtg were delineated by testing Vtg constructs for interaction with the receptor. The Vtg region containing a motif known to be homologous to positively charged LR-binding motifs in apoB and apoE (e.g. RKLRKRLL in human apoE), shown originally to bind to the human LDL receptor (see section 3.1.1.), was identified as essential for the binding to the receptor. Interestingly, the three N-terminal LA repeats of the tilapia receptor harbor the most acidic residues of

the binding domain, suggesting at least a partial mediation of binding via electrostatic attraction, but charge-changing mutations had no effect on the interaction. Given the rather indirect yeast two-hybrid assay which does not reveal relative binding affinities, further detailed direct evaluation of interactions between Vtg and its receptor is required.

Nevertheless, all of the above studies have now firmly established the fact that at least in all oviparous vertebrates (if not in all oviparous species), VtgRs are LR family members, and predict that these receptors contain 8 clustered LA repeats constituting the binding domain. Depending on the yolk composition, the genes specifying yolk precursor lipoprotein components and receptor specificities appear to have coevolved towards maximal efficacy of yolk deposition via these multiligand receptors, a key step in the reproductive effort of oviparous animals.

5. VITELLOGENIN PROCESSING AND CLEAVAGE INTO THE OOCYTE AFTER ENDOCYTOSIS

Once VTG is secreted by hepatocytes, its dimerized form is transported via the bloodstream to oocytes, where it is finally internalized in growing oocytes. Vtg enters ovarian follicles through capillaries of the theca layer, reaches the granulosa layer, and has contact with the oocyte surface through the pore canals of the zona radiata (Busson-Mabillot, 1984, Wallace, 1985; Ndiaye et al., 2006). As previously described, Vtg is selectively sequestered by growing ovarian follicles due to receptor-mediated endocytosis. Specific oocyte VtgRs are clustered in clathrin-coated pits (see Chapter 1 for details). Coated vesicles fuse with Golgian lysosomal-like multivescicular bodies (MVB) in the outer ovoplasm (Sire et al., 1994) and Vtg is proteolytically cleaved into smaller yolk proteins. The MVB contain the aspartic protease cathepsin D (Sire et al., 1994) and possibly other enzymes, which participate in the transformation of Vtg into yolk derivative proteins (Carnevali et al., 1999a; Hiramatsu et al., 2002c). The involvement of cathepsin D in Vtg proteolysis was first demonstrated in *Xenopus* (Opresko and Karpf, 1987a) and chickens (Retzek et al., 1992; Yamamura et al., 1995). Cathepsin D immunolocalization in rainbow trout oocyte showed that endocytosed Vtg is co-localized with cathepsin D in MVB (Sire et al., 1994). During *in vitro* experiments performed at a pH of 3.5, sea bream Vtg was converted into related egg yolk proteins using a homologous cathepsin D (Carnevali et al., 1999b). Similar experiments were performed using charr Vtg as substrate digested with bovine cathepsin D in a mildly acidic environment (Hiramatsu and Hara, 1996). In both experiments, the digestion generated a spectrum of proteins with an apparent molecular mass similar to that found in natural yolk. In both trout and sea bream, the level of cathepsin D enzymatic activity was especially intense after the onset of vitellogenesis (Sire et al., 1994; Carnevali et al., 1999b) and enzyme activity may be correlated to transcript level (Brooks et al., 1997).

Lipovitellin I and II, phosvitin, and β-component were generated by the enzymatic cleavage of Vtg by cathepsin D (Figure 1; and Chapter 4) and direct

amino acid sequence data mapped the precursor-product relationships of Vtg to the derived yolk proteins (Hiramatsu and Hara, 1996; Matsubara et al., 1999; Hiramatsu et al., 2002a, b; LaFleur et al., 2005; Sawaguchi et al., 2005a). In the LLT module, a possible posttranslational-processing site is located in the linking segment between the α-helical and the β-pleated sheet domains as demonstrated for lamprey Vtg during the creation of lipovitellin subunits (Sharrock et al., 1992). An additional proteolytic cleavage site is present close to the C1 motif (Sharrock et al., 1992; Babin et al., 1999), leading to the intraoocytic release of VWD module in the yolk. This is named YGP40 in chickens (Yamamura et al., 1995) and β-component in teleost fish species (Babin 1987a; Matsubara and Sawano, 1995; Hiramatsu and Hara, 1996; Matsubara et al., 1999; LaFleur et al., 2005; Sawaguchi et al., 2005a). Lipovitellin subunits and phosvitin are stored in yolk globules or platelets, while β-component encompasses the C-terminal VWD module of Vtg remaining in the cytoplasm soluble fraction (Babin 1987a; Tyler et al., 1988; Matsubara et al., 1999). These (lipo) proteins may be used as a nutrient for developing embryos. In addition to this primary Vtg cleavage, the major effectors for oocyte hydration are free amino acids generated by a partial secondary proteolysis of Vtg-derived proteins (Wallace and Begovac, 1985, Matsubara and Sawano, 1995; Thorsen et al., 1996; Matsubara and Koya, 1997; LaFleur et al., 2005; Carnevali et al., 2006; Raldua et al., 2006) through potential consensus PEST sites for proteolytic degradation (LaFleur et al., 2005) together with the accumulation of inorganic ions (LaFleur and Thomas, 1991; Fabra et al., 2006). The second processing of lipovitellin during oocyte maturation was found to be associated with cathepsin L in the gilthead sea bream (Carnevalli et al., 1999a) and with cathepsin B in the killifish (Raldua et al., 2006). Egg buoyancy after spawning due to water uptake is important for marine fish with pelagic eggs (see Chapter 12 for details).

6. TRANSPORT TO THE OOCYTE OF NEUTRAL LIPIDS, RETINOIDS, AND CAROTENOIDS

6.1. Origin of Yolk Neutral Lipids

In addition to their role in plasma or hemolymph lipid transport between somatic tissues, extracellular LLTP, mainly Vtg, apoB, and apoCr, facilitate the massive desposit of yolk reserves inside the oocytes of most oviparous species. ApoB, apoLp-II/I, and possibly apoCr-containing lipoproteins are involved in neutral lipid deposition in the oocyte after receptor-mediated endocytosis on the same, or similar oocyte-specific receptor used by Vtg. As demonstrated in birds, cathepsin D catalyses the intraoocytic fragmentation of both apoB and Vtg (Retzek et al., 1992; Elkin et al., 1995) after binding and endocytosis by the same oocyte-specific receptor belonging to the LDL receptor superfamily (Bujo et al., 1994). In these bird species, VLDL possesses two major apolipoproteins, apoB and apoVLDL-II; the latter is synthesized by the liver under the strict

control of estrogens (Chan et al., 1983). The presence of apoVLDL-II results in a considerable decrease of VLDL sensitivity toward lipoprotein lipase (LPL), enabling these lipoproteins to be incorporated into growing oocytes (Perry and Gilbert, 1979; Griffin et al., 1982; Schneider et al., 1990). Even though no direct proof exists, the ovarian lipolysis of circulating lipoproteins is probably a main source of neutral lipids stored in the form of lipid (oil) globule(s) for growing fish oocytes containing one, several or numerous oil globules (Wallaert and Babin, 1992, 1994; Wiegand, 1996). This is suggested by the considerable increase in LPL (Lindberg and Olivecrona, 2002) and salt-resistant lipase activities in the trout ovary during the exogenous vitellogenesis (Black and Skinner, 1987), and the high expression of LPL transcript in the fish ovary (Kwon et al., 2001). LPL in this species is greatly activated by VLDL and, to a lesser extent, by high-density lipoprotein (HDL) (Skinner and Youssef, 1982).

6.2. Retinoids and Carotenoids

Eggs of oviparous vertebrates contain retinols, retinals, and retinyl esters in addition to carotenoids and their relative abundance changes between the different taxonomic groups (Lubzens et al., 2003). Since retinoids and their carotenoid precursors are not synthesized *de novo* by vertebrates, they must be derived from dietary sources, stored in the maternal organism, and transported to oocytes during vitellogenesis. Retinoids originate in the form of retinyl-esters from animal products and carotenoids originate from plant food. Retinoids are important regulatory signaling molecules during embryonic development and therefore, should be present in the eggs of oviparous animals that develop independently of the maternal organism. The specific importance of vitamin A (retinol) lies in its regulation of a large array of vertebrate functional processes, including reproductive processes. Its activity is performed by conversion within cells, to an active form, retinoic acid that serves as a ligand in regulating transcription (see Mangelsdorf et al., 1994; Means and Gudas, 1995 for reviews).

It has been reported that retinal is the predominant form of vitamin A in fish eggs (see Lubzens et al., 2003 for a review) and may be bound to Vtg derivatives. In some marine fish species, retinals constitute almost 100% of all retinoids in the egg and it has been suggested that this form of storage is associated with the absence of lipid globules (Irie and Seki, 2002). Fish eggs also contain retinols and retinyl-esters and their abundance is higher in eggs of freshwater species compared with marine fish, reaching over 30% in trout or salmon (Plack, 1964). Retinol and retinyl-esters were detected in fish eggs in the fat soluble fraction (Irie and Seki, 2002).

Retinol is transported in the vertebrate circulatory system bound to the plasma retinol-binding protein 4 (RBP4). The molecular and/or biochemical characterization of this protein has been performed in different teleost fish species including the rainbow trout (Sammar et al., 2001) and zebrafish (Tingaud-Sequeira et al., 2006). The pathway(s) for transporting retinoids into

developing oocytes in oviparous vertebrate species have not been fully resolved and the chicken is the best studied model so far. The retinol constituting the major form of retinoids stored in the chicken egg is internalized by the chicken oocyte bound to its serum protein transport complex consisting of RBP4 and transthyretin (TTR), and the accumulation of RBP4–TTR was suggested to be a receptor-mediated process as revealed by their presence in clatherin-coated vesicles of the oocyte (Vieira and Schneider, 1993 Vieira et al., 1995a, b). In teleost fish, RBP4 probably transports retinol without forming a complex with TTR, in contrast to RBP4 of frogs, birds, and mammals (see Lubzens et al., 2003 for a review). In contrats to Vtg, the relative abundance of RBP4 in the plasma was found similar in male and female fish, and these did not change in the plasma of females at progressively advanced stages of vitellogenesis or in 17β-estradiol treated fish (Sammar et al., 2001, 2005). These results suggest differences in the regulation of *rbp4* and *vtg* expression during vitellogenesis, suggesting that RBP4 may not be the main transporter protein for retinoids to fish egg.

Fish eggs also contain carotenoids (Ando and Hatano, 1987) that may reach the oocytes by plasma lipoproteins and these carotenoids could be converted to retinoids within the oocyte or during embryonic development. At least three carotenoid-carrying lipoprotein (CCL) fractions (LDL, HDL, and VHDL including Vtg) were identified in the serum of chum salmon (Ando et al., 1986a; Ando and Hatano, 1988). The CCL from the HDL fraction (Babin 1987b) was identified in males and females and was associated with carotenoids transport from the muscle to the integument, while the VHDL fraction was identified as Vtg (Ando et al., 1986b). As previously pointed out for neutral lipids and their constituents, it is not known whether the other lipoproteins are taken up by the oocyte or just deliver their lipids and liposoluble constituents without being sequestered in the process.

7. FUTURE PROSPECTS

Eggs of oviparous animals have to contain all the nutrients required for the normal development of the embryos following fertilization, until the commencement of exogenous feeding, after hatching. While a multitude of novel molecular data on teleost vitellogenesis has been added during the last two decades there still remain several questions that should be addressed in the future. One of the intriguing question lies in the regulation of protein synthesis in the liver, of proteins transported to the ovary such as the yolk precursor protein, Vtg. The posttranslational modifications of this complex molecule requires the coordinated availablity of ligands such as lipids, retinal, calcium, and others, suggesting that it entails multigene regulatory processes and they await future investigations. Most of all, we must make progress, through molecular biological approaches, in obtaining much more detailed structural and functional informations about VtgRs and LRs in general, and their ligands, and from a much wider range of teleost and non-teleost fish species. The clarification of the

relationships between the small, medium-sized, and large members of the LR supergene family, and possibly the discovery of yet unknown relatives, will unravel the history and principles of evolution of these biologically extremely important genes. In addition and as demonstrated in chicken, VtgR also binds and transports major yolk components other than Vtg such as VLDL (Bujo et al., 1994), alpha(2)-macroglobulin (Jacobsen et al., 1995), riboflavin-binding protein (Mac Lachlan et al., 1994), and complement component C3 (Recheis et al., 2005). It remains to be determined to which extent such coordinated transport of nutrients to the growing oocytes occurs during fish oogenesis. As we have seen, animals can express several of these related receptor genes, i.e. produce several similar proteins, commonly in different tissues, but possibly also in one and the same cell type. Redundancy in gene products (provision of backup systems) is one of the biological means to protect from genetic disease and/or to equip an organism for specialized tasks through similar proteins. The results described here reveal the roles of multiple and possibly redundant genes in oocyte growth, the key to reproduction in oviparous species. We have gained some insights into, but must learn much more about, the regulation of the individual genes in order to be able to understand exactly why several similar gene products are expressed in a single organism, in particular following whole-genome duplication in teleost fishes. Specifically, do common regulatory elements exist in genes of receptors and ligands that trigger growth of the germ cell? Which of these receptors and ligands are expressed during embryonic development? Do posttranslational modifications of ligands determine their cellular fate (i.e. receptor recognition)? What, if anything, can the similarity of oviparous species' Vtgs to apoB tell us? What are the mechanisms at the ovarian follicle level of transport to the oocyte of neutral lipids, retinoids, carotenoids, and hormones? What is the biological purpose of limited intraoocytic proteolysis instead of complete degradation of Vtg? Hopefully we will soon be able to answer some of these questions by studying oocyte-specific events in heterologous cell systems amenable to manipulation or in transgenic oviparous animals, e.g. zebrafish or medaka.

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CHAPTER 3

ZEBRAFISH OVARIAN FOLLICLE TRANSCRIPTOME

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1. INTRODUCTION

The ability of an oocyte to develop into a viable embryo depends on the accumulation of specific maternal information and molecules, such as RNAs and proteins. Folliculogenesis and oogenesis include the formation of ovarian follicles, the initiation and completion of meiosis, and the accumulation of specific information and molecules such as RNAs, proteins, or imprinted genes in the female germ cells to sustain embryo development to the stage where zygotic gene activation takes over (Matova and Cooley, 2001; Eichenlaub-Ritter and Peschke, 2002; Duranthon and Renard, 2003). As in other vertebrates (Matova and Cooley, 2001), somatic gonadal cells in ray-finned fish species surround a single oocyte to establish a follicle (Selman and Wallace, 1989; Selman et al., 1993; Chapter 1). Oocyte

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growth, particularly in oviparous species, is characterized by intense deposition of RNAs and proteins. These maternal factors can be stored for very long periods of time until their use during embryonic development as the entire folliculogenesis process, from primary growth to post-vitellogenic stage, takes a few days in some species (e.g. in zebrafish) and months (e.g. trout) or years (e.g. sturgeons) in other species.

The aim of this chapter is to review our knowledge of the ovary/follicle molecular phenotype of teleost fish species with particular emphasis on zebrafish. Since large numbers of follicles at different developmental stages are easily obtained year round in zebrafish, this species offers an excellent alternative model for analysing some fundamental aspects of ovarian development and regulation (Ge et al., 2005), as well as identifying conserved maternal factors (Pelegri, 2003, see also Chapter 6), which are important in early stages of embryo development.

2. GLOBAL GENE EXPRESSION PROFILING OF TELEOST FISH OVARIES AND FULLY GROWN FOLLICLES

2.1. Methods for Gene Expression Profiling

A number of methods are currently used for gene expression profiling. They differ in scale, economy, and sensitivity. The delineation of the transcriptome of teleost fish ovaries has been evaluated using large-scale expressed sequence tags (ESTs) sequencing (Boguski et al., 1993) of cDNA libraries of zebrafish (Zeng and Gong, 2002; Li et al., 2004) and Atlantic salmon (Davey et al., 2001; Rise et al., 2004), subtractive hybridization of cDNA libraries (Wang and Brown, 1991) of medaka (Kanamori, 2000), or microarray-based analyses of rainbow trout (von Schalburg et al., 2005) gonads. While these methods give an idea of transcript abundance or enrichment in a specific tissue, a few genes expressed at high levels usually represent a large proportion of the total transcripts and are thus more frequently represented in the EST database (Chen et al., 2002; Knoll-Gellida et al., 2006). In addition, quantitative comparison of EST libraries requires compensation for systematic biases in cDNA generation (Liu and Graber, 2006). Serial analysis of gene expression (SAGE) based on the enumeration of directionally reliable short cDNA sequences (tags), provides qualitative as well as quantitative analysis of a large number of genes in a defined tissue (Velculescu et al., 1995; Gowda et al., 2004). It has been demonstrated that the SAGE method is reproducible (Dinel et al., 2005), provides an unbiased, quantitative report of gene expression, that may be correlated with microarray data (Ibrahim et al., 2005; van Ruissen et al., 2005), and seems more efficient than EST-based methods for discovering novel genes and spliced variant transcripts (Chen et al., 2002). However, one limitation of the SAGE method is the presence of transcripts that produce multiple matched tags (see as an example:

Knoll-Gellida et al., 2006). This technique has been widely applied in human studies and various SAGE tags/SAGE libraries have been generated from different cells/tissues, including human oocytes (Neilson et al., 2000; Stanton et al., 2002), silkworm eggs (Huang et al., 2005) and zebrafish fully grown ovarian follicles (Knoll-Gellida et al., 2006), and successfully identified differentially expressed genes in human ovarian carcinomas and normal ovarian surface epithelium (Peters et al., 2005). Localization of selected maternal transcripts in teleost oocytes according to the polarization of the oocyte along the animal–vegetal axis may be visualized using whole-mount *in situ* hybridization enable the determination of the cellular distribution of the maternal transcripts inside the oocyte cytoplasm (Figure 1; see also Chapter 6). This screening procedure may be performed at large scale (Thisse et al., 2004).

2.2. Transcriptome of Zebrafish Fully Grown Follicles

The transcript repertoire, named ZEBRAOV, determined using the SAGE method on zebrafish fully grown ovarian follicles is an accurate picture of gene expression on both qualitative and quantitative levels and gives a global expression profile of transcripts (Knoll-Gellida et al., 2006). The list of these experimental SAGE tags and their relative frequencies were deposited in the NCBI gene expression and hybridization array data repository (GEO)

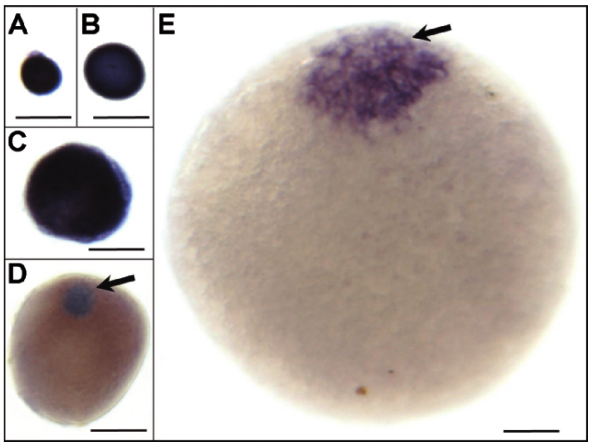

Figure 1. Localization of metallothionein 2 (*mt2*) transcripts in zebrafish fully grown follicles as revealed by whole-mount *in situ* hybridization. Stage-specific polarized distribution of *mt2* short transcript isoform in stage IB (A, B), II (C), early stage III (D), and stage IV (E) follicles. The *met2* hybridization signal detected at early stages was widely distributed in the ooplasma, whereas it concentrated at the animal pole from early stage III to the end of vitellogenesis. For stages III and IV, the animal pole, oriented toward top of page, is indicated by an arrow. Scale bar = 100 μm. (*See Color Plates*).

(http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE3679. Sequencing of 27,486 SAGE tags identified 11,399 different tag species, classified into 3,437 UniGene (ftp://ftp.ncbi.nih.gov/repository/UniGene/) clusters with tags in position at sense or antisense corresponding mRNA sequence in the presence of a polyadenylation signal and poly(A) tail, including 3,329 tag species with an occurrence greater than one. Identified transcripts expressed at over 0.15% of the mRNA population in zebrafish fully grown ovarian follicle as evaluated by the presence of their corresponding SAGE tag were listed in Table 1. Comparative analysis of transcriptional activity, using the ZEBRAOV SAGE tag database and dbEST (http://www.ncbi.nlm.nih.gov/dbEST/index.html) libraries currently available for zebrafish ovaries, revealed a globally similar pattern between ZEBRAOV and the ID.9767 library. However, a clearly different quantitative pattern was obtained with library ID.15519, due to an over-representation of the number of EST sequences attached to a small number of unique transcripts. Consequently, some of the abundant transcripts found by the SAGE method were not detected in library ID.15519. For example, a transcript moderately similar to zygote arrest 1 (ZAR1), with a domain similar to the atypical homeodomain (PHD) finger motif found in ZAR1 in vertebrate species, including zebrafish (Wu et al., 2003), is highly expressed in ZEBRAOV, moderately in library ID.9767, and not in library ID.15519. In a second example, the transcript of signal sequence receptor beta (*ssr2*), also called translocon-associated protein beta, was detected at high levels with SAGE, and very low levels with dbEST ovary library sequencing. This protein is part of the translocon-associated protein (TRAP) complex required for the translocation of nascent polypeptides into the lumen of the endoplasmic reticulum, and the corresponding zebrafish *ssr2* mRNA is maternally supplied to the egg (Mangos et al., 2000). ZEBRAOV database is around 1.8 times more sensitive than EST sequencing in detecting transcripts with a characterized maternal genetic contribution, even if the number of cDNA clones sequenced to generate the ZEBRAOV SAGE tags database was around 21 times lower (Knoll-Gellida et al., 2006). However, this number is not sufficient to identify some of these transcripts, as demonstrated with the maternal-effect *vasa* transcript that was not detected in ZEBRAOV. The *vasa*-like genes are expressed in the germ cells of many animal species (Raz, 2000), including zebrafish oocytes and early-stage embryos (Bratt et al., 1999; Krovel and Olsen, 2004). Furthermore, the presence of unmatched tags in the SAGE library generated from ovarian follicles indicates the presence of genes in the zebrafish genome or spliced variant transcripts that had not previously been identified by EST data. A broader snapshot of gene expression was therefore obtained by SAGE, as previously reported (Chen et al., 2002; Dinel et al., 2005). It should be pointed out that some of these unidentified transcripts are largely expressed in the zebrafish fully grown follicle transcriptome.

Table 1. Identified transcripts expressed at over 0.15% of the mRNA population in zebrafish fully grown ovarian follicle as evaluated by the presence of their corresponding SAGE tag

Tag N°	Tag (CATG+)	Nbr	Rank	GenBank	Cluster	Gene	Description
1	AAATAAACGC	284	R1	BM141044	Dr.12439		Transcribed locus similar to rhamnose-binding lectin
2	TAAGACATCC	226	R1	BC045879	Dr.1109	*bactin2*	Beta-actin2
3	CAGTGCTTTT	209	R1	BM531116	Dr.46793	*h2b*	Transcribed locus similar to H2B histone family
4	TCCTTTGTCT	142	R1	BC049475	Dr.47289	*mt2*	Metallothionein 2
5	ACCGTTTTTA	130	R1	BC078260	Dr.26975	*cldnd*	Claudin d
6	CAAAGGAAAC	124	R1	BC055519	Dr.47296		Zgc:66160
7	CTAGCCTAAT	122	R1cr	BM861564	Dr.5628		Similar to contains element MSR1 repetitive element/ ZP1-related
8	GTGAAACCTG	119	R1	AF068772	Dr.31066	*hsp90b*	Heat shock protein 90-beta
9	TTGATGAGGG	113	R1	BC044190	Dr.729	*ldhb*	Lactate dehydrogenase B4
10	TAAAACCAAA	108	R1	AF057040	Dr.25213	*bactin1*	Beta-actin1
11	TATTTCACTA	98	R1	BC045894	Dr.1043	*atp5g*	ATP synthase, H+ trans., mit. F0 complex, subunit c (subunit 9)
12	GATATTTAAC	94	R1	BI979482	Dr.47212		Transcribed locus, moderately similar to thymosin beta 10
13	CATTTTCTAA	90	R1cr	BM571854	Dr.19916	*zp2.3*	Egg envelope protein ZP2 variant B [zp2.3]
13	CATTTTCTAA	90	R1	BC093133	Dr.23439	*zp2.4*	Zp2.4 protein
14	ACAACTAAAA	89	R1	BC071411	Dr.989		Zgc:56419 similar to stress-associated endo. reticulum protein 1
15	AGAAACGTTT	77	R1	BC067692	Dr.26977	*zp3b*	Zona pellucida glycoprotein 3b
16	GTGGTGTTGA	75	R1	NM_131331	Dr.30322	*zp3*	Zona pellucida glycoprotein 3

(*continued*)

Table 1. Continued

Tag N°	Tag (CATG+)	Nbr	Rank	GenBank	Cluster	Gene	Description
17	CAATCATATT	75	R1	BM533765	Dr.13590		Transcribed locus, moderately similar to zygote arrest 1
18	CAAATGTTTT	71	R1	CK699865	Dr.29860		Transcribed locus
19	CAGTACTGCG	66	R1	AY561514	Dr.30264	*rpl3*	Ribosomal protein L3
20	CCATAAGTGC	66	R1	BC054596	Dr.1012		Retinol dehydrogenase 10
21	TAAAGTCGCC	65	R1	BM778414	Dr.1428	*rps15a*	Ribosomal protein S15a
22	GTCTACACGT	64	R1cr	BI325627	Dr.7383		Zgc:73301 similar to cytochrome oxidase III
23	CTTGTTGGAA	62	R1	BQ260230	Dr.29755		Wu:fe38a04 similar to RL31_HUMAN 60S ribosomal protein L31
24	GGATTCGGAC	61	R1	BM102431	Dr.23788	*gstp1*	Glutathione S-transferase pi
25	GGGTGCTTTT	61	R1	CO351922	Dr.14015		Im:7140333 weakly similar to latrophilin; lectomedin-1
26	TGCTCCATAC	59	R1cr	BC095579	Dr.5555	*ccna1*	Cyclin A1
27	AGGCTGTCTG	55	R1	BC055524	Dr.24903		Zgc:66168 similar to ubiquitin; 40S ribosomal protein S27a
28	TGTTCTGTAT	55	R1	BC095694	Dr.5890		Zgc:112226 similar to cathepsin S
29	TCGATGATGG	54	R1cr	BM103997	Dr.26808	*rpl10a*	Ribosomal protein L10a
30	TTAATAAAAG	53	R1	NM_198809	Dr.5605	*tubb2*	Tubulin beta 2
31	AGGAAAGCTG	52	R1	BC071384	Dr.1335	*rpl36*	Ribosomal protein L36
32	TGCTGCTTGT	51	R1	BC045278	Dr.6496	*fth1*	Ferritin, heavy polypeptide 1
33	ATCAATGTGA	50	R1	BC075915	Dr.1209	*ssr2*	Signal sequence receptor, beta
34	TCTGTTTAAC	47	R1	BC049058	Dr.11308	*rplp0*	Ribosomal protein, large, P0
35	AAGCCCATTA	46	R1	BC048027	Dr.4947	*cirbp*	Cold-inducible RNA-binding protein

(*continued*)

Table 1. Continued

Tag N°	Tag (CATG+)	Nbr	Rank	GenBank	Cluster	Gene	Description
36	GTTCCTTGGC	46	R1	BQ617300	Dr.1334	*rps25*	Ribosomal protein S25
37	TAAATGAGAT	42	R1	BC054688	Dr.12480		Clone IMAGE: 5604190, weakly similar to Cd27-binding protein

Nbr is the number of times a tag was counted in ZEBRAOV; Rank, is tag position at sense (R1) or antisense (R1cr) corresponding mRNA sequence; Cluster, cluster accession number of zebrafish UniGene database Build#87.

3. COMPARISON OF THE TRANSCRIPTOME OF ZEBRAFISH OVARIAN FOLLICLES WITH OVARY/EGG FUNCTIONAL GENOMIC DATA FROM OTHER ANIMALS

Comparison of the transcriptome of zebrafish fully grown follicles as evaluated by the SAGE method with ovary/egg transcriptomes available for other animal species revealed both similarities and differences. SAGE revealed the presence of several tags corresponding to novel transcripts, some highly expressed in the zebrafish fully grown follicle transcriptome and well conserved in vertebrates (Table 2). As expected, some of the most abundant transcripts identified in zebrafish, corresponding to some ribosomal proteins or translated to housekeeping genes, including beta-actins, and tubulins, or well-known ovary-enriched proteins, like zona pellucida (ZP) protein isoforms or cyclins, are well conserved in the ovarian transcriptomes of other fish species (Knoll-Gellida et al., 2006). Homologous highly expressed transcripts were also recovered in mammals and *Xenopus* transcriptomes and, to a lesser extent, in silkworm, nematode, sea urchin, and amphioxus egg profiles. Some of these transcripts are members of multigene families that may be widely expressed in the zebrafish fully grown follicle transcriptome, e.g. ZP protein isoform transcripts. This high transcript level may be restricted to a few members of other gene families, as illustrated with claudin genes. Claudins, the major tight junction transmembrane proteins, are members of the tetraspanin protein superfamily that mediate cellular adhesion and migration. Numerous claudin genes have been identified in zebrafish (Kollmar et al., 2001) but only the *cldnb* transcript was recovered at very high levels in ZEBRAOV and library ID.9767, and *cldng* in library ID.9767. Some of the claudin isoform transcripts of maternal origin are then downregulated in the early stages of zebrafish embryogenesis (Lo et al., 2003).

The most abundant transcript in zebrafish fully grown follicles belongs to a large conserved protein family containing one domain with sequence similarities

Table 2. The most abundant annotated SAGE tags of zebrafish fully grown follicles and the presence of UniGene or homologous UniGene clusters in zebrafish and vertebrate ovary/unfertilized egg cDNA libraries, respectively

Tag N°	Cluster*	Description	Zebra-fish	Trout	Salmon	Fugu	Xenopus	Human
1	Dr.12439	Transcribed locus similar to rhamnose-binding lectin/ Latrophilin-2	**Y***	**Y***	**Y***	n.i.	**Y**	**Y**
2	Dr.1109	Beta-actin2	**Y***	**Y**	N	**Y***	**Y**	**Y***
3	Dr.46793	Transcribed locus similar to H2B histone family	**Y***	n.i.	n.i.	n.i.	N	**Y**
4	Dr.47289	Metallothionein 2	**Y**	n.i.	Y	N	N	N
5	Dr.26975	Claudin d	**Y***	N	n.i.	n.i.	**Y***	**Y**
6	Dr.47296	Zgc:66160	**Y***	N	n.i.	n.i.	**Y**	N
7	Dr.5628	Similar to contains element MSR1 repetitive element/ ZP1-related	**Y***	N	N	N	**Y**	**Y**
8	Dr.31066	Heat shock protein 90-beta	**Y**	N	**Y**	n.i.	**Y**	**Y***
9	Dr.729	Lactate dehydrogenase B4	**Y**	N	N	n.i.	**Y**	**Y***
10	Dr.25213	Beta-actin1	**Y***	**Y**	N	**Y***	**Y**	**Y***
11	Dr.1043	ATP synthase, H+ transporting, mit.F0 complex, subunit c (subunit 9)	**Y**	**Y***	**Y***	**Y***	**Y**	**Y**
12	Dr.47212	Transcribed locus, moderately similar to thymosin beta 10	**Y***	N	n.i.	n.i.	**Y**	**Y**
13	Dr.19916	Egg envelope protein ZP2 variant B [zp2.3]	**Y***	**Y**	N	N	n.i.	N
13	Dr.23439	Zp2.4 protein	**Y***	**Y**	N	N	n.i.	N
14	Dr.989	Zgc:56419 similar to stress-associated endoplasmic reticulum protein 1	**Y**	N	n.i.	n.i.	**Y**	**Y**
15	Dr.26977	Zona pellucida glycoprotein 3b	**Y***	**Y***	**Y***	N	**Y**	**Y**
16	Dr.30322	Zona pellucida glycoprotein 3	**Y***	**Y***	**Y***	N	**Y**	**Y**
17	Dr.13590	Transcribed locus, moderately similar to zygote arrest 1	**Y**	**Y**	n.i.	N	**Y**	N
18	Dr.29860	Transcribed locus	**Y**	N	n.i.	n.i.	n.i.	n.i.
19	Dr.30264	Ribosomal protein L3	**Y**	N	N	n.i.	N	Y*
20	Dr.1012	Retinol dehydrogenase 10	**Y**	N	n.i.	n.i.	**Y**	**Y**

(*continued*)

Table 2. Continued

Tag N°	Cluster*	Description	Zebra-fish	Trout	Salmon	Fugu	Xenopus	Human
21	Dr.1428	Ribosomal protein S15a	**Y**	N	N	n.i.	N	**Y**
22	Dr.7383	Zgc:73301 similar to cytochrome oxidase III	**Y**	n.i.	n.i.	n.i.	n.i.	n.i.
23	Dr.29755	Wu:fe38a04 similar to RL31_HUMAN 60S ribosomal protein L31	**Y**	N	n.i.	n.i.	n.i.	n.i.
24	Dr.23788	Glutathione S-transferase pi	**Y**	N	n.i.	n.i.	N	**Y**
25	Dr.14015	Im:7140333 weakly similar to latrophilin; lectomedin-1	**Y**	n.i.	n.i.	n.i.	**Y**	**Y**
26	Dr.5555	Cyclin A1	**Y**	N	n.i.	n.i.	**Y***	**Y**
27	Dr.24903	Zgc:66168 similar to ubiquitin; 40S ribosomal protein S27a	**Y**	**Y**	**Y**	**Y***	**Y**	**Y**
28	Dr.5890	Zgc:112226 similar to cathepsin S	**Y***	N	N	n.i.	N	N
29	Dr.26808	Ribosomal protein L10a	**Y**	N	N	N	N	**Y***
30	Dr.5605	Tubulin beta 2	**Y***	N	**Y**	n.i.	**Y**	**Y***
31	Dr.1335	Ribosomal protein L36	**Y**	N	N	N	**Y**	**Y**
32	Dr.6496	Ferritin, heavy polypeptide 1	**Y**	**Y**	**Y***	**Y**	**Y**	**Y***
33	Dr.1209	Signal sequence receptor, beta	**Y**	N	n.i.	n.i.	**Y**	**Y**
34	Dr.11308	Ribosomal protein, large, P0	**Y**	N	n.i.	N	**Y**	**Y***
35	Dr.4947	Cold inducible RNA binding protein	**Y**	N	**Y**	n.i.	**Y**	**Y**
36	Dr.1334	Ribosomal protein S25	**Y**	**Y**	n.i.	**Y**	**Y**	**Y**
37	Dr.12480	Clone IMAGE: 5604190, weakly similar to Cd27 binding protein	**Y***	N	n.i.	n.i.	**Y**	**Y**

UniGene Build#87. The homologous UniGene clusters in other vertebrate species were found with UniGene tool or Blast search. Y, UniGene or homologous UniGene cluster identified in the library. Y, UniGene or homologous UniGene cluster identified in the library with an occurrence >0,15%. N, the UniGene or homologous UniGene cluster was not detected in the library. n.i., an homologous UniGene cluster was not recovered in UniGene. The presence of homologous clusters in at least four compared vertebrate species or in common between zebrafish and the two selected tetrapod species, are underlined. The vertebrates dbEST cDNA libraries accession numbers used for comparison were: ID.9767 for zebrafish (*Danio rerio*); ID.15587 for rainbow trout (*Oncorhynchus mykiss*); ID.15459 for Atlantic salmon (*Salmo salar*); ID.11967 for Fugu (*Takifugu rubripes*); ID.9909 for *Xenopus tropicalis*; and ID.4908, ID.10552, and ID.5611 for human (*Homo sapiens*).

to the galactose/rhamnose-binding lectin domain found in numerous proteins with sugar-binding properties. This domain was initially characterized in sea urchin (*Anthocidaris crassispina*) egg lectin (SUEL) (Ozeki et al., 1991). It was then characterized in rhamnose-binding lectins of rainbow trout eggs (*Oncorhynchus mykiss*), which consist of two homologous SUEL domains repeated in tandem (Tateno et al., 1998). It has been suggested that this domain plays a role from egg maturation to fertilization (Tateno et al., 2001). Rhamnose-binding lectin in catfish (*Parasilurus asotus*) is composed of three tandem-repeat domains homologous to the SUEL lectin domain (Hosono et al., 1999). A cysteine-rich domain homologous to the SUEL protein has been also identified in the N-terminal part of mammalian latrophilin-2 precursor protein (Lelianova et al., 1997).

The SAGE approach also revealed numerous transcripts highly expressed in zebrafish that were not previously known to be significantly expressed by zebrafish ovaries, including *mt2*, *hsp90b*, *ldhb*, *atp5g*, *fth1*, *cirbp, rplp0*, and 40S ribosomal protein S27a (Tables 1 and 2). The relative abundance of molecules stored in oocytes may differ between species but some of the abundant transcripts found in zebrafish follicles are common highly expressed transcripts in vertebrate ovaries/unfertilized eggs (Table 2). It is noteworthy that almost all ribosomal protein transcripts identified from the SAGE tags, expressed at over 0.15%, were recovered below this limit from the two selected zebrafish ovary cDNA libraries. In some cases, these differences may be related to the loss of these small size transcripts after size selection of cDNAs during construction of the libraries, a process that did not occur using the SAGE method.

Short *mt2* transcript is a very good example of the quantitative as well as qualitative original data obtained after SAGE analysis. We found that *mt2* was very abundantly expressed in zebrafish oocytes, at a level ten times higher than that previously inferred from analysis of zebrafish ovary dbEST libraries (Knoll-Gellida et al., 2006). This difference in *mt*2 transcript levels may be due to the loss of this small size transcript during cDNA library construction. An enrichment of this transcript in fully grown oocytes *versus* ovaries is less plausible due to the asynchronous development of zebrafish ovaries, containing oocytes at different stages in development (Selman et al., 1993). In addition, whole-mount *in situ* hybridization demonstrated a strong stage-dependent *mt2* polarized hybridization signal in the cytoplasm of zebrafish oocytes (Figure 1). These data are consistent with the metallothionein activity content of zebrafish oocytes (Riggio et al., 2003a) and the presence of this transcript before the mid-blastula transition of the embryo (Chen et al., 2004). Metallothionein transcripts were also recovered from sea urchin egg and salmon ovary dbEST libraries, as well as lizard ovarian follicles (*Podarcis sicula*), with the highest level in ovulated eggs (Riggio et al., 2003b). Expression of the rat *Mt2* gene is also strongly regulated during primordial follicle assembly and development in rat ovaries (Kezele et al., 2005). SAGE may also help to distinguish between the expressions of several isoforms at the 3′-end of a transcript. In the same

UniGene *mt2* cluster, a second *mt2* transcript, identified with its *in silico* SAGE tag, contained an identical sequence in the coding region but a long untranslated 3′ part. This long transcript was not expressed in zebrafish fully grown follicles. It should be noted that differential expression of 3′-end transcript isoforms was easily identified using the SAGE method, as also demonstrated with the *ccnb2* transcript.

In addition to the *mt2* short transcript, abundant transcripts of heavy chain ferritins, including *fth1,* related to metal binding, were also detected in ZEBRAOV. This is in accordance with a disproportionately high number of salmon ovary assembled ESTs seen in GO categories related to heavy metal (copper, iron, and zinc) (Rise et al., 2004) and the presence of ferritin H mRNA in rainbow trout (*O. mykiss*) eggs (Aegerter et al., 2005). Homologous genes to zebrafish *fth1* are expressed in all vertebrate ovary dbEST libraries available at UniGene, with very high relative levels in salmon, swine, dog, and human libraries. Ferritin-containing inclusions were demonstrated in yolk platelets of schistosome (*Schistosoma mansoni*) (Schussler et al., 1995), a species in which a female-specific yolk ferritin transcript is expressed at high levels in the vitellarium (Schussler et al., 1996). Ferritin also occurs in amphibian (Brown and Caston, 1962; Kandror et al., 1992) and snail (von Darl et al., 1994) eggs. It should be noted that high-level expression of ferritin H-chain mRNA is observed in metastatic human ovarian tumours (Tripathi et al., 1996).

The second significant difference in transcript abundance between zebrafish fully grown follicle transcriptomes as evaluated by SAGE and the profile defined in ovary cDNA libraries concerned the *hsp90β* transcript (Table 2). Extensive molecular characterization, including zebrafish transcripts, and biochemical studies have revealed that vertebrate members of the heat shock protein 90 (HSP90) family play a post-translational regulatory role within the cell by interacting with several important cellular signalling molecules and transcription factors, such as steroid receptors, and modulating their activity (Krone et al., 1997). Homologous transcripts are highly expressed in mouse and human ovaries (Table 2) and a strong HSP90 immunoreactivity was demonstrated in rat primordial germ cells (Ohsako et al., 1995). This signal was also detected in both male and female pre-meiotic germ cells. HSP90 was also identified as one of the highly abundant proteins in mature mouse eggs and is strongly associated with the plasma membrane (Calvert et al., 2003). In addition, *hsp83* transcript, the *Drosophila* homologue of the mammalian Hsp90 family of regulatory molecular chaperones, is present at high levels through the end of oogenesis and both maternal and zygotic transcripts are spatially restricted during early embryo development (Ding et al., 1993). All these data are consistent with the high transcript level of *hsp90β* in zebrafish ovaries, whereas a high number of *hsp90β* cDNA clones was observed in the library generated from testes but not ovaries (Zeng and Gong, 2002). The large discrepancy in the relative level of *hsp90β* transcripts observed between the SAGE and cDNA approaches may be related to an enrichment of this transcript in the terminal stages of folliculogenesis.

Other transcripts highly expressed in zebrafish follicles and consistently represented in vertebrate ovarian transcriptomes are transcripts of ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (ATP5G), cold-inducible RNA-binding protein (CIRBP), and lactate dehydrogenase B4. *atp5g* is highly expressed in fish ovaries and the encoded protein is one of the chains of the nonenzymatic membrane component (F0) of mitochondrial ATPase in mitochondrial membrane. CIRBP apparently plays an essential role in cold-induced suppression of cell proliferation (Fujita, 1999). One of the *Xenopus* CIRBP homologues is a major RNA-binding protein in fully grown oocytes and may be involved in translational regulation via modulation of oocyte ribosomal function (Matsumoto et al., 2000). Lactate dehydrogenase B transcripts are widely distributed in animal ovarian transcriptomes, with high levels found in mice and humans. It has been previously demonstrated that lactate dehydrogenase B mRNA is one of the most abundant transcripts in fully grown mouse oocytes (Roller et al., 1989). Lactate dehydrogenase mRNA appears to be translated efficiently during oocyte growth and then downregulated during maturation and after fertilization (Cascio and Wasserman, 1982).

4. COMPARISON OF ZEBRAFISH FOLLICLE PROTEIN REPERTOIRE DEDUCED FROM SAGE WITH THE PROTEIN REPERTOIRE ISOLATED AFTER PROTEOMIC ANALYSIS

The egg is a transcriptionally inactive cell and, as such, is a storehouse of maternal mRNA and proteins required for fertilization and initiation of zygotic development. However, many of the proteins comprising the animal egg proteome have yet to be identified, as very few large-scale proteome analyses have been performed (see also Chapter 4). The proteins extracted from fully grown follicles were resolved by one dimensional SDS-PAGE or two-dimensional PAGE, subjected to in-gel tryptic digestion, followed by tandem mass spectrometry (MS/MS) identification of the resulting peptides (Aebersold and Mann, 2003). The protein repertoire determined was then compared with the repertoire deduced from ZEBRAOV (Table 3). As expected, the zebrafish follicle protein repertoire, determined by proteome analysis, identified ribosomal proteins, ZP family protein members, components of the cytoskeleton, chaperonins, heat shock proteins, and vitellogenin (Vtg) derivatives, but also some proteins not previously reported in ovary protein repertoires, e.g. a Sjogren syndrome antigen B homologous protein (Table 3). This RNA-binding protein binds to several small cytoplasmic RNA molecules, known as Y RNAs, and may stabilize these RNAs, preventing degradation (Deutscher et al., 1988). At least eight proteins, out of a total of 38 deduced using the SAGE assigned transcript method and expressed at over 0.15%, were identified by proteome analysis. The identification of abundant mRNAs without the corresponding translated proteins may be due to insufficient proteome delineation and/or the presence of oocyte stage-specific maternal transcripts, stored inside the oocyte cytoplasm and translated during

Table 3. Comparison of zebrafish follicle protein repertoire deduced from SAGE with the protein repertoire isolated by proteomic analysis

Tag (CATG+)	Nbr[1]	GenBank	Cluster[2]	Gene	Protein name	UniProt accession number
TAAGACATCC	226	BC045879	Dr.1109	*bactin2*	Beta-actin2	Q7ZVI7, Q6NWJ6
TAAAACCAAA	108	AF057040	Dr.25213	*bactin1*	Beta-actin1	
TTGATGAGGG	113	BC044190	Dr.729	*ldhb*	Lactate dehydrogenase B4	Q9PVK4, Q803U5
		BM571854	Dr.19916	*zp2.3*	Egg envelope protein ZP2 variant B [zp2.3]	Q98SS2
CATTTTCTAA	90					
		BC093133	Dr.23439	*zp2.4*	Zp2.4 protein	Q502R0
AGAAACGTTT	77	BC067692	Dr.26977	*zp3b*	Zona pellucida glycoprotein 3b	O12989, Q6NW78
GTGGTGTTGA	75	NM_131331	Dr.30322	*zp3*	Zona pellucida glycoprotein 3	Q9PWC7, Q6NW70
TTAATAAAAG	53	NM_198809	Dr.5605	*tubb2*	Tubulin, beta, 2	Q6TGT0, Q6P5M9, Q6IQJ2
TCTGTTTAAC	47	BC049058	Dr.11308	*rplp0*	Ribosomal protein, large, P0	Q7ZUG3, Q6P5K3
CTTAAATTGA	40	BC095386	Dr.30628	*gapd*	Gapdh protein (Fragment)	Q5XJ10
TTCTTTCTGT	36	BM778256	Dr.23435	*zp2.2*	Egg envelope protein ZP2 variant A [zp2.2]	Q98SS3
CCACAGGGCA	35	CO917383	Dr.5705	*rps3*	Ribosomal protein S3	Q6TLG8
GCTGCCTTGG	34	NM_199795	Dr.23436	*tuba4l*	Tubulin alpha 3; Alpha tubulin-like protein	Q802Y6, Q6TGS5
AGGATCGAGG	30	BI844143	Dr.25678	*eno3*	Enolase 3, (beta, muscle)	Q568G3, Q6TH14
TACAAGGCTT	28	AY394968	Dr.18315	*eef1g*	Elongation factor 1-gamma	Q6PE25, Q6NYN8
TTCTCCCTGT	25	NM_001003844	Dr.28231	*rpl6*	60S ribosomal protein L6	Q6DRC6, Q567N5
TGCTGAAAGT	16	NM_213058	Dr.26116	*hspa5*	Heat shock 70 kDa protein 5	Q7SZD3, Q6P3L3
CCCGGTGGAT	15	BC045841	Dr.24802	*hspa8*	Heat shock protein 8	Q7ZVJ1, Q6TEQ5, Q6NYR4
GTGTAAGTGA	15	NM_212772	Dr.28850	*tuba8l*	Tubulin, alpha 2	Q6TNP9
CTCAAAGATG	12	BG305824	Dr.25868		Hypothetical protein zgc:64133 (L-lactate dehydro-activity)	Q7T334

(*continued*)

Table 3. Continued

Tag (CATG+)	Nbr[1]	GenBank	Cluster[2]	Gene	Protein name	UniProt accession number
TCTTCTGAAA	12	CO352070	Dr.34146	*zp3al*	Zona pellucida glycoprotein 3a, like	Q5TYP2
		CO351980	Dr.25594	*zp3a*	Zona pellucida glycoprotein 3a	Q5TYP5
TTTTTTTTGA	11	BC056719	Dr.24206	*cct4*	Chaperonin containing TCP1, subunit 4 (Delta)	Q6PH46, Q6P123
TCTTGGTTAA	9	NM_200153	Dr.6969		Hypothetical protein zgc:55944 (aminopeptidase I activity)	Q803B5
TGTTGATGAA	8	NM_199547	Dr.3463	*ssb*	Sjogren syndrome antigen B (Autoantigen La)	Q7ZTI0
TGAAGCACTT	8	NM_212718	Dr.18319		ZPA domain containing protein [si:dkeyp-50f7.2]	Q8JIX8, Q5TYX2
AGAAGGAGGA	6	BC055516	Dr.20938	*sod1*	Superoxide dismutase [Cu-Zn]	O73872
TCTGAGCTGA	4	AY398308	Dr.4751	*aldh2*	Mitochondrial aldehyde dehydrogenase 2 family [aldh2l]	Q6TH48, Q6NWJ9
AACCACTCAA	4	BC050953	Dr.7644	*cct2*	Chaperonin containing TCP1, subunit 2 (Beta)	Q6PBW6
ACACAAAATA	4	BQ263267	Dr.25009	*vg1*	Vitellogenin 1	Q8JH36, Q8JH37, Q90YN8
TGCTCAAAAT	4	BC045933	Dr.134	*cct7*	Chaperonin-containing T-complex protein 1 eta subunit	Q8JHG7 Q7ZTS3
ATTGTATGGT	3	BM860549	Dr.7343		Acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain	Q7ZTH8, Q6NYM4, Q502R3
ATGTGGATCC	3	CK673334	Dr.28241	*rpl7a*	Ribosomal protein L7a	Q6PBZ1

CATTGTATAT	3	BM101604	Dr.11543		Zgc:103482	Q5XJA5
CCGACCCTAC	3	BC083399				
TTCTCCCTCA	3	BC091659	Dr.10133		Zgc:110766 (translation elongation factor activity)	Q5BJ17
AAGATGGAAA	3	BQ285089	Dr.24685		Hyp.prot.zgc:111961 (hydro-translo.F-type ATPase complex)	Q4VBK0
TTTTGTACAA	2	NM_200174	Dr.8999		Hypothetical protein zgc:55702	Q7ZVR7
TCACAGGCCA	2	CO919203	Dr.29735	*naca*	Nascent polypeptide-associated complex alpha subunit	Q8JIU7
AAGCGGAGCT	2	BC059705	Dr.28202		Hypothetical protein zgc:73404 (Peptidase)	Q6NSN3, Q6PBH6
CAAGTGTGAT	1	BI843130	Dr.20270		Wu:fi20c07 Hypothetical protein (fragment) (Pentaxin)	Q7SZ53, Q5XJ77, Q4V8X3
CCTGATCTGT	1	NM_201052	Dr.1065	*rpsa*	Ribosomal protein SA	Q803F6
AGGGCTGGAA	1	NM_201290	Dr.6928	*cct6a*	Chaperonin containing TCP1, subunit 6A (Zeta 1)	Q7ZYX4
TTGAACTCCA	1	BQ419723	Dr.28864	*serpina1*	Novel protein similar to Alpha-1 antiproteinase (Serpina1)	Q5SPJ1, Q5SPJ4
Non-detected						
TGGCTTTCAA	0	NM_200446	Dr.831	*chia*	Eosinophil chemotactic cytokine	Q7SYA8, Q6TH31
CTGGACCAGC	0	NM_131804	Dr.10788	*nots*	Nots protein	Q5PR42, Q9DDE2

[1]Nbr is the number of times a tag at R1 or R1cr position was counted in ZEBRAOV.
[2]UniGene Build#87.

early embryo development. There were several proteins distributed in over one spot position after 1D-, 2D-PAGE separation and MS/MS (Knoll-Gellida et al., 2006). While some of them, e.g. creatine kinase (CK), were present in closely isoelectric focusing located spots, suggesting the presence of isoforms or post-translational modifications, the distribution of other spots, e.g. Vtg derivatives, indicates a cleavage of precursor proteins with the presence of lower-molecular-weight derivative fragments (see Chapter 4). CKs play crucial roles in intracellular energy transfer and expression of a CK brain-type isoenzyme during oogenesis has been demonstrated in rodents (Iyengar et al., 1983; Naumoff and Stevenson, 1985). *ckb* mRNA is shown to be maternally supplied in zebrafish embryos (Dickmeis et al., 2001).

Comparison of the zebrafish follicle protein repertoire deduced from SAGE with the protein repertoire isolated after proteomic analysis revealed that some abundant transcripts identified by their SAGE tags, but not previously reported to be present in abundance in fish ovaries, had corresponding proteins. This was the case of lactate dehydrogenase B4 and, to a lesser extent, ribosomal protein large P0 (Tables 2 and 3). Comparison also revealed that *bactin1* and *bactin2* transcripts were differentially expressed in zebrafish ovarian follicles, but their protein sequences were not resolved due to the very high sequence conservation of these duplicated gene copies. On the other hand, some ZP family protein members could be discriminated on the protein level, while the same SAGE tags were generated with *zp2.3* and *zp2.4* or *zp3a* and *zp3al* transcripts, due to the high sequence similarities of the 3′-end untranslated part of these transcripts.

Oocyte growth, particularly in oviparous species, is characterized by intense deposition of RNAs and proteins, not necessary of the same nature and origin. These maternal factors can be stored for very long periods of time until their use during embryonic development. Comparison of transcriptome and proteome data revealed that transcript levels provide little predictive value with respect to the extend of protein abundance, taking into account the fact that the protein identification approach used detects relatively abundant proteins from the biological extract, while the mRNA abundance evaluated by SAGE tag frequency varied by over two orders of magnitude (Table 3). Transcript profiling provides a measure of RNA abundance, which may be affected not only by transcription levels but also by RNA processing and degradation. Moreover, not all transcripts are translated and RNA abundance may not correspond to protein levels. High transcription and translation rates during folliculogenesis and oocyte growth are followed by differential translational silencing and degradation of many mRNA species, especially at the end of the oocyte growth phase (Eichenlaub-Ritter et al., 2002; Schultz, 2002). The identification of zebrafish follicle proteins, e.g. pyruvate kinase and enolase I, by proteome analysis, with very low corresponding transcript levels but very high homologous transcript counts in human ovary transcriptomes used as an external reference, suggests a downregulation of the quantity of these transcripts and storage of the proteins at the end of zebrafish folliculogenesis.

A comparison of transcriptome and proteome data revealed two proteins encoded by *chia* and *nots* without corresponding transcripts in ZEBRAOV (Table 3). *chia* is related to the multiple chitinases genes identified in rainbow trout and Japanese flounder (Kurokawa et al., 2004), as well as, to a lesser extent, the acidic mammalian chitinase precursor in humans (Boot et al., 2001). While the molecular functions of these proteins are related to chitin binding and chitinase activity, the functionality and origin of the protein identified in zebrafish fully grown follicles remains to be determined. However, the presence of a small amount of *chia* transcript in multiple follicular stage zebrafish cDNA libraries ID.9767, ID.15519 supports a stage-specific transcription of this gene during zebrafish folliculogenesis as previously demonstrated by the downregulation of the transcription of some fish maternal genes, e.g. Vtg/very-low density lipoprotein receptor (Perrazolo et al., 1999) at the end of oogenesis. A high rate of protein deposition has also been observed during oocyte growth in oviparous species via a receptor-mediated endocytosis of exogenous precursors (see Chapter 2). The presence of an abundant protein in the repertoire without a corresponding transcript in ZEBRAOV may be due to endocytosis of the protein from the plasma to the oocyte. Zebrafish *vg1* and *vg3* are mainly expressed in the liver and, to a lesser extent, in several non-liver tissues, including the adipocytes associated with several organs, such as ovaries (Wang et al., 2005). This may explain the presence of a limited amount of *vg1* and *vg3* transcripts in ZEBRAOV (Table 3). These precursor proteins are synthesized outside oocytes during vitellogenesis, specifically incorporated in the oocyte by receptor-mediated endocytosis and cleaved into yolk proteins (See Chapters 1 and 2). The identification of nothepsin in Zebrafish fully grown follicles by proteome analysis although no transcript was detected in the ovary by Northern blot (Riggio et al., 2000), EST sequencing of ovary cDNA libraries, or SAGE (Knoll-Gellida et al., 2006), strongly suggests an extraovarian origin for this enzyme that may be present in the plasma of females undergoing vitellogenesis. Zebrafish *nots* encodes a paralogous aspartic proteinase related to endoproteolytic proteinases, such as cathepsin D, cathepsin E, and pepsin. This gene is specifically expressed in the liver under estrogenic control (Riggio et al., 2002) and the sexual dimorphic expression of *nots* may be related to the reproductive process.

5. CONCLUSIONS

A complete sequence data set of maternal mRNA stored in zebrafish germ cells at the end of oogenesis has been recently provided (Knoll-Gellida et al., 2006). This catalogue contains highly expressed transcripts that are part of a vertebrate ovarian expressed gene signature. The delineation of the transcriptome of teleost fish ovaries has also been evaluated using large-scale EST sequencing of cDNA libraries (Davey et al., 2001; Zeng and Gong, 2002; Li et al., 2004; Rise et al., 2004), subtractive hybridization (Kanamori, 2000), and microarray-based

analyses (von Schalburg et al., 2005). The molecular phenotype described provides groundwork for future experimental approaches aimed at identifying functionally important stored maternal transcripts and proteins involved in oogenesis and early stages of embryo development or transcriptional events and molecular pathways disrupted by environmental toxicans.

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CHAPTER 4

PROTEOMICS ANALYSIS OF THE DEVELOPING FISH OOCYTE

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1. INTRODUCTION

This chapter describes the implementation of the new proteomics technologies to the study of the protein repertoires of developing oocytes. Proteomics is a new analytical field aimed at simultaneously characterizing entire protein repertoires of biological systems. Using proteomics, comprehensive lists of the protein constituents of cells and organisms can be established. Even more importantly for developmental biology, changes in the relative amounts of the proteins and in the patterns of their posttranslational modifications can be followed. Here we will

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review the way proteomics technologies based on one or two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), multidimensional high-performance liquid chromatography (HPLC) and mass spectrometry were employed for following molecular changes in oocyte development. Indeed, proteomes of different developmental stages of oocytes of different species have been studied, revealing both the similarities and the differences. This chapter will allude to the effects of hormonal cues and cryopreservation on the protein repertoires of oocytes and their influence on viability and fertilization failures.

2. THE SHIFTING PROTEIN PATTERNS DURING OOCYTE DEVELOPMENT

The molecular changes taking place in oocytes during development from early stages of oogenesis to the mature oocytes ready to be laid, are well preserved among different species. Such preservation of the molecular events among diverse organism paves the way to identification of evolutionary conserved factors, which are likely to be very significant to the developmental process or serve as important maternal factors. Following these molecular events and comparing them between diverse organisms is crucial for the understanding of the common developmental pathways on one hand, and the details of adaptations to different environments and conditions on the other.

3. PROTEOMICS METHODOLOGY

To analyze complex protein patterns as those of the developing oocytes the new tools of proteomics are needed. Analytical proteomics enables the simultaneous analyses of changes in proteins patterns and in posttranslational modifications (PTMs) in any type of protein containing sample (Aebersold and Mann, 2003; Patterson and Aebersold, 2003; Zhu et al., 2003; Görg et al., 2005) such as the developing oocytes (Coonrod et al., 2002). Thus proteomics is emerging as one of the most powerful new tool for research in developmental biology similarly to other fields.

The advances in proteomics stem from the introduction of new instrumentations, methodologies and direct access to large gene and protein databanks. The most important among these technologies is mass spectrometry (MS). It involves accurate measurements of the masses of proteins or their derived proteolytic peptides and is followed by vacuum-phase fragmentation of the proteolytic peptides and mass fingerprinting of the resulting fragments (tandem mass spectrometry: MS/MS). Since proteins and peptides are nonvolatile molecules, their introduction into the gas phase of the mass spectrometers is achieved by interfacing liquid chromatography through electrospray ionization (ESI) or dried-down peptide samples through matrix assisted laser desorption ionization (MALDI) (Aebersold and Mann, 2003). The measured masses of the peptides and the masses of their fragmentation products are matched to the calculated masses of all the protein sequences in the data banks, resulting in a relatively straightforward identification of those proteins whose amino acid sequences are known. Powerful bioinformatics tools and modern

computers fitted with the genes, proteins and peptides databases (Nesvizhskii and Aebersold, 2004) speed up the data analysis processes and facilitate large-scale analyses of entire proteomes using minute amounts of analyzed protein extracts.

The vast complexity of the protein pools extracted from oocytes necessitates their pre-fractionation into less complex sub-fractions to facilitate the identification of the individual peptides. 2D-PAGE and multidimensional-chromatography are the most appropriate tools to achieve this goal. The majority of the sample's proteins can be resolved by 2D-PAGE in a single-step. Once resolved by 2D-PAGE and stained, each stained spot contains one, or a few proteins at most. These can now be identified relatively easily by in-gel proteolysis, extraction of the proteolytic peptides from the gel pieces, followed by MS/MS of the resulting peptides as described above (Aebersold and Mann, 2003). Most importantly, the patterns of proteins observed by 2D-PAGE can be used for comparisons of the protein repertoires between different samples, such as protein extracts of maturing oocytes. Performing 2D-PAGE is relatively simple and inexpensive and does not require large amounts of protein. The relative amounts of the proteins in each spot can be compared by digitizing the images of the stained 2D gels and quantization of the optical density in each spot. Shifts in the patterns of PTMs are also followed by 2D-PAGE, since they influence the both the isoelectric point and the size of the proteins. Thus, PTMs cause the appearance of stained protein spots adjacent to the spots of unmodified proteins. When western blots of 2D-PAGEs of oocyte proteins are probed with mono-specific antibodies directed against one or a few of the oocytes' proteins, a high sensitivity can be obtained, much beyond the sensitivity of stained gels (Hiramatsu et al., 2002).

While 2D-PAGE is most appropriate for monitoring changes in the protein repertoires of biological sample, cutting each individual gel spot and identifying its protein content is laborious and time consuming. Therefore, two other approaches are commonly used for the analysis of the entire repertoire of proteins present in a complex sample.

The first is based on following changes in protein repertoires in developing tissues or cells using multidimensional chromatography (such as multidimensional protein identification technology, MudPIT) (Figure 1) (Link et al., 1999). Comparing of chromatography patterns overcome many of the inherent problems of 2D-PAGE, such as detection of membrane proteins, very large and very small proteins, and very basic and very acidic proteins (reviewed in Wang and Hanash, 2003). MudPIT starts with proteolysis of the entire protein mixture, followed by separation of the resulting peptides by ion-exchange chromatography. Next, each fraction of the salt steps is separated by reversed-phase (RP) HPLC and interfaced directly into the mass spectrometer using capillary RP-HPLC connected through the ESI interface (Link et al., 1999; Wolters et al., 2001) (reviewed in Wu and MacCoss, 2002; Wang and Hanash, 2003).

Another very appropriate approach for the analysis of complex protein repertories is based on running the protein in 1D-SDS-PAGE and then cutting gel slices after staining the gel. The proteins in each of the gel slices are proteolyzed *in situ* and

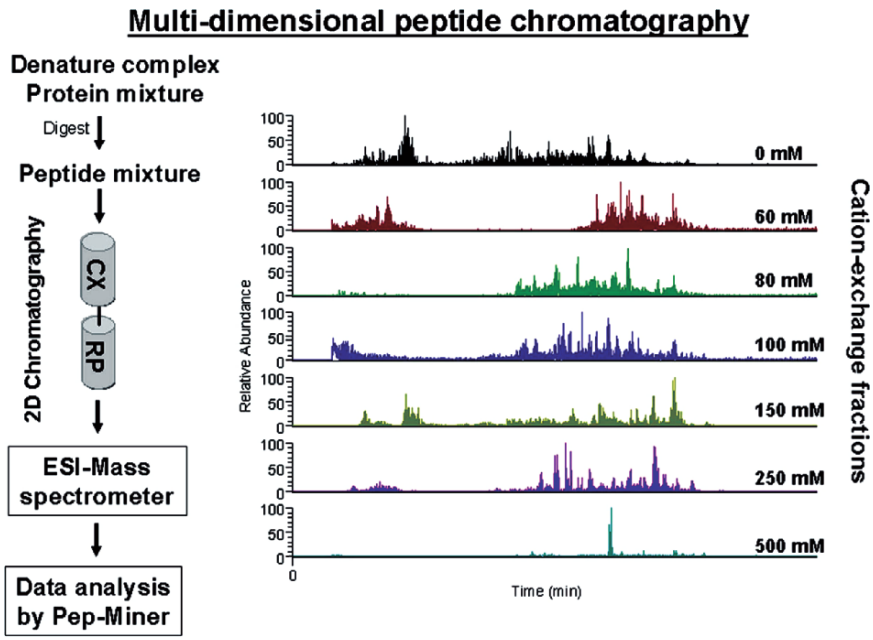

Figure 1. Two-dimensional chromatography of tryptic peptides of oocytes proteins. Total protein extracts of zebrafish oocytes were proteolyzed with trypsin and the peptides were separated in the first dimension on a disposable strong cation exchange columns and on the second dimension on uRP-HPLC connected to MS/MS. Each chromatography displays the total-ion-current chromatogram (TIC) for each of the salt elutions of the peptides. (*See Color Plates*).

the resulting peptides are analyzed and identified by MS/MS (Figure 2; Table 1). This approach facilitates the identification of the entire protein repertoire of the sample, particularly significant for membranal and basic proteins (Ostrowski et al., 2002).

4. OOCYTE PROTEOMICS

One application of the proteomics technologies has been to study oocyte development. Indeed, proteomics technologies were already used to study the cellular biology of oocytes in a number of species, including humans (Trounson, 2003), mice (Coonrod et al., 1999; Calvert et al., 2003), pigs (Ellederova et al., 2004; Huo et al., 2004), fish (Rime et al., 2004) and insects (Lee et al., 2000; Tufail and Takeda, 2002). The foremost goals of most of these studies were cataloguing the oocytes' proteins (Coonrod et al., 2002). These were followed by studies of how the protein repertoires, changed in their relative amounts, in their PTM patterns and in their intra-oocyte location during maturation (Calvert et al., 2003).

Oocytes develop through schemes that include physical growth, structural changes, proteins synthesis, and protein imports. During development, the aggregation states of some of the proteins and their patterns of PTMs change

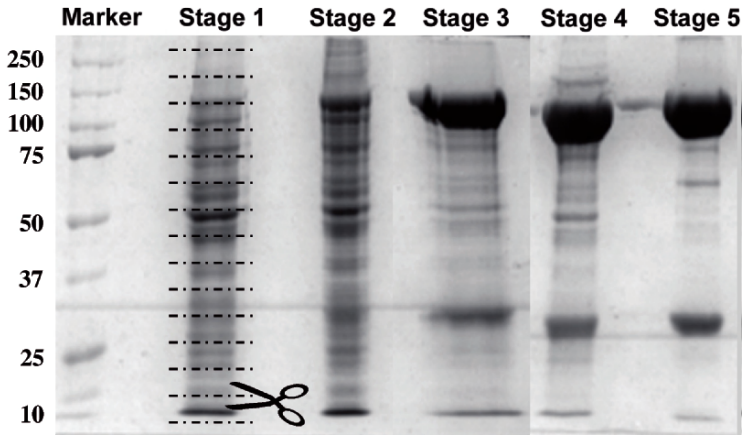

Figure 2. Analysis of oocytes proteins by SDS-PAGE, gel slicing and mass spectrometry. Proteins extracts of stages I to IV zebrafish oocytes were separated by SDS-PAGE, the gels were stained with Coomassie and 14 gel slices from each lane were cut and in-gel proteolyzed with trypsin. The resulting peptides were identified by μLC-MS/MS (summary of results in Table 1).

Table 1. Statistics of protein identification of zebrafish oocytes using SDS-PAGE and gel slicing. Proteins extracted from zebrafish oocytes at stages I–IV were identified as described in Figure 2. The list of the identified proteins and the number of unique proteins for each developmental stage of the oocytes are indicated (Ziv, Gattengo, Chapovetsky, Wolf, Barnea, Lubzens and Admon, in preparation.)

Oocyte stage	Number of identified proteins	Unique proteins
I	38	3
II	55	14
III	30	2
IV	17	0
Total	66	19

dramatically (Selman et al., 1993; Banoub et al., 2003; Banoub et al., 2004). During the past few years, with the introduction of genomics, proteomics, and bioinformatics, the study of developmental processes has taken a new molecular level direction. These studies are often based on the simultaneous analyses of gene and protein expression patterns instead of one gene or protein at a time. Proteomics is being implemented for oocyte research to follow changes in protein repertoires of oocytes as some proteins are being imported into the oocytes, where they are further modified. Such changes cannot be followed by genomics (or transcriptomics) technologies, and therefore, need to rely solely on proteomics for elucidation. Indeed, studies of the proteomes of oocytes of a number of species have resulted in catalogues of proteins expressed by oocytes in general (Coonrod et al., 1999; Lee et al., 2000; Coonrod et al., 2002; Tufail and Takeda, 2002; Calvert et al.,

2003; Trounson, 2003; Ellederova et al., 2004; Rime et al., 2004) and even in different developmental stages or intra-oocyte locations (Coonrod et al., 2004).

Proteomics was also used to identify different porcine oocyte proteins that are possibly involved in nuclear reprogramming of somatic cells. Novak et al. used *in vitro* systems to incubate biotin-labeled porcine oocyte proteins with isolated somatic cell nuclei, this way inducing the association of the oocyte proteins with the nuclei previously isolated from the somatic cells. This analysis resulted in the identification of oocyte proteins that accumulated in the nuclei and therefore could be involved in nuclear reprogramming of somatic cells nuclei during early embryonic development. They used a novel biotin-based affinity-binding system to recover the oocyte proteins associated with the nuclei and 2D-PAGE for separation and visualization of labeled oocyte proteins. The proteins of interest were identified using both MALDI-time of flight (TOF) and ESI-LC-MS (Novak et al., 2004).

The proteomes of mouse oocyte were established, including the sequences of 30 of 80 labeled surface proteins. Nine highly abundant molecular chaperones that were localized to the oolemma of the mature mouse egg were also identified (Calvert et al., 2003; Coonrod et al., 2004).

In one of our current study, we used proteomics technologies to analyze the protein repertoires of both gilthead sea bream (*Sparus aurata*) and of zebrafish (*Danio rerio*) oocyte at different developmental stages and consequently identified a large number of proteins from both species (Gattegno, Chapovetsky, Lubzens and Admon, in preparation, and Figure 3). Our current data-set includes more than 600 different zebrafish oocyte proteins.

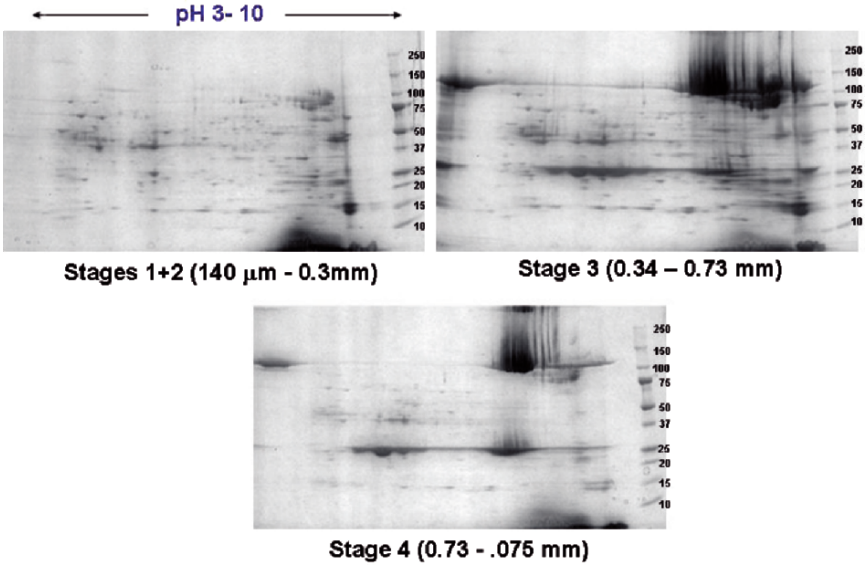

Figure 3. 2D-PAGE analysis of zebrafish oocytes proteins. The total protein extract of stage I, II, III, and IV of the zebrafish oocytes were resolved by 2D-PAGE. Labeled proteins are those observed at elevated level at stages I and II. Selected proteins were cut from the gel, digested by trypsin in the gel and identified by μLC-MS/MS.

5. THE STUDY OF FISH OOCYTE VIABILITY USING PROTEOMICS

One of the uses of proteomics involves searching for correlations between oocyte viability and their associated protein repertoires. As oocyte age, or lose viability due to treatments such as cryopreservation, their protein patterns should reflect this abrupt or gradual change in viability. Indeed, Rime and colleges applied proteomics tools to study the correlation between the decrease in egg quality and the aging of postovulatory oocyte in rainbow trout (*Oncorhynchus mykiss*) coelomic fluid (CF). Proteins accumulating in the CF during the postovulatory period were analyzed using MALDI-TOF and some were identified as lipovitellin II fragments. They proposed that as the oocyte age during the postovulatory period, they release egg protein fragments that accumulate in the CF fluid. These proteins can be associated with a decrease in egg quality and therefore can be used as biomarkers for this process (Rime et al., 2004). We have followed the effect of cryopreservants on the repertoire of oocyte proteins. We noticed that very small changes appear as a result of treating fish oocytes with concentrations of methanol and DMSO, agents usually employed in cryopreservation. These results indicate that the loss of viability of these oocytes apparently involves physical changes and not a major change in the protein repertoire.

6. PROTEOMICS OF OOCYTES OF ORGANISMS WITH YET UNSEQUENCED GENOMES

The genomes of only a few egg-laying organisms have been sequenced already, and, as expected, the amino acid sequences of the vast majority of their proteins are not available in the protein databanks. Identification of proteins extracted from unsequenced organisms using MS/MS analysis requires the use of either fully *de novo* sequencing or the use of similarity-based identifications. Similarity-based identifications involves looking for homology between the measured MS/MS spectra of peptides obtained after proteolysis of protein extracts from the organism with the unsequenced genome and the calculated MS/MS of the entire set of peptides produced by simulation of proteolysis of the complete protein data bank (Shevchenko et al., 2001; Habermann et al., 2004). Homology analyses are capable of increasing the throughput of protein identification without the need to manually sequence *de novo* each of the proteins. Liska et al. (2004) have used functional proteomics in order to overcome this limitation in *Xenopus laevis*. They used sequence-similarity methods to analyze *Xenopus* microtubule-associated proteins, and successfully identified more than 40 unique microtubule-bound proteins as well as additional new proteins.

7. 2-D MAP OF THE VITELLOGENIN-DERIVED PROTEINS IN OOCYTES

Protein maps from oocytes of different species and from different developmental stages have been obtained mostly by using 2D-PAGE (Coonrod et al., 2002; Calvert et al., 2003). The most prominent protein present in fish oocytes

are the vitellogenin (Vtg)-derived proteins. As the oocytes progress through their different development stages, the amounts of Vtg products increase and become processed into the yolk proteins; lipovitellin I and II and phosphvitin. The Vtgs are mostly phosphorylated and glycosilated before their import into the oocyte and their processing in the oocyte include mainly proteolysis (Wallace and Selman, 1990; Selman et al., 1993). While the Vtg and its products are easily identified in the protein preparation, their large amounts and multiple forms and sizes mask and complicate the identification and analysis of other oocyte proteins. This situation is very similar to that encountered while attempting to analyze serum or plasma proteins of animals and humans. The very large amounts of albumin and antibodies present in serum mask and hinder the analyses of other less abundant proteins (Anderson et al., 2004). To circumvent this hurdle, attempts were made to deplete the Vtg products from the oocytes before the analyses of less abundant proteins. This is not a simple task since immunoaffinity depletions of oocyte protein extracts from the very abundant Vtg products are limited by lack of monoclonal antibodies (mAb) directed against the different forms of the protein. The presence of the different yolk proteins as multiple derivatives of Vtg with variable sizes and different PTMs further complicates these attempts.

The different forms of the yolk proteins shift dramatically during oocyte growth, not only in their total amounts but also in their size and isoelectric points. These proteins are transported from its synthesis site in the liver though specific receptors into the oocytes. Vtg is proteolyzed in the oocyte into the smaller derived yolk proteins (in teleosts, these yolk proteins include lipovitellin and phosvitin, a protein heavily phosphorylated on it serines stretch). These changes are apparently needed for accumulation and storage of the protein, lipids, sugars, and phosphate within the oocytes (Selman et al., 1993). When the Vtg-derived proteins are degraded into their constituent amino acids, they are used as building blocks for synthesis of the developing embryo cells. In some species, specifically the pelagic species, this processing is appropriately timed to increase the osmotic pressure in the oocyte leading to an increased uptake of water uptake through aquaporins (Fabra et al., 2005) and corresponding increase in buoyancy (Selman et al., 1993).

Proteomics technologies based on 2D-PAGE, western blots of proteins separated on 2D-PAGE (Hiramatsu et al., 2002) and phosphopeptide analysis are very useful for establishing the vitellogenin map (Watts et al., 2003). Vtg processing has been studied in a number of species: insects such as cockroach (Tufail and Takeda, 2002) and cicada (Lee et al., 2000), marine shrimp (Avarre et al., 2003), teleost species, such as, masu salmon, greenback flounder, Atlantic salmon, rainbow trout, zebrafish, medaka, red sea bream and white perch (Matsubara et al., 1999; Hiramatsu et al., 2002; Watts et al., 2003; Wood and Van der Kraak, 2003; Tong et al., 2004; Sawaguchi et al., 2006), and in chicken (Luo et al., 1997). Proteomics methods were used to characterize rainbow trout Vtg protein in its intact form, and then analyzed its derived tryptic and

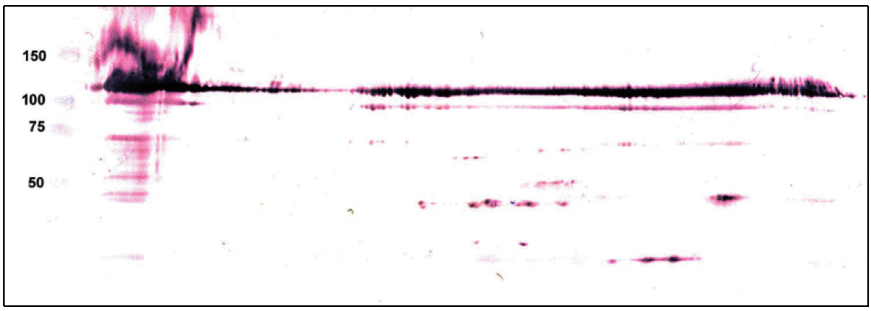

Figure 4. 2D-PAGE Western analysis of zebrafish oocytes proteins. The total protein extract of stage IV of the zebrafish oocytes were resolved by 2D-PAGE and transfer to nitrocellulose membrane. The membrane was developed with rabbit anti-Vtg polyclonal antibodies. (*See Color Plates*).

cyanogen bromide peptides by MALDI-TOF-MS and ESI quadrupole (Q)-TOF (Banoub et al., 2004). The same group also characterized the Atlantic salmon Vtg by *de novo* sequencing it with both ESI and MALDI-based MS/MS (Banoub et al., 2003). To demonstrate the multitudes of forms of Vtg-derived proteins present in the oocyte of a fish, the example in Figure 4 displays an anti-Vtg antibodies western blot of total zebrafish oocyte at stage IV (Gattegno et al., in preparation).

8. OOCYTE DEVELOPMENT AND MOLECULAR STAGING BY PROTEOMICS

Until recently, oocyte development was mostly followed by visual inspection of the different stages of the oocytes, either under a microscope (Selman et al., 1993; Maack and Segner, 2003), or by following the activity of enzymes involving with the maturation process with their corresponding maturation stages. Oocyte growth and development are accompanied by changes in the opacity of the oocyte, as well as changes in color, size, and density. These changes are mostly attributed to the accumulation of Vtg products, to their aggregation into protein aggregates, to their processing into small peptides, and finally into free amino acids. Molecular staging of the oocytes can be performed using transcriptomics and proteomics. It is based on establishing genes and proteins expression patterns as representing specific stages of oocyte development. The gene expression patterns discussed in Chapter 3 in this book (Knoll-Gellida et al., 2006) are example of the transcriptomic-based approach and its comparison with proteomic profiling (recent review of molecular staging in farm animals (Sirard et al., 2003). Molecular staging based on proteomics approach was demonstrated in pigs by Ellederova et al. (2004). Proteomics-based molecular staging is able to include in the analysis those proteins produced outside the oocytes (such as Vtg products, which are synthesized in the liver).

Ellederova et al. (2004) used proteomics tools to analyze pig oocyte proteins during *in vitro* maturation (IVM) using 2D-PAGE for separation, and visualization of oocyte proteins and MS for the identifications. Comparative analysis was used to identify unique protein patterns of different staged *in vitro* mature oocytes. They noticed that the expression of antiquitin (D7A1) increased during first meiosis and second meiosis compared to germinal vesicle *in vitro* maturated oocytes. Such differentially expressed proteins may be useful as biomarkers of oocyte IVM and quality.

We have also followed the changes in the forms of Vtg-derived proteins in sea bream and in zebrafish during oocyte development and identified by MS/MS many of the Vtg-derived protein spots on a 2D-PAGE. We therefore propose 2D-PAGE as a useful approach for establishing comprehensive maps of Vtg-derived proteins in oocytes during their development (Gattegno et al., in preparation).

9. SINGLE OOCYTE PROTEOMICS

The exquisite sensitivity of modern genomics and proteomics technologies enables the analyses of the transcriptomes and the proteomes of single oocytes. The significance of this ability relates to the possibility of studying the variability among oocytes of the same apparent stage and correlating the visible changes taking place during maturation with molecular level changes in protein repertoires. The oocytes are visualized and photographed under the microscope, followed by extraction and analysis of their proteins. Each of the different maturation stage proteins are resolved by 2D-PAGE, with each gel image representing the protein repertoire of a single oocyte. Each gel reveals the differences among individual oocytes and correlates protein patterns with morphology. We have analyzed single oocytes of zebrafish and of sea bream that were morphologically defined at developmental stage III. They differed significantly in their protein repertoires, most notably, in their processing of the Vtg-derived proteins. Earlier stage oocytes are more difficult to analyze due to their small size and limited amount of protein content. Oocytes that were morphologically defined at stage IV displayed a very similar protein repertoire, since Vtg is very abundant at this stage, and as previously mentioned, it masks many of the other proteins in the sample (Gattegno et al., in preparation).

10. CONCLUSION

The use of proteomics for cataloguing of oocyte proteins, following the changes in the proteins repertoire during development and determining the variability between individual oocytes is just beginning. It is likely to enhance our understanding of oocyte biology in the near future and to facilitate medical and biotechnology advances in reproductive biology and animal farming as the technology will be implemented into routine use.

11. ACKNOWLEDGMENT

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CHAPTER 5

OOCYTE ZONA PELLUCIDA PROTEINS

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1. INTRODUCTION

All vertebrate species produce an egg surrounded by an acellular vitelline envelope. In mammals the envelope is transparent and called the zona pellucida. The zona pellucida consists of 2–4 major proteins (Wassarman, 1988; Lefièvre et al., 2004).

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As these proteins that are the main constituents of the zona pellucida (eggshell) the proteins have been named zona pellucida proteins (ZPs). In teleosts these protein are mainly synthesized in the liver (Hamazaki et al., 1985, 1989; Lyons et al., 1993), although in carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*) the ZPs are synthesized in the ovary (Chang et al., 1996). This ovary expression of ZP proteins has also been shown for chicken, mouse, and *Xenopus*. In chicken, the synthesis of ZPs occurs in the granulosa cells (Waclawek et al., 1998), while mouse (Epifano et al., 1995a) and *Xenopus* (Yamaguchi et al., 1989) synthesize ZPs in the oocytes.

At the onset of growth, the oocyte is enclosed in a single layer of granulosa cells. The gonadotropins released at this stage will induce the granulosa cells to increase in size and numbers and form a thick cell layer. At the same time, the mesenchyme cells of the ovary differentiate into thecal cells forming a thick outer cell layer separated from the granulose cells by a basal lamina. During the growth phase, the oolemma will form microvillies, which extend between the plasma membrane and the granulosa cells. Formation of the vitelline envelope (zonagenesis) starts during oocyte growth. Microvilli are formed from the plasma membrane of the oocyte, and they stretch towards the surrounding granulosa cells. The synthesis of the eggshell starts at the base of these microvilli (Tesoriero, 1977). The eggshell is a thick structure consisting of proteins and glycoproteins (Cotelli et al., 1988). The structural complexity and macromolecular composition differ from species to species. The development of the gilthead sea bream (*Sparus aurata*) oocyte and its vitelline envelope are shown in Figure 1. During the early phases, the vitelline envelope consists of four identifiable layers. The outermost layer is reduced during development of the oocyte and does not appear to be present at stage IV. Following hydration of the oocyte at stage V, the vitelline envelope alters its appearance and becomes a striated structure.

The ZPs forming the acellular egg envelope surrounding the teleost oocyte (Hyllner and Haux, 1992) has many different functions such as uptake of nutrients, functional buoyancy (Podolsky, 2002), protection of the growing oocyte, species specific sperm binding, guidance of the sperm to the micropyle (Dumont and Brummet, 1980) and does also possess bactericidal properties (Kudo and Inoue, 1989). In addition, the eggshell protects the developing embryo until implantation in placental mammals or until hatching in teleosts.

In the present review we will describe the current knowledge of ZP proteins with special emphasis on their synthesis, regulation, assembly and function in teleost. We will also describe differences observed between teleost species and between teleosts and other animal groups.

2. NOMENCLATURE

The nomenclature of this unique group of structural proteins is very ambiguous. These proteins have been called zona pellucida (ZP) proteins, zona radiata proteins (ZRP), and vitelline envelope proteins (VEP). Not only does the terminology differ between mammalian vertebrates, amphibians, and birds, but

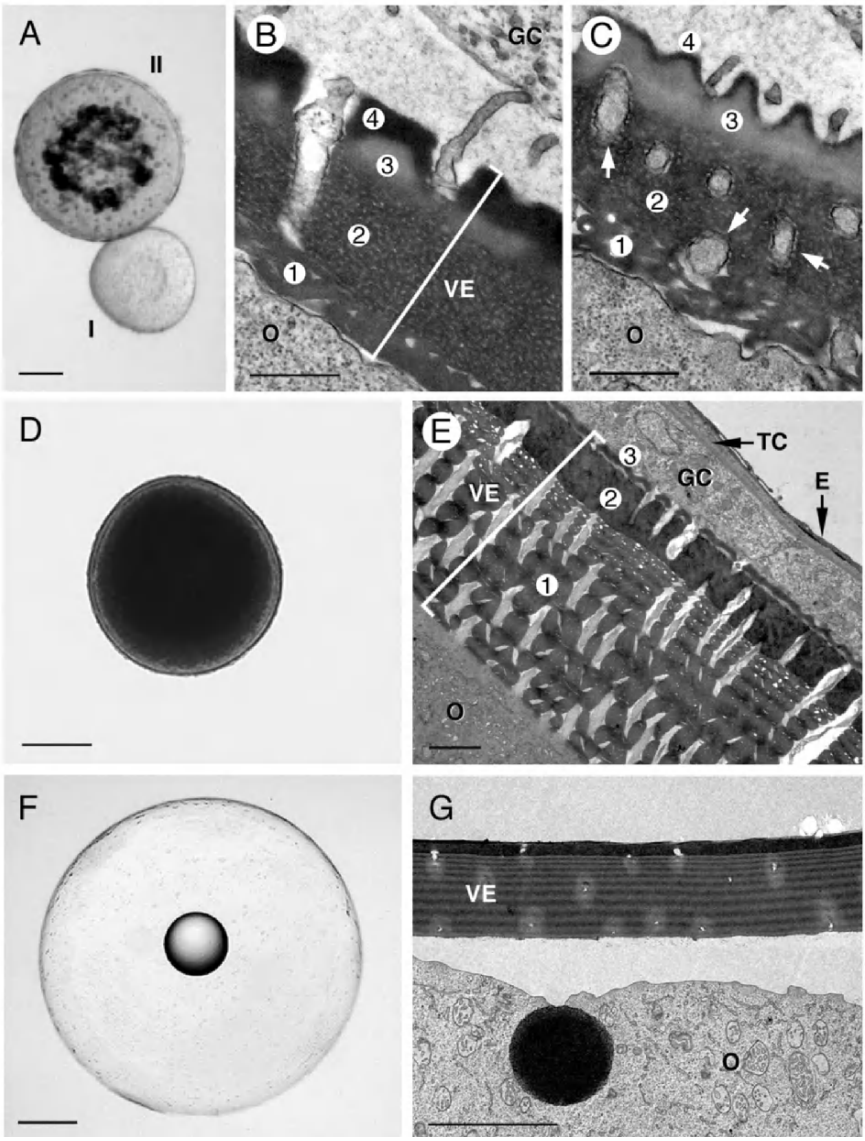

Figure 1. Ultrastructure of the vitelline envelope (VE) in ovarian follicles in the gilthead seab ream. Stereomicrsocope images of the developing oocytes are shown at stage I and II (A), stage IV (D) and stage V (F) oocytes. Electron micrographs of stage I oocytes shows 4 distinct VE layers (B) while only 3 layers are observed in stage II oocytes (C) and stage IV oocytes (E). Layer 1 is less compact than layer 2 and the outermost layer 3 appears granular. At stage V the oocyte microvilli have retracted and the VE appears striated. The arrows in panel C shows the presence of the microvilli. Bars (in micrometer [μm]): 50 (A), 0.5 (B and C), 200 (D and F), 2 (E), and 5 (G). O, oocyte; GC, granulosa cells; TC, theca cells. (Images by J. Cerda).

it also differs between various teleosts species. Over the years, the nomenclature for describing the eggs of teleosts has gone through considerable variation. Terms used for describing the egg envelope include chorion, egg capsule, eggshell, primary membrane, radiata membrane, zona pellucida, zona radiata, vitelline membrane, and vitelline envelope (Groot and Alderdice, 1985).

With the discovery of novel ZP proteins from many different species, it has become apparent that there is a need for a revision of the nomenclature of eggshell proteins. As a recent review suggests standardization of the nomenclature (Spargo and Hope, 2003) we will use the suggested nomenclature in the present review. The ZP proteins may be divided into ZPA (no teleost members have so far been found to belong to this group), ZPB, ZPC, and ZPX (no mammalian genes belong to this group) and the phylogenetic relationships are described in a recent study of chicken ZP genes (Smith et al., 2005).

3. MOLECULAR CHARACTERIZATION OF ZP

This section will deal with the genes encoding the ZP proteins, the primary structure of the proteins, protein assembly and function. In addition, the implications of conserved and diverged sequences will be discussed.

3.1. Zona Pellucida Genes

Several ZP genes from various vertebrate species have been isolated and characterized (Kinloch et al., 1988, 1990; Chamberlin and Dean, 1989; Lyons et al., 1993; Epifano et al., 1995b; Taya et al., 1995; Yamasaki et al., 1996; Chang et al., 1996, 1997; Mold et al., 2001; Lee et al., 2002a, b; Harris and Piersen, 2003; Kanamori et al., 2003; Smith et al., 2005). The complexity found in genomes of different species believed to be generated mainly by gene duplication processes. In higher vertebrates the ZP genes are present as single copy loci in the genome in contrast to certain teleost species where there are several copies of the ZP genes probably as a result of gene duplication (Mold et al., 2001; Conner and Hughes, 2003; Kanamori et al., 2003). However, some genes demonstrate a higher divergence than others when comparing closely related species. The majority of these genes are considered to be involved in sexual reproduction processes and in particular the vertebrate ZP genes appear to be under positive Darwian selection (Makalowski and Boguski, 1998; Swanson et al., 2001; Swanson and Vacquier, 2002).

3.2. Gene Organization

Four groups of egg envelope proteins containing the ZP domain have been identified in vertebrates namely ZPA, ZPB, ZPC, and ZPX (Spargo and Hope 2003; Smith et al., 2005). ZPA, ZPB, and ZPC were all identified in mammals, whereas ZPX have been identified in *Xenopus*, chicken, and fish

(Kanamori, 2000; Lindsay et al., 2001, 2002; Smith et al., 2005). Hitherto no ZPA genes have been found in fish. The only vertebrate where all 4 ZP encoding genes have been identified so far is the chicken (Smith et al., 2005).

Teleost fish appears to have multiple copies of ZPB-related genes while the ZP genes are present as single copy loci in the genome of higher vertebrates (Connor and Hughes, 2003). In addition teleost fish appears to have an expanded family of ZPB and ZPC genes (Mold et al., 2001; Kanamori et al., 2003; Smith et al., 2005) possibly due to an evolutionary duplication event.

Several ZP-domain protein-encoding genes have been identified in the teleost medaka (*Oryzias latipes*) (Kanamori, 2000; Lee et al., 2002b). These genes display dual site of expression with expression in the oocyte or the liver. The localization and function of the oocyte-expressed ZP gene products are yet to be established. So far the egg envelope of the medaka has been shown to consist of 3 proteins, choriogenin L (ZPC), choriogenin H (ZPB), and choriogenin H minor (ZPB) (Murata et al., 1995; Sugiyama et al., 1998; Lee et al., 2002b), which are expressed in the liver in response to estrogen (Murata et al., 1994, 1997). The genes encoding ZPBs are linked to the gene coding for ZPC. Several of the other genes encoding ZP proteins are linked together in the medaka genome (Kanamori et al., 2003). The gene organization appears to be somewhat conserved within different teleost species. The ZPB encoding genes of medaka (Kanamori et al., 2003), carp (Chang et al., 1997), winter flounder (*Pseudopleuronectus americanus*) (Lyons et al., 1993) and zebrafish (Mold et al., 2001) have a total of 8 exons where the ZP domain is encoded by 6 of these 8 exons. However, the liver expressed medaka gene encoding ZPB (choriogenin H) have been shown to contain only 7 exons (Lee et al., 2002b). The multiple copies of teleost ZPB genes show high levels of similarity at the DNA level except in the first exon where a repeat domain is present in some species (Connor and Hughes, 2003; Kanamori et al., 2003). This domain is of variable length and is rich in proline, glutamine, and lysine residues. However, this repeat domain is not present in all identified teleost ZPB genes. The zebrafish ZPB genes have probably arisen from a series of gene duplication events since they are clustered together in a head-to-tail pattern in the genome (Mold et al., 2001).

Similar to teleost ZPB genes most ZPC encoding genes have 8 exons, however teleost genes do not contain the transmembrane domain (TMD) present in mammalian genes. The number of exons in the various ZPC genes of medaka varies between 8 and 10 (Lee et al., 2002b; Kanamori et al., 2003). In addition the ZP domain in medaka ZPC is encoded by 7 instead of 6 exons. Carp and medaka ZPC differs in site of expression, where medaka ZPC is expressed in the liver or the oocyte (Murata et al., 1994, 1997; Kanamori et al., 2003) compared to the oocyte expression of carp ZPC (Chang et al., 1996). Thus it is possible that not only ZPB but also ZPC genes are expressed at dual sites in several species of teleost fish.

The medaka ZPX1 is encoded by 21 exons and similar to the ZPB the ZP domain is encoded by 6 of these 21 exons. The TMD present in avian,

amphibian, and mammalian ZPs are lacking from both *Xenopus* and medaka ZPX1 (Lindsay et al., 2001; Kanamori et al., 2003). In addition, the ZPX also lack the trefoil domain found in vertebrate ZPBs. Evidence suggests that there is a ZPX gene present in the human genome, however, the ZP domain encoding part of the gene appears to have been deleted (Smith et al., 2005).

Recently, the genes encoding chicken ZP were characterized and it was discovered that the genes encoding ZPA, ZPB1, ZPB2, ZPC, and ZPX1 were located on chromosomes 14, 5, 6, 10, and 3 within the chicken genome (Smith et al., 2005). In human ZPA, ZPB1, ZPB2, and ZPC are located on chromosome 16, 11, 1, and 7 respectively. The murine genes encoding ZPA, ZPB1, ZPB2, and ZPC are located on chromosomes 7, 19, 13, and 5 in the mouse genome. When analyzing the chromosomal context of the ZP genes, there appear to be conserved synteny between these three different species.

The conservation of the ZP domain in such evolutionary divergent species as teleost fish, amphibians, birds, marsupials (Mate and McCartney, 1998; McCartney and Mate, 1999), and mammals suggests that not only has this protein domain been duplicated in vertebrates but that it has been conserved and used as an egg envelope protein in species that diverged several million years ago.

3.3. Phylogenetic Relationships

Given that oocytes of all vertebrate species are covered by an acellular egg envelope and since the proteins forming this egg envelope share common characteristics between different species it appears that at least one ZP gene must have evolved during the very early stages of vertebrate evolution. This ancestral gene then gave rise to the different ZP genes found in vertebrates today. Recent studies provide support for the theory that the first occurrence in the ZP gene evolution was a gene duplication event (Spargo and Hope, 2003). This duplication may have given rise to both the ancestral ZPC gene and the precursor of the ZPA, ZPB, and ZPX subfamilies (Figure 2). This precursor was then duplicated at least three times during evolution, thus giving rise to the ancestral ZPX, ZPA, and ZPB genes. These duplications events took place early during vertebrate evolution, almost certainly before evolution of the first amphibians since every vertebrate lineage has ZP genes. Subsequently, duplication events have taken place in several lineages, with the most notable occurring early during evolution of the amniotes and giving rise to the ZPB1 and ZPB2 groups within the ZPB subfamily. Human, mouse, chicken, and *Xenopus* ZPA are orthologues, but so far no ZPA gene has been found in fish although phylogenetic data implies that it was once present but may have been lost through deletion events (Spargo and Hope, 2003).

Human, mouse, and chicken ZPB1 and ZPB2 are all orthologues judging from conserved synteny and the levels of homology (Smith et al., 2005). Recent findings indicate that the mouse orthologue of the human ZPB2 gene are present in the murine genome but that it does not encode a continuous open

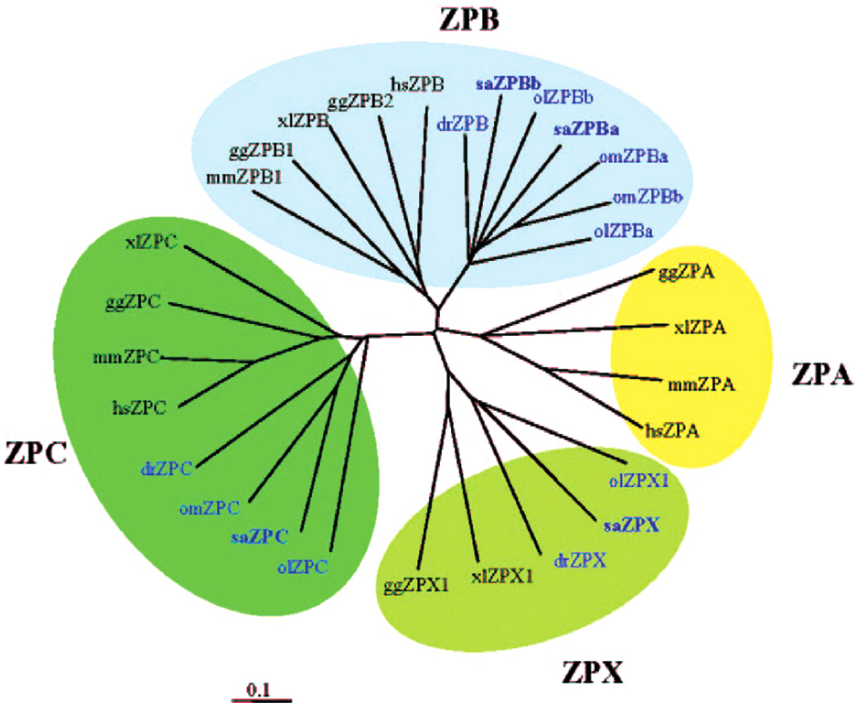

Figure 2. ZP protein sequence similarity analysis displayed in a phylogenetic tree. Clades containing ZPA, ZPB, ZPC, and ZPX are circled. Mouse (*Mus musculus*) mmZPA (nm_011775), mmZPB1 (nm_009580), mmZPC (x14376); human (*Homo sapiens*) hsZPA (m90366), hsZPB (nm_021186), hsZPC (nm_007155); chicken (*Gallus gallus*) ggZPA (xm_424608), ggZPB1 (aj289697), ggZPB2 (ab025428), ggZPC (ab031033), ggZPX1 (aj698915), African clawed frog (*Xenopus laevis*) xlZPA (af038151), xlZPB (u449950), xlZPC (u44952), xlZPX1 (af225906); rainbow trout (*Oncorhynchus mykiss*) omZPBa (af231706), omZPBb (af231707), omZPC (af231708); zebrafish (*Danio rerio*) drZPB (af331968), drZPC (af095457); Japanese medaka (*Oryzias latipes*) olZPBa (af128808), olZPBb(d89609), olZPC (af128813), olZPX1 (af128807); gilthead sea bream (*Sparus aurata*) saZPBa (ay928800), saZPBb (ay928798), saZPC (x93306), saZPX (ay928799). (*See Color Plates*).

reading frame thus making it improbable that a functional protein will be expressed (Lefièvre et al., 2004). The ZPB1 and ZPB2 genes are closely related to each other and may not be present in other species than mammals and birds. It has been suggested that teleost ZPB-homologous genes are not orthologous of the mammalian ZPBs. Instead the teleost genome may contain multiple copies of genes that are equally related to ZPB1 and ZPB2 (Conner and Hughes, 2003). There may have been a duplication event of the ancestral ZPB1/ZPB2 gene in the teleost lineage resulting in 2 paralogous genes. A switch to liver specific expression of one of the resulting genes could then have followed this duplication event thus explaining the dual site of synthesis found within teleost species.

The gene encoding the medaka eggshell ZPC has 8 exons (Lee et al., 2002b), identical to the human (Chamberlin and Dean, 1990) and mouse ZPC gene (Kinloch et al., 1988). A gene orthologous to the medaka ZPC has been found in Fugu (Kanamori et al., 2003) displaying identical exon-intron organization.

The ZPX genes are present in lower vertebrates such as amphibians, fish, and birds, but they are not present in mammals (Smith et al., 2005). It has been suggested that the ZPX genes was lost through evolutionary mutations in these rapidly evolving genomes. The recent findings of new orthologues and paralogues among the different ZP genes in various vertebrate species indicate a higher degree of complexity within the ZP gene family than what was perhaps previously anticipated.

3.4. Zona Pellucida Proteins

The proteins forming the egg envelope in vertebrates are glycoproteins that were first identified using SDS-PAGE analysis of egg envelope components. The mouse eggshell consists of 3 main ZP proteins (Wassarman et al., 1999) and it has been accepted as a model for the eggshell of higher vertebrates. However recent studies have shown that there are 4 ZP glycoproteins expressed in humans (ZPA, ZPB1, ZPB2, and ZPC) and that all 4 proteins can be found in the eggshell (Hughes and Barrat, 1999; Lefièvre et al., 2004).

It has been shown that the polypeptide primary structures of ZP proteins are similar to such an extent that human ZPs may replace mouse ZP counterparts as components of the mouse egg envelope (Rankin et al., 1998). Perhaps even more intriguing is the fact that mouse ZPs appear to be sufficiently conserved throughout evolution to be incorporated into the *Xenopus* egg envelope (Doren et al., 1999).

The egg envelope in most teleost species is a thick structure consisting of proteins and glycoproteins (Cotelli et al., 1988). The structural complexity and macromolecular composition differ from species to species. Thus, eggshell probably reflects adaptations to different ecological conditions under which the egg develops (Stehr and Hawkes, 1979). The major molecular constituents of the egg envelope in teleost fish are 3–4 proteins with molecular masses between 47 and 129 kDa (Begovac et al., 1989; Hamazaki et al., 1985, 1989; Oppen-Berntsen et al., 1990; Brivio et al., 1991; Masuda et al., 1991; Hyllner et al., 1991, 1994, 1995). The relationship of the ZPs to other proteins is not clear, but evidence suggests that the ZPs belong to a separate class of structural proteins (Hagenmeier, 1985; Hyllner et al., 2001). All ZP proteins contain a ZP domain that can also be found in many components of the extracellular matrix and membrane proteins (reviewed in Sutton et al., 2002).

3.5. Protein Structure

The protein structure of the different vertebrate ZPs is very similar. Nearly all vertebrate ZP proteins have an N-terminal signal peptide and a TMD at their

C-terminus. In between these regions there is a conserved ZP domain (Bork and Sander, 1992). In several species the ZP domain in ZPB are preceded by a so-called trefoil domain, rich in cysteins (Bork, 1993). In addition, some teleost species have a proline- and glutamine-rich repetitive domain in the N-terminal part of the protein (Hyllner et al., 2001; Lee et al., 2002b; Kanamori et al., 2003). The length and number of repeats varies between different species and the function of these repeats has not yet been revealed. However, there are theories that these proline- and glutamine-rich repeats are involved in the hardening of the eggshell at fertilization. It has been shown that components of the hatching enzyme in medaka release proline-rich polypeptides from the eggshell (Lee et al., 1994). It is thus probable that these N-terminal repetitive domains may function as a substrate for the hatching enzyme in some teleost species.

The ZP domain is a common feature shared by different filament or matrix forming proteins probably reflecting its importance in protein polymerization. Sequences corresponding to this domain can also be found in invertebrates (Chung et al., 2001; Jazwinska et al., 2003; Roch et al., 2003) suggesting an evolutionary ancient origin of the ZP domain. The ZP domain consists of about 260 amino acid residues and it contains 10–12 conserved cysteine residues (Bork and Sander, 1992). In a study on rainbow trout (*Oncorhynchus mykiss*) for ZP proteins, it was recently shown that the cysteines are involed in intramolecular disulfide bridge formation (Darie et al., 2004). While all ZPA and ZPB proteins contain 10 cysteines in the ZP domain there are an additional 2 cysteines in teleost ZPB genes. These 2 cysteines (Cx and Cy) form one disulfide bond. Interestingly, the teleost ZPX genes, that closest match the ZPA genes, do not contain the Cx and Cy cysteins. In addition, the ZP domain displays conservation between species regarding hydrophobicity, polarity, and turn-forming tendency, indicating a common three-dimensional structure. Although the function of this domain is largely unclear, a recent study has demonstrated its importance in polymerization of ZP glycoproteins (Jovine et al., 2002). It has been shown that the C-terminal TMD and the short cytoplasmatic tail of the mouse ZPA and ZPC are important for assembly of the egg envelope in mice. They are however not required for secretion of mouse ZPA and ZPC. The fact that the majority of teleost ZP proteins are synthesized in the liver, and subsequently transported via the bloodstream to the growing oocyte may explain the absence of the TMD in the ZP proteins of these species.

3.6. Protein Assembly and Function

Formation of the teleost egg envelope (zonagenesis) starts during oocyte growth. The ZP matrix formation is initiated during the previtellogenic stage of the oocyte (Anderson, 1967; Tesoriero, 1977; Abraham et al., 1984; Rizzo and Bazzoli, 1991; Cruz-Höfling and Cruz-Landim, 1993; Andrade et al., 2001; Fausto et al., 2004). The plasma membrane of the oocyte forms microvilli, which stretch towards the surrounding granulosa cells. The synthesis of the egg envelope starts at the base

of these microvilli (Tesoriero, 1977). The ZP precursor proteins are synthesized, modified with oligosaccharides, secreted, and assembled into crosslinked filaments that exhibit a structural repeat (Wassarman et al., 2004a). Nascent ZP glycoproteins are incorporated into secretory vesicles that will fuse with the oocyte plasma membrane, and the nascent ZP glycoproteins will be deposited into the innermost layer of the thickening ZP.

It has been postulated that the mammalian ZP matrix is composed of heterodimers consisting of 2 out of the 3 major glycoproteins, ZPA and ZPC, while the third, ZPB, functions as a crosslinker between the filaments (Greve and Wassarman, 1985; Wassarman, 1988; Wassarman et al., 1999, 2004a). We recently analyzed the composition of the zebrafish eggshell and found that there are 2 ZPB proteins present for each ZPC protein. In addition there are ZPX protein present in the egg shell and the relative ration of ZPB:ZPC:ZPX is 8:4:1 (Modig et al., 2006).

The nascent mammalian precursor ZP protein consists of an N-terminal signal sequence, a ZP domain and a furin cleavage site followed by a hydrophobic C-terminal (Wassarman et al., 2004a). The C-terminal propeptide contains a transmembrane region (TMD) and a short cytoplasmatic tail. The N-terminal signal sequence directs the ZP protein to the endoplasmatic reticulum and is then cleaved off. The ZP precursor proteins are subsequently modified with N- and O-linked oligosaccharides. Before the ZP proteins are incorporated into the egg envelope, the propeptide is proteolytically processed at the conserved furin cleavage sites (Qi et al., 2002). In mammals the consensus for the furin cleavage site in ZP proteins is R-X-K/R-R (Jovine et al., 2002), while in teleosts the consensus site appears to be K/R-X_n-K/R (Darie et al., 2004). Studies suggest that this proteolytical processing is necessary for enabling the secretion of the ZP proteins (Williams and Wassarman, 2001; Qi et al., 2002). Nascent mammalian ZP glycoproteins are subsequently incorporated into secretory vesicles. These vesicles will then fuse with the egg plasma membrane and the nascent ZP glycoproteins will be deposited into a three-dimensional extracellular matrix around the growing oocyte. The liver-expressed teleost ZP glycoproteins will be secreted and transported in the blood stream to the ovary where they are incorporated into the inner layer of the thickening eggshell.

The ZP domain present in vertebrate ZPs is believed to be involved in the assembly of the egg envelope. Recent studies show that the TMD is essential for C-terminal processing of the ZPs and suggests that the filaments of the different ZP-domain proteins may share a common three-dimensional architecture (Jovine et al., 2002, 2004).

As in mammals, the rainbow trout N-terminal signal sequence is cleaved off, resulting in Q23 (ZPBa), Q21 (ZPBb), and Q23 (ZPC) being the N-terminal amino acid in mature ZP protein (Darie et al., 2004). In mammals the C-terminal TMD anchors the ZP proteins in the membrane where the assembly takes place. While the teleost ZP proteins lack a TMD, they have other sequences in common with mammalian ZP proteins. Thus, in front of the C-terminal sequence there is a

conserved furin-like cleavage site in all ZP proteins. In the C-terminal sequence of fish ZP proteins there is an external hydrophobic patch (EHP) present similar to that of mammalian ZP proteins. It has been suggested that the EHP interacts with an internal hydrophobic patch (IHP) and that polymerization of ZP proteins require that the C-terminal sequence is cleaved of thereby removing the interaction between EHP and IHP and allowing IHP to form intermolecular bridges (Jovine et al., 2004). The conservation of these interacting regions with the ZP proteins may indicate that the process forming the vertebrate eggshell is similar in teleosts and mammals even though teleost ZP proteins lack the TMDs present in both the mammalian and amphibian counterparts. A schematic representation of teleost ZPB, ZPC, and ZPX proteins and their conserved domains are shown in Figure 3.

The ZPC have been implicated to function as the primary sperm receptor in mice (Bleil and Wassarman, 1980; Florman and Wassarman, 1985; Ringuette et al., 1986). The human ZPA have been shown to be important for secondary sperm binding and the penetration of the sperm into the ZP (Tsubamoto et al., 1999). In *Xenopus* ZPA (gp69/64) has been shown to bind sperm (Tian et al., 1997, 1999).

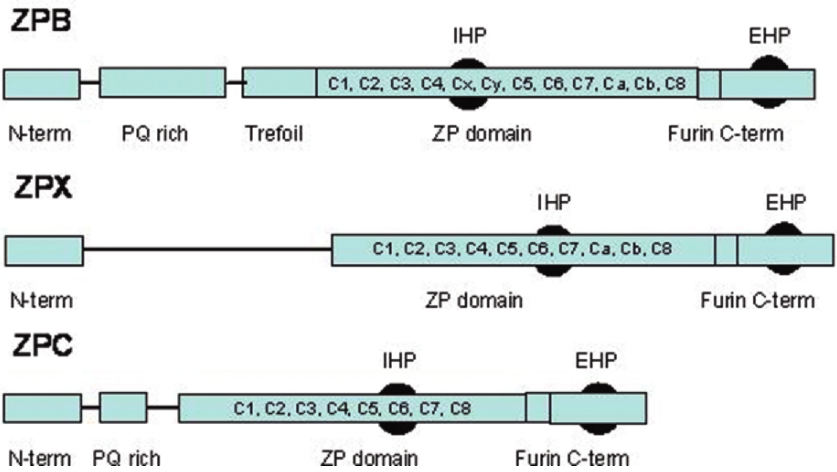

Figure 3. Schematic representation of the teleost ZP protein domains. All ZP proteins contain an N-terminal signal sequence that is cleaved of in the mature protein. There is a PQ rich domain in ZPB and ZPC proteins that appear to be lacking in ZPX proteins. The ZP domain contains 8–12 conserved cysteins. All ZP contain C1–8 while ZPB and ZPX contain an additional 2 cysteins Ca and Cb, and these are in common with mammalian ZP proteins. In the teleost ZPB proteins there are 2 additional cysteins (Cx and Cy) that are not present in mammalian ZPA or ZPB or in teleost ZPX. The cysteins create intramolecular sulphydryl bridges. Downstream of the ZP domain, there is a furin-like cleavage site in all ZP proteins followed by a C-terminal domain that is cleaved off in the mature protein. In addition to this there are two hydrophobic patches, one internal (IHP) and one located in the C-terminal domain (external; EHP). The IHP sequence has been proposed to participate in polymerization of ZP proteins. (*See Color Plates*).

The roles of the individual eggshell components in the fertilization event of teleost eggs are not as well established as in other vertebrate species. In teleost fertilization the sperm gains entry to the oocyte through the micropyle, thus the need for sperm binding and acrosome reaction is diminished. Consequently, it appears that the ZP have more of a structural role in teleost species when compared to the mammalian situation.

4. REGULATION OF ZP EXPRESSION

The regulation of ZP genes varies among different vertebrates, probably reflecting the differences in oogenesis, fertilization mechanisms, and embryonic development between species. The formation of fertilizable oocyte is a process requiring coordination between several different factors and cell types. Mammals and fish display differences in the transcriptional and translational regulation of eggshell components. Furthermore the sites of synthesis of eggshell components vary from species to species. In the majority of vertebrate species the ZP genes are exclusively transcribed in the developing oocyte. However, among birds and fish there are exceptions, with species displaying expression of ZP genes in the liver and granulosa cells (Figure 4). Teleosts produce a large number of eggs during their reproductive cycle and although the size of the eggs may differ considerably between different species still most fish species have to produce a large amount of ZP proteins during that period. Depending on the teleost species the specific regulatory processes governing the synthesis of these proteins may differ. However, in contrast to the oocyte expression of ZPs in mammalian species, where the synthesis of different components of the eggshell is regulated on a transcriptional level, the regulation of the liver expression of ZPs in teleost species appears to be under hormonal control.

4.1. Regulation of Mammalian and Teleost ZP Proteins

In mammals the transparent ZP matrix consists of 3–4 major glycoproteins (Greve and Wassarman, 1985; Wassarman, 1988; Wassarman et al., 2004a; Lefièvre et al., 2004). The formation of the ZP matrix indicates the beginning of follicle growth (Wolgemuth et al., 1984). The germ cells maintain their contact with the somatic cells through cellular processes, which cross the ZP to form gap junctions between the two cell types. In mouse, the expression of the ZP genes is restricted to the oocyte where the proteins are posttranslationally modified (Bleil and Wasserman 1980; Greve et al., 1982; Shimizu et al., 1983; Liang et al., 1990, 1997; Litscher et al., 1999; Dean, 2004). ZP expression in mice is restricted to a growth period of 2–3 weeks, during which the ZP matrix grow to about 6.5 μm in width (~3 ng protein) (reviewed by Wasserman et al., 2004b). There is a coordinated expression of the ZP genes, and ZP transcripts in mouse have been detected as early as embryonic day 18 (E18) (Epifano et al., 1995b; Dean, 2004). The ZP proteins are modified to sulfated glycoproteins within the oocyte and are secreted

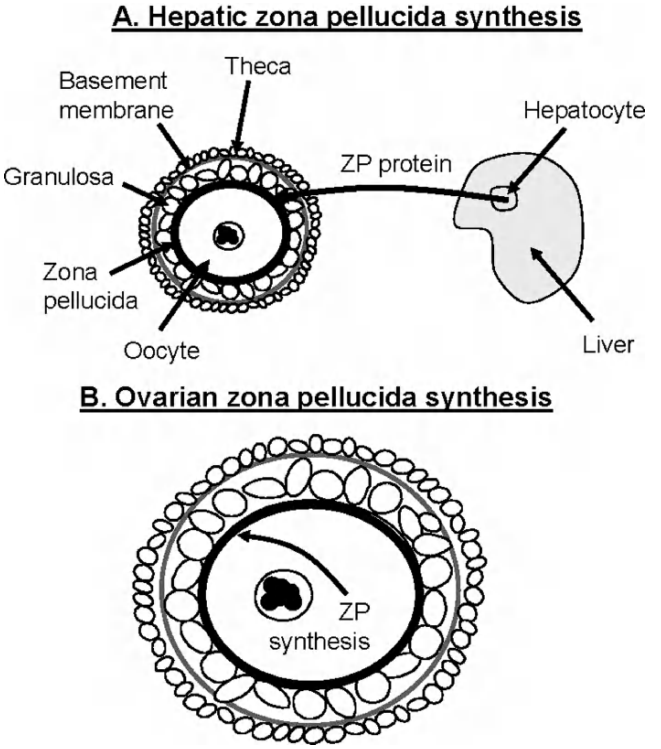

Figure 4. A schematic description of the sites of synthesis of ZP protein in fish. ZP proteins are synthesized in liver and oocytes. In some instances synthesis occur in both locations. (A) In many teleost species, including the gilthead sea bream, the zona pellucida protein are synthesized in the hepatocytes in the liver and thereafter transported to the oocyte where they are incorporated into the ZP. (B) ZP proteins are exclusively synthesized in the oocytes of zebrafish. In rainbow trout, ZPC is synthesized in both the liver and oocyte. This is also the case for ZPBa and ZPX in the gilthead sea bream.

to the surface were the 120–140 (ZPA), 180–200 (ZPB), respectively 83 kDa (ZPC) proteins are assembled into a matrix (Shimizu et al., 1983; Dean, 2004).

A regulatory element, a so-called E-box (CANNTG), that binds basic helix-loop-helix (bHLH) transcription factors (Murre et al., 1989) is located upstream of the transcription start site and is involved in the regulation of all 3 major mouse ZPs (Liang et al., 1990, 1997; Epifano et al., 1995a). The protein "Factor in the germline alpha" (FIGα), is a germ cell-specific bHLH transcription factor implicated in postnatal oocyte-specific gene expression and has been shown to bind as a heterodimer with the ubiquitous transcription factor E12 to the E-box (Liang et al., 1997). FIGα can be detected as early as mouse embryonic day 13 (Soyal et al., 2000). In human FIGα is expressed in ovarian follicles and is detected in oocytes up to mature metaphase II stage, while little or no expression is detected in preimplant embryos (Huntriss et al., 2002).

Mouse lines lacking FIGα have been established and although embryonic gonadogenesis appears normal, primordial follicles are not formed and the massive depletion of oocytes results in shrunken ovaries and female sterility (Soyal et al., 2000). Furthermore FIGα null females do not express ZPA, ZPB, or ZPC. However, although the presence of FIGα is necessary for ZP gene expression it is not sufficient to bring about the expression of these proteins *in vivo* (Liang et al., 1997). Thus, FIGα seems to be important for the coordinated expression of the ZP genes in mammals whereas other factors are needed for activation of the ZP genes.

The synthesis of ZP proteins in fish occurs in the liver (Chang et al., 1996), or in the ovary (Chang et al., 1997), or in both (Hyllner et al., 2001). It was recently proposed that the dual site of ZP synthesis is the result of a polyploidization event during evolution (Conner and Hughes, 2003). Liver expression of ZP proteins is not restricted to fish but has also been shown in chicken (Bausek et al., 2000). In several teleost species the synthesis of ZPs takes place in the liver (Hamazaki et al., 1985, 1989; Oppen-Berntsen et al., 1992a, b; Lyons et al., 1993; Shimizu et al., 1998,) under the control of 17β-estradiol (Hamazaki et al., 1987; Hyllner et al., 1991; Westerlund et al., 2001; Berg et al., 2004). Precursors to ZP proteins (pZP) are induced in the liver by estrogen and are transported into the ovary via the bloodstream. ZP precursors in the serum have been measured in estrogen injected Sakhalin taimen (*Hucho perryi*) and masu salmon (*Oncorhynchus masou*) (Shimizu et al., 2000; Fujita et al., 2004). In addition 17β-estradiol have proven to be an effective inducer of ZP protein expression in male and juvenile fish of several different teleost species (Hyllner et al., 1991; Murata et al., 1991; Larsson et al., 1994, 2002; Westerlund et al., 2001; Berg et al., 2004) in much the same manner as it induce vitellogenin (Vtg).

While estrogen regulation of ZP is widespread among teleosts, it was shown that the estrogenic response could be altered by cortisol in Arctic char (*Salvelinus alpinus*) (Berg et al., 2004). The known stress hormone, cortisol, was potentiating the estrogen-dependent ZP induction. This is however not general in fish as neither gilthead sea bream nor zebrafish ZP expression is altered by cortisol (Modig et al., 2006). Although estrogens appear to be a major inducing factor for teleost ZP expression, androgens have been suggested to regulate ZPs in fish (Japanese eel, *Anguilla japonica*) and birds (Japanese quail, *Coturnix japonica*) (Miura et al., 1998; Pan et al., 2001). In cell cultures testosterone induce high ZPC mRNA expression in quail, while 11-ketotestosterone suppressed ZP mRNA transcriptions in eel.

The site of ZP synthesis varies among species and between different ZPs in the same fish. In addition, the dual expression of the rainbow trout ZPC (Hyllner et al., 2001) and gilthead sea bream ZPBa and ZPX (Modig et al., 2006) in both liver and ovary adds to the complexity. The expression of ZPs in ovaries has been shown in carp and zebrafish (Chang et al., 1997; Wang and Gong, 1999; Del Giacco et al., 2000; Mold et al., 2001). Like mammalian ZPs, both zebrafish ZPB and ZPC have potential phosphorylation sites, but

compared to mammalian ZPs zebrafish ZPB and C contain fewer glycosylation sites (Wang and Gong, 1999). The reason and mechanisms for synthesizing egg envelope components in two different sites are not known. It is difficult to compare different teleost since they have different spawning seasons, spawning frequency, reproductive strategy, number of eggs, and thickness of the envelope, as well as that the natural seasonal cycle of sex-steroids in the fish vary, but this may also account for the differences in expression site and regulation observed for fish ZPs. Therefore, the characterization of ZP gene promoters from species with different spawning strategies and dual expression patterns are of importance in order to understand the regulation of ZPs.

Not all fish species have ZP proteins that are regulated by estrogen receptors. E-boxes have been identified in the ZP promoters of winter flounder (Lyons et al., 1993), carp (Chang et al., 1997), zebrafish (Mold et al., 2001; Onichtchouk et al., 2003), and medaka (Kanamori et al., 2003). Recently, a cluster of 3 genes coding for ZPB was isolated in zebrafish, these genes were expressed in the ovary of female zebrafish (Mold et al., 2001). The presence of E-boxes was shown for 2 of the 3 ZPB promoters. The expression of the zebrafish ZPB mRNA varied during the reproductive cycle, with a high expression at 2–3 h and 1 day post-ovulation (Mold et al., 2001). The presence of FIG-α in medaka (Kanamori, 2000) suggests that the regulatory pathways for ZP expression may be conserved between mammals and fish. FIGα can be induced in male juvenile medaka exposed to 17α-ethynylestradiol, and the normal expression of FIGα in juvenile females was reduced by androgen exposure (Scholz et al., 2003) indicating that estrogen regulates FIGα in medaka. Surprisingly, none of the zebrafish ZPB promoters contained estrogen responsive elements (EREs) (Mold et al., 2001) and are insensitive to estrogen induction (Liu et al., 2006), thus implicating that the estrogen/ER complex may not be directly regulating zebrafish ZPB expression. This was confirmed in a recent study, showing that estrogens did not regulate ZPB or ZPC in zebrafish (Modig et al., 2006). In this study cortisol and testosterone were also unable to affect ZP mRNA levels in the ovaries. Thus, estrogenic control of ZP synthesis is not a universal model for fish. While liver expression appears to be regulated by estrogen, ovarian expression can either be under estrogenic control, directly or via FIGα (as observed for medaka) or independent of estrogen as seen for zebrafish (Table 1). Apart from estrogens, other hormones such as cortisol and androgens, have been shown to alter estrogen-dependent regulation of ZP proteins in some species.

5. ZONA PELLUCIDA PROTEINS AS BIOMARKERS IN ENVIRONMENTAL TOXICOLOGY

Several recent reports have indicated the ability of estrogenic compounds, present in the environment, to disrupt different parameters involved in reproduction (Panter et al., 1998; Larsson et al., 1999; Folmar et al., 2001). Salmonid species

Table 1. Regulation and site of expression of fish ZP proteins

Species	Accession number	Name	Site of expression	Regulation	Reference
Gilthead sea bream (*Sparus aurata*)	AY928800 AY928799 AY928798	ZPBa ZPX ZPBb	Liver, Ovary Liver, Ovary Liver	E_2	Modig et al. (2006)
	CAA63709	gp49 (ZP3, ZPC)	Liver	E_2	Del Giacco et al. (1998)
Rainbow trout (*Oncorhynchus mykiss*)	AF407574	Zona radiata protein	Liver	E_2	Arukwe et al. (2002)
	AF231706 AF231707 AF231708	VEPα (ZPBa) VEPβ (ZPBb) VEPγ (ZPC)	Liver Liver Liver, Ovary	E_2	Hyllner et al. (2001)
Arctic char (*Salvelinus alpinus*)	AY426715 AY426716 AY426717	ZPα (ZPBa) ZPβ (ZPBb) ZPγ (ZPC)	Liver	E_2 and E_2 + F	Berg et al. (2004)
Japanese medaka (*Oryzias latipes*)	BAA13994	Choriogenin H (ZPB)	Liver	E_2	Murata et al. (1997)
Regulation mediated through FIGα	BAA76901	Choriogenin H minor (ZPB)	Liver		Sugiyama et al. (1998)
	AAM47575	Choriogenin L (ZPC)	Liver	EE_2	Lee et al. (2002)
	AAD38905	ZPB-domain containing protein	Ovary	EE_2[1]	Kanamori (2000)
	AAN31186 AAN31187 AAN 31188-92	ZPAX ZPB ZPC1-5	Ovary	EE_2[1]	Kanamori et al. (2003)
Zebrafish (*Danio rerio*)	NP571405 NP571406	ZP2 (ZPA) ZP3 (ZPC)	Ovary	Not E_2 regulated[2]	Wang and Gong (1999)
Carp (*Cyprinus carpio*)	CAA96572 -5 L41639	ZP2 ZP3	Ovary		Chang et al. (1996, 1997)
Goldfish (*Carassius auratus*)	CAA96576 AAB41819	ZP2 ZP3A	Ovary		Chang et al. (1996, 1997)
Japanese eel (*Anguilla japonica*)	AB016041 AB016042	eSRS3 (ZP2) eSRS4 (ZP3)	Ovary	11-KT ↓ (male)	Miura et al. (1998)

[1]Scholz et al. (2003); [2]Modig et al. (2006)

E_2, 17β-estradiol; EE_2, 17α-ethinylestradiol; 11-KT, 11-ketotestosterone.

have been used as sentinels for the presence of estrogenic substances in water. The induction of Vtg is predominantly used as a biomarker, although recently the use of ZP proteins has been suggested as an alternative indicator for estrogenic endocrine-disrupting substances (Arukwe et al., 1997, 2001, 2002; Celius and Walther, 1998; Celius et al., 1999; Oppen-Berntsen et al., 1999).

Studies have shown that the expression of ZPs precedes that of Vtg, thus implicating a higher sensitivity for estrogenic induction (Westerlund et al., 2001). Both Vtg and ZPs are transported in the circulatory system to the ovary of several teleost species, thus being readily detected in blood plasma. Although the regulation of ZP genes to a large extent is unknown, the ZPs have recently been implicated as sensitive biomarker of endocrine disruption in fish, for example swordfish, bluefin tuna, rainbow trout, and medaka (Desantis et al., 2005; Fossi et al., 2004; Thomas-Jones et al., 2003; Lee et al., 2002a, b).

17β-estradiol has been shown to rapidly induce the expression of ZPs. Already after 6 h there is a significant increase in all 3 major ZPs mRNAs in liver of Arctic char (Westerlund et al., 2001). This rapid induction has lead to the usage of ZP proteins as biomarkers of estrogenic exposure, arguing for a higher sensitivity than V (Arukwe et al., 1997; Celius and Walther, 1998; Celius et al., 2000). However, several aspects of ZP protein regulation suggest that these proteins may not be ideal as indicators of estrogenic exposure. The basal level expression of ZPs has been implicated to be higher than that of Vtg. In juvenile Arctic, char the basal level expression of ZPBb was readily detected both on mRNA and protein level (Westerlund et al., 2001). In adult fish the ZP mRNAs were readily detected, even though the level of 17β-estradiol was relatively low. In addition, the ZPBb expression was shown to be highly variable and independent of the sex of juvenile fish. The ZP mRNA expression was restricted to liver of female rainbow trout with the exception of ZPC, which was also detected in the ovary. Interesting to note is the expression of all 3 ZP mRNAs in male liver (Hyllner et al., 2001). The expression of ZPB mRNA and protein has been shown for juvenile fish, independent of sex or body weight. The mechanism behind this early and sex-independent expression is not known.

In an attempt to evaluate the value of ZP as a biomarker for endocrine disruption there are several parameters to take into consideration. First, stress and cortisol have been shown to potentiate estrogen-induced ZP expression in Arctic char (Berg et al., 2004), making it difficult to draw a correlation between amount of endocrine disrupting pollutions and synthesized ZP proteins at least in salmonid species. Second, it has been shown in some species, such as Arctic char and eelpout (*Zoarces viviparus*), that the water temperature reflects on ZP expression in the fish. High temperature results in lower levels of 17β-estradiol in Arctic char and male eelpout and therefore the expression of ZP is reduced under these conditions (Larsson et al., 2002; Berg et al., 2004). To make the scene more complex, androgens have also been suggested as ZP regulators in fish (Miura et al., 1998). Furthermore, in fish where ZPs are expressed in the oocyte (pipefish, (*Syngnathus fuscus*), Begovac and Wallace, 1989; carp, Chang et al., 1997;

zebrafish, Islinger et al., 2003), no induction or very low induction of ZP expression in response to estrogens have been observed. Recently it was shown that ZP expression in female zebrafish was not effected of 17β-estradiol, cortisol, or testosteron (Modig et al., 2006), however, a slight increase of ZPB mRNA expression has been shown in testis after 17 days exposure to 17α-ethinylestradiol (Islinger et al., 2003). Taken together these studies indicate that this species is not suitable as an endocrine-disrupting model animal, despite a one-point measurement of elevated ZP expression in male zebrafish.

6. EGGSHELL FUNCTION

The vitelline envelope or eggshell has several physiological functions that are important for both the fertilization of the oocyte and the protection of the growing embryo. In addition to providing mechanical protection to the developing embryo, the eggshell also allows for embryonic respiration. In this section we will describe the proposed functions of the ZP proteins with respect to their antimicrobial properties and their involvement in the fertilization event.

6.1. Antimicrobial Properties of ZP Proteins

There is evidence of the vitelline envelope having bactericidal properties, thereby protecting the egg from bacterial pathogens (Kudo and Inoue, 1989; Kudo, 1992, 2000). Following fertilization the vitelline envelope is transformed into the fertilization envelope (FE). The FE hardens and becomes resistant to various drugs, enzymes, fungus, and bacteria. It has been shown that the FE has bactericidal properties and is effective against both Gram-positive and Gram-negative bacteria (Kudo, 2000). Extracts from FE have been shown to be effective against bacteria, such as *Aeromonas hydophilia* and *Vibrio anguillarum*. FE also protect against fungus as demonstrated by the fungicidal activity of FE extracts on *Saprolegnia parasitica*. The bactericidal effect have been suggested to be associated with the presence of phospholipase D, lysozyme, proteinases, and DNases present in the FE (Kudo, 2000).

6.2. Fertilization and ZP Proteins in Mammals and Teleosts

The eggshell is also involved in different fertilization event, such as sperm binding and sperm guidance (Yanagimachi, 1992). In contrast to mammals, the sperm of teleost fish does not have to penetrate the egg envelope in order to reach the cell membrane and fertilize the egg. The teleost egg has a funnel-shaped micropyle, which traverses the egg envelope at the animal pole (Nagahama, 1983). Fertilization takes place when a single sperm travels through the micropyle and reaches the egg cell. The micropyle is only wide enough to let one sperm reach the egg. When the egg is activated by the sperm, the micropyle closes. In salmonids a process called water activation takes place mediated through a change in the egg

envelope (Blaxter, 1969), which brings about the closure of the micropyle. Following activation or fertilization the egg absorbs water, the perivitelline space is formed and the egg envelope hardens. Studies have shown that the permeability of fertilized eggs in Atlantic salmon (*Salmo salar*) is high during water hardening. After that permeability is low up until the eyed stage (Blaxter, 1969).

Molecules present on the surface of sperms and eggs have been suggested to be responsible for species-specific interactions between the gamets. The ZPC have been implicated to function as a sperm receptor in mice (Bleil and Wassarman, 1980; Florman and Wassarman, 1985; Ringuette et al., 1986). In mammalian ZPC two serine residues are conserved (Ser-332 and Ser-334) and these serine residues are proven to be involved in sperm binding (Chen et al., 1998). However, these two serine residues are not conserved in teleost ZPC (Hyllner et al., 2001), possibly reflecting the differences between mammalian and teleost fertilization. The sperm receptor associated functions of the ZPC in mice may include gamete adhesion and activation of the acrosome reaction (Wassarman, 1990). In teleost fish the sperm travels through the micropyle in order to reach the egg plasma membrane and subsequently fertilize the egg. Thus, the sperm-binding ability of mammalian ZPA and ZPC need not be conserved in teleost species. However, in *Xenopus* the sperm binding is implicated to depend upon the oligosaccharide chains of the envelope protein ZPA (gp69/gp64) (Tian et al., 1997), which is a homologue of the mammalian ZPA (Tian et al., 1999). The homologous molecules of mouse ZPC have also been identified in lower vertebrates such as amphibians (*Xenopus laevis*) gp43/ 41 (Kubo et al., 1997; Yang and Hedrick, 1997) and medaka choriogenin L (Murata et al., 1995).

In Pacific herring it has been shown that a component of the vitelline envelope is responsible for the activation of sperm motility in this species (Pillai et al., 1993; Griffin et al., 1996). Recently it was shown that this component, named sperm motility initiation factor (SMIF), induced calcium influx, sodium efflux and membrane depolarization in herring sperm (Vines et al., 2002). Sperm motility was shown to be dependent on the extracellular sodium levels and could be activated in the absence of SMIF in low-sodium water. SMIF is a 105 kDa basic glycoprotein localized to the micropyle region of the oocyte. Thus, in herring the sperm require direct contact with the micropylar region of the oocyte in order to become motile. The SMIF gene remains to be characterized.

7. SUMMARY AND FUTURE PROSPECTS

In the present review we have described the present knowledge of the regulation and function of ZP proteins in teleosts. While a multitude of novel information has been added lately, there still remain several questions that should be addressed in the future. While there is ample data on estrogen regulation of teleost ZP, it still remains to be shown how FIGα and other unknown

transcription factors are involved in the regulation of ZP in oocytes in species like the zebrafish. The emerging differences in regulation of ZP among different teleost species are also of interest to address in order to better understand mechanisms of envelope synthesis and function. The newly identified ZPX genes that are now found in many teleosts are interesting as their structure is different from ZPB. Also, in light of their low abundancy in the envelope, it would be interesting to know how they participate in the forming of the vitelline envelope, and which functions they confer to the structure. Finally, it remains to be shown how the sperm can locate the micropyle and what molecular signals are involved in this process.

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CHAPTER 6

MATERNAL FACTORS IN FISH OOGENESIS AND EMBRYONIC DEVELOPMENT

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1. INTRODUCTION

Maternal factors are essential for early animal development, including fish species. The teleost fish *Danio rerio*, commonly known as zebrafish, has become a valuable model system to study fish (and vertebrate) biological processes due to its potential to combine genetic, embryological and molecular methods. This chapter attempts to summarize current knowledge on the role of maternal factors in this organism, although studies on the subject carried out in other fish species, when available, are also included.

As the name implies, maternal genes are defined as those required in the adult female for the development of the offspring. This dependency on maternal genes derives from the fact that the earliest steps in embryonic development are driven by factors produced during oogenesis, which are stored in the form of mRNA, protein, or any other biomolecule. Upon egg activation and fertilization, such factors become usable for embryogenesis, sometimes after a process of activation involving translation or protein modification. Fish, as most other animals, have a substantial period of time in embryonic development which occurs prior to the activation of the zygote's own genes. The initiation of the zygotic gene program normally occurs during the blastula stages, at the so-called midblastula transition (MBT), and it appears to be precisely a new set of (zygotically derived) regulators that allow the embryo to proceed to later stages and undergo the cell-specific programs characteristic of gastrulation. Prior to the MBT, the entire developmental program depends solely on maternal products. The MBT constitutes a period of time in which the importance of maternal products gradually subsides and zygotic gene products become the primary factors driving development. However, a role for maternal products, often in conjunction with zygotic products derived from the same gene, has also been demonstrated in the zebrafish for a variety of developmental processes that occur after the MBT.

Much of this chapter relates information we have learned from the analysis of genetic mutants affecting early development. The function of such genes can be deduced when the effect (phenotype) in the offspring depends on the genetic constitution (genotype) of the mother. In the case of strictly maternal-effect

genes, the phenotype depends solely on the genotype of the mother and is independent of the genotype of the embryo itself. In the case of maternal–zygotic genes, the function of the gene depends on gene products derived both maternally and zygotically. In this case, the phenotype of the embryo depends on the genotype of both the mother and the embryo. For simplicity, we refer to the affected embryos, which are derived from adult females that are homozygous mutant (and, in the case of maternal–zygotic genes, also homozygous mutant in the zygote), as mutant embryos.

In many cases the function of maternal factors in the developing embryo depends on a precise plan of storage and localization that is initiated during oogenesis. Therefore, we will also discuss maternally driven processes required for embryogenesis in the context of their expression during oogenesis.

2. PRODUCTION AND ACCUMULATION OF MATERNAL PRODUCTS DURING OOGENESIS

2.1. Oogenesis and the Animal–Vegetal Pattern of Polarity

Most maternal products appear to be produced endogenously by the oocyte during its development (see Chapters 1–3), although some products are taken up by the oocyte from the surrounding tissue (see Chapters 1 and 2). Oocyte growth is massive, as the oocyte increases a few hundred fold in volume, and shows increases in RNA and protein content of several orders of magnitude (Chaudhuri and Mandal, 1980; Selman et al., 1993). At the same time, the oocyte differentiates along the animal–vegetal axis. These events are discussed below for the zebrafish oocyte, according to the staging by Selman et al. (1993). Other fish have a similar overall pattern of development, although some differences are notable. For example, there is a greater role for hydration and yolk protein processing in marine species during oocyte maturation. Similarly, lipid droplets are present in the oocytes of other fish species, particularly many teleosts, but are lacking in the zebrafish, although lipids are contained within the zebrafish yolk (Malone and Hisaoka, 1963). A general analysis of fish oogenesis can be found elsewhere (Guraya, 1969; Selman and Wallace, 1989; Wallace and Selman, 1990) (see also Chapter 1). The stages of oogenesis in the zebrafish are as follows (see also Figure 1):

2.1.1. Primary growth stage (Stage I)

During this stage, oocytes begin to produce maternal products and grow in size. They also initiate meiosis and arrest during prophase I (Hisaoka and Firlit, 1962). Initially (prefollicle phase, stage 1A, oocyte diameter 7–20 μm) the oocytes lie together in a space lined by a single layer of somatic (prefollicular) cells and begin to undergo chromosome condensation characteristic of the first meiosis. At the same time, nucleoli appear in the nucleus, or germinal vesicle, of the oocyte, and these nucleoli begin to accumulate both in the periphery and inner membrane of the nucleus. These nucleoli are thought to be involved in the

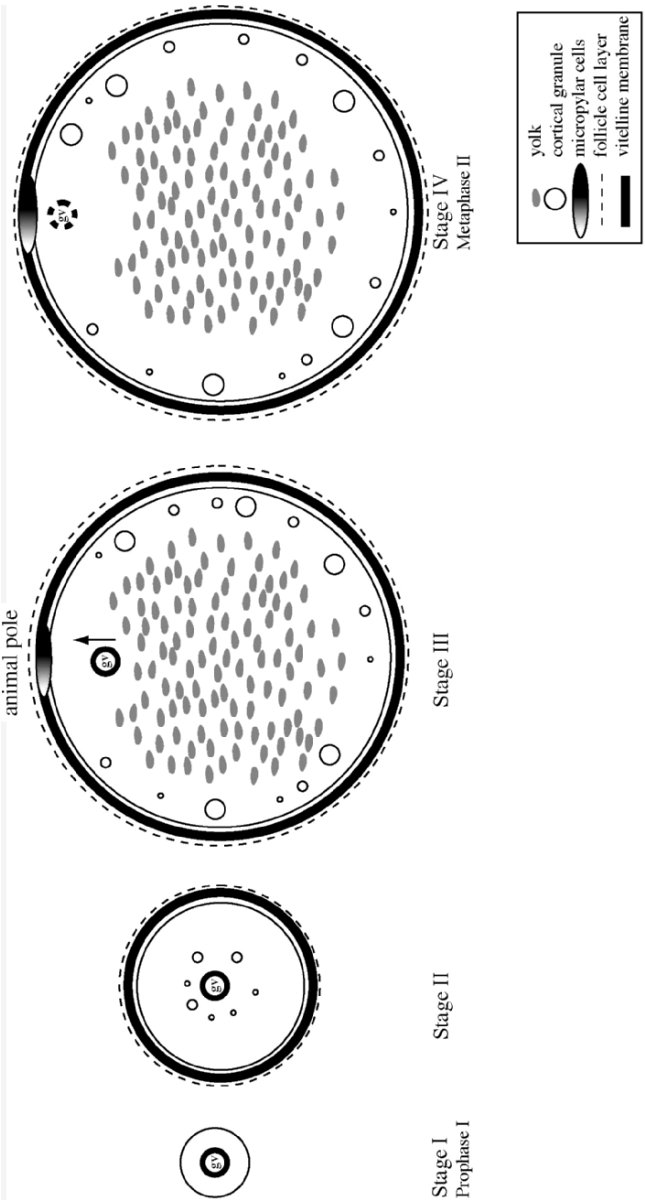

Figure 1. Diagram representing the stages of oogenesis. (See text for details.)

production of ribosomal mRNAs (Wallace and Selman, 1990), which will be used for the production of oocyte proteins.

Prior to entry into the diplotene stage of meiosis, the oocyte becomes surrounded by a single layer of follicle cells (follicle phase, stage 1B, follicle diameter 20–140 μm). Chromosomes now decondense, and the oocyte arrests at the diplotene stage of meiosis I. The decondensed chromosomes acquire a lampbrush morphology, involving extended DNA loops containing large amounts of mRNA and protein (Baumeister, 1973). Coincident with the appearance of this chromosomal morphology, and presumably aided by this chromatin conformation, the oocytes at this stage begin to increase their rates of transcription (Baumeister, 1973). Once the oocyte becomes surrounded by cells in the follicular layer, microvilli extend from the cell membrane of the oocyte towards the follicle cells and vice versa, until these microvilli reach and contact each other. Electron microscopy shows that these connections contain adherens junctions, desmosomes, and gap junctions (Kessel et al., 1985; Cerda et al., 1993), which may facilitate not only cell adhesion and cohesiveness to the complex, but also the uptake of small molecules by the oocyte. At the same time, patches of electron-dense material begin to appear in the space between the oocyte and the follicle cell. These patches gradually expand, apparently primarily driven by secretion of oocyte components, to form a contiguous vitelline envelope (Yamagani et al., 1992).

During this stage, subcellular structures such as mitochondria, Golgi, and endoplasmic reticulum, which are important for the production of maternal factors, start to become abundant in the oocyte. A heterogeneous complex of structures, known as Balbiani's vitelline body (also known as the yolk body) also develops. This contains mitochondria, annulate lamellae, Golgi bodies, endoplasmic reticulum and "nuage," the electron-dense fibrogranular material associated with germ cells and germ plasm material (Clérot, 1976; Kessel et al., 1984). Although the function of this structure is not well understood, it may be analogous to a structure of similar morphology in amphibian oocytes, the mitochondrial cloud, which has been shown to be involved in the transport and localization of factors, including germ plasm components, at the vegetal pole of the oocyte (Forristall et al., 1995; Kloc and Etkin, 1995; Chang et al., 2004). Similar vegetally localized products have been observed in zebrafish oocytes (see section 2.2.), and it is possible that the Balbiani's body is involved in the localization of these factors.

2.1.2. Cortical alveolus stage (Stage II, follicle diameter 140–340 μm)

This stage is marked by the appearance of bound vesicles, the cortical alveoli, in the ooplasm. These vesicles will become anchored at the cortex of the oocyte and will undergo exocytosis during egg activation, thus triggering changes in the vitelline membrane as part of the fertilization reaction. The production of factors by the oocyte continues, as reflected by the presence of up to 1,500 nucleoli in a single oocyte (Hisaoka and Firlit, 1962), mitochondria, annulate lamellae and lysosome-like bodies. At this stage, the vitelline membrane contains three separate layers and fully surrounds the oocyte, although microvilli traverse these layers at specialized pores and continue to provide a direct connection between the oocyte and the surrounding follicle.

2.1.3. Vitellogenesis stage (Stage III, follicle diameter 340–690 μm)

During this stage the oocyte acquires large amounts of vitellogenin (Vtg), which will be set aside in the yolk cell after fertilization as a source of energy for the developing embryo. This factor, one of the few known to be supplied exogenously to the oocyte, is produced by the liver and acquired by the oocyte through endocytosis from the plasma fluid (see Chapter 1 and 2). The accumulation of yolk in the oocyte also results in the centripetal displacement of cortical alveoli, which begin to acquire their final location at the cortex of the oocyte. This stage has the highest rate of protein increase during oogenesis, apparently accounted for largely by the increase in the uptake of Vtg (Selman et al., 1993). However, towards the end of this stage, the number of nucleoli decreases, in anticipation of the decreased rates of endogenous production of maternal factors during the following stages.

During the vitellogenesis stage, a polarity has already been initiated along the so-called animal–vegetal axis of the egg. The animal pole will eventually contain the female pronucleus and the site of sperm entry, and will be where the embryonic cells form during meroblastic cleavage. The opposite, or vegetal, pole of the egg lacks those structures, and during early embryogenesis, will contain the yolky mass. This existing animal–vegetal polarity is indicated by the presence of specialized follicle cells, the micropylar cells, at a site of vitelline material accumulation that will develop into the micropylar canal, where the sperm will enter the oocyte at the animal pole. In addition towards the end of the vitellogenesis stage the oocyte nucleus begins to move toward the animal pole region. Thus, during this stage, morphological landmarks indicate that the establishment of oocyte polarity is already ongoing, an observation corroborated by the localization patterns of mRNAs in the oocyte (section 2.2.).

2.1.4. Oocyte maturation (Stage IV, follicle diameter 690–730 μm)

This stage is characterized by the movement of the oocyte nucleus toward the animal pole of the egg, the resumption of meiosis, including the breakdown of the oocyte nuclear membrane, and the production of the first polar body. The oocyte nucleus acquires its position at the animal pole of the oocyte and arrests at metaphase of meiosis II. During this stage the oocyte continues to enlarge about 10–15%, although much of this enlargement may be caused by proteolysis of yolk proteins and hydration, rather than continued high rates of transcription and/or translation of maternal products. At this stage the microvilli that allow communication between the follicle cells and the oocyte through the vitelline membrane retract, reflecting the end of uptake of exogenous products by the oocyte.

2.1.5. Ovulated, mature egg (Stage V; egg diameter: 730–750 μm)

This stage involves the extrusion of the oocyte away from the oocyte/follicle complex and into the lumen of the ovary, where it is available for release during mating. Ovulation is thought to be triggered by hormonal stimulation, which itself is provoked by the mating behavior of the male.

2.2. Patterns of mRNA Localization

The expression of specific maternal mRNAs during oogenesis can be detected as early as Stage I. However, while mRNAs at this early stage are uniformly distributed in the oocyte, at later stages some mRNAs become localized to specific regions of the oocyte. Transcripts fall into four classes depending on their localization at later stages (Howley and Ho, 2000; see also Figure 2 and Chapter 3).

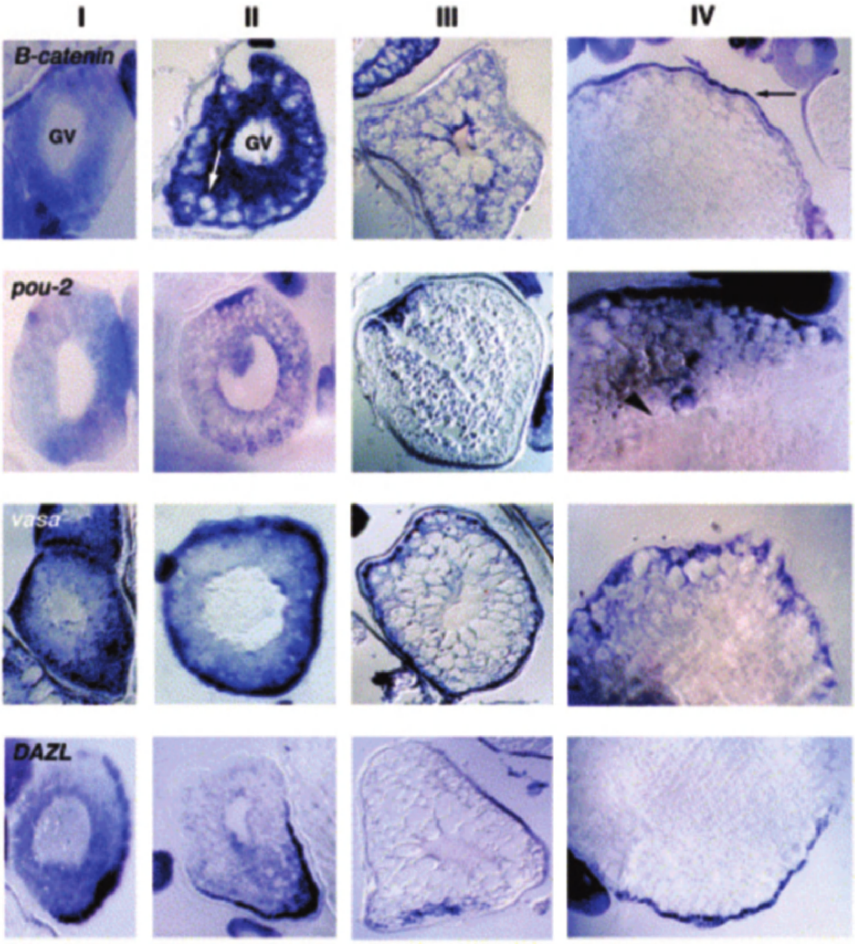

Figure 2. Patterns of mRNA localization during oogenesis (stages I–IV). mRNAs represent the various classes of observed patterns: ubiquitous (*β-catenin*) and localized to the animal region (*pou-2*), the entire cortex (*vasa*) or the vegetal cortex (*DAZL*). (Adapted from C. Howley and R.K. Ho with permission from Elsevier.) (*See Color Plates*).

2.2.1. Uniformly localized transcripts

Various mRNAs, including those for the genes *activin receptor type II, β-catenin, cdc25, goosecoid, p62, snail, sox19, stat3*, and *ryk*, remain uniformly distributed in the ooplasm (Bally-Cuif et al., 1998).

2.2.2. Animally localized transcripts

Transcripts for mRNAs for some genes, such as *cth1, cyclin-B, notch, PABP, pou-2, taram-A*, and *zorba* become localized to the animal region during stages II and III (Howley and Ho, 2000), reflecting the earliest signs of oocyte differentiation along the animal–vegetal axis of the oocyte. Transcripts for *vg1* have been reported to localize to this same region at a somewhat later stage, stage IV (Bally-Cuif et al., 1998). The localization of these transcripts is in a wedge-like region, reflecting a specialized cytoplasm in the animal pole. This cytoplasmic region also can be distinguished from the remainder of the oocyte by an enrichment in microtubules (Bally-Cuif et al., 1998) and, at later stages, the presence of the oocyte nucleus (Selman et al., 1993), the formation of the sperm site of entry and a reduced number of cortical alveoli (Hart and Donovan, 1983). Studies of the *zorba* mRNA suggest that the anchoring of this mRNA depends on an intact microfilament network. It is not clear, however, whether this requirement is directly at the level of mRNA anchoring or reflects a more indirect need for cytoplasmic integrity.

2.2.3. Vegetally localized transcripts

A small number of mRNAs, for the genes *deleted in azoospermia (daz)* and *Bruno*, have been shown to become localized to the cortical region of the vegetal pole of the oocyte during stages Ib and II, respectively (Maegawa et al., 1999; Suzuki et al., 2000), again reflecting an existing animal–vegetal polarity in the early oocyte. mRNAs for these two genes localize during early embryogenesis to the germ plasm (see section 4.3.) and they may therefore be involved in the specification of the primordial germ cells. The localization of both of these mRNAs in the vegetal region is at the cortex, which is rich in actin (Becker and Hart, 1996), and treatments that interfere with actin function result in the detachment of these mRNAs from the cortex (Suzuki et al., 2000).

2.2.4. Cortically (but ubiquitously) localized transcripts

Transcripts for the genes *vasa* and *eomesodermin* have also been shown to become cortically localized during stage II, although in the case of these genes the localization occurs in the entire cortex around the oocyte (Braat et al., 1999; Howley and Ho, 2000; Knaut et al., 2000; Bruce et al., 2003). Like *DAZ* and *bruno*, *vasa* RNA also localizes to the zebrafish germ plasm and is thought to have a role in germ-line development, while *eomesodermin* has been shown to be involved in the specification of the endoderm at later stages of development. The cortical localization of *vasa* mRNA during late oogenesis has also been demonstrated in the tilapia *Oreochromis niloticus* (Kobayashi et al., 2000).

2.3. Patterns of Protein Localization and Accumulation

Our knowledge of the localization patterns of proteins during oogenesis relies on a limited set of examples. These appear to reflect functions during oogenesis, and, when these functions are completed, the proteins are either stored for later use during embryonic development or degraded. For example, α- and β-catenins and E-cadherins form part of cell adhesion junctions, and these proteins localize in the oocyte cortex at the sites of contact between the oocyte and the follicle cells (Cerda et al., 1999). As these connections are dissolved during oocyte maturation, however, this pattern of protein localization disappears, and proteins are stored as uniformly distributed complexes in the oocyte. On the other hand, protein for another member of the catenin family, plakoglobulin, is stored in the oocyte as free protein.

Another example of a known protein localization pattern is that of the Zorba protein, which is ubiquitously distributed in the oocyte during stages Ib and II, but becomes localized during stage III to the animal pole of the oocyte (Bally-Cuif et al., 1998). This localization pattern mirrors the localization of the *zorba* mRNA. It is unclear, however, whether the enrichment in the animal region at later stages is caused by transport of the initially ubiquitous protein to the animal region or by translation of the localized mRNA coupled to protein degradation in other regions. The Zorba protein contains an RNA recognition motif, and it is possible that this motif is involved not only in its function but also its anchoring at the animal region.

The Vasa protein, whose mRNA is part of the zebrafish germ plasm at early stages of embryonic development, is another type of factor with a known subcellular localization pattern in developing oocytes. The Vasa protein localizes to perinuclear patches during oogenesis (Braat et al., 2000; Knaut et al., 2000), a localization pattern that may be relevant to the development of the germ line. During oocyte maturation, the Vasa protein becomes delocalized in the embryo until it appears in a perinuclear pattern again at the sphere stage (cell cycle 13; Knaut et al., 2000; Wolke et al., 2002). In the tilapia, the Vasa protein also appears diffuse in the cytoplasm (Kobayashi et al., 2002). Thus, in these two fish species, the cytoplasmic Vasa protein distribution is different from that of its cortically localized mRNA.

2.4. Oogenesis Mutants

Although screens designed to identify mutations affecting oogenesis have been reported (Bauer and Goetz, 2001), an analysis of these mutations has not been documented. A screen for recessive maternal-effect mutations that affect early embryos has led to the identification of several mutations that appear to affect the process of oocyte maturation. Oocytes from wild-type females exhibit a substantial change, from opaque in immature (stage IV) oocytes to clear in mature, ovulated eggs (stage V). However, females homozygous for four mutations, *over easy*, *ruehrei*, *sunny side*, and *souffle*, produce eggs with the opaque appearance characteristic of immature eggs (Dosch et al., 2004; Table 1). Protein analysis of

Table 1. Genes with a strictly maternal-effect contribution[1]

Class	Process affected	Mutant	References
Oogenesis	Oocyte maturation	*over easy*	Dosch et al. (2004)
		ruehrei	Dosch et al. (2004)
		souffle	Dosch et al. (2004)
		sunny side	Dosch et al. (2004)
	Oocyte polarity	*bucky ball*	Dosch et al. (2004)
		p6cv	Dosch et al. (2004)
Egg activation / fertilization	General egg activation	*jump start*	Dosch et al. (2004)
		p11cv	Dosch et al. (2004)
	Reduced ooplasmic streaming	*dp14nb*	Dosch et al. (2004)
		emulsion	Dosch et al. (2004)
		under repair	Pelegri et al. (2004)
	Pronuclear fusion	*futile cycle*	Dekens et al. (2003); Pelegri et al. (2004)
General cellular requirements	Initiation of mitosis	*atomos*	Dosch et al. (2004)
		cobblestone	Pelegri et al. (2004)
		indivisible	Dosch et al. (2004)
		irreducible	Dosch et al. (2004)
	Pattern of membrane deposition and mitosis	*barrette*	Pelegri et al. (2004)
	Initiation of cytokinesis	*acytokinesis*	Kishimoto et al. (2004)
	Completion of cytokinesis	*nebel*	Pelegri et al. (1999; 2004)
	Cell adhesion/membrane formation[2]	*aura*	Pelegri et al. (2004)
		cellular atoll	Dosch et al. (2004)
		cellular island	Dosch et al. (2004)
		janus[3]	Abdelilah et al. (1994)
		weeble	Pelegri et al. (2004)
	DNA segregation (chromosomal bridges in mutant)	*golden gate*	Pelegri et al. (2004)
		kwai	Pelegri et al. (2004)
		screeching halt	Wagner et al. (2004)
	DNA segregation (loss of DNA in mutant)	*bo peep*	Pelegri et al. (2004)
		waldo	Pelegri et al. (2004)
Pattern formation	Dorsal axis induction	*brom bones*	Wagner et al. (2004)
		hecate	Pelegri et al. (2004)
		ichabod	Kelly et al. (2000)
		tokkaebi	Nojima et al. (2004)
Gastrulation	Gastrulation (arrest at 50% epiboly)	*betty boop*	Wagner et al. (2004)
	Gastrulation (slow)	*bedazzled*[4, 5]	Wagner et al. (2004)
		mission impossible	Pelegri et al. (2004)
		poky[4]	Wagner et al. (2004)
		slow[4]	Wagner et al. (2004)

(*continued*)

Table 1. Continued

Class	Process affected	Mutant	References
Other body plan mutants	Tail formation	*bobtail*	Kishimoto et al. (2004)
		pug	Wagner et al. (2004)
	Head morphogenesis	*pollywog*	Wagner et al. (2004)
	Ventral tail fin morphology	*blistered*	Wagner et al. (2004)

[1]In addition to mutations in the genes presented in this table, 36 additional maternal-effect mutations leading to a pleiotropic degeneration phenotype have been described (Wagner et al., 2004).
[2]Completion of cytokinesis depends on the deposition of internal membrane containing cell adhesion molecules. Mutants may therefore have an underlying defect in late cytokinesis.
[3]*janus* leads to double blastula embryos with normal membrane integrity and may therefore specifically affect early cell adhesion.
[4]Mutations affecting early processes such as DNA segregation can result in embryonic phenotypes that manifest during gastrulation (Pelegri et al., 2004). The indicated mutants have not been thoroughly tested for early cellular defects and may be potentially affecting such early processes.
[5]Mutations in *bedazzled* lead in addition to the sloughing of cells during gastrulation, suggestive of a defect in cell adhesion.

the major yolk protein reveals that a shift to lower molecular weight forms, also characteristic of oocyte maturation, does not occur normally in these mutants. This suggests that these mutations may affect genes in a pathway involved in egg maturation. In addition, two other maternal-effect mutations, *bucky ball* and *p6cv*, affect the localization of maternal mRNAs along the animal–vegetal axis, at least in mature, ovulated oocytes, as well as the redistribution of the ooplasm upon egg activation (Dosch et al., 2004; see section 3.2.). It is therefore possible that these mutants affect the determination of the animal–vegetal axis during oogenesis. Further work will be necessary to identify additional factors that drive the various stages and processes that occur during oogenesis.

3. EGG ACTIVATION

Egg activation, which occurs upon contact with a hypotonic solution such as water, and is usually, but not necessarily, linked to sperm entry, is characterized by a series of changes in the mature oocyte. On a superficial level, the egg changes from a flattened, somewhat oblong shape to a rounded shape, likely due to an increase in turgidity, and the chorionic membrane expands away from the egg. At the subcellular level, exocytosis of the cortical alveoli, which are anchored at the cortex in mature oocytes, occurs and their contents are released to the space between the oocyte outer membrane and the vitelline membrane (Becker and Hart, 1996, 1999). These released factors in turn will lead to the restructuring of the vitelline membrane and its expansion (Yamagani et al., 1992). Another process triggered by egg activation is the resumption of meiosis, so that the

oocyte chromosomal material, arrested in metaphase of meiosis II since the maturation stage, produces a second polar body and a haploid nucleus (Streisinger et al., 1981; Selman et al., 1993; Dekens et al., 2003). The membranes around the oocyte allow sperm entry in the specialized actin-rich micropylar canal at the animal pole of the egg within the first minutes after egg activation (Hart and Donovan, 1983; Hart et al., 1992). In the context of maternal factors important for early development, egg activation is also of primary importance because it leads to the redistribution of these factors toward the forming cell. Thus, we will discuss in detail various aspects of egg activation and maternal product redistribution.

3.1. Redistribution of Maternal Products During Egg Activation

The most obvious process of redistribution consists of the segregation of yolk granules and ooplasm, which are initially intermingled in the mature oocyte, during the early embryonic cell cycles (Figure 3; see also Figure 4(a)). This process will result in the formation of a yolk-free cell at the animal pole of the oocyte. Ooplasmic segregation is thought to be triggered by the periodic propagation of calcium waves from the animal pole of the oocyte to the base of the forming cell, or blastodisc (Leung et al., 1998). These calcium waves may trigger the local contraction of the actin-rich network and the accumulation of actin at the base of the blastodisc (Leung et al., 2000). Ooplasmic segregation occurs primarily through internal channels, or axial streamers, which contain the bulk of the ooplasm as it moves towards the forming cells. In the zebrafish, the formation of axial streamers, as well as the streaming itself depends on microfilament,

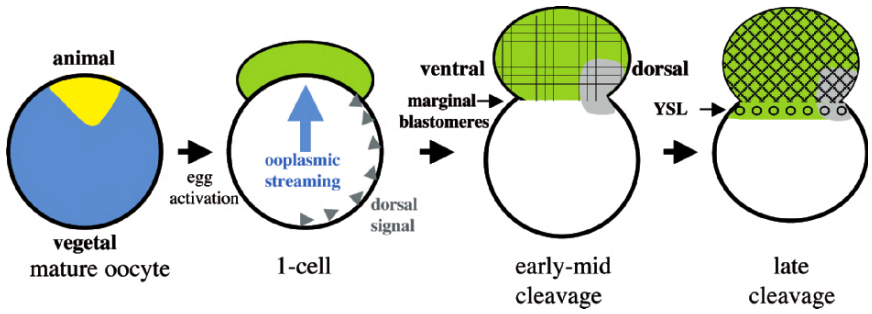

Figure 3. Redistribution of maternal factors during egg activation. Ooplasmic streaming through axial streamers within the forming yolk cell allows ubiquitously distributed factors (blue) to accumulate in the forming cell at the animal pole. Cortical translocation of a dorsal signal originally localized to the vegetal pole of the oocyte results in its movement (arrowheads) to the animal pole along one side of the embryo. This side becomes the prospective dorsal region during the blastula stage (grey). Factors already localized at the animal region during oogenesis (yellow) remain in that region after activation. The yolk syncytial layer (YSL) forms from the most marginal blastomeres, thus allowing a direct connection of nuclei in this layer to the yolk cell. See text for details. (*See Color Plates*).

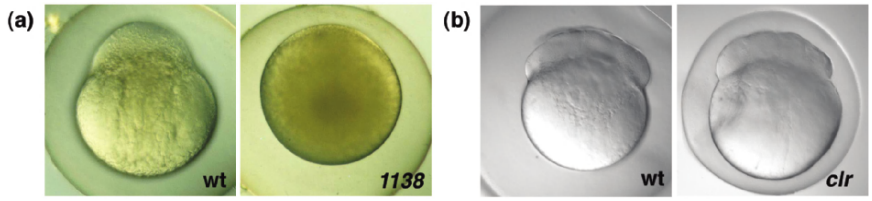

Figure 4. Mutations affecting egg activation. A mutation in the line 1138 exemplifies defects in cell lifting, presumably due to a defect in ooplasmic streaming. In the case of the mutation *claustro (clr)*, ooplasmic streaming is normal but chorion expansion is reduced. (Adapted from F. Pelegri et al., with permission from Wiley-Liss, Inc.) (*See Color Plates*).

but not microtubule, function (Katow, 1983; Hart and Fluck, 1995; Leung et al., 2000). However, it is not clear how global this requirement is among fish species because in medaka fish it is the microtubule network that has been shown to be required for bulk ooplasmic streaming and the consequent formation of the blastodisc (Abraham et al., 1993; Webb et al., 1995).

Although microtubule function does not appear to be important in the transport of the bulk of the ooplasm in the zebrafish activated egg, some studies indicate that microtubules are indeed involved in the transport of some cytoplasmic components. The mRNA for the gene *squint*, which is uniformly distributed in the mature egg, is translocated during egg activation toward the forming blastodisc in a manner dependent on microtubules, but not microfilaments (Gore and Sampath, 2002). This is reminiscent of the microtubule-dependent requirement for rapid movement of small inclusions observed in the medaka fish activated egg (Webb et al., 1995). It appears therefore that both microfilament- and microtubule-based cytoskeletal networks are involved in the segregation of ooplasmic components, but that the dependence on their function for bulk ooplasmic flow varies in different fish species.

Molecular studies have indicated similar mechanisms involved in the redistribution of specific mRNAs during egg activation. This has been best studied in the case of mRNAs present in the egg that, with the exception of those already enriched in the animal region of the mature oocyte, translocate toward the forming blastodisc during egg activation. mRNAs uniformly distributed in the mature oocyte accumulate after egg activation in the forming blastodisc together with the bulk of the ooplasm. It remains to be tested whether, as is the case for the *squint* mRNA, their movement is dependent on microtubule function. Those mRNAs localized at the vegetal pole of the mature oocyte, such as *daz* and *bruno-like*, have been shown to also translocate toward the forming blastocyst along axial streamers, where they will later accumulate at the location of the zebrafish germ plasm. Another germ plasm component, the *vasa* mRNA, which is initially localized in the cortex around the mature oocyte, also translocates toward the forming blastodisc (see section 4.3.).

As described in section 4.5., embryological studies have also indicated the presence of factors involved in dorsal axis induction, at the vegetal pole of the mature oocyte, which are translocated toward the forming blastodisc in a microtubule-dependent process during egg activation. This movement appears to be mimicked by fluorescent beads injected at the vegetal pole of the oocyte, which show a saltatory, microtubule-dependent pathway of movement toward the blastodisc along the prospective dorsal region of the cortex (arrowheads in Figure 3).

These examples reflect a variety of processes involved in the redistribution of maternal factors during egg activation. It is likely that additional processes will be discovered as the localization of other maternal products is studied in detail. In addition, potential changes in the distribution of maternally derived organelles, such as the ER and mitochondria, during egg activation (Speksnijder et al., 1993; Volodina et al., 2003), need to be determined in fish species.

3.2. Mutations Affecting Egg Activation or Fertilization

Several maternal-effect mutations have been reported to affect various aspects of egg activation. Mutations in *jump start* and *p11cv* result in multiple defects, including a failure to undergo the characteristic increase in egg turgidity, ooplasmic segregation, and at least partially, cortical granule release (Dosch et al., 2004). Therefore, these mutations may affect genes required for the initiation of egg activation. Other mutations appear to have more specific defects. Three mutations, *under repair*, *emulsion* and *dp14nb*, have been reported to lead to defects in the segregation of the ooplasm, such that mutant eggs exhibit a reduction in the lifting of the blastodisc and separation between the yolk and the ooplasm (Dosch et al., 2004; Pelegri et al., 2004; see Figure 4(a)). These mutations may affect the actin network involved in ooplasmic segregation, or possibly, events such as calcium waves that may organize the actin network. Another mutation, in the gene *claustro*, results in eggs that undergo normal changes in the egg morphology but show a reduced expansion of the chorionic membrane (Figure 4(b)). As there is no evidence for remaining cortical alveoli-derived vesicles in *claustro* eggs, this mutation may affect a component normally released by cortical alveoli that is involved in the restructuring of the vitelline membrane. Finally, as mentioned previously, mutations in *bucky ball* and *p6cv* result in eggs in which the cytoplasm segregates radially rather than in the vegetal to animal direction. It is possible that these two mutations affect the direction of the actin network, whose reorganization is triggered by egg activation and is required for streaming. However, at least in the case of *bucky ball*, the localization of maternal mRNAs in oocytes is also affected, suggesting that the defect in this mutant occurs at an earlier stage, during the determination of animal–vegetal egg polarity in oogenesis.

A mutation in the gene *futile cycle* results in a defect in the meeting of the pronuclei immediately after fertilization (Dekens et al., 2003; Figure 5(b)). Sperm can enter the egg and deliver the male pronucleus, but the female and

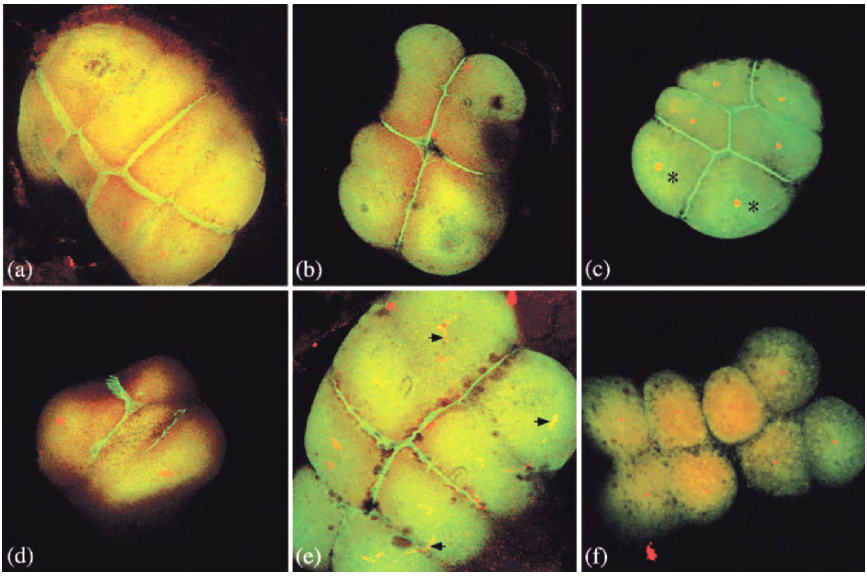

Figure 5. Mutations affecting early cellular processes. Confocal images of 8-cell stage embryos, labeled with an antibody against β-catenin protein, which labels cell adhesion junctions at the newly formed membranes (green) and the DNA dye propidium iodide (red). The embryo in (a) shows the normal appearance of these markers. A mutation in *futile cycle* results in defects in pronuclear fusion (b). A fraction of cells (asterisks) in *cobblestone* mutants do not initiate mitosis (c, note larger size of affected cells). A mutation in *barrette* affects nuclear division and the pattern of membrane deposition (d). *golden gate* mutants show abnormal DNA bridges (arrowheads) indicative of defects in chromosome segregation during mitosis (e). A mutation in *aura* results in defects in the deposition of adhesive membrane at the furrow (f). (*See Color Plates*).

male pronucleus remain separate and do not fuse. This suggests that there are active nuclear transport processes involved in pronuclear fusion during fertilization. Typically, the sperm provides a pair of centrioles, which can act as a microtubule organizing center. It will therefore be interesting to determine whether a microtubule-dependent process is involved in such pronuclear movements. Of interest, *futile cycle* mutant embryos undergo a relatively normal program of cellular division in spite of the fact that most cells lack a nucleus, indicating that the machinery that drives cell cleavage can function independently of DNA.

4. EARLY EMBRYONIC DEVELOPMENT

4.1. Overview of Morphological Development

The first 24 h of zebrafish development, during which the major body axes and germ layers are formed, are characterized by distinct patterns of cell division and cell movements, which allow us to further subdivide this period

(Kimmel et al., 1995; Figure 6). The cleavage stage (0–128 cells) is characterized by regular synchronous cleavages. These are incomplete for the cells at the blastula margin, which remain connected by cytoplasmic bridges. Cleavage is followed by the blastula stage (128 cells – onset of gastrulation). In addition to continued cell division, this period is also characterized by the formation of the yolk syncytial layer (YSL), the occurrence of the MBT, and, soon afterward, the beginning of epiboly. The YSL is a multinucleate layer formed by the collapse of the membranes of cells closest to the blastoderm margin (the marginal cells) which contains signaling molecules important for the induction of the germ layers (Mizuno et al., 1996; Ober and Schulte-Merker, 1999; Rodaway et al., 1999). The MBT marks the onset of zygotic transcription in the embryo, which is

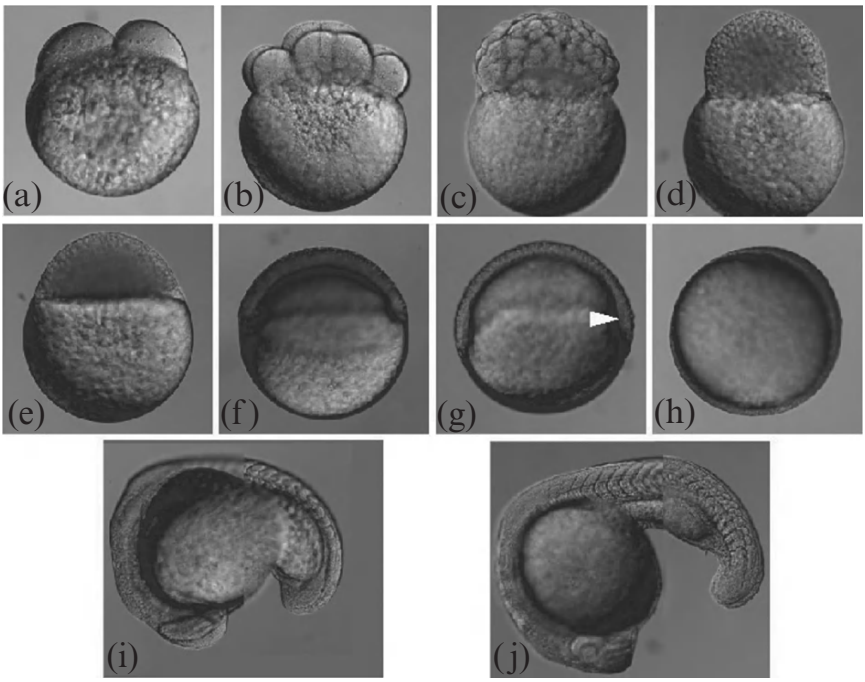

Figure 6. Early zebrafish development. Stages shown are 2-cell (0.75 hrs p.f. (a)), eight-cell (1.25 hrs p.f. (b)), 128-cell (2.25 hrs p.f.(c)), 1,000-cell (3 hrs p.f. (d)), high (3.3 hrs p.f. (e)), 30% epiboly (4.7 hrs p.f. (f)), 60% epiboly (7 hrs p.f. (g)), 90% epiboly (9 hrs p.f. (h)), 16 somites (17 hrs p.f. (i)), pharyngula (24 hrs p.f. (j)). Meroblastic cleavage leads to the formation of a blastula at the animal pole of the oocyte (a)–(d). Epibolic movements of cells towards the vegetal pole result in the cells covering the yolk cell (f)–(h). During 50% epiboly, cells begin to migrate towards the dorsal axis (dorsal convergence) and form a thickening, or shield (white arrowhead). Other processes that occur during gastrulation result in the formation of the various germ layers through involution and the extension of the dorsal axis. During the somite stages and thereafter (i)–(j), the basic body plan has already been set up and is further refined through organogenesis. All views are side views animal up for (a)–(h), and anterior left and dorsal up for (i)–(j)

apparent by a dramatic increase in new RNA synthesis. In addition, at the MBT, the cell cycle lengthens and cells become motile. Cell motility becomes apparent during epiboly when the blastodisc spreads over the yolk. The beginning of involution at the margin at 50% epiboly marks the start of the gastrula period and initiates the formation of an embryo with the basic embryonic layers: ectoderm, mesoderm, and endoderm. At the same time, directed migration, or convergence, of cells towards the dorsal side of the embryo leads to the formation of a thickened structure at this site known as the shield. Within 10 h of development, the embryo exhibits clear axes of polarity and its embryonic germ layers have already been determined. Additional cellular divisions, morphogenesis and cell differentiation processes result in the further regionalization of these structures and tissues. Genetic analysis has shown the involvement of maternal factors in all of these processes.

4.2. Genes Required for General Cellular Requirements

A number of maternal-effect genes have been shown to be involved in basic cellular processes. Perhaps the most apparently obvious of such processes is that of cell division. Visible defects in cell division can be caused by either a defect in the initiation of mitosis, thus affecting both karyokinesis and cytokinesis, or solely in the process of cytokinesis.

The genes *irreducible*, *indivisible*, and *atomos* may be part of the first class of genes, as mutations in them cause a strictly recessive, maternal effect that results in embryos with neither karyokinesis nor cytokinesis (Dosch et al., 2004). Similarly, the mutation in *cobblestone* results in cells that do not undergo mitosis, although only a fraction of the cells are affected, typically in one side of the embryo (Pelegri et al., 2004; Figure 5(c)). Previous reports have indicated that p38 kinase activity is required for cellularization in only one side of the embryo (Fujii et al., 2000), suggesting the unexpected possibility that specific cellularization pathways may be required in different embryonic regions. A mutation in *barrette* results in an irregular pattern of furrowing and addition of new membrane (Pelegri et al., 2004; Figure 5(d)), suggesting that this gene is involved in the precise positioning of the cleavage plane pattern. Eggs derived from *barrette* mutant mothers also lack a normal number of DNA masses, indicating that *barrette* mutant eggs have additional defects in either fertilization or mitosis.

A second class of cell division mutants includes those that undergo karyokinesis but fail to undergo cytokinesis. These in turn can be divided into two types, depending on whether the mutation affects the initiation or the completion of cytokinesis. Mutations in the gene *acytokinesis* (ack^{kt5}) affect the initiation of cytokinesis (Kishimoto et al., 2004); two other mutations, ack^{kt62} and ack^{kt119} recovered in the same screen have been reported to have the same phenotype and map at the same location, and it is possible that these are alleles of the same gene, if not identical mutations). Maternal mutants for *acytokinesis* lack proper actin reorganization during the early divisions, including deposition of actin at

the forming furrow. This gene may thus be involved in actin rearrangements involved in the induction of the furrow or the contraction of the actomyosin ring, which initiates cellularization.

Other genes, such as *nebel* and *aura*, are required for the completion of cytokinesis (Pelegri et al., 2004; Figure 5(e)). In these maternal mutants, furrow contraction initiates, but the furrow never fully matures to form a membrane wall (or septum) between the daughter cells. Consequently, the daughter cells either remain separate from each other or fuse after furrow regression. Staining for membrane markers suggests that these mutations affect the deposition of membrane at the furrow. This membrane is thought to contain adhesive molecules (Jesuthasan, 1998), so that the defect results not only in reduced septum formation, but also reduced adhesion between daughter cells. The mutation in *nebel* has been shown to affect the organization of the furrow microtubule array (FMA), an array of microtubules parallel to each other and perpendicular to the plane of the furrow (Pelegri et al., 1999). Moreover, inhibition of microtubule function during late cytokinesis leads to cytokinesis defects similar to those caused by the *nebel* mutation (Jesuthasan, 1998; Pelegri et al., 1999). These studies show an involvement of the FMA in the localized exocytosis at the furrow that is essential for the formation of new cellular membranes.

Other mutations, such as *janus*, *cellular island*, *cellular atoll*, and *weeble* (Abdelilah et al., 1994; Dosch et al., 2004; Pelegri et al., 2004), also affect the process of cellularization, although their primary characterization has not addressed whether their defect occurs in early or late cytokinesis. Of interest, the mutation in *janus* results in the formation of two groups of blastomeres in the embryos, resulting from cellularization defects during the first three cellular divisions (Abdelilah et al., 1994). It is possible that the first three cellular divisions in the development of the zebrafish embryo are distinct from later divisions, as they involve larger cells and exhibit the formation of slow waves of intracellular calcium that develop along their furrows during cytokinesis (Chang and Meng, 1995; Webb et al., 1997; Créton et al., 1998). Similar slow calcium waves at the forming furrows have been observed in the medaka fish early embryo (Fluck et al., 1991).

Another type of early defect involves the segregation of DNA. Five maternal-effect mutations, in the genes *golden gate*, *kwai*, *screeching halt*, *waldo*, and *bo peep*, have been shown to lead to embryos with defects in DNA segregation (Pelegri et al., 2004; Wagner et al., 2004). Embryos from homozygous *golden gate*, *kwai and screeching halt* mothers form abnormal DNA bridges that often span two cells (Figure 5(d)). Mutations in the genes *waldo* and *bo peep* do not result in DNA bridges, but do lead to the presence of small supernumerary masses of DNA in the embryo, which possibly correspond to missegregated chromosomes. Of interest, mutations in these genes do not result in embryonic arrest until the onset of epiboly or later. This is likely due to the fact that at these stages the embryo begins to rely on zygotic products, and DNA loss results in the absence of large sets of genes in various cells. Consistent with this scenario,

mutations with similar underlying defects, such as *golden gate* and *kwai*, or *waldo* and *bo peep*, can result in embryonic lysis at different stages of development, and the severity of the live phenotype (in terms of how far into development the embryos can survive) appears to correlate with the severity of the DNA segregation phenotype. The ability of zebrafish embryos to undergo early cleavages even with an incomplete DNA content (as underscored by the phenotype of the fertilization mutant *futile cycle* (Dekens et al., 2003), indicates that some mutations that result in defects in epiboly (Wagner et al., 2004; see section 4.6.), which have not been analyzed for subcellular defects at earlier stages, may actually have defects in DNA segregation. This emphasizes the need to carry out subcellular analyses of zebrafish maternal mutants in order to determine the primary defect caused by the mutation. In addition to these mutations, 36 mutations leading to widespread, apparently pleiotropic defects and cell death, have been reported (Wagner et al., 2004).

4.3. Segregation of the Germ plasm

The germ plasm is a specialized cytoplasm that can be recognized under the electron microscope due to its electron-dense nature, and enrichment in fibrils and mitochondria. This specialized structure contains maternally supplied RNA, proteins, and other components that specify the germ line (reviewed in Wylie, 1999). In other organisms such as *Caenorhabditis elegans* and *Drosophila*, mutations in genes that affect germ plasm formation lead to defects in the formation in the primordial germ cells (PGCs). Germ plasm appears to have a similar role in the zebrafish, since, as described below, germ plasm components of maternal origin exhibit a highly regulated pattern of segregation, and become part of the PGCs. Thus, one would expect maternally derived factors in the zebrafish to be important for PGC determination, and indeed ablation of the sites at the furrow where germ plasm accumulates (see below) results in defects in PGC determination (Hashimoto et al., 2004). Current studies, using MO-mediated knockdowns, have shown a role for some germ plasm components, including the gene products of *nanos* (Köprunner et al., 2001) and *dead end* (Weidinger et al., 2003), in the migration and viability of PGCs, although it is unclear whether these functions are maternally or zygotically derived. So far, however, no study has shown a role for any particular zebrafish gene product in PGC specification. Here, we summarize the stereotypic pattern of segregation of these maternal components from the mature egg to the late cleavage stages, when cell transplantation experiments show that the PGCs have already been specified (Ciruna et al., 2002; Waskiewicz et al., 2002). For the role of PGC-specific genes in germ-cell migration and maintenance, the reader is referred to previous reviews (Wylie, 2000; Raz, 2004).

As cleavage begins, germ plasm accumulates at the furrows of the first and second cleavage divisions. Transcripts for several genes, *vasa* (Yoon et al., 1997), *nanos* (Köprunner et al., 2001), *dead end* (Weidinger et al., 2003), *DAZ*

(Hashimoto et al., 2004), *Bruno-like* (Hashimoto et al., 2004), and *askopos* (Blaser et al., 2005), have been shown to localize to this region, and electron microscopy has shown this region has the characteristic appearance of germ plasm material (Knaut et al., 2000). At least in the case of *vasa*, early recruitment appears to involve various steps that may allow the gradual aggregation of germ plasm material (Yoon et al., 1997; Braat et al., 1999; Pelegri et al., 1999; Knaut et al., 2000). An early step involves a transient accumulation around the periphery of the first cell. Subsequently, as the furrows begin to form, products such as *vasa* RNA are recruited at the forming furrow in a rod-like structure. This recruitment may be at least partially independent of furrow formation, as mutants which fail to initiate furrow ingression still show some *vasa* mRNA accumulation at the site where the furrows would have presumably formed (Kishimoto et al., 2004). A later step involves the further aggregation of the material at the distal ends of the mature furrow. This latter process has been shown to be dependent on the function of the FMA, whose ends appear to be embedded in the germ plasm material (Pelegri et al., 1999). By the 32-cell stage, each of the four germ plasm aggregates ingresses into a single cell and then segregates asymmetrically to a single daughter cell during the cleavage stages (Yoon et al., 1997; Braat et al., 1999; Knaut et al., 2000). This maternally driven asymmetric segregation pattern ceases at the sphere stage, somewhat coincident with the activation of the zygotic genome, but independently of zygotic gene function (Knaut et al., 2000). In summary, maternally derived germ plasm exhibits a highly regulated pattern of segregation and is essential for PGC determination. However, the precise role of specific germ-line components in PGC specification remains to be elucidated.

An evolutionary study has compared the pattern of localization of *vasa* mRNA at the furrows in the teleost lineage (Knaut et al., 2002). Species in the ostariophysan lineage such as the zebrafish, Fegrade's danio, carp and tetra, exhibit a pattern of localization at the furrow. On the other hand, all euteleost species tested, medaka (see also Shinomiya et al., 2000), trout, and rainbow fish, lack furrow-specific *vasa* mRNA localization and instead contain diffuse mRNA in the cytoplasm. Importantly, the butterfly fish, a basal species in the teleost lineage, shows a localized *vasa* mRNA pattern similar to that of ostariophysans (Knaut et al., 2002). Moreover, the 3′UTR sequence is highly conserved among localized ostariophysan *vasa* RNAs but not among nonlocalized euteleost *vasa* RNAs. These data suggest that *vasa* mRNA localization at the furrows is a basal feature of the teleost lineage, and that euteleosts have lost this feature.

4.4. Segregation of other Maternal RNAs During Early Cleavage

Although most RNAs known to segregate during the early cleavages are germ plasm components, several studies have reported other maternal RNAs that exhibit specialized segregation patterns during these cellular cycles. Maternal RNA

coding for the transcription factor *eomesodermin*, segregates during the first cellular cycle to the marginal most region of the blastodisc (Bjornson et al., 2005). Notably, maternal *eomesodermin* function is important for the induction of the mesendodermal germ-cell layer during gastrulation, which itself is derived from the marginal-most cells of the blastula. Thus, a segregation program at the one-cell stage results in an enrichment of a maternal RNA in the region of the blastula where function will be needed later during gastrulation.

In a pattern somewhat complementary to that of *eomesodermin,* maternal RNA for the gene *mago nashi,* which codes for a conserved protein of unknown function, segregates to the internal-most cells of the early blastula (Pozzoli et al., 2004). Interestingly *mago nashi* expression later in embryogenesis occurs primarily in neural tissues (Pozzoli et al., 2004), which are derived from the internal cells that inherit the *mago nashi* RNA (Kimmel et al., 1990). Thus, in this case, it is also possible that the early segregation of maternal RNA allows for the enrichment of a product required in the descendant cells, in this case potentially involved in neural development.

4.5. Determination and Patterning of the Embryonic Axes

Determination and patterning of the axes is a fundamental step in early vertebrate development that lays the framework for later developmental decisions in the embryo. With respect to the embryonic axes, our knowledge of the establishment of the dorsoventral pattern is the most advanced and has been shown to involve three signaling pathways – the Wnt/β-catenin pathway, the Wnt/Ca^{2+} pathway, and a TGF-β pathway.

Dorsoventral patterning in zebrafish depends initially on the translocation of an as yet unidentified dorsal signal inferred by ablation studies to be localized in the cortex of the vegetal pole of the early embryo (Mizuno et al., 1999; Ober and Schulte-Merker, 1999). The alignment of a microtubule array in the yolk is required both for the transport of particles from the vegetal pole and for the formation of a dorsal axis suggesting that the initial dorsal signal must move in a microtubule-dependent manner from the vegetal pole to the presumptive dorsal region (Jesuthasan and Strähle, 1997). Embryological manipulations in early embryos of *Fundulus* (Oppenheimer, 1936), loach (Kostomarova, 1969), and goldfish (Tung et al., 1945; Mizuno et al., 1997) are also consistent with the presence of a dorsal signal in the vegetal yolk of the oocyte which is subsequently transported to the blastoderm. The translocated dorsal signal is thought to result in the localized activation of the so-called canonical Wnt/β-catenin signaling pathway (reviewed in Pelegri 2003). Initiation of the pathway leads to the stabilization and nuclear localization of β-catenin on the presumptive dorsal side. β-catenin interacts with a member of the tcf/LEF family of transcription factors to activate dorsal gene expression. A maternal-effect mutation in the gene *ichabod* results in a block in the Wnt/β-catenin pathway at the level of β-catenin nuclear localization and leads to ventralization of the embryo (Kelly et al., 2000).

Similarly, *tokkaebi*, another zebrafish recessive maternal-effect mutation causing ventralization, also prevents the nuclear localization of β-catenin (Nojima et al., 2004). Rescue experiments using mRNA expression suggest that *tokkaebi* affects a more upstream component of the Wnt/β-catenin pathway than *ichabod*, namely the regulation of the protein complex responsible for β-catenin degradation. However, the precise nature of this regulation remains to be identified.

The Wnt/β-catenin and Wnt/Ca^{2+} pathways act antagonistically in different contexts (Weidinger and Moon, 2003). Activation of the Wnt/Ca^{2+} pathway by a Wnt ligand through a G-protein coupled receptor results in the hydrolysis of phosphotidylinositol to inositol triphosphate (IP_3), which interacts with an IP_3 receptor on the endoplasmic reticulum to promote Ca^{2+} release. Modulation of the levels of Ca^{2+} and the Wnt/Ca^{2+} pathway in developing cells has been shown to affect dorsoventral patterning by regulating Wnt/β-catenin activity. For instance, in zebrafish, increasing Ca^{2+} levels through a chimeric serotonin receptor inhibits the axis-induction ability of exogenous Wnt8 ligand (Slusarski et al., 1997) and injection of a constitutively active calcium-dependent calmodulin kinase (CamKII) enlarges ventral-specific gene expression domains (Westfall et al., 2003a). Conversely, treatment with pharmacological inhibitors including the phosphoinositide cycle inhibitor L-690,330, the PLC-inhibitor U-73122, or inhibitors of Ca^{2+} release from the endoplasmic reticulum result in ectopic dorsal gene expression domains (Westfall et al., 2003a, b). Recent studies have implicated Wnt5a as the potential endogenous ligand that triggers Wnt/Ca^{2+} activity in early development, as a fraction of zebrafish embryos lacking maternal and zygotic Wnt-5a protein exhibit an expansion of dorsal cell fates at the expense of ventral fates (Westfall et al., 2003a, b). The antagonism of Wnt/β-catenin signaling by Wnt/Ca^{2+} activity suggests that Ca^{2+} released from the endoplasmic reticulum may act through Ca^{2+}-sensitive targets to modulate canonical Wnt signaling. The recessive maternal-effect mutation *hecate* causes an increase in the frequency of Ca^{2+} transients during the blastula stages and a reduction in dorsal gene expression with a corresponding expansion of ventral tissues (Lyman Gingerich et al., 2005). Analysis of the *hecate* mutation suggests a role for this gene in Ca^{2+} modulation, potentially in the Wnt/Ca^{2+} pathway, and supports a role for Ca^{2+} in the regulation of Wnt/β-catenin signaling.

A third pathway important for dorsoventral patterning involves ligands of the TGF-β family, reflecting that induction of ventral cell fates may not be simply a default state in the absence of activation of dorsal gene expression. Embryos depleted of maternal and zygotic Radar, a TGFβ factor of the BMP family, develop extra dorsal tissues at the expense of ventral tissues (Sidi et al., 2003). The binding of TGF-β ligands such as radar to receptors is thought to phosphorylate and activate Smad proteins, which activate ventral-specific genes. This, in turn, activates a subset of Bmp genes, which are expressed zygotically in ventral regions of the embryo (Hammerschmidt and Mullins, 2002). Mutations in the

zygotic gene coding for the secreted protein Ogon also have a maternal contribution and reveals a role for this protein in BMP signaling. Surprisingly, considering its homology to the Wnt pathway regulator Frizzled, Ogon does not appear to regulate Wnt signaling, and rather modulates BMP signaling through a negative feedback loop (Yabe et al., 2003). The identification of mutants affecting dorsoventral patterning in a number of pathways indicates that the initiation and maintenance of the dorsoventral axis is complex and highlights the importance of maternal factors in initiating processes whose resultant phenotype may not be observed until after the MBT. A fourth mutation showing a strict maternal effect, in the gene *brom bones*, has also been reported to affect axis induction (Wagner et al., 2004), although a role for this gene in the various pathways involved in dorsoventral patterning needs to be determined.

Few maternal factors necessary for the determination of the anteroposterior axis have been identified, possibly because, due to its establishment at later developmental stages, anteroposterior patterning may be less dependent on maternal products. The anteroposterior pattern is established by the onset of gastrulation, possibly by a signal from the yolk cell (Koshida et al., 1998; Nikaido et al., 1999). Again, the identification of maternal mutants whose phenotype is not apparent until well after the MBT highlights the importance of maternal factors for initiating relatively late pattern formation events. A maternal-effect mutation in the gene *pug* results in a near complete loss of the tail, as well as additional defects in the maintenance of gene expression characteristic of the midbrain-hindbrain boundary (Wagner et al., 2004), and a mutation in *bobtail* has also been reported to result in tail truncation (Kishimoto et al., 2004). Further research is needed to determine whether these mutant phenotypes derive from a general misspecification of the anteroposterior axis or more specific defects in posterior development. In addition, maternal contribution for the zygotic genes *tcf-3*/*hdl* and *lazarus*/*pbx4* has been shown to play a role in anterior brain specification and rhombomere identity, respectively (Kim et al., 2000; Waskiewicz et al., 2002).

4.6. Germ Layer Specification and Morphogenesis

Just after 50% epiboly, the appearance of the germ ring around the margin of the developing embryo indicates that involution has started (Kimmel et al., 1995). As epiboly continues, two distinct layers of the deep cells of the blastoderm can be identified: the epiblast and the hypoblast, which, at the end of gastrulation, will have been specified as the ectoderm and the mesendoderm, respectively. The movements of the YSL, deep cell layers, and enveloping layer (EVL) are normally tightly coordinated during epiboly and gastrulation.

Some mutations appear to affect all gastrulation movements. The gene *betty boop* is required for progression past 50% epiboly and may be necessary for the initiation of gastrulation (Wagner et al., 2004). At 50% epiboly, *betty boop* mutant embryos show a constriction of the margin followed by lysis of the yolk

cell. Mutations in the genes *mission impossible* and *poky*, on the other hand, lead to generally slowed epiboly movements, as compared to wild-type embryos, and lysis before epiboly is complete (Pelegri et al., 2004; Wagner et al., 2004).

Several mutations have been shown to uncouple the movements of various layers with respect to the others, demonstrating that the control of their migration during epiboly, although coordinated, depends on separate sets of genes. In *slow* and *bedazzled* mutant embryos, epibolic movements of the deep layer are more severely affected than those of the EVL (Wagner et al., 2004). This phenotype is reminiscent of that caused by zygotic mutations in the *half-baked* gene, which lead to defects in epiboly of the deep cells, but not the YSL or the EVL (Kane et al., 1996). Dominant maternal effects caused by mutations in the *half-baked* gene also suggest a role for the maternal *hab* product in epiboly movements (Kane et al., 1996). Thus, it appears that the movements of the EVL, the YSL, and the deep layers during epiboly and gastrulation are tightly regulated, at least in part by maternal factors. Of interest, both *bedazzled* and *half-baked* mutant phenotypes are associated with the presence of loose cells in the embryo, suggestive of a defect in cell adhesion. This possibility has been further substantiated in the case of *half-baked* gene, which has been recently shown to encode an embryonic cadherin (Kane et al., 2005).

Other maternal-effect mutations have been shown to lead to defects in the formation of the body plan, which become apparent at later stages of development. A mutation in *blistered* results in an enlargement of the ventral tail vein (Wagner et al., 2004). Although this phenotype is reminiscent of weak ventralization phenotypes, no apparent ventralization is observed in this mutant during gastrulation, suggesting a different cause for this defect. In addition, a mutation in *pollywog* results in a failure to elevate the head off the yolk at the 24–48 h stage (Wagner et al., 2004).

5. THE TRANSITION BETWEEN MATERNAL AND ZYGOTIC CONTROL

5.1. The Midblastula Transition

Before the MBT, cells alternate between synthesis and mitosis with no intervening gap phases (Zamir et al., 1997). The first cell cycles are highly synchronous, but become metasynchronous and are initiated in waves originating from the animal pole starting at the eighth cell cycle. In the zebrafish, the MBT encompasses cell cycles 10 through 14 and is characterized by the gradual appearance of the G1 phase of the cell cycle (and correspondingly, cell cycle lengthening), a loss of cell synchrony, activation of transcription, and the appearance of cell motility (Kane et al., 1992; Kane and Kimmel, 1993). As in other organisms, the MBT has been shown to depend on the nucleocytoplasmic ratio of individual cells (Kane and Kimmel, 1993; Zamir et al., 1997; Dekens et al., 2003), although the precise mechanism for detecting this ratio and for initiating the changes

associated with the MBT remains unknown. It appears, however, that part of this mechanism depends on new zygotic transcription, as treatment of embryos with the transcriptional inhibitor actinomycin D abolishes the appearance of G1 phase at the MBT (Zamir et al., 1997).

Lack of zygotic transcription before cell cycle 10 may not be applicable to all genes. A systematic analysis of transcript abundance revealed that out of 16,416 genes studied, 125 increased in abundance prior to the MBT (Mathavan et al., 2005). Work by Leung et al. (2003) demonstrates that while mRNA for the Wnt/β-catenin target gene *bozozok* is not maternally provided, it becomes detectable in dorsal cells at the 512 cell stage. Thus, transcription of *bozozok* begins before the bulk activation of the zygotic genome at the MBT. This suggests that a subset of genes, possibly those crucial for early inductive events, such as axis induction and the MBT itself, may be activated during this pre-MBT period.

5.2. Genes with Maternal–Zygotic Contributions

Even once zygotic transcription has been initiated, it is clear that maternal factors remain important for developmental events. Such a maternal requirement can be deduced when the embryonic phenotype is more severe when both the maternal and zygotic genetic constitutions are mutant than when either contribution is mutated separately. When both the mother and the embryo (i.e. the maternal and zygotic contributions) are homozygous for the mutation, embryos are referred to as maternal–zygotic (MZ) mutants. Testing the mutant germ line for zygotic genes requires the creation of a mutant germ line. This can be done in a relatively simple manner when a zygotic mutation is not fully lethal, such that some homozygous individuals can become fertile females, as in the case of *headless*/*tcf* (Kim et al., 2000), *ogon* (Hammerschmidt et al., 1996; Miller-Bertoglio et al., 1999; Wagner and Mullins, 2002), and *schmalspur* (Pogoda et al., 2000). Identification of genes with both a maternal and a zygotic contribution is more difficult when the zygotic mutant phenotype is fully lethal, as homozygous individuals do not reach fertile adulthood. In these cases, rescue of the zygotic phenotype with injection of factors that rescue or bypass the affected pathway allows for the production of viable homozygous adults. For example, injection of *bmp2* RNA rescues *swirled* mutant embryos (Kishimoto et al., 1997). An alternative strategy has been germ-line replacement, where mutant PGCs are transplanted into wild-type hosts at the late blastula stages to create chimeric individuals that are viable (by virtue of their wild-type soma) but carry a mutant subset of germ cells that allow the production of maternally mutant progeny eggs (Ciruna et al., 2002; Waskiewicz et al., 2002).

The increased severity of the phenotype of MZ embryos compared to zygotic mutants may be indicative of an early requirement for the maternal product to pre-pattern the embryo, or of partially redundant functions between the maternal and zygotic contributions. A number of zygotically required genes, when

mutated in the germ line, have been shown to have maternal contributions. For example, embryos deficient in both maternal and zygotic *dicer* have much more severe developmental defects than zygotic *dicer* mutants as the maternal product can function in pre-microRNA processing for up to 10 days (Giraldez et al., 2005). This is also the case for several genes that act in the specification of mesendoderm via a Nodal signaling cascade, such as the one-eyed pinhead (oep) and *schmalspur* (*sur*)/FoxH1/FAST1 genes. MZ mutants for oep (*MZoep*) exhibit a more severe phenotype than zygotic mutants. In addition to the loss of endoderm, *MZoep* mutants completely lack head and trunk mesoderm, indicating the importance of both maternal and zygotic *oep* gene products for mesendoderm induction (Gritsman et al., 1999). A similar MZ requirement has been demonstrated for the *sur*/*FoxH1*/*FAST1* gene, although, in this case, a more limited phenotype suggests that this gene acts at a more downstream step in the Nodal signaling cascade (Pogoda et al., 2000; Sirotkin et al., 2000).

A maternal contribution for zygotic genes has also been demonstrated for several genes involved in brain patterning. While *spiel ohne grezen*/*pou5f1*/*pou2* zygotic mutants have decreased expression of gene markers normally induced at the mid–hindbrain boundary, *MZspg* mutants lack expression of these gene markers and, in addition, lack endoderm (Lunde et al., 2004). Similarly, the maternal contribution of *headless*/*tcf* is required for the development of anterior brain structures (Kim et al., 2000). *lazarus*/*pbx* is another zygotic gene with a demonstrated maternal contribution to brain development, in this case, in the patterning of the hindbrain (Waskiewicz et al., 2002).

In some cases, the maternal and zygotic functions for a given gene appear to be required for different functions. This is the case for the *landlocked* mutant, which encodes *scribble1*. Zygotic *scribble1* function, but not maternal function, is essential for motor neuron migration. However, the MZ mutant has impaired convergent extension movements and zygotic *scribble1* can only partially compensate for the loss of the maternal contribution (Wada et al., 2005). In the case of the gene *yobo,* maternal and zygotic functions appear to be completely separable. Absence of zygotic *yobo* function leads to defects in pigment formation, but reduction of maternal *yobo* function results in strict maternal-effect defects in epibolic movements (Odenthal et al., 1996). Thus, maternal and zygotic contributions can be used either redundantly in the same process or separately in different processes.

The removal of the maternal contribution through the creation of a mutant germ line demonstrates that the maternal gene product can partially compensate for the loss of the zygotic gene product. Interestingly, however, in these cases, removal of the maternal contribution alone (i.e. in embryos carrying at least one copy of the wild-type allele) does not lead to apparent phenotypes. This indicates that for these zygotic genes, the primary function of the gene can be fully carried out by its zygotic expression and the maternal product provides a

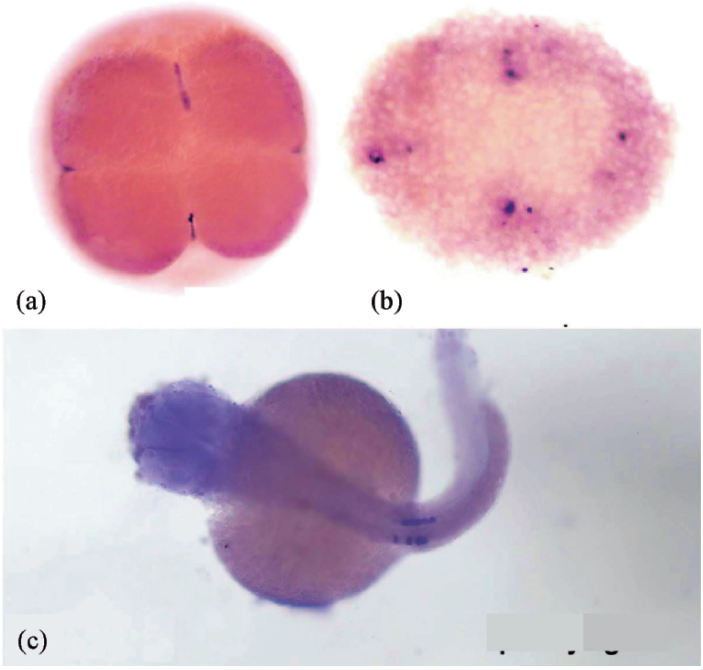

Figure 7. mRNA localization pattern of the *vasa* RNA in the germ plasm and primordial germ cells. (a) *vasa* mRNA, together with other components (see text), localizes to the furrow of the first and second cell cleavages, allowing to visualize the zebrafish germ plasm. Cells that inherit the germ plasm become the primordial germ cells, here visualized as four clusters at the late blastula stage (b) and in the prospective gonadal region in a 24 h embryo (c). (*See Color Plates*).

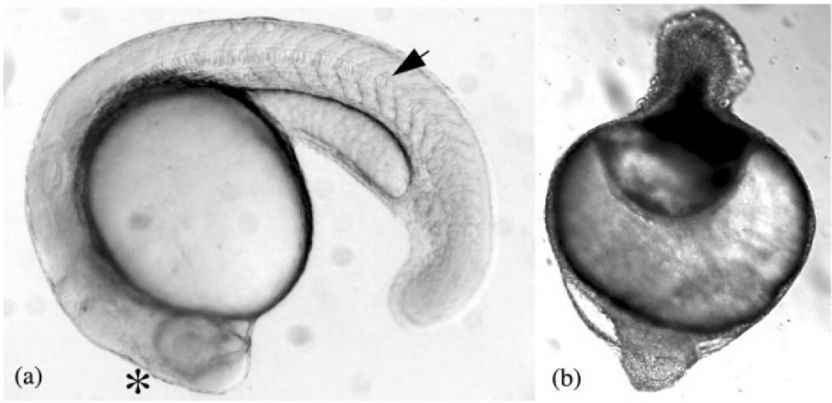

Figure 8. Axis induction defects in *hecate* mutant embryos. (a) wild type 24 h embryo, posterior to the right and dorsal up. Normal anterior and dorsal structures are indicated (asterisk: head; arrowhead: notochord). (b) *hecate* mutant embryo with a strong axis induction defect. The mutant embryo lacks normal dorsoanterior structures and appears radially symmetric (posterior down).

Figure 9. MZ contribution of the *one-eyed pinhead (oep)* gene. (a) wild-type embryo. (b) Zygotically mutant *oep* embryos derived from heterozygous mutant mothers (*Zoep* phenotype), showing cyclopia and a general curvature of the embryo. (c) Zygotically mutant *oep* embryos derived from homozygous *oep* mutant mothers (*MZoep* phenotype), showing a lack of mesendodermal derivatives along the trunk (e.g. v-shaped somites indicated in (A) and (B) with arrowheads). Side views of 24 h embryos with anterior to the left and dorsal up. (Images kindly provided by Dr. Alex Schier, Harvard University). (*See Color Plates*).

supportive redundant role. Whether this is a general phenomenon for zygotic genes awaits the analysis of the maternal contributions of additional zygotic mutations, as well as of potential MZ interactions for mutations found by virtue of their maternal effect.

6. CONCLUSIONS AND PROSPECTS

Molecular, embryological and genetic studies have shown that maternal factors are essential for the earliest stages in development for all animal species, including fish species such as zebrafish, goldfish, and medaka. Maternal factors are indispensable prior to the activation of the zygotic genome at the midblastula transition, but have also been shown to have important functions after this transition. The transition itself from maternal to zygotic control of development appears to be governed at least partially by maternal cues. Initially vaguely defined as activities inferred from embryological studies, the identity and function of these factors are being uncovered by the isolation and analysis of maternal-effect mutations, as well as the molecular identification of the affected genes. Similarly, genes known for their zygotic effect are being analyzed through germ-line replacement for a potential maternal functional contribution (Ciruna et al., 2002; Waskiewicz et al., 2002). Such forward genetic approaches are an invaluable entry point to the molecular pathways involved in oogenesis and early embryogenesis. The development of complementary reverse genetic approaches is currently underway, as genes whose expression is enriched during oogenesis are identified molecularly (Wen et al., 2005) and methodologies are developed in fish and other vertebrates that identify mutations in specific genes (Wienholds et al., 2002) or interfere with genes expressed during oogenesis (Nasevisius and Ekker, 2000; Stein et al., 2003; Babb and Marrs, 2004).

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CHAPTER 7

GONADOTROPIC REGULATION OF OOCYTE DEVELOPMENT

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1. TELEOST GONADOTROPINS

Vertebrate reproduction is regulated by the gonadotropic hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), both synthesized and secreted by the pituitary gland. Their coordinated action enables fine-tuning of ovarian and testicular function. FSH in certain fish (e.g. salmonids) has a dominant role in the initiation of gametogenesis and regulation of gonadal growth, whereas LH is dominant mainly during gonadal maturation and spermiation/ovulation.

Gonadotropin (GtH) duality is well established in tetrapod vertebrates, in seven teleostean orders (Anguilliformes, Cypriniformes, Cyprinodontiformes, Siluriformes, Salmoniformes, Perciformes, and Pleuronectiformes), and has recently been reported even in the more ancient fish taxa, the Dipnoi (Quérat et al., 2004), Chondrostei (Quérat et al., 2000), and Elasmobranchii (Querat et al., 2001). Phylogenetic studies (Li and Ford, 1998; Querat et al., 2000) demonstrated that the piscine GtHs, previously known as GtH I and GtH II are directly related to the tetrapod FSH and LH, respectively. This concept was further confirmed based on the chemical traits of the GtHs and their differential action during the reproductive cycle (Swanson and Dittman, 1997).

1.1. Structural and Evolutionary Considerations

The GtH hormone family includes an additional member, chorionic gonadotropin (CG) produced exclusively by placental trophoblasts of primates and horses, and is essential for normal pregnancy. All GtHs are heterodimers, sharing a common α-subunit and differing in their β-subunits. Both subunits exhibit high content of cysteine residues (C), 10 in the α- and 12 in the β-subunits, forming multiple intramolecular disulfide bonds known to determine the tertiary structure of the molecule. Crystalographic studies of human CG (Lapthorn et al., 1994; Wu et al., 1994) and human FSH (Fox et al., 2001) revealed that the basic scaffold of the α and β-subunits consists of a "cystine knot" motif. The latter is formed by three disulfide bonds, which delineate an elongated structure of three β-hairpin loops. However, the β-subunit is distinguished by a "seat-belt" configuration formed by an additional disulfide bond. This motif enfolds one of the α loops, thus stabilizing the heterodimer (Figure 1). In addition, each subunit contains an asparagine (N)-linked glycosylation sequon encoded by a triad of amino acids ($NX^{T}/_{S}$). Two sequons are present in the α, FSHβ and CGβ-subunits, and only one in LHβ. The N-linked carbohydrates are highly important for structural as well as functional characteristics of the molecules, i.e. folding, subunit assembly, heterodimer secretion, metabolic clearance rate, interaction with the specific receptor and signal transduction (Matzuk et al., 1996; Matzuk and Boime, 1988a, b, 1989; Boime and Ben-Menahem, 1999; Ulloa-Aguirre et al., 2001).

Phylogenetic analyses indicate that teleost FSHβ sequences are more divergent than those of the LHβ, and suggest that the changes in the former have occurred

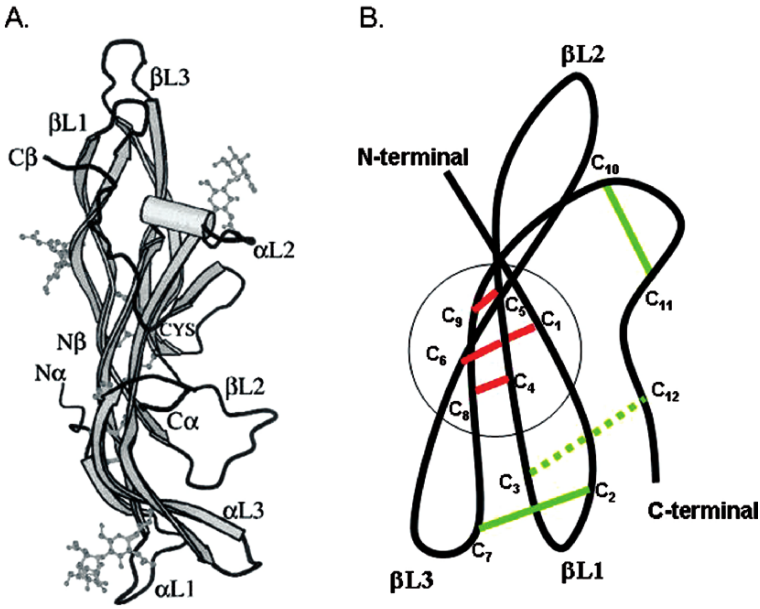

Figure 1. (A) Ribbon diagram showing the crystal structure of human FSH. The α-subunit (light gray ribbon) and the β-subunit (dark gray ribbon) demonstrate the typical fold of "cystine-knot" cytokines. Subunit N- and C-termini, as well as main loops (e.g. αL1, αL2, αL3, βL1, βL2, βL3) are identified. The carbohydrate and the disulfide bonds in both subunits are represented by ball-and-stick models. (B) Schematic structure of human FSH β-subunit showing disulfide bond pairings. The "cystine knot" motif is circled, and the "seatbelt" configuration that distinguishes the β-subunit from the α-subunit is marked with dashed line. The figure was prepared by using the program MOLSCRIPT. (Kraulis, 1991.) (*See Color Plates*).

by periodical bursts (Rosenfeld, 2003). Such a pattern of slow basal rate with bursts of rapid change typifies other pituitary hormones, including growth hormone (Wallis, 1996), and prolactin (Wallis, 2000), but differs from that characterizing the mammalian FSHβ (Wallis, 2001). Interestingly, the LHβ data exhibit completely distinct patterns; with a slow basal rate of evolution among all teleost lineages, and episodes of rapid change in mammals, especially in *Perissodactyla*, *Artiodactyla*, and *Primates* (Wallis, 2001).

Zooming in on the N-terminal of the teleosts FSHβ sequences shows an unexpected divergence of the most conservative amino acid residues, namely, asparagine (N), which establishes the N-linked glycosylation sequon, and cysteine (C) (Figure 2). It would appear that the representative of lower classes of fish, i.e. dogfish, sturgeon and eel, stayed closer to the ancestral pattern seen in tetrapods, which consists of 12 C and two N-linked putative glycosylation sites. In general, the representatives of the superorder *Ostariophysi* (e.g. cyprinids and catfish) possess an additional C at the N-terminal, probably due to relocation of

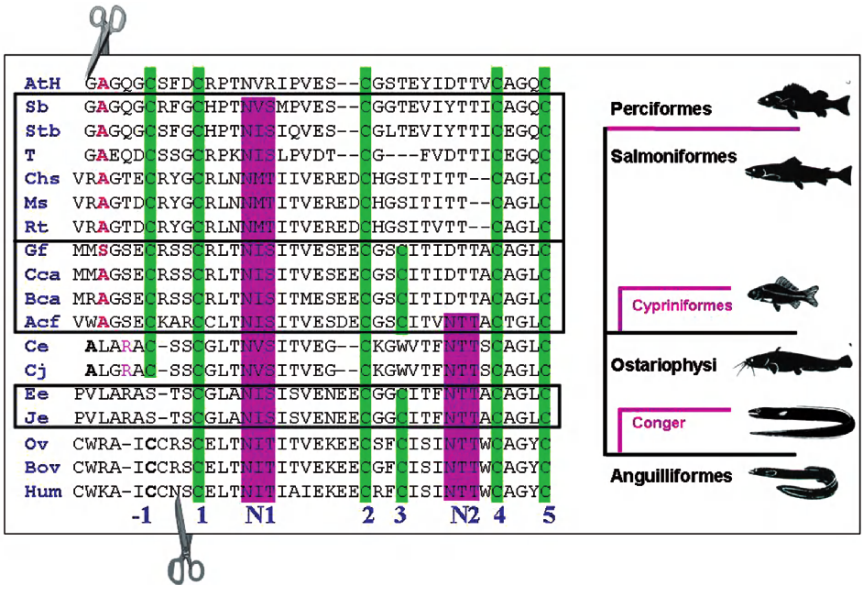

Figure 2. (Right panel) Evolutionary model for the FSHβ molecules in teleosts. The pink branches represent the lineages exhibiting semiparallelism. (Left panel) Multiple sequence alignment of the FSHβ N-terminal. The sequences include a small portion of the signal peptide enclosing the tentative cleavage site (indicated by scissors). Cysteine residues and putative N-linked glycosylation sites are highlighted with green and pink background, respectively. (*See Color Plates*).

the signal-peptide cleavage site, making a total of 13 C residues. Salmonid and perciform fish possess the most divergent FSHβ sequences consisting of a single N-linked putative glycosylation site and 12 C residues. The second N and the fourth C (counting from the N-terminal) were replaced by other amino acids as a result of nonsynonymous substitutions. Noticeably C residues arrangement, which does not resemble any of the aforementioned patterns, was identified in zebrafish FSHβ sequence (So et al., 2005), whereas a complete absent of N-linked putative glycosylation sites, is represented by the Atlantic halibut FSHβ sequence (Weltzien et al., 2003a). The physiological roles of the observed structural changes are not yet clear. However, it is argued that the C residues alteration affects the formation of the seatbelt, and consequently narrows the gap in the β-subunit through which the loop of the α-subunit is straddled. This change is known to increase heterodimer stability (Xing et al., 2004).

1.2. Cellular Localization

Vertebrate GtHs are mainly produced in the gonadotropic cells of the adenohypophysis. The mammalian gonadotrophs constitute approximately 20% of adenohypophysial cells, and span three cell populations. The majority (60%)

produces both FSH and LH, whereas the minority represents monohormonal gonadotrophs producing either LH (18%) or FSH (22%). Unlike the situation in mammals, teleost pituitary consists only of two monohormonal cell populations (FSH or LH producing cells) which are distinguished by their specific location in the proximal pars distalis. The cells producing FSH occupy a position adjacent to the somatotrophs, surrounding the nerve ramification, while those producing LH occupy a more peripheral position of the proximal pars distalis (Figure 3). The spatial distribution of the gonadotroph cells was first established in salmonids (Nozaki et al., 1990; Naito et al., 1991), and was further confirmed in other fish species, including: tilapia (Melamed et al., 1998; Parhar et al., 2003a), tuna (Kagawa et al., 1998a), gilthead sea bream (Elizur et al., 2000), African catfish (Vischer et al., 2003a), Atlantic halibut (Weltzien et al., 2003b), and zebrafish (So et al., 2005).

Recently, using *in situ* hybridization (ISH) and RT-PCR, which expanded detection sensitivity, it was shown that both GtHs are being produced, albeit to a lesser extend, in some extra-pituitary tissues including brain, gonads, kidney, and liver (Parhar et al., 2003b; Wong and Zohar, 2004; So et al., 2005). Studies with tilapia and sockeye salmon suggested that the brain derived GtHs may control their own synthesis and release through their actions on GnRH neurons and also on gonadotrophs (Parhar et al., 2003b). Other studies with the gilthead sea bream revealed temporal expression of ovarian derived GtHs, and demonstrated their enhanced expression and secretion in response to GnRH stimulation (Wong and Zohar, 2004). It was postulated that the ovarian GnRH-GtH axis might have a role in teleost intraovarian communication between oocytes and ovarian follicular cells. It is worth mentioning, that extra pituitary GtHs

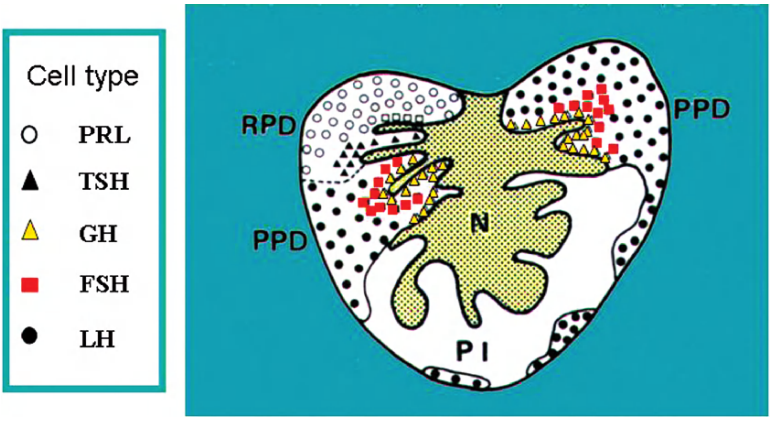

Figure 3. Cartoon representing a typical teleost adenohypophysis, with relative localization of the gonadotrophs, LH and FSH producing cells. *Abbreviations*: GH – growth hormone; PRL – prolactin; TSH – thyroid-stimulating hormone; N – neurohypophysis; PI – pars intermidia; PPD – proximal pars distalis; RPD – rostral pars distalis. (*See Color Plates*).

were also detected in mammals including humans (Emanuele et al., 1981; Goldsmith et al., 1983; Markkula et al., 1995, 1996; Zhang et al., 1995), however, their physiological roles are still to be clarified.

2. ROLE OF PITUITARY GtHs IN FEMALE REPRODUCTION

Until the mid-1980s it was believed that the pituitary of fish produces a single GtH. However, since the pioneering isolation of two distinct GtHs from salmon pituitaries (Kawauchi et al., 1989) and the detection of this duality in other fish species (Swanson and Dittman, 1997; Li and Ford, 1998; Querat et al., 2000, 2001, 2004) considerable effort have been made to explore the specific function of the two GtHs in teleost reproduction. Nevertheless, our understanding is still far from being comprehensive, mainly due to the variety of reproductive strategies found among fishes.

The following account briefly surveys current knowledge on two common reproductive strategies among oviparous fish. The first model is represented by fish with a group-synchronous ovarian development, characterized by two oocyte populations: primary oocytes, from which the later stages will be recruited and developmentally homogeneous oocytes that will ovulate and spawned as a single batch. The second model is represented by multi-spawner fish, with an asynchronous ovarian development (Figure 4), having at the same time several generations of oocytes, each at a different stage of development.

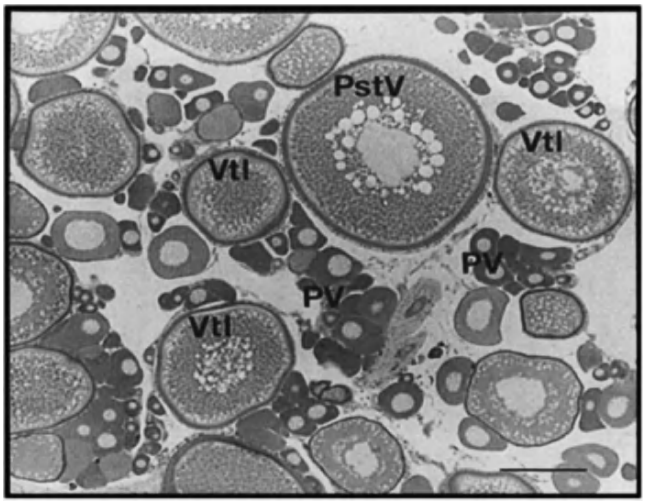

Figure 4. Histological section of gilthead sea bream ovary demonstrating asynchronous oocyte development. *Abbreviations*: PV – previtellogenic oocyte; Vtl – vitellogenic oocyte**;** PstV – postvitellogenic oocyte.

2.1. Models Typifying a Synchronous Ovarian Development

Accumulated data from ISH and immunocytochemical (ICC) analyses has demonstrated that FSH-producing cells are widespread in the pituitaries of sexually immature and vitellogenic fish, while cells producing LH are more abundant in the pituitaries of mature fish (Nozaki et al., 1990; Naito et al., 1991). The temporal profiles of circulating FSH and LH in salmon and trout further support the suggestion that FSH has a predominant role during early oogenesis while LH function is restricted to the phase of oocyte maturation and ovulation. In female trout plasma FSH increases at the onset of oocyte secondary growth (i.e. early stages of vitellogenesis), then declines to a basal level and rises again prior to ovulation (Prat et al., 1996; Breton et al., 1998; Davies et al., 1999; Gomez et al., 1999; Santos et al., 2001). Such an accord between FSH plasma levels and vitellogenic phase in the rainbow trout was also observed when vitellogenesis was induced by shifting photoperiod regime (Bon et al., 1999). Following spawning (or after manual stripping of ovulated eggs), plasma FSH levels increase again during recruitment of a new oocytes generation (Breton et al., 1998; Chyb et al., 1999), whereas, the circulating levels of LH remain undetectable or low throughout most of the reproductive cycle, rising just prior to ovulation (Prat et al., 1996; Breton et al., 1998; Davies et al., 1999; Gomez et al., 1999; Santos et al., 2001). Similar patterns, albeit at the LHβ and FSHβ gene expression levels, were reported in other fishes with synchronous ovary the striped bass (Hassin et al., 1999), and in the viviparous rockfish, (Kim et al., 2005). Yet, it is important to note that among non-salmonid group synchronous spawners, and even among salmonid species there are many exceptions exhibiting variations of the aforementioned generalized GtH patterns. For example, in the coho salmon (Swanson et al., 1991) the plasma FSH levels remain high throughout vitellogenesis but decline later, being low during oocyte maturation and ovulation without showing the "two-peak pattern" as in trout. Another example illustrates a different pattern for LH in the African catfish (Schulz et al., 1997), black carp (Gur et al., 2000), and sea bass (Mateos et al., 2003). In these species, LH levels are already detectable in juveniles, steadily increasing concomitant with gonadal growth and reach climax upon maturation.

Studies aimed at verifying the activities of purified FSH and LH have shown that both GtHs can stimulate, in a dose- and time-dependent manner, steroid production by the salmonid ovarian tissue (Suzuki et al., 1988; Swanson et al., 1989; Planas et al., 1997, 2000; Montserrat et al., 2004). Nevertheless, their steroidogenic potency varies according to the state of gonadal development (Planas et al., 1997, 2000). LH and FSH have similar potencies in stimulating estradiol (E2) production during early phases of oogenesis; yet, LH is more potent during later stages of the process (Suzuki et al., 1988; Planas et al., 1997). In addition, using coho salmon intact follicles and isolated follicular layers, it was found that LH exclusively stimulates the production of the maturation-inducing steroid (MIS), 17,20βP, in granulose layers isolated from developmentally sorted

oocytes (i.e. from late vitellogenesis until the completion of GVBD) (Planas et al., 2000). Other exclusive activities for LH include the induction of GVBD in intact follicles (Planas et al., 2000) and, as suggested by Jalabert (2005), quality preservation of ovulated eggs when these are retained a long time in the body cavity. The FSH exclusive functions that complement those of LH in salmonid females are: (i) triggering the uptake of vitellogenin by the growing oocytes (Tyler et al., 1991); (ii) stimulating the development of a new cohort of follicles, as indicated by the rainbow trout unilateral ovariectomy (Tyler et al., 1997); and (iii) augmenting LH function, probably by upregulating the expression of LH receptors, during the acquisition of maturational competence (Bobe et al., 2003, 2004). Another function attributed to FSH is stimulation of cytochrom P-450 aromatase activity and gene expression (Montserrat et al., 2004). However, in order to attribute it exclusively to FSH a similar experiment has to be conducted with LH.

2.2. Models Typifying an Asynchronous Ovarian Development

Unlike the extensive data describing the gonadotropic interplay that regulates synchronous ovarian development in salmonid species, reports describing the situation in multiple spawners are by far fewer, being based merely on hormonal profiles of LH and/or temporal transcript levels of the GtH-subunit genes. So far, all studies with multiple spawners indicate the expression of both FSHβ and LHβ genes as from the very early phases of gonadal development, yet, their seasonal profiles exhibit a certain degree of species variations (reviewed by Yaron et al., 2001, 2003; Swanson et al., 2003). In general, these variations could be classified within three major models describing: (i) a close correlation between the related transcript levels and the GSI values, with the FSHβ mRNA peak at the early stages of gonadal development and ebb in fish with higher GSI values, whereas those of LHβ are high in fish with most developed gonads (tilapia: Melamed et al., 1997); (ii) a parallel fluctuation for both FSHβ and LHβ transcripts positively correlated with the progression of ovarian maturity (blue gourami: Jackson et al., 1999; goldfish: Sohn et al., 1999; Japanese flounder: Kajimura et al., 2001; gilthead sea bream: Meiri et al., 2004); and (iii) a pattern characterized by high levels for LHβ transcripts and circulating LH from the beginning of oogenesis to spawning, and low levels for FSHβ transcripts throughout ovarian maturation (red sea bream: Gen et al., 2000, 2003; Kagawa et al., 2003). The first two models reflect, more or less, the nature of the asynchronous ovarian development and emphasize the necessity of the complement action of FSH and LH, to support the development of oocytes at all stages, coexisting during the spawning season. The third model, however, is fairly exceptional inasmuch as it suggests predominant roles for LH and negligible roles (if any) for FSH, all through the reproductive cycle (Gen et al., 2003).

Functional analyses using native or recombinant GtHs, and the homologous *in vitro* ovarian bioassays, highlighted several similarities shared by multi- and

synchronous-spawners, including (i) capacity of both LH and FSH to stimulate the production of E2 (tuna: Okada et al., 1994; red sea bream: Tanaka et al., 1995; gilthead sea bream: Meiri et al., 2000; killifish: Lin et al., 2004); (ii) the capacity of FSH to augment LH function (gilthead sea bream: Meiri et al., 2000; zebrafish: So et al., 2005); and (iii) exclusive abilities of LH to elicit MIS (killifish: Lin et al., 2004), to induce final oocyte maturation as well as the development of oocyte maturational competence (red sea bream: Kagawa et al., 1998b), and preserve the quality of fully mature oocytes and ovulated eggs (gilthead sea bream: Meiri and Rosenfeld, 2004). Nevertheless, many examples indicating dissimilarity between the multi- and synchronous-spawners were reported as well. For instance, the carp FSH and LH were found to be equipotent in stimulating GVBD in the goldfish (Van der Kraak et al., 1992), and LH but not FSH, was found to stimulate cytochrom P-450 aromatase activity and transcript levels in the red sea bream (Kagawa et al., 2003).

In view of the above, it is apparent that teleost gonadotropic interplay exhibits a wide range of variations, which crosses over most noticeable categories including the species' phylogenetic affiliation, its reproductive mode, or any combination of the two. This can be well exemplified by the distinct patterns observed in striped bass (Hassin et al., 1999) and sea bass (Mateos et al., 2003), both of which are group-synchronous spawners and members of the family Moronidae. Many attempts to explain this enigma (Swanson et al., 2003; Yaron et al., 2003; Yaron and Sivan, 2006) pointed out the rapid change of the FSH molecule during the evolution of teleosts lineages and the concurrent changes in cognate GtHs receptors (see next paragraph) as major effectors. These changes seem to allow teleostean FSH to slightly drift from its classical activities, leaving these to be fulfilled by its counterpart, LH. Hence, the red sea bream model seems to attest an extreme situation in which LH takes over most of functions attributed to FSH.

3. TELEOST GTH RECEPTORS

3.1. Structural Characteristics

The GtHs, like other members of the glycoprotein hormone family, stimulate target tissues through receptors displayed on the cell surface. These receptors belong to a large family of G protein-coupled receptors (GPCRs), yet, they are distinguished with an exceptionally large extracellular domain, arranged as leucine-rich repeats (LRR) (Milgrom et al., 1997; Hai et al., 1999). Recently, genomic studies in model organisms, including invertebrates, unveiled some new members of the LRR-GPCR family, which implies a common ancestral origin for these receptors (reviewed by Park et al., 2005).

The general structure of the LRR-GPCRs (Figure 5) consists of four major components (reviewed by Fan and Hendrickson, 2005; Moyle et al., 2005). The first component is a leucine-rich domain (LRD), mainly responsible for hormone

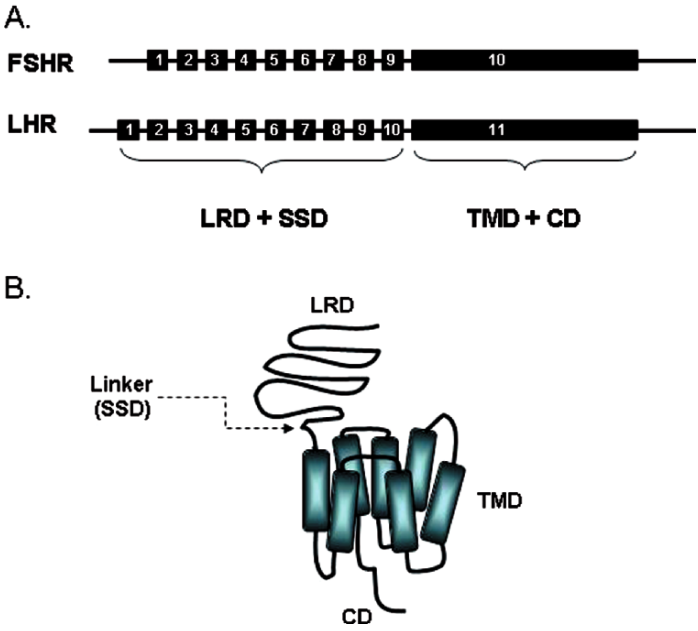

Figure 5. Structural characteristics of the GtHR genes (A) and proteins (B). The gene xons are represented by black boxes and are numbered (10 and 11 exons in the genes encoding for FSHR and LHR, respectively). *Abbreviations*: CD – cytoplasmic domain; LRD – leucine-rich repeat domain; SSD-signaling-specificity domain; TMD – transmembrane domain. (*See Color Plates*).

recognition and binding. The LRR are composed of alternating α-helices and β-sheets that provide an extensive contact surface with other proteins. Additionally, the LRR motif is flanked at its N- and C-terminal ends by cysteine clusters stabilizing the whole structure via disulfide bridges. The second component is a "linker" formed by varying portions of the C-terminal region of the extracellular domain. In some models describing receptor–ligand interactions, it is thought to serve as a "hinge" that places the LRD/ligand complex in an appropriate position for interaction with other parts of the receptor (Fan and Hendrickson, 2005), whereas in other models it is considered as a signaling-specificity domain (SSD) reflecting its roles in ligand binding and signal transduction (Moyle et al., 2004). The third component is a transmembrane domain (TMD) of seven-membrane traversing α-helices connected by three extracellular and three intracellular loops. The forth component is a short cytoplasmic domain (CD) that forms, together with the cytoplasmic parts of the TMD, the interaction domain with G proteins. The genes encoding for the FSH receptor (FSHR) and the LH receptor (LHR) consist of 10 and 11 exons, respectively. In higher vertebrates, the last exon within these genes encodes for the TMD and

CD, while other exons encode for the extracellular domain (Simoni et al., 1997; Dufau, 1998). Both genes exhibit multiple splice variants, differing in their extracellular domain, which may have different functions (Ji and Ji, 1991; Tena-Sempere et al., 1994).

The dual nature of fish GtHRs was first established in salmonids by using ligand-binding assays (Yan et al., 1992). Since then, the cDNAs encoding for both GtHR types have been cloned from several fish species including: amago salmon (Oba et al., 1999a, b), African catfish (Bogerd et al., 2001; Vischer and Bogerd, 2003), channel catfish (Kumar et al., 2001; Kumar and Trant, 2001), zebrafish (Laan et al., 2002, Kwok et al., 2005), medaka (Hirai et al., 2003), gilthead sea bream (Wong and Zohar, 2004), sea bass (Rocha et al., 2004), Japanese eel (GeneBank accession numbers: AY742794 and AY742795), and tilapia (GeneBank accession numbers: AB041762, AB041763). Phylogenetic analyses revealed that the fish GTHRs are genetically comparable to tetrapod FSHR and LHR, sharing higher homology to their mammalian counterparts than to each other (reviewed by Kumar and Trant, 2001; Oba et al., 2001).

3.2. Tissue Distribution and Expression Profiles

In the mammalian ovary FSH binds exclusively to FSHRs in granulosa cells, whereas LH binds its cognate receptors in thecal cells (Griswold et al., 1995). Nevertheless, this model describing "two cell types for two GtHs" was found to be inapplicable to teleost ovary. Ligand-binding studies carried out with coho salmon vitellogenic oocytes defined similar binding site to both FSH and LH within theca and granulosa layers (Miwa et al., 1994). In postvitellogenic and preovolatory oocytes the FSH/LH-binding sites were confined to the theca layer, whereas exclusive binding sites for LH were restricted to granulosa layer. Similar results that were achieved in the amago salmon further corroborate the existence of two-receptor types for salmonind GtHs (Oba et al., 1999b). Studies in rainbow trout using RT-PCR analyses showed an increased expression of FSHR during the acquisition of maturation competence (FMC), with no differential expression for cognate LHR (Bobe et al., 2003, 2004). Since LH is the determining factor stimulating the production of MIS and the induction of meiosis resumption in salmonids, the physiological relevance of FSHR at the stage of FMC needs to be clarified.

In tilapia, a multi-spawner species, the transcript profiles of FSHR and LHR (Oba et al., 2001), appear to follow the expression patterns of the respective FSHβ and LHβ, ligand-specific subunits (Melamed et al., 1996). Accordingly, the FSHR transcripts reach maximal levels during early vitellogenesis, decrease at late vitellogenesis and increase again after maturation; whereas those of LHR are low at early vitellogenesis, gradually increase during the process and peak in fully grown oocytes. In zebrafish, like in tilapia, the expression of FSHR is closely associated with vitellogenesis while those of LHR are associated with final oocyte maturation (Kwok et al., 2005). The aforementioned

GTHR transcript profiles, which emphasize the predominant roles of FSH and LH in the respective stages of vitellogenesis and final oocyte maturation, should not be surprising since most reproductive modes studied so far in fish were found to rely on some degree of complementary function of FSH and LH. In this regard, it should be of great interest to trail FSHR and LHR profiles in the red sea bream exemplifying a unique model in which LH is dominating both during vitellogenesis and final oocyte maturation (Gen et al., 2000, 2003).

Unlike the amago salmon, in which the tissue expression of FSHR and LHR was found to be highly specific to gonads (Oba et al., 1999a, b), in catfish (Kumar et al., 2001), sea bass (Rocha et al., 2004), and zebrafish (Kwok et al., 2005) the related transcripts were detected in some extra-gonadal tissues including kidney and liver. The extragonadal expression of GTHR was reported also in bullfrog (Kubokawa and Ishii, 1987) and human (Pabon et al., 1996; Rao Ch et al., 2004). These results suggest some nonclassical roles for LH and FSH, which are still to be explored.

Interestingly, ISH analysis revealed that in addition to LHR and FSHR, TSHR is also being expressed in the fish gonads, which would suggest its direct role in gametogenesis (Hirai et al., 2000; Kumar et al., 2000).

3.3. Receptor–Ligand Specificity

In mammals the interactions of LH and FSH with cognate receptors are highly specific (Braun et al., 1991; Tilly et al., 1992). Nevertheless, equivalent receptor–ligand interactions in teleosts demonstrate merely a loose specificity. All studies carried out so far with fish species including salmonids (Yan et al., 1992, Miwa et al., 1994, Oba et al., 1999a, b), catfish (Bogerd et al., 2001; Vischer and Bogerd, 2003; Vischer et al., 2003b), and zebrafish (So et al., 2005) show a similar trend, in which the FSHR is capable of binding both FSH and LH albeit with some preference to the homologous ligand.

Another peculiar aspect typifying the GTH–GTHR interactions in vertebrates including teleosts is the ability of heterologous GTHs to trigger biological activities that do not necessarily reflect those of endogenous hormones. For example: chicken LH effectively binds the rat FSHR (Iwasawa et al., 1998), and pregnant mare serum gonadotropin (PMSG), the LH-like hormone, efficiently binds both the LHR and FSHR in rats (Licht et al., 1979). In zebrafish, bovine FSH and LH were found to reciprocate their ligand–receptor specificity compared to endogenous hormones, inasmuch as the bovine LH is highly specific to zebrafish LHR, whereas bovine FSH effectively binds both zebrafish GtHR types (Kwok et al., 2005).

In view of the above situation, many models have challenged the puzzling manner in which the glycoprotein hormones interact with their receptors. The basic model hypothesized that all three glycoprotein hormones (LH, FSH, and TSH) contact a similar region of the curved in surface of the LRD, whereas charge interactions regulate ligand–receptor binding specificity (reviewed by

Remy et al., 1996). Accordingly, the hormone contacts the LRD via parts of the α-subunit C-terminus, β-subunit loop 2, and most of the "determinant loop" (i.e. residues between C_{10} and C_{11} forming the small seatbelt loop). The linker in this model functions as a "hinge" that turns the LRD/ligand complex so that a second surface of the ligand can interact with the TMD, upon which cell signaling is triggered. Nevertheless, among many other shortcoming of this model, it fails to explain the promiscuous characteristics of fish FSHR that binds both LH and FSH despite their structural dissimilarities. These dissimilarities affect the overall conformation of the respective seatbelt loops (see above) known to be essential for receptor selectivity in mammals (Campbell et al., 1991; Dias et al., 1994; Moyle et al., 1994; Grossmann et al., 1997) as well as in fish (Vischer et al., 2003c, d, 2004).

A recent model suggests an alternative interacting unit consisting of three receptor components: the LRD, SSD, and TMD (reviewed by Moyle et al., 2004, 2005). In this model the glycoprotein hormones match up with their receptors via a pocket formed by both the LRD and SSD. These interactions evoke structural change of the TMD, which, in turn, promotes cell signaling. The specific features of the pocket (i.e. repeated motifs within the LRD) seem to tolerate at least two orientations for effective ligand binding. The specific docking site for LH is situated at the central region of the LRD, whereas the respective docking sites for FSH and TSH are situated at the C-terminus of the LRD (reviewed by Moyle et al., 2004, 2005; Bogerd et al., 2005). In parallel, different parts of each ligand contribute to receptor-binding specificity. For example, within the seatbelt loop, the residues between C_{10} and C_{11} are more important for LH binding, while residues between C_{11} and C_{12} are more important for FSH and TSH binding (Moyle et al., 1994, 2004, 2005; Vischer et al., 2004). The aforementioned dual ligand-binding capacity has the potential for independent evolvement of each docking site, without demolishing receptor activity. Moreover it allows multifactorial interactions, including the use of a specific receptor for several functions, and/or the activation of several receptors by the same hormone.

In view of the above, it would appear that Moyle's model, together with the common hypothesis regarding the expansion of the glycoprotein hormone family and their receptors through gene duplication (reviewed by Park et al., 2005), provide a rational mechanism through which ligand–receptor coevolved and eventually created multiple tissue-specific endocrine systems in vertebrates. Nevertheless, these models fall short of anticipating what would be the functional fate of FSH in teleosts. The rapid changes of the hormone-specific β-subunit (FSHβ) across teleost lineages concomitant with FSHR loosen ligand-binding specificity, and the overriding importance of LH during preovulatory follicular development in some fish species, attest some degree of subfunctionalization. Yet, bearing in mind that there is no apparent selective pressure to maintain redundancy of two hormones (e.g. FSH and LH) performing the same activities (Rastogi and Liberles, 2005), such subfunctionalization of fish

FSH may serve as a transition state to neo-functionalization rather than as a terminal fate.

4. POST-RECEPTOR SIGNALING SYSTEMS

4.1. Developmentally Related GtH Response

In fish, as in other nonmammalian vertebrates, ovarian paracrine-signaling cascades activated by GtHs reinforce steroidogenesis along with vitellogenesis, and promote a remarkable growth of the oocyte itself. Excluding species like killifish and medaka, the steroidogenic process during vitellogenesis involves the synthesis of testosterone (T) in theca cells and its conversion by aromatase to E2 in granulosa cells (Nagahama, 1994; Nagahama et al., 1994). As ovarian maturation approaches, a pituitary LH surge triggers a switch in steroidogenic activity of the two cell layers, so that granulosa cells synthesize the MIS (a C21-steroids) instead of E2 (Nagahama, 1994; Nagahama et al., 1994; Selman et al., 1994). This switch from E2 to MIS production leads to completion of oocyte maturation, and release of a fertilizable egg.

In salmonids and other teleosts there is ample evidence indicating that LH, rather than FSH, is capable of stimulating the production of MIS (Planas et al., 1997, 2000; Kagawa et al., 1998b), most likely via highly specific interaction with the LHR, which appears on granulosa cells only at the preovulatory stage (Yan et al., 1992, Miwa et al., 1994). Nevertheless, at earlier stages, i.e. during vitellogenesis, the relative roles of teleost FSH and LH are less perceptible, largely because both can stimulate *in vitro* the production of ovarian T and E2 (Suzuki et al., 1988; Swanson et al., 1989; Van der Kraak et al., 1992; Planas et al., 1993, 1997; Planas and Swanson, 1995), and because both can activate FSHR (reviewed by Bogerd et al., 2005) displayed on theca and granulosa cells throughout oogenesis (Yan et al., 1992; Miwa et al., 1994).

The fact that GtHs support a changeable activity throughout the follicular development raises a fundamental question concerning how the cellular responses to FSH and LH are altered. This enigma is even further emphasized in teleost species that do not exhibit the classic follicular model of "two-cell types for two-GtHs." Considering the multiple pathways involved in mediating and modulating GtH signals (see below), it was postulated that binding of different ligands to the same receptor type may stimulate divergence signaling pathways, and consequently may dictate altered cell functions (Planas et al., 1997). Following the same rational, altered cell functions could also be the outcome of a change in the relative abundance of the FSHR and LHR on the follicular cells, as detected by ligand-binding assays in mature salmonid oocytes (Miwa et al., 1994; Oba et al., 1999b), and by RT-PCR analyses in rainbow trout (Bobe et al., 2003, 2004) and goldfish (Kwok et al., 2005). However, further research should be conducted to verify these hypotheses, and to elucidate the nature of GtH–GtHR interactions and their consequences on follicular cell functions in teleost species.

4.2. cAMP-Dependent and cAMP-Independent Signal Transduction Mechanisms

The canonical post-receptor signaling systems that convey FSH and LH action on theca and granulosa cells are based on adenylyl cyclase (AC), cAMP production and activation of protein kinase A (PKA) (Figure 6). In teleosts too, *in vitro* studies with ovarian fragments have shown activation of the cAMP/PKA pathway by GtHs (Fontaine et al., 1972; Idler et al., 1975; Salmon et al., 1985; Van der Kraak, 1992; Planas et al., 1997), and vice versa, stimulation of ovarian steroidogenesis by exposure to cAMP analogues (e.g. dbcAMP) or by exposure to forskolin, a direct activators of AC (Bogomolnaya and Yaron, 1984; Nagahama et al., 1984, 1991; Tan et al., 1986; Kanamori and Nagahama, 1988; Van der Kraak and Chang, 1990). Recently, transient transfection studies that have used heterologous cell lines over-expressing teleost FSHR or LHR

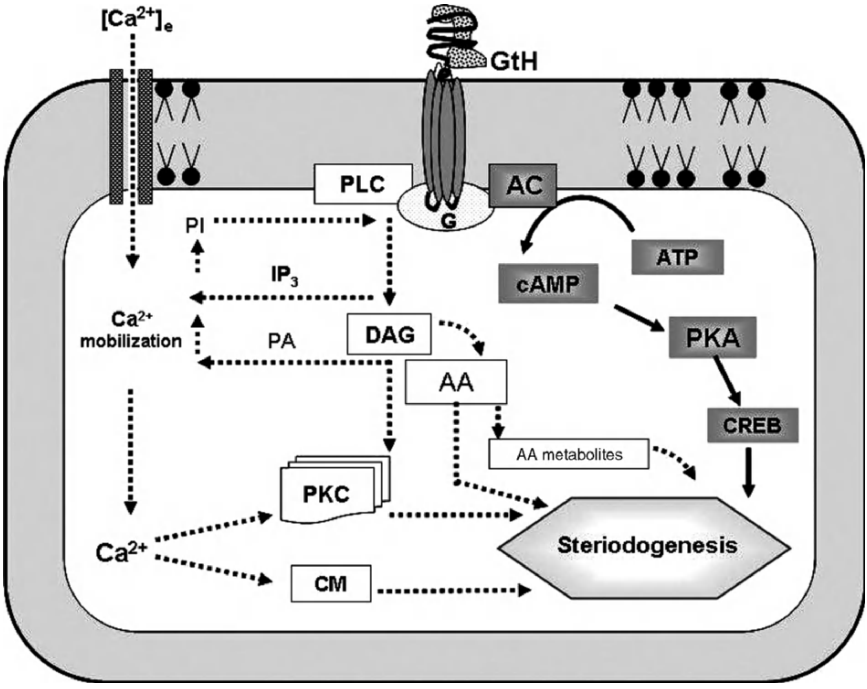

Figure 6. Major intracellular signal transduction pathways known to mediate GtH action into theca and granulosa cells. The full and dashed arrows represent cAMP-dependent and cAMP-independent signal transduction mechanisms, respectively. *Abbreviations*: AA – aracidonic acid; AC – adenylate cyclase; $[Ca^{+2}]_e$ – extracellular calcium entry trough a voltage-sensitive calcium channel; Ca^{+2} – intracellular calcium stores; CM – calmodulin; CREB – cAMP-response element binding protein; DAG – diacylglycerol; IP_3 – inositol 1,4,5 trisphosphate; PKA – protein kinase A; PKC – protein kinase C; PLC – phospholipase C.

provided solid evidence that the activation of both receptor types is coupled to the cAMP/PKA signaling pathway (Bogerd et al., 2001; Vischer and Bogerd, 2003; Zmora et al., 2003; Rocha et al., 2004; Kwok et al., 2005). In these studies, a ligand-stimulated cAMP production and/or ligand-activation of a chimeric reporter gene that contains a *cis*-acting cAMP response element (CRE) were demonstrated.

Binding of GtHs to their receptors can also activate various cAMP-independent mechanisms, including phospholipase C (PLC), arachidonic acid (AA), Ca2+/calmodulin, and chloride ions (reviewed by Van der Kraak and Wade, 1994; Cooke, 1999). These signaling systems may operate independently or may interact with the cAMP–PKA and/or protein kinase C (PKC) pathways (Figure 5). Accumulating data have corroborated the involvement of cAMP-independent signaling in teleost fish as well. In this regard, Ca^{2+} mobilization from extracellular compartment through voltage-sensitive calcium channels, and from intracellular stores via inositol-1,4,5-triphosphate (IP_3; metabolite of the phosphoinositide cycle) and calmodulin (Ca^{2+} binding protein) were found to mediate GtH effects on ovarian steroidogenesis in goldfish (Van der Kraak, 1991; Van der Kraak and Jacobson, 1991; Van der Kraak and Wade, 1994), and in Atlantic croaker (Benninghoff and Thomas, 2005). The latter study with Atlantic croaker highlighted ovarian aromatase as a key candidate for the Ca^{2+} regulation. It is noteworthy that ovarian responsiveness to Ca^{2+} may completely change during the course of follicle development. For example, *in vitro* studies with goldfish follicles that were sorted according to developmental stages have shown that increased intracellular concentrations of Ca^{2+} downregulated the hCG-stimulated steroid production in follicles at the vitellogenic stage, but upregulated the respective process in prematurational follicles (Srivastava and Van der Kraak, 1994). Developmental modulation of the LH and FSH steroidogenic effects by PKC/Ca^{2+} was also detected in the rainbow trout (Planas et al., 1997). It was suggested that PKC directly interferes with the FSH signaling in vitellogenic follicles, but does not interfere with the signal cascade triggered by the LH in prematurational follicles. Other studies indicated that AA, the active metabolite of the PLA_2 on membrane phospholipids, stimulates ovarian production of T in goldfish (Van der Kraak and Chang, 1990, Mercure and Van Der Kraak, 1996), and enhances the GtH-induced oocyte maturation in sea bass (Sorbera et al., 2001). Nevertheless, the latter effect was not observed in Atlantic croaker (Patino et al., 2003).

In addition to the aforementioned GtH-signal transduction pathways, emerging data indicate that GtHR, like other GPCRs can also interact with various cytoplasmic scaffold and adapter proteins, which can link the receptors to a range of signaling intermediates and intracellular effectors (reviewed by Hall and Lefkowitz, 2002; Dias et al., 2005). Although of a preliminary nature, these findings amass multiple options that provide a good platform for fine-tuning and modulating the GtH stimuli.

4.3. Intrafollicular Interactions and Potential Modulators of the Gonadotropic Stimuli

The follicles of all studied vertebrates, from fish to mammals, exemplify a dynamic microenvironment, in which the somatic granulosa/theca cells and the oocyte communicate with each other through a network of locally produced factors (reviewed by Hsueh et al., 2000; Hillier, 2001; Matzuk et al., 2002; Ge, 2005). This network enables mediation of the gonadotropic signals from the somatic cells, in which GtH receptors are expressed, toward the oocyte, and also aids modulation of these signals via interactions with developmentally specific factors produced by the somatic cells and/or by the oocyte itself. Some of the well-characterized participants of the aforementioned network are the growth and differentiation factors such as insulin-like growth factor-I and II (IGF-I; IGF-II), epidermal growth factor (EGF), and members of the transforming growth factor β (TGF β) superfamily, e.g. activin, inhibin and follistatin (reviewed by Hiller, 2001). Studies with various fish species have suggested a possible paracrine role for the IGF system during the acquisition of follicular maturational competence and during oocyte maturation (Kagawa et al., 1994; Weber and Sullivan, 2001; Thomas et al., 2001; Bobe et al., 2003, 2004). An extensive work on zebrafish has characterized the entire ovarian activin system, including its signaling components (Wang and Ge, 2003a). Three lines of evidence indicate the involvement of the activin system in transducing the gonadotropic signal: (i) recombinant goldfish activin β enhanced the stimulating effects of GtH (e.g. hCG) and MIS (e.g. 17α,20β-DHP), and promoted *in vitro* maturational competence in zebrafish oocytes (Pang and Ge, 1999); (ii) follistatin, which is a specific binding protein of activin, has blocked both the activin- and GtH- induced oocyte maturation (Pang and Ge, 2002); (iii) activin and follistatin synthesis were found to be upregulated by the GtH-induced cAMP–PKA pathway (Wang and Ge, 2003a, b). The expression patterns of the activin system components within intact zebrafish follicles enabled deducing their network interactions (Wang and Ge, 2003c). It was postulated that activin, mainly produced in the follicle cells, affects the oocyte in a paracrine manner, whereas follistatin that is predominantly produced in the oocyte, modulates activin function within the follicle. Similarly, characterization of the EGF system in zebrafish follicle has suggested that EGF, which is largely produced in the oocyte under the regulation of GtH-induced cAMP–PKA pathway, serves as a paracrine signal from the oocyte to modulate the function of the somatic follicle cells, exclusively expressing the EGF receptors (Wang and Ge, 2004). Figure 6 schematically illustrates hypothetical regulatory loops between the oocytes and the follicle cells (reviewed by Ge, 2005). However, it should be noted that this field of research is relatively new, and many new peptides are continually emerging and now being recognized as potential factors in mediating GtH action in a paracrine/autocrine manner in the follicle, adding up missing parts to the puzzling intrafollicular communication network.

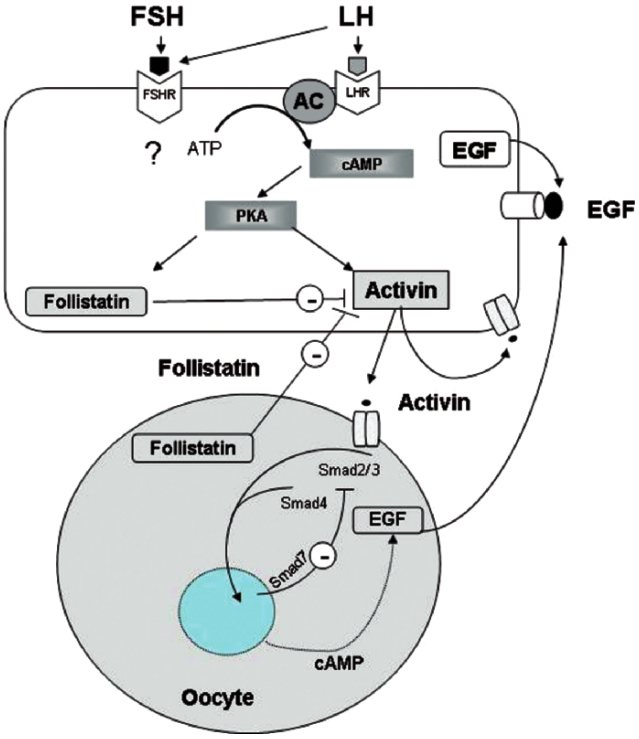

Figure 7. Tentative regulatory interplay between follicle cells and the oocyte. Data obtained so far in zebrafish, suggest that activin mainly produced in the follicle cells, affects the oocyte, which in turn produces follistatin and EGF. The latter feedback-regulate follicle cells function. (for more details see Ge, 2005.) (*See Color Plates*).

5. CONCLUSIONS AND PROSPECTS

During the last two decades, the availability of molecular cloning techniques has helped establish the dual nature of the gonadotropic action also in fish. Yet, due to the variety of reproductive strategies found among fishes, the specific functions of the GtHs in general, and in particular those of the "newly discovered" member, the FSH, are still ambiguous. In salmonids (synchronous spawners), which represent a relatively simple reproductive model, the FSH is predominant during early oogenesis and is thought to promote sex steroid synthesis, follicular differentiation, and gonadal growth. LH at a later phase induces steroidogenic changes that trigger the production of MIS, and consequently direct gamete maturation and spawning. Nevertheless, the role of FSH in multiple spawners is fairly blurred, largely because both GtHs: (i) appear to be present throughout the reproductive cycle; (ii) can stimulate the *in vitro* production of ovarian T and E2; and (iii) activate FSHR displayed on theca and granulosa cells during oogenesis. This ambiguity is further stressed in multiple spawners

like the red sea bream, where LH seems to override FSH action all through the reproductive cycle.

Another puzzle relates to the changeable activity of the GtHs during the follicular development. GtHs support the synthesis of E2 during vitellogenesis, while at the time of maturation they support MIS synthesis. Considering that teleosts do not exhibit the classic follicular model of two-cell types for two-GtHs, it was suggested that binding of different ligands to the same receptor type may stimulate divergence signaling pathways with the result of altered cell functions. In this respect, altered cell functions could also be the outcome of a change in the relative abundance of the FSHR and LHR on the follicular cells. However, to verify these hypotheses further research should be conducted.

It is expected that the ongoing use of fish recombinant GtHs and GtH-Rs for function-structure studies, along with future establishment of transgenic "gain-of function"/ "loss of function" mutants of model fish species such as zebrafish and medaka, will help understanding the nature of GtH–GtHR interactions and their affect on follicular cell functions in fish.

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CHAPTER 8

SEX STEROID HORMONE RECEPTORS IN FISH OVARIES

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1. INTRODUCTION

The ovarian follicle is not only the major site of synthesis of estrogens, androgens, and progestins in female vertebrates, but is also an important target for sex steroid hormones. Both classic (genomic) and rapid, nongenomic actions of

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estrogens, androgens, and progestins have been described in the ovaries of a variety of vertebrate species, as well as the nuclear and membrane receptors thought to mediate these effects. The teleost ovary has proven to be a particularly useful model for investigating nongenomic steroid actions and the receptors on the plasma membrane through which they act. However, a comprehensive understanding of the precise physiological functions of many steroid receptors in teleost ovaries is currently lacking. In this chapter recent developments in steroid receptor research in the teleost ovary are briefly reviewed and some major gaps in our knowledge of their roles in ovarian function identified.

2. NUCLEAR STEROID RECEPTORS

The classic genomic mechanism of steroid hormone action involves passage of steroids through the plasma membranes of target cells where they bind to specific intracellular receptors called nuclear receptors. The activated ligand receptor complex then binds to response elements on target genes to alter their transcriptional activity. These genomic actions of steroid hormones involving new mRNA and protein synthesis are typically slow, taking hours to days to complete (Mechanism 1, Figure 1). Nuclear steroid hormone receptors are ligand-activated transcription factors that belong to the of nuclear receptor superfamily that includes thyroid hormone receptors, vitamin D_3 receptors, retinoic acid receptors, and a number of orphan receptors, whose ligands remain unknown. In the inactive state, these steroid receptors reside in the cytosol or nucleus of target cells, in close association with multiple inhibitory proteins, such as members of the heat shock protein family. Upon ligand binding, the inhibitory proteins dissociate from the receptors which undergo a conformational change and form dimers. Recent evidence suggests that nuclear steroid receptor subtypes can form both homo- and heterodimers. For example, ERα can either dimerize with another ERα or an ERβ (Cowley et al., 1997; Pace et al., 1997). The hormone/receptor complex is then translocated to the chromatin where it interacts with nuclear cofactors and specific hormone response elements on target genes to alter their rates of transcription.

Members of the nuclear steroid receptor superfamily share a similar structure, with each receptor having five distinct domains (Mangelsdorf et al., 1995). At the N-terminal region resides a transactivation domain (TAD) (A/B) which is responsible for recruitment of coactivator molecules during transcription via the activation domain, AF-1. The DNA-binding domain (DBD, domain C) is the region responsible for association of the receptor with appropriate response elements on target genes. This region also plays a role in receptor dimerization and contains a nuclear localization sequence (NLS) which targets the receptor to the nucleus. The DBD is highly conserved and contains two zinc fingers which facilitate receptor–chromatin binding and are unique to nuclear receptors, compared to other DNA-binding proteins (Mangelsdorf et al., 1995).

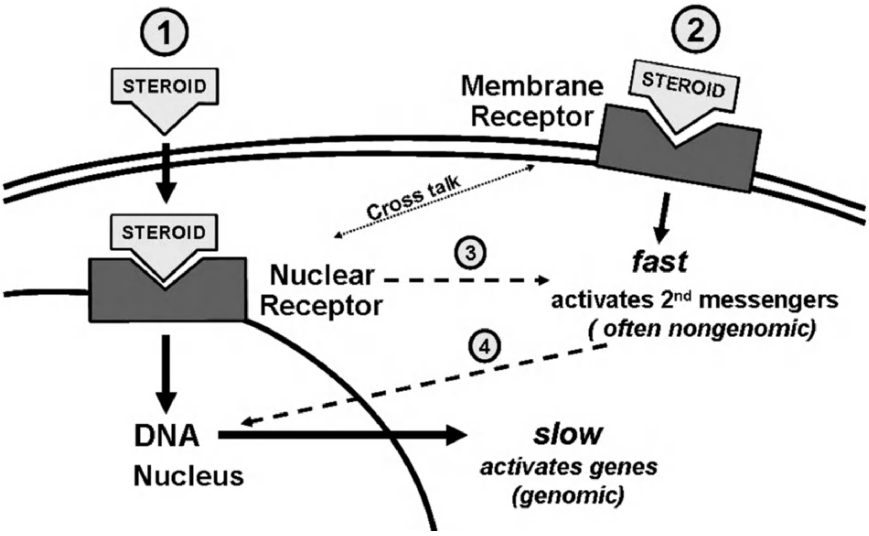

Figure 1. Major receptor-mediated mechanisms of steroid action at target cells.
1. Classic mechanism of steroid action via binding to intracellular nuclear steroid receptors, translocation to the nucleus and binding to hormone response elements of genes resulting in alterations in their rates of transcription (genomic mechanism). This mechanism, involving new mRNA and protein synthesis, is relatively slow and occurs over a timeframe of hours to days.
2. Alternative mechanism of steroid action via binding to receptors on the cell surface, called membrane receptors, resulting in activation of ion channels or intracellular second messengers. This mechanism is often rapid may not involve alterations in gene transcription (nongenomic). The identities of most steroid membrane receptors are unresolved. Some membrane receptors are nuclear receptor-like while others, such as the ovarian progestin membrane receptor, are novel proteins unrelated to the nuclear steroid receptors.
There is also the possibility of crosstalk between these two signaling pathways.
Additional mechanisms of steroid action that have been identified involve activation of intracellular second messengers through the intracellular nuclear receptor (3) and alterations in gene transcription via steroid membrane receptors (4).

A third domain, the hinge region (D) is variable among the different nuclear receptor types. The ligand-binding domain (LBD, domain E) is highly conserved and is located towards the C-terminal region. This domain is essential for the recognition of specific ligands and inducing conformational changes associated with formation of receptor dimers. The LBD also contains a NLS as well a TAD, AF-2, which is responsible for recruitment of coactivators. Finally, there is a highly variable C-terminal region (F) at the end of the receptor molecule.

Although all classes of steroid receptors contain AF-1 and AF-2 domains, some of them have additional TADs. For example, certain progesterone receptor isoforms contain a third TAD, AF-3, whereas most estrogen receptors contain only AF-1 and AF-2. Recent evidence suggests that the efficiency of target gene transcription can be greatly influenced by coactivator or corepressor

binding to transactivation motifs. These coregulators are believed to affect gene transcription by binding to motifs that become available upon agonist binding (Edwards, 2000). For example, members of the p160 family of proteins, called steroid receptor coactivators (SRCs) are known to bind ligand-activated receptors and enhance gene transcription. SRCs can recruit additional coactivator proteins, such as p300/CBP, which even further enhance gene transcription. Association of coactivator proteins enhances gene transcription by stabilizing the transcriptional complex which includes the ligand-activated receptor, RNA polymerase II and other coregulatory proteins (Edwards, 2000). Taken together, these findings demonstrate that steroid hormone signaling involves a complex integration of various intracellular components. Recently, the importance of coregulatory proteins and a wide variety of intracellular proteins which participate in the steroid regulation of transcription has become widely recognized and will likely lead to a clearer understanding of the mechanisms of nuclear steroid receptor function as a whole.

2.1. Nuclear Progestin Receptors

The major progestin hormones produced by teleost ovaries differ from those in other vertebrates in that they possess multiple hydroxyl groups on the side chain of the progesterone nucleus. Two of these progestins, 17,20β-dihydroxy-4-pregnen-3-one (17,20βDHP) and 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S), are the most abundant and biologically active in most teleost species. Moreover, binding moieties with high binding affinities for these progestins have been identified in ovarian subcellular preparations, indicating the presence of specific receptors for 17,20βDHP and 20β-S in teleost ovaries.

The teleost nuclear progestin receptor (nPR), was first characterized biochemically in the ovaries of spotted sea trout (*Cynoscion nebulosus*) (Pinter and Thomas, 1995). The nPR is present in the sea trout ovary throughout gonadal recrudescence (Pinter, 1995) and while the hormonal regulation of the nPR has not been clearly demonstrated in teleosts, other vertebrate nPRs contain estrogen response elements in the promoter region (Kastner et al., 1990), indicating that receptor expression may be regulated by estrogen. Full-length nPR cDNAs, analogous to PR-B in other vertebrates, have been cloned from the Atlantic croaker (*Micropogonias undulatus*), the Nile tilapia (*Oreochromis niloticus*), and the Japanese eel (*Anguilla japonica*) (Todo et al., 2000). Only one isoform of the nPR has been identified in teleosts, while up to two truncated isoforms of the PR-B, PR-A, and PR-C, originating from alternative transcription of the same gene, have been identified in the human and chicken (Schrader and O'Malley, 1972; Kastner et al., 1990; Wei et al., 1990; Sartorius et al., 1994; Wei and Miner, 1994; Wei et al., 1996; Wei et al., 1997). An additional functional subtype of the progesterone receptor, PRβ, encoded by a second gene, has been identified in the Japanese eel (Ikeuchi et al., 2001; Ikeuchi et al., 2002). As expected, the teleost nPR sequences share high sequence homology with other vertebrate nPRs in the DBD (85% sequence identity) (Ikeuchi et al., 2002).

However, although the sequences of their LBD are highly conserved amongst teleosts, they differ from those of nPRs of other vertebrates. It has been suggested that these differences in the nPR LBD sequences are related to the different progestin hormones that activate the nPRs in fish (Todo et al., 2000). Saturation and Scatchard analyses of [^{3}H]17,20βDHP binding in spotted sea trout and Japanese eel membrane preparations indicate that the cytosolic receptor has a single high-affinity (K_d 1.89 and 2.44 nM, respectively), low-capacity (B_{max} 1.80 pmol/g ovary for the sea trout) binding-site characteristic of nPRs (Pinter and Thomas, 1995; Ikeuchi et al., 2002). Relatively high amounts of nPR-binding activity have been measured in fully recrudesced gonadal tissues and lower amounts in the gills and liver of spotted sea trout, whereas no specific [^{3}H]17,20βDHP binding was detected in the brain, muscle, or plasma (Pinter and Thomas, 1995). Further studies on the steroid specificity of the receptor indicate that, while the progestin receptor in teleost fish demonstrates other binding characteristics similar to those of progesterone receptors in other vertebrates, it has a different steroid specificity profile demonstrating higher affinity for 17,20βDHP and 20β-S than for progesterone (Pinter and Thomas, 1997). It is widely recognized that upon binding to progestogens, nPRs form homo- or heterodimers and act as transcription factors that bind progesterone response elements (PRE) on the promoter regions of progesterone responsive genes. In other vertebrate models it is known that transcriptional efficiency is further modulated by coactivator proteins which are recruited to the transactivation functions of the progesterone receptors (Rowan and O'Malley, 2000), but there is nothing known about the coactivators or corepressors of the nPRs in teleosts.

Cellular localization of the nPR within the teleost ovary has not been reported to date, although preliminary evidence indicates that the receptor is present in the follicle cells in Atlantic croaker (unpublished observation), in agreement with previous evidence suggesting that the nPR initiates ovulation in sciaenid fishes. 20β-S, the maturation-inducing steroid or substance (MIS) in the spotted sea trout, was shown to induce ovulation *in vitro* in a dose-dependent manner (Pinter and Thomas, 1999). In addition, 20β-S-stimulated ovulation was inhibited by the addition of blockers of RNA (actinomycin D) and protein (cycloheximide) synthesis, indicating that progestin induction of ovulation is via a genomic mechanism. The third piece of evidence indicating that the nPR mediates ovulation in this species is that 17,20βDHP is a more potent inducer of ovulation than 20β-S. Binding studies on the sea trout nPR shows that 20β-S has a relative binding affinity for the nuclear receptor only 65% that of 17,20βDHP (Pinter and Thomas, 1997), whereas 20β-S has a higher binding affinity for the membrane receptor than 17,20βDHP (Thomas and Das, 1997). Together these data indicate that progestins are acting through the nPR, and not the membrane progestin receptor, to initiate ovulation. A similar situation is described for the ovulation of yellow perch (*Perca flavescens*) oocytes. In this perciform teleost 17,20βDHP has been shown to induce ovulation *in vitro* (Goetz and Theofan, 1979). The ability of progestin to induce ovulation was also shown to be

dependent on the transcription of RNA indicating a genomic mechanism of action and suggesting involvement of the nPR (Theofan and Goetz, 1981). Additional studies in the yellow perch have demonstrated that progestins upregulate prostaglandin E and F synthesis in the follicle suggesting a mechanism for progestin initiation of ovulation (Goetz, 1997).

We still have a poor understanding of the roles nuclear progesterone receptors in teleost ovaries but the nPRs are clearly involved in ovulation. Clues from other vertebrate models may help to give direction to further characterization of other functions of these receptors. For example, while it is not known whether progestins have a role in ovarian development in fishes, PR-A knockout mice have been shown to have ovarian abnormalities in addition to other impairments of the reproductive and neuroendocrine systems (Conneely et al., 2003).

2.2. Nuclear Androgen Receptors

Androgens such as testosterone (T), 5α-dihydrotestosterone (DHT), and 11-ketotestosterone (11-KT) play important roles in sexual differentiation and development of secondary sex characteristic in male vertebrates. The teleost ovary produces large amounts of androgens, and circulating androgen levels in females are often greater than those of estrogens during ovarian recrudescence. The major ovarian androgen, testosterone, serves as the precursor for estrogen biosynthesis, but studies have shown that androgens are also involved in other physiological functions in female vertebrates, such as bone metabolism, cardiac growth, and regulation of the immune system (Ikeda et al., 2005; Michael et al., 2005; Syed and Khosla, 2005; Ahmadi and McCruden, 2006). However, the identification of androgen-binding activities in ovarian cytosolic extracts of coho salmon and Atlantic croaker characteristic of nuclear androgen receptors (nAR) (Fitzpatrick et al., 1994; Sperry and Thomas, 1999a, b), indicates that androgens also exert direct hormonal actions in the teleost ovary, although their physiological roles are unknown.

In most vertebrates only one functional nAR gene has been identified, but in several teleost species, such as rainbow trout (*Oncorhyncus mykiss*), Japanese eel, astato tilapia (*Haplochromis burtoni*), Nile tilapia, and the mosquitofish (*Gambusia affinis*), two androgen receptor (AR) subtypes, ARα and ARβ, have been identified (Ikeuchi et al., 1999). The duplication in the Japanese eel is probably the result of the gene duplication event that occurred in other gene families in teleosts (Amores et al., 1998), including the nuclear estrogen receptor (nER)β (Hawkins et al., 2000). It has been suggested that the high degree of homology between the two rainbow trout ARs is the results of the tetraploidy that occurred in the salmonids (Takeo and Yamashita, 1999). Both AR subtypes have been identified in ovarian tissues of rainbow trout. Sequence analysis of the teleost nARs shows that the DBD and LBD regions of both subtypes display high homology (80%) to these regions of human, rat, mouse, and *Xenopus* nARs. While the teleost ARα N-terminal TAD shows a high sequence

similarity (85%) to the nAR found in tetrapods, the ARβ TADs have only about 20% sequence similarity to other vertebrate TADs. Functional studies of ARα and ARβ have so far only been performed in a few species. In rainbow trout it was found that only one of the subtypes is functional (ARα), while both ARα and ARβ were found to be active in the Japanese eel (Ikeuchi et al., 1999; Takeo and Yamashita, 1999). The subtypes also differ in steroid specificity between species. In Japanese eel it has been shown that DHT and 11-KT are equally potent in activating both nARs, while T has much lower potency (Ikeuchi et al., 1999). In contrast, the red sea bream AR is equally activated by T and 11-KT, while the rainbow trout ARα was found to be activated to the same extent by DHT, 11-KT, and T (Takeo and Yamashita, 1999; Touhata et al., 1999). The mammalian nAR contains three autonomous regions responsible for transactivation for the nuclear receptor, two strong, constitutively active, ligand independent activation functions (AF-1 and AF-5) found in the N-terminal TAD and a weaker ligand-dependent activation function (AF-2) located in the LBD (Gronemeyer and Laudet, 1995; Olsson et al., 2005). Activation of AF-1, AF-2, and AF-5 are dependent on coactivator molecules to initiate gene transcription (Onate et al., 1998; Bevan and Parker, 1999) and so structural differences in the AF regions might possibly explain the observed differences in the steroid specificities of the different teleost AR subtypes for transactivation functions (Heinlein and Chang, 2002). Clearly, the significance of the structural differences in the ARβ TADs in teleosts requires further investigation.

The physiological roles of steroids in regulating nAR expression in vertebrates remain unclear. AR mRNA levels have been found to either increase (Mora et al., 1996; Cardone et al., 1998; Larsson et al., 2002) or decrease (Tetsuka and Hillier, 1996) after androgen treatment. It has also been shown that treatment with estradiol-17β increases abundance of the nAR in croaker brains (AR1), suggesting that crosstalk between nAR and nER might occur (Larsson et al., 2002), possibly through coactivators. Numerous nAR coactivators that affect functions such as ligand specificity and DNA-binding capacity have been identified. Although AR normally functions as a homodimer, it has been shown that certain coactivators might induce heterodimer formation between AR and other receptors. These heterodimers can lead to crosstalk between receptors causing nonspecific ligand-induced receptor activation. For example the p160 proteins, ARA55 and ARA70, have been shown to mediate an increase in nAR transcriptional activity when activated by estradiol-17β and progesterone (Miyamoto et al., 1998; Fujimoto et al., 1999). Other members of the p160 family, such as ARA54, ARA267-α, Smad-3, and AIB1, have also been well characterized as nAR coregulators (Yeh et al., 1999).

In Atlantic croaker the nAR-binding properties have been extensively characterized using various natural and synthetic androgens (Sperry and Thomas, 1999a, b, 2000). The biochemical characterization of the binding of different androgen ligands to the receptors led to the finding of two different isoforms of AR (AR1 and AR2) in the Atlantic croaker and the kelp bass (*Paralabrax clathratus*)

(Sperry and Thomas, 1999a, b). The two isoforms were found to display both differences in binding as well as different distribution patterns. AR1, with the highest affinity for T, was only found in brain tissue while AR2, which displayed highest affinity to DHT, was found both in brain and gonads. These differences in tissue distribution and ligand specificity of the two nARs suggest that AR1 and AR2 have different physiological functions. It is not known, though, whether the AR1 and AR2 proteins are the products of different genes or the result of posttranslational modifications, which has been suggested as a cause of the tissue-specific differences observed in ligand activation of the nAR (Wolf et al., 1993; Peterziel et al., 1999).

Although the functions of nARs in teleosts ovaries are unknown, there is circumstantial evidence that they have important roles in gametogenesis in other vertebrate species. In mice, chicken and *Xenopus laevis* androgens appear to be involved in oocyte maturation, but it has also been shown that androgens stimulate atresia and apoptosis in mice ovaries (Drummond, 2006; Katoh et al., 2006). Aromatase levels were decreased in fathead minnow ovaries after exposure to antiandrogens and environmental contaminants which disrupt endocrine disrupting contaminants (EDCs) function, suggesting a role for nARs in the regulation of estrogen biosynthesis in the teleosts ovary and the nAR as a site for interference by EDCs (Ankley et al., 2005). The finding that these EDCs can bind to the Atlantic croaker and kelp bass ovarian nARs (AR2) (Sperry and Thomas, 1999b) is consistent with this mechanism of EDC interference.

The current lack of comprehensive information on the identity and cellular distribution of nARs in teleost ovaries and on their patterns of expression throughout oogenesis and in response to hormonal treatment have prevented the development of a clear understanding of nAR functions in teleost ovarian physiology. Further studies to characterize nARs in teleosts ovaries, as well as additional investigations of ovarian actions of androgen will be required to make significant progress in our knowledge of the roles of nARs in gonadal physiology in female teleosts.

2.3. Nuclear Estrogen Receptors

Estrogens play an important role in numerous developmental, reproductive, and behavioral physiological processes in vertebrates and are mediated by specific receptors expressed in target tissues. Studies in mammalian models have suggested that there were two nER subtypes in vertebrates; ERα and ERβ (Kuiper et al., 1996). However, in recent years, there has been a reclassification of teleost estrogen receptors with the discovery of three distinct estrogen receptor subtypes encoded by different genes (Hawkins et al., 2000; Menuet et al., 2002). Like mammals, teleosts possess a single ERα subtype which shows approximately 90% and 60% similarity to mammalian ERα DBDs and LBDs, respectively. However, unlike mammals and other vertebrates, which have a single ERβ subtype, teleosts have two ERβ subtypes; ERβa (formerly known as ERγ) and

ERβb (Hawkins and Thomas, 2004). These two receptors are similar to mammalian ERβs, showing greater than 91% homology to the human ERβ DBD and approximately 65% to the LBD. To date ERα, ERβa, and ERβb have been found in Atlantic croaker, zebrafish, largemouth bass, European sea bass, fathead minnow, goldfish, and seabream (Tchoudakova et al., 1999; Halm et al., 2004; Sabo-Attwood et al., 2004; Filby and Tyler, 2005; Pinto et al., 2006).

All three teleost nERs have been shown to bind estrogens with high affinity in a manner which is characteristic of steroid hormone receptors (Hawkins et al., 2000; Menuet et al., 2002). For example, *in vitro* synthesized Atlantic croaker ERα, ERβa, and ERβb have K_ds of 0.61, 0.38, and 0.40 nM, respectively (Hawkins and Thomas, 2004). These data are consistent with the affinity of nER for estradiol-17β in the liver and testis of this species (Loomis and Thomas, 1999). A similar pattern has been demonstrated in zebrafish, where K_ds for the three estrogen receptor subtypes synthesized *in vitro* range 0.42–0.75 nM (Menuet et al., 2002). Furthermore, nER-binding properties have been extensively characterized in croaker and zebrafish using various natural and synthetic estrogens. In croaker, competition analyses using fusion proteins have shown that the top five competitors for all ER subtypes are estradiol-17β, diethylstilbesterol (DES), 4-hydroxytamoxifen (TOH), ICI164, and ICI182,780 (Hawkins and Thomas, 2004). However, the ERα and ERβb display a higher affinity for synthetic estrogens, whereas ERβa shows the highest affinity for natural estrogens, such as estrone and estriol. In zebrafish, both estradiol-17β and DES were shown to be strong agonists for all three nER subtypes using a luciferase reporter assay, whereas ICI164 and TOH were shown to antagonize estradiol-17β activation in all three subtypes (Menuet et al., 2002). Taken together, these data corroborate nER-binding data from other vertebrate species (Kuiper et al., 1997; Matthews et al., 2000; Pinto et al., 2006).

In a recent study, Hawkins et al. (2005) were able to show that changes in the amino acid composition of the LBDs of croaker nERs were responsible for observed differences in the binding characteristics of estrogenic compounds. As one example, mutation of ERβaPhe^{396} to methionine or isoleucine, which corresponds to the structure of ERα and ERβb, respectively, was successful in shifting the binding characteristics of ERβa to that of ERα and ERβb (Hawkins and Thomas, 2004). These data demonstrate that highly conserved amino acid profiles of LBDs are crucial in regulating estrogen binding in the different receptor subtypes and are likely of strong evolutionary and functional significance.

Investigations into the expression of nERs in teleost tissues have shown that the three subtypes have overlapping yet differential expression. In Atlantic croaker, ERα and ERβb are primarily expressed in the liver, with low expression in the ovary and brain (Hawkins et al., 2000). In contrast, ERβa is highly expressed in the ovary, with little expression detected in the liver and other tissues. Similar expression patterns have been described in largemouth bass, fathead minnow, and seabream (Sabo-Attwood et al., 2004; Filby and Tyler, 2005;

Pinto et al., 2006). Taken together, binding and localization data suggest that ERβa plays a crucial role in development and function of the teleost ovary.

In the brain, differential expression of nERs has been demonstrated in zebrafish and Atlantic croaker suggesting that the different nER subtypes may play distinct roles in behavior and development. For example, all three zebrafish nERs are expressed in ventral portion of the parvicellular area of the preoptic nucleus (PPa), with ERβb being more broadly expressed and ERα and ERβa showing more distinct localization to the ventral region (Menuet et al., 2002). In croaker, expression of nERs in this region differs, with ERα and ERβa being more broadly expressed and ERβb showing localization to the ventral portion of the PPa, suggesting a possible divergence in function of nERs in the brain of teleosts (Hawkins et al., 2005). However, the neuroendocrine functions of teleost nERs are currently unknown.

Although much attention has been focused on the characterization of nER subtypes in fish, their individual physiological roles in reproduction remain unclear. Of particular importance, however, is the hepatic synthesis of the yolk protein vitellogenin (Vtg), which is mediated by estradiol-17β in oviparous vertebrates and is crucial for proper oocyte development. Numerous studies are available which demonstrate that this process is mediated predominately through ERα (Flouriot et al., 1996; Teo et al., 1998). The role of nERs in ovarian function is less clear in fish models. Estradiol-17β has been shown to act in an anti-apototic manner to inhibit follicular atresia in both preovulatory and vitellognic cultured rainbow trout oocytes (Janz and Van der Kraak, 1997; Wood and Van der Kraak, 2002). Localization studies described above suggest that ERβs are expressed at higher concentrations in the ovary than ERα. In the fathead minnow and the largemouth bass ERβs, in particular ERβa, appear to be involved in ovarian development as it is highly expressed during early ovarian differentiation and development (Filby and Tyler, 2005). These data are consistent with studies in mammalian models, in which recent work is beginning to shed light on the individual roles of ERα and ERβ in the ovary. Studies using estrogen receptor α knockout (αERKO) mice have shown that ERα plays an important role in the estrogen-mediated negative feedback of luteinizing hormone secretion from the pituitary (Schomberg et al., 1999; Couse et al., 2003). The absence of ERα leads to hypergonadism, increased steroidogenesis and subsequent disruption of function of the ovary. ERβ on the other hand has been suggested to be an important mediator of the proper development of the ovarian follicle. Specifically, βERKO mice show poor granulosa cell differentiation as well as the inability to properly respond to a gonadotropin surge necessary for ovulation (Couse et al., 2003). Although similar experiments in fish will be complicated by the presence of two distinct ERβs, the studies presented above should provide a good foundation for the exploration of the distinct roles of the nER subtypes in the ovarian development and function in fish.

Hormonal regulation of nERs is controlled in large part by estrogens themselves. In the rat, it has been shown that treatment of ovariectomized females

with estradiol-17β results in an increase in ERα expression in the pituitary and the liver (Shupnik et al., 1989). However, in the same experiment a decrease in uterine ERα expression was observed after estrogen treatment suggesting tissue-specific effects of estradiol-17β in receptor regulation. In rainbow trout hepatocyte primary cultures, ERα expression is also upregulated by estradiol-17β treatment and is correlated with increased Vtg production (Flouriot et al., 1996). Changes in ERα concentrations were determined to occur at both the transcriptional and posttranscriptional levels. In zebrafish, studies on ERα, ERβa, and ERβb expression have shown that these three genes are differentially regulated. Specifically, ERα mRNA is upregulated in response to estrogen, whereas ERβb expression decreased and ERβa remained unchanged (Menuet et al., 2004). Furthermore, luciferase reporter assays have demonstrated that ERα expression was mediated primarily by ERα itself, along with some transcriptional activation via ERβa (Menuet et al., 2004). However, there is a lack of information on the hormonal regulation of the three nER subtypes in fish ovaries. Data from mammalian models suggest that ERβ plays a larger role in the ovary than ERα and localization studies of teleost ERβs suggest that this phenomenon is conserved in vertebrates (Kuiper et al., 1997). Thus, further investigations into the hormonal regulation and role of the two teleost ERβs in the teleost ovary are warranted.

Despite extensive characterization of the three nERs in two fish species the exact roles of nERs in mediating the diverse actions of estrogens at target tissues remain to be unclear. As one component of this, the role of coregulator proteins remains to be determined in teleost models, although it has been suggested that teleost nERs recruit similar coactivators as mammalian nERs (Mezaki et al., 2001). Thus, it is likely necessary to use studies in mammalian models as a basis for further nER studies in fish. Mammalian nERs have been shown to contain two primary TADs; AF-1 and AF-2 (Hall et al., 2001). Studies have demonstrated that AF-2 interacts with steroid receptor coactivator (SRC) members of the p160 family of coregulators in both ERα and ERβ. However, it has been suggested that the regulatory roles of AF-2 differ for the two subtypes (Hall and McDonnell, 1999). In ERα the AF-1 domain has been shown to recruit SRC proteins, but may additionally bind different coregulators, such as CPB/p300 (Webb et al., 1999). Interestingly, the presence of a functional AF-1 domain in ERβ varies in mammalian models, with mouse ERβ having a functional AF-1 domain and human ERβ lacking one due to divergence of the N-terminal regions of these subtypes (Cowley and Parker, 1999; Hall and McDonnell, 1999). Such findings indicate that regulation of nER activity by coregulatory proteins differs according to species and subtype. Although there are numerous reports of AF-1 and AF-2 domains in teleost nERs, further studies should provide interesting insight into the function of nER coregulators.

The presence of three distinct estrogen receptors opens the possibility of novel action of EDCs in teleosts. Although many studies have shown the ability of xenoestrogens to induce Vtg synthesis in both males and females, more recent

work has shown that xenoestrogens can also induce sex reversal, disrupt gonadal development and even impair reproductive behavior in teleosts (Tyler et al., 1998). Furthermore, binding of xenoestrogens to nERs has been demonstrated in teleosts (Loomis and Thomas, 1999; Gale et al., 2004). Since xenoestrogens have different binding characteristics for individual nER subtypes, there is potential for tissue-specific effects depending on expression patterns of nERs for a given tissue (Gale et al., 2004). This has been demonstrated in radial glial cells transfected with zebrafish nERs as aromatase expression was shown to be differentially regulated by the three nERs in response to xenoestrogens (Le Page et al., 2006). Although many studies are available which describe physiological effects of xenoestrogens, how these chemicals interact with nERs to elicit these effects is not clear at present.

Finally, teleosts are unique in that they are the only vertebrate group described to date which possess three distinct nERs. Thus, they provide a valuable opportunity to explore the evolutionary and functional significance of nERs. Although there is much to be learned in terms of nER function in teleosts, recent evidence in mammalian models suggests that there is a high degree of conservation of nER function in vertebrates. Thus, studies of mammalian nERs will likely lead to a stronger understanding of nER function in fish. This is especially true in the teleost ovary, where little information is currently available for the role of nERs in ovarian function.

3. MEMBRANE STEROID RECEPTORS

It is now broadly accepted that numerous actions of steroid hormones cannot be explained by the classic genomic mechanism of steroid action mediated via nuclear steroid receptors involving new mRNA and protein synthesis which is relatively slow, typically occurring over a timescale of hours to days (Revelli et al., 1998a; Watson and Gametchu, 1999; Falkenstein et al., 2000). Many research groups have shown that steroids also act at the cell surface of numerous target tissues and cell types to initiate rapid activation of intracellular signaling pathways, alterations in calcium concentrations occurring within seconds (Blackmore et al., 1991), and of other second messengers such as cyclic nucleotides and MAPkinase, within a few minutes (Filardo et al., 2000; Zhu et al., 2003a) (Mechanism 2, Figure 1). Evidence has accumulated that these rapid steroid actions are mediated by binding to specific receptors in the plasma membranes of the target cells (Revelli et al., 1998a, b; Watson and Gametchu, 1999; Falkenstein et al., 2000; Thomas, 2000b; Norman et al., 2004). Specific membrane receptors for estrogens have been identified in numerous vertebrate tissues including mammalian breast (Thomas, 2005a), hypothalamic, pituitary, and uterine tissues (Pappas et al., 1994; Monje and Boland, 1999; Qiu et al., 2003) and fish testes (Loomis and Thomas, 2000); for androgens in lymphocytes and fish ovaries (Braun and Thomas, 2004), and for progestogens in fish and amphibian oocytes (Patino and Thomas, 1990; Maller, 1998) in bovine ovaries

(Rae et al., 1998), in human and fish sperm membranes (Blackmore, 1996; Thomas et al., 1997, 2005b), and for progesterone metabolites in breast cancer cells (Weiler and Wiebe, 2000). However, despite intensive research in many laboratories over the past 25 years, the identities of most steroid membrane receptors remain unresolved and surrounded by controversy (Watson, 2003). This lack of comprehensive information on the identity and molecular structure of membrane steroid receptors has prevented substantial progress being made in our understanding of critical molecular aspects of steroid actions via this alternative mechanism and a more widespread appreciation of its significance. Recent research in fish ovaries, however, has led to the discovery of novel progestin membrane receptors of likely widespread physiological importance in vertebrate reproduction. In addition, estrogen and androgen membrane receptors have been biochemically characterized in fish gonadal tissues.

3.1. Membrane Progestin Receptors

3.1.1. Progestin induction of oocyte meiotic maturation

One of the most extensively investigated and well-characterized models of nongenomic steroid actions initiated at the cell surface is the induction of oocyte meiotic maturation in fish and amphibians by progestin hormones (Nagahama et al., 1994; Maller, 1998). At the end of the ovarian cycle, when oocyte growth due to incorporation of Vtg is complete and the environmental conditions are appropriate for spawning, a surge in gonadotropin secretion induces the final stage of oocyte development, termed oocyte maturation, which results in the formation of fully mature oocytes that can be fertilized to produce viable offspring. Gonadotropin induces the final phase of oocyte maturation, called the germinal vesicle breakdown phase (Patiño et al., 2001), in fish and amphibians indirectly by inducing the synthesis of MIS by the ovarian follicles. The MISs in turn bind to specific receptors on the oocyte plasma membrane to trigger a cascade of intracellular second messengers leading to the resumption of meiosis and completion of oocyte maturation. Novel progestin and 11-deoxycorticosteroid hormones (C21 steroids) with multiple hydroxyl groups on the side chain have been identified in teleost fishes that act as MISs to induce oocyte maturation in *in vitro* bioassays (Scott and Canario, 1987). The MIS has been positively identified as the progestin 17,20βDHP in amago salmon, *Onchorynchus rhodurus* (Nagahama and Adachi, 1985) and is the likely MIS in a wide variety of salmonid, cyprinid, and other teleost orders (Scott and Canario, 1987), whereas the progestin 20β-S has been positively identified as the MIS in Atlantic croaker (Trant and Thomas, 1989) and is probably the major MIS in many other scaeinid and perciform species, such as spotted sea trout, striped bass (*Morone saxatilis*), and black porgy (*Acanthropagus schlegeli*) (Trant and Thomas, 1989; King et al., 1994a, b; Yueh et al., 2005). Other 21 carbon steroids, such as 11-deoxycortisol, are synthesized by the ovaries of several

flatfishes and can induce oocyte maturation and therefore may function as MISs in these species. It is possible that multiple MISs contribute to the induction of oocyte maturation in many teleost species because often more than one of these C21 steroids with maturation-inducing activity are detected in ovarian and plasma samples (King et al., 1994a). Several lines of evidence indicate MIS action on teleost oocytes is not via the classical steroid mechanism through binding to intracellular nuclear steroid receptors, but instead is through receptors on the oocyte plasma membrane (Nagahama et al., 1994). Firstly, the MIS acts on the cell surface and microinjection of oocytes with the MIS is ineffective in triggering maturation (Nagahama, 1987). Moreover, induction of oocyte maturation by the MIS requires protein synthesis but is not blocked by transcription inhibitors, indicating a nongenomic mechanism of action (Jalabert, 1976; DeManno and Goetz, 1987). In addition, the action is rapid; a few minutes exposure to the MIS *in vitro* is often sufficient to initiate oocyte maturation, although the entire process may take a day to complete (Thomas and Das, 1997). Finally, progestin membrane receptors have been identified in teleost ovaries which are the likely mediators of these nongenomic MIS actions.

3.1.2. Membrane progestin receptors

The presence of a binding site for progestins on ovarian plasma membranes that fulfils the major criteria for its designation as the MIS receptor was first described in spotted sea trout (Patino and Thomas, 1990). The receptor displays high affinity (K_d 3.5 nM), limited capacity (0.1–1.0 pmol/g ovary), displaceable, specific binding for [^{3}H]20β-S, with rapid rates of association and dissociation, typical of steroid membrane receptors. The binding is specific for progestins and 20β-S has a binding affinity fold higher than that of 17,20βDHP (Patino and Thomas, 1990). In addition, there is a close correlation between the relative binding affinities of various progestins and 11-deoxycorticosteroids for the spotted sea trout progestin membrane receptor and their agonist or antagonist activities in an *in vitro* oocyte maturation bioassay (Thomas and Das, 1997). Thus, these results provide convincing evidence that the progestin membrane receptor identified on sea trout membranes is the intermediary in 20β-S induction of oocyte maturation in this species. Receptors with high-binding affinities for progestins have since been identified in ovarian and oocyte membranes prepared from rainbow trout, arctic char (*Salvelinus alpinus*), and yellow tail (*Seriola quinqueradiata*) (Yoshikuni et al., 1993; Rahman et al., 2002; Berg et al., 2005). Consistent with the likely identity of the MIS in salmonids fishes as 17,20βDHP, the receptors in rainbow trout and arctic char show higher binding affinities for this progestin than for any other putative MISs (Yoshikuni et al., 1993; Berg et al., 2005).

Interestingly, ovarian membrane progestin receptor concentrations increase two- to threefold in spotted sea trout during oocyte maturation *in vivo* (Patino et al., 2001). Receptor concentrations are also upregulated in fully grown sea trout ovarian follicles *in vitro* during the initial "priming" (steroid-independent)

stage of gonadotropin induction of oocyte maturation, when oocyte maturational competence develops (Thomas et al., 2001). Moreover similar increases in ovarian or oocyte progestin membrane receptor concentrations have been observed in rainbow trout, arctic char, and yellow tail after gonadotropin treatment (Yoshikuni et al., 1993; Rahman et al., 2002; Berg et al., 2005). The finding that the increase in receptor concentrations *in vitro* is associated with the oocytes becoming responsive to the MIS and able to complete oocyte maturation suggests it is of physiological importance during this process.

3.1.3. Signaling pathways activated by progestin membrane receptors

Although many of the intracellular signaling molecules, such as maturational promotion factor, involved with the later stages of oocyte maturation in teleosts have been well characterized (Nagahama et al., 1994), until recently equivalent information was lacking on the early events initiated upon binding of the MIS to the progestin membrane receptor. A decrease in cAMP levels has been reported in response to MIS in teleost oocytes (Jalabert and Finet, 1986; Haider and Chaube, 1996). Initial studies in rainbow trout suggested that this decrease in cAMP levels associated with MIS induction of oocyte maturation involved activation of a pertussis toxin (PTX)-sensitive inhibitory G protein (G_i), although oocyte maturation could not be blocked by microinjection with PTX (Yoshikuni and Nagahama, 1994). However, PTX was found to be effective in blocking MIS maturation of Atlantic croaker oocytes (Thomas et al., 2002). Recent studies in spotted sea trout have provided direct evidence that binding of the MIS to its receptor activates a PTX-sensitive inhibitory G protein and that activation of this pathway is necessary for the completion of oocyte maturation (Pace and Thomas, 2005a). First, it was demonstrated that the MIS, 20β-S, downregulates adenylyl cyclase activity in sea trout oocyte membranes in a steroid-specific manner, and that this action is blocked by PTX. Microinjection of PTX into oocytes was also shown to block MIS induction of oocyte maturation in this species. Both of these results suggest the MIS activates a G_i protein. A follow-up experiment showed that MIS treatment increases [^{35}S]GTPγS binding to ovarian membranes, confirming that the steroid activates a G protein (Barr and Manning, 1999), and a subsequent immunoprecipitation study showed that the increased [^{35}S]GTPγS binding was associated with the alpha subunits of inhibitory G ($G_{i\ 1–3}$) proteins. A characteristic of G-protein coupled receptors (GPCRs) is that treatment with excess guanine nucleotides such as GTPγS decreases ligand affinity by reducing G protein coupling to the receptor through depleting the pool of inactive G proteins able to couple to it, or decreases ligand binding by reducing the number of binding sites on the membrane (Orchinik et al., 1992; Chidiac, 1998). Treatment of receptors coupled to inhibitory G proteins with PTX causes inactivation and uncoupling of G proteins (West et al., 1985) and therefore has a similar effect as GTPγS, decreasing ligand affinity for the receptor (Tucker et al., 2000). Treatments with both GTPγS and PTX decreased binding of [^{3}H]20β-S to sea trout ovarian

membranes (Pace and Thomas, 2005a). Similarly, a decrease in [^{3}H]17,20βDHP binding to rainbow trout oocyte membranes was observed after treatment with PTX (Yoshikuni and Nagahama, 1994). It is concluded from these studies that the membrane progestin receptor in sea trout and also probably in rainbow trout is directly coupled to inhibitory G-proteins.

These findings indicate MIS activation of an inhibitory G protein results in downregulation of adenylyl cyclase activity through the α and/or βγ subunits of the G protein leading to a reduction in protein kinase A (PKA) activation. Inhibition of PKA appears to be sufficient to induce oocyte maturation in the Indian catfish, *Clarius batrachus* (Haider and Baqri, 2002), whereas the cell permeable PKA inhibitors (Rp-cAMP and KT5720) were ineffective in inducing or enhancing MIS-induced oocyte maturation in Atlantic croaker (Pace and Thomas, 2005b). In croaker, however, inhibitors of the phosphatidylinositol 3-kinase (PI3K)/Akt signal transduction pathway (PI3K inhibitors: Wortmannin and LY294002; Akt inhibitor: ML-9) completely blocked MIS-induced oocyte maturation (Pace and Thomas, 2005b). This pathway has previously been implicated in hormonal induction of oocyte maturation in striped bass (Weber and Sullivan, 2001). On the basis of these results a model is proposed whereby the βγ subunits of the heterotrimeric G protein also recruit PI3K to the plasma membrane to catalyze the formation of phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 in turn binds to serine/threonine kinase Akt resulting in its activation and subsequent alteration of downstream effectors such as phosphodiesterases (PDEs), which breakdown cAMP (Pace and Thomas, 2005b). Thus, MIS binding to its receptor and activation of an inhibitory G protein could potentially lead to decreased intracellular concentrations of cAMP via multiple signaling pathways to release the oocytes from meiotic arrest and complete maturation.

3.1.4. Identification of a novel cDNA in sea trout ovaries encoding a putative progestin membrane receptor

Recently a novel cDNA was discovered in a spotted sea trout ovarian cDNA library which has the major characteristics of the membrane progestin receptor mediating meiotic maturation of spotted sea trout oocytes (Zhu et al., 2003b). Previous attempts to obtain highly purified preparations of steroid membrane receptors for amino acid sequencing using classical protein purification approaches had been unsuccessful due to instability of the purified receptors. Therefore, a combination of partial receptor purification, antibody generation, and expression library screening was used to identify likely cDNA candidates for the membrane progestin receptor (Thomas et al., 2002). First, solubilization procedures were developed that were effective in extracting most of the receptor from membranes but did not interfere with the radioreceptor assay which was used to track the receptor protein through the subsequent purification steps. After removal of both very low- and high-molecular weight components by filtration, the solubilized receptor preparation was fractionated on a diethylamino ethanol

(DEAE) anion exchange column and eluted with a stepwise NaCl gradient. The majority of the [^{3}H]20β-S binding activity eluted in the first fraction contained only a single major protein band on SDS-PAGE with a molecular weight of ~40 kDa and only minor amounts of other proteins. This fraction was used to immunize mice for the generation of monoclonal antibodies. The monoclonal antibodies were subsequently screened for their ability to detect the membrane progestin receptor in a novel double antibody receptor capture assay (Thomas et al., 2002). One positive antibody, PR10-1, which detected a 40 kDa band in fraction 1, was used to screen a spotted sea trout cDNA expression library prepared from ovaries containing fully grown follicles primed with gonadotropin to enhance progestin membrane receptor expression. A novel cDNA was detected, unrelated to any previously described gene, which encoded a protein of ~40 kDa with 352 amino acids. The characteristics of this novel gene and its protein were subsequently investigated to determine whether they were consistent with its identity as a membrane progestin receptor.

3.1.5. Characteristics of the novel putative sea trout membrane progestin receptor

It was considered that the following eight criteria needed to be satisfied for designation of the novel sea trout gene and its protein as a membrane progestin receptor: plausible structure, tissue specificity, subcellular localization, steroid binding, signal transduction, hormonal regulation, biological relevance, and multiplicity (Zhu et al., 2003a, b). First, hydrophilicity and transmembrane analysis of the deduced amino acid sequence showed the protein has seven transmembrane domains, which is characteristic of GPCRS, the largest class of membrane receptors (proposed model shown in Figure 2). Northern blot analysis showed that greatest expression of the mRNA for the novel sea trout receptor, called stmPRα, occurred in reproductive and neuroendocrine tissues (ovaries, testes, pituitary, and brain), consistent with the known regulatory roles of progestins in reproductive and endocrine functions. Immunocytochemistry of ovarian follicles, using a polyclonal antibody directed against a peptide fragment in the N-terminal region of stmPRα, indicated strong immunostaining only peripherally in the region of the oocyte plasma membrane, with practically no staining intracellularly or in the follicle cells surrounding the oocytes, as expected for the receptor mediating MIS induction of oocyte maturation. Western blot analysis of ovarian membranes showed two immmunoreactive bands, one at ~40 kDa, the predicted size of stmPRα, and another at approximately twice that size, which is presumably represents a dimer of the protein. Strong immunoreactive bands (~40 and 80 kDa) have also detected using the stmPRα antibody in Western blots of sea trout sperm plasma membranes, where a progestin membrane receptor has previously been characterized using radioreceptor assay techniques (Thomas et al., 1997). Of course it is critical to demonstrate that the recombinant protein can bind progestins with the characteristics of a hormone receptor. High affinity (K_d 30 nM), limited capacity (B_{max}

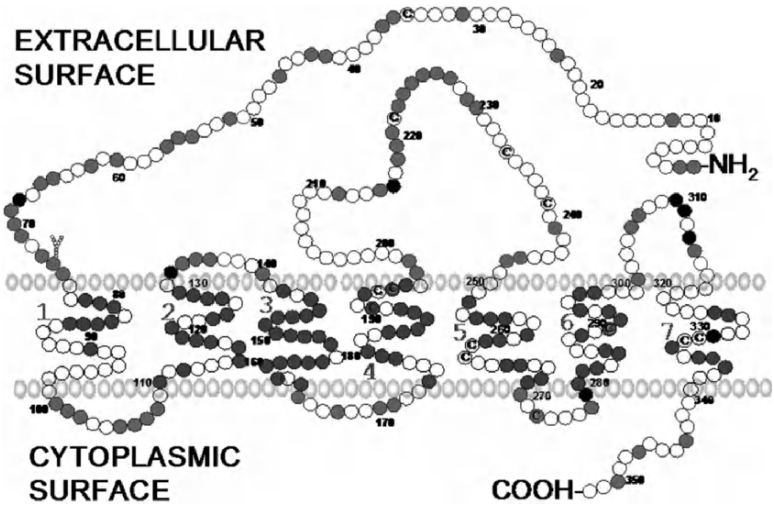

Figure 2. Proposed model for insertion of the spotted sea trout mPRα in the plasma membrane with the N-terminal on the outside of the cell. Residues identical with other vertebrate mPRαs are shown by the grey filled circles. Residues diagnostic of this clade are shown as filled black circles. Y: potential N-linked glycosylation site; C: cysteine residues. (Reproduced from Zhu et al., 2003b; with permission.)

0.49 nM), displaceable, and specific binding for progestins typical of steroid membrane receptors was initially demonstrated with the recombinant protein produced in a bacterial (*Escherichia coli*) expression system (Zhu et al., 2003b), and subsequently confirmed in membranes prepared from human breast cancer cells (MDA-MB-231 cells) transfected with the sea trout cDNA. Rapid activation of signal transduction pathways in the transfected human cells was observed after treatment with physiological concentrations of progestins, but not after treatment with estrogens and androgens. Treatment with 20β-S caused a decrease in adenylyl cyclase activity which was reversed by pretreatment with activated PTX, but not after pretreatment with inactive PTX, which suggested stmPRα activates an inhibitory G protein (Zhu et al., 2003b). Subsequent studies have confirmed that stmPRα is coupled to and activates an inhibitory G protein, consistent with its identity as the MIS receptor. The patterns of stmPRα protein expression in oocytes at various stages of development and in response to hormonal treatments also support a role for the receptor during oocyte maturation. Concentrations of stmPRα are elevated in fully grown oocytes and show a further increase in response to gonadotropin treatment during oocyte maturation (Zhu et al., 2003b), similar to the pattern of changes in 20β-S receptor-binding activity during oocyte maturation described previously (Thomas et al., 2001). Direct evidence of a requirement for stmPRα in oocyte maturation by microinjection of antisense oligonucleotides could not be obtained with sea trout follicle-enclosed oocytes which are relatively small and fragile and did not

tolerate the microinjection procedure. Therefore, phosphothioate and morpholino antisense oligonucleotides were generated to the homologous receptor identified in zebrafish (zfmPRα), which has larger oocytes suitable for microinjection. Injection of both types of antisense oligonucleotides, but not sense or missense ones, during gonadotropin induction of priming blocked the subsequent response of zebrafish oocytes to 17,20βDHP, most of them failing to mature after treatment with the MIS (Zhu et al., 2003b). It is concluded from these studies that mPRα is the MIS receptor in these two teleost species.

3.1.6. Characteristics of mPRs in other species

Subsequently, 13 closely-related genes were identified in other vertebrates that could be separated into three clades on the basis of sequence identity and phylogenetic analysis, called α, β, and γ subtypes, which are primarily localized in reproductive tissues in mammals (ovary, testis, placenta, uterus), brain, and renal tissues, respectively (Zhu et al., 2003a). Moreover, saturation and Scatchard analyses showed that the mammalian recombinant α, β, and γ soluble recombinant proteins produced in an prokaryotic expression system also have high affinity (K_d 20–30 nM), saturable (B_{max} 0.3–0.5 nM), single-binding sites specific for progestins (Zhu et al., 2003a). Recent results indicate that mPRα and mPRβ are present in human myometrial tissues and are coupled to inhibitory G-proteins (Karteris et al., 2006). Interestingly, the first evidence for crosstalk between the mPRs and the nuclear progesterone receptor (nPR) was obtained in the human myocytes. The results show that progesterone, acting through a PTX-dependent pathway, can decrease nPR transactivation by altering the expression of a coactivator, SRC-2. These findings provide a plausible explanation of how a functional progesterone withdrawal can occur in the presence of high-circulating levels of progesterone and suggest a critical role for the mPRs in the actions of progesterone in women at term (Karteris et al., 2006).

Since their discovery in spotted sea trout and zebrafish, mPR subtypes have been identified in other teleost species. The α subtype has been identified in both the oocytes and sperm of Atlantic croaker and appears to have a role in hormonal induction of maturation of both female and male gametes in this species (Pace, 2005b; Thomas, 2006). The mPRα subtype has also been cloned from a goldfish, *Carassius auratus*, ovarian cDNA library (Tokumoto et al., 2006). Western blotting using a specific goldfish mPRα antibody demonstrated the presence of an immunoreactive band of the predicted molecular weight (~40 kDa) in oocyte plasma membranes. Similar to the previous results in spotted sea trout, mPRα protein expression in fully grown goldfish oocytes was upregulated by treatment with gonadotropin and was associated with the development of oocyte maturational competence. Moreover, microinjection of mPRα antisense morpholino oligonucleotides into goldfish oocytes blocked both the induction of oocyte maturation in response to the MIS and the upregulation of mPRα protein levels (Tokumoto et al., 2006). Thus the results obtained with representatives of two distantly related teleosts families, the Scaenidae and

Cypriniformes, support the suggestion that mPRα acts as an intermediary in MIS induction of oocyte maturation in teleosts. The finding that microinjection with antisense oligonucleotides to zebrafish mPRβ also partially blocks oocyte maturation in this species suggests this subtype is also involved in MIS signaling during this process (Thomas et al., 2004). The three mPR subtypes have also been cloned from a channel catfish (*Ictalurus punctatus*) ovarian cDNA library (Kazeto et al., 2005). Although the pattern of changes observed in gene expression of the mPR subtypes (α, β, and γ) in catfish ovaries during the final stages of gametogenesis and in response to hormonal treatment suggests that gonadotropin induction of oocyte maturation does not involve upregulation of the mPR message in oocytes, it does not preclude posttranslational regulation of mPR protein expression by gonadotropin (Kazeto et al., 2005). In fact, differential upregulation by gonadotropin of mPRα protein levels in the absence of changes in mRNA expression has already been described during maturation of both goldfish and croaker oocytes (Pace and Thomas, 2005a; Tokumoto et al., 2006).

The mPRs are likely involved in other functions of progestins in teleosts because they are expressed in a wide variety of tissues (Zhu et al., 2003a; Kazeto et al., 2005), including sperm and the hypothalamus, where progestins have been shown to exert rapid, nongenomic actions (Thomas, 2004, 2006). Recently, mPRα has been identified in ovarian follicle cells of Atlantic croaker (unpublished observation). Thus, the MIS could potentially act via nontraditional as well as though genomic mechanisms in ovarian follicle cells during oocyte maturation and ovulation in teleosts. The possible functions of the mPRs in the follicle cells surrounding teleost oocytes and their potential interactions with the nPR are interesting research questions to address in the future.

3.2. Membrane Androgen Receptors

Rapid, cell surface-initiated, nongenomic actions of androgens and the membrane receptors thought to mediate these effects have been described in a variety of vertebrate tissues. For example, androgen stimulation of calcium influx initiated at the cell surface has been observed in mouse T cells which lack a nAR through a PTX-sensitive pathway, suggesting the involvement of an inhibitory G protein (Lieberherr and Grosse, 1994; Wunderlich et al., 2002; Benten et al., 2004). Moreover, androgen binding to plasma membranes has been partially characterized in these T cells and also in mammalian endothelial cells, osteoblasts, prostate cells, glial cells, and macrophages (Lieberherr and Grosse, 1994; Benten et al., 1999; Armen and Gay, 2000; Kampa et al., 2002; Somjen et al., 2004). Recently, a nontraditional action of androgen was identified in Atlantic croaker ovaries. The androgens dihydrotestosterone, 11-ketotesterone and the synthetic androgen mibolerone inhibited gonadotropin-stimulated estradiol-17β production from Atlantic croaker ovarian fragments *in vitro*, while treatment with the nAR antagonist, cyproterone acetate, did not reverse the

androgen inhibition (Braun and Thomas, 2003). The finding that dihydrotesterone conjugated to bovine serum albumin, which cannot pass through the cell membrane, exerted similar inhibitory effects on estrogen production suggests this androgen action is initiated at the cell surface. Furthermore, co-treatment with actinomycin D did not block the inhibitory response, indicating that the androgens are acting through a nongenomic mechanism (Braun and Thomas, 2003). Taken together, these findings suggest that the androgens could be acting through a nontraditional androgen receptor.

Subsequently, an androgen membrane receptor was extensively characterized in Atlantic croaker ovaries (Braun and Thomas, 2004). The ovarian membrane protein has a high affinity (K_d 15.3 nM), limited capacity (B_{max} 2.8 pmol/mg protein) and a displaceable single-binding site specific for androgens, all characteristics of steroid receptors. The steroid specificity of the croaker ovarian membrane receptor differs from that of the nARs, showing no affinity for R1881 and mibolerone, whereas they bind to the nAR previously characterized in the ovary of this species (Sperry and Thomas, 1999a; Braun and Thomas, 2004). The lack of agreement between the nongenomic activity of milobolerone and its binding to ovarian membranes raises the possibility that the inhibitory effects of androgens on estrogen production may be mediated by a different uncharacterized androgen membrane receptor. Interestingly, the membrane androgen receptor displays high-binding affinity for progesterone, although R5020 and RU486 and the fish progestins 20β-S and 17,20βDHP do not compete for androgen binding to the receptor (Braun and Thomas, 2004, Thomas et al., 2006). Moreover, the rates of ligand association/dissociation to the membrane receptor are much more rapid than they are to the nAR, further suggesting that the membrane bound androgen receptor is unrelated to nARs.

Recently it was shown that testosterone treatment of ovarian cell membranes causes G-protein activation, which is in agreement with the earlier findings in T cells (Lieberherr and Grosse, 1994), indicating that the mAR might be a GPCR (Thomas et al., 2006). Separation of solubilized ovarian membrane proteins on a DEAE column, and subsequent determination of the approximate molecular weight of the major protein present in the eluted fraction containing the androgen-binding activity indicates it has a molecular weight of approximately 40 kDa, within the molecular weight range of GPCRs (Thomas et al., 2006). Currently the identity of the ~40 kDa protein in croaker ovaries with androgen-binding activity is being investigated.

3.3. Membrane Estrogen Receptors

Nongenomic, cell surface-initiated actions of estrogens in teleost gonads were first described in the mature testis of the Atlantic croaker in which a membrane estrogen receptor was also fully characterized (Loomis and Thomas, 1999). It was observed that estrogen decreases hCG-stimulated androgen synthesis in the testis via a nongenomic estrogen mechanism, since it is not blocked by

transcription and translation inhibitors, and is cell-surface mediated (Loomis and Thomas, 2000). Binding studies on the membrane-localized receptor confirmed that it bound estradiol-17β and DES with high affinity as well as the antiestrogens, tamoxifen and ICI 182,780. In general, the binding characteristics of the membrane estrogen receptors are similar to the nER in this species (Loomis and Thomas, 2000). However, the finding that ICI 182,780 does not act as an antiestrogen, but instead decreases testicular androgen production, suggests that the membrane estrogen receptor mediating these effects may be unrelated to the nER, but its identity remains unknown. Cell-surfaced initiated, rapid estrogen actions identified in mammalian models include increases in cAMP in estrogen-responsive breast cancers (Aronica et al., 1994) and calcium flux in the developing human oocyte (Tesarik and Mendoza, 1995). In some models the nER has been identified in the cell membrane (Razandi et al., 1999) and shown to associate with caveolin, a membrane scaffolding protein (Razandi et al., 2002). Recently, an orphan G-protein coupled receptor, GPR30, first identified in the MCF7 human breast cancer cell line (Carmeci et al., 1997) and associated with estrogen signaling through adenylyl cyclase and transactivation of epidermal growth factor receptor (Filardo, 2002; Filardo et al., 2002; Filardo and Thomas, 2005), was shown to have all the characteristics of a high affinity estrogen membrane receptor (Thomas, 2006). Binding of estrogens, tamoxifen and ICI 182,780 to GPR30 results in activation of a stimulatory G protein (G_s) and increases in adenylyl cyclase activity (Thomas et al., 2005a). Preliminary evidence has been obtained that GPR30 is also expressed in croaker ovaries. The finding that exposure of croaker ovarian membrane fractions to estradiol causes an increase in cAMP levels suggests a similar mechanism of estrogen action in this tissue, possibly through GPR30 (Thomas, 2006). However, despite these recent advances we only have a rudimentary understanding of the nongenomic actions of estrogens in the teleost ovary and the identities of the receptors through which they are mediated. Investigations of the functions of GPR30 in the croaker ovary and testis could provide valuable insights of its involvement in estrogen signaling in vertebrate gonads.

4. ENDOCRINE DISRUPTION OF NONGENOMIC STEROID ACTIONS

Teleost gametes and gonads have proven to be excellent models for examining the potential of environmental estrogens (xenoestrogens) and other contaminants to interfere with nontraditional steroid actions (Thomas, 2000a). The first clear evidence for chemical interference with this mechanism was obtained with Atlantic croaker oocytes, which failed to undergo oocyte maturation in response to 20β-S in an *in vitro* assay in the presence of several xenoestrogens (Ghosh and Thomas, 1995). Subsequently, it was shown that this antagonism of MIS action by xenoestrogens was through binding to the membrane progestin receptor

(Das and Thomas, 1999). Interestingly, several xenoestrogens have been shown to interfere with progestin stimulation of sperm motility in this species by the same receptor-mediated mechanism (Thomas et al., 1998; Thomas and Doughty, 2004). The identification of the novel mPR involved in oocyte maturation enables the interactions of xenoestrogens with membrane steroid receptors to be investigated for the first time at the molecular level. Xenoestrogens also have the potential to interfere with the actions of estrogens through binding to the membrane estrogen receptor. The ortho, para derivative of DDT (o′,p′-DDE), binds to membrane estrogen receptor recently identified in human breast cancer cells, GPR30, and has estrogenic activity in cells expressing the receptor (Thomas, 2006). Recently GPR30 has been detected in croaker ovaries (unpublished observation). In croaker testes the decrease in androgen production by estrogens was mimicked by Kepone, nonylphenol, and 2,2′, 5′-PCB-4-OH. Moreover, several xenoestrogens including o,p′-DDT, o,p′-DDE and 2,2′,5′-PCB-4-OH have also been shown to bind to the membrane estrogen receptor identified in this tissue (Loomis and Thomas, 1999).

5. CONCLUDING REMARKS

Considerable progress has been made over the last few years in identifying both nuclear and membrane sex steroid receptors in teleost ovaries. Unfortunately, fewer advances have been made on the ovarian actions of steroids and the functions of many of these receptors remain unclear. More comprehensive studies on the cellular distribution of steroid receptors in teleost ovaries, their patterns of expression during gonadal differentiation and the reproductive cycle, and their hormonal regulation will be required to formulate testable hypotheses of their physiological functions. The more extensive information on steroid actions in mammalian ovaries should also provide clues of the likely roles of these receptors in teleosts. However, elucidating the precise functions of the estrogen, androgen, and progestin nuclear and membrane receptors in teleost ovaries throughout the reproductive cycle will be a major challenge that will likely require an extensive research effort over the next decade by reproductive physiologists. It is hoped that by the time this topic is next reviewed we will have a better understanding of the roles of sex steroid receptors in regulating the functions of the teleost ovary.

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CHAPTER 9

HORMONAL REGULATION OF FOLLICULAR ATRESIA IN TELEOST FISH

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1. INTRODUCTION

In fish, cyclic ovarian development is synchronized by hormones that are predominantly produced by the brain, pituitary, and ovary. These hormones act to promote early development of ovarian follicles, vitellogenesis, oocyte growth, differentiation, maturation into a preovulatory oocyte, and finally the release of a group of eggs through the process of ovulation. The brain and pituitary comprise the neuroendocrine level of control and are constantly regulated through feedback mechanism by ovarian hormones (see Chapters 7 and 8).

Ovarian development and growth results from the positive balance between follicular development and atresia. Follicle growth is characterized by several stages (see Chapter 1), including: (1) Primary oocyte growth, which involves formation of the zona radiata, granulosa, and thecal cell layers; (2) Formation of lipid bodies, cortical alveoli, and expression of polysialoglycoproteins; (3)

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Vitellogenesis, leading to ovarian follicle growth; in fish, increase in oocyte size is mostly due to the uptake of vitellogenin (Vtg), which is produced by the liver and is then processed and clived as yolk proteins by the oocyte (a process different from mammals) (see Chapter 2); (4) Oocyte maturation, initiated by the surge of gonadotropins, which stimulate the production of the maturation inducing hormone and resumption of meiosis (see Chapter 11); (5) Ovulation: oocytes are arrested at the metaphase stage of meiosis II and expelled to the body cavity (Nagahama, 1983; Nagahama et al., 1995; Tyler et al., 1996; Jalabert, 2005). Although follicular atresia is a common event in the mammalian ovary, the presence of atretic follicles in the teleost ovary is frequently associated with environmental stress or changes in hormone levels (Saidapur, 1978; Nagahama, 1983, 1994; Nagahama et al., 1995). Four morphological stages of follicular atresia have been identified in teleosts: (1) oocyte fragmentation and hypertrophy of follicular or granulosa cells and/or thecal cells; (2) follicular cell invasion of the space occupied by the oocyte and elimination of cellular remnants; (3) granulosa cell degeneration and appearance of orange pigment; (4) degeneration of the atretic follicles (Saidapur, 1978; Nagahama, 1983). During follicular atresia, there are also changes in plasma composition. For example, the yolk material in the oocytes that fail to ovulate are reabsorbed, leading to increased circulatory yolk proteins associated with high-density lipoproteins (HDL) in rainbow trout (Babin, 1987). In the common carp, as follicular atresia progresses, there is accumulation of triglycerides as well as cholesterol and/or its esters, and this phenomenon has been attributed to follicular degeneration (reviewed in Saidapur, 1978; Guraya, 1986). Fatty acid-binding protein 11 mRNA level is correlated to ovarian follicle atresia in *Solea senegalensis* and this transcript may be a useful molecular marker for determining cellular events and environmental factors that regulate follicular atresia in teleost fish (Agulleiro et al., 2007). Resorption of the yolk in atretic follicles results in formation of corpora atretica, apparently due to the action of phagocytic follicular cells, which degenerate afterwards (Saidapur, 1978; Babin, 1987). In the fish corpora atretica, there is evidence for the expression of steroidogenic enzymes, indicating possible secretory role (reviewed in Saidapur, 1978; Guraya, 1986), although a number of investigators failed to detect synthesis of steroid hormones (reviewed in Guraya, 1986). In this context, the endocrine role of corpora atretic in fish remains unresolved.

While in mammals ovarian follicular atresia is closely associated with apoptosis (Kaipia and Hsueh, 1997; Morita and Tilly, 1999; Matova and Cooley, 2001; Hussein, 2005), similar relationship has not been established in fish. A recent study, however, demonstrated occurrence of apoptosis in postovulatory follicles of a teleost species (Drummond et al., 2000). In mammals, more than 95% of the follicles present at birth degenerate during life and only a few hundreds will eventually ovulate. The majority of the remaining follicles undergo atresia (Kaipia and Hsueh, 1997). In fish, most of the follicles ovulate in each reproductive cycle either as a whole or in several batches, depending on the species (Nagahama, 1983). Atresia occurs only if follicles fail to ovulate due to the disruption of the reproductive process. In fish as well as mammals, hormonal signals regulate the

initiation of follicular maturation and atresia (Nagahama, 1994; Nagahama et al., 1995). This review provides a brief overview of the hormonal coordination of brain–pituitary–gonadal axis, endocrine and paracrine regulation of synchronized follicular development, and examines the possibility that apoptosis may be the mechanism underlying ovarian follicular atresia in fish.

2. HORMONAL COORDINATION OF THE BRAIN–PITUITARY–GONADAL AXIS

Gonadotropin-releasing hormone (GnRH) is a decapeptide, best known for its regulation of the synthesis and release of gonadotropin hormones (GtHs) (Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH)), which in turn, stimulate the gonadal function in all vertebrates. Following the demonstration of novels forms of GnRH molecules in chicken (chicken GnRH-I; King and Millar, 1982) and salmon (salmon GnRH; Sherwood et al., 1983), numerous studies have demonstrated the presence of at least 24 molecular variants of GnRH in various vertebrate and invertebrate species (for more recent reviews see Lethimonier et al., 2003; Iwakoshi et al., 2002; Adams et al., 2003; Morgan and Millar, 2004). At the present time, however, the function of these GnRH variants in protochordates and invertebrates remains uncertain. Multiple forms of GnRH are detected in all vertebrate species, including the ubiquitous chicken cGnRH-II, which is present in species that evolved 500 million years ago. In this context, the brain of most species tested contains at least two GnRH variants, while the brain of certain teleosts contains three or more GnRH variants (Lethimonier et al., 2003). In addition to its action on pituitary cells, there is also clear evidence that GnRH and GnRH receptors (GnRH-R) are expressed in various extrapituitary tissues, including ovaries and testes, as well as in tumor cells derived from various tissues, where they are postulated to exert a paracrine function (Hsueh et al., 1996; Habibi, 1999; Habibi and Matsoukas, 1999; Imai and Tamaya, 2000; Habibi et al., 2001).

The existence of different GnRH forms implies that functionally distinct GnRH receptor types may also be present. Studies have demonstrated the existence of three types of GnRH receptors (Type I, Type II, and Type III), which differ in both, structure and ligand selectivity, in the pituitary and extrapituitary tissues of a number of vertebrates. In bullfrog, the presence of three genes encoding GnRH-R has been demonstrated (Wang et al., 2001), although other amphibians such as *Xenopus laevis* may only have two receptor forms (Troskie et al., 2000). In mammals, two different types of GnRH-R have been isolated (Type I and Type II), one without the C-terminal tail (Type I) and the other (type II) containing a cytoplasmic tail domain (Millar et al., 2001; Neill et al., 2001). However, some mammalian species may only have one functional form of GnRH-R (Type I) despite the presence of two distinct GnRH-R genes because of a frame shift that results in a premature stop codon (Morgan and Millar, 2004). Similarly, there is evidence for the presence of multiple forms of GnRH-Rs in fish (Lethimonier et al., 2003) and their coupling to different intracellular signaling pathways (Klausen et al., 2002). Recently, three molecular forms of

GnRH-R have been demonstrated in various cell types of the pituitary gland of tilapia (Parhar et al., 2005), and there is a report on identification of a third novel form of GnRH-R in medaka (Okubo et al., 2003). Furthermore, five different forms of GnRH-R cDNA have been described in masu salmon (Jodo et al., 2003). However, there is little information on their ligand selectivity and functional significance at the present time.

In addition to GnRH, other neuromodulatory factors including, steroids, catecholamines, amino acids, and gonadal peptides regulate the production of FSH and LH. For instance, dopamine is known to inhibit basal and GnRH-induced GtH release from gonadotropes in a number of fish species. These topics have been previously reviewed and will not be discussed here (Peter and Yu, 1997; Peter and Chang, 1999; Chang and Habibi, 2003; Yaron et al., 2003).

3. THE ROLE OF GONADAL HORMONES IN THE CONTROL OF PITUITARY GONADOTROPIN PRODUCTION

Fish are seasonal spawners and undergo distinct annual reproductive cycles in response to environmental cues. Sexual maturity and gonadal development is associated with increased circulating levels of gonadotropins and the gonadal steroids (Kobayashi et al., 1987, 1988; Habibi et al., 1989; Huggard et al., 1996, 2002). In fish, the circulating concentrations of sex steroids such as testosterone and estradiol (E2) are important factors controlling the preovulatory GtH surge (for review, see Peter and Yu, 1997; Yaron et al., 2003). Direct stimulatory effects of steroids have been demonstrated in a number of fish species including goldfish (Huggard et al., 1996, 2002), rainbow trout (Xiong et al., 1994), European silver eels (Querat et al., 1991), coho salmon (Trinh et al., 1986), European eels (Dufour et al., 1983), and juvenile rainbow trout (Crim et al., 1981). In goldfish, treatment with testosterone and estrogen have been shown to stimulate the mRNA levels of both alpha, and LH-β and FSH-β subunits with varying sensitivity at different stages of the gonadal cycle (Huggard et al., 1996, 2002). There is also evidence for significant variability in the response to gonadal steroids in other fish species, depending on both, the stage of sexual maturity and the reproductive season. Contrary to the results observed in cells from sexually immature rainbow trout, treatment of pituitary cells from spawning rainbow trout with E2 did not affect LH-β transcript levels (Xiong et al., 1994), and in mature rainbow trout, injection with E2 was found to reduce circulating LH levels (Billard et al., 1977). In African catfish, treatment with an aromatizable androgen was found to reduce GtH release, while it stimulated LH synthesis (De Leeuw et al., 1986). In general, pituitary cells from teleosts have stronger aromatase activity than those from mammals (Callard et al., 1990, 1993). In goldfish, the activity of the aromatase enzyme is maximal during the spawning season when the circulating steroid levels are relatively high, suggesting that estrogenic effects are important at this time (Pasmanik and Callard, 1988). While aromatization is important (Trudeau et al., 1993; Trudeau and Peter, 1995), there is evidence that androgens

alone also affect gonadotropin production since treatment of goldfish pituitary fragments with a nonaromatizable androgen was found to stimulate LH subunit mRNA levels (Huggard et al., 1996). In goldfish *in vivo* and *in vitro* treatment with E2 was found to stimulate all three GtH subunit mRNA levels with significant seasonal variations (Huggard et al., 2002). In addition, E2 was found to influence the GnRH-induced GtH subunit mRNA levels in a seasonally dependent manner (Huggard et al., 2002). These findings demonstrate a significant interaction between the hormones produced by the brain, pituitary, and gonadal tissues, which is ultimately responsible for the observed cyclic changes in circulating hormones and variations in the sensitivity of the pituitary or brain to gonadal steroids and peptides.

4. ENDOCRINE AND PARACRINE REGULATION OF SYNCHRONIZED FOLLICULAR DEVELOPMENT: GnRH AS A CENTRAL PLAYER IN FOLLICULAR DEVELOPMENT

In each reproductive cycle, a large number of oocytes develop as a group and undergo synchronous maturation and ovulation either as a whole or in several batches depending on the species. Environmental cues including temperature, photoperiod, lunar cycle, or the amount of rainfall are perceived and conveyed as endogenous stimuli to regulate endocrine system to synchronize growth and reproduction. Early stages of ovarian development are the result of vitellogenesis, starting with the mobilization of lipids from storage sites, the synthesis of Vtg in the liver, and its deposition in oocytes. It has been known for many decades that the absence of hormone-derived vitellogenesis caused by hypophysectomy results in ovarian atresia (reviewed in Saidapur, 1978; Guraya, 1986). In fish, vitellogenesis is primarily regulated by hormones of pituitary and ovarian origin. Numerous studies have demonstrated the importance of estrogens and GtHs in the control of vitellogenesis in fish (for a review see Polzonetti-Magni et al., 2004). In fish, and probably in many other species, GnRH peptides regulate synchronized follicular development at two separate levels: the pituitary and the ovary. Hypothalamic GnRH exerts an important hypophysiotropic function, resulting in increased secretion of GtH and GH (Klaussen et al., 2002; Chang and Habibi, 2003), which in turn stimulate steroidogenesis, vitellogenesis, and gametogenesis. On the other hand, GnRH peptides produced in the gonads (Pati and Habibi, 1998) regulate steroidogenesis and apoptosis in the ovary and testis (Andreu-Vieyra and Habibi, 2000; Andreu-Vieyra, et al., 2005) and work in concert with other hormones to maintain gonadal synchronicity. The latter is a relatively new hypothesis, which is based on recent studies carried out in goldfish and seabream. In this regard, there is unambiguous evidence demonstrating the presence of GnRH and GnRH-R in the gonadal tissue of fish and other vertebrate species (Pati and Habibi, 1992, 1993, 1998; Habibi et al., 1994, 2001; Gazourian et al., 1997; Nabissi et al., 1997). Further studies have provided strong support for the hypothesis that locally produced GnRH plays an autocrine/paracrine role

in the regulation of gonadal function. In fish, ovarian extract was shown to contain compounds with GnRH-like activity (Habibi and Pati, 1993; Habibi et al., 1994; Nabissi, et al., 1997; Habibi, 1999). In these studies, treatment of goldfish cultured pituitary fragments with ovarian extracts was found to stimulate LH release and subunit gene expression, which could be inhibited by co-treatment with a GnRH antagonist. Furthermore, serially diluted ovarian extract was found to specifically bind to GnRH-R in the goldfish ovary and pituitary. High-performance lipid chromatography (HPLC) analysis of goldfish ovarian extracts revealed the presence of two fractions with high GnRH-like activity: an early eluting peak and a second peak that co-eluted exactly with sGnRH. Amino acid analysis and sequencing confirmed the presence of a decapeptide with identical primary structure as salmon GnRH (Pati and Habibi, 1998). In that study, however, the early eluting compound with GnRH-like activity could not be identified, suggesting the presence of either a novel form of GnRH or a GnRH variant resulting from differential splicing of GnRH transcripts (Pati and Habibi, 1998; Nabissi et al., 2000). The presence of compounds with GnRH-like activity was also demonstrated in the ovary of African catfish (Habibi et al., 1994) and seabream (Nabissi et al., 1997). In spite of extensive attempts to purify GnRH peptides from the ovary, cGnRH-II peptide, which is present in the goldfish brain, was not detected in goldfish ovarian extracts suggesting that cGnRH-II expression in the ovary of this species is either very low or transient. All GnRH peptides are produced as immature precursors, composed by GnRH associated peptide (GAP), a signal peptide, and the GnRH sequences. Proteolytic cleavage of the immature precursor renders the active GnRH form. In recent years, characterization of complementary DNA (cDNA) sequences encoding the precursor of GnRH molecules made it possible for many investigators to study the expression of gonadal GnRH genes in various mammalian and nonmammalian species (Sakakibara, 1989; Oikawa et al., 1990; Goubau et al., 1992; Millar and King, 1994; Peng et al., 1994; Lin and Peter, 1996; Millar et al., 1997; Nabissi et al., 2000). While the general structure of the GnRH moiety is highly conserved, the GAP and signal peptide regions display less than 50% similarity among different forms. Furthermore, GnRH transcripts were found to contain intronic sequences and undergo differential splicing suggesting the production of different size precursor GnRH molecules in the ovary of mammals (Goubau et al., 1992) and seabream (Nabissi et al., 2000) as well as other fishes (Lethimonier, et al., 2003; Morgan and Millar, 2004).

The extensive study of GnRH action in the ovary of goldfish has led to a better understanding of the physiological significance of gonadal GnRH peptides. There is clear evidence demonstrating that GnRH agonists influence meiotic maturation, as defined by germinal vesicle breakdown (GVBD) and H1 kinase activity, steroidogenesis, and apoptosis. Both endogenous forms of GnRH, sGnRH and cGnRH-II, were found to stimulate GVBD *in vitro* as well as histone H1 kinase activity, which is an indicator of maturation promoting factor (MPF) activation (Pati and Habibi, 2000; Pati et al., 2000). However, sGnRH, which is found in the ovary (Pati and Habibi, 1998; Figure 1), was found to

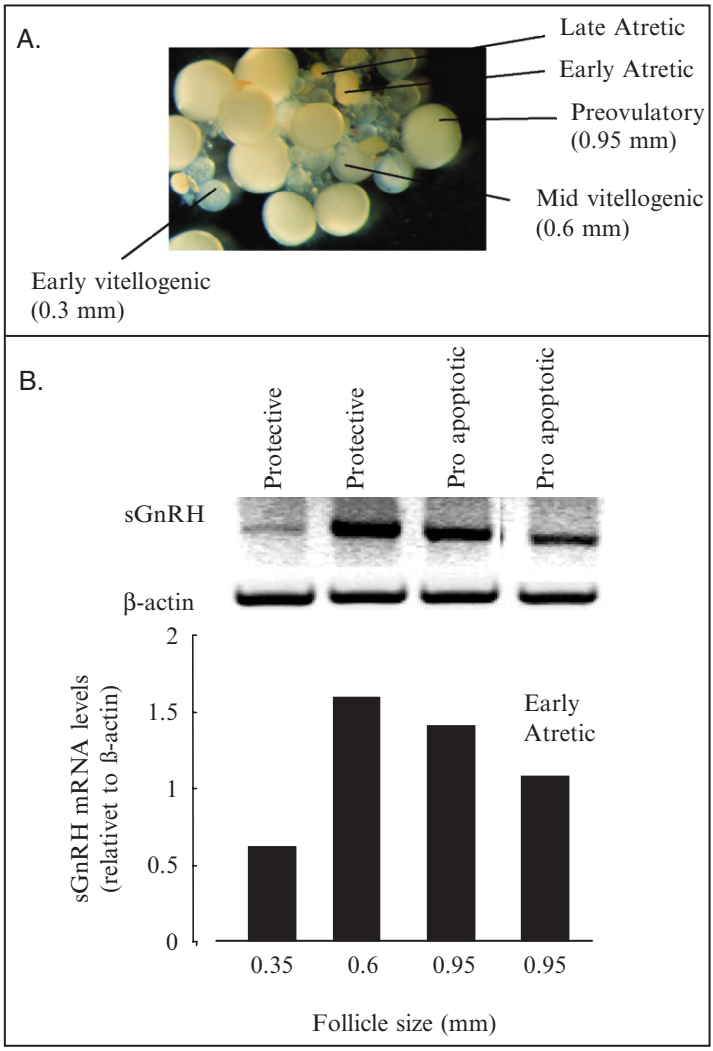

Figure 1. Morphological appearance and expression of salmon GnRH transcript in goldfish ovarian follicles at different stages of maturity; 0.35 mm: early vitellogenic; 0.6 mm: mid-vitellogenic; 0.95 mm: mature, preovulatory. (B) Values are expressed relative to b-actin levels to correct for total amount of RNA. The quantified values represent the mean of two observations. Early atretic follicles were obtained from the ovaries that failed to ovulate and showed signs of atresia (appearance of orange pigment and signs of degeneration (A). (*See Color Plates*).

inhibit GtH-induced GVBD and steroidogenesis, while cGnRH-II had no effect on GtH-induced responses (Pati and Habibi, 2000). These findings suggest the involvement of different receptor-activated pathways mediating the stimulatory and inhibitory actions of sGnRH in the goldfish follicles (Pati and Habibi, 2002). The seemingly opposite actions of GnRH in gonadal tissues have been

difficult to reconcile. However, recent studies on GnRH-induced apoptosis in the goldfish ovary and testis have helped to clarify GnRH gonadal function and have provided a base for better understanding the functional significance of GnRH in follicular development and atresia.

5. APOPTOSIS AND FOLLICULAR ATRESIA: IS GnRH AN ATRETOGENIC FACTOR IN TELEOSTS?

Despite the fact that the morphological characteristics of follicular atresia in the fish ovary have been known for more than three decades, the molecular mechanisms underlying stress-induced atresia have remained largely unknown. The vast majority of the available information about follicular atresia comes from studies carried out in mammalian models. It is currently accepted that atresia is a hormonally controlled apoptotic process, since it displays all morphological features described for this type of cell death (Hughes and Gorospe, 1991; Tilly et al., 1991; Hsueh et al., 1994; Palumbo and Yeh, 1994, 1995, Hussein, 2005). Apoptosis or programmed cell death is characterized by biochemical and morphological changes including chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies (Hacker, 2000). Apoptosis also involves the sequential activation of a cascade of caspases, a set of cysteine-dependent aspartyl-directed enzymes that are present in the cell as inactive zymogens (Enari et al., 1998). In particular, caspase-3 has been shown to activate a DNAse that is responsible for the "DNA ladder," a pattern of low molecular DNA fragments observed after gel electrophoresis (Minaretzis et al., 1995; Enari et al., 1998). Induction of apoptosis can occur via a receptor mediated or receptor independent pathway, which may involve mitochondrial factors. Fas is one of the "death receptors," which has been shown to participate in the induction of apoptosis in ovarian and endometrial tumor cells as well as uterine leiomyomas and other cell types (Imai and Tamaya, 2000). Activation of fas by its ligand (fas ligand (fasL)) leads to the activation of the caspase cascade and ultimately to DNA fragmentation and cell death (Beyaert et al., 2002; Wajant, 2002; Figure 2). On the other hand, different stressors have been shown to cause changes in mitochondrial membrane potential and to stimulate the release of several mitochondrial factors, including caspase-9 and apoptosis activating factor 1 (Apaf-1), which in turn activate the caspase cascade (Strasser et al., 2000). The B-cell lymphoma (Bcl) family of proteins, which include pro-apoptotic proteins such as Bax and anti-apoptotic proteins such as Bcl-2 and Bcl-xL, regulate the release of mitochondrial factors. The death receptor and mitochondrial pathways can act independently or synergistically depending on the pro-apoptotic stimuli (Hussein, 2005; Figure 2).

Apoptosis has been demonstrated in the ovary of several fish species, including goldfish and rainbow trout, both *in vivo* and *in vitro* (Janz and Van der Kraak, 1997; Wood and Van der Kraak, 2001), and in response to the presence of endocrine disrupting chemicals (Janz et al., 1997). Moreover, the various components of the apoptotic machinery, including effector caspases such as

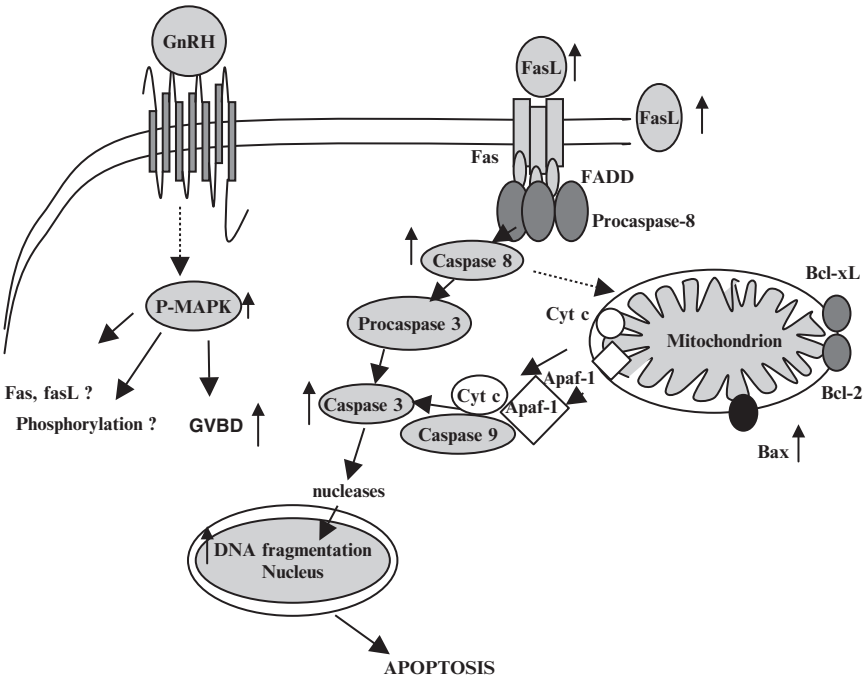

Figure 2. Proposed mechanism of GnRH-induced apoptosis in goldfish gonadal tissues. In peri-ovulatory ovaries, GnRH interaction with its receptor activates the MAPK pathway, which may lead to increases in Fas and fas ligand (fasL), protein phosphorylation and resumption of meiosis (germinal vesicle breakdown (GVBD). An increase in fas/faL and their interaction will recruit caspase-8, which associates with fas and cleaves pro-caspase-3, which, in turn can activate a nuclease responsible for DNA fragmentation. Activation of caspase-8 may lead to changes in mitochondrial membrane potential, causing the release of mitochondrial factors such as cytochrome c (cyt c) and apoptosis activating factor 1 (Apaf-1). These factors are required for caspase-9 activation, which in turn can activate caspase-3, amplifying the apoptotic signal. In addition, changes in the levels of the Bcl 2 family of proteins may contribute to mitochondrial factor release. In this case, increases in the pro-apoptotic protein Bax will offset its balance with anti-apoptotic proteins Bcl2 and Bcl-xL, further increasing cyt c and apaf-1 release. Upright arrows show changes in protein levels that have been demonstrated in goldfish testes and/or ovaries in response to GnRH treatment.

caspase-3, have been shown to be present in the ovary of mammals and birds (reviewed in Andreu-Vieyra and Habibi, 2000; Johnson and Bridgham, 2000). Although caspase tissue distribution in teleost gonads remains unknown, we have recently detected caspase-3 activity in the goldfish testes and ovary (Andreu-Vieyra et al., 2005; Andreu-Vieyra and Habibi, unpublished data). More recently, studies have focused on the regulation of apoptosis in the ovary and several factors have been shown to provide protection against gonadal cell apoptosis under different conditions (Andreu-Vieyra and Habibi, 2000). For example, GtHs and E2 have been shown to prevent apoptosis in the mammalian

ovary under a variety of conditions (Billig et al., 1993; Chun et al., 1996; Kaipia and Hsueh, 1997; McGee et al., 1997; Andreu-Vieyra and Habibi, 2000; Markstrom et al., 2002; Wood and Van der Kraak, 2002). Similarly, *in vitro* treatment of rainbow trout follicles with epidermal growth factor, E2, and gonadotropins has been shown to suppress DNA fragmentation induced by serum deprivation (Janz and Van der Kraak, 1997; Wood and Van der Kraak, 2002), suggesting evolutionary conservation of the survival factors involved in the regulation of the ovarian function. However, much less is known about the identity of either local or systemic pro-apoptotic factors. GnRH is one of the few hormones that have been proposed to participate in follicular atresia in mammals. The expression of GnRH and its receptors have been demonstrated in the ovary of fish, amphibians, and mammals (Hsueh and Erickson, 1979; Minaretzis, et al., 1995; Whitelaw et al., 1995; Fraser et al., 1996; Nabissi et al., 1997; Yu et al., 1998; Illing et al., 1999; Von Schalburg and Sherwood, 1999), and several studies have provided support for both direct inhibitory and stimulatory actions of GnRH in the ovary of several species (Hillensjo and LeMaire, 1980; Hsueh et al., 1980; Jones and Hsueh, 1980; Chieffi et al., 1991; Takekida et al., 2000; Habibi et al., 2001). However, GnRH has been proposed to be primarily an atretogenic factor in mammals, since treatment with GnRH agonists has been shown to induce DNA fragmentation in the rat ovary as well as rat, porcine, and human granulosa cells (Hillensjo and LeMaire, 1980; Banka and Erickson, 1985; Yoshimura et al., 1990; Billig et al., 1993). There are many similarities in the paracrine/autocrine actions of GnRH in the fish ovary and testis. A recent study by Andreu-Vieyra et al. (2005) tested the hypothesis that GnRH plays a differential regulatory role during male gamete maturation in goldfish. The results clearly demonstrate that GnRH-induced DNA fragmentation only occurs at the late stages of spermatogenesis mediated by increases in the levels of fas and fasL-like proteins, as well as the activities of caspase-3 (an executioner caspase) and caspase-8 (a death receptor activated caspase) (Andreu-Vieyra et al., 2005). In addition, GnRH treatment increased the expression of the pro-apoptotic Bax (Andreu-Vieyra and Habibi, unpublished data), suggesting the involvement of both mitochondrial and receptor pathways in GnRH mediated apoptosis (Figure 2). In the goldfish ovary, GnRH was found to exert a survival action on mid-vitellogenic follicles without influencing GtH-stimulated functions. In fully mature preovulatory follicles, however, GnRH was found to induce apoptosis after 24 h of treatment, which was blocked in the presence of GtH (Andreu-Vieyra, 2003; Andreu-Vieyra and Habibi, unpublished data). Initial studies of GnRH paracrine action in gonads were primarily focused on direct effects of GnRH on meiotic maturation and steroidogenesis (Habibi et al., 1988, 1989; Habibi and Pati, 1993; Pati and Habibi, 2000). Resumption of meiosis in response to GnRH has been observed in rabbit and rat oocytes in both healthy and atretic follicles (Hillensjo and LeMaire, 1980; Banka and Erickson, 1985; Yoshimura et al., 1990), opening the possibility that GnRH-induced meiotic maturation maybe part of the oocyte programmed cell death process, rather than an ovulatory response. In this regard, pseudomaturation,

GVBD and polar body extrusion have also been observed in mouse atretic follicles (Sato et al., 2006). Although GnRH appears to induce true meiotic maturation in the absence of GtH as indicated by activation of H1 kinase (Pati and Habibi, 2000), it is possible that reinitiation of meiosis may be a transient process occurring in parallel to apoptosis. There is no indication that ovarian follicles that undergo meiotic maturation induced by GnRH in the absence of GtH can actually be ovulated and fertilized to form fish embryos. In this context, studies carried out in the invertebrate starfish indicate that, in the absence of fertilization, oocyte apoptosis occurs 10 h after resumption of meiosis via the mitogen activated protein kinase (MAPK) pathway (Kishimoto, 2004). This could also be the case for goldfish oocytes, since GnRH is known to activate the MAPK pathway (Klaussen et al., 2002) and neither caspase-3 activity nor DNA fragmentation could be detected in goldfish follicles at times earlier than 24 h after GnRH treatment (Andreu-Vieyra and Habibi, unpublished data). Since GnRH has been shown to oppose gonadotropin effects on mature goldfish follicle steroidogenesis, a high ratio of GtH/GnRH, which is achieved during the narrow period of ovulation, is likely to be necessary for reinitiation of meiosis. In this context, apoptosis would be the default fate for oocytes that resume meiosis in response to GnRH and in the absence of the GtH ovulatory surge, which would lead to follicle demise. Communication between granulosa cells and the oocyte has been shown to be essential for maintenance of the oocyte meiotic arrest in some teleosts (Cerdà et al., 1993). Thus, an alternative scenario in which GnRH treatment may disrupt communication between granulosa cells and the oocyte, leading to resumption of meiosis and cell death, cannot be discarded. In the future, more studies should be carried out to test the novel hypothesis that GnRH-induced reinitiation of meiosis in the absence of GtH is a transient process occurring in oocytes triggered to undergo apoptosis by GnRH. Taken together, these results strongly suggest that GnRH may be involved in the induction of apoptosis during gonadal regression resulting from lack of the appropriate ovulatory surge of GtH.

Based on these findings, Habibi et al. (2001) proposed a model in which sGnRH plays a role in the maintenance of ovarian synchronicity. This model is expanded here as shown in Figure 3. The observed protective action of sGnRH in mid-vitellogenic follicles indicates that the presence of GnRH may be critical in protecting the follicles against apoptosis and premature meiotic maturation, which maybe induced by other local or systemic factors. The presence of GnRH at the early stage of folliculogenesis appears to be important to maintain synchronous development of growing follicles. At this early stage, follicles will be further protected by the presence of increasing levels of E2 (Andreu-Vieyra, 2003; Andreu-Vieyra and Habibi, unpublished data). There is evidence that sGnRH is present in mid-vitellogenic as well as fully mature goldfish follicles (Figure 1). There is further evidence that growing follicles that reach mature preovulatory stage become sensitive to the pro-apoptotic action of GnRH (Andreu-Vieyra and Habibi, 2000; Andreu-Vieyra, 2003; Andreu-Vieyra and Habibi, unpublished data). In the normal ovary, however, the pro-apoptotic

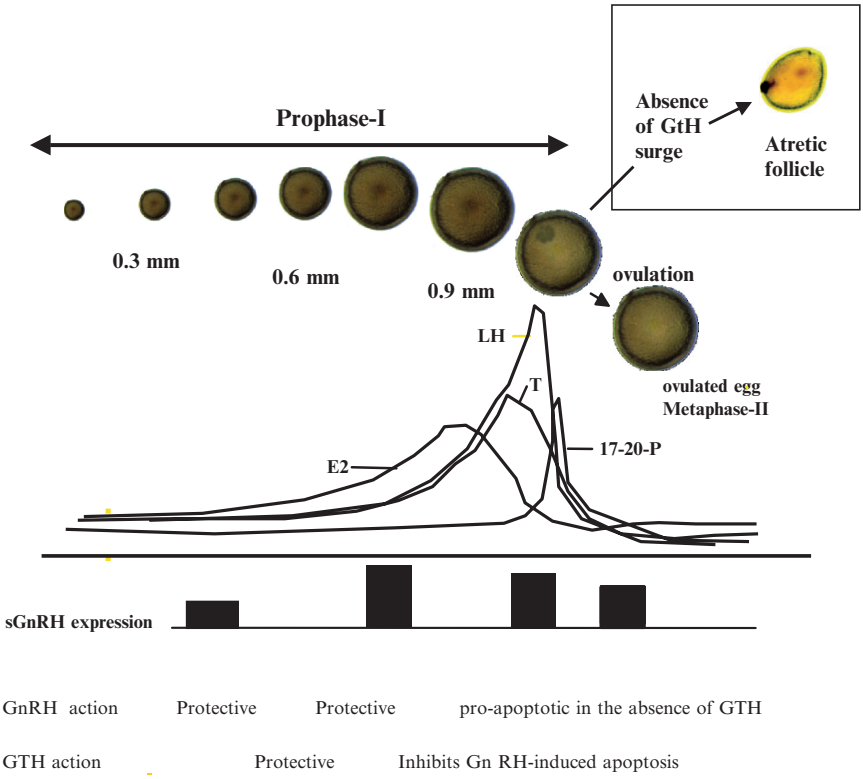

Figure 3. Proposed mechanism for the control of ovarian folliculogenesis by locally produced GnRH. During folliculogenesis, estradiol (E2) levels and GnRH expression gradually increase. Up to midvitellogenesis, our *in vitro* studies have shown that GnRH treatment has no effect on follicle survival. At midvitellogenesis, both E2 and GnRH appear to have pro-survival actions, although the mechanism underlying this GnRH effect remains unknown. As folliculogenesis progresses, the ovulatory surge of LH (GtH-II) results in a shift in steroidogenesis, which increases the production of the maturation-inducing hormone 17 alpha,20 beta-dihydroxy-4-pregnen-3-one (17,20-P) (Nagahama, 1997). GnRH transcript levels remain high through folliculogenesis and preovulatory follicles are susceptible to undergo apoptosis upon GnRH treatment. Our studies show that GTH opposes GnRH-pro-apoptotic actions *in vitro*, suggesting the possibility of a similar situation *in vivo*. We propose that GnRH can act as an atretogenic factor in the fish ovary in situations where GTH levels fail to raise.

action of GnRH is effectively blocked by LH, which is produced in increasing amounts during late vitellogenesis. Therefore, GnRH induction of apoptosis in the fish ovary of fish will only be possible if the ovulatory surge of LH fails to occur, either as a result of inappropriate environmental cues or the presence of endocrine disrupting chemicals in the environment. In this context, conditions that favor sGnRH induction of apoptosis in mature follicles, will eventually lead to ovarian follicular atresia (Figure 3).

6. PERSPECTIVES

Although much progress has been made in the understanding of follicular development in teleosts, there are still many questions that remain to be answered. The identification of GnRH as a central player in ovarian follicle growth and demise is significant. However, other unknown factors may contribute to or modulate GnRH actions. These factors may be produced by the somatic component of the follicle or by the oocyte itself.

There is increasing evidence that cellular events or factors produced in the oocytes contribute to the fate of ovarian follicles in mammals. For example, apoptosis in mammalian primordial and small antral follicles originates first in the oocyte and then extends to the somatic cell layers of the follicle (Morita et al., 1999; Eppig, 2001). However, the opposite is true in large antral follicles (Eppig, 2001). Although this process has not been investigated in fish follicles, it has been recently demonstrated that fish oocytes produce factors that modulate follicular functions, and that proteolysis of yolk proteins may lead to oocyte cell death (Wang and Ge, 2003; Wood and Van der Kraak, 2003). These findings support the view that gametes participate in the regulation of fish follicular development and apoptosis. Undoubtedly, more studies are needed to further test these possibilities and to identify other factors that maybe involved in the regulation of follicular atresia. Despite the limited available information, a picture is emerging in which GnRH plays an important role in the regulation of follicular development and apoptosis in the fish ovary.

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CHAPTER 10

ECOTOXICOLOGICAL EFFECTS OF ENDOCRINE DISRUPTING COMPOUNDS ON FISH REPRODUCTION

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1. INTRODUCTION

Public concern about the potential effects of endocrine disrupting chemicals (EDCs) on wildlife and human health has heightened experimental paradigms to assess the mechanistic effects of exposure to these compounds. The worry stems from the known effects caused in humans by exposure of pregnant mothers to the synthetic estrogen, diethylstilbestrol (DES) to help them avoid miscarriage (Milhan, 1992). The effects were observed 20 years after the initial exposure of the mothers and resulted in a higher than normal chance of inducing rare endocrine-related cancers as well as other reproductive abnormalities in their progeny.

The effects of exposure to EDCs is much more difficult to quantify and evaluate compared to other types of toxicants because the endocrine system of all vertebrates, including fish, is rather complex. The compounds in question are diverse in their structures and difficult to identify and they can be present in the environment in large quantities. They include pharmaceuticals such as ethinylestradiol (EE_2), plasticizers such as bisphenol A and phthalates, surfactants such as the alkylphenols (APs), and organochlorine pesticides, among others. They work by either mimicking the molecular effects of endogenous hormones or by altering hormone homeostasis, interfering with the synthesis, metabolism, and/or excretion of endogenous hormones. It is now clear that EDCs work *havoc* during critical windows of exposure, mostly during development and reproduction. The most sensitive stages are the early life stages undergoing sex hormone dependent tissue differentiation. Exposure at these times results in irreversible changes in tissue differentiation, altering the sex and long-term reproductive potential of offspring. Vinclozlin and methoxychlor exposures cause epigenetic effects in rats, as recently demonstrated (Anway et al., 2005). Guerrero-Bosagna et al. (2005) reviewed the mechanisms involved in this type of transgenerational effects. Exposure of adults, on the other hand, appears to be reversible, with endocrine disruption lasting only as long as the exposure.

The impact of EDC exposure in fish at the individual level in adults uses vitellogenin (Vtg) induction in males as the key endpoint. Irreversible effects on gonadal development have also been described worldwide, including the widespread occurrence of intersex in wild populations of roach (*Rutilus rutilus*) and gudgeon (*Gobio gobio*) in the UK (Jobling et al., 1998; van Aerle et al., 2001), flounder (*Platichthys flesus*) (Kirby et al., 2004) and carp (*Cyprinus carpio*) (Sole et al., 2003). Exposure of fish to EDCs in laboratory studies have

resulted in intersexed fish (Gimeno et al., 1996, 1997; Gronen et al., 1999) with ova-testis and other gonadal pathologies being described (Sumpter and Jobling, 1995; Jobling et al., 1996; Christenson and Strauss, 2000; Zillioux et al., 2001). In addition to the feminization described above, there are studies showing masculinization of fish, for example the extension of a gonopodium in female mosquitofish (*Gambusia* spp.) exposed to paper mill effluents (Parks et al., 2001). In other studies, fish treated in the laboratory with exogenous hormones caused complete cessation of reproduction and this has accounted for the multifaceted changes observed in the field, including presence of undifferentiated and intersex gonads (Patyna et al., 1999; Koger et al., 2000; Urushitani et al., 2002), altered secondary sex characteristics (Sone et al., 2005) and disruption of egg production (Ankley et al., 2003, 2004; Jensen et al., 2004), among other endpoints. Most of these effects are long-term, irreversibly affecting progeny (Iguchi et al., 2001).

There is still much research required to understand the mechanisms by which EDCs affect fish embryos, early life stages, and reproductive adults. In addition to soluble nuclear hormone receptors, it is now clear that sex hormones can also bind directly to membrane receptors and affect nongenomic actions. For example, contaminants such as zearalenone (a phytoestrogen), bisphenol A, polychlroniated biphenyls, and organochlorine pesticides (such as chlordane and methoxychlor) can at very low concentrations (0.1 μM) directly interfere with the action of progestin (17,20 β, 21-trihydroxy-4-pregnen-3-one or 20β-S), a hormone responsible for final maturation of oocytes and sperm motility (Thomas and Doughty, 2004).

2. ENDOCRINE DISRUPTING CHEMICALS (EDCs) AND THEIR MODES OF ACTION

2.1. Receptor-Mediated and Nonreceptor-Mediated Effects

Hormones and their mimics can alter homeostasis through both receptor-mediated and receptor-independent pathways (Figure 1). Receptor-mediated pathways can either impact signal transduction pathways through membrane-bound receptors and thus alter cellular homeostasis and cause effects in just a few minutes, or they can involve intracellular receptors resulting in alterations in gene transcription which are evidenced several hours postexposure. Examples of nonreceptor-mediated pathways include alterations in the synthesis, metabolism, and excretion of endogenous hormones. Both pathways are important for long-term sex hormone homeostasis and reproduction in fish.

Receptor-mediated pathways have received more attention than other pathways, with special emphasis given to estrogen receptor (ER)-mediated pathways (Figure 2). EDCs can act both as agonists and as antagonists, sometimes changing their mode of action depending on their concentration. For example,

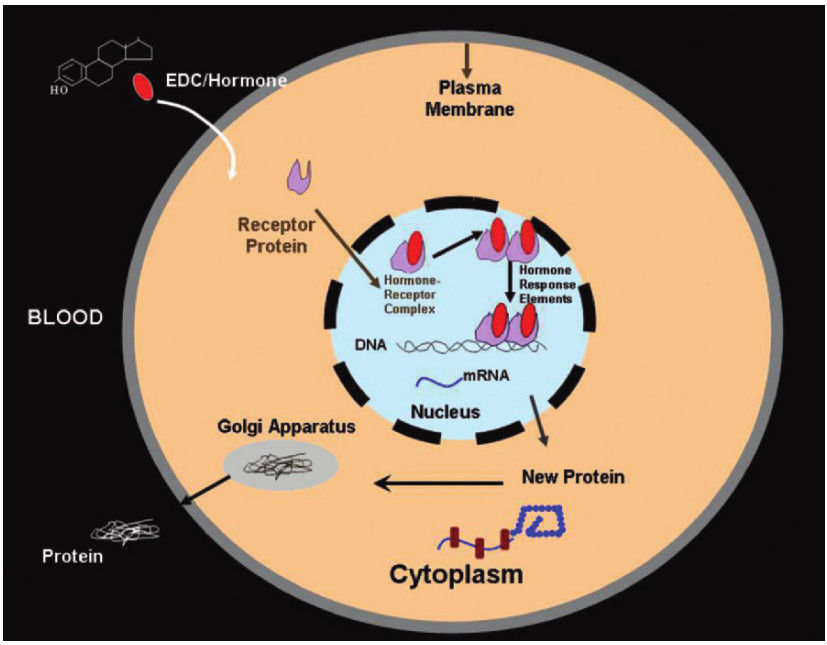

Figure 1. Endocrine disrupting chemicals (EDCs) can exert their effects via receptor-mediated mechanisms. In this example, an EDC interacts with cellular receptors to elicit effects. (*See Color Plates*).

bisphenol A can act as a 17-β-estradiol (E_2) agonist at high concentrations and as an antagonist at low concentrations (Safe et al., 2001). In addition, EDCs do not always follow a "typical" dose–response curve (Figure 3). There is excellent documentation now for effects of EDCs at very low doses with U-shaped curves being reported in animals exposed to these compounds (e.g. Pollack et al., 2003), similar to dose–response curves observed with endogenous hormones. Different modes of action at the low and high concentrations may be responsible for the U-shaped curves, as mentioned above and by feedback inhibition of endocrine function at high concentrations.

How environmental contaminants affect reproduction is a question that still needs considerable experimentation. One possible theory is that environmental contaminants bind to sex hormone receptors and disrupt their programmed gene activation cascades by inducing gene expression at inappropriate times. For instance, there is an ordered sequence of E_2-controlled gene transcription events in female fish undergoing oogenesis that depend on plasma E_2 levels and other cues such as temperature, oxygen availability, and light cycles. Among the genes that are activated as a result of this process are the ER itself, Vtg, the egg yolk precursor protein (Pakdel et al., 1991; Bowman et al., 2002), and the zona radiata proteins (ZRPs) that actually precede the synthesis of

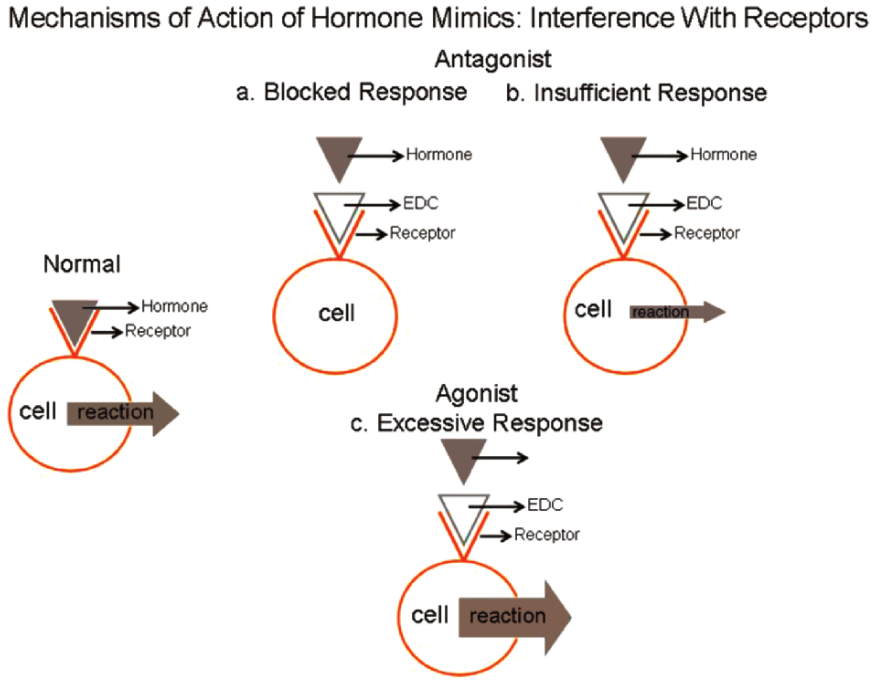

Figure 2. Mechanisms of action of endocrine disrupting chemicals (EDCs) acting on steroid hormone receptors. Upon interaction of EDCs with cellular receptors, two outcomes are possible: (1) EDC can act as an antagonist and induce a total cessation or a reduction of response (a) and (b); or (2) it can act as an agonist eliciting an enhanced response (c). (*See Color Plates*).

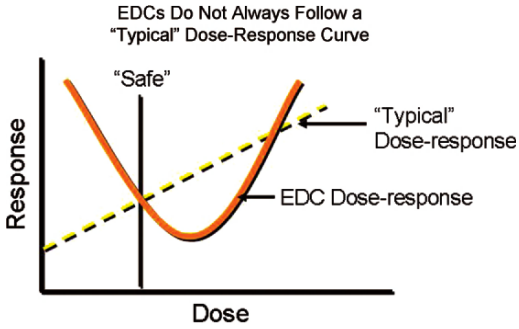

Figure 3. Endocrine disrupting chemicals (EDCs) do not always follow a typical dose–response curve, and U-shaped curves are common after exposure to these chemicals or to endogenous hormones. An explanation for this type of dose–response curve is that there are different modes of action at the low and high concentrations, for example, feedback inhibition of endocrine function occurs at high concentrations. (*See Color Plates*).

Vtg (Arukwe and Goksoyr, 2003). The three fish ERs (α, $\beta_{a,}$ and β_b) alone or in combination may control different subsets of genes. Research with mammalian models indicates, for example, that E_2 mimics may bind differently to the ERα and β receptors, acting as agonists in one case and antagonists in another (Paech et al., 1997; Safe et al., 2001). This complicates the issue of determining the risks of exposure to environmental pollutants in fish populations. In addition, it is still not totally understood how the pleiotropic effects of these gene induction pathways and potential modulation by EDCs give toxic outcomes and this area requires additional research. Still lacking are studies demonstrating clear mechanisms of disruption linked to altered physiological parameters. In addition to the soluble sex hormone receptors, it is now clear that sex steroid hormones and their mimics also bind to membrane-bound receptors to enact immediate changes in signaling via nongenomic pathways (Thomas and Doughty, 2004). Specific hormone membrane receptors have been identified in fish testis and ovaries for E_2 (Loomis and Thomas, 2000; Thomas-Jones et al., 2003), testosterone (T) (Braun and Thomas, 2004), and progestins (Patiño and Thomas, 1988).

2.2. Synthesis and Metabolism of Endogenous Hormones

The reproductive biology of teleost fishes follows a well-defined cycle regulated by exogenous environmental cues such as photoperiod and temperature, and endogenous hormonal cues. The latter process is dependent on the coordinated actions of hormones associated with the brain–hypothalamus–pituitary–gonad axis (HPG). The hypothalamus controls the synthesis and release of gonadotropin-releasing hormone (GnRH), resulting from neural stimulation from the central nervous system. This messenger hormone is responsible for controlling the synthesis and release from the pituitary of the primary teleost gonadotropin hormones: follicle-stimulating hormone (FSH, previously known as GTH-I in the fish literature) and luteinizing hormone (LH, previously known as GTH-II) (Jalabert, 2005). FSH is typically involved in stimulating events leading to vitellogenesis or spermatogenesis and early gonadal development, whereas LH is mostly responsible for stimulating events leading to final oocyte maturation and ovulation in females and spermiation in males. In addition, gonadotropins stimulate steriodogenesis or the synthesis of sex steroid hormones (androgens, estrogens, and progestins), which, in turn, act on target tissues to regulate gametogenesis (Nagahama, 1994; Schulz et al., 2001). In the majority of teleosts, E_2 and the androgens T and 11-ketotestosterone (11-KT) are the primary sex steroids responsible for regulating gametogenesis, in females and males, respectively. Increases in plasma concentrations of these hormones are associated with the onset of seasonal reproductive activity.

In female fish, circulating FSH increases during early oocyte development and binds to receptors on follicles, which synthesize T and allow

aromatization to result in the formation of E_2. Subsequently, E_2 is released by the follicular cells into the blood from were it can enter into steroid sensitive tissues. In the liver, E_2 binds to ER to initiate a cascade of events resulting in the production of hepatic Vtg, a precursor to egg yolk protein (Wahli et al., 1981). Vtg moves from the liver into the blood and eventually through endocytosis into growing oocytes. As oocyte development continues, levels of FSH begin to decrease and at the same time levels of LH begin to increase. Receptors for LH are found predominately on the granulosa cells of follicles and binding stimulates the synthesis and release of progestins, which play a role in final gamete maturation and ovulation (Basu and Bhattacharya, 2002; Thomas et al., 2004; Jalabert, 2005). Similarly, in male fish, FSH is typically elevated throughout spermatogenesis and decreases at the time of spawning, when LH becomes elevated. These gonadotropins stimulate proliferation of spermatogonia as well as the synthesis of androgens required for gametogenesis in male fish (Nagahama, 1994).

Environmental contaminants may also disrupt the endocrine system by altering hormone homeostasis, which is tightly controled by hormone synthesis, metabolism, and excretion. The first and rate-limiting step in steroid synthesis is the transport of cholesterol to the inner mitochondrial membrane by the steroidogenic acute regulatory protein or StAR (Stocco and Clark, 1997). The conversion of cholesterol to the sex hormones proceeds through several ordered reactions. The first reaction is the conversion of cholesterol to pregnenolone throught the action of cytochrome P450 side chain cleavage enzyme (CYP-450ssc). Pregnenolone is then converted into T through a series of enzymatic actions. Testosterone is then converted into E_2 by the action of aromatase or to 11-KT by the action of 11β-Hydroxysteroid dehydrogenases (11-βHSD) (Kusakabe et al., 2003). As already discussed, 11-KT is the main androgen in male fish responsible for regulation of spermiation and expression of secondary sex characteristics (Hu et al., 2001). EDCs target each of these steps. For instance, there is evidence that StAR is susceptible to the action of the imidazole antifungal drugs, econazole, and miconazole, as well as to other known EDCs (Walsh et al., 2000a, b, c).

Metabolism of steroids is primarily accomplished by phase I CYP-450 enzyme-mediated hydroxylation and phase II enzyme-mediated conjugation with sulfate and uridine diphosphoglucuronic acid (UDPGA) (Matsui et al., 1974; Waxman, 1988), although dehydrogenation is also an important metabolic pathway (Martel et al., 1992). The liver is the primary site of steroid metabolism with some lesser activity in other tissues (Sonderfan et al., 1989). A number of studies have demonstrated that compounds that modulate the synthesis or metabolism of steroids are responsible for changes in circulating steroid levels (Wilson and LeBlanc, 1998; Coulson et al., 2003). It is clear that EDCs can also affect the enzymatic activities of proteins that normally degrade endogenous hormones. For example, 5 alpha reductase activity of common carp testicular microsomes is highly sensitive to dicofol, organotins, and phthalate

(Thibaut and Porte, 2004). EDCs disrupt these pathways by specific ways, as elucidated below.

2.3. Vitellogenesis and Zonagenesis as E_2-Regulated Events

Vitellogenesis in oviparous fish is the principle event contributing to the massive growth of oocytes, due to a rapid uptake of the egg yolk precursor Vtg (Wallace and Selman, 1985). The liver synthesizes Vtg under the control of ER and secretes it into the blood stream. The Vtg receptors on the surface of oocytes are specific for Vtg and take it up into the oocytes. A series of specific proteases cleave Vtg into smaller yolk proteins, which later form yolk granules. This material accounts for about 90% of the protein content of mature oocytes. The yolk granules are stored during oogenesis and serve as a nutrient source for embryonic development (Wahli et al., 1981). Vitellogenesis ceases once the oocytes reach their fully developed size. The next phase is the period of oocyte maturation. During this time, follicles increase in volume due to hydration and accumulation of other vital proteins. Protein uptake stops at the time of germinal vesicle breakdown (GVB). The follicle, however, continues to increase in volume by hydration. The timing of ovulation is species-specific and takes place when follicles reach a specific size (Wallace and Selman, 1980).

The fish egg envelope consists of two distinct layers: a thin outer layer formed around the oocytes during later stages of previtellogenic development, and an inner thicker layer, called zona radiata (ZR), which occupies most of the egg envelope (Sugiyama et al., 1999; Arukwe and Goksoyr, 2003). The synthesis of the zona layer precedes vitellogenesis (Celius and Walther, 1998a) and is under the control of E_2 (Arukwe and Goksoyr, 2003; Berg et al., 2004). In the Japanese medaka (*Oryzias latipes*) ZR proteins consist of two major subunit groups, ZI-1, 2 and ZI-3. Like for Vtg, most fish species synthesize these proteins in the liver from where they proceed into the blood. They form the envelope of eggs. However, in some species it is clear that the ovary itself is the site of synthesis for some of these proteins (Mold et al., 2001; Onichtchouk et al., 2003). There is ample evidence that EDCs disrupt synthesis of these proteins and that they may have specific sensitivities to certain pesticides (Celius and Walther, 1998b).

2.4. Types of EDCS and known Mechanisms of Action

The list of chemicals that affect the endocrine and reproductive systems of teleost fish includes heavy metals; organochlorine, triazine, and organometal pesticides; fungicides; persistent halogenated pollutants (polychlorinated biphenyls (PCBs), dibenzo-*p*-furans, and dibenzo-*p*-dioxins, and polybrominated flame-retardants); synthetic and natural estrogens and androgens, found in complex effluents such as sewage, confined animal feeding operations (CAFOs), and paper mill effluents. As already discussed, most mechanistic studies of EDCs with fish have focused on receptor-mediated modes of action.

Furthermore, most of the research has centered on the interaction of these chemicals with the ER. However, it is now clear that endocrine disruption can occur at many steps throughout the HPG axis, all the way from inhibition of secretion of the glycopeptide hormones in the brain to effects in liver, gonads, and other target organs. Thus, when evaluating the effects of EDCs in fish oogenesis and reproduction, it is important to consider all possible mechanisms of action, including the role of other important hormone receptors, such as the androgen receptor (AR) or membrane-bound receptors, as well as receptor-independent mechanisms. This review will focus on the effects of EDCs on oocyte and sperm maturation, as well as on the development of early life stages.

2.4.1. Cadmium

Cadmium (Cd) is a persistent neurotoxic heavy metal released to the environment by both natural (weathering of minerals, forest fires, and volcanic eruptions) and anthropogenic (emissions from mining and smelting operations and from urban and agricultural runoff) related sources. Once in water ecosystems, this heavy metal accumulates in sediments and aquatic organisms, including fish. Cd is toxic to freshwater fish. Salmonids are among the most sensitive fish species to the effects of this heavy metal. Mechanistically, studies with fish support the idea that Cd behaves as an antiestrogenic EDC through several signaling pathways, including direct inhibition of steroidogenesis in the gonad and adrenal gland, decreased production of E_2-stimulated genes by the liver, and through neurological effects that occur at the central nervous system level. For instance, in rainbow trout (*Oncorhynchus mykiss*) Cd inhibits T production in Leydig cells of male gonads, and progesterone (P), E_2 and T production in ovarian cells (Kime, 1984; Leblond and Hontela, 1999). Exposure to Cd also decreases *in vitro* sex steroid production in Japanese medaka and brook trout (*Salvelinus fontinalis*) (Sangalang and O'Halloran, 1972, 1973; Tilton et al., 2003). In female common carp, Cd also inhibits gonadotropin-stimulated steroidogenesis and ovarian maturation (Mukherjee et al., 1994). In addition to depressed gonadal steroidogenesis, Cd interferes with adrenal steriodogenesis in teleost fish, disrupting the signaling pathway in the step prior to pregnenolone synthesis within the mitochondria (Lacroix and Hontela, 2004). Inhibition of steroidogenesis occurs through several mechanisms including reduction of cholesterol uptake by the mitochondria (Piasek and Laskey, 1999), inhibition of CYP-450 enzymes in liver and gonad (Singhal et al., 1987), and alterations in calcium homeostasis within the steroidogenic cells (Verbost et al., 1987; Mathias et al., 1998).

Cd can affect Vtg gene expression by replacing Zn^{2+} in the DNA-binding domain of the ER thereby reducing its transcription activity (Le Guevel et al., 2000) and by reducing hepatic ER mRNA levels (Vetillard and Bailhache, 2005). Specifically, Cd inhibits E_2-stimulated transcription and translation of Vtg (Pereira et al., 1993; Olsson et al., 1995; Hwang et al., 2000; Vetillard and Bailhache, 2005). In the brain, Cd reduces the expression of ERα and increases

the expression of GnRH in a dose-dependent manner (Jadhao et al., 1994; Vetillard and Bailhache, 2005).

In addition, Cd accumulates in the liver and yolk, interfering with oogenesis, ovoposition, and egg case formation (Brown et al., 1994; Olsson et al., 1995). Interestingly, there is higher uptake of Cd by the liver and oviduct in fish co-treated with E_2 (Olsson et al., 1995).

In summary, Cd can act as a potent endocrine disrupter in fish, affecting different tissues and gene expression. Recent studies have shown that Cd behaves like an antiestrogenic endocrine disrupter, inhibiting steroidogenesis and hepatic female-specific protein production, and is capable of inducing neurological changes at the central nervous system level.

2.4.2. Pesticides

Organochlorine Pesticides. Organochlorine pesticides (OCPs) comprise a large group of structurally diverse compounds historically used to control agricultural pests and vectors of human disease. Because of their significant potential threat to human and wildlife health, their use was banned in the USA, starting in the early 1970s. Nevertheless, because of their early widespread use and high chemical stability and lipophilicity, they continue to bioaccumulate in animal tissues, and are currently one of the most common and widely distributed types of pollutants worldwide. In fact, all old-use OCPs including chlorinated ethanes such as DDT and derivatives, and methoxychlor; cyclodienes such as chlordane, dieldrin, endosulfan, heptachlor, and toxaphene; and other chlorinated cyclic hydrocarbons such as hexachlorocyclohexane appear in the latest National Priority List (NPL) (US EPA, 2003a) developed by the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) as substances that require immediate attention.

Exposure of fish to OCPs can result in endocrine disruption of reproduction. For instance, the cyclodienes (including dieldrin, toxaphene, and chlordane) and dichlorodiphenylethane (e.g. p,p′-DDE) pesticides have been reported to cause a myriad of negative reproductive effects in many fish species (e.g. decreased sex hormone concentrations, spawning, fertility, hatchability, and survival of early life stages) (reviewed by Gross et al., 2002).

The mode of action for OCPs differs not only among the different classes, but also between different metabolites within a class. Extensive evidence has accumulated on the endocrine disrupting capability of OCPs, mostly from work done with DDT and its metabolites (o,p′-DDE and p,p′-DDE). In fish, results from *in vivo* and *in vitro* studies indicate that o,p′-DDE binds to the ER, but has much lower estrogenic potency compared to E_2 (Spies et al., 1996; Garcia et al., 1997; Nimrod and Benson, 1997; Matthews et al., 2000). Other prominent OCPs such as methoxychlor (especially the methoxychlor metabolites), dieldrin, and endosulfan, are also able to bind to the ER of fish (Matthews et al., 2000; Tollefsen, 2002). These chemicals compete with endogenous E_2 for steroid-binding sites on target cells and sex steroid-binding proteins of fish in a dose-dependent and

competitive manner (Gale et al., 2004; Tollefsen et al., 2004). In males, estrogenicity of DDT metabolites has been widely studied, with numerous reports showing vitellogenic induction and increase expression of egg proteins (choriogenins) (Mills et al., 2001; Larkin et al., 2002, 2003a; Leaños-Castañeda et al., 2002; Okoumassoun et al., 2002).

Besides binding to the ER, OCPs can also bind to the AR in several fish species (including goldfish, *Carassius auratus*; guppies, *Poecilia reticulata*; and white sturgeon, *Acipenser transmontanus*). OCPs in this case primarily act as antiandrogens, preventing the transcription of T-regulated genes and resulting in demasculinization (Kelce et al., 1995; Wells and Van der Kraak, 2000; Baatrup and Junge, 2001; Foster et al., 2001; Bayley et al., 2002). In addition, several OCPs (p,p′-DDT, o-p′-DDT, chlordane, and methoxychlor) have been reported to block the action of the progestin 17,20β, 21-trihydroxy-4-pregnen-3-one, the enzyme responsible for upregulating sperm motility in fish, through interactions with the membrane receptor for progestin (Thomas and Doughty, 2004). Other mechanisms of action of OCPs include their ability to induce aromatase (this has been shown in male rats dosed with p,p′-DDE (You et al., 2001), and to induce the ovarian synthesis of maturation-inducing hormones (20α and 20β-hydroxysteroid dehydrogenases) in common carp (Thibaut and Porte, 2004).

The ability of OCPs to induce antiestrogenic effects in female teleost fish has received little attention so far. This is of great ecological significance, since competitive binding of some of these weak E_2 agonists to the ER could reduce E_2 function, leading to impaired gonadal development and decreased vitellogenesis, and ending in the production of poor quality eggs and decreased reproductive success. This prediction is based on recent studies showing a significant down regulation of egg quality-related genes (Vtgs and choriogenins) in adult female largemouth bass (*Micropterus salmoides*) exposed to p,p′-DDE (Larkin et al. 2002, 2003b), and other antiestrogenic effects in female fish exposed to toxaphene and endosulfan (Chakravorty et al., 1992; Fahraeus-Van Ree and Payne, 1997). Toxaphene exhibits antiestrogenic effects in birds (Ratnasabapathy et al., 1997) and in human cell lines (Arcaro et al., 2000).

Causes for this general steroid suppression by OCPs seem to be compound-specific, with p,p′-DDE directly inhibiting steroid synthesis (Chedrese and Feyles, 2001), and toxaphene decreasing aromatase activity as well as steroidogenesis in general (Yang and Chen, 1999; Chen et al., 2001). However, exposure to OCPs have also resulted in the down regulation of genes involved in many other nonestrogen-dependent pathways suggesting the involvement of other mechanisms as well (Larkin et al., 2002).

Altered sex steroid concentrations in fish environmentally and experimentally exposed to OCPs could result from the disruptive effects of these chemicals on hypothalamic or pituitary gonadotropin hormones. As already discussed, gonadotropins serve as stimulating hormones for the synthesis and secretion of sex steroids, and any alteration in normal concentrations of the gonadotropins

could ultimately lead to decreased sex steroid production. DDT exposure of Kelp bass (*Paralabrax clathratus*) significantly depressed concentrations of LH during their summer spawning period, compared to bass collected from a reference site (Spies et al., 1996). In another study, Mozambique tilapia (*Oreochromis mossambicus*) treated with endosulfan showed necrosis of pituitary gonadotroph cells (Shukla and Pandey, 1986).

Methoxychlor is a structural analog of DDT. In mammalian systems, methoxychlor and its metabolites, OH-MXC and HPTE, appear to be specific ERα agonists, ERβ antagonists (Gaido et al., 1999, 2000), and AR antagonists (Maness et al., 1998). All three appear to reduce the ability of animals to synthesize sex steroids (Chedrese and Feyles, 2001; Crellin et al., 2001; Pickford and Morris, 2003), and modulate CYP-P450 expression (Schlenk et al., 1997; Li and Kupfer, 1998; Mikamo et al., 2003). Methoxychlor also inhibits GnRH synthesis in cultured hypothalamic cells by competitively binding with the ER in these cells and thus preventing GnRH production (Gore, 2002).

Triazine Pesticides. One of the most common chemical substances currently detected in US waters is the triazine pesticide atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine). First marketed in 1959, atrazine currently is the most widely used herbicide in the USA. In order to diminish early growth of weeds, application of this herbicide occurs in April and May. Farmers apply approximately 50 million pounds of atrazine per year in the USA, much of which controls weeds in agricultural crops such as sugarcane, corn, sorghum, and soybeans (US EPA, 2003b). Because of its frequent use, atrazine is now the most common contaminant of ground, surface, and drinking water in the USA (Seybold et al., 1999). Fate and transport studies have shown that atrazine can enter water systems through several pathways, including runoff, spray drift, contaminated groundwater, atmospheric deposition, and sediment/soil deposition (US EPA, 1997). Due to its high mobility from soil, the concentration of atrazine in surface water is highest in field runoff, with concentrations peaking following early major storms, which occur a few weeks after application (Wauchope, 1978). The persistence or half-life of atrazine in water systems is highly variable, and can range from just a few days (3–12 days) in small estuarine systems (Jones et al., 1982) to close to a year (300 days) in larger lake systems (Muir et al., 1978).

Because of its high water solubility, leaching potential, and its widespread use, atrazine poses a significant risk to exposed aquatic organisms. Indeed, adverse effects of atrazine on survival, growth, and reproduction of aquatic organisms occur in numerous field and laboratory studies (reviewed by US EPA, 2003b). Although the mechanism(s) of action of atrazine in vertebrates is not well understood, recent evidence implicates atrazine as an endocrine disruptor, eliciting estrogenic and antiestrogenic effects through nonreceptor-mediated mechanisms (ATSDR, 2003).

Much of the research concerning the mode of action of atrazine comes from experiments conducted with laboratory rodents, with scarce data currently available for fish. The primary target of this herbicide in mammalian models has

been the female reproductive system. Atrazine alters estrus cyclicity, and induces developmental effects (decreased fetal weight, abortion, and altered development) in exposed female rats and mice at rates ranging from 7 to 300 mg/kg/day (ATSDR, 2003). Detailed mechanistic studies have demonstrated that atrazine may disrupt the endocrine system of mammals primarily through its action on the central nervous system, through suppression of the production of LH and prolactin (PR) by the hypothalamus (Wetzel et al., 1994; Cooper et al., 2000). The pituitary releases LH and PR in response to GnRH from the hypothalamus, which in turn are regulated by the interactions of several ligands with the gamma-aminobutyric acid receptor (GABA). In studies with rats, atrazine interferes with the binding of some of these ligands to the GABA receptor (Shafer et al., 1999). Disruptions of the HPG axis would explain the observed lack of ovulation, persistent high-plasma E_2 levels, and persistent estrus in female rats and mice dosed with atrazine. In addition, atrazine directly inhibits Leydig cell T production (Friedmann, 2002). These nonreceptor-mediated mechanisms are consistent with results from *in vitro* studies with several mammalian models that have failed to demonstrate a great affinity of this herbicide for the ER (Tennant et al., 1994; Connor et al., 1996; Tran et al., 1996; Aso et al., 2000). Atrazine does not bind to ER in channel catfish (*Ictalurus punctatus*), as demonstrated in experiments using recombinant ERα and ERβ (Gale et al., 2004).

Another nonreceptor-mediated mechanism of action reported for this herbicide involves stimulation of CYP-450 aromatase enzyme (CYP19) activity (Sanderson et al., 2000). Since aromatase is the rate-limiting enzyme involved in the conversion of androgens to estrogens, its induction could result in overall estrogenic effects. Male amphibians exposed to atrazine become "demasculinized," probably through activation of this activity. For instance, laboratory studies with the African clawed frog (*Xenopus laevis*) showed that male larvae exposed to at least 1 ppb (part per billion) atrazine throughout development suffered from impaired laryngeal growth (Hayes et al., 2002a) and induced several gonadal deformities (Hayes et al., 2003). Because similar effects have been observed in three other frog species from different genera (and different families: Pipidae, Ranidae, and Hylidae), it was concluded that this effect of atrazine could be generalized to anurans and not restricted to a single (or a few related) species (Hayes et al., 2002b, 2003; Hayes, 2004).

Atrazine has also been reported to inhibit aromatase activity in human cell lines (Oh et al., 2003), which would lead to the production of antiestrogenic effects. To this date, however, this mode of action has not been reported in fish exposed to atrazine.

Organometal Pesticides. Most metals in their inorganic form are insoluble in water, and thus, their bioavailability and potential toxicological effects are low. However, exactly because of this reason, chemical modification of some metals increases their toxicity for use as pesticides through the addition of organic ligands. Tributyltin (TBT) is a good example of this, showing a great efficiency as an algicide, miticide, and fungicide. Its greater use over the years

has been as a marine antifouling agent, applied to large ships and marine structures for prevention of algae growth. This has lead to the contamination and bioaccumulation of TBT in marine aquatic environments around the world.

Interestingly, over a decade of field and laboratory studies have documented that TBT behaves as a potent endocrine-disruptor. Indeed, one of the best-documented cases of endocrine disruption comes from work done with marine gastropods exposed to TBT. Masculinization of female gastropods (mostly seen with the dogwhelk, *Nucella* spp.) occurs after exposure to environmental concentrations of TBT. They develop male sex organs (penis and *vas deferens*) over the oviductal tissues (Spooner et al., 1991; Horiguchi et al., 1997; Matthiessen and Gibbs, 1998), a condition known as "imposex." Depending on the species and doses attained, spermatogenesis supplants oogenesis leading to abnormal breeding activities and, in many cases, sterility. Complete suppression of oogenesis in marine snails has been reported at concentrations as low as 3–5 pptr (parts per trillion, Gibbs et al., 1988). Thus, it is not surprising that exposure to TBT has been implicated as the predominant cause for population declines of prosobranch snails worldwide (Depledge and Billinghurst, 1999). Competitive inhibition of aromatase CYP19A by TBT causes the development of imposex in female snails (Spooner et al., 1991; Oberdörster and McClellan-Green, 2002). TBT may also interfere with sex steroid metabolism, inhibiting the formation of T and E_2 sulphur conjugates (Ronis and Mason, 1996; Morcillo et al., 1998; Ohkimoto et al., 2005).

How TBT affects the endocrine systems of teleost fish remains comparatively unknown. As an aromatase inhibitor, exposure of fish to TBT can result in altered sexual development in juvenile fish. In fact, inhibition of fish CYP1A by TBT has been the subject of several *in vivo* and *in vitro* studies (Fent and Stegeman, 1993; Fent and Bucheli, 1994; Kawano et al., 1996; Morcillo and Porte, 1997; Morcillo et al., 2004). More recently, irreversible masculinization of female juvenile fish exposed to TBT has been reported in zebrafish (*Danio rerio*) (McAllister and Kime, 2003) and Japanese flounder (*Paralichthys olivaceus*) (Shimasaki et al., 2003). These studies suggest a direct link between suppression of the aromatase gene and sex reversal in fish. Of great ecological significance is the fact that in both of these studies very low doses (0.1–1.0 pptr) induced effects. Similarly to what has been observed with marine gastropods, fish exposed to TBT also respond with an inhibition of T and E_2 conjugation and sulfation (Morcillo et al., 2004; Thibaut and Porter, 2004) and sex steroid synthesis (Thibaut and Porte, 2004). A decline in sperm motility has also been reported in male fish exposed to TBT, and has been attributed to a decrease on adenosine triphosphate (ATP) content in semen (Rurangwa et al., 2002).

The effects of TBT in human tissue culture cells (choriocarcinoma or ovarian granulosa) suggest a different mechanism of action through direct binding to the retinoid X receptor (RXR) and the peroxisome proliferators activated receptor (PPAR) and direct effects at the level of transcription (Nakanishi et al., 2006). Whether fish respond in similar manner to TBT is unclear, making this an area that still needs investigation.

Vinclozolin. Vinclozolin (3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione) is a dicarboximide fungicide widely used to control a variety of fungal pathogens on turf grass, vegetables, and fruit crops. Vinclozolin does not function as an endocrine disruptor *per se*, but its metabolites M1 and M2 work as AR antagonists in mammals, preventing transcription of androgen-dependent genes (Wong et al., 1995; Kelce and Wilson, 1997). In fish, studies evaluating the antiandrogenic effects of these metabolites have so far failed to demonstrate a great affinity for the AR. For instance, Wells and Van der Kraak (2000) reported no affinity of M1 and M2 for the brain and gonad ARs of goldfish or of rainbow trout, and Makynen et al. (2000) found no affinity using the fathead minnow (*Pimephales promelas*). Nevertheless, several studies have shown demasculinizing effects in fish exposed to vinclozolin, including delayed sexual maturation, reduced expression of secondary sex characteristics, gonadal development, sperm counts, and suppressed courtship behavior, in a manner consistent with antiandrogen action (Baatrup and Junge, 2001; Bayley et al., 2002, 2003; Kiparissis et al., 2003). Furthermore, these studies concluded that vinclozolin is capable of disrupting the sexual phenotype of male fish in a manner similar to the therapeutic antiandrogen, flutamide. The lack of binding affinity of the vinclozolin metabolites M1 and M2 to ARs in fish may be due to the structure of ARs in fish. There is evidence suggesting that AR structures vary not only among different teleost species, but also between organs within a species (Wells and Van der Kraak, 2000). It follows then that multiple AR isomers showing distinct steroid- and xenobiotic-binding specificities are likely present in fish. Indeed, two distinct ARs (AR1 and AR2) have been identified in the brain and gonads of kelp bass and Atlantic croaker (*Micropogonias undulatus*), and only AR2 was found to bind to the antiandrogens M1 and M2 (Sperry and Thomas, 1999).

In summary, exposure of fish to pesticides can result in a myriad of negative endocrine effects including inhibition or suppression of GnRH and gonadotropin hormones, ER and AR binding, and altered sex steroid synthesis and metabolism, all of which could ultimately lead to decreased reproductive output and to significant population-level effects, as discussed in more detail under section 3. Definitive causes and mechanisms of endocrine disruption and reproductive effects following pesticide exposure in fish are still under investigation.

2.4.3. Persistent organic pollutants

Halogenated Aromatic Hydrocarbons. Because of their widespread occurrence and substantial biological activity, halogenated aromatic hydrocarbons such as PCBs, polychlorinated dibenzofurans (PCDFs), and polychlorinated dibenzo-*p*-dioxins (PCDDs) comprise one of the most important classes of contaminants in the environment. Some chemicals in this class cause adverse biological effects after binding to an intracellular cytosolic protein called the aryl hydrocarbon receptor (AhR). Toxic responses such as thymic atrophy, weight loss, immunotoxicity, and acute lethality, as well as induction of CYP-4501A (CYP1A),

correlate with the relative affinity of PCBs, PCDFs, and PCDDs for the AhR. In addition, these compounds are EDCs due to their ability to induce antiestrogenic effects in several tested animal models. It is likely that the antiestrogenicity of CYP1A-inducing compounds results from crosstalk inhibitory interactions among the AhR and ER receptors (Spink et al., 1990; Safe and Krishnan, 1995; Kharat and Saatcioglu, 1996). Since both of these receptors play crucial roles during several key reproductive events in fish (i.e. Vtg and CYP1A gene activation and expression), their interaction may lead to adverse reproductive effects through disruptions of E_2-regulated physiological processes. The mechanisms of action as well as the reproductive toxicity of these compounds in many vertebrate taxa have been well documented and summarized in several review articles (e.g. Safe, 1994, 1999; Boening, 1998; Birnbaun and Tuomisto, 2000; Gross et al., 2002), and thus will not be discussed here. However, we will discuss in detail a new emergent class of halogenated contaminants, the polybrominated diphenyl ethers (PBDEs), which have recently been associated with endocrine disrupting effects as described in more detail below.

Polybrominated Diphenyl Ethers (*PBDEs*). An emergent class of contaminants is the PBDEs, which are used as brominated flame-retardants (BRFs). There are close to 175 different types of flame-retardants, with the BFRs being the largest market group because of their low cost and high performance. Although PBDEs are extremely stable, debromination occurs in the environment to change PBDEs to lower congeners. For instance, laboratory and field studies have shown that commercial mixtures of PBDEs are biotransformed or metabolized into lower bromine congeners by biota (Akutsu et al., 2001; Burreau et al., 2004; Stapleton et al., 2004; Zhu and Hites, 2004; Eljarrat et al., 2005). Overall, these results suggest that BDE-209 is not stable in fish tissues and has the potential to debrominate to less brominated BDE congeners. This is of great toxicological importance because lower brominated congeners (4–7 bromines) are far more bioaccumulative and persistent (De Wit, 2002). Studies in fish show that penta-BDE congeners continue to be present. Considering the high release of BDE-209 into the environment, it is plausible that significant formation of low brominated compounds will continue to form in the environment, and fish appear to be a crucial component of this transformation phenomenon.

Despite the widespread use of BRFs and their rapid increase in tissues of fish, wildlife, and human receptors over the last 10 years, their toxicity and mode of action remains unknown. There is some indication that juvenile salmonids and carp exposed to PBDEs have increased mortality of early life stages and decreased growth rates and lipid content (Stapleton et al., 2004). PBDEs have also been reported to be weakly estrogenic, with potencies of about 10^{-6} relative to E_2 (Meerts et al., 2001). Similar to the effects elicited by hydroxylated PCBs, PBDE toxicity involves effects on thyroid function due to the structural similarities between these compounds and thyroid hormones. From studies conducted using mammalian models, PBDEs function as EDCs due to their ability to interact directly with thyroid gland function and regulation, thyroid hormone

metabolism, and thyroid hormone transport mechanisms (Brouwer et al., 1998). These mechanisms of action of PBDEs have been more or less, confirmed in feeding studies with rats and mice. They have resulted in thyroid hyperplasia and tumors; induction of liver glucoronidase activity resulting in increased elimination rates of thyroxine (T_4) and triiodothyronine (T_3); and in competitive binding of PBDEs to thyroid hormone receptor sites and to thyroid transporter proteins (transthyretin) (Kitamura et al., 2002; Zhou et al., 2002; Stoker et al., 2003; Birnbaum and Staskal, 2004). Although there are no studies evaluating thyroid toxicity due to PBDEs in fish, the highly conserved thyroid system across vertebrates, would suggest that similar toxicity effects might be occurring in this group of vertebrates.

In summary, PBDEs are a new emergent class of compounds that behave very similarly to PCBs in terms of their fate, stability, and bioaccumulation properties. Their concentrations in tissues of fish, wildlife, and humans have increased exponentially over the last decade. There is considerable evidence indicating that PBDEs are EDCs due to their ability to interact with thyroid hormone homeostasis as indicated by their ability to bind competitively to thyroid hormone receptor sites and to thyroid hormone transporter proteins. Alterations in thyroid homeostasis in fish embryos and fry could lead to growth retardation and malformations. However, the toxicological profile of these compounds in fish remains largely unknown, and there is a requirement for additional mechanistic and toxicological studies to assess the threat of PBDEs to fish populations.

2.4.4. Pharmaceutics and sewage effluents

Effluents coming from sewage treatment plants are capable of inducing estrogenic effects in male fish due to their ability to bind to ERs and induce the production of Vtg. This leads to an increase in the incidence of hermaphrodism and "feminization" of male fish (Hansen et al., 1998; Jobling et al., 1998; Sumpter, 1998; Tyler and Routledge, 1998; Liney et al., 2005). The mechanisms involved in these endpoints are still murky and research to elucidate them is required. For example, it is clear that while exposure of early life wild roach males to effluents from sewage treatment facilities causes permanent feminization in a dose-dependent manner (Liney et al., 2005), no permanent effects on adult male fish are observed. However, adult fish obtained from a population that exhibited intersex showed an increased amount of intersex when exposed to these effluents. This study suggests that the generation of ovo-testis is much more complex than originally thought and may have an epigenetic component (Liney et al., 2005).

Comprehensive chemical analyses of sewage effluents using a combination of chemical fractionation and biological screening techniques (mainly recombinant yeast assays), have identified several estrogenic active compounds, including natural estrogens (E_2); synthetic estrogens widely used in birth controls pills (ethinylestradiol, EE_2); alkylphenolic chemicals (resulting from the breakdown of nonionic surfactants); and plasticizers (bisphenol-A, BPA). In addition,

potent synthetic anabolic androgens (17β-trenbolone) emerge downstream from CAFOs (Ankley et al., 2003).

Synthetic steroidal estrogens, such as EE_2, may be causing the greatest estrogenic effects in fish inhabiting streams contaminated with sewage effluents. This is because synthetic estrogens are highly potent hormones, and thus concentrations in the pptr range are capable of inducing biological effects. For instance, EE_2 induces Vtg synthesis in male rainbow trout at concentrations as low as 0.1 pptr (Purdom et al., 1994). This is of great ecological relevance, since concentrations of EE_2 in natural streams and rivers are at least this high (Routledge et al., 1998). Women are the primary source of natural and synthetic estrogens in sewage effluents, either because of their excretion of inactive conjugates during menstrual cycling and pregnancy or because of the use of contraceptive pills. In male fish, EE_2 is a potent ER-agonist. It induces the expression of several female-specific proteins, including Vtg and ZR proteins (Bowman et al., 2000; Denslow et al., 2001; Lattier et al., 2002; Lee et al., 2002; Palace et al., 2002; Seki et al., 2002; Van den Belt et al., 2002; Islinger et al., 2003; Orn et al., 2003; Robinson et al., 2003; Thomas-Jones et al., 2003; Thorpe et al., 2003; Werner et al., 2003; Versonnen and Janssen, 2004). Furthermore, studies with sheepshead minnow (*Cyprinodon variegatus*) have shown that exposure of male fish to this synthetic E_2 induces a cDNA fingerprint pattern of expression almost identical to fish exposed to E_2 (Denslow et al., 2001) and other estrogenic xenobiotics, such as nonylphenol (Larkin et al., 2003a). As already mentioned, EE_2 has a much higher estrogenic potency (5- to 66-fold higher) compared to E_2 (Rose et al., 2002; Thomas-Jones et al., 2003; Thorpe et al., 2003).

Recent studies with zebrafish have also shown that EE_2 is capable of enhancing the expression of CYP19A2, the aromatase responsible for conversion of androgens to estrogens in the brain (Scholz and Gutzeit, 2000; Andersen et al., 2003a; Kazeto et al., 2004). A recent report described a previously unrecognized mechanism of estrogenicity of EE_2 in plaice (*Pleuronectes platessa*) (Brown et al., 2004). In this study, although male plaice exposed to EE_2 responded with an increased induction of Vtg and ZR proteins, females responded with a down regulation of egg proteins, probably due to feedback inhibition of pituitary hormones due to the increased level of overall E_2 concentration. A decline in the production of egg proteins could lead to the production of poor quality eggs, and thus decreased reproductive success as discussed in more detail under section 3.

17-β-trenbolone is a steroidal androgen that has found its way into the environment through cattle feedlots, since it is an anabolic growth promoter (Ankley et al., 2003). Trenbolone exhibits high affinity for fish ARs and has multiple effects on fathead minnow reproduction (Ankley et al., 2003). Trenbolone is not aromatizable by CYP19 aromatase and thus has pure androgen-like qualities, different from many androgens. Exposure of sexually mature females to trenbolone results in their masculinization, including the production of male

secondary sex characteristics. This exposure also results in the reduction of Vtg, potentially leading to poor quality eggs. The mechanism by which trenbolone affects Vtg synthesis is not clear, but suggests significant crosstalk between the estrogen- and androgen-regulated gene expression mechanisms.

Alkylphenol ethoxylates (APEs) are effective nonionic surfactants serving as components of industrial and domestic detergents, emulsifiers, pesticide formulations, cosmetics, and paints. APEs are common components of sewage effluents, and during effluent treatment, are biodegraded by bacteria to form the APs nonylphenol and octylphenol. Sewage effluents might contain over 100 ppb APs (Nakada et al., 2004), although most streams surveyed in the UK and in the USA have contained equal or less than 10 and 0.1 ppb of APs, respectively (Naylor, 1985; Blackburn and Waldock, 1995). Because of their liphophilicity, both nonylphenol and octylphenol are capable of bioaccumulating in fish adipose tissues. Both have estrogenic properties in fish, although they bind to the ER with an affinity 2,000 times less compared to E_2 (Routledge and Sumpter, 1996). Nevertheless, several studies have reported increased Vtg protein or mRNA production in male and/or juvenile fish exposed to these compounds (Jobling et al., 1996; Lech et al., 1996; Nimrod and Benson, 1996; Madsen et al., 1997; Christiansen et al., 1998; Christensen et al., 1999; Arukwe et al., 2000; Shioda and Wakabayashi, 2000a, b; Sohoni et al., 2001; Hemmer et al., 2002; Van den Belt et al., 2003b; Cardinali et al., 2004; Ishibashi et al., 2004; Rasmussen and Korsgaard, 2004; Rasmussen et al., 2005). In juvenile Atlantic salmon (*Salmo salar*), nonylphenol has also been reported to induce the synthesis of eggshell ZR proteins (Arukwe et al., 2001), hepatic ER (Yadetie et al., 1999), and LHβ mRNA (Yadetie and Male, 2002). ZRPs have also been up regulated in male largemouth bass and Japanese medaka exposed to nonylphenol (Lee et al., 2002; Larkin et al., 2003a). APs and APEs elicit endocrine disrupting effects mainly through receptor-mediated mechanisms, binding to the ER and activating the transcription of E_2 responsive genes, as shown by many *in vitro* and *in vivo* studies (Jobling and Sumpter, 1993; White et al., 1994; Soto et al., 1995; Routledge and Sumpter, 1996; Min et al., 2003; Yamaguchi et al., 2005). P-substituted phenols are the most potent estrogens (Smeets et al., 1999). In a recent study with channel catfish, octylphenol and nonylphenol showed greater affinity for ERα compared to ERβ (Gale et al., 2004). Another indication of their ability to compete with endogenous E_2, is the fact that APs are also capable of displacing E_2 from the sex steroid-binding site of sex steroid-binding proteins of fish in a dose-dependent and competitive manner (Tollefsen, 2002; Tollefsen et al., 2004).

In addition, APs can induce reproductive alterations through nonreceptor-mediated mechanisms of action. Recent studies have shown that APs can exert effects *in vivo* by directly interacting with cellular enzyme targets leading to alterations of both synthesis and metabolic clearance pathways of sex hormones in fish. For instance, studies with zebrafish and Japanese medaka have shown that nonylphenol is capable of enhancing the expression of CYP19 the key

steroidogenic enzyme responsible for conversion of androgens to estrogens (Min et al., 2003; Kazeto et al., 2004). Inhibition of sulfotransferases, the enzymes responsible for converting active estrogenic steroids to inactive steroid sulfates, have also been reported in fish exposed to APs (Kirk et al., 2003; Thibaut and Porte, 2004). In contrast, nonylphenol enhances ovarian synthesis of maturation-inducing hormones (20α and 20β-hydroxysteroid dehydrogenases) (Thibaut and Porte, 2004). Other nonreceptor-mediated modes of action of APs include changes in the posttranscriptional regulation of Vtg mRNA processing (Ren et al., 1996) and increases in the rate of apoptosis of Sertoli cells, nurse-like cells that provide support and facilitate transport of maturing sperm into the testicular deference duct (Raychoudhury et al., 1999). Male sheepshead minnows also appear to exhibit non-ER mediated mechanisms of action for nonylphenol (Larkin et al., 2003b). In these studies, fish responded with a significant up regulation of ubiquitin conjugating enzyme 9 compared to fish treated with E_2 alone.

Bisphenols are a group of diphenylalkanes commonly used in the production of polycarbonate plastic (used in a variety of common products including digital media, electric equipment, and reusable food and drink containers) and epoxy resin plastics (used in many engineering applications such as electrical laminates, composites, paints and adhesives, as well as in a variety of protective coatings). They consist of two phenolic rings joined by a carbon bridge and two methyl groups in the case of BPA, one of the most environmentally prevalent types of bisphenols. BPA is an estrogen mimic, a quality known since the 1930s (Dodds and Lawson, 1938). Since then, numerous *in vitro* and *in vivo* studies with fish have confirmed it as an estrogen by showing increased induction of female-specific proteins and mRNA (Vtg and eggshell proteins) by males (Milligan et al., 1998; Celius et al., 1999; Islinger et al., 1999; Smeets et al., 1999; Arukwe et al., 2000; Shilling and Williams, 2000; Ishibashi et al., 2001; Lee et al., 2002; Paris et al., 2002; Pait and Nelson, 2003; Van den Belt et al., 2003b). These studies have confirmed the ability of BPA to interact directly with the ER, and with plasma sex steroid-binding proteins (Tollefsen, 2002; Tollefsen et al., 2004). Comparative studies evaluating the estrogenic capability of several EDCs have reported that BPA has a lower estrogenic potential compared to EE_2 and APs (Paris et al., 2002; Yamaguchi et al., 2005), but higher when compared to estrogenic OCPs such as o,p′-DDT (Tollefsen et al., 2003). A recent study with Atlantic croaker reported that BPA was also capable of blocking the action of a progestin (17,20β, 21-trihydroxy-4-pregnen-3-one), the enzyme responsible for upregulating sperm motility in fish (Thomas and Doughty, 2004). BPAs can also induce reproductive alterations through nonreceptor-mediated mechanisms of action, such as enhanced expression of CYP19 genes (Min et al., 2003).

In summary, sewage effluents contain several classes of estrogenic bioactive compounds, including natural and synthetic estrogens (E_2 and EE_2), steroidal androgens such as trenbolone, nonionic surfactants (nonylphenol and octylphenol), and plasticizers (BPA). Extensive evidence with several fish species has

accumulated over the last 5 years implicating these compounds as EDCs, mainly through ER- or AR-mediated mechanisms of action. Indeed, male and reproductively immature fish exposed to these estrogenic chemicals respond with an induction of several female-specific proteins, including Vtg, eggshell proteins, and ER. ER-mediated effects on the transcription of brain aromatase and possible associated alterations of the neuroendocrine system needs further study. In addition, these xenoestrogens can induce reproductive alterations through nonreceptor-mediated mechanisms of action, through cell signaling pathways that can affect sex steroid biosynthesis and metabolism. More importantly, and as discussed in more detail under section 3, these molecular and biochemical changes have been linked to reduced reproductive success and population-level effects in several fish species.

2.4.5. Paper mill effluents

Over the past 20 years, a great body of work has focused on the effects of paper mill effluents on multiple biochemical and physiological parameters of fish. Altered reproductive function is the most meaningful responses seen in these studies. Specifically, fish exposed to these effluents respond with alterations in sex steroid biosynthesis and metabolism, gonadal development, sexual maturation, and development of secondary sex characteristics. Despite the extensive knowledge on the effects of paper mill effluents on fish reproduction, there is sparse information on the chemical(s) that are responsible for such changes. Identification of causative bioactive agents in these effluents has proven extremely difficult, because paper mill effluents are complex mixtures, known to contain several hundred different types of compounds. Furthermore, variations in wood furnish, in the pulping and bleaching process, and in the treatment of effluents between mills and within mills over time, leads to the production of many different effluent compositions. Nevertheless, all pulping protocols involve the separation and discharge of natural wood components, such as resin and fatty acids, plant sterols, and sugars, which generally undergo bacteriological degradation and sedimentation in settling and aeration ponds. In addition, depending on the type of bleaching techniques used, these effluents may also contain more persistent and bioaccumulative classes of compounds, such as chlorinated phenolics and guaicols.

In the last few years, it has become apparent that chlorinated compounds might not be the primary key players in the observed effects, since exposure to unbleached paper mill effluents, black liquor, and mechanical pulping effluents causes the same reproductive alterations (Lehtinen, 2004). Bioassay-based fractionation studies have shown that paper mill effluents contain a broad range of compounds that have weak estrogenic activity. These include industrial nonionic surfactants (nonylphenol ethoxylates) (Lee and Peart, 1999) and natural wood components such as fatty acids (Mercure and Van der Kraak, 1995), lignin derivatives (e.g. polyphenolics) (Hewitt et al., 2002), isoflavones (genistein) (Kiparississ et al., 2001), resin acids and plant sterols (MacLatchy and Van der

Kraak, 1995; MacLatchy et al., 1997; Tremblay and Van der Kraak 1998, 1999; Lehtinen et al., 1999). Indeed, several reports have implicated the plant sterol β-sitosterol, as a possible significant factor contributing to the negative reproductive effects observed in fish exposed to paper mill effluents. Although β-sitosterol can induce estrogenic effects in male fish, binding to the ER and promoting expression of the Vtg gene (Mellanen et al., 1996; Tremblay and Van der Kraak, 1998), most effects associated with this phytoestrogen have been antiestrogenic in nature. For instance, female goldfish treated with β-sitosterol respond with reductions in plasma sex steroids and decreased gonadal T and pregnenolone production (MacLatchy et al., 1997). Furthermore, overall suppression of steroidogenesis and gonadal development by paper mill effluents has been a consistent finding from research conducted with many different fish species and effluent types (Munkittrick et al., 1992a,b; McMaster et al., 1991, 1995, 1996). Results from our own laboratories have also shown similar antiestrogenic effects in female largemouth bass exposed to paper mill effluents (Sepúlveda et al., 2002, 2003, 2004b). The reproductive effects observed in largemouth bass (i.e. declines in sex steroids, Vtg, and gonad development) are suggestive of exposure to compound(s) capable of causing an overall inhibition of reproductive function, rather than receptor-mediated activity (i.e. E_2 or androgen mimicry). Indeed, preliminary differential display studies using livers from bass exposed to this same effluent indicate changes that are different from the ones induced after injections with E_2 (Denslow et al., 2004). The absence of Vtg induction in male bass exposed to this effluent is also indicative of exposure to chemical(s) that lack a strong estrogenic potential. Antiestrogenic activity of paper mill effluents has also been detected using mammalian *in vitro* recombinant receptor/reporter bioassays (Zacharewski et al., 1995).

Other compounds present in paper mill effluents that depress reproductive function in fish include phenol and sulfide. Both of these chemicals inhibited the uptake of radiolabeled cholesterol into carp ovary from the peripheral circulation and its ovarian conversion to progesterone and pregnenolone (Mukherjee et al., 1991).

There is also evidence suggesting that compounds present in paper mill effluents are capable of mediating endocrine responses through the AR (Svenson and Allard, 2004). For instance, female mosquitofish (*Gambusia affinis*) inhabiting a stream contaminated with paper mill effluents were masculinized showing both physical secondary sexual characteristics (fully developed gonopodium) and reproductive behavior of males (Howell et al., 1980; Bortone and Davis, 1994). Masculinization of female fish likely is due to the action of androgenic hormones that result from the biotransformation of plant sterols (and also cholesterol and stigmasterol) by bacteria such as *Mycobacterium* (Denton et al., 1985; Howell and Denton, 1989). Although research has demonstrated uptake of androgenic compounds by fish, there has been little success associating androgenic compounds with masculinization effects. For instance, downstream from a mill that produces masculinizing effects, water and

sediments were tested in binding assays using the human AR (Parks et al., 2001; Jenkins et al., 2003), suggesting that androstenedione was present and responsible for the binding. More quantitative analyses of these effluents, however, indicated that the bioactive components did not include androstenedione since this compound eluted in fractions, which did not induce human AR activity and expression (Durhan et al., 2002; Ellis et al., 2003). In addition, several bioassay-based fractionation studies, have failed to support androstenedione and T as active androgens that bind the AR and masculinize females (Jenkins et al., 2001, 2003).

Paper mill effluent targets several key steps along the HPG axis of fish. Fish from exposed sites had significantly lower plasma LH levels and showed depressed responsiveness of sex steroids and 17,20β-dihydroxy-4-pregnen-3-one (a maturation-inducing steroid) after GnRH injections (Van der Kraak et al., 1992). In these studies, fish also had lower circulating levels of T glucoronide, which would be suggestive of altered peripheral steroid metabolism. Similarly to observations under *in vivo* conditions, *in vitro* incubations of ovarian follicles collected from paper mill effluent-exposed females also showed reduced steroid production (McMaster et al., 1995). The similarities between both types of studies suggest that reductions in plasma steroid levels are mainly due to alterations in ovarian steroid production. In addition, exposure to paper mill effluents causes apoptotic DNA fragmentation and increased expression of a 70 kDA heat-shock protein in oocytes from exposed pre-spawning females. Coupled with a decrease in sex steroids, this effect may explain the observed decreased gonad weights and delayed sexual maturity (Janz et al., 1997).

In summary, exposure of fish to paper mill effluents results in a myriad of negative reproductive effects including altered pituitary function; reduced gonadal biosynthesis and peripheral metabolism of sex steroids; and altered development of secondary sex characteristics. Effects of paper mill effluents vary depending on the chemical composition of the mixture under study, and can range from exerting mostly antiestrogenic/antiandrogenic effects, to causing masculinization of female fish (androgenic effects). Expression of Vtg (or other female-specific proteins) is not consistently reported for fish exposed to paper mill effluents, despite the fact that these effluents contain phytoestrogens, such as β-sitosterol.

2.5. Fish Models to Study the Effects of EDCs

Many fish species serve as models to study the effects of EDCs on reproduction and early life stages. These come from a variety of habitats and exhibit a variety of reproductive paradigms. For a good comparative work, research on evaluation of sensitivity, mechanisms of action, and effects on early life stages should employ several species. The models of choice for laboratory exposures are small fish species such as the fathead minnow, zebrafish, Japanese medaka, or mummichog (*Fundulus heteroclitus*), all of which are fractional spawners and provide

eggs throughout the year for easy experimentation. Indeed, there is a substantial database for evaluation of toxicologic endpoints and physiological outcomes for these smaller fish species. Their life histories are well studied and documented, and many standard protocols exist for their use in ecological risk assessment (ERA). Large groups of researchers have selected these models across the globe. A disadvantage of many of these fish models is that they do not inhabit most contaminated streams and lakes, and thus report poorly on the wider ecological effects of contaminant exposure.

A variety of large fish species including rainbow trout, salmon, flounder, sea bream (*Sparus aurata*), roach tilapia and largemouth bass also serve to show both the commonality of EDC function among these fish as well as the differences. The rainbow trout is the number one model for environmental toxicology, based on a recent examination of the number of citations in the EcoTox database. The Atlantic salmon is also a highly studied model. It is an anadromous fish that hatches in freshwater, spends most of its adult life in the sea, returns to freshwater to spawn, and thus presents a unique opportunity to study the interactions between contaminants released into rivers and a life at sea. These larger fish species are interesting because they usually spawn only once a year and their reproductive cycles are synchronous. Exposing these fish to EDCs at different times during their reproductive cycles gives researchers the opportunity to dissect out the different mechanisms by which these types of contaminants exert their effects.

Small Fishes. Fathead minnows inhabit many areas of the country, both freshwater lakes and the margins of rivers and they offer several advantages in terms of captive breeding. Therefore, they are an ideal model for testing many different hypotheses in relation to establishing modes of action and effects of EDCs in fish. Indeed, their overall sensitivity to the effects of environmental contaminants has made them a standard test species for aquatic toxicology since the 1960s (Mayer and Ellersieck, 1986). In addition, they are sexually dimorphic, have a short reproductive cycle, spawn year-round, and their reproductive physiology is very well known (Jensen et al., 2001). A continuous, fractional reproductive cycle allows for the conduction of multiple reproductive experiments per year (rather than single spawning experiments with seasonal spawners) and thus for a faster and more efficient way of assessing effects and molecular mechanisms of action of EDCs. The fathead minnow has been identified as an excellent model for evaluating the effects of EDCs (e.g. AR and ER agonists and antagonists), and as such has been added to the endocrine screening battery being developed by the US EPA Endocrine Disrupter Screening and Testing Advisory Committee, EDSTAC (Ankley et al., 2001; Gray et al., 2002; Ankley and Villeneuve, 2006). Finally, the fathead minnow is an excellent genomics model (Miracle et al., 2003) with a gene chip (>22,000 genes) currently under development.

The sheepshead minnow and the mummichog are excellent models for estuarine work in North America. Natural populations of mummichogs along the east coast of the USA serve to understand mechanisms of adaptation and

development of resistance in fish exposed to pollutants (Meyer et al., 2003; Hahn et al., 2004). The sheepshead minnow works as a testing model by EPA and is an accepted model for risk assessment. Both models are excellent for reporting on this environmental niche and are equivalent models.

Zebrafish and Japanese medaka have become widely used model organisms to assess the impairment of reproduction in aquatic vertebrates exposed to EDCs, particularly in Europe and Asia, respectively. There is a tremendous database on the genetics of zebrafish and it is quite useful as a model, especially since genome sequence is available. Zebrafish excel in this regard due to their small size and short generation time. They serve in numerous partial and whole life cycle tests to determine effects on gonadal development, sex ratios, fertilization rates, and hatching success after exposure to different chemicals. Additionally, the Japanese medaka serves as an attractive model to study sexual differentiation. As for many other fish, the sex of medaka is XX/XY-coded. However, the phenotypic sex changes depending on the presence of androgens or estrogens applied during sensitive periods of embryonic or juvenile development (Kobayashi and Iwamatsu, 2005). Detection of histopathological effects is possible because of the characterization of gonadal differentiation and morphology in these fish. Furthermore, cDNAs of sex-specifically transcribed genes like aromatase, choriogenin H, ER, and CYP-450 have been cloned and sequenced and can be used to analyze the effects of EDCs on gene expression (Fukada et al., 1996; Murata et al., 1997).

Large Fishes. Many large fish species serve as exceptional models in ecotoxicology. Here we are summarizing work on largemouth bass, only as an example of how these models help to evaluate the mechanisms of action and effects of EDCs in seasonal spawners. Other important large fish models include the salmonids, such as the rainbow trout and the Atlantic salmon, sea bream, roach and several species of tilapia.

The largemouth bass is a fish of great ecological and commercial importance throughout the USA. Bass are group-synchronous spawners, an act triggered by a rise in water temperature during the spring months (Clugston, 1966). Since largemouth bass are long-lived aquatic predators, they bioaccumulate significant concentrations of lipophilic persistent compounds (Sepúlveda et al., 2004a). Humans routinely consume them and thus their contamination poses a problem for human health. There are several lines of evidence suggesting their populations have declined due to environmental contaminants. For instance in Florida, population declines have been reported for largemouth bass on Lake Apopka and to a lesser degree on many other lakes in Florida (Benton et al., 1991; Benton and Douglas, 1996). Finally, over the last decade, we have gathered substantial basic physiological and reproductive information for this species. In addition, we have developed a microarray gene chip, which will be crucial for future interpretation of endocrine disrupting studies in this species (Denslow et al. 1996; Orlando et al., 1999; Bowman et al., 2002; Gross et al., 2002; Larkin et al., 2002; Sepúlveda et al., 2002; Smith et al., 2002).

3. BIOINDICATORS OF ADVERSE EFFECTS IN FISH REPRODUCTION

It is possible to measure biomarkers of effect at different levels of biological organization from the molecular to the full ecosystem (Figure 4). Although it is important to assess whether exposures cause adverse effects on fish populations and ultimately on ecosystem health, it is often very difficult to determine these endpoints, because at the usual environmental concentrations, very high exposure times are required. By the time a response is noted, the ecosystem has sustained irreparable damage and perhaps some species are close to extinction. Furthermore, it is hard to distinguish effects of exposure to EDCs from other confounding variables including general changes in the environment such as, water quality (temperature, dissolved oxygen, and pH), food availability, and genetics and adaptability of fish. Thus, there is great interest in discovering biomarkers that can predict ecosystem damage at much earlier times, in order to start remediation earlier to avert a disaster. Responses at the molecular, subcellular, and cellular levels occur with low-exposure concentrations and appear

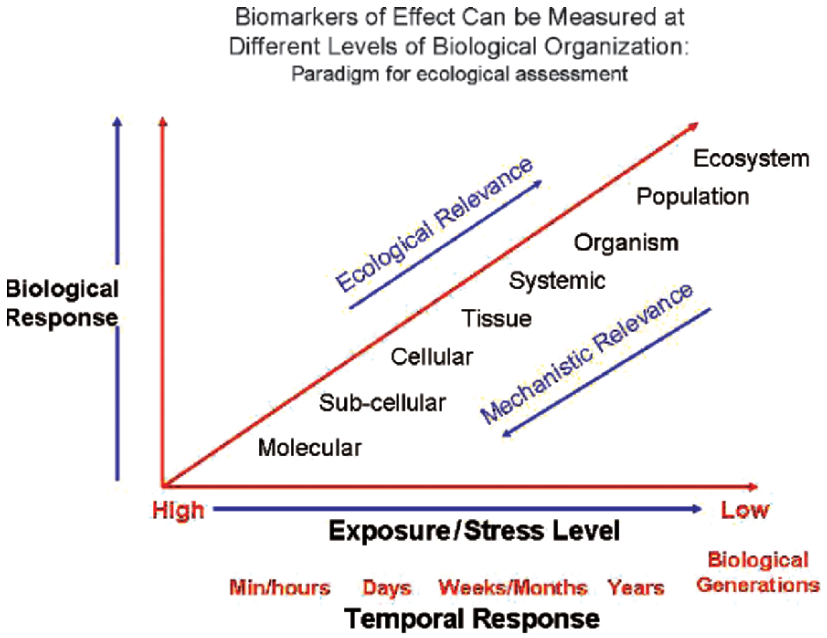

Figure 4. Endocrine disrupting chemicals (EDCs) can exert effects at different levels of biological organization, ranging from the molecular to the ecosystem levels. Effects at low levels of biological organization have high mechanistic significance, whereas effects at higher levels of biological organization have high ecological relevance. The figure also illustrates the time needed for the different responses to occur, ranging from minutes to days for effects measured at the subcellular levels, to several generations in the case of changes at the ecosystem level. (*See Color Plates*).

within hours to days. There is considerable interest to identify sensitive biomarkers at these levels that will predict ecosystem health. Cellular and molecular biomarkers have high mechanistic relevance, because they pinpoint exactly how the specific exposure has changed biochemical pathways.

A new paradigm in biology, termed "Systems Biology" (Idekar et al., 2001) seeks to define whole organism changes in response to environmental and chemical factors. In the case of toxicant exposure, this paradigm has been designated "Systems Toxicology" (Waters and Fostel, 2004). With the availability of genomics and proteomics methodologies, it is now possible to determine mechanistic effects with whole animal exposures, looking at the changes in biochemical pathways that lead to toxicity.

3.1. High Ecological Significance

An important challenge currently facing the field of aquatic toxicology is to clearly identify and quantify population-level effects in fish exposed to EDCs. It has only been in the last five years or so, that information about mechanisms of action and threshold tissue concentrations have related to population-level effects. From the previous background information on the modes of action of EDCs on fish, it follows that exposure to these compounds can elicit significant reproductive effects. Changes at the molecular, biochemical, and cellular levels correlate with responses at the organ and organism levels. More importantly, several field epidemiological studies have also demonstrated that exposure of fish to EDCs can lead to population and even ecosystem perturbations (e.g. Miller and Ankley, 2004; Orlando et al., 2004). Simplistically, we can list a chronology of potential reproductive events after exposure of fish to EDCs as follows. First, exposure of sexually differentiated fish to EDCs results in a decrease in the bioavailability of sex hormones and gonadotropins, which lead to impaired gonadal development, altered reproductive behaviors, and decreased fecundity and fertility. In females, declines in the production of E_2 results in altered vitellogenesis, which causes detrimental effects on oogenesis and egg quality, ultimately leading to developmental abnormalities, increased embryo and sac fry mortality, and even spawning inhibition. These reproductive changes are usually reversible (activational) with animals being capable of reverting to normal after the exposure ceases. However, exposure of EDCs during the period of sex differentiation can result in irreversible structural (organizational) changes leading to altered reproductive output and permanent (irreversible) masculinization or feminization. Depressed spawning and fertility, coupled with altered sex ratios and decreased survival of early life stages could in turn affect recruitment and have significant population-level effects. Although most studies published to date have explored the effects of clear estrogenic and androgenic chemicals on the female's reproductive physiology, recent studies have began to evaluate effects on males, and measurable effects on sperm quantity and quality have been observed. It is clear that this field of research is still

in its infancy and that many more years of work are still needed to understand the potential effects of these compounds in populations of aquatic organisms. The following is a review of ecologically relevant reproductive endpoints adversely affected in fish exposed to EDCs.

3.1.1. Reproductive behavior, gonad development, spawning, fecundity, and fertility

In vertebrates, a multitude of external (e.g. photoperiod and temperature) and internal (e.g. hormonal levels) stimuli influence behavioral responses. The different sensory organs perceive vision, olfaction, audition, electrical stimulus, and taste, and the anterior portion of the brain integrates these with the appropriate locomotor activity (Heath, 1995). Thus, changes on behavior represent a high level of integration and complexity. Nevertheless, even minor changes in normal behavior, can lead to serious alterations in reproductive physiology. This area has not received much attention in relation to endocrine disrupting effects on fish, and only a few studies appear in print so far. As already discussed, sex steroids play a key role in the development and maintenance of sexual functions, including courtship behavior, and alterations in their synthesis and/or metabolism could lead to altered reproductive behaviors. A severe disruption in the male guppy's courtship behavior was observed after exposures to the antiandrogens vinclozolin (1–10 μg/mg) and p,p′-DDE (0.1–1.0 μg/mg) (Baatrup and Junge, 2001). In this study, there was an inhibition of male orientation towards females and of their sigmoid display. Bayley et al. (2002, 2003) also reported a significant reduction in the intensity of sexual display in guppies exposed to vinclozolin and p,p-DDE. In another study with this same species, exposure to the xenoestrogen EE_2 (112 ng/L) reduced intermale competition for female copulations, and this decrease in courtship behavior was accompanied by reductions in sperm counts, testis weight, and decreased expression of secondary sex characteristics (Kristensen et al., 2005). Similar suppression in courtship and aggressive behaviors have been observed in fathead minnow, Japanese medaka, threespined stickleback (*Gasterosteus aculeatus*), and sand gobies (*Pomatoschistus minutus*) males exposed to environmental relevant concentrations of EE_2 (Bayley et al., 1999; Bell, 2001; Majewski et al., 2002; Robinson et al., 2003; Balch et al., 2004). Other EDCs known to have altered male reproductive behavior include OCPs (Matthiessen and Logan, 1984), octylphenol (Bayley et al., 1999; Gray et al., 1999a,b; Nirmala et al., 1999), TBT (Nakayama et al., 2004), and atrazine (Moore and Lower, 2001). In the latter study, male Atlantic salmon exposed to 0.5–2 μg/L atrazine showed reduced sensory responses to the female pherohormone prostaglandin $F_{2\alpha}$.

Gonad Growth. Abnormal gonadal growth is an indicator of changes along the HPG axis of fish. Thus, changes in the synthesis, metabolism, and/or receptor interactions of GnRH, GTHs, and sex steroids could lead to reduced gonad weights or gonadosomatic indices (GSIs). In addition, xenobiotics could have direct toxicity effects on the gonads. GSI is defined as the weight of the gonad

divided by the weight of the fish. This represents a cost-effective and easy way to quantify reproductive development in fish. There are several good examples of decreased GSIs in fish following exposure to EDCs, and thus this biomarker continues to show enough sensitivity for evaluating reproductive effects due to these chemicals. As already discussed, however, it lacks specificity since many different factors influence gonad maturation and size. There are significant reductions in ovarian and testicular somatic indices in oviparous fish exposed to xenoestrogens, although most studies so far have reported declines in testicular weights. Chronic exposure to estrogen mimics such as octylphenol and nonylphenol (Jobling et al., 1996; Le Gac et al., 2001; Toft and Baatrup, 2001; Kang et al., 2003; Cardinali et al., 2004; Rasmussen and Korsgaard, 2004), EE_2 (Van den Belt et al., 2001; Schultz et al., 2003; Pawloski et al., 2004; Tilton et al., 2005), and BPA (Sohoni et al., 2001) inhibits testicular growth in adult male fish. In some of these studies, males also produced less milt and fewer offspring compared to untreated fish, suggesting a direct effect of decreased GSIs on fertilization rates. Marked reductions in testicular mass have also been observed in viviparous fish (eelpout, *Zoarces viviparus*) dosed with octylphenol (Rasmussen et al., 2005). Other EDCs that have induced decreased GSIs in male fish include o,p′-DDT (Mills et al., 2001), Cd (Das, 1988; Pereira et al., 1993; Mukherjee et al., 1994; Tilton et al., 2003), and atrazine (Bringolf et al., 2004). In addition, declines in female and male GSIs have been a consistent finding in several species of fish exposed to paper mill effluents (Munkittrick et al., 1992a; McMaster et al., 1996; Sepúlveda et al., 2002, 2003). In females, declines in ovarian growth have been reported after exposures to vinclozolin (Makynen et al., 2000) and EE_2 (Scholz and Gutzeit, 2000).

Spawning, Fecundity and Fertility. In females, declines in gonad size and alterations in normal gonad maturation can reduce the number of eggs spawned (here referred to as fecundity), whereas in males it can result in alterations in sperm production (here referred to as fertility). EE_2 is an EDC frequently studied in relation to effects on fecundity and fertilization rates. The lowest observable effect concentration (LOEC) for effects on fecundity and fertility was reported in zebrafish, where a lifelong exposure to 5 ng/L EE_2 caused a 56% reduction in fecundity and complete population failure with no fertilization (Nash et al., 2004). Altered sexual differentiation caused infertility since males lacked functional testes. As has been demonstrated in many other studies with EDCs, this study highlighted the importance of "timing" of exposure, since exposure of adult fish for only 40 days to the same dose of EE_2 had no impact on reproductive success (Nash et al., 2004). A slightly higher threshold concentration of EE_2 (10 ng/L) was reported in this same species by Van den Belt et al. (2001) who found a dose-related reduction in the number of females capable of spawning. These authors also reported a complete inhibition of spawning at levels of 25 ng/L EE_2. Similar sensitivities have been detected in sand gobies, with close to a 90% reduction in fecundity and fertility when exposed to 6 ng/L EE_2 (Robinson et al., 2003). Studies with fathead minnows, Japanese medaka, and

rainbow trout have reported LOECs of EE_2 for fecundity and fertility ranging from 10 to 100 ng/L (Scholz and Gutzeit, 2000; Schultz et al., 2003; Pawloski et al., 2004). Emphasizing again the importance of timing of exposure, decreased egg fertilization rates were detected in fathead minnows at much lower concentrations (0.3 ng/L EE_2) when exposure covered the period from egg fertilization to adulthood (Parrott and Blunt, 2005).

Other estrogenic compounds that affect fish fecundity and fertility include the APs nonylphenol and octylphenol and BPA. In Japanese medaka, egg production and fertility were decreased by both nonylphenol (101–184 µg/L) (Kang et al., 2003) and octylphenol (20–230 µg/L) (Gronen et al., 1999). Significant declines (40–75%) in total sperm counts also occurred after exposure of fish to BPA (274–1,280 µg/L) (Haubruge et al., 2000; Sohoni et al., 2001). In fathead minnows, females dosed with 1,280 µg/L BPA showed diminished egg production (Sohoni et al., 2001). Antiandrogens, such as p,p′-DDE and vinclozolin have also induced declines in fecundity and fertility. For instance, p,p,-DDE (0.01 and 0.1 µg/mg) and vinclozin (0.1 and 10 µg/mg) induced >60% reduction in sperm counts in guppies exposed from juvenile to adults (Bayley et al., 2002). Sperm declines correlated with delayed sexual maturation, reduced display coloration, smaller gonopodium, and reduced sexual behavior indicating a significant demasculinization in these fish. In another study with this same species, vinclozolin administered at a rate of 1.8–180 mg/kg from 8 to 14 weeks of age also resulted in the decline of sperm counts, sexual display behavior, and fertility (Bayley et al., 2003). Fertilization rates and fecundity were diminished in male fish exposed to OCPs (Burdick et al., 1964; Macek, 1968; Smith and Cole, 1973; Monod, 1985; Cross and Hose, 1988; Hose et al., 1989) and TBT. The latter chemical is known to affect fertilization rates in adult Japanese medaka exposed to 1 µg/g body weight daily for 3 weeks (Nakayama et al., 2004). In addition, in zebrafish, TBT (0.1–100 ng/L for 30 and 70 days) decreased sperm motility and morphology, showing a higher incidence of sperm lacking flagella (McAllister and Kime, 2003). An important conclusion from this study was that effects of TBT on zebrafish early life only became apparent at adulthood and were irreversible even after 5 months in clean water.

Finally, effects of paper mill effluents on eggs and sperm production have been quite variable mainly related to differences in the chemical composition of effluents tested. Some studies have shown dramatic effects on eggs including reduced fecundity, smaller egg size, and poorer fertilization of eggs (Landner et al., 1985; Vuorinen and Vuorinen 1985; Munkittrick et al. 1991). Whereas in others, there is equal or greater fertilization rates and no effects on egg hatchability, even though the fish demonstrated indices associated with reproductive dysfunction such as reduced concentrations of sex steroids, gonad and egg size, and sperm motility (McMaster et al., 1995). Exposure to these types of effluents in several other field and laboratory studies did not alter fecundity (Karås et al., 1991; Adams et al., 1992; Swanson et al., 1992; Kovacs et al., 1996). However, exposure of fathead minnows to 10% bleached sulfite mill effluent for an entire

life cycle resulted in reduced fecundity while exposure of females to ≥30% effluent resulted in complete failure to produce eggs (Parrott et al., 2004).

3.1.2. Egg quality and survival, growth and development of embryos and fry

Early life stages are the most sensitive to the effects of xenobiotics compared to adults. However, the effects of parental exposure to EDCs on offspring survival, growth and development remain unknown. In addition, although many EDCs affect growth, and development of early life stages, the exact modes of action for many of these chemicals are unclear and may be the result of nonendocrine-mediated pathways. For instance, these chemicals may have direct toxicity on early life stages of fish. Indeed, direct exposure of gravid females to the more lipophilic EDCs, such as PCBs, OCPs, and TCDD results in maternal transfer followed by developmental toxicity of fish embryos (Russell et al., 1999). In the case of OCPs, numerous laboratory and field studies have linked exposure to DDT and derivatives to early life stage mortality. For instance, Burdick et al. (1964) found that lake trout (*S. namaycush*) eggs containing DDT concentrations of 10 mg/kg or greater resulted in 50% or greater mortality of sac fry and that 4.75 mg/kg of DDT produced 15% sac fry mortality. Similarly, feeding brook trout with sublethal concentrations of DDT (1.0 and 2.0 mg/kg per week over a period of 156 days) resulted in sac fry mortality (Macek, 1968). Egg concentrations of 1,600 μg/kg DDT or greater cause developmental alterations, such as vertebral deformities, bone erosions, and hemorrhages and increased embryo mortality (Smith and Cole, 1973). White croaker (*Genyonemus lineatus*) also exhibited increased embryo mortality when ovarian DDT concentrations were 4 mg/kg and above (Cross and Hose, 1988; Hose et al., 1989). Mortality among the sac fry from the DDT treated trout corresponded to the release of DDT residues from yolk fat, during the developmental period when fry depend on yolk reserves for nutrition. Winter flounder (*Pseudopleuronectes americanus*) exposed to aqueous sublethal concentrations of DDT induced fry vertebral deformities at time of hatch (Smith and Cole, 1973). Fry, which hatched from eggs containing DDT concentrations equal to or exceeding 2.39 mg/kg demonstrated deformities including erosion and hemorrhaging at the vertebral junctions. Our own studies with female largemouth bass exposed to p,p′-DDE have shown complete inhibition of spawning at ovarian concentrations >5 mg/kg (Sepúlveda et al., University of Florida, 2000).

Maternal transfer of TCDD to developing eggs also causes developmental toxicity. Signs of TCDD-induced developmental toxicity resemble blue sac disease, which is an edematous syndrome characterized by yolk sac and pericardial edema, subcutaneous hemorrhages, craniofacial malformations, retarded growth, and death at swim-up stage (Cooper, 1989). Effects of TCDD on fry survival are significant at egg doses ranging from 50 to 5,000 pg/TCCD/g (Boening, 1998), with salmonids (such as the lake trout) being considered extremely sensitive to TCDD developmental toxicity (Walker and Peterson, 1991; Walker et al., 1991).

Other examples of maternally transferred EDCs include TBT and phytoestrogens. In a study with Japanese medaka, TBT (1–25 μg/g for 21 days) maternally transferred into the developing eggs induced early life stage mortality and alterations on eye development (Nakayama et al., 2005). TBT has also induced embryo and swim-up stage mortality in Japanese flounder (Nirmala et al., 1999). Maternal transfer of wood-derived phytosterols (primarily β-sitosterol) has resulted in increased egg mortality and larval deformities (Lehtinen et al. 1999, Mattsson et al. 2001).

EDCs can also affect development, growth, and survival of early life stages through alterations in egg quality via disruption of vitellogenesis or indirectly through changes in other biochemical pathways or processes. There is no doubt that egg composition profoundly influences embryonic growth and survival. The types, amounts, and relative proportions of lipids and fatty acids, protein, water, vitamins, and minerals present in eggs are crucial for the normal development of fish embryos (Speake et al., 1994; Bell and Sargent, 2003; Royle et al., 2003; Tveiten et al., 2004). In oviparous fish, large lipoglycophosphoproteins or Vtgs transport essential nutrients to the developing oocyte. In addition to providing the major source of nutrition during embryonic and early life stage development, Vtgs also serve as transporters of immunoglobulins and hormones to the oocyte (Babin, 1992; Specker and Sullivan, 1994; Montorzi et al., 1995; Picchietti et al., 2001). Thus, it is expected that a decline in circulating levels of these proteins in females exposed to EDCs could result in alterations in egg composition and thus in embryo survival.

As already discussed under section 2.2, EDCs can decrease circulating levels of Vtg in females through several mechanisms, including alterations in steroid synthesis and/or metabolism; by directly binding to the ER ligand-binding domain thereby reducing transcription activity and protein expression; and/or by reducing hepatic ER mRNA levels. Results from our laboratories support this hypothesis, since exposure of female largemouth bass to OCPs (p,p′-DDE and dieldrin) results in low circulating levels of E_2 with concomitant decreases in the expression of several genes involved in reproduction (Garcia-Reyero et al., 2006). Treatment of female bass with p,p′-DDE results in decreases in mRNA expression of Cyp 19, ERα, ERβa, and AR in the gonad and ERβa and Erβb in the liver (Garcia-Reyero et al., 2006). Dieldrin decreases ERs βb and βa in both the gonad and the liver of females as well as Cyp1A in the liver (Garcia-Reyero et al., 2006). This combination of effects would result in a significant down regulation of egg quality related genes such as Vtgs and choriogenins in the liver (Larkin et al. 2002, 2003b). Exposure of females of other fish species to the OCPs toxaphene and endosulfan causes similar antiestrogenic effects (Chakravorty et al., 1992; Fahraeus-Van Ree and Payne, 1997). The displacement of E_2 from the ER and from sex steroid-binding proteins in parent females by many of the so-called xenoestrogens (EE_2, APs, and BPA) could also lead to similar antiestrogenic effects, however most studies have focused on estrogenic effects in males. Nevertheless, several full life cycle tests with EE_2, APs, and BPA

have reported decreased hatching and swim-up success in offspring produced from adult exposed females, suggesting a possible link between exposure to xenoestrogens and decreased egg quality (Shioda and Wakabayashi, 2000a; Zilloux et al., 2001; Hill and Janz, 2003; Versonnen and Janssen, 2004; Tilton et al., 2005). This was indeed what was observed in female plaice exposed to EE_2, which responded with a down regulation of Vtg and ZR proteins (Brown et al., 2004). Viviparous fish species also exhibited alterations in the distribution of essential nutrients from maternal tissues to developing embryos. For instance in the eelpout, a marked reduction in calcium and free amino acids was detected in the ovarian sac and ovarian fluid after exposures to octylphenol (Rasmussen et al., 2002). These changes were correlated to a decline on embryo growth.

Other classes of EDCs known to affect growth, development, and survival of early life stages of fish include atrazine (Beliles and Scott, 1965; Macek et al., 1976; Dionne, 1992; Grande et al., 1994; Whale et al., 1994) and the heavy metal Cd (Sanggalang and O'Halloran, 1972; Woodworth and Pascoe, 1982; Hatakeyanna and Yasuno, 1987; Michibata et al., 1987; Brown et al., 1994; Cheng et al., 2000). In addition, there is some evidence suggesting that exposure of fish to paper mill effluents can also lead to disruption of embryos and fry. For instance, exposure of fathead minnows to 20% paper mill effluent in the laboratory affected both survival from larvae to adult, and growth (Kovacs et al., 1995a, 1995b, 1996). In an investigation with perch (*Perca fluviatilis*), Karås et al. (1991) reported no effects on fecundity and egg mortality values in fish from a paper mill effluent-exposed area, but fry hatched from this site were smaller and had a greater frequency of abnormalities, which resulted in fewer fry and fewer young-of-the-year fish. Studies with largemouth bass have also shown decreased fry survival and increased deformities in offspring produced by females exposed chronically to ≥10% effluents (Sepúlveda et al., 2003).

3.1.3. Gonad differentiation, sex ratios, and secondary sex characteristics

In toxicology, the dose and length of exposure, as well as the potency of the chemical tested largely determine the observed physiological responses of exposed organisms. For EDCs, however, knowing the developmental stage at which the exposure took place is also of great relevance. This is because exposure to estrogen and androgen mimics during critical windows of enhanced sensitivity, such as the periods of sexual differentiation of early life stages or gonad maturation in adults, can result in long-term and many times irreversible sexual abnormalities. The finding of an unexpected high frequency of intersexed fish (i.e. having both testicular and ovarian gonadal tissues, also known as ova-testis) triggered research on the effects of environmental contaminants on sex differentiation of fish in English rivers receiving large loads of sewage effluents (Jobling et al., 1998; Routledge et al., 1998). Since then, several other species of fish sampled from other streams polluted with sewage effluents also exhibited high incidences of intersex gonads (Allen et al., 1999; Harshbarger et al., 2000; Vigano et al., 2001).

Potent synthetic and natural estrogenic and androgenic compounds have been isolated and identified from these effluents. Furthermore, recent studies have shown that intersexuality in free-ranging male fish can decrease milt volume and sperm motility leading to decreased fertilization rates (Jobling et al., 2002). Field observations were followed by laboratory studies evaluating the effects of different classes of estrogenic and androgenic compounds on sex differentiation and gonadal development of fish. Results from these studies have shown clear evidence that some environmental chemicals are indeed capable of disrupting the development of male and female phenotypes by interfering with the normal action of sex steroids. More importantly, threshold concentrations at which these effects occur fall well within levels currently observed in the environment. The following is a review of some of the more significant findings so far.

The majority of studies on the effects of xenoestrogens on male gonad development and differentiation have focused on EE_2. Seminal studies with zebrafish point to several aspects of male gonad development that is sensitive to EE_2 exposure. (1) The timing of exposure is critical for the observed effects, even though some of the observed effects are reversible during most stages of gonadal development and gametogenesis. (2) Feminization of male gonads can occur at very low EE_2 threshold concentrations (≤3 ng/L). (3) The observed changes can lead to significant population-level effects due to declines in fertilization rates and skewed sex ratios biased towards females in affected populations. Maack and Segner (2004) nicely illustrated the importance of timing of exposure in a study in which zebrafish were exposed to 10 ng/L EE_2 during three stages of gonadal differentiation. The first stage was the juvenile hermaphroditic stage when gonads display morphology of an immature ovary (~15–42 days postfertilization (dpf)). The second stage was the gonad transition stage when the gonad differentiates into either testes or ovary (43–71 dpf). Finally, the third stage was that of premature testicular and ovarian development (72–99 dpf). Exposure during the gonad transition period induced delay in onset of spawning and a significant reduction in fecundity and fertilization success, whereas exposure during the hermaphroditic or premature phases had no impact on reproductive parameters. A similar window of sensitivity to the effects of EE_2 was reported by Andersen et al. (2003b) who exposed zebrafish to EE_2 (15 ng/L) during critical periods of development and complete feminization was observed in groups exposed from 20 to 60 days post-hatch (dph). Hill and Janz (2003), who exposed zebrafish from 2 to 60 dph to EE_2, found that no male fish were produced at exposures of 10 ng/L EE_2, compared to a 45% male incidence in the control group. Thus, it appears that at least in zebrafish, the gonad transition stage is the most sensitive period to the persistent effects of developmental estrogen exposure. The importance of timing of exposure was also recognized in another study, where lifelong exposure of zebrafish to 5 ng/L EE_2 caused complete population failure with no fertilization due to the lack of functional testes, whereas the same level of exposure for up to 40 days in mature adults had no impact on reproductive success (Nash et al., 2004).

Zebrafish studies provide information on the issue of permanent vs. irreversible gonad damage. In the first study, zebrafish were exposed to 3 ng/L EE_2 from fertilization until all-ovary stage of gonad development (42 dpf) or from fertilization until reproductive stage (118 dpf) (Fenske et al., 2005). Early life exposure to EE_2 led to a lasting Vtg induction, but did not affect sex ratios or reproductive output. However, although the full life cycle exposure resulted in 100% feminization and total reproductive inhibition, 26% of this all-female cohort developed fully differentiated testes later on. Van den Belt et al. (2003a) provided a second example. Zebrafish embryos were exposed to EE_2 (0.1–25 ng/L) until 3 months postfertilization, and a dose-dependent increase in the number of fish with no macroscopic recognizable gonads was observed. After a period of 5 months in clean water, all fish had developed normal gonads, although a reduced number of eggs were still found in females exposed to 10 or 25 ng/L EE_2 (Van den Belt et al., 2003a). In a third example, zebrafish exposed to either 3 or 10 ng/L EE_2 during the gonad transition stage presented with ovaries in all individuals examined at 71 dpf. However, the effect was reversible since fish with testes appeared at 190 dpf (Maack and Segner, 2004). Finally, Hill and Janz (2003) found no male fish when zebrafish were exposed to EE_2 from 2 to 60 dph to 10 ng/L EE_2. The same fish when examined at 160 dph exhibited no significant departure from a 1:1 sex ratio. These results suggest that exposure of zebrafish to EE_2 during sexual differentiation and early gametogenesis arrests the developmental transition of gonads of genetic males, but after exposure ceases, males are able to accomplish testicular differentiation.

A good model for evaluating the effects of EE_2 on sex differentiation is the Japanese medaka. Similar to results obtained with zebrafish, exposure to EE_2 induces partial and complete sex reversal, as well as delayed spermatogenesis. For instance, nanoinjection of medaka eggs with 0.05 ng/egg EE_2 results in retardation of spermatogenesis in adults and complete phenotypic sex reversal of XY males to XY females occurs at a slightly higher dose (0.5 to 2.5 ng/egg) (Papoulias et al., 2000). Complete sex reversal of XY male medaka to females was also observed at waterborne exposure of 100 ng/L EE_2 (Scholz and Gutzeit, 2000). Incomplete sex reversal resulting in the formation of intersexed individuals occurred after exposure of 5 dph medaka to 10 ng/L EE_2, however these fish were still capable of reproductive behavior and could fertilize eggs (Balch et al., 2004). Thus, it appears that in at least some fish species, the presence of oocytes in testicular tissues may not directly influence reproductive capability. Male Japanese medaka exposed to ≥64 ng/L EE_2 also formed ova-testes (Seki et al, 2002). Prenatal exposure of Japanese medaka to EE_2 produced permanent changes in hepatic ER and Vtg content in the adults resulting from exposure during gametogenesis. This resulted, however, in a diminished response of males to subsequent estrogen exposure (Foran et al., 2002).

Feminization of male fish has also been reported from juvenile three-spined stickleback treated with EE_2 (0.05 µg/L) from hatching to 39–58 dph (Hahlbeck et al., 2004). In this study, EE_2 induced a total reversal of the gonad type,

identified using a male sex-linked PCR gonad marker. The authors concluded that in this species, the process of sex differentiation is most sensitive to the influence of external steroids during the first 2 weeks after hatching. Full life cycle studies with fathead minnows have so far yielded one of the LOECs for EE_2 feminization effects on males. In a study published by Lange et al. (2001), fathead minnows were exposed to EE_2 (0.2–64 ng/L) from newly fertilized embryos (<24 h) to 305 days of age. Male fish exposed to 4 ng/L EE_2 failed to develop normal secondary sex characteristics, and testicular tissue. The sex ratio in this group was 84 F:5 M (compared to 50 F:50 M in the control group) with an 11% incidence of ova-testes.

Exposure to other EDCs has also resulted in alterations in gonad differentiation of male fish. For example, exposure of zebrafish to nonylphenol (10–100 μg/L) between 1 and 60 dph resulted in adverse gonadal effects (Hill and Janz, 2003). In particular, fish showed a concentration-dependent increase in undeveloped gonads at 60 dph. Intersex has also been induced in other fish species exposed to APs, BPA (Gimeno et al., 1997; Dreze et al., 2000; Kelly and Di Giulio, 2000; Yokota et al., 2000; Metcalfe et al., 2001), and vinclozolin (Kiparissis et al., 2003). In the latter study, Japanese medaka were exposed either to the antiandrogenic vinclozolin (2,500 μg/L) or to the vinclozolin formulation Ronilan (1,000 and 5,000 μg/L) from hatch to 100 dph. Exposure resulted in a low incidence (2–7%) of gonadal intersex, with a few scattered oocytes developing within testicular lobules.

The effects of TBT on sex differentiation of teleost fish have produced contradictory results. In the Korean rockfish (*Sebastes schlegeli*), a higher ratio of female to male (5.4:1) was induced in undifferentiated fry (51 dph) treated with 100 μg/g TBT, compared to a 1.3:1 sex ratio in the control group (Lee et al., 2003). In a separate study, male Japanese medaka dosed with TBT at a rate of 1 μg/g body weight daily for 3 weeks also showed signs of feminization, with an increased incidence of ova-testes (Nakayama et al., 2004). On the other hand, and in agreement with what has been observed in marine snails, TBT caused masculinization of genetically female Japanese flounder (Shimasaki et al., 2003), and a male biased population in zebrafish exposed to TBT (0.1 ng/L) for 70 days (McAllister and Kime, 2003).

Besides altering gonad development and differentiation, EDCs can also elicit effects on the expression of secondary sex characteristics. Demasculinizing effects occur in several species of fish exposed to xenoestrogens. For instance, in a full life cycle test with fathead minnows, the most sensitive early response (which occurred at 60 dph) was an increase in the incidence of the ovipositor index (a female secondary sex characteristic) and was observed in fish exposed to ≥3.5 ng/L EE_2 (Parrot and Blunt, 2005). Skewed sex ratios (towards females) and demasculinization of males were evident at 160 dph, and occurred at extremely low EE_2 concentrations (0.3 and 0.9 ng/L). There are other examples of demasculinization as well. Male sand gobbies exposed to 6 ng/L EE_2 show a reduction in the development of urogenital papilla (Robinson et al., 2004). Male

gobbies exposed to octylphenol show a reduction in the size and color intensity of the sexually attractive orange spots (Baatrup and Junge, 2001; Toft and Baatrup, 2001). In addition, male medaka exposed to 100 ng/L BPA exhibit the formation of a female-like anal fin (Tabata et al., 2001).

Perhaps one of the strongest and better-documented cases of altered secondary sex characteristics after exposure to EDCs has to do with paper mill effluents. Changes in secondary sex characteristics of fish exposed to different types of pulp and paper mill effluents include both precocious and delayed maturation, as well as feminization and masculinization. Among these alterations, masculinization is the most consistently reported effect across field and laboratory studies. The Eastern mosquitofish (*G. holbrooki*) was the first species recorded as masculinized by exposure to pulp and paper mill effluent (Howell et al., 1980). Sampling of Eastern mosquitofish downstream from a pulp and paper mill discharge in Eleven Mile Creek, Florida, revealed masculinization of females, which possessed a male-like gonopodium (the copulatory organ used to inseminate females in this livebearing species) and which displayed male reproductive behavior. In addition, all males exhibited precocious development of secondary sex characters and reproductive behavior. Photomicrographs indicated elongation, segmentation, and intermittent terminal differentiation of female anal fins similar to the male gonopodium. After significant process changes at the Eleven Mile Creek mill, however, quantification of the response (anal fin length) and statistical comparison to females from a reference stream showed masculinization remained (Cody and Bortone, 1997). Similar alterations of secondary sex characteristics occurred in Eastern mosquitofish sampled from another effluent-receiving stream in Florida, the Fenholloway River. Recently, exposure of fish to water in Rice Creek, the receiving stream for the effluents discharged by a paper mill plant in central Florida resulted in masculinization of female mosquitofish and two additional species (least killifish, *Heterandria formosa* and sailfin molly, *P. latipinna*) (Bortone and Cody, 1999). As further evidence, Bortone and Cody (1999) detected a statistically significant increase in the ratio of anal fin length to fish standard length, and inferred a distance/dose-dependent response downstream of pulp mill effluent.

In contrast, controlled exposures of mosquitofish to effluent dilutions have provided mixed evidence for masculinization. No masculinization was detected in Western mosquitofish (*G. affinis*) exposed to paper mill effluents. However, female mosquitofish exposed to high concentrations of phytosterols (~0.1–0.5 g/L of stigmastanol and β-sitosterol) combined with the bacterium *Mycobacterium smegmatis* induced male-like gonopodial development within 2 weeks (Denton et al., 1985; Howell and Denton, 1989). The male-like gonopodial structures, however, did not elongate to the length of normal male gonopodia, but developed terminal differentiations. Based on these results, the authors hypothesized that female mosquitofish were being masculinized when exposed to androgens formed by bacterial degradation of phytosterols present in pulp and paper mill effluents. This was confirmed by controlled exposure of adult

female mosquitofish to 15–100% effluent (McCarthy et al., 2004). In this study, effluents from four out of seven pulp mills tested and from one sewage treatment facility induced masculinization. Among the four pulp mill effluents, two induced masculinization relatively quickly (within 3 weeks) while the other two required 24 weeks of exposure.

Studies with fathead minnows have reported both precocious and delayed maturation of secondary sex characteristics after exposure to paper mill effluents (Kovacs et al., 1995a; Parrott and Wood 2002; Parrott et al., 2003, 2004). The latter authors also reported masculinization (≥10% effluent) and feminization (≥32% effluent) in fathead minnows exposed to a bleached sulfite effluent. In these studies, exposure of fertilized fathead minnow eggs to ≥30% bleached sulfite mill effluent through 140 dph resulted in masculinization of females (presence of nuptial tubercles and dorsal fin dots) and in feminization of males (evidenced as an increase of males with ovipositors) (Parrott et al., 2003, 2004). Masculinization of fathead minnows has also been reported after exposure to other bleached kraft mill effluents (Kovacs et al. 1995a) but not to effluents released from a thermo-mechanical pulp mill (Kovacs et al., 1995b).

An important conclusion from all these studies was that, at least for female mosquitofish, potential for reproduction did not appear impaired by exposure to pulp and paper mill effluent, since normal ovaries lacking testicular tissue were consistently reported in masculinized females (Howell et al,. 1980; McCarthy et al., 2004). Fathead minnows, on the other hand, might respond differently to this type of exposure, since males showing feminized external characteristics also lacked differentiated testes (Parrott et al., 2003, 2004).

3.2. High Mechanistic Relevance

Biomarkers that provide information on biochemical mechanisms affected by EDCs include levels of plasma hormones and Vtg, changes in tissue structure (histology) and now more recently, changes in gene and protein expression, as measured by genomics (microarrays) and proteomics, respectively. These biomarkers were discussed in detail in the sections relating to modes of action of EDCs.

3.2.1. Plasma hormone levels

While there is tremendous seasonal variability in the exact levels of sex steroid hormones in plasma, a useful parameter is the ratio of sex hormones (E/T or E/11-KT). During prime reproductive season, female fish normally have high E/T ratios (>1), whereas males have low ratios (<1), making it easy to distinguish them from each other. However, when contaminants affect either steroidogenesis or sex hormone metabolism, the ratios overlap and become indistinguishable (Figure 5). This parameter is an important measurement when trying to determine the mechanisms of action of contaminants, especially through nongenomic pathways. For example, in Florida, adult largemouth bass

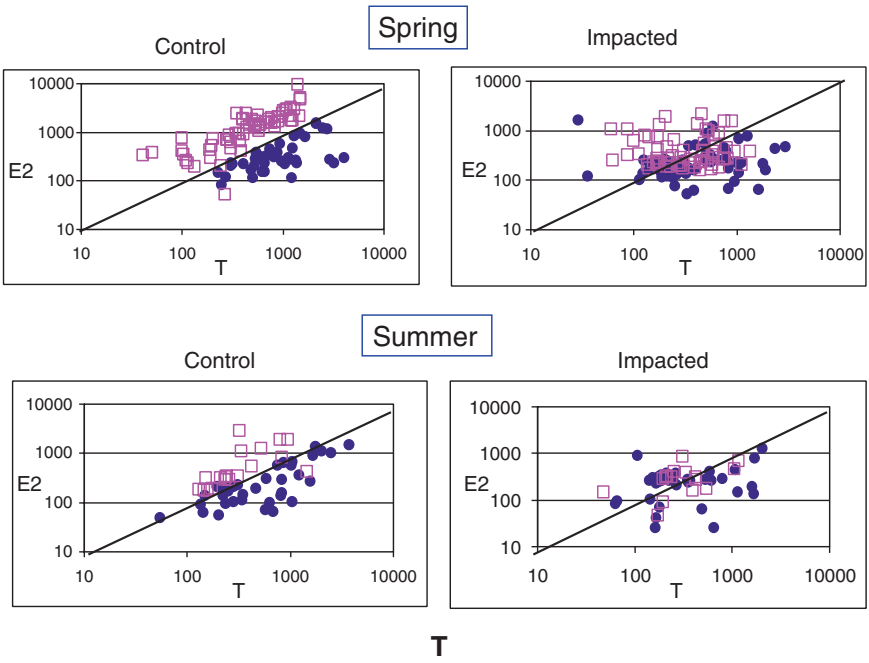

Figure 5. Measurement of circulating hormone levels in largemouth bass during the spring at the height of the reproductive season and in the summer when hormone levels are at their lowest. The graphs plot the concentrations of E_2 and T for each fish for samples collected at a control site and a site that is highly contaminated with p,p′-DDE, dieldrin, chlordanes, and toxaphene. (*See Color Plates*).

stocked in reclaimed and reflooded agricultural lands (or "muck farms") contaminated with several types of OCPs have decreased sex steroid concentrations (E_2 and 11-KT) compared with bass sampled from reference sites (Johnson, 1999). Seasonal studies have also shown little to no cyclicity in bass sampled from these muck farms, suggesting an overall depression on steroidogenesis and a potential detrimental effect on spawning success. This decline in plasma steroid hormones was mimicked in laboratory studies using largemouth bass exposed to both p,p′-DDE and dieldrin in their diet following a 50-day exposure period, as compared to bass fed a control diet (Muller et al., 2004; Garcia-Reyero et al., 2006). Similar declines in plasma steroid concentrations have been reported in male summer flounder (*P. dentatus*) dosed with o,p′-DDT (Mills et al., 2001).

Definitive cause(s) and mechanism(s) for the effect of OCPs on circulating sex hormone levels are still under investigation. A recent study suggests that contaminant-induced changes in enzymes involved in steroidogenesis (StAR and CYP19) as well as changes in enzymes involved in hormone metabolism (CYP-3a and CYP-1A) may be responsible (Garcia-Reyero et al., 2006).

Many of the steps leading to oocyte growth and maturation depend on sex steroid hormones, as discussed above. Thus, decreases in circulating levels of hormones would affect these processes directly. As expected, bass in reflooded muck farms that are high in OCPs show a decline in plasma steroid hormones and show poor recruitment as assessed by the number of young of the year (Benton et al., 1991; Benton and Douglas, 1994, 1996). This suggests that the decline is plasma steroid hormones may be a good bioindicator of effects at the population level.

Exposure to paper mill effluents also cause declines in circulating sex steroids in fish (Adams et al., 1992; Hodson et al., 1992; Gagnon et al., 1994; McMaster et al., 1995, 1996; Munkittrick et al., 1998). Concomitant with these decreases, are decreases in gonadal sizes, changes in secondary sexual characteristics, and decreases in egg clutches and sizes (McMaster et al., 1996). The specific effects vary greatly among species and among paper mill effluents. The differences are largely attributed to the complexity of different paper mill effluents, which depend on the type of wood furnish used and the processes for extracting paper fibers, as described above, and on the sensitivities of different fish species. Nevertheless, dramatic changes in ratios of circulating sex hormones in females and males are early warning signals of exposure to EDCs.

3.2.2. Plasma vitellogenin and zona radiata proteins

Several studies document the value of measuring plasma Vtg and ZR proteins in male fish as biomarkers of exposure for EDCs. It is now clear that there is a proportional dose–response effect between exposure to estrogenic compounds and elevated levels of these proteins in the blood of male fish, which normally have only trace amounts of the proteins (Rose et al., 2002; Nozaka et al., 2004; Panter et al., 2002, 2004; Nilsen et al., 2004;). More recently, some researchers have also examined females and have seen depression or total suppression of vitellogenesis by exposure to EDCs. The importance of Vtg to egg quality and the future success of progeny should not be underestimated. Vtg is the main nourishment for developing embryos and fry. Without it, they will not survive.

3.2.3. Histopathology

Xenoestrogens induce several gonadal pathologies in fish. Adult fathead minnows exposed to 1–3 ng/L EE_2 exhibit reduced size of parenchymatic areas in ovaries and ultrastructural changes in testes (Pawlowski et al., 2004). Similarly, fathead minnow males sampled from a lake experimentally treated with EE_2 (range of EE_2 water concentrations of 4–8 ng/L) had widespread testicular fibrosis and an overall inhibition of testicular development (Palace et al., 2002). Interestingly, these fish also contained a 9,000-fold higher concentration of the egg yolk precursor Vtg, compared to males sampled prior to the EE_2 treatment. Similar effects were observed in pearl dace (*Margariscus margarita*) another species collected from the same lake. In this case, edema was observed in the ovaries and males exhibited inhibited development of testicular tissue and

kidney lesions. In addition, fish exhibited intersex (Palace et al., 2006). In this case, Vtg levels in both males and females increased by 4,000-fold compared to fish collected prior to the addition of EE_2 to the lake (Palace et al., 2006). Male medaka (Metcalfe et al., 2001) and male sheepshead minnow exposed to EE_2 (Zillioux et al., 2001) exhibited testicular fibrosis with a simultaneous decrease in the density of mature spermatozoa. Androgen antagonists (vinclozolin) are also capable of inhibiting spermatogenesis, indicating that this effect on testicular development can occur because of exposure to both estrogen agonists and androgen antagonists (Kiparissis et al., 2003).

Sertoli cell hypertrophy with a concomitant decline of germ cells, was observed in the Japanese eel (*Anguilla japonica*) exposed to both 11-KT and nonylphenol (Miura et al., 2005). Viviparous fish also exhibit blockage of spermatogonial mitosis. For instance, adult male guppies exposed to octylphenol, BPA, and p′p-DDE responded with an almost complete cessation of testicular spermatogenesis, which included reduced number of spermatogenic cysts and an increased number of spermatozonia in ducts (Kinnberg and Toft, 2003; Kinnberg et al., 2003). In the eelpout, another viviparous fish, octylphenol (50–100 μg/g) affected testicular and Sertoli cell structure and induced degeneration of lobular structures, with the majority of treated fish showing trapped sperms in parts of the seminiferous lobules and sperm ducts (Rasmussen and Korsgaard, 2004; Rasmussen et al., 2005). Interestingly treatment of eelpout with a potent antiestrogen (ZM 189,154) abolished histological effects on testicular structure, providing evidence that at least some of these testicular abnormalities are mediated through the ER.

Histological analyses of testes from Cd chronic exposures in fish exhibit degeneration of mature spermatocytes, absence of spermatids and spermatozoa, and severe necrosis of Leydig cells (Das, 1988). Other reported testicular abnormalities in fish exposed to this heavy metal include presence of macrophage aggregates, cysts, and interstitial/epithelial tissue proliferation (Bielek and Viehberger, 1983). Male fish exposed to a mixture of octylphenol and Cd had testis with few to no mature spermatozoa (Karels et al., 2003). In some studies, exposure of fish to APs has resulted in decreased testicular growth and degeneration of seminiferous tubules (Jobling et al., 1996; Christiansen et al., 1998).

Exposure of female fish to EE_2 can also lead to altered ovarian histology. For instance, female adult zebrafish exposed to EE_2 (10–25 ng/L) for 24 days responded with altered ovarian histology and morphology after only 3 days of exposure (Van den Belt et al., 2001). Furthermore, there was a negative correlation between Vtg induction and GSI, demonstrating that excessive Vtg induction in female fish exposed to xenoestrogens can result in decreased reproductive output. Similarly, post-parturition ovaries from adult female guppies exposed to octylphenol, BPA, and p′p-DDE, showed an altered morphology suggesting an inhibition of yolk deposition (Kinnberg et al., 2003). Another important pathology commonly observed in female fish exposed to EDCs is ovarian atresia. Atresia is a normal process at the post-ovulatory stage of ovarian development,

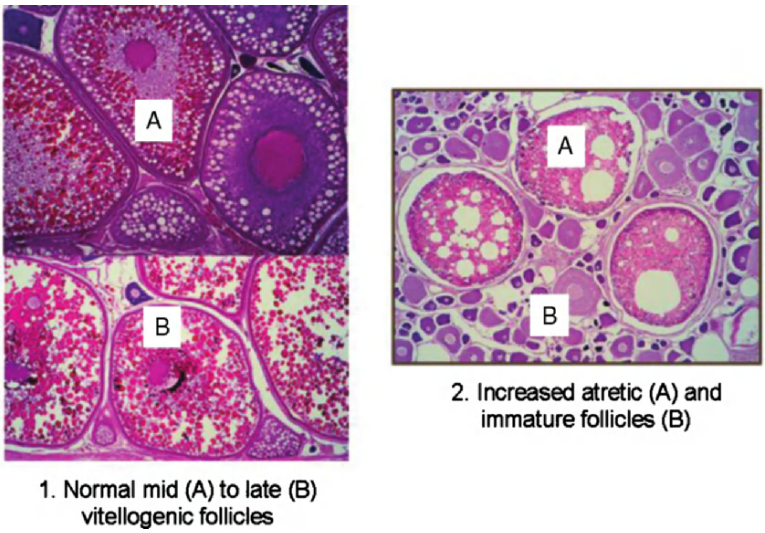

Figure 6. Histological changes observed in ovaries of female largemouth bass exposed chronically (56 days) to paper mill effluents (>40%). Note the presence of atretic follicles and overall depression of follicular development in ovaries from exposed females (2) compared to controls (1). (*See Color Plates*).

but not for ovaries in previtellogenic or early vitellogenic stages (Guraya, 1973). Female medaka exposed to high doses of vinclozolin (2,500 µg/L) exhibit a higher incidence of ovarian atresia (Kiparissis et al., 2003). Our studies with paper mill effects on ovarian morphology of female largemouth bass have also detected higher rates of atresia and an overall depression of follicular development (Figure 6).

Exposure of fish during development results in morphological and ultrastructural gonadal alterations. In zebrafish exposed to the xenoestrogens nonylphenol (≥100 µg/L) and EE_2 (≥1 ng/L) between 2 and 60 dph, caused a concentration-dependent suppression of gametogenesis, with gonadal pathologies (ovarian atresia) being detected over 8 months later (Weber et al., 2003).

3.2.4. Gene expression profiles and molecular tools to assess contaminant effects

A change in the normal physiology of an animal due to exposure to contaminants is ultimately a result of changes at the molecular and cellular levels. These molecular changes can be detected using gene arrays (also called microarrays), a research tool where thousands of genes specific to an organism are spotted onto a solid support matrix and queried with mRNA samples from controls and exposed animals (Figure 7). The amount of signal observed on the array is proportional to the amount of mRNA present in the tissues. If the exposure has up regulated the expression of certain genes, then they will

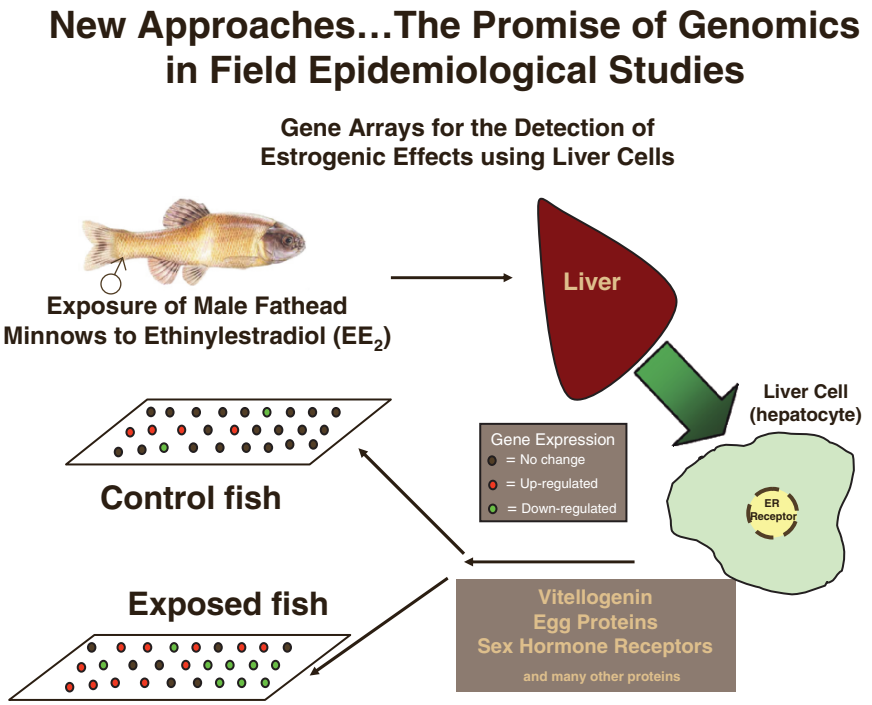

Figure 7. Gene arrays made on glass slides with probes for fish genes (either cDNAs or oligonucleotides) determine the relative expression levels of multiple genes following an exposure to a contaminant. In the example shown, the signal comes from total RNA extracted from liver of control fish and fish exposed to EE_2, copied into cDNA, labeled with fluorescent dyes, and then hybridized to the arrays. The level of expression for each gene is proportional to the intensity of the fluorescent dye. Fish treated with EE_2 exhibit up regulation of Vtg and other estrogen-regulated genes. (*See Color Plates*).

be overrepresented in the pool of mRNAs compared to their levels in control tissues. Microarrays have been shown to work exceptionally well for receptor-mediated effects of EDCs.

Experiments by our research group, and others using gene array technology have shown that classes of chemicals, and perhaps even individual chemicals, are likely to display their own "chemical signatures" (Hamadeh et al., 2002; Larkin et al., 2002, 2003a; Waters and Fostel, 2004), in terms of the specific genes that are activated upon exposure. With this technology, it may be possible to detect and differentiate exposure among individual contaminants and their mixtures at impacted sites.

Several fish genomes have been sequenced including zebrafish, two species of pufferfish (*Takifugu rubripes* and *Tetraodon nigroviridis*) and there are several large databases of expressed sequences for medaka (Schartl et al., 2004), carp (Gracey et al., 2004), and salmon (Rise et al., 2004; Renn et al., 2004). Microarrays serve for experiments designed to test the response of whole

animals to a toxicant using a "systems biology" approach. However, it is imperative to document changes at the tissue level caused by the toxicants. This is referred to as "phenotypic anchoring" (Tennant, 2002) and includes integrating changes in gene expression with traditional endpoints, for example changes in gonadal structure, known to be evidence of adversely affected reproduction and health. Work by Moggs et al. (2004) with rodents treated with EE_2 is a beautiful example of how these technologies serve to determine temporal changes in gene expression that account for changes in tissue structure and function.

Molecular- and cellular-based assays are more sensitive than ecological assays and may be able to predict adverse reproductive effects in fish at much lower chemical concentrations and much sooner than traditional assays. If it were possible to determine the risk of exposure earlier in the process, it might be easier to maintain a healthy environment. However, molecular markers cannot exist alone; they are only useful if one can make the link to adverse effects on the individual and/or population. Thus, much work is still required to validate the molecular approaches and to link them to adverse changes in whole organisms and in populations.

3.3. Ecological Risk Assessment (ERA)

There is now substantial evidence describing the effects of EDCs on reproduction in a number of different fish species. Many endpoints have also been assessed, ranging from physiological responses (e.g. Vtg induction), through histological changes (e.g. induction of intersex) to population-level effects (e.g. reproductive success). Several of these endpoints have formally entered into the ERA framework, developed by the US EPA (1992) (Figure 8). The first step in the ERA process is that of problem formulation. Here is where the assessment endpoints, measures of effects, timing, and a definition of what should be protected are clearly stated. The next step is an analysis plan, which focuses on ecosystem and receptor characterization, and exposure and effects assessment. The final step is risk characterization, which seeks to establish and quantify the risk or probability of effects (or response) based on exposure.

Toxicological effects research has historically focused on responses at lower levels of biological organization (molecules to organisms) while one of the primary interests of ecotoxicologists is to predict effects and provide guidance for effects at the population level. Indeed, consensus is growing that for estimating ecological hazards of environmental contaminants population-level effects are preferable, compared to individual-level adverse responses (Tanaka and Nakanishi, 2001). One of the most comprehensive measures of population-level effects is the intrinsic rate of natural increase (r). Individual survivorship and fecundity data obtained from standard life cycle tests serve to calculate r-values using an age-classified matrix model. An r-value below an acceptable minimum would indicate that a population will not proliferate and will likely go extinct

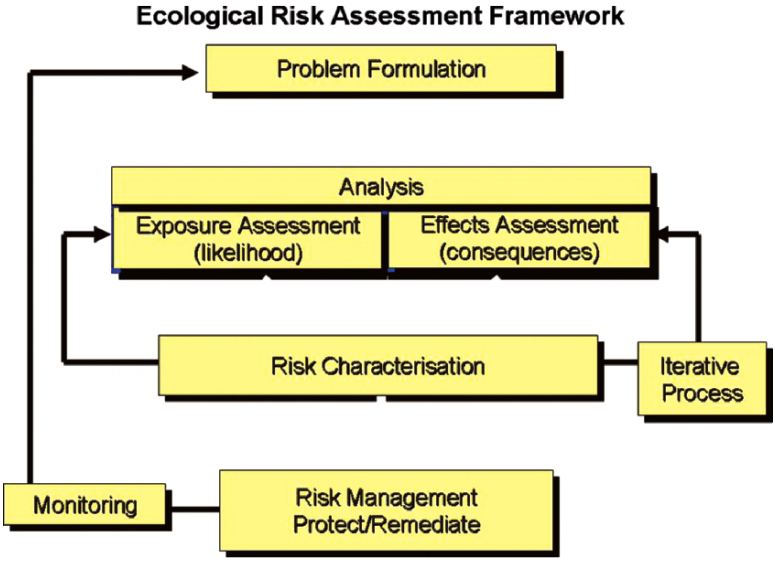

Figure 8. Diagram summarizing the most important steps in ecological risk assesment (ERA). (*See Color Plates*).

(Foley, 1994). Exposure–response models for use in ERA derive from population growth rate estimates paired with contaminant concentration data (i.e. exposure).

Other important issues that relate to ERA include measurements of uptake of chemicals by fish; length, duration and timing of exposure, as well as pharmacokinetic considerations. In determining the environmental thresholds for EDCs, the measured effects of prolonged exposure are most relevant from studies that monitor tissue concentrations (or maximum acceptable toxicant concentration (MACT)). As an example of an ERA, we will illustrate the work done with EE_2. To determine risk, one must first determine concentrations for no adverse effects (or no observable adverse effect level (NOAELs)). These concentrations vary, depending on the fish model used, since sensitivities to endocrine disruption are different among different fish species. For instance, NOAELs for fecundity or fertilization success have been reported from a number of partial or full life cycle tests with fathead minnow (1 and 10 ng/L) (Lange et al., 2001), and zebrafish (0.3–5 ng/L) (Van den Belt et al., 2001). To calculate population-level effects life table responses (i.e. survival of different age classes and fecundity) are required. For fathead minnows, EE_2 concentrations estimated to reduce r to zero (ErC100) were estimated to be ~3 ng/L (Grist et al. 2003). As already discussed, this value is below the NOAELs reported for this chemical in this species.

Europe, Japan, and the USA have contributed to establishing test guidelines for EDCs for the Organization for Economic Cooperation and Development

(OECD). The strategy developed to characterize EDCs integrates both laboratory and field exposures (Hutchinson and Pickford, 2002). Determination of the potential for endocrine disruption for over 87,000 compounds requires a multitiered testing approach. The strategy for tier 1 utilizes Vtg induction in a 14-day exposure fish-screening paradigm.

Higher tier tests include both partial and full life cycle tests to determine various different reproductive endpoints. The majority of experiments will utilize small fish such as fathead minnow, medaka, and zebrafish and will occur in the laboratory. However, it will also be important to assess the effects of compounds on the reproductive cycle of wild fish, especially looking at effects during the spawning period and during larval–embryonic development. In the latter case, the main endpoints will be tissue differentiation, growth, whole body Vtg and development of intersex, or ova-testis. In all of these levels, Vtg is a key endpoint because of the strong connection to its appearance in the plasma with ER-dependent mechanisms of action.

4. CONCLUSIONS

There is no longer any doubt that EDCs found in the environment can affect fish reproduction. Laboratory-based exposures validate observations in the field. The effects of endocrine disruptors occur from the molecular level up through organismal effects and to population effects. While there is still much research required in this area, new technologies relying on global changes in gene and protein expression coupled to changes in physiology will add much information regarding the specific steps affected by these contaminants and will provide insight into fundamental biochemical mechanisms involved in reproduction. It is now clear that EDCs target both receptor-dependent and receptor-independent processes. The full application of gene expression studies to the ecological arena will require cooperation among researchers and support from national and international governmental funding bodies.

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CHAPTER 11

REGULATORY MECHANISMS OF OOCYTE MATURATION AND OVULATION

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1. INTRODUCTION TO OOCYTE MATURATION AND MPF

1.1. Oocyte Maturation and Meiotic Cell Cycle

1.1.1. Oocyte growth and maturation in meiotic cell cycle

Oocytes are produced in the ovaries by the entry of mitotically proliferating oogonia into meiosis. In vertebrates, including fishes, oocytes stop their meiotic cell cycle at prophase I, during which they grow by the accumulation of substances, such as yolk and mRNAs, necessary for early embryonic development (see Chapters 1–3). These prophase I-arrested oocytes are still immature and unable to be fertilized even when they reach their fully grown stage. Hormonal stimulation allows the oocytes to resume meiosis and proceed to metaphase II, where meiosis stops again. These metaphase II-arrested oocytes are now mature (called ova or eggs) and can undergo embryonic development when fertilized. The process from prophase I arrest to metaphase II arrest is termed oocyte maturation in the field of biological sciences or final oocyte maturation in the field of fisheries sciences (Figure 1).

During the course of maturation, oocytes undergo drastic morphological changes in accordance with the progression of meiosis. These include chromosome condensation, germinal vesicle breakdown (GVBD), and first polar body expulsion that intervenes between spindle formations at metaphases I and II. The germinal vesicle (GV), the oocyte nucleus, is generally located at the center of the oocyte. In response to hormonal stimulation, it migrates to the animal

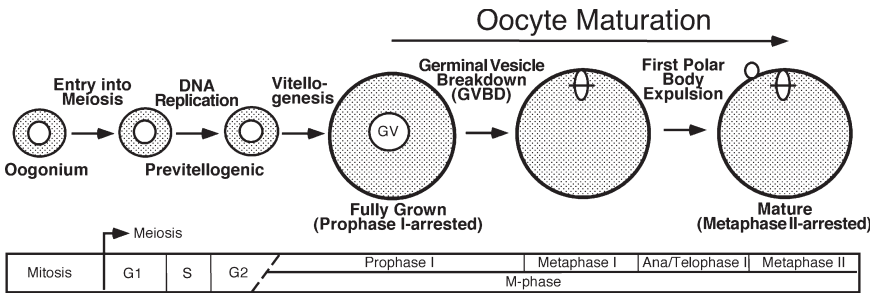

Figure 1. Oocyte growth, oocyte maturation, and meiosis. Oocytes produced by the entry of mitotically proliferating oogonia into meiosis stop their meiotic cell cycle at prophase I, during which they grow by the accumulation of yolk (vitellogenesis). The prophase I-arrested oocytes are immature. Upon hormonal stimulation, the immature oocytes resume meiosis and proceed to metaphase II, at which stage they mature. *Abbreviations*: GV – germinal vesicle; GVBD – germinal vesicle breakdown.

pole, where GVBD takes place. As maturation proceeds, therefore, the GV becomes visible under a dissecting microscope and then disappears. GVBD is usually regarded as a hallmark of the progress of oocyte maturation.

1.1.2. GTH-MIH-MPF

Oocyte maturation is induced by sequential actions of three major substances: gonadotropic hormone (GTH) secreted from the pituitary gland, maturation-inducing hormone (MIH) synthesized in and secreted from follicle cells surrounding the oocytes, and maturation-promoting factor (MPF) produced and activated in the oocyte cytoplasm (Yamashita, 2000). As in the case of mammals, in which two GTHs (LH and FSH) exist, GTH comprises two types in fishes (GTH I and GTH II). GTH I is responsible for oocyte growth, and GTH II is responsible for oocyte maturation, stimulating follicle cells to synthesize and secrete MIH.

MIH is a steroid that interacts with a membrane-bound receptor on the oocyte surface. Since the first identification of 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) as an MIH in the amago salmon *Oncorhynchus rhodurus* (Nagahama and Adachi, 1985), this steroid has been reported to be a natural MIH in other fishes: Indian catfish (Haider and Rao, 1992), killifish (Petrino et al., 1993), medaka (Fukada et al., 1994), yellowtail (Rahman et al., 2002), and chub mackerel (Matsuyama et al., 2005). A derivative of 17, 20βP (17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S)) is also known to be a natural MIH in several fishes: Atlantic croaker (Trant et al., 1986; Trant and Thomas, 1989; Patiño and Thomas., 1990), spotted seatrout (Thomas and Trant, 1989), striped bass (King et al., 1994), and puffer fish (Matsuyama et al., 2001). Both 17,20βP and 20β-S act as MIHs in kyusen wrasse (Matsuyama et al., 2002) and red seabream (Ohta et al., 2002).

The MIH signal received on the oocyte surface is transduced to the cytoplasm, finally resulting in the formation and activation of MPF. MPF was first purified from mature oocytes of the African clawed frog *Xenopus laevis* as a 200 kDa complex containing 32 and 45 kDa proteins (Lohka et al., 1988). The 32 kDa protein is a *Xenopus* homolog of Cdc2, the serine/threonine protein kinase encoded by the fission yeast *Schizosaccharomyces pombe cdc2*$^{+}$ gene, and the 45 kDa protein is a *Xenopus* counterpart of Cyclin B, which was first discovered in early embryos of marine invertebrates. Later, MPF was also purified from mature oocytes of starfish (Labbé et al., 1989) and the carp *Cyprinus carpio* (Yamashita et al., 1992a), and it has been confirmed that MPF exhibits a universal molecular structure as a complex of Cdc2 and Cyclin B in any species (Cdc2 is also designated as Cyclin-dependent kinase1, Cdk1).

1.2. Characteristics and Molecular Entity of MPF

1.2.1. Generality of MPF in eukaryotes

Direct injection of MIH into the oocyte cytoplasm cannot induce oocyte maturation, while injection of the cytoplasm from maturing oocytes stimulated by MIH induces maturation of the recipient oocyte. This is because MPF is formed and activated inside the oocyte cytoplasm after the MIH signal is received on the oocyte surface. In fishes, as in amphibians, protein synthesis in the oocyte is required for GTH- or MIH-stimulated maturation but not for MPF-stimulated maturation (Yamashita et al., 1992a). Furthermore, maturation is initiated much faster in MPF-stimulated oocytes than in GTH- or MIH-stimulated oocytes (Kondo et al., 1997). It is therefore clear that MPF is the substance acting most downstream of oocyte maturation.

In contrast to GTH and MIH, the action of MPF is universal among species. For example, MPF obtained from pachytene microsporocytes of a higher plant (lily, *Lilium longiflorum*) can induce GVBD and chromosome condensation when injected into *Xenopus* oocytes (Yamaguchi et al., 1991). Moreover, *Xenopus* oocyte maturation can be induced by injection of MPF extracted from mammalian culture cells and yeasts at mitotic metaphase (Kishimoto et al., 1982; Tachibana et al., 1987). Conversely, MPF derived from eggs acts on nuclei of somatic cells, causing chromosome condensation (Miake-Lye and Kirschner, 1985). Thus, MPF is present in all eukaryotic cells and functions as the dominant factor to promote M-phase of the cell cycle, irrespective of meiosis and mitosis. At present, therefore, MPF is known as M-phase-promoting factor rather than maturation-promoting factor.

1.2.2. Molecular characterization of MPF

Since a large number of carp eggs can be obtained easily, fish MPF was first purified from carp (Yamashita et al., 1992a). The purified carp MPF exhibits about 100 kDa in native gel-filtration chromatography. Immunoblotting analyses with antibodies raised against recombinant goldfish Cdc2 and Cyclin B have

demonstrated that the purified MPF consists of 34 kDa Cdc2 and 46 or 48 kDa Cyclin B. Although the purified MPF contained Cdk2, its behavior did not correspond to MPF activity. It has therefore been concluded that Cdk2 is not a component of MPF.

The finding that Ca^{2+} - and cyclic AMP (cAMP)-independent histone H1 kinase activity increases in accordance with oocyte maturation was first reported in starfish (Picard et al., 1985; Sano, 1985). This activity was analyzed along with MPF purification in starfish (Labbé et al., 1989) and carp (Yamashita et al., 1992b). In both cases, the histone H1 kinase behaved similarly to MPF throughout purification, demonstrating that the H1 kinase and MPF are the same entity.

Following the purification of MPF from carp eggs, fish MPF has been partially purified from the catfish *Clarias batrachus* (Balamurugan and Haider, 1998) and the perch *Anabas testudineus* (Basu et al., 2004), and the results obtained confirm that MPF consists of Cdc2 and Cyclin B. In addition to the cDNA clone encoding goldfish Cyclin B (Hirai et al., 1992a), Cyclin B cDNAs have been isolated from other fishes, including the zebrafish *Danio rerio* (Kondo et al., 1997), the medaka *Oryzias latipes* (Mita et al., 2000), the bullseye puffer *Sphoeroides annulatus* (Gutierrez et al., 2003), and the Japanese eel *Anguilla japonica* (Kajiura-Kobayashi et al., 2004). As in other animal species (Kotani et al., 2001), several subtypes of Cyclin B molecules are present in medaka (Cyclins B1 and B2) and eel (Cyclins B1, B2 and B3), and the expression patterns of the subtypes are different (Mita et al., 2000; Kajiura-Kobayashi et al., 2004). However, the functional differences of subtypes in fish oocyte maturation remain to be elucidated.

1.3. MPF Actions

Although the molecular entity of MPF has become clear, its action mechanisms still remain elusive despite extensive studies (Ubersax et al., 2003; Tyers, 2004). Indeed, only a few proteins have been identified as *in vivo* substrates of MPF to date, in spite of the fact that biochemical identification of the substrates is prerequisite for understanding the action mechanisms of MPF. Here, we refer to several candidate substrate proteins for MPF during fish oocyte maturation.

1.3.1. Lamin B3

The nuclear membrane of the GV is underlain by the nuclear lamina that is constructed from a class of intermediate filament proteins called lamins. The disassembly of GV lamina, which might be triggered by phosphorylation of lamin proteins, is indispensable for the occurrence of GVBD. For the first step toward understanding the action of MPF on GVBD during fish oocyte maturation, Yamaguchi et al. (2001) have collected the nuclear lamina from goldfish GVs that were isolated by a newly established bulk-isolation method and biochemically characterized the isolated lamin proteins. The GV lamina of goldfish oocytes mainly consists of lamin B3, as in *Xenopus*. According to the partial amino acid

sequences of GV lamins, a cDNA clone encoding goldfish lamin B3 has been isolated and the entire amino acid sequence of goldfish lamin B3 has been deduced. Cdc2, the catalytic subunit of MPF, is known to phosphorylate lamins in mitotically dividing cells and induce the disassembly of nuclear envelope at M-phase. Although it is not clear whether the phosphorylation of GV lamins by Cdc2 is responsible for GVBD during oocyte maturation, goldfish lamin B3 retains the amino acid sequence phosphorylated by Cdc2, suggesting that lamin B3 is a candidate substrate for Cdc2 responsible for GVBD (Yamaguchi et al., 2001).

1.3.2. Microtubules

Dramatic changes in the organization of cytoskeletal components are required to promote the series of morphological events during oocyte maturation, such as GV migration, GVBD, and spindle formation (Kotani and Yamashita, 2005). Two protein kinases, MPF and mitogen-activated protein kinase (MAPK), play a central role in these morphological changes during oocyte maturation in amphibians (Kotani and Yamashita, 2002). Although the involvement of these kinases in cytoskeletal organization of fish oocytes remains to be investigated, changes in the organization of microtubules in goldfish oocytes during maturation determined by using a confocal laser scanning microscope have been described in detail with discussion of the molecular basis of GV migration from the center of the oocyte to the animal pole during maturation (Jiang et al., 1996).

1.3.3. Eukaryotic polypeptide chain elongation factor 1γ

Eukaryotic polypeptide chain elongation factor 1γ (EF-1γ), which is responsible for elongation of the polypeptide chain during protein synthesis on the ribosome, has been reported to be a major substrate for MPF (Belle et al., 1995). The possibility that EF-1γ is a substrate of MPF during goldfish oocyte maturation has been investigated (Tokumoto et al., 2002). The amino acid sequence of goldfish EF-1γ deduced from its cDNA showed that, unlike the *Xenopus* counterpart, the Cdc2-phosphorylation site is not conserved in this protein. However, phosphorylation analyses have demonstrated that goldfish EF-1γ is phosphorylated by MPF. This finding suggests that EF-1γ is a substrate for MPF and that its phosphorylation plays a role in protein synthesis during oocyte maturation in goldfish.

2. FORMATION AND ACTIVATION OF MPF DURING OOCYTE MATURATION

2.1. Signal Transduction Pathway From MIH to MPF

2.1.1. MIH receptor

Although the presence of the MIH receptor on the oocyte surface had long been suggested, its biochemical entity remained to be characterized. A breakthrough in this standstill has been made recently; a membrane progestin receptor has

been identified and characterized in fish oocytes (Zhu et al., 2003a, b). This receptor mediates nongenomic actions of steroids and has characteristics of G-protein-coupled receptor (for details, see Chapter 8).

Like an endogenous MIH, diethylstilbestrol (DES) known as an endocrine disrupting substance, has been reported to induce fish oocyte maturation (Tokumoto et al., 2004). An anti-MIH receptor antibody blocks DES-induced oocyte maturation as well as MIH-induced maturation, implying the interaction of DES with the MIH receptor. Tokumoto et al. (2004) have also identified the molecular structure that is essential for the receptor to recognize MIH, providing a new insight into molecular mechanisms of MIH reception on oocytes.

In contrast to fish oocytes, it has been reported that the classical nuclear progesterone receptor is involved in *Xenopus* oocyte maturation (Bayaa et al., 2000; Tian et al., 2000; Bagowski et al., 2001; Lutz et al., 2001; Liu et al., 2005). However, the involvement of the classical MIH receptor during oocyte maturation is still controversial in this species (Maller, 2001; Hammes, 2004).

2.1.2. G-protein and cAMP

It is not clear how the signal received by the membrane-bound MIH receptor on the oocyte surface is transmitted to the cytoplasm, where MPF is formed and activated during fish oocyte maturation. It is likely that pertussis toxin-sensitive inhibitory G-protein (G_i) is involved in the signal transduction pathway in fish oocyte maturation (Yoshikuni and Nagahama, 1994; Oba et al., 1997). In contrast, it has been suggested that stimulatory G-protein (G_s) is involved in *Xenopus* oocyte maturation, which is repressed by progesterone stimulation (Luts et al., 2000; Sheng et al., 2001; Romo et al., 2002; Wang and Liu, 2004).

Levels of intracellular cAMP in fish oocytes decrease upon MIH stimulation (Jalabert and Finet, 1986; Finet et al., 1988; Haider and Chaube, 1995; Cerdà et al., 1998). Drugs that elevate cAMP levels of oocytes, such as adenylate cyclase activators and phosphodiesterase inhibitors, prevent 17,20βP-induced oocyte maturation, suggesting a negative role of cAMP in induction of fish oocyte maturation (DeManno and Goetz, 1987a, b; Haider and Chaube, 1996; Haider, 2003). It is likely that a decrease in the activity of protein kinase A caused by the decrease in intracellular cAMP level plays an important role in induction of oocyte maturation in fish. It is still not clear what molecules follow protein kinase A and lead to oocyte maturation (Patiño et al., 2003).

2.1.3. Proteasome

Proteasome is a large nonlysosomal protease and plays a key role in protein destruction that controls various cell functions, including cell cycle progression. It has two forms, 20S and 26S proteasomes; the former is latent and the latter is active (Tokumoto et al., 1995a, b). The 26S proteasome acts on proteins that have been specifically marked for destruction by the covalent attachment of ubiquitin.

Diisopropyl fluorophosphate (DFP), a serine protease inhibitor, blocks the activity of 26S proteasome in goldfish oocyte maturation and stops the oocytes at two distinct stages. The first DFP-sensitive stage is prior to migration of the GV to the animal pole, when MPF is inactive, and the second DFP-sensitive stage is just before GVBD, when MPF is abruptly activated. These results suggest that 26S proteasome functions at several steps of the signal transduction pathway from MIH to MPF in goldfish oocyte maturation (Tokumoto et al., 1997a, 2000; Tokumoto, 1999; Horiguchi et al., 2005).

2.2. Molecular Mechanisms of MPF Formation and Activation

2.2.1. MPF formation during goldfish oocyte maturation

Behavior of the components of MPF, Cdc2 and Cyclin B, has been investigated in detail during goldfish oocyte maturation (Hirai et al., 1992a; Katsu et al., 1993). Cdc2 is present in immature oocytes, and its protein content is constant during oocyte maturation. Cyclin B is absent in immature goldfish oocytes despite the presence of its mRNA. Cyclin B protein is newly synthesized from its mRNA stored in the oocyte during the process of maturation and the synthesized Cyclin B is bound to preexisting Cdc2, forming active MPF after phosphorylation of Cyclin B-bound Cdc2 (Figure 2).

Even under the conditions of protein synthesis inhibition, injection of a recombinant Cyclin B protein in immature goldfish oocytes induces GVBD (Katsu et al., 1993). Thus, the synthesis of Cyclin B in response to 17,20βP is sufficient to induce oocyte maturation in goldfish. Although the necessity of

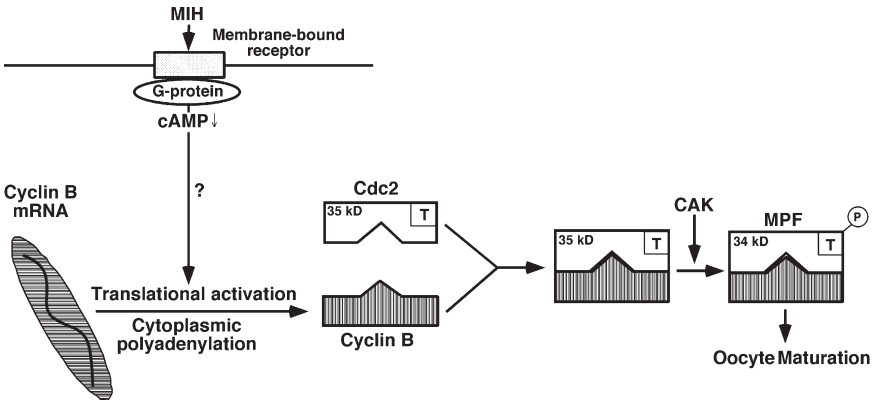

Figure 2. MPF formation and activation in oocytes without Cyclin B (pre-MPF). Immature oocytes contain monomeric Cdc2, and Cyclin B is synthesized by translational activation of the masked mRNA after MIH stimulation received by the surface receptor. After complex formation of the preexisting Cdc2 and the *de novo* synthesized Cyclin B, Cdc2 is activated by CAK-catalyzed T161 (T) phosphorylation.

Cyclin B protein synthesis for inducing oocyte maturation has not been confirmed in fish, the necessity has been demonstrated for the Japanese brown frog *Rana japonica*, in which Cyclin B is absent in immature oocytes and is newly synthesized during oocyte maturation, like in fish (Ihara et al., 1998). The absence of Cyclin B proteins in immature oocytes has been demonstrated not only in goldfish but also in other fishes, including carp, catfish, loach, lamprey, and zebrafish (Tanaka and Yamashita, 1995; Kondo et al., 1997). Therefore, the initiation of Cyclin B synthesis via translational activation of the dormant mRNA stored in immature oocytes is a key step for initiating oocyte maturation in these species.

2.2.2. MPF activation during goldfish oocyte maturation

MPF activation is regulated by biochemical modifications (phosphorylation and dephosphorylation) of Cdc2 in maturing oocytes. Phosphorylation states of Cdc2 were investigated during goldfish oocyte maturation (Yamashita et al., 1995). In maturing oocytes, activation of Cdc2 is associated with its phosphorylation and mobility shift on SDS-PAGE from 35 to 34 kDa. The activating phosphorylation site of Cdc2 is threonine 161 (T161). Substitution of T161 with a nonphosphorylatable residue results in suppression of both kinase activation and the electrophoretic mobility shift from 35 to 34 kDa. It is therefore concluded that T161 phosphorylation of Cdc2 causes MPF activation, coincident with the change in apparent molecular mass from 35 to 34 kDa, during goldfish oocyte maturation (Figure 2).

2.3. Involvement of Cell Cycle Regulators in Oocyte Maturation

2.3.1. Cdk-activating kinase (CAK)

CAK contains Cdk7 as a catalytic subunit and has a kinase activity that phosphorylates T161 of Cdc2. CAK does not phosphorylate monomeric Cdc2 but phosphorylates T161 of Cdc2 bound to Cyclin B. CAK is active throughout the processes of oocyte growth and maturation (Kondo et al., 1997). Consequently, the binding of newly synthesized Cyclin B to preexisting Cdc2 during oocyte maturation results in immediate phosphorylation of T161 of Cyclin B-bound Cdc2 by CAK, forming active MPF. Thus, the presence of CAK is required for activating MPF during oocyte maturation, but it does not play a key role in regulation of MPF activation during maturation.

2.3.2. Cdk2

Given that the kinase activity of Cdk2 increases during goldfish oocyte maturation as in the case of Cdc2 (Hirai et al., 1992b), the involvement of Cdk2 and its partner, Cyclin E, in inducing maturation was investigated (Yoshida et al., 2000). Immunoprecipitation experiments demonstrated complex formation of Cyclin E with Cdk2, but not with Cdc2, in oocytes. However, injection of Cyclin E mRNA into immature oocytes did not cause GVBD or MPF activation,

despite the fact that the injection induced the activation of Cdk2 in the oocytes. In addition, injection of a Cdk2 inhibitor had no effect on MPF activation and GVBD in MIH-treated oocytes, although the injection inhibited the kinase activity of Cdk2 in the oocytes. These results indicate that Cdk2 activity is neither sufficient nor necessary for initiating goldfish oocyte maturation. In mammals, however, Cdk2 is thought to be essential for completion of prophase I of the meiotic cell cycle, according to the results obtained from Cdk2-knockout mice (Ortega et al., 2003).

2.3.3. Cyclin A

Cyclin A is a regulatory subunit common to Cdc2 and Cdk2 in the mitotic cell cycle, but its function during oocyte maturation remains a mystery. Cyclin A is present in both immature and mature oocytes of goldfish, and its protein levels do not change remarkably during goldfish oocyte maturation induced by 17,20βP. Goldfish Cyclin A forms a complex with Cdc2, but not with Cdk2, in mature oocytes. While the kinase activity of Cyclin A-bound Cdc2 appears at the time of GVBD, it increases drastically after the completion of the first meiotic division. Microinjection of goldfish Cyclin A mRNA into immature goldfish oocytes does not induce GVBD. These results suggest that Cyclin A associated with Cdc2 may play an important role in steps after GVBD in goldfish oocyte maturation (Katsu et al., 1995).

Recently, it has been reported that Cyclin A-bound Cdk2 activity is required for the function of classical nuclear progesterone receptors in mammalian cultured cells (Narayanan et al., 2005). Although the involvement of nuclear progesterone receptors in oocyte maturation through a nongenomic activity remains to be clarified, it may be worth investigating whether Cyclin A/Cdk2 contribute to oocyte maturation via the level of progesterone reception rather than the level of cell cycle machinery.

Silver crucian carps, which reproduce by gynogenesis, produce unreduced diploid eggs by inhibiting the first meiotic division (Yamashita et al., 1993). Recently, a new member of the Cyclin A family in fish, Cyclin A2, has been isolated by subtractive suppression hybridization between the gynogenetic silver crucian carp and gonochoristic color crucian carp (Xie et al., 2003). The expression of Cyclin A2 mRNA is more abundant in gynogenetic carp oocytes than in gonochoristic carp oocytes, whereas the expression levels of Cyclin B mRNA in the two species are similar. Cyclin A2 protein is detected throughout the process of oocyte maturation in gynogenetic silver carp, while it appears only after hormone stimulation in gonochoristic color carp. Cyclin A2 may contribute to the inhibition of the first meiosis in gynogenetic crucian carp.

2.3.4. Cdc25

Cdc25 is a phosphatase that dephosphorylates the inhibitory phosphorylation on threonine 14/tyrosine 15 (T14/Y15) of Cdc2, thereby activating MPF at the G2/M-phase transition of the mitotic cell cycle. As stated above, Cyclin B is

absent in goldfish immature oocytes. Therefore, neosynthesis of Cyclin B and the following activation of MPF through T161 phosphorylation of Cdc2 are required for oocyte maturation upon MIH stimulation in this species (Figure 2). In contrast to goldfish, *Xenopus* immature oocytes contain an inactive MPF called pre-MPF, which consists of Cyclin B-bound Cdc2 phosphorylated by both T161 and T14/Y15. The phosphorylation of T14/Y15 of Cdc2 inactivates MPF that is phosphorylated on T161. In *Xenopus*, therefore, MIH (progesterone) stimulates Cdc25 to dephosphorylate T14/Y15, yielding active MPF that consists of Cyclin B-bound Cdc2 phosphorylated only on T161 (Figure 3: see also Schmitt and Nebreda, 2002; Haccard and Jessus, 2006).

Pre-MPF is absent in immature oocytes of goldfish, carp, catfish, zebrafish, and lamprey (Tanaka and Yamashita, 1995; Kondo et al., 1997). Recently, however, the presence of pre-MPF and the possible involvement of Cdc25 in MPF activation have been reported in a freshwater perch, *A. testudineus* (Basu et al., 2004). Since the molecular mass of *Anabas* Cyclin B is much smaller than the molecular masses of known Cyclin B proteins (*Anabas* Cyclin B is 30 kDa whereas goldfish Cyclin B is 46–48 kDa), investigations should be carried out to determine whether the 30 kDa protein is actually *Anabas* Cyclin B. Nevertheless,

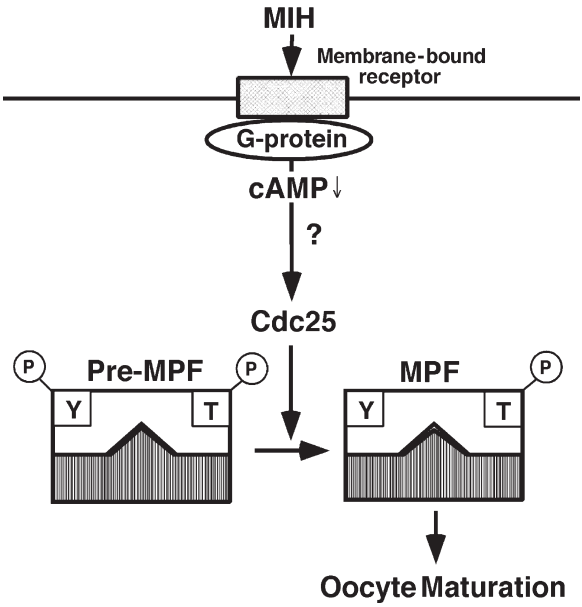

Figure 3. MPF activation in oocytes with pre-MPF. Cdc2 molecules that comprise pre-MPF are phosphorylated on both T14/Y15 (Y) and T161 (T). The MIH signal received by the surface receptor induces Cdc25-catalyzed pre-MPF activation through T14/Y15 (Y) dephosphorylation of Cyclin B-bound Cdc2. Biochemical pathways involved in this type of MPF activation has been extensively investigated in *Xenopus*.

it is plausible that the molecular mechanisms of MPF formation and activation in *Anabas* differ from those in other fishes examined so far, as discussed later.

2.4. Translational Control of Cyclin B mRNA

2.4.1. Structural changes in Cyclin B mRNA during oocyte maturation

In species that contain no pre-MPF in immature oocytes, dormant Cyclin B mRNA present in immature oocytes must be translationally activated for induction of oocyte maturation. To obtain an insight into the mechanisms of translational activation of Cyclin B mRNA, *in situ* hybridization analysis was performed during oocyte growth and maturation in zebrafish (Kondo et al., 2001).

In previtellogenic oocytes, Cyclin B mRNA is uniformly distributed throughout the cytoplasm (Figure 4A). As oocytes grow, however, Cyclin mRNA moves toward the future animal pole and localizes to the animal cortex in fully grown immature oocytes (Figure 4B). In contrast, Cyclin B mRNA in mature oocytes stimulated by 17,20βP disperses throughout the cytoplasm. When oocytes are

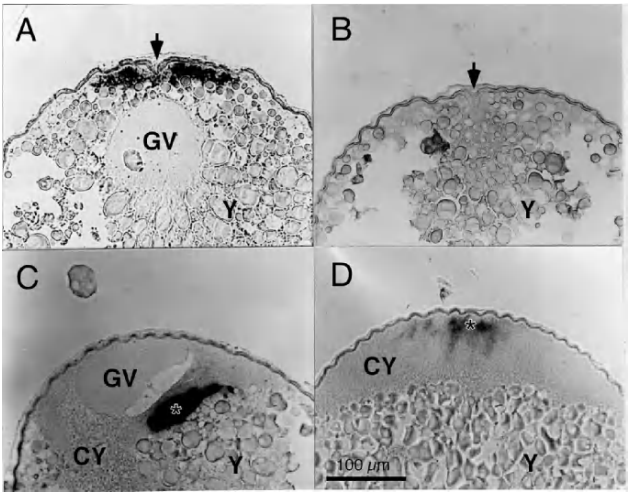

Figure 4. Localization of Cyclin B mRNA in immature and mature zebrafish oocytes. (A) A fully grown immature oocyte showing localization of Cyclin B mRNA along the cytoplasm at the animal pole identified by the presence of a micropyle (indicated by arrowhead) but not in other regions, including the germinal vesicle (GV) and yolk (Y). (B) A mature oocyte treated with 17,20βP showing the absence of Cyclin B mRNA along the cytoplasm at the animal pole (arrowhead indicating a micropyle as a marker of the animal pole). (C) Cyclin B mRNA in immature oocytes stratified by centrifugation on a Ficoll density gradient. The centrifugation segregated the oocyte cytoplasm (CY), including the germinal vesicle (GV), from the metaplasm consisting mainly of yolk (Y). Cyclin B mRNA is seen in the cytoplasmic layer of immature oocytes, existing as an aggregated form (asterisk). (D) Cyclin B mRNA in mature oocytes stratified by centrifugation on a Ficoll density gradient. Cyclin B mRNA exists as indistinct matter (arrowhead) in the cytoplasmic layer (CY) of a mature oocyte.

centrifuged in a density gradient of Ficoll, Cyclin B mRNA is distinctly aggregated in the cytoplasmic layer of immature oocytes (Figure 4C), while it is found as indistinct matter in that of mature oocytes (Figure 4D). The aggregation of Cyclin B mRNA present in immature oocytes immediately disperses just prior to the initiation of Cyclin B protein synthesis and of GVBD (Kondo et al., 2001). These results strongly suggest that the 17,20βP-induced morphological change in Cyclin B mRNA from the aggregated form to the dispersed form is responsible for translational activation of the mRNA during zebrafish oocyte maturation.

2.4.2. Cytoplasmic polyadenylation of mRNA

There is a growing body of evidence that cytoplasmic polyadenylation has an important role in the regulation of maternal dormant mRNAs stored in oocytes (Barnard et al., 2004). Polyadenylation (elongation of the poly (A) tail) of Cyclin B mRNA during oocyte maturation upon stimulation by 17,20βP has been examined using goldfish (Katsu et al., 1999). The poly (A) tail of Cyclin B mRNA in mature oocytes is about 100 nucleotides longer than that in immature oocytes. Elongation of the poly (A) tail of Cyclin B mRNA occurs at the same time as that of GVBD during oocyte maturation. Moreover, cordycepin, an inhibitor of poly (A) tail elongation, blocks 17,20βP-induced oocyte maturation. Taken together, these results suggest that the 17,20βP-induced Cyclin B mRNA translation requires elongation of the poly (A) tail, but it is still unknown how 17,20βP stimulates the polyadenylation.

2.4.3. Molecular basis of translational control

When *in vitro* transcribed unmasked Cyclin B mRNA is injected into immature zebrafish oocytes, GVBD occurs without stimulation of 17,20βP (Kondo et al., 1997). The presence of unmasked Cyclin B mRNA in the oocytes is thus sufficient for induction of oocyte maturation. Translation of Cyclin B mRNA stored in immature oocytes is probably prohibited by masking proteins, and 17,20βP induces the release of mRNA from its masking proteins, which triggers the translational activation. Although the finding that Y box protein is bound to Cyclin B mRNA in immature oocytes implies translational repression of Cyclin B mRNA by this protein in goldfish (Katsu et al., 1997), the molecular mechanisms of masking and unmasking of Cyclin B mRNA in fish oocytes are completely unknown.

There has been impressive progress in understanding regulatory mechanisms of cytoplasmic polyadenylation-induced translational activation of Cyclin B mRNA during oocyte maturation of *Xenopus*, in which the control is mediated by a sequence called cytoplasmic polyadenylation element (CPE) present in the 3′ untranslated region (3′ UTR) of Cyclin B mRNA (Nakahata et al., 2003; Barnard et al., 2004; Pascreau et al., 2005; Padmanabhan and Richter, 2006). As mentioned above, goldfish Cyclin B mRNA is polyadenylated and inhibition of its polyadenylation prevents MIH-induced oocyte maturation. In addition, the 3′ UTR of goldfish Cyclin B mRNA contains CPE-like motifs (Katsu et al., 1999).

These results suggest that, as in the case of *Xenopus*, CPE-mediated cytoplasmic polyadenylation regulates Cyclin B mRNA translation during goldfish oocyte maturation. To obtain tools for investigating molecular mechanisms of translational activation of Cyclin B mRNA in fish oocytes, Nakahata et al. (2001) isolated cDNA clones encoding goldfish poly (A) polymerase (PAP), which catalyzes elongation of the poly (A) tail, and those encoding goldfish poly (A) binding protein (PABP), which binds to and stabilizes the elongating poly (A) tail. They also succeeded in producing specific antibodies against goldfish PAP and PABP. Further studies using these probes should disclose the molecular basis of Cyclin B mRNA translation during fish oocyte maturation.

2.5. Oocyte Competence

Although fully grown oocytes mature in response to MIH, growing (previtellogenic and vitellogenic) oocytes cannot respond to MIH. Since the injection of active MPF into growing oocytes can induce GVBD, the failure of growing oocytes to mature by MIH treatment must be due to a deficiency in the mechanisms prior to MPF action. Hormonal control of oocyte competence, including the interaction of follicle cells and oocytes, will be dealt with in Chapter 7. Here, we focus on the mechanisms that operate within the oocytes.

Using zebrafish as an experimental model, Kondo et al. (1997) investigated which parts of the mechanisms are not yet established in growing oocytes. They showed that growing fish oocytes contain concentrations of Cdc2 and Cdk7 proteins and Cyclin B mRNA similar to those in fully grown oocytes, and that, as in fully grown oocytes, injection of a recombinant Cyclin B protein or *in vitro* transcribed Cyclin B mRNA induces GVBD in growing oocytes through the activation of Cdc2. These results suggest that the unresponsiveness of growing fish oocytes to MIH stimulation is not due to a deficiency in the components of MPF itself or its regulatory machinery such as Cdk7 and Cdc25 but that it is due to a deficiency in the events leading to Cyclin B mRNA translation, including 17,20βP reception on the oocyte surface, subsequent signal transduction pathways, and release from translational repression of Cyclin B mRNA that is accompanied by a structural change in the mRNA (Kondo et al., 2001). In contrast to such species as zebrafish and goldfish, which contain no pre-MPF in immature oocytes, there is a different reason for the incompetence in *Xenopus*, which contains pre-MPF in immature oocytes. The oocyte competence in this species is dependent on a positive feedback loop that allows MPF auto-amplification in growing oocytes (Karaiskou et al., 2004).

2.6. Generality and Species-Specificity in MPF Formation and Activation

Despite the generality of MPF structure and function, the existence of species-specific mechanisms of MPF formation and activation during oocyte maturation has been recognized (Yamashita et al., 2000). As mentioned above,

pre-MPF is absent in immature oocytes of fishes except for a freshwater perch (Basu et al., 2004). In these species, therefore, MPF is newly produced by translational activation of Cyclin B mRNA, and the resulting MPF is activated by T161 phosphorylation (Figure 2). Although not confirmed yet, it is likely that pre-MPF present in immature oocytes of the freshwater perch consists of Cyclin B-bound Cdc2 phosphorylated on both T161 and T14/Y15. Hormonal stimulation probably activates Cdc25, leading to the dephosphorylation of the inhibitory phosphorylation of T14/Y15 and the production of active Cdc2 phosphorylated only on T161 (Figure 3).

In amphibians except for *Xenopus*, it has been reported that immature oocytes of both Anura (*R. japonica* and *Bufo japonicus*) and Urodela (*Cynops pyrrhogaster*) contain no pre-MPF (Tanaka and Yamashita, 1995; Sakamoto et al., 1998), as in the case of many fishes. A recent study showed that, as in *Xenopus*, pre-MPF is present and is activated during oocyte maturation in a urodele amphibian, axolotl (Vaur et al., 2004). However, immature axolotl oocytes have very low levels of pre-MPF and exhibit progressive and slow Y15 dephosphorylation of Cdc2. It is therefore possible that Cyclin B synthesis also contributes to MPF activation in the axolotl oocytes, as in the case of fish and other amphibians except *Xenopus*. MPF activation in the axolotl oocytes could represent an intermediate category between the two extreme models, the *Xenopus* model and the goldfish model. The differences in MPF activation between the two might be due to intracellular compartmentalization of molecules responsible for MPF activation such as Cdc25 (Vaur et al., 2004) as well as the presence and absence of pre-MPF in immature oocytes.

3. MOLECULAR MECHANISMS FOR MAINTAINING FERTILIZABILITY OF EGGS

3.1. Meiotic Arrest of Eggs at Metaphase II

3.1.1. MII-arrest and release from it upon fertilization

The meiotic cell cycle of mature oocytes (eggs) of many vertebrates is stopped at metaphase II (MII-arrest). A key component that exerts MII-arrest is a product of c-*mos* proto-oncogene, Mos (Sagata et al., 1989; Sagata, 1996). Mos inhibits Cyclin B destruction, thereby stabilizing the MPF activity, through a pathway that includes MAPK. Release from MII-arrest is triggered by a transient increase in cytosolic free Ca^{2+} that occurs universally upon fertilization of vertebrate eggs (Tunquist and Maller, 2003; Kishimoto, 2005; Schmidt et al., 2006).

MII-arrested eggs maintain a high level of Cdc2 kinase activity. When they are fertilized or artificially activated, the level of kinase activity abruptly decreases, coincident with Cyclin B destruction (Iwamatsu et al., 1999). Mature *Xenopus* oocytes maintain a high level of MPF activity even when their protein synthesis is inhibited by cycloheximide. In striking contrast, cycloheximide treatment of mature goldfish oocytes induces partial decrease in both MPF

activity level and Cyclin B protein content. Moreover, the cell cycle of the treated oocytes is released from metaphase II and proceeds to anaphase II, where it stops again. Thus, cell cycle progression is also partial in cycloheximide-treated goldfish oocytes, indicating that MPF activity, Cyclin B protein level and cell cycle progression are closely linked to each other (Katsu et al., 1993).

3.1.2. MPF inactivation

The high level of MPF activity in MII-arrested eggs decreases rapidly and the cell cycle proceeds from metaphase to anaphase after fertilization or egg activation. Inactivation of MPF is caused by Cyclin B degradation through a ubiquitin-dependent proteolytic pathway (Tunquist and Maller, 2003; Kishimoto, 2005; Schmidt et al., 2006). To investigate the mechanism of Cyclin B degradation upon egg activation, 26S proteasome was purified from goldfish oocytes and its role in the regulation of Cyclin B degradation was examined (Tokumoto et al., 1997b).

The purified 26S proteasome can digest a recombinant goldfish Cyclin B (49 kDa) at lysine 57 (K57) independent of ubiquitination, producing a 42 kDa truncated form. The 42 kDa Cyclin B is also produced by the digestion of native Cyclin B forming a complex with Cdc2. This truncated Cyclin B appears transiently before the complete disappearance of Cyclin B, when eggs are released from the MII arrest by egg activation. Mutant Cyclin B, in which K57 was converted to arginine (K57R), is resistant to digestion by the 26S proteasome. Full-length Cyclin B is completely degraded in *Xenopus* egg extracts, but the mutant K57R Cyclin B is not. These results have revealed the sequence of the biochemical reactions that occur during Cyclin B degradation upon goldfish egg activation: an initial ubiquitin-independent restricted digestion of Cyclin B at K57 by the 26S proteasome allows the truncated Cyclin B to be ubiquitinated, and then the ubiquitinated Cyclin B becomes a target of further complete destruction by ubiquitin-dependent activity of the 26S proteasome, leading to inactivation of MPF in the eggs.

3.2. Functions of Mos and MAPK in MII-Arrest

The kinase activity of Mos, together with that of MAPK activated by Mos, is involved in maintaining the MII-arrest in *Xenopus* eggs (Tunquist and Maller, 2003). The function of Mos in goldfish oocyte maturation stimulated by 17,20βP was investigated (Kajiura-Kobayashi et al., 2000). Mos is absent in immature oocytes, appears before the onset of GVBD, and increases to a maximum level in mature oocytes. After fertilization, it disappears. Activity level of MAPK increases in accordance with the accumulation of Mos during oocyte maturation and reaches a maximum in mature oocytes. Injection of goldfish c-*mos* mRNA, as well as of *Xenopus* c-*mos* mRNA, into one blastomere of 2-cell-stage goldfish or *Xenopus* embryos inhibits cleavage and induces metaphase arrest in the injected blastomeres. These results indicate that, similarly to *Xenopus*, Mos contributes to the MII-arrest in goldfish eggs.

3.3. Application of MPF to Fisheries

Establishment of a simple and versatile method for estimating egg quality required for embryonic survival is in great demand in many fields of fisheries sciences; however, reliable biochemical markers for fish egg quality are presently unavailable. Recently, Aegerter et al. (2004) have shown that postovulatory aging of rainbow trout oocytes is associated with the abundance of several mRNAs (Cyclin B, insulin-like growth factors I and II, and insulin-like growth factor receptor Ib) within the oocytes, but their method, which includes poly A plus RNA extraction, reverse transcription, and real-time PCR, is too complicated for wide use as a method for estimating egg quality in the field.

MPF activity could be used as a marker for estimating egg quality. MPF activity, which is measured as activity phosphorylating histone H1 as an exogenous substrate, oscillates according to the meiotic cell cycle of the oocyte, peaking at metaphase and dropping at interphase (Yamashita et al., 1992b). Thus, mature oocytes arrested at MII exhibit a high level of MPF activity. When inseminated, MPF activity decreases dramatically, and the cell cycle is liberated from metaphase and proceeds to interphase. Accordingly, it can be assumed that eggs that exhibit a low level of MPF activity are released from MII-arrest; thereby, they would show only a low rate of embryonic development even when inseminated. Although the correlation between MPF activity and developmental competence of eggs should be investigated, it is possible that measurement of MPF activity of eggs will enable determination of their quality. Usual methods for measuring kinase activity require radioisotopes, which hinder convenient use of such methods. It might be possible to measure kinase activity more simply by using an antibody recognizing the peptide sequence phosphorylated by MPF specifically (Yamashita et al., 1992b). Since MII-arrest is dependent on Mos and MAPK, as well as MPF, it might be possible to estimate egg quality by measuring the levels of Mos and MAPK activities.

In the case of porcine, prolonged *in vitro* culture of mature eggs accelerates the aging of eggs, resulting in an increase in the rate of parthenogenetic activation of the eggs. It has been reported that aging of porcine eggs is correlated decrease in the level of MPF (histone H1 kinase) activity (Kikuchi et al., 1995, 1999). It is thought that the quality of aged porcine eggs can be determined by measuring MPF activity.

4. REGULATORY MECHANISMS OF OVULATION

4.1. Introduction to Ovulation

Ovulation, the event occurring subsequent to oocyte maturation and hydration, is a dynamic process that results in the release of mature oocytes from their follicular cells. Following disruption of microvillar connections between the follicular wall and the oocyte (follicular separation) that occurs during oocyte maturation, the ovulation process involves opening of the follicular wall

surrounding the oocyte (follicular rupture) and active expulsion of the oocyte through the opening site (Cerdà et al., 1999; Bolamba et al., 2003). These processes are regulated by a number of molecules, including proteases, protease inhibitors, progestational steroids, eicosanoid, catecholamines, and vasoactive peptides (Goetz et al., 1991). Using various experimental systems, especially with mammals, many investigations have been carried out to understand the molecular mechanisms involved in ovulation, but there are still many unresolved issues, including the biochemical pathways that link the initial stimulation by hormones to the final acting molecules, probably proteinases, essential for follicular rupture in ovulation.

The ease of manipulating fish oocytes and ovarian fragments *in vitro* makes fish an excellent experimental model for investigating regulatory mechanisms of ovulation. Ovulation can be induced *in vitro* by gonadotropin and MIH, although the extent of response to the hormones varies from species to species. For example, fully grown immature oocytes of yellow perch (*Perca flavescens*) undergo both GVBD and ovulation *in vitro* when stimulated by MIH, whereas those of brook trout (*Salvelinus fontinalis*) are never ovulated *in vitro*, although maturation (GVBD) can be induced. Ovulation does occur spontaneously *in vitro* if the oocytes have already undergone GVBD prior to their removal from the fish. It is thus likely that certain key processes prerequisite for ovulation are not stimulated *in vitro* by MIH. It is possible that ovulation is a more delicate process than is oocyte maturation, artificial induction of ovulation therefore requiring certain circumstances that allow the oocyte to undergo accurate biological reactions leading to ovulation. Whereas oocyte maturation is regulated by nongenomic action of MIH, ovulation is regulated by genomic mechanisms; that is, ovulation requires transcriptional activity accompanied by new mRNA synthesis (Theofan and Goetz, 1981; Pinter and Thomas, 1999). This may be one of the reasons why induction of ovulation is more difficult than that of maturation.

Injection of MPF into immature fish and amphibian oocytes induces oocyte maturation (GVBD) but not ovulation (Yamashita, unpublished data). Although both oocyte maturation and ovulation are promoted by MIH, the pathway leading to oocyte maturation via MPF and that leading to ovulation are clearly distinct. However, these two pathways should be communicated to each other for ensuring the correct timing of maturation and ovulation (Liu et al., 2005).

4.2. Molecular Mechanisms of Ovulation

Although molecular mechanisms of ovulation in fish have been studied in several species to date, the biochemical pathway that ends in digestion of the follicular wall is still not clear. Nevertheless, it is well known that arachidonic acid and its metabolites, including prostaglandins, are involved in ovulation in fish.

In yellow perch, indomethacin, an inhibitor of prostaglandin endoperoxide synthase, blocks 17,20βP-induced ovulation at low levels, and its inhibition correlates with the inhibition of prostaglandin synthesis in the ovary. This finding, in addition to other supportive findings, suggests the necessity of prostaglandin synthesis for induction of ovulation (Bradley and Goetz, 1994; Goetz and Garczynski, 1997).

On the basis of the notion that genes involved in ovulation are upregulated at the time of ovulation, a subtractive cDNA screening has been carried out by using cDNAs from oocytes at GVBD and from those at ovulation, and a gene that has significant sequence homology to a group of mammalian protease inhibitors (antileukoproteinases) has been isolated as an upregulating gene at ovulation (Goetz and Garczynski, 1997). Although its actual function remains to be investigated, this gene might contribute to the regulation of proteolysis at the time of ovulation.

Using specific inhibitors and activators, involvement of arachidonic acid and protein kinase C in MIH-induced ovulation has been investigated pharmacologically in the Atlantic croaker *Micropogonias undulates* (Patiño et al., 2003). The results suggest that ovulation in this species requires cyclooxygenase and lipoxygenase metabolic pathways that involve protein kinase C, arachidonic acid, and its metabolites, prostaglandins. Although the presence of similar signalling pathways has been suggested in yellow perch, the relative position of each component in the pathway seems to be different from that in Atlantic croaker (Bradley and Goetz, 1994).

Ovulation is finally carried out by follicular rupture that is caused by dissolution of the extracellular matrix of the follicular wall. It is therefore reasonable to assume that proteolytic enzymes are involved in the final process of ovulation. Indeed, a variety of proteinases have been proposed as executing enzymes to rupture the follicle, but the proteinases that are essential for follicular rupture in ovulation have not yet been identified, despite much effort. Recently, Ogiwara et al. (2005) have succeeded in identifying a biochemical process most proximal to follicular rupture using an *in vitro* ovulation system of medaka. This study will make a breakthrough in understanding the molecular mechanisms that regulate the final step of ovulation via the actions of proteinases and their inhibitors.

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CHAPTER 12

PHYSIOLOGICAL AND MOLECULAR BASIS OF FISH OOCYTE HYDRATION

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1. INTRODUCTION

As in other lower vertebrates, teleost oocytes growing within the ovary pass through a series of developmental stages that eventually culminate in the production of a mature female gamete or egg. During most of its time they are temporarily arrested in meiotic prophase I, and energy expenditures are concentrated on the synthesis and uptake of various substances (e.g. vitellogenin (Vg)) required by the developing oocyte and subsequent embryo development. After oocyte growth, meosis resumes as the large nucleus or germinal vesicle breaks down (GVBD), half the chromosomes are eliminated into a small polar body by unequal cytokinesis, and the remainder becomes aligned in second meiotic metaphase at the animal pole. During this process, termed "meiotic maturation", or "oocyte maturation", ovulation generally occurs. Shortly after second meiotic metaphase is achieved, the oocyte becomes "activable" or capable of being fertilized, and hence becomes an egg.

Marine teleost oocytes also undergo a significant increase in volume due to rapid water uptake during the maturation process previous to ovulation. This physiological mechanism occurring in teleosts, known as oocyte hydration, is unique among vertebrates since terminal oocyte growth due to swelling has been documented only in the cecropia moth oocyte (Telfer and Anderson, 1968). The mechanism of oocyte hydration was described in marine teleosts more than 100 years ago but the underlying molecular and cellular events have been ignored for many years. However, it is now recognized that this process has enormous physiological and ecological implications, especially in marine teleosts, in which the exceptionally high water content of the eggs is the principal cause of their buoyancy in the ocean, thereby improving the survival and dispersal of early embryos.

During the last years a number of physiological, cellular, and molecular studies have notably contributed to the knowledge on "why" and "how" water is driven into the fish oocyte, contributing to our understanding of the operant mechanisms involved in fish embryo survival both in the wild and under captive conditions. In this chapter, these studies will be reviewed to give an overview of our current knowledge of the physiological basis of fish oocyte hydration, and focus will be on cellular and molecular mechanisms recently uncovered which indicate that oocyte water uptake in fish is indeed a highly controlled process.

2. OOCYTE HYDRATION AND EGG BUOYANCY: PELAGIC VS. BENTHIC EGGS

In 1891, Fulton published a monograph on fecundity in marine fish and noted that those fish which produce pelagic eggs (i.e. eggs that float in seawater), the egg itself is remarkably transparent. Moreover, in pre-spawning or gravid

females, he found whitish or opaque oocytes within the ovary extending up to a certain size together with transparent eggs of a much larger size. He reasoned that a rapid increase in size must occur as a terminal phase of oocyte growth. These observations were addressed in detail in a later publication, and Fulton (1898) concluded:

> The most important point . . . is that the final change in the maturation of the pelagic ovum, while still within the ovary, is accompanied by a comparatively rapid and relatively great accession of a water fluid, of low density, from without, which dissolves the yolk-spherules, is associated with the dissolution of the germinal vesicle, and the definite formation of the periblast, distends the ovum to three or four times its former volume, thinning the capsule correspondingly, renders it of crystalline transparency, and reduces its specific gravity so that it is enabled to float in sea-water of ordinary density – in other words, to become pelagic.... The process is different from the ordinary slow growth of the eggs within the follicle. It is a rapid physical or physico-chemical change which is associated with the dissolution of the germinal vesicle and the rearrangement of the chromatin for fertilisation – the phase making the completion of ovarian growth and the preparation of the egg for fertilisation – but which has, so to speak, in the pelagic egg, been seized upon and exaggerated by natural selection for another purpose – namely to enable eggs to float and become widely dispersed . . . I have also been able to establish . . . that this change likewise occurs in the demersal eggs of Teleosteans at maturation, but to a less extent, the quantity of fluid absorbed being much smaller in amount, and the yolk-spheres remaining comparatively little affected.

He provided several examples for a variety of genera and documented a three- to four-fold increase in volume during oocyte maturation in marine fish that spawn pelagic eggs (Table 1).

The significance of Fulton's observations, later extended by Milroy (1898), is that about 67–75% of the final egg size in marine teleosts is rapidly achieved during oocyte maturation, resulting in a profound alteration or transformation of the structure of the oocyte as it becomes an egg. Over the last decades, changes in the oocyte volume during meiotic maturation have been reported in many teleost ovaries or ovarian follicles. These changes have ranged from slight in most freshwater and euryhaline species, to several folds in marine species. Thus, high increases in oocyte volume (3.1- to 8.4-fold) are associated to marine species that produce pelagic eggs in seawater, while lower oocyte volume increases (1.0- to 3.0-fold) are observed in species that produce benthic, non-buoyant eggs (Table 1). These species are termed pelagophils and benthophils, respectively.

In pelagic eggs, as well as in marine organisms in general, density reduction to become buoyant in seawater must be accomplished by the accumulation of substances less dense than seawater (Phleger, 1998). In early studies, the significant content of lipid of teleost eggs was implied or assumed to play a hydrostatic function or that it was primarily responsible for egg buoyancy in marine teleosts (e.g. Balon, 1977; Eldridge et al., 1983; Tocher and Sargent, 1984). However, the problem was compounded by the fact that some pelagic eggs, such as those of turbot (*Scophthalmus maxima*) for instance, possess discrete oil globules, while others, such as those of plaice (*Pleuronectes platessa*), do not (Craik

Table 1. Relative changes in oocyte volume (mm^3), and/or water content (% in wt) during meiotic maturation in pelagophil and benthophil teleosts

Species	Immature		Mature		Volume increase (×)	References
	Volume	Water	Volume	Water		
Freshwater benthophil						
Carassius auratus	0.5	68	0.5	75	1.0	Clemens and Grant (1964); Greeley et al. (1986)
Danio rerio	0.17		0.21		1.2	Selman et al. (1993)
Esox lucius		65		72		Craik and Harvey (1984)
Oncorhynchus mykiss		52		56		Craik and Harvey (1984)
Oryzias latipes	0.5	76	0.8	81	1.4	Hirose (1976)
Perca fluviatilis		79		85		Craik and Harvey (1986)
Plecoglossus altivelis[1]	0.4	60	0.7	79	1.8	Hirose et al. (1974); Chen et al.(2003)
Marine benthophil						
Clupea harengus		66		74		Craik and Harvey (1986)
Cyprinodon variegatus	0.3		0.9		2.8	Greeley et al. (1986)
Dormitator maculatus	0.011		0.014		1.3	Greeley et al. (1986)
Fundulus heteroclitus	1.3	61	2.9	81	2.2	Greeley et al. (1991)
F. Majalis	2.57		8.18		3.0	Greeley et al. (1986)
Gasterosteus aculeatus	0.7	74	1.4	80	2.1	Wallace and Selman, (1979); Craik and Harvey (1986)
Gobionellus boleosoma	0.008		0.019		2.4	Greeley et al. (1986)
Labrus bergylta	0.16	71	0.36	76	2.7	Finn et al. (2002b)
Limanda yokohamae	0.1	69	0.2	82	1.7	Oshiro and Hibiya (1981b)
Pomatoschistus minutus		70		68		Craik and Harvey (1986)
Spinachia spinachia		75		77		Craik and Harvey (1986)
Marine pelagophil						
Aspitrigla cuculus	0.52		1.95		3.8	Fulton (1898)
Callionymus lyra	0.18	76	0.52	90	2.9	Fulton (1898); Craik and Harvey (1986)
Centropristis striata	0.1	54	0.4	93	3.7	Cerdà et al. (1996); Selman et al. (2001)
Ctenolabrus rupestris	0.05	62	0.34	93	8.4	Finn et al. (2002b)
Cynoscion nebulosus	0.02	67	0.1	76	4.6	LaFleur and Thomas (1991)
Eutrigla gurnardus	0.38		1.60		4.2	Fulton (1898)
Gadus morhua	0.4	71	1.6	92	4.0	Craik and Harvey (1987); Thorsen and Fyhn (1991)

(*continued*)

Table 1. Continued

Species	Immature		Mature		Volume increase (×)	References
	Volume	Water	Volume	Water		
Hippoglossus hippoglossus	3.4	63	14.1	90	4.1	Finn et al. (2002a)
Micropogonias undulatus	0.1	73	0.2	83	3.8	LaFleur and Thomas (1991)
Microstomus kitt	0.2		1.0		6.3	Thorsen and Fyhn (1996)
Molva molva	0.18	64	0.66	89	3.7	Fulton (1898); Craik and Harvey (1987)
Mugil cephalus	0.2	60	0.5	85	2.9	Watanabe and Kuo (1986)
Pleuronectes platessa	0.9	68	3.3	93	3.7	Fulton (1898); Craik and Harvey (1987)
Scomber scombrus	0.32		1.02		3.2	Fulton (1898)
Scophthalmus maxima	0.18		0.56		3.1	Fulton (1898)
S. Rombus	0.27		1.15		4.2	Fulton (1898)
Sparus aurata	0.1	63	0.5	92	4.5	Fabra et al. (2005)
Solea vulgaris	0.38		1.44		3.8	Fulton (1898)
Takifugu rubripes	0.29		1.02		3.5	Chuda et al. (1998)
Tautogolabrus adspersus	0.1		0.3		4.9	Wallace and Selman (1981)
Trachinus vipera	0.27		1.20		4.4	Fulton (1898)
Verasper mooseri	0.7	69	2.8	91	4.1	Matsubara and Koya (1997)

[1]The highest changes in volume and water content of the oocyte occur during ovulation and oviposition, rather than during oocyte maturation.

and Harvey, 1987). Furthermore, comparative studies indicated that the ovaries from benthophil species have lipid contents, expressed in terms of dry weight, which are equal to or greater than those of pelagic eggs (Tocher and Sargent, 1984).

By contrast, when the water content of teleost ovaries containing large vitellogenic or prematurational oocytes was compared with that of mature eggs, water uptake emerged as the primary cause for the oocyte volume increase. The relative water content of pelagic eggs increases up to 76–93% from 54–76% before maturation, whereas the eggs of marine and freshwater benthophil teleosts show lower increases in relative water content, usually from 53% to 79% in prematuration oocytes to 56–85% in mature eggs (Table 1 and references therein). Therefore, in general, water uptake during oocyte maturation can account for more than 95% of the observed oocyte volume

increase. Thus, it is apparent that water rather than lipid is accumulated in pelagic eggs to dilute the yolk mass of the oocyte, decreasing its specific gravity, so that the spawned egg becomes buoyant in seawater. However, more than a half of pelagic eggs contain oil globules (Russell, 1976; Ahlstrom and Moser, 1980) that may also contribute to buoyancy, and in cases where the eggs show an elevated number of oil droplets, these appear to be less hydrated (Riis-Vestergaard, 2002).

2.1. Ecological Implications

The positive buoyancy of pelagic eggs in seawater confers clear advantages in the ocean, since buoyancy allows more efficient oxygen exchange between the developing embryos and the atmosphere and increases their survival (Mellinger, 1994). The egg specific gravity also affects the dispersal and vertical distribution of pelagic eggs within the water column by currents, and thus it may influence the reproductive success at different hydrographic conditions (Craik and Harvey, 1987; Mellinger, 1994; Nissling et al., 2003). The physiological and ecological importance of the mechanism of egg hydration in marine teleosts is also illustrated by the observation that some species that reproduce in areas were salinity is low, or is subject to high fluctuations, have the ability to adjust the buoyancy of the eggs to the environment. This mechanism has been extensively studied in the reproducing populations of flounder (*Platichthys flesus*), plaice (*P. platessa*), and cod (*Gadus morhua*) of the deep basins of Baltic Sea. In these areas, there is a halocline at 50–80 m depth with denser, more saline (10–18 ppt) deep water which is only partially mixed with the less saline (6–8 ppt) surface water (Nissling et al., 1994). The deep water is exchanged mainly during periods of saline water inflow from the North Sea, and due to the irregularity of these inflows, stagnant water with unfavourable oxygen conditions prevails for years in the bottom layers. Consequently, neutral buoyancy of the eggs at a salinity of 11–14 ppt is critical to avoid the low oxygen levels of the deepest layers (Nissling and Westin, 1991), and this is accomplished by increasing the egg size and their relative water content to 96–97% with respect to 91–93% of the marine counterparts (Solemdal, 1967, 1973; Thorsen et al., 1996). Such adaptation seems to be of a genetic nature achieved over long-term selection and are specific for the Baltic populations (Solemdal, 1973; Thorsen et al., 1996).

Water content of oocytes in benthophil freshwater fish also increases from 52–79% to 56–85% during meiotic maturation (Table 1). However, the use of water by pelagic eggs as a means of achieving egg buoyancy would clearly be impossible in species that spawn in freshwater, whereas lipid, being less dense than the medium, may be used for this purpose. For instance, in eggs of species such as silver carp (*Hypophthalmichthys molitrix*), density reduction is achieved by the presence of greatly enlarged perivitelline space, many times the volume of the yolk. In Pacific gudgeon (*Paraleucogobio soldatovi*), density is reduced by extensive hydration of the chorion, and in Chinese perch (*Siniperca chua-tsi*) by

the presence of a large oil droplet. Such adaptations, while reducing the density, do not render the egg buoyant in freshwater (Soin, 1964). Similarly, in marine teleosts that spawn in freshwater, such as striped bass (*Morone saxatilis*), the eggs show large oil globuli and seem to have an exceptional high lipid content (55% dry weight), although they require a current to remain suspended and alive (Stevens, 1966; Mangor-Jensen et al., 1993). True egg buoyancy in freshwater is seen in few species, as Amur snakehead (*Ophiocephalus argus*) and gourami (*Colisia lalia*), in which an enormous oil droplet occupies two-thirds of the egg volume allowing the eggs to develop at the surface of stagnant, oxygen-depleted waters (Soin, 1964).

The water content of benthic eggs is considerably lower than that of pelagic eggs. Since the eggs of benthophil species do not float in seawater, what could be the purpose of oocyte hydration in these species? One possible explanation was suggested by Greeley et al. (1991) by considering the spawning habitats of some of these species, such as the killifish (*Fundulus heteroclitus*) which exhibits a semilunar reproductive cyclicity. The killifish and other members of the family Cyprinodontidae spawn their eggs into the crevices of plant stems and empty mussel shells or into sandy substrates that are generally flooded only during the fortnightly occurrences of the high spring tides (reviewed by Taylor, 1984). Thus, the fertilized eggs of these fish actually incubate and undergo embryonic development while out of water, albeit in relatively protected sites, and then hatch when reflooded on consecutive sets of spring tides. An extreme example of this unusual reproductive behaviour is shown by semi-annual South American killifishes, such as *Austrofundulus limnaeus,* which is able to survive in intense dehydrated areas of coastal and inland savanna regions during months (Podrabsky et al., 2001). Therefore, these unique reproductive strategies suggest that the primary reason for oocyte hydration in these species is to provide an adequate supply of water for embryonic development in a quite atypically arid (for a fish) environment.

3. PHYSIOLOGICAL MECHANISMS FOR WATER UPTAKE DURING OOCYTE MATURATION

Once oocytes reach their full-grown size, they become competent to respond to the maturation-inducing steroid (MIS), secreted by the associated follicle (granulosa) cells in response to a gonadotropin surge, by undergoing meiosis resumption (see Chapter 11). During this process, besides the nuclear events leading to GVBD and initiation of the second meiotic division, the oocyte undergoes apparent morphological changes (Figure 1). In pelagophil species, the oocytes progressively enlarge in volume as a result of a remarkable hydration, their cytoplasm becomes "stippled", and eventually appear translucent, showing oil droplets in variable number and of different size depending on the species (Figure 1A). By contrast, in marine benthophil species the oocytes show less volume increase during maturation and a typical coalescence of a high number of oil droplets from the periphery

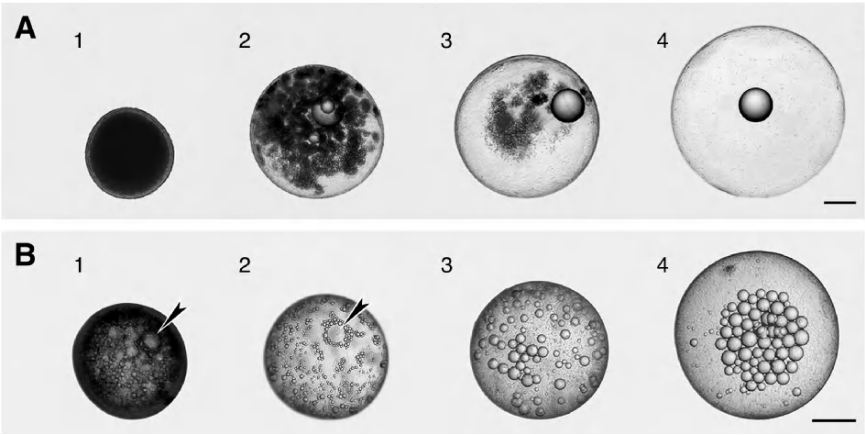

Figure 1. Teleost follicle-enclosed oocytes undergoing maturation and hydration *in vitro*. (**A**) Follicles from a pelagophil species, the gilthead sea bream (*Sparus aurata*) (Bar, 200 μm). (**B**) Follicles from a benthophil species, the killifish (*Fundulus heteroclitus*). The germinal vesicle (arrowheads) is easily distinguished in killifish prematuration oocytes and it is not longer visible as oocyte maturation proceeds (Bar, 500 μm). (1) prematuration oocytes; (2–3) oocytes undergoing maturation; (4) mature oocytes prior to ovulation.

towards one pole of the oocyte, and at the same time, oocytes become more translucent with respect to prematuration oocytes (Figure 1B).

The morphological changes occurring during oocyte maturation and hydration *in vivo* may be reproduced *in vitro* by stimulating post-vitellogenic follicle-enclosed oocytes with gonadotropin preparations (e.g. human chorionic gonadotropin, hCG), MISs, and a variety of hormones (e.g. insulin growth factor I), using appropriate culture media. These media, which generally have been the "FO" solution (Wallace and Selman, 1978; Thorsen and Fyhn, 1996), Leivovitz L-15 diluted to a variable extent (McPherson et al., 1989; Cerdà et al., 1993, 1996; Matsubara et al., 1995; Thorsen and Fyhn, 1996; Selman et al., 2001; Fabra et al., 2005) and Dulbecco's medium (LaFleur and Thomas, 1991), are hypotonic, allowing positive response of oocytes to the hormones by undergoing GVBD and swelling to an extent similar to that occurring *in vivo*. In some cases, however, oocytes hydrating *in vitro* show somewhat reduced swelling response when compared with oocytes hydrating *in vivo* (Wallace and Selman, 1978; McPherson et al., 1989; Cerdà et al., 1996), which possibly occurs by the slightly higher tonicity of the medium used and/or the occurrence of GVBD in smaller oocytes *in vitro* as compared with the *in vivo* situation (Wallace and Selman, 1978). This effect may also be due by the absence of the amino acid taurine in the culture medium, which uptake by the oocyte may be part of the water driving mechanism during swelling

(Thorsen and Fyhn, 1996). Nevertheless, the procedures *in vitro* have confirmed that the process of oocyte hydration is hormonally triggered and that it occurs concomitantly with the resumption of meiosis.

3.1. Role of Free Amino Acids as Osmotic Effectors

Over 20 years ago, Wallace and co-workers (Wallace and Selman, 1985; Wallace and Begovac, 1985) first described in the teleost *F. heteroclitus* an additional proteolytic event of Vg-derived yolk proteins, following original incorporation of Vg into growing oocytes (see Chapter 2), associated with the maturation and concomitant hydration of the oocyte. This finding, unique among oviparous vertebrates (Byrne et al., 1989), was later extended to other pelagophil and benthophil teleosts, and it became apparent that the extent of yolk proteolysis is well correlated with the extent of oocyte hydration during maturation (Greeley et al., 1986) (Figure 2). Thus, in pelagophil species, which produce highly hydrated eggs, proteolysis of yolk proteins is most pronounced than in benthophil fish, resulting in the generation of smaller proteins that may possess some osmotic significance (Wallace and Selman, 1985; Wallace and Begovac, 1985; Greeley et al., 1991).

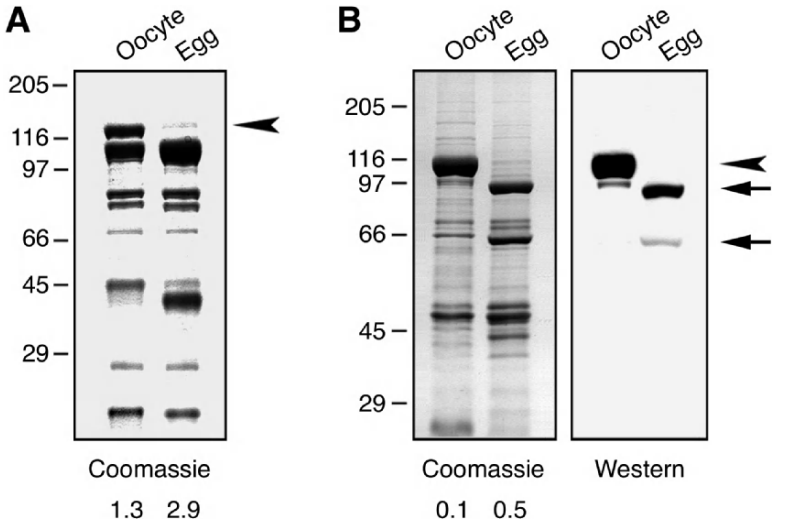

Figure 2. Yolk protein profile of oocytes and eggs from benthophil (*F. heteroclitus*) (**A**) and pelagophil (*S. aurata*) (**B**) teleosts observed by SDS-polyacrylamide gel electrophoresis. Yolk proteins are stained with coomassie blue (A) and detected by Western blot using an anti-sea bream Vg antiserum (B). The arrowheads indicate lipovitellin components of ~122 and 103 kDa in killifish and sea bream oocytes, respectively, whereas the arrows indicate smaller peptides apparently formed from the proteolysis of yolk proteins. Molecular mass values are provided on the left in kilodalton, and oocyte volumes (mm^3) are indicated below each lane.

3.1.1. Pelagophil teleosts

Many organisms, including fish (King and Goldstein, 1983), employ free amino acids (FAAs) for volume regulatory purposes. Thus, FAAs and other organic osmolytes were suggested to play a role during water uptake by maturing teleost oocytes (Oshiro and Hibiya, 1981a, b; Wallace and Begovac, 1985; Wallace and Selman, 1985; Greeley et al., 1986; Watanabe and Kuo, 1986). The evidence for such involvement was indirectly shown by Craik and Harvey (1987) who demonstrated pronounced increases in the content of total ninhydrin-positive substances (interpreted as FAAs) and water during the formation of the pelagic eggs of the plaice and cod. They further suggested that the breakdown of yolk proteins into FAAs during oocyte maturation contributed to the osmotic pressure for water influx into the oocyte. This hypothesis is supported by the findings of Thorsen et al. (1993) and Thorsen and Fyhn (1996) who determined the FAA content in oocytes and eggs from pelagophil and benthophil fish, both *in vivo* and *in vitro*, and found an increased production of FAAs during oocyte maturation, which was not caused by transport of FAAs from an extracellular source. A larger FAA pool in pelagic eggs in comparison with that in benthic eggs was also noted.

Thorsen et al. (1993, 1996) described a correlation between the FAA increase and the proteloysis of Vg-derived yolk proteins during oocyte maturation, together with a similar amino acid profile between the FAA pool and certain yolk proteins of the oocyte, with the exception of urea which more likely reflected the further metabolism of released amino acids. These observations thus indicated that FAAs probably originate from the oocyte yolk proteins. Maturation-associated proteolysis of yolk proteins has been described in many pelagophil teleosts both *in vivo* and *in vitro* (Carnevali et al., 1992, 1993; Okumura et al., 1995; Matsubara et al., 1995, 1999; Matsubara and Sawano, 1995; Thorsen and Fyhn, 1996; Cerdà et al., 1996; Matsubara and Koya, 1997; Reith et al., 2001; Selman et al., 2001; Finn et al., 2002a, b; Fabra et al., 2005) (Figure 2B). In some of these species, a positive relationship between yolk protein proteolysis, and FAA and water content of oocytes has also been confirmed (Matsubara and Koya, 1997; Reith et al., 2001; Selman et al., 2001; Finn et al., 2002a, b), confirming the role of FAAs as important osmotic effectors for water uptake during oocyte hydration. Table 2 summarizes the data on FAA content of oocytes and eggs of representative pelagophil and benthophil teleosts. These data indicate that during the transformation of the prematuration oocyte into an egg, the absolute amount (nmol/individual) of FAAs increases by 9.1- to 15-fold in pelagic eggs, whereas in benthic eggs this increase and the final concentration of FAA is much lower. In some pelagic eggs, however, because of the pronounced hydration, the final concentration of FAA is reduced by about 43%, which may indicate that either other low-molecular weight substances helped to drive water into the oocyte (see below) and/or most of the FAA initially present

Table 2. Total amount and concentration of free amino acids (faas) of prematuration oocytes and eggs in pelagophil and benthophil teleosts

	FAAs[1]				
	nmol/ind.		Mm		
Species	Oocytes	Eggs	Oocytes	Eggs	Reference
Freshwater benthophil					
Plecoglossus altivelis[2]	22	72	14	19	Chen et al. (2003)
Marine benthophil					
Crenilabrus melops	7	27	54	96	Finn et al. (2002b)
Fundulus heteroclitus	48	141	40	34	Greeley et al. (1991)
Labrus bergylta	6	9	27	41	Thorsen et al. (1993)
Labrus mixtus	5	6	40	27	Finn et al. (2002b)
Marine pelagophil					
Centropristis striata	5	48	208	118	Selman et al. (2001)
Ctenolabrus rupestris	5	40	96	119	Finn et al. (2002b)
Gadus morrhua	17	223	49	143	Thorsen et al. (1993)
Hippoglossus hippoglossus	280	2,564	82	182	Finn et al. (2002a)
Microstomus kitt	18	165	60	154	Thorsen et al. (1993)
Pleuronectes platessa	36	649	33	175	Thorsen et al. (1993)
Verasper moseri[3]	3	46		149	Matsubara and Koya (1997)

[1]The concentrations are calculated from the given oocyte and egg volumes and FAA content.
[2]Data on FAA content are given as nmol/mg dry weight.
[3]Data on FAA content are given as µg/ind.

in the prematuration oocyte are not osmotically active (i.e. are present in a bound form) (Selman et al., 2001).

The role of FAAs as osmotic effectors during oocyte hydration is also reinforced by other indirect and direct evidences. First, pelagophil fish that spawn in brackish waters of the Baltic sea, which increase the egg diameter and water content with respect to their oceanic counterparts, also show an increased intracellular content of FAA (Thorsen et al., 1996). Second, buoyant eggs obtained by hormonally induced females show higher FAA and water content, but similar lipid content, than non-buoyant eggs (Seoka et al., 2003). And finally, the inhibition of yolk proteolysis during the maturation of marine oocytes *in vitro* prevents the production of FAAs and reduces the hydration of the oocyte, providing a direct demonstration of the role of FAAs during water uptake (Selman et al., 2001) (Figure 3). However, the level of FAA in mature pelagic eggs generally accounts for approximately half of their osmolality (Thorsen et al., 1993; Finn et al., 2002a), which implies that low-molecular weight effectors other than FAA may be also accumulated in the pelagic egg and be osmotically active (see below).

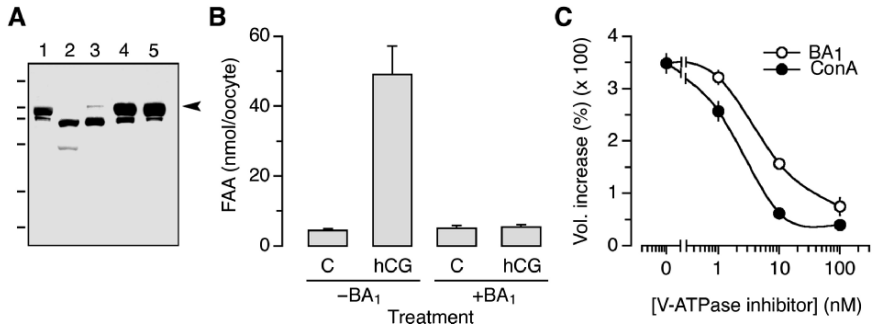

Figure 3. Inhibition of yolk proteolysis, generation of FAAs, and oocyte hydration in pelagophil teleosts. (**A**) Western blot of gilthead sea bream (*Sparus aurata*) yolk proteins of control (lane 1) and MIS-stimulated ovarian follicles in the absence (lane 2) or the presence of 1, 10, and 100 nM bafilomycin A_1 (BA_1), an inhibitor of the vacuolar ATPase (V-ATPase) (lanes 3, 4, and 5, respectively), using a sea bream Vg antisera. Note that the degradation of a yolk protein of ~100 kDa, possibly lipovitellin, is inhibited by treatment with BA_1. The position (bars) of molecular weight markers is indicated on the left (from top to bottom: 200, 116, 97, 66, 45, and 29 kDa). (**B**) Effect of BA_1 on the generation of FAAs during hCG-induced oocyte maturation in the black sea bass (*Centropristis striata*). (**C**) The treatment of follicles with BA_1 and concanamycin (ConA), another V-ATPase inhibitor, prevents the hydration of sea bream oocytes. (Data in (B) and (C) are from Selman et al., 2001.)

3.1.2. Benthophil teleosts

Few studies have investigated the FAA profile of marine benthophil teleosts (Table 2). Some of these works indicated almost no change in the yolk protein electrophoretic profile or in the FAA pool during oocyte maturation (Greeley et al. 1991; Finn et al., 2002b), which would fit with the reduced or null hydration of these oocytes. However, in some benthophil species, such as the killifish and corkwing (*Crenilabrus melops*), a limited proteolysis of Vg-derived yolk proteins and a 2.9- to 3.8-fold increase in FAA content can be detected (Wallace and Begovac, 1985; Greeley et al. 1986, 1991; Finn et al., 2002b) (Figure 2A). In these fish, as well as in other benthophil species, the amino acid taurine is by far the most abundant amino acid found in the FAA pool (Greely et al., 1991; Thorsen et al., 1993; Thorsen and Fyhn, 1996; Finn et al., 2002b), unlike in pelagic eggs where its amount corresponds only 2–4% of the total FAA content (Thorsen et al., 1993; Thorsen and Fyhn, 1996). Taurine is the amino acid most often associated with volume regulatory strategies, and because is not a building block for protein synthesis, intracellular concentrations of this organic solute can change without perturbing the normal functioning of the cell (Lang et al., 1998). Taurine has been implicated in volume regulation of several teleost cell types (reviewed by Fugelli and Thoroed, 1990), and because is the most abundant FAA in the benthic oocyte, it probably fulfills a similar role during the normal course of follicle development in benthophil teleosts (i.e. regulates responses to anisosmotic conditions). However, at least in the killifish, is very unlikely that taurine is contributing

significantly to the stage-specific volume increases associated with oocyte maturation (Greeley et al., 1991). Although the total amount of taurine does increase somewhat during maturation, water influx was found to be far greater, resulting in lower taurine concentrations during the course of maturation. Furthermore, the minor increases of taurine that occur during maturation seem to cease at GVBD, while follicle volume (and water content) continue to rise (Greeley et al., 1991). However, direct comparisons of taurine content in isolated oocytes at distinct hydration stages have not been done in other benthophil species in addition to the killifish, and therefore the role of taurine during oocyte hydration in benthic teleosts remains to be fully elucidated.

The low but significant increase of FAAs in some benthic eggs have the potential to be effective osmotic effectors for oocyte hydration. In fact, the total oocyte content of FAA in these species increased steadily through oocyte maturation, suggesting that these substances could be involved in driving the associated water uptake (Greeley et al., 1991; Chen et al., 2003). This hypothesis, however, was contradicted by McPherson et al. (1989) who demonstrated that proteolysis of yolk proteins in killifish follicle-enclosed oocytes cultured *in vitro* occurred in both K^+-containing and K^+-free media, while hydration of the oocyte was suppressed in K^+-free media. These findings thus indicate that yolk protein proteolysis is not a primary osmotic effector in killifish oocytes (McPherson et al., 1989). Furthermore, other data also argue against a major role for FAAs for oocyte hydration. First, the combined concentrations of non-taurine FAAs in follicles relative to oocyte water were actually only maintained at a constant level during maturation (Greeley et al., 1991; Chen et al., 2003). Second, the combined concentrations of non-taurine FAAs in follicles were relatively low in comparison with the concentrations of inorganic ions (less than half of the concentrations of Na^+ and K^+) (Greeley et al., 1991). Therefore, unlike in pelagophil fish, the contribution of FAAs to oocyte hydration in marine benthophil teleosts, at least in the killifish, seems to be low.

3.2. Role of Inorganic Ions and Other Low-Molecular-Weight Osmotic Effectors

Early studies in benthophil teleosts, suggested that an intracellular increase of inorganic ions, K^+ and Na^+ in particular, may provide the primary osmotic stimulus for water influx into the maturing oocyte. For instance, in the medaka (*Oryzias latipes*), follicle Na^+ and water content was reported to increase during maturation and ovulation *in vitro* (Hirose, 1976), and in tilapia (*Oreochromis niloticus*) and ayu (*Plecoglossus altivelis*), hormonally induced ovulation is also preceded by increased ovarian Na^+ and water content (Hirose and Ishida, 1974; Babiker and Ibrahim, 1979).

Further studies in the killifish presented convincing evidence for the involvement of Na^+ and K^+ in the volume increases and water uptake of maturing oocytes *in vivo* (Greeley et al., 1991). Both ions are present in relatively high

concentrations in post-maturation follicles and eggs, being higher than that of FAAs, and changes in the concentration of K^+ in particular closely parallel changes in oocyte osmolality during maturation. These observations have also been confirmed by experiments *in vitro* (Wallace et al., 1992), which indicate that the follicle contents of Na^+ and K^+ rose during oocyte maturation and associated volume increase, the increase in K^+ being twice that of Na^+, and that the K^+ influx exceeds that of water resulting in a net increase in the concentration of this cation. Wallace et al. (1992) also demonstrated in the killifish by using Na^+ and K^+-free mediums that oocyte hydration, but not maturation, is strictly dependent on the concentration of external K^+, suggesting that this cation is the major cause of the uptake of osmotically obligated water during oocyte maturation. However, in other benthophil species that spawn in freshwater or in low salinity areas, such as the ayu, a steady increase in the concentrations of Na^+ but not of K^+ occurs during maturation and ovulation *in vivo* (Chen et al., 2003). Interestingly, an additional increase in Na^+ concentration is observed in ayu oocytes during oviposition, when a higher increase in oocyte volume and water content is noted. These recent observations, together with early reports, could suggest a preferential uptake of Na^+ or K^+ into the oocyte for hydration among benthophil teleosts depending on the salinity of the spawning sites chosen by the different species (Chen et al., 2003). This hypothesis, however, remains to be demonstrated.

In pelagophil teleosts, inorganic ions, especially K^+ and Cl^-, as well as inorganic phosphorus (Pi), have also been implied in the increase of oocyte osmolality during maturation and hydration (Milroy, 1898; Craik and Harvey, 1984, 1986, 1987; Watanabe and Kuo, 1986; Thorsen and Fyhn, 1991; LaFleur and Thomas, 1991; Selman et al., 2001; Finn et al., 2002a) (Table 3). In the mullet (*Mugil cephalus*), the K^+ content of ovarian pieces was found to rise during maturation and hydration (but in absolute terms only: the K^+ concentration relative to the increasing water remains fairly constant), while the Na^+ content remains about the same (and hence declines in concentration) (Watanabe and Kuo, 1986). In two species of sciaenid fishes, the Atlantic croaker (*Micropogonias undulatus*) and spotted sea trout (*Cynoscion nebulosus*), LaFleur and Thomas (1991) gathered evidences for an increase of K^+ but not of Na^+ during oocyte hydration *in vivo*, although they also reported increases in Mg^{++} and Ca^{++}. Experiments *in vitro* indicated that oocyte hydration was dependent on external K^+. Selman et al. (2001) also found an accumulation of K^+ and Na^+ associated with oocyte hydration in the black sea bass (*Centropristis striata*), the absolute amount of K^+ being approximately double than that of Na^+. A recent study on individual oocytes of Atlantic Halibut (*Hippoglossus hippoglossus*) has shown that K^+, Cl^-, total ammonium (NH_4^+), and Pi accounted for part of the transient hyperosmolarity of the oocytes responsible for water uptake (Finn et al., 2002a). In these oocytes, the K^+ content raises mainly after ovulation, following a rapid increase of Cl^- and Pi during the early phases of oocyte hydration. It thus appears that in pelagophil teleosts FAAs derived from proteolysis of yolk proteins contribute ~50% of the oocyte osmolality and ions (K^+, Cl^-, Pi, and NH_4^+) make up the balance (Finn et al., 2002a).

Table 3. Total amount and concentration of low-molecular weight effectors in prematuration oocytes (O) and eggs (E) in pelagophil and benthophil teleosts are calculated from the given oocyte and egg volumes and ion content

	Na^+				K^+				
	nmol/ind.		mM		nmol/ind.		Mm		
Species	O	E	O	E	O	E	O	E	Reference
Plecoglossus altivelis[1]	70	176	116	221	50	39	128	10	Chen et al. (2003)
Marine demersal									
Fundulus heteroclitus	25	85	25	40	49	180	42	80	Wallace et al. (1992)
Marine pelagic									
Centropristis striata	5	9	214	22	4	21	200	51	Selman et al. (2001)
Cynoscion nebulosus	2	3	72	30	3	7	110	61	LaFleur and Thomas (1991)
Micropogonias undulatus	1	2	60	35	2	7	70	65	LaFleur and Thomas (1991)
Hippoglossus hippoglossus	125	150	37	11	300	1,200	88	85	Finn et al. (2002a)

[1]Data on Na^+ and K^+ content is given in nmol/mg oocyte.

It is important to note, however, that in benthophil species the combined concentrations of FAAs including taurine, K^+ and Na^+ account also for at most ~55% of the measured osmolality of the maturing oocyte (Greeley et al., 1991, Chen et al., 2003). Thus, there is still some room for the involvement of other osmotic effectors in the hydration mechanism. Unmeasured ions, such as Cl^-, Ca^{++}, Mg^{++}, HCO_3^-, HPO_3^-, etc., and nitrogen waste compounds such as NH_4^+, could be also playing osmotic roles as it occurs in pelagophil teleosts. In fertilized eggs of the herring (*Clupea harengus*) the concentration of Cl^- reaches about 60–70% of the combined total concentration of Na^+ and K^+ in mature oocytes of other benthophil species, and thus the combination of these ions would account for the final osmolality of the mature oocyte (Hølleland and Fyhn, 1986). Furthermore, hydrating oocytes of ayu show a 16-fold increase in the concentration of Ca^{++} during oviposition, although reaching approximately one-fourth of the Na^+ levels (Chen et al., 2003).

3.2.1. Mechanisms for ion uptake into the oocyte

Concomitant with yolk proteolysis, Craik (1982) and Craik and Harvey (1984, 1986) noted a pronounced loss of protein-associated phosphorus during oocyte maturation in several marine teleosts and proposed that protein-linked phosphate may be utilized as an energy source during the massive water uptake that simultaneously occurred. These results implied that an ATPase/cation pump was involved to drive K^+ ions into the oocyte against a concentration gradient

from the blood plasma in the capillary bed of the ovarian follicle (the most likely source of ions) which has a high Na^+/K^+ ratio. Experimental evidences in support of this hypothesis, however, were not provided until LaFleur and Thomas (1991) found a role of Na^+, K^+-ATPase during the hydration of Atlantic croaker and spotted sea trout oocytes. In these species, Na^+, K^+-ATPase activity increases threefold and the concentration of the enzyme increased by 50% in ovarian tissue during oocyte hydration. In addition, incubation of croaker follicle-enclosed oocytes with ouabain and amiloride, known blockers of Na^+, K^+-ATPase, and Na^+-channels, inhibited gonadotropin-induced oocyte hydration *in vitro*. In the benthophil fish ayu, a Na^+, K^+-ATPase system has also been inferred to play a role during the active transport of Na^+ into the oocyte by the time of oviposition (Chen et al., 2003).

The regulation and cellular localization of Na^+, K^+-ATPase enzymes involved in oocyte hydration in the fish ovarian follicle are however unknown. LaFleur and Thomas (1991) suggested that gonadotropin may regulate the activity and concentration of the Na^+, K^+-exchange pump by acting on the somatic cells and/or in the oocyte. Although this mechanism was not demonstrated, it would be consistent with the known "priming" action of gonadotropin in teleost ovarian follicles, which allows the oocyte to become responsive to the MIS by undergoing meiosis resumption. This process apparently occurs through the stimulation of the synthesis and translocation of the MIS receptor into the oocyte plasma membrane (Patiño et al., 2001; Zhu et al., 2003), and thus additional effects on the synthesis and/or regulation of Na^+, K^+-ATPase enzymes by gonadotropin(s) in somatic cells or the oocyte can also be considered. This potential regulation would be consistent with the direct effect of gonadotropin on Na^+ and K^+ transport in sperm duct epithelium of brook trout (*Salvelinus fontinalis*) (Marshall, 1989). However, a direct action of the MIS on the oocyte Na^+, K^+-ATPase pumps through the activation of the maturation enzymatic machinery cannot be ruled out. It is clear therefore that the precise hormonal mechanism by which Na^+, K^+-ATPase enzymes are regulated during oocyte hydration remains to be elucidated.

In the benthophil killifish, where a correlation between K^+ uptake and an increase in oocyte volume and water content have been demonstrated (Greeley et al., 1991; Wallace et al., 1992), experimental evidence for an involvement of a Na^+, K^+-ATPase is lacking. Wallace et al. (1992) found that relatively high concentrations of ouabain (10^{-5} M) had no effect on oocyte hydration *in vitro*, neither had different ion channel blockers (R.A. Wallace, personal communication). Based on this observation, a typical Na^+, K^+-ATPase is apparently not involved in killifish oocyte hydration, which would agree with the fact that K^+, as well as Na^+, are accumulated within the oocyte during hydration. Alternative mechanisms may be therefore implied, such as the Na^+–K^+–$2Cl^-$ cotransport system (Hoffman, 1985; Geck and Heinz, 1986) that is believed to function in the volume regulatory mechanisms of various somatic cells. However, the occurrence in killifish follicles of an unusual Na^+, K^+-ATPase not sensitive to

ouabain, together with Na^+-channels to facilitate a net influx of Na^+ into the oocyte (Bulling et al., 2000), could also be considered.

In general, the uptake of Na^+ into hydrating fish oocytes may occur presumably through passive diffusion, flowing inward down its concentration gradient from the blood plasma, while K^+ would need to be specifically transported into the oocyte. The changes in the colloid osmotic pressure due to the abundant yolk proteins of the oocyte, as well as specific Na^+-channels, might also be involved in these ions movements. For the movement of Cl^-, vacuolar proton-ATPases (V-ATPase) which typically move H^+ and Cl^- across lysosomal and endosomal membranes (Nelson and Harvey, 1999) have been proposed (Finn et al., 2002a). However, none of these mechanisms have been demonstrated and thus how the inward influx of Na^+ and Cl^- (and in some cases also of K^+) is brought about in hydrating fish oocytes is still subject to conjecture.

3.2.2. Role of cell–cell communication for K^+ translocation into the oocyte

During the search for the cellular mechanisms that might be involved in the translocation of K^+ ions into the killifish oocyte, Wallace et al. (1992) reported two remarkable observations. First, the ability of MIS-treated follicle-enclosed oocytes to hydrate was dependent on the specific stage of maturation regardless of the presence or absence of external K^+. Second, manually defolliculated oocytes, resulting in denuded oocytes free of associated theca and granulosa cells, loses the ability to hydrate in K^+-containing medium in response to steroid stimulation. One of the main events that is known to occur during oocyte maturation in lower vertebrates is the withdrawal of microvillar and macrovillar processes by the oocyte and overlying granulosa cells, respectively, so that the intercellular contact is broken as the oocyte prepares for ovulation. Thus, this mechanism was proposed as an elegant explanation for the gradual loss of the follicle ability to hydrate, which was consistent with the observation that the connection oocyte-granulosa cells is essential for oocyte hydration in killifish (Wallace et al., 1992).

In both freshwater and marine teleosts, intercellular communication between the oocyte and overlying granulosa cells is mediated by different cell–cell contact structures (see Chapter 1). Among these structures, heterologous gap junctions (GJs) are formed between the oocyte and granulosa cells, through which molecules with a molecular mass <1,200 Da can pass from one cell to the other (Figure 4). Therefore, it is conceivable that K^+ sequestered by the follicle cells may be translocated to the maturing oocyte via heterologous GJs which are dissociated as maturation reaches completion (Wallace et al., 1992). By using special fluorescent dyes, such as Lucifer yellow (LY) which is cell impermeable and cannot pass between cells except through functional GJs, Cerdà et al. (1993) provided consistent evidences supporting this hypothesis in killifish. Injection of LY into the oocyte readily passed to the overlying granulosa cells, demonstrating effective dye coupling and therefore the presence of functional heterologous GJs (Figure 5). Passage of LY, and hence intercellular communication, was

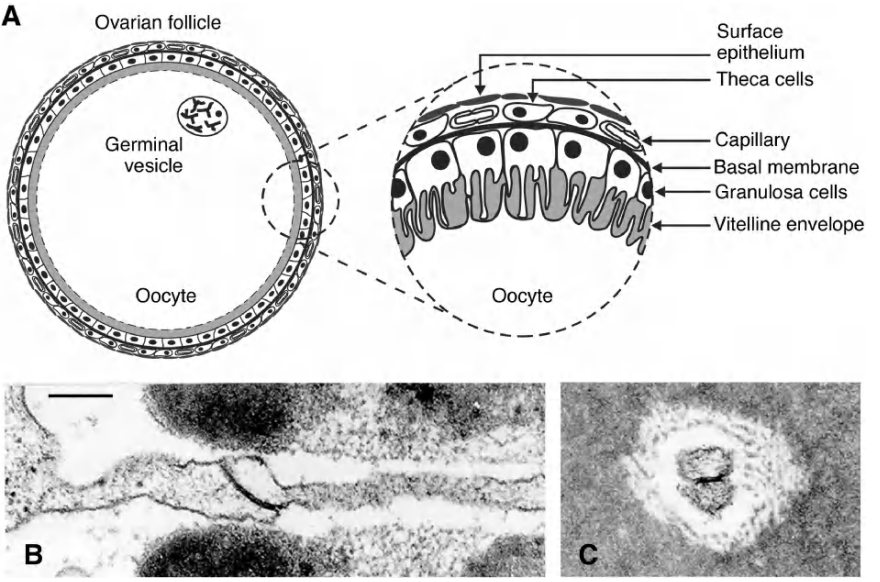

Figure 4. Cell–cell communication within the teleost ovarian follicle. (**A**) Schematic representation of the structure of an ovarian follicle showing the oocyte and the associated somatic cell layers. (**B** and **C**) Electron micrographs of gap junctions (GJs) formed at the level of microvilli crossing the vitelline envelope that extend from the oocyte and granulosa cells in zebrafish (*Danio rerio*) ovarian follicles ((B) longitudinal section of the vitelline envelope; (C) cross-section of vitelline envelope). Magnification bar, 0.25 μm.

inhibited by the tumour-promoting phorphol ester phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, and by 1-octanol which are known uncouplers of GJs (Figure 5B). Both PMA and octanol also inhibited oocyte hydration initiated by the MIS (Figure 5C), which provides further support for a role of GJs for the translocation of K^+ ions from the granulosa cells to the maturing oocyte. These findings thus suggest that the passage of K^+ into the killifish oocyte could be a passive process from the granulosa cells to the oocyte through connecting GJs, although the exact mechanism by which this cation is presumably accumulated in granulosa cells is not known.

In pelagophil oocytes, a similar GJs-mediated mechanism for the translocation of K^+, possibly accumulated in granulosa cells by the Na^+, K^+-ATPase system, have not been yet demonstrated. However, dye transfer between the oocyte and granulosa cells is observed in both incompetent and competent fully-grown vitellogenic ovarian follicles of Atlantic croaker (Yoshizaki et al., 2001), which as in the killifish suggest the presence of functional GJs. Also, in both croaker and red sea bream (*Pagrus major*), gonadotropin priming of ovarian follicles for the acquisition of oocyte maturational (and hydration) competence is accompanied by an increase of GJs proteins (connexins) mRNAs and of intercellular

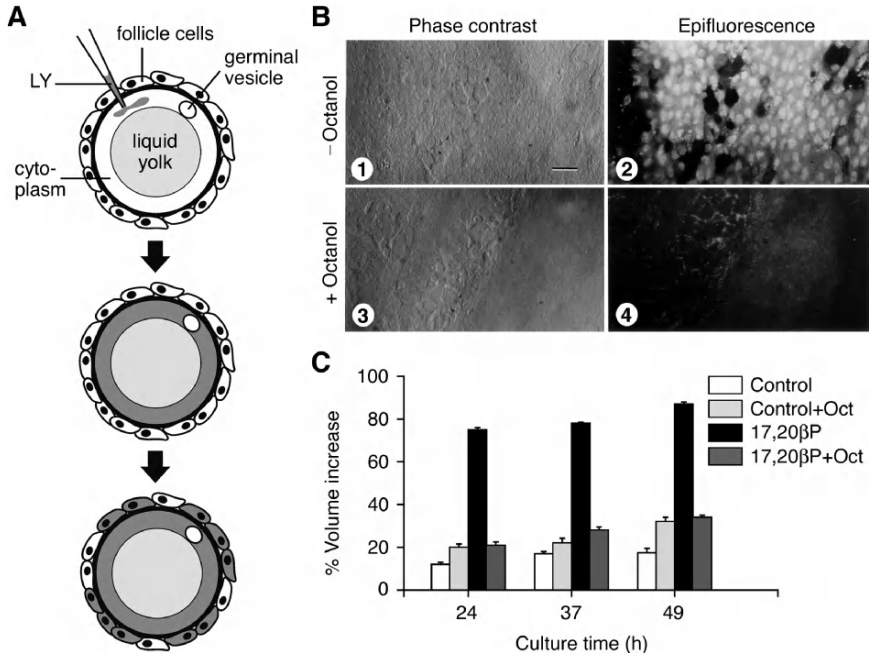

Figure 5. Heterologous gap junctions (GJ) in *F. heteroclitus* ovarian follicles. (**A**) Procedure for testing GJ communication between the oocyte and granulosa cells by the transfer of Lucifer Yellow (LY). After microinjection of LY into the oocyte, it is observed whether the dye is transferred through functional GJ to the overlying granulosa cells (presence of "dye coupling"). (**B**) Effective dye coupling between the oocyte and granulosa cells (panels 1 and 2) and inhibition by 1.5 mM of the GJ inhibitor octanol (panels 3 and 4). Panels 1 and 3 show phase-contrast images of vitelline envelope/granulosa cell preparations; panels 2 and 4, the same preparations examined for epifluorescence. The patchy fluorescence in panel 4 is due to pieces of oocyte cytoplasm associated with the vitelline envelope. Magnification bar, 20 μm. (**C**) Effect of octanol on 17, 20βP-induced oocyte swelling *in vitro*. (B) and (C) show data from Cerdà et al. (1993).

contacts between the oocyte and granulosa cells (Chang et al., 2000; Choi and Takashima, 2000; Yoshizaki et al., 2001; Bolamba et al., 2003). Therefore, these observations would be consistent with a potential role of GJs for K^+ translocation into the maturing oocyte also in pelagophil teleosts.

4. PROCESSING OF YOLK PROTEINS DURING OOCYTE MATURATION AND HYDRATION

In teleosts, as in other oviparous vertebrates, very low-density lipoproteins and Vgs are produced in the liver under estrogen regulation, secreted into the bloodstream and incorporated into growing oocytes by receptor-mediated endocytosis (see Chapter 2). During this process, Vgs are cleaved into smaller molecular

weight polypeptides, known as the yolk proteins, lipoproteins (lipovitellins (Lvs)), highly phosphorylated proteins (phosvitin and phosvetes) and β′component, that will be stored in the oocyte throughout the growth period. During oocyte maturation in marine teleosts, a second proteolytic event on yolk proteins is activated by the MIS before ovulation, which signifies the source of FAAs for oocyte hydration.

4.1. Ultrastructural Changes of Yolk Inclusions During Oocyte Maturation

During oocyte growth, Vg-derived yolk proteins are initially stored in yolk platelets or, most commonly among teleosts, in fluid-filled yolk spheres, also called "yolk globules" or "yolk bodies", forming within the peripheral ooplasm. It is important to note that the term "yolk vesicles" or "yolk vacuoles" are misnomers since these structures present in small oocytes correspond to nascent cortical alveoli that do not contain yolk *per se* and therefore should not be confused with yolk-containing inclusions (Wallace and Selman, 1981; Selman et al., 1986; Masuda et al., 1986; Selman et al., 1988). In pelagophil teleosts, the membrane-bound yolk globules usually maintain their integrity throughout oocyte growth, and yolk proteins stored in the globules initially accrue within crystalline inclusions that can be visualized at the electron microscopy level (Flegler, 1977; Lange, 1980; Kjesbu and Kryvi, 1989; Selman and Wallace, 1989; Selman et al., 2001) (Figure 6). During oocyte maturation and hydration, yolk globules fuse centripetally, eventually forming a single and central mass of fluid yolk less electron-dense at the electron microscopy level which displaces the ooplasm into a peripheral rim surrounding the yolk mass (Wallace and Selman, 1981; Oshiro and Hibiya, 1982; Iwamatsu et al., 1988; Kjesbu and Kryvi, 1993; Selman et al., 2001). During this process, the ordered crystalline structures within the globules generally disassemble, which confers on such oocytes their characteristic transparency (Yamamoto, 1957; Oshiro and Hibiya, 1982; Wallace and Selman, 1981; Iwamatsu et al., 1988; Kjesbu and Kryvi, 1993; Selman et al., 2001) (Figure 1A and Figure 6C–F). Both yolk globule fusion and disassembling of crystalline inclusions during maturation might be K^+ dependent (Selman et al., 2001).

In the oocytes of marine benthophil species, the fusion of generally non-crystalline yolk globules into a single yolk mass can occur at different oocyte stage depending on the species. For instance, this occurs relatively soon after the initial formation of yolk spheres as in sticklebacks (*Gasterosteus aculeatus* and *Apeltes quadracus*; Wallace and Selman, 1981), or during the later stages of vitellogenesis as in the goldfish (Yamamoto and Yamakaki, 1961), sheepshead minnow (*Cyprinodon variegatus*; Selman and Wallace, 1982; Wallace, 1985), and killifish (Selman and Wallace, 1986). In the latter species, the fusion of yolk spheres is generally accelerated and terminated during oocyte maturation, resulting in a single mass of fluid yolk that occupies most of the oocyte cytoplasm and displaces the cortical alveoli to the periphery (Selman and Wallace, 1986; Selman et al., 1988). However, in some freshwater benthophil fish, such as the

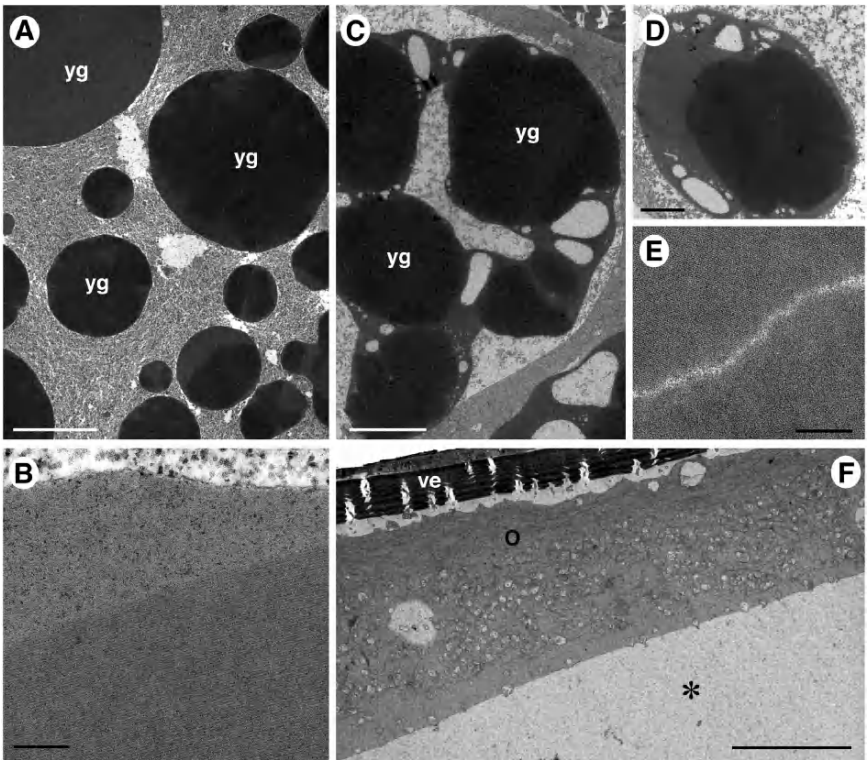

Figure 6. Transmission electron micrographs of yolk globules in the ooplasm of the pelagophil teleost gilthead sea bream. (**A**) Membrane-limited yolk globules with irregular shaped crystalline inclusions in a post-vitellogenic oocyte (Bar, 5 μm). (**B**) Increased higher magnification micrograph of a portion of a crystalline inclusion within a membrane-bound yolk globule (Bar, 0.2 μm). (**C**) Transformation of spherical yolk globules into a single fluid-filled yolk mass during oocyte maturation (Bar, 5 μm). (**D**) Detail of a fragmenting yolk globule (Bar, 2 μm). (**E**) Higher magnification of a fragmented crystalline inclusion within fusing yolk globules (Bar, 0.2 μm). (**F**) Cortical region of a mature and hydrated oocyte showing only a thin rim of ooplasm surrounding a single mass of fluid yolk (asterisk), which is devoid of crystalline inclusions and appears flocculent (Bar, 10 μm). *Abbreviations*: yg – yolk globule; ve – vitelline envelope; O – ooplasm.

zebrafish (*Danio rerio*), yolk globules contain large crystalline inclusions, which lose their structure and develop an homogeneous interior during oocyte maturation (Yamamoto and Oota, 1967; Ulrich, 1969; Selman et al. 1993). In this case, yolk globules do not fuse into a single yolk mass, and yolk bodies in matured oocytes appear identical to those in eggs but they have a slightly larger size and smoother and less angular contour than the yolk inclusions in prematurational oocytes (Figure 7).

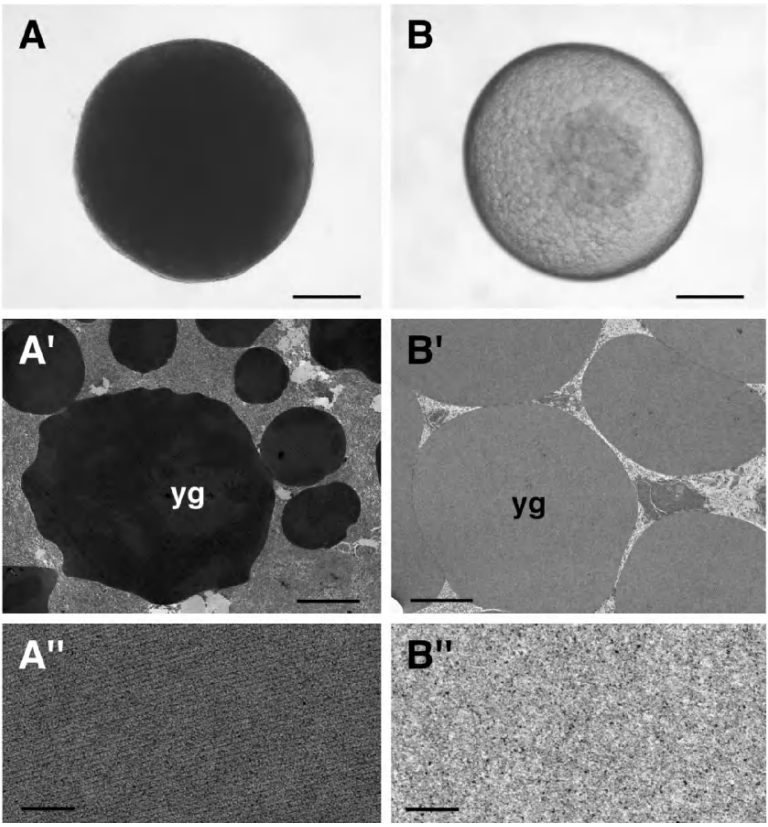

Figure 7. Morphological and ultrastructural changes of yolk inclusions during oocyte maturation in the freshwater benthophil teleost *Danio rerio*. (**A**–**B**) Photomicrographs of manually isolated ovarian follicles containing immature (A) and mature (B) oocytes (Bars, 200 μm). (**A′**–**B′**) Low-magnification electron micrographs of yolk globules (yg) in immature (A′) and mature (B″) oocytes. The crystalline nature of the yolk bodies is not apparent at this magnification (Bars, 5 μm). (**A″**–**B″**) Higher magnification micrographs of crystalline (A″) or homogeneous (B″) yolk in immature and mature oocytes, respectively (Bars, 0.2 μm).

4.2. Pattern of Yolk Protein Processing in Pelagophil and Benthophil Teleosts

The ultrastructural changes of yolk globules that can be observed during teleost oocyte maturation are associated to biochemical modifications of the yolk proteins. These modifications include an extensive or limited proteolysis of Lvs, phosvitins, and β′ components, derived from both Vg1 and Vg2, into smaller peptides and FAAs (Wallace and Selman, 1985; Wallace and Begovac, 1985; Matsubara and Sawano, 1995; Matsubara et al., 1995; Matsubara and Koya, 1997).

The coding sequence of vertebrate *vg* genes is arranged in linear fashion with respect to yolk protein domains as follows: NH_2-Lv heavy chain (LvH)-phosvitin (polyserine domain)-Lv light chain (LvL)-COOH (Nardelli et al., 1987) (Figure 8A). Recent evidences in pelagophil teleosts indicate that yolk proteins are differentially processed during oocyte maturation. In barfin flounder (*Verasper moseri*), Matsubara et al. (1999) have demonstrated that two different LvH components, derived from Vg1 and Vg2 (also called VgA and VgB, respectively) are found in post-vitellogenic oocytes. During oocyte maturation, dimeric LvH2 is dissociated into a monomer, whereas LvH1 is completely proteolytically degraded into FAAs. In addition, phosvitins and β′ components derived from both Vgs are extensively degraded during maturation, while LvL1 and LvL2 appear to be partially processed (Figure 8B). These authors further suggested that the degradation of LvH1 substantially contributes to the FAA pool utilized for water uptake and oocyte hydration, while LvH2 and LvLs are mainly stored in the oocyte as an energy and nutrient source for subsequent embryonic development (Matsubara et al., 1999; Ohkubo and Matsubara, 2002).

Two forms of LvH derived from Vg1 and Vg2 have also been found in the oocytes of another pelagophil teleost, the haddock (*Melanogrammus aeglefinus*), in which LvH1 is completely degraded during egg formation while LvH2 undergoes a slight reduction in size (Reith et al., 2001). In this species, the FAA profile of eggs is strikingly similar to the amino acid profile of LvH1, which is consistent with the significant contribution of this yolk component to the FAA pool of ovulated eggs (Reith et al., 2001). The yolk protein profile of oocytes and eggs from other studies indicate that many pelagophil teleosts apparently have two different Lvs, potentially derived from two different *vg* genes, since they have similar SDS-polyacrylamide gel patterns as those found in barfin flounder and haddock (Carnevali et al., 1992, 1993; Okumura et al., 1995; Matsubara et al., 1995; Matsubara and Sawano, 1995; Cerdà et al., 1996; Thorsen and Fyhn, 1996; Matsubara and Koya, 1997; Fyhn et al., 1999; Selman et al., 2001; Finn et al., 2002a, b; Fabra et al., 2005). Therefore, these data may suggest a correlation between an increase in FAAs and the degradation of LvH1 during the maturation and hydration of pelagophil oocytes, implying that LvH1 is the major source of FAA in all marine teleosts (Reith et al., 2001).

However, in some marine benthophil species as the killifish, where two forms of Vg were also isolated and sequenced (LaFleur et al., 1995a, b), the Vg-yolk protein precursor/product relationships and further processing during oocyte maturation and hydration seem to be different to those described for pelagophil teleosts. In this species, the two major high-molecular weight yolk proteins of immature oocytes (122 and 103 kDa, respectively) correspond to LvH1, whereas Vg2 seem to play only a minor role in yolk protein formation (LaFleur et al., 2005). During oocyte maturation, only the higher LvH1 (122 kDa) and phosvitins are completely degraded (Wallace and Selman, 1985; Wallace and Begovac, 1985; Greeley et al., 1986; LaFleur et al., 2005), while only a slight

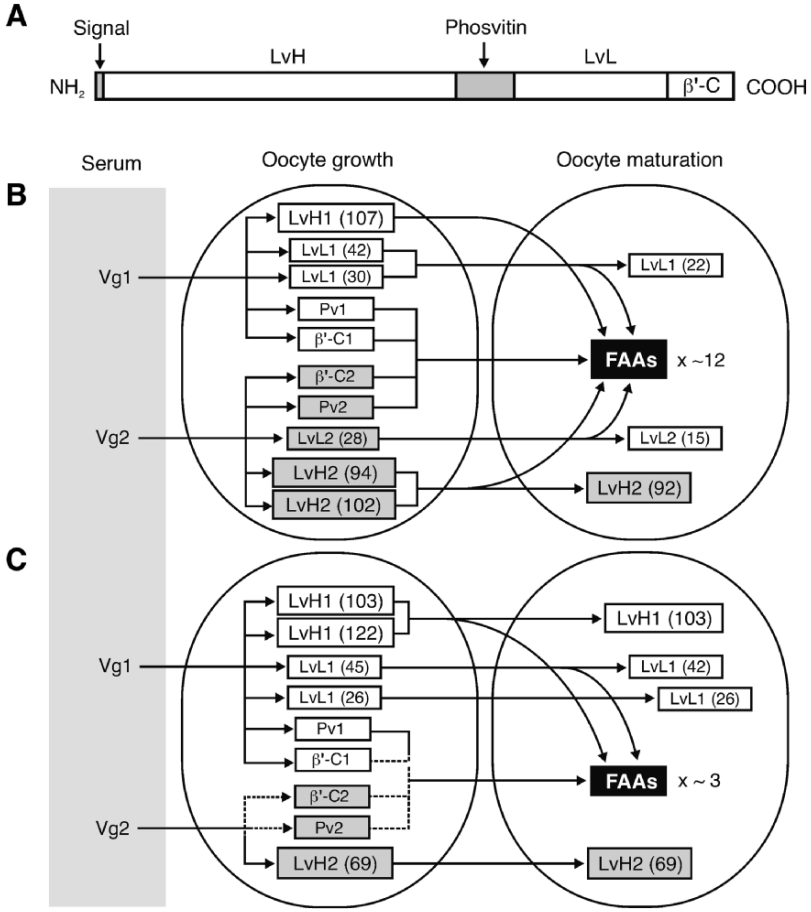

Figure 8. Processing of vitellogenin (Vg) and Vg-derived yolk proteins (YPs) during oocyte growth and maturation. (**A**) Amino acid sequence of Vg showing the domains corresponding to lipovitellin heavy chain (LvH), phosvitin (Pv), lipovitellin light chain (LvL) and β′-component (β′–C). (**B**–**C**), Flow chart describing molecular alterations of Vg1, Vg2, and YPs in pelagophil (B) and benthophil (C) fish. Dotted lines indicate hypothetical pathways. Proteolysis of YPs results in approximately 12- and 3-fold increase in the absolute amount of free amino acids (FAAs) in pelagophil and benthophil oocytes, respectively. The apparent molecular mass of LvH and LvL is indicated in parenthesis. (Modified from LaFleur et al., 2005.)

increase in FAAs is observed (Greeley et al., 1991; Wallace et al., 1992) (Figure 8C). Interestingly, two LvH1 have also been found in vitellogenic ovaries of another benthophil teleost, the white perch (*M. americana*) (Hiramatsu et al., 2002b). However, in the zebrafish, where almost no oocyte hydration is noted and therefore there is no need for FAA to drive water uptake (Tables 1 and 2), a limited proteolysis of a most likely Lv-related yolk protein is also detected (Selman et al., 1993). Therefore, it is apparent that Vgs are differentially

processed into yolk proteins in pelagophil and benthophil teleosts, and that the presence of LvH1 in benthophil oocytes, and its complete or limited degradation during meiotic maturation, does not necessarily correlate with a dramatic increase in FAAs and subsequent hydration.

Both LvH1 and LvH2 in haddock appear to span the same region of their corresponding Vg precursors, which clearly undergo the same proteolytic processing steps to generate LvHs in post-vitellogenic oocytes (Reith et al., 2001). However, during oocyte maturation and hydration complete degradation of LvH1, but only C-terminal processing of LvH2, occurs. In killifish, both LvHs derived from Vg1 also appear to be differentially processed during oocyte maturation (LaFleur et al., 2005). Apparently, differences in the amino acid sequence, secondary structure, or compartmentation of the LvHs within the same or separated yolk globules, might allow the protease(s) that are activated or synthesized during oocyte maturation to distinguish between the two LvHs. Interestingly, in the killifish a proteolytic consensus signal (PEST site; Rogers et al., 1986) has been found near the C-terminus of the 122 kDa LvH1 that becomes degraded during maturation, which is missing from its stable, but truncated twin sequence, the 103 kDa LvH1 (LaFleur et al., 2005). The 122 kDa LvH1, once generated from Vg1, is stable until oocytes undergo maturation, so presumably the proteolysis-triggering PEST site only becomes exposed to the appropriate protease at this time due to a change in intracellular compartmentation. However, PEST sites are found in Vg1 sequences from other cyprinodontiform teleosts, such as common carp (*Cyprinus carpio*) and medaka (*O. latipes*), and in petromyzontiformes, such as silver lamprey (*Ichthyomyzon unicuspis*), but they are missing in both Vg1 and Vg2 sequences from marine pelagophil teleosts. Thus, the mechanisms underlying the differential processing of LvHs during oocyte maturation in teleosts are still unclear.

4.2.1. Role of lysosomal cysteine proteinases during yolk protein proteolysis

Cysteine proteinases, particularly cathepsins L and B, and aspartic proteases, such as cathepsin D, are the major degradative enzymes in the yolk of invertebrate and vertebrate embryos (Fagotto, 1990a, b; Mallya et al., 1992; Cho et al., 1999; Gerhartz et al., 1999; Yoshizaki et al., 1998; Yamahama et al., 2003; Yoshizaki et al., 2004). Evidence available in marine and freshwater teleosts may indicate a similar scenario (Murakami et al., 1990; Sire et al., 1994; Carnevali et al., 1999b, 2001b; Kestemont et al., 1999; Hiramatsu et al., 2002a). However, much less work has been carried out to identify the proteases involved in the proteolysis of Vg-derived yolk proteins during teleost oocyte maturation and hydration. Recent studies by Carnevali et al. (1999a) on the pelagophil gilthead sea bream (*Sparus aurata*) have found high levels of cathepsin D and B activities in isolated early vitellogenic oocytes, whereas cathepsin L activity is higher than that of cathepsin B and D in follicles at mid-vitellogenesis stage. Isolated cathepsin L from the sea bream ovary was able to cleave purified Lv *in vitro* under appropriate conditions, and based on this data the authors suggested that

cathepsin L is most likely the protease involved in the oocyte maturation-associated proteolysis of Lv *in vivo* (Carnevali et al., 1999a). These data might agree with further observations in gilthead sea bream indicating high cathepsin L activity in fertilized buoyant eggs (Carnevali et al., 2001a), and with other studies in trout showing high cathepsin L mRNA levels in late vitellogenic oocytes (Kwon et al., 2001).

In benthophil teleosts, however, the enzymatic machinery involved in yolk protein processing during oocyte maturation appears to be different. In the killifish, the enzyme activity of cathepsin L shows a dramatic decrease during oocyte maturation both *in vivo* and *in vitro* (LaFleur et al., 2005), despite that its mRNA increases approximately threefold during MIS-induced meiotic maturation (Fabra and Cerdà, 2004). By contrast, the activity of cathepsin B increases transiently during maturation, coincident with the time of maximum degradation of the 122 kDa LvH1 (LaFleur et al., 2005). These observations thus suggest that cathepsin B, rather than cathepsin L, is the major protease involved in LvH1 degradation in killifish oocytes. Interestingly, a similar pattern of cathepsin B and L enzyme activities has been reported during late maturation of zebrafish oocytes *in vivo* (Cionna et al., 2004). The cause for these apparent differences between pelagophil and benthophil species regarding the specific protease(s) involved in the degradation of LvH components remain however intriguing. The fact that in these species LvH1 and LvH2 seem to be processed differently during vitellogenesis and oocyte maturation, and that the FAAs derived from this proteolysis appear to play distinct roles during hydration, are factors that could be related.

4.2.2. Acidification of yolk inclusions and cathepsin activation

During oocyte growth, yolk proteins are stored within yolk platelets or globules under mildly acidic conditions maintained by a V-ATPase (Mallya et al., 1992; Fagotto and Maxfield, 1994a). In the embryo of most animals, a second V-ATPase-mediated acidification of yolk inclusions triggers further digestion of the stored proteins. Thus, in insects, the major acid proteases are stored in the yolk bodies as a latent, acid-activable proenzymes (Fagotto, 1990b; Cho et al., 1999; Yamahama et al., 2003). These are initially neutral, but they become acidic during development, causing maturation and/or activation of the proenzymes, procathepsin L or procathepsin B, and yolk degradation (Fagotto, 1990b; Nordin et al., 1991; Cho et al., 1999; Yamahama et al., 2003). Acidification of yolk platelets and its relationship with the onset of yolk proteolysis has also been reported in the clawed frog (*Xenopus laevis*) (Fagotto and Maxfield, 1994b) and sea urchin (Mallya et al., 1992), where in the latter the activity of a cathepsin B-like enzyme is regulated by changes in pH.

In teleosts, recent findings suggest that a similar pH-mediated mechanism might control the onset of maturation-associated yolk processing in both pelagophil and benthophil species. In the pelagophil black sea bass, Selman et al. (2001) reported that bafilomycin A_1, an inhibitor of V-ATPase at the

nanomolar range, blocks proteolysis of Lv, generation of FAAs and concomitant oocyte hydration of hCG-treated ovarian follicles *in vitro*. However, bafilomycin A_1 does not prevent GVBD or the disassembly and fusion of yolk crystalline inclusions during maturation (Selman et al., 2001). Similar effects of bafilomycin A_1 have been recently obtained in gilthead sea bream oocytes (see Figure 3A, C). These results are thus consistent with the hypothesis that V-ATPase-regulated acidification of yolk compartments is necessary for appropriate yolk hydrolysis in pelagophil teleosts. The K^+ translocation into the oocyte during maturation, which would not be affected by bafilomycin A_1, may be responsible for yolk crystal disassembly (Selman et al., 2001). This latter process may serve to render yolk proteins available to protease action, although the specific mechanism by which these enzymes may be activated is still unknown.

In killifish, the V-ATPase inhibitors bafilomycin A_1 and concanamycin A prevent acidification of yolk globules as directly determined by fluorescent microscopy using the cell-permeable acidic pH probe N-(3-((2, 4-dinitrophenyl)-amino propyl)-N-(3-aminopropyl)-methylamine, dihydrochloride) (DAMP) (Raldúa et al., 2006) (Figure 9). In this species, however, acidification of yolk globules is already noted in post-vitellogenic oocytes before maturation, which is consistent with the occurrence of yolk globule fusion, possibly associated with the processing to some extent of yolk proteins, during the later stages of vitellogenesis (Selman and Wallace, 1986). The activation of oocyte maturation *in vitro* by the MIS potentiates the activation of procathepsin B into active enzyme and accelerates the hydrolysis of the 122 kDa LvH1 (Raldúa et al., 2006). Bafilomycin A_1-induced increase of internal pH during oocyte maturation *in vitro* inhibits cathepsin B activation and enzyme activity, preventing yolk proteolysis, thus confirming that this protease is most likely the enzyme regulated by V-ATPase responsible for LvH1 degradation in killifish. However, it is unknown whether cathepsin B is located in the ooplasm or within yolk structures, as cathepsin D (Sire et al., 1994), where it might be activated through maturation of the proenzyme by the action of another cysteine proteinase or simply by changes in pH (i.e. autoprocessing). Cathepsins are normally delivered to lysosomes as proenzymes (Hasilik, 1992), and since bafilomycin A_1 has been found to suppress indirectly the fusion of lysosomes into target vacuoles (Pillay et al., 2002), an inhibition of the delivery of cathepsin B to yolk globules during MIS-stimulated oocyte maturation is another potential mechanism that cannot be ruled out. In any event, the details of cathepsin B and L regulation in fish oocytes, including the mechanism by which the V-ATPase becomes activated by hormonal stimulation, remain to be elucidated.

4.2.3. Role of yolk proteolysis in benthophil teleosts

Marine benthophil and freshwater fish show varying degrees of Lv degradation during oocyte maturation (Greeley et al., 1986; Iwamatsu et al., 1992; Selman et al., 1993; Finn et al., 2002b). Given that in these species low or almost no hydration of the oocyte is noted, and that the Lv-derived pool of FAAs is

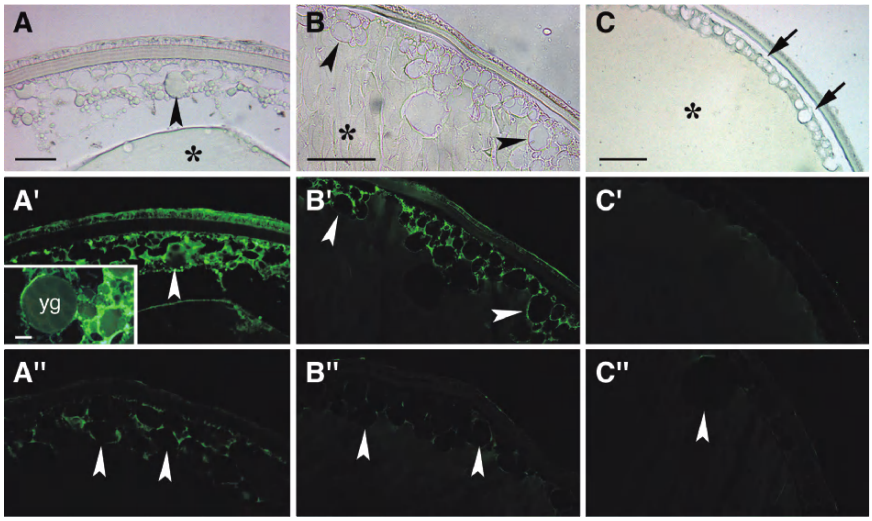

Figure 9. Light microscopy (**A–C**) and fluorescent images of *in vitro* maturing *F. heteroclitus* oocytes in the absence (**A′-C′**) or presence (**A″–C″**) of 100 nM bafilomycin A_1 (BA_1), labelled with the cell-permeable acidic pH probe 3-((2, 4-dinitrophenyl)-amino)propyl)-N-(3-aminopropyl)-methylamine, dihydrochloride) (DAMP). (**A′–A″**) Prematuration oocytes show acidic compartments surrounding the yolk globules (inset), and this acidification is reduced by BA_1 treatment. (**B′–B″**) Oocytes undergoing oocyte maturation show BA_1-sensitive acidic compartments located in a more peripheric area than in immature oocytes, where the fusion of the yolk globules into the central mass of fluid yolk takes place. (**C′–C″**) Yolk globule acidification is no longer visible in mature oocytes, when the fusion of yolk inclusions and proteolysis of yolk proteins finishes. Arrowheads point yolk globules (yg), and the asterisk indicates the central mass of fluid yolk. In C, the arrows point cortical alveoli at the periphery of mature oocytes. Scale bars, 50 μm (A), 20 μm (A′, inset), 100 μm (B and C). (Data from Raldúa et al., 2006) (*See Color Plates*).

modest, the physiological significance of this process is not well understood. In these cases, however, the degradation of Lv may be mainly related to the generation of FAAs and small peptides that are believed to serve as a major substrates for energy production via aerobic metabolism and for protein synthesis during embryogenesis (Fyhn and Serigstad, 1987; Fyhn, 1989; Rønnestad and Fyhn, 1993; Rønnestad et al., 1992, 1993; Finn et al., 1995; Ohkubo and Matsubara, 2002). The multiple functions of FAAs during fish egg and embryo development has been reviewed by Wright and Fyhn (2001).

Recent findings in the killifish, however, suggest that proteolysis of LvH1 during meiosis resumption may also play an important role during oocyte hydration, in contrast to what was previously suggested (McPherson et al., 1989). In this species, inhibition of LvH1 proteolysis *in vitro* in K^+-containing mediums, through the suppression of yolk globule acidification by bafilomycin A_1, reduces K^+ influx into the oocyte and strongly diminishes subsequent hydration (Raldúa et al., 2006). These surprising observations are however consistent with

Ling's association-induction hypothesis on the behaviour of small ions within cells (Ling, 1990), which implicates that chemical modification of yolk proteins in the oocyte could have profound effects on the ion-binding properties of the resulting peptides newly exposed to an aqueous environment. Therefore, bafilomycin A_1-mediated inhibition of LvH1 cleavage may reduce the generation of new K^+-binding sites, thus preventing the accumulation of K^+ in the oocyte, and hence reducing the increase of the osmotic pressure. This mechanism would thus imply the passive diffusion of K^+ from capillary beads to the oocyte, most likely through heterologous GJs (Cerdà et al., 1993), and would not require the presence of a Na^+, K^+-ATPase to accumulated K^+ in follicle cells, which has not been found in killifish ovarian follicles (Wallace et al., 1992). Therefore, proteolysis-facilitated accumulation of K^+ in the oocyte, along an electrochemical gradient, would be the mechanism for the hydration of killifish oocytes. Although this system needs to be directly documented, it would suggest that proteolysis of yolk proteins is essentially involved in the process of oocyte hydration in both benthophil and pelagophil teleosts, reinforcing the notion that the hydration of fish oocytes is generated by the oocyte itself rather than by the associated follicle cells.

5. MECHANISMS FOR WATER TRANSPORT INTO THE FISH OOCYTE

The increase of the osmotic pressure of the maturing oocyte, as a result of the accumulation of FAAs and inorganic ions, signify the major driving force for oocyte hydration in marine teleosts. According to what was long assumed in physiology, water transport into the oocyte was predicted to occur by simple diffusion through the lipid bilayers. However, the recent discovery of molecular water channels, or aquaporins (AQPs), in virtually every living organism, from bacteria to humans (reviewed by Agre et al., 1993, 2002), has recently lead to the investigation of the molecular mechanisms involved in water transport in fish oocytes.

5.1. The Aquaporin Gene Superfamily

AQPs are molecular channels that transport water along an osmotic gradient, where the direction of water flow is determined by the orientation of the gradient. These channels consist of six transmembrane domains, which are connected by five loops (A–E) and have their N- and C-termini located intracellularly (Figure 10). One molecule consist of two repeats, which are 180° mirror images of each other. Each repeat contains the highly conserved asparagine–proline–alanine (NPA) motifs (in loops B and E), which is the hallmark of the major intrinsic proteins (MIP) family to which AQPs belong. The folding of the loops B and E is important for the formation of the water pore (Fujiyoshi et al., 2002; Harries et al., 2004), and the Cys^{189} located close to loop E is responsible for the

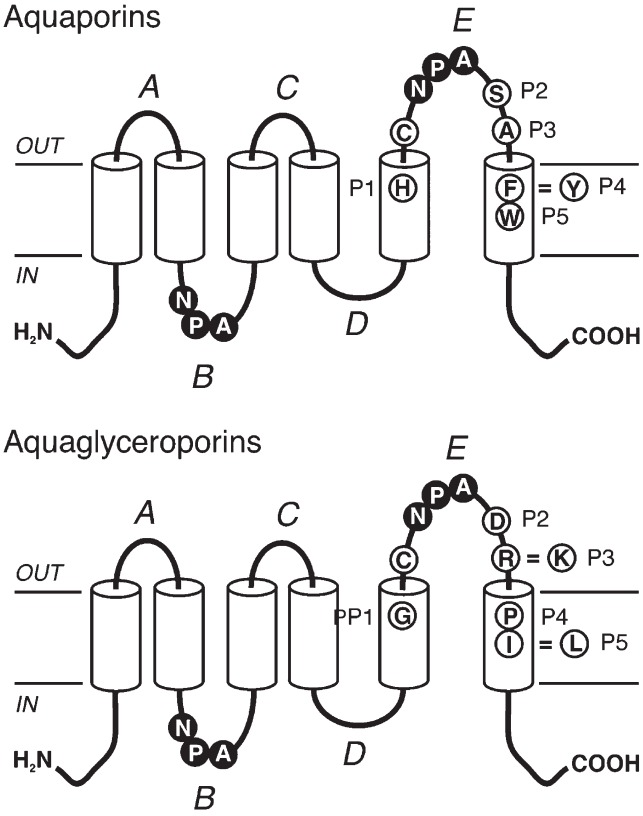

Figure 10. Topological models for aquaporins and aquaglyceroporins showing the six transmembrane helices and the two NPA motifs located in loops B and E. The P1–P5 residues conserved in each of the two aquaporin clusters are indicated (=, denotes functionally conserved substitutions). (Based on Stahlberg et al., 2001)

inhibition of AQP water permeability by submillimolar concentrations of Hg^{2+} (Preston et al., 1993; Shi and Verkman, 1996). Although most AQPs form homotetramers, in which one or two monomers are glycosilated, radiation inactivation and other studies have shown that in AQPs each monomer is an independent water channel (Walz et al., 1994; Kamsteeg et al., 2000; Sui et al., 2001).

Homology cloning by degenerated oligonucleotide polymerase chain reaction (PCR) based on the amino acid sequences of AQP1 and other members of the MIP family, have been employed to isolate different AQP members. At least, approximately 13 different AQPs have been identified in mammals (Agre et al., 2002), and about 38 MIP-related sequences, divided into four types, PIP, TIP, NIP, and SIP, are found in plants (Chrispeels et al., 2001). The AQPs all are about 30 kDa and form at least two subgroups based on their permeability

properties when heterologously expressed in *X. laevis* oocytes: those that are water-selective channels (AQP0, AQP1, AQP2, AQP4, AQP5, and AQP8), and those that are also permeable to small solutes, such as glycerol and urea (also called aquaglyceroporins; AQP3, AQP7, AQP9, and AQP10) (Agre et al., 2002). In general, the AQPs and aquaglyceroporins can be distinguished based on five particular amino acids, called P1–P5, surrounding the narrowest region of the pore in loop E (Stahlberg et al., 2001) (Figure 10). Aquaporin-6 (AQP6) is a particular AQP since, although weakly transports water, it is also an ion gated channel with intermediate selectivity for anions, and both anion and water permeabilities are activated by low pH and low concentrations of $HgCl_2$ (Yasui et al., 1999a, b; Holm et al., 2004). The functional repertoire of AQPs however is also extended to other solutes. For instance, AQP9 is also permeable to arsenite, $As(OH)_3$ (Liu et al., 2002), and a wide variety of non-charged solutes (Tsukaguchi et al., 1999), AQP1 can transport CO_2 which is of physiological importance when the CO_2 gradient across a membrane is small (Blank and Ehmke, 2003; Uehlein et al., 2003), and AQP8 and other plant AQPs (TIP2) can transport NH_4^+/NH_3 (Jahn et al., 2004). Other AQP-like channels with poorly conserved NPA motifs (AQP11 and AQP12) have also been recently found in mammals and other multicellular organisms, named superaquaporins as they belong to the AQP superfamily, but their functional properties are yet not well characterized (Morishita et al., 2004).

5.1.1. Physiological roles of aquaporins in vertebrates

In mammals, AQPs are widely distributed in different tissues and cell types, including the ovary, with important water transport or secretory roles (reviewed by Borgnia et al., 1999; Takata et al., 2004). However, despite its extensive tissue distribution, the physiological significance of most of AQPs is still to be elucidated, in part due to the unavailability of AQP specific inhibitors. One exception is the role of AQP2 in the mammalian kidney, where it is known that this water channel mediates water reabsorption and urine concentration in the renal collecting duct in response to the arginine–vasopressin hormone (reviewed by Deen et al., 2000). The use of transgenic knockout mice in which individual AQPs have been selectively deleted by targeted gene disruption has provided recently new insights into the role of AQPs in kidney, eye, and brain physiology (reviewed by Verkman, 1999, 2000, 2003; Manley et al., 2004).

In non-mammalian vertebrates, such as fish and amphibians, much less is known on AQP distribution and function. In amphibians, AQP1 homologues have been cloned from the urinary bladder of *Rana esculenta* (Abrami et al., 1994) and *Bufo marinus* (Ma et al., 1996), and more recently, three different AQPs, AQP-h1, AQP-h2, and AQP-h3, have been cloned from the ventral skin of the tree frog, *Hyla japonica* (Tanii et al., 2002; Hasegawa et al., 2003). Aquaporin-h1 (AQP-h1) is homologous to mammalian AQP1, whereas both AQP-h2 and AQP-h3 are most similar to mammalian AQP2 and seem to be vasotocin-regulated playing a role in water balance of the frog body (Hasegawa

et al., 2003). In the oocyte of *X. laevis*, Virkki et al. (2002) have recently identified a novel aquaglyceroporin strongly expressed in the oocyte and fat body, named AQPxlo, which presents unique permeability properties to glycerol and urea, but its physiological role is unknown.

In teleosts, within the completed or nearly completed genomes, such as zebrafish, fugu (*Takifugu rubripes*) and "smooth" pufferfish (*Tetraodon nigroviridis*), about fifteen different sequences per species related to the MIP family can be identified, including unusual AQPs related to mammalian super-aquaporins. This might indicate a similar distribution of AQPs in teleosts as in higher vertebrates, although their specific functional properties have not been yet characterized. In the killifish, however, an AQP0 homologue has been cloned which is specifically expressed in the lens and shows an unusual higher water permeability as compared with human AQP1, and it is weakly permeable to CO2 but not to glycerol or urea (Virkki et al., 2001). In the gilthead sea bream, a novel aquaglyceroporin paralogue permeable to glycerol and urea, with homology to mammalian AQP3 and AQP10, named Glp, has been cloned from a kidney cDNA library (Santos et al., 2004). The Glp is highly expressed in the posterior region of the gut and kidney (Santos et al., 2004), and interestingly, orthologues of this water channel are found in other teleosts, such as fugu (Santos et al., 2004), zebrafish, sea bass, and *Tetraodon*, which suggest that teleosts may have evolved unique aquaglyceroporins after divergence from the tetrapod lineage.

Recent studies in the European eel (*Anguilla anguilla*) and Japanese eel (*A. japonica*) have isolated several cDNAs classified as putative AQP3 and AQP1 since the functional properties of the encoded proteins are unknown (Cutler and Cramb, 2002; Aoki et al., 2003). Nevertheless, both AQPs show different pattern of mRNA expression, while AQP3 is highly expressed in the gill with low levels in the eye, oesophagus, and intestine, the AQP1 mRNA is widely distributed. Semi-quantitative analysis of gene expression showed that the transition from freshwater to seawater acclimation increases AQP1 mRNA in the intestine (Aoki et al. 2003), whereas AQP3 expression in gills strongly decreases (Cutler and Cramb, 2002). These observations suggest that AQP1 may play a pivotal role in regulating water absorption by the intestine, which is an important osmoregulatory organ in teleosts (Wilson et al., 2002). Aquaporin-3 has also been immunolocalized in chloride cells from the gill in the eel, as well as in the Osorezan dace (*Tribolodon hakonensis*) (Lignot et al., 2002; Hirata et al., 2003). In the eel, AQP3 expression seems to be downregulated following seawater acclimation, whereas in the dace transfer of fish to extremely acidic water induces its expression.

5.2. Role of Aquaporins During Fish Oocyte Hydration

Pelagophil teleosts that spawn buoyant eggs highly hydrated offer an excellent experimental model for the investigation of the potential role of AQPs during fish oocyte hydration. In the gilthead sea bream, Fabra et al. (2005) found that

the swelling of the oocyte *in vitro* after stimulation with the MIS, 17α, 20β-dihydroxy-4-pregnen-3-one (17, 20βP), occurred progressively for about 10 h, which reflected a 4.5- and 1.4-fold increase in oocyte volume and relative water content, respectively (Fabra et al., 2005; Table 1). Oocyte hydration, however, appeared to be accelerated within ~2 h following the complete proteolysis of major yolk proteins (Lv), which is presumably time of maximum osmotic pressure within the oocyte. Therefore, the relatively rapid swelling of the sea bream oocyte during meiotic maturation would be compatible with a role of water channels during this process.

To test the possible involvement of AQPs during oocyte hydration, degenerate oligonucleotide primers were designed to the highly conserved amino acid regions of the MIP gene superfamily (Preston, 1997) and employed to isolate AQP cDNAs from the sea bream ovary. This approach resulted in the isolation of a full-length cDNA bearing six potential transmembrane domains and two NPA motifs, the conserved signature of the AQP superfamily (Fabra et al., 2005). The amino acid sequence of the cDNA showed the highest identity to mammalian AQP1, as well as the specific residues surrounding the narrowest region of the pore in loop E that are conserved among water-selective AQPs but not in aquaglyceroporins. Accordingly, the cDNA heterogeneously expressed in *X. laevis* oocytes was selectively permeable for water (but not permeable for glycerol and urea), which could be inhibited by mercury chloride ($HgCl_2$) and reversed with β-mercaptoethanol (βME) (Figure 11), thus showing similar functional characteristics as for the mammalian AQP1 (Preston et al., 1992, 1993). However, the identity found between the newly isolated sea bream cDNA and mammalian AQP1 was slightly lower (60%) than the identity found between putative teleost AQP1 sequences available in the public databases and the mammalian orthologues (62–66%). In addition, unlike the mammalian AQP1 which is ubiquitously expressed in adult tisues (Nielsen et al., 1993a, b), the mRNA of sea bream AQP was found to be predominantly expressed in the ovary, with some expression in gills, kidney, and distal intestine (Fabra et al., 2005). Based on these unique features, it was concluded that the protein encoded by the sea bream ovarian cDNA was a novel AQP paralogue unknown in vertebrates, and it was named the *S. aurata* AQP1 of the ovary (SaAQP1o).

The SaAQP1o water channel has been further localized in the sea bream ovary by using a specific affinity-purified antisera. A strong immunoreaction was observed exclusively in the oocyte and not in the surrounding somatic cells, and it was first noted in the cortical cytoplasm of early vitellogenic oocytes (Fabra et al., 2005, 2006). As oocytes advance into vitellogenesis, SaAQP1o appears to be translocated towards a more peripheral area of the oocyte. When vitellogenesis resumes and the oocyte reaches the fully grown stage, SaAQP1o seems to be completely translocated within a thin layer just below the oocyte plasma membrane. During oocyte maturation, and more specifically at the time when maximum proteolysis of Lv and the fastest period of oocyte swelling occurs, both light and electron microscopy show that SaAQP1o is translocated

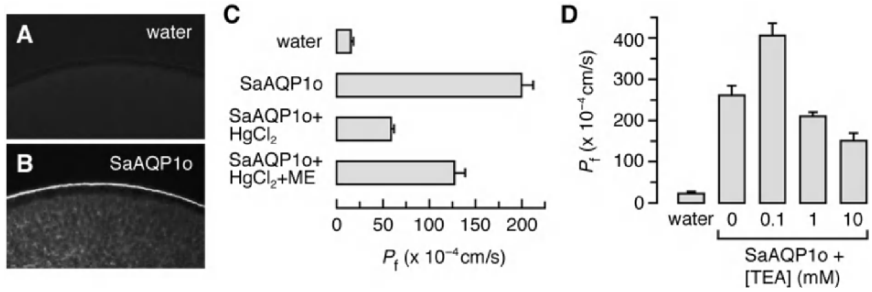

Figure 11. Heterologous expression of SaAQP1o in *Xenopus laevis* oocytes. (**A–B**) Immunofluorescence microscopy of water-injected (A) and SaAQP1o-expressing (B) *X. laevis* oocytes using an antisera against SaAQP1o. Note in (B) that SaAQP1o peptides are translocated into the oocyte plasma membrane. (**C–D**) Osmotic water permeability (P_f) of *X. laevis* oocytes injected with water or SaAQP1o cRNA (C, 1 ng; D, 0.5 ng). (C) Inhibition of water permeability by 1 mM $HgCl_2$ and recovery by 15 min of exposure of oocytes to 5 mM (βME) immediately after mercury treatment. (D) Effect of increasing doses of tetraethylamonium (TEA) on SaAQP1o-mediated water transport. (From Fabra et al., 2005.)

further into the oocyte microvilli crossing the vitelline envelope, a location where it can potentially mediate water transport into the oocyte (Figure 12).

The characteristic pattern of subcellular localization of SaAQP1o during oocyte maturation and hydration has provided circumstantial evidence for a role of this channel mediating water influx into the oocyte. However, to assess the functional relevance of this finding, *in vitro* incubations of ovarian follicles undergoing oocyte maturation in the presence of known AQP inhibitors have been carried out. In addition to $HgCl_2$, the ion channel blocker tetraethylammonium (TEA) was also employed, since this non-mercurial compound reduces SaAQP1o-mediated water transport in *X. laevis* oocytes (Fabra et al., 2006) (Figure 11D), similarly as it occurs for mammalian AQP1 (Brooks et al., 2000; Yool et al., 2002). The treatment of maturing sea bream follicles with $HgCl_2$ effectively inhibited oocyte volume increase in a dose-dependent manner, and such inhibition was reversed by βME, which may indicate an specific effect of $HgCl_2$ on an AQP involved in oocyte water permeability (Fabra et al., 2005). Similarly, the presence of relatively high doses of TEA (10 mM) reduced the swelling of the oocyte during maturation (Fabra et al., 2006). However, low doses of TEA weakly but significantly stimulated sea bream oocyte hydration, but interestingly, the same effect was observed in *X. laevis* oocytes expressing SaAQP1o (Figure 11D). Altogether, these data indicated that the pattern of water permeability of maturing sea bream oocytes in response to $HgCl_2$ and TEA strongly resembled that of *X. laevis* oocytes expressing SaAQP1o. Therefore, these findings have provided evidences for the notion that SaAQP1o is essentially involved in the mechanism of water uptake into the sea bream oocyte. However, both $HgCl_2$ and TEA are effective but unspecific inhibitors of AQPs, and consequently the involvement of SaAQP1o in oocyte hydration awaits direct demonstration by specific inhibitors of SaAQP1o function.

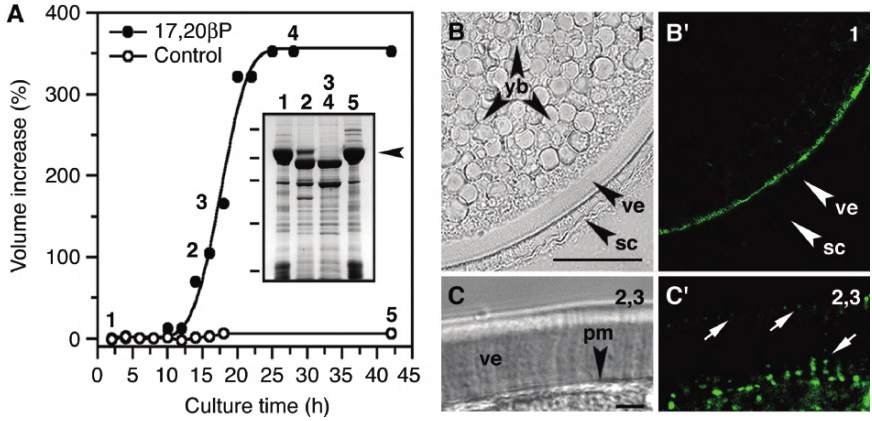

Figure 12. Time-course of oocyte hydration, proteolysis of major yolk proteins, and immunolocalization of SaAQP1o in the oocyte of gilthead sea bream during MIS-activated meiotic maturation *in vitro*. (**A**) Representative volume changes over time of a single oocyte treated with 0.1 mg/mL of MIS (17, 20βP) or ethanol vehicle (control). The inset shows an SDS-PAGE of follicle protein extracts (5 µg/lane) at specific hydration stage (named 1–5). The position (bars) of molecular weight markers is indicated on the left (from top to bottom: 200, 116, 97, 66, and 45 kDa). The arrowhead indicates degradation of lipovitellin of ~100 kDa. (**B–C**) Corresponding immunolocalization of SaAQP1o in oocytes at stage 1 and 2–3. Phase-contrast (**B′–C′**) and immunofluorescencen (**B′–C′**) images. In stage 1 (**B′–B′**), SaAQP1o is located within a thin layer below the oocyte plasma membrane, while during oocyte maturation and hydration, stage 2–3 (**C′–C′**), when proteolysis of the major yolk proteins is complete, SaAQP1o staining is observed within the oocyte microvilli crossing the vitelline envelope (arrows). *Abbreviations*: yb – yolk bodies; gv – germinal vesicle; ve – vitelline envelope; sc – somatic cells; pm, plasma membrane. Scale bars, 50 µm (1), 5 µm (2, 3). (Adapted from Fabra et al., 2005.) (*See Color Plates*).

5.2.1. The teleost AQP1o water channels

By homology cloning based on degenerate oligonucleotide primers, cDNA library screening and genome searching, additional amino acid sequences for fish water-selective AQPs have been recently identified (Tingaud-Sequeira et al., in preparation). Using these new sequences, the phylogenetic analysis of AQP1-like water channels among vertebrates now reveals the presence of two subgroups of AQP1-like channels in teleosts (Figure 13). The first group is composed by orthologues of mammalian AQP1 strictly speaking, which share 64–67% identity with mammalian, avian, and amphibian sequences. The other group is formed by several ovarian AQP1-like proteins related to SaAQP1o that are found in different marine and catadromous species, such as fugu, Senegal sole (*Solea senegalensis*) and European eel, and in one freshwater species, the catfish (*Ictalurus punctatus*). These sequences share 58–62% identity with other vertebrate AQP1, thus slightly lower than that of the teleost AQP1, and only 63–70% identity with teleost AQP1 orthologues. The identity among AQP1o sequences is however quite heterogeneous, from 60% to 82%, the identity between Perciformes (sea bream) and Tetraodontiformes (fugu) being the highest. Most

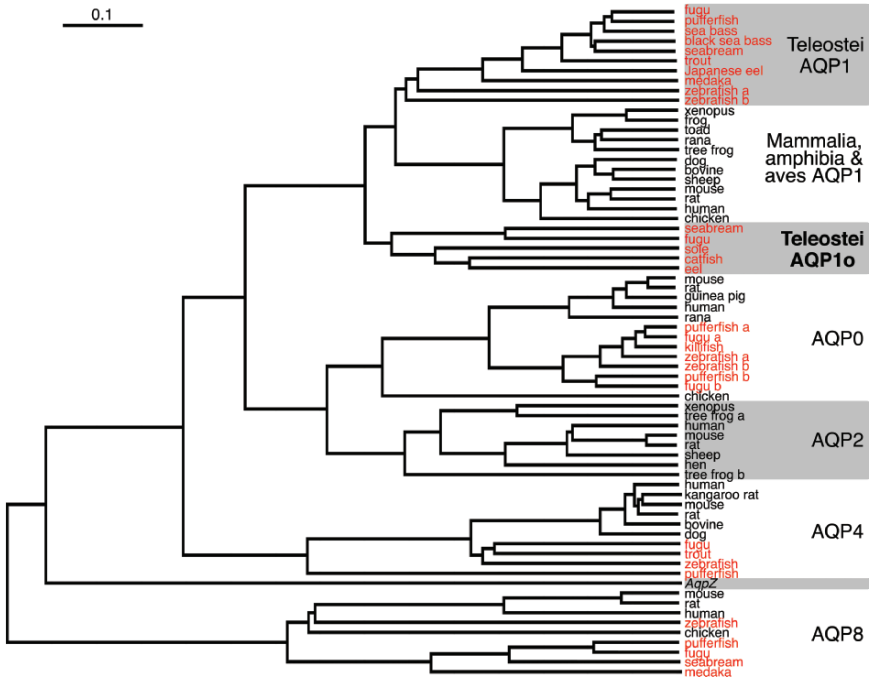

Figure 13. Phylogenetic relationships of water-selective aquaporins in vertebrates. Only the amino acid sequence between the two NPA motifis were used for multiple amino acid sequence alignments. Phylogenetic analyses were performed with the neighbour-joining method, and the *Escherichia coli* water-selective aquaporin homologue (*AqpZ*) was included as outgroup. The fish sequences are highlighted in red, and the scale bar represents genetic distance between homologues.

remarkably, however, is that the teleost SaAQP1o-related AQPs cluster into a distant and separate group from other vertebrate AQP1 proteins, including fish, and show larger branches than those of normal fish AQP1. These findings thus indicate the specific and fast evolution of a new subfamily of ovarian AQPs in teleosts, most likely derived from a common AQP1-like ancestral gene.

The AQP1o-related water channels have been found in marine teleosts from different orders, such as Perciformes, Anguilliformes, Tetraodontiformes, and Pleronectiformes, that all produce hydrated eggs. Catfish (Siluriformes) live in freshwater, but although they do not produce buoyant eggs, in some species oocytes hydrated during maturation (K.P. Joy, unpublished data). Therefore, the expression of an AQP1o-related channel in the ovary of these species may be consistent. The apparent rapid evolution of ovarian AQP1o proteins might be helped by an ancient whole-genome duplication event that probably occurred just before the teleost radiation (Postlethwait et al., 2000, 2004). This mechanism can allow a fast development of one gene copy into a specialized function

(as shown here for SaAQP1o during oocyte hydration), while the old function will be maintained by the remaining AQP1. This may be exemplified by the finding of two AQP1-like and AQP0 proteins in zebrafish which might be the result of the early genome duplication that occurred in the zebrafish lineage (Postlethwait et al., 2000). It is known that during early evolution, fish evolved from seawater to freshwater, but later, during the Jurassic period, fish are thought to have re-entered into seawater (Long, 1995). This transition likely demanded certain osmotic adaptations, and among them the hydration of the oocyte mediated by the proteolysis of yolk proteins, has been suggested as a key step during the evolution of marine teleosts to alleviate the passive water loss of the developing embryo imposed by the hyperosmotic seawater (Fyhn et al., 1999). Aquaporin-1o water channels might have developed in pelagophil teleosts to regulate water uptake into the oocyte for the same purpose, and also to render the eggs buoyant in seawater, thereby improving the survival of the embryo in the ocean. However, AQP1o peptides are also found in the distal intestine, an important osmoregulatory organ in teleosts, which suggest that this channel may have other osmoregulatory functions in marine fish yet unknown (Raldúa et al., unpublished data). Thus, the discovery of the AQP1o subfamily of water channels may illustrate how marine teleosts have evolved specific molecular mechanisms during its evolution to adapt to a hyperosmotic environment.

6. CONCLUSIONS AND PERSPECTIVES

The biological process of egg hydration in fish is a unique and fascinating mechanism of which the molecular processes involved have only started to be uncovered. However, the studies carried out during the last decades allow to establish a basic hypothetical model for fish oocyte hydration integrating early and recently identified mechanisms for both the generation of osmotic solutes and water transport (Figure 14). As indicated in this model, the main osmotic effectors for water uptake into the fish oocyte are both organic and inorganic solutes. In early studies, the generation of a yolk protein-derived FAA pool was mainly associated with pelagophil species, whereas the accumulation of ions was usually associated with benthophil species. The most recent studies, however, strongly suggest that FAAs and ions play equal important roles during oocyte hydration in both pelagophil and benthophil species. Thus, the main osmotic effectors for oocyte hydration would be conserved among teleosts, although different species may employ these solutes differently depending on the production of buoyant or non-buoyant eggs, and the requirements of their specific habitat. Nevertheless, the specific molecular mechanisms for solute generation in the oocyte still need to be elucidated, such as the hormonally controlled cellular processes for selection and degradation of yolk proteins, or the mechanisms for specific ion translocation into the maturing oocyte.

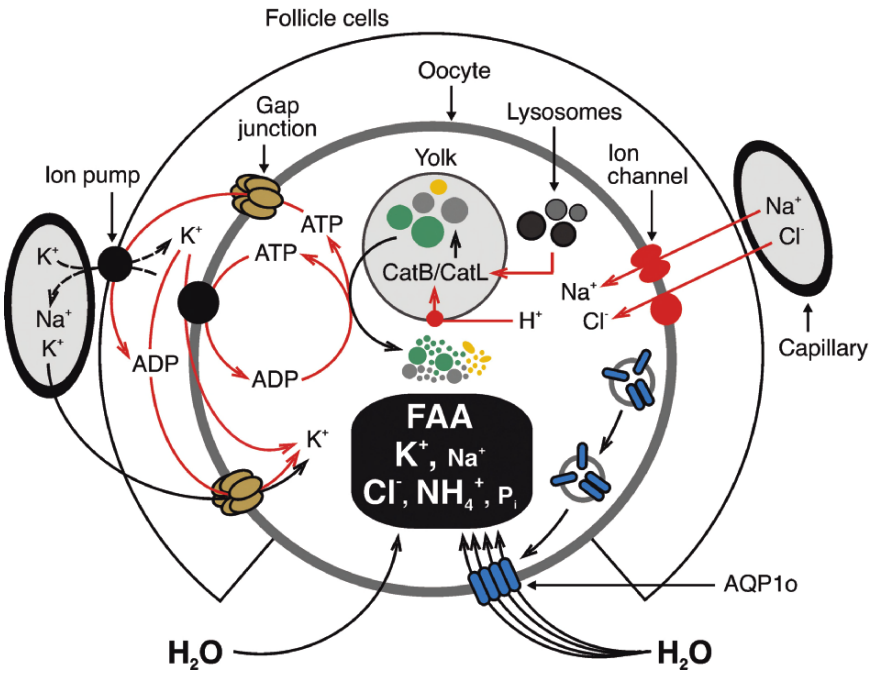

Figure. 14. Integrated model of molecular and cellular mechanisms involved in solute generation and water uptake during oocyte hydration in pelagophil and benthophil teleosts. In red are indicated postulated mechanims based on experimental evidences in fish oocytes or obtained from other biological systems. The major yolk components, lipovitellin, phosvitins, and β′-component, are indicated in green, grey, and yellow. *Abbreviations*: ATP – adenosine triphosphate; ADP – adenosine diphosphate; AQP1o – aquaporin-1o; CatB – cathepsin B; CatL – cathepsin L; FAA – free amino acids. (*See Color Plates*).

The recent discovery of AQP1o in marine teleosts and its role during the hydration of sea bream oocytes has uncovered one of the key molecular mechanisms involved in water transport into the oocyte. These findings suggest that fish oocyte hydration is indeed a highly controlled mechanism based on the interplay between protein hydrolysis and ion accumulation to create an osmotic gradient, and SaAQP1o to regulate water influx. Remarkably, AQP1o-related water channels have been found in pelagophil teleosts, suggesting that AQP1o proteins might be specialized in hormone-induced oocyte hydration in these species. The physiological significance of the AQP1o-regulated mechanism is however not yet fully understood although this system may be required by teleosts with group-synchronous ovarian development, such as fugu, sea bream, and sole, in which sequential batches of oocytes are recruited into maturation (and hydration) and subsequent ovulation during the spawning season. Further studies will be required to elucidate the possible hormonal regulation of AQP1o synthesis, trafficking and/or function in the ovary, as well as the existence of AQP-mediated mechanisms in the oocyte of benthophil teleosts.

The process of fish oocyte hydration affects egg viability and embryo survival, and therefore it has important implications for the controlled reproduction of fish in captivity and mass production of larvae (Kjørsvik et al., 1990). Low hydrated eggs frequently do not develop into viable embryos and there are some evidences indicating that the level of hydration may affect egg fertilizability (e.g. Carnevali et al., 1999b). Consequently, a high percentage of buoyant eggs is currently used as a marker of good egg quality, despite that the physiological basis for poor or good egg quality in fish remains poorly understood. The investigation of the operant mechanisms during oocyte hydration can also contribute to fill this gap, as well as to identify valid markers for egg viability in marine fish (Maarstoel et al., 1993; Carnevali et al., 2001a). In addition, the definition of the role of AQP1o genes, as well as their intracellular regulatory pathways, will identify new targets and tools for the long-term preservation of marine fish gametes and embryos, since their high water content is one of the biological barriers that has severely impaired successful cryopreservation (see Chapter 14). Thus, the development of novel cryopreservation technologies based on AQP1o function may contribute with more efficient methods for the long-term preservation of fish eggs and embryos which would be of great value for both aquaculture and conservation biology.

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CHAPTER 13

FERTILIZATION AND EGG ACTIVATION IN FISHES

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1. INTRODUCTION

The basic morphological and biochemical aspects of fertilization are highly conserved and make use of principles common to most metazoan animals. However, fertilization of the fish oocyte occurs in a wide variety of environmental circumstances and the details for bringing sperm and egg together, as well as for transforming the fertilized oocyte into a developing embryo can differ greatly. Adaptations for activating a yolk-rich oocyte typically found in fish species and establishing polarity of the embryo involve some processes that appear to be unique to fish oocytes. Such unique aspects of fish fertilization provide opportunities to understand mechanisms that are more difficult to detect in oocytes from other types of animals. The following sections will review the current knowledge relating to strategies of fertilization, mechanisms of gamete interaction, and egg activation in fish species.

2. FERTILIZATION STRATEGIES

Fertilization in fish can occur either inside or outside of the female reproductive tract (internally or externally). Most species engage in broadcast fertilization

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(external) where sperms are released into the surrounding water to find their way to the egg; a strategy that is facilitated by the aquatic environment. While mating behavior can assure species-specificity in fish that fertilize internally, additional mechanisms have developed in order to overcome the added challenge of maintaining specificity in externally fertilizing fish. These include agents released by the eggs that hyperactivate motility of same species sperm, and species-specific sperm-egg adhesion components (Iwamatsu, 2000).

External fertilization requires that the sperm find its way to the micropyle of an unfertilized egg. A key initial step is the acquisition of motility by sperm, which have been stored in an immotile state in the testis. Motility acquisition occurs through a variety of mechanisms. The most common is a response to changes in the osmolarity, pH, or the concentration of Na+, K+, or Ca^{2+} that occur upon spawning (Morisawa, 1994). In addition to these environmental factors, polypeptides or small molecules produced by the oocyte can stimulate sperm motility. SMIFs or sperm motility initiating factors synthesized in follicle cells and accumulated in the egg chorion have been shown to play a role in the activation of sperm motility in several species. Pacific Herring SMIFs, also known as HSAPs (herring sperm activating proteins) are tightly associated with the micropyle region and appear to be structurally related to trypsin inhibitor like proteins. They stimulate sperm motility by inducing a Ca2+ influx, Na+ efflux and membrane depolarization of the sperm (Vines et al., 2002). It is thought that HSAPs interact with prolyl endopeptidase on the surface on the sperm tail to activate motility (Yoshida, 1999). In addition to protein activators of sperm motility, recent work in the fathead minnow system has shown that nitric oxide produced by the oocyte can enhance sperm motility (Creech et al., 1998). Given the frequency with which redundant mechanisms are found in reproductive biology, it would not be surprising if both oocyte-derived peptides, as well as nitric oxide or other small molecules function to enhance the probability that the sperm will contact the oocyte. In addition to diffusible factors, fixed components of the chorion may have a role in sperm activation (see below).

3. SPERM–EGG INTERACTIONS

Once the fertilizing sperm has been activated, it must find the sperm entry site on the egg plasma membrane. In order to do this it must first interact with the egg's extracellular matrix, the chorion. The chorion is a highly structured egg envelope, made mostly of proteins and glycoproteins, that plays a large role in sperm–egg interactions as well as later protecting the developing embryo. The chorion is a "complex of several concentric lamellae" that consists of a "wave-shaped fibrillar component embedded in an amorphous matrix" (Hosokawa, 1985). It contains two layers, the zona radiata interna and externa, whose thickness varies depending on the mode of reproduction (viviparous fish chorions have thinner layers). In addition, the outer layer of the chorion surrounding the

micropyle is specialized and, it has been shown to help direct sperm to the sperm entry site. (Amanze and Iyengar, 1990)

The chorion also acts as a barrier to sperm and presents a single passage, the micropyle, by which sperm can gain access to the egg plasma membrane. The plasma membrane subadjacent to the micropyle is invaginated slightly and is specialized with elongated microvilli (Hart and Donovan, 1983). The micropyle is grooved, and data gathered by Amanze and Iyengar (1990) show that fertilizing sperm travel preferentially along these grooves into the micropylar pit. Although the morphology of the sperm and egg are not usually a factor in maintaining species-specificity, micropyle size can sometimes contribute to the expulsion of heterologous species sperm. Once the sperm has past through the micropyle, it binds to the plasma membrane (Hart, 1990). The binding of rainbow trout sperm to the plasma membrane is mediated by strong carbohydrate–carbohydrate interactions between deaminated neuraminyl ganglioside ((KDN)GM3) and a Gg3-like epitope, both of which are found only in the area surrounding the micropyle (Yu et al., 2002). Detailed electron microscope data show that actual sperm–egg fusion begins between the microvilli of the sperm entry site and the sperm plasma membrane. Fusion will not occur in the absence of extracellular calcium ions (Iwamatsu et al., 1985).

Once the sperm has fused with the plasma membrane, the cortical reaction (below) severs the associations between the plasma membrane and the chorion creating a perivitelline space (Hart and Yu, 1980). Concurrently, the fertilization cone is formed from microvilli as the egg cortex reach up and draw the sperm further into the egg (Kudo, 1983). The early cone collapses and a later fertilization cone is formed and persists for 3–4 min before disappearing as the sperm is completely incorporated into the oocyte (Iwamatsu and Ohta, 1981). The result of this process is that the sperm plasma membrane has been incorporated into the egg plasma membrane, and the cytoplasmic contents of the sperm are inside of the egg. As the late fertilization cone disappears, the sperm nucleus begins its migration inward and the male and female pronuclei eventually meet at the center of the blastodisc.

As with every other oocyte studied, the binding and/or fusion of the sperm to the oocyte plasma membrane triggers a depolarization of the plasma membrane resting potential. In one well-studied example (*Oryzias latipes*), the plasma membrane resting potential is about –47 mV until the sperm penetrates the micropyle whereupon depolarization drives the potential to +4 mV for an period of approximately 20 s (Nuccitelli, 1977, 1987). The depolarization is followed by a long hyperpolarization (at least 2 min) 31 mV in amplitude during which K+ permeability increases (Ito, 1962; Nuccitelli, 1980).

The plasma membrane depolarization is followed by an intracellular Ca^{2+} transient wave (discussed below) which originates in the cortical cytoplasm near the micropyle. The cortical layer, or cortex, lies just below the chorion and is enriched in F-actin bundles (Hart and Collins, 1991; Hart and Fluk, 1995), it is also rich in endoplasmic reticulum, which functions in the

regulation of calcium signaling. Release of calcium from the endoplasmic reticulum network in the egg cortex triggers exocytosis of cortical alveoli in a wave termed the cortical reaction. The cortical reaction starts at the animal pole about 9 s after fertilization and spreads across the egg secreting the cortical vesicle contents into the perivitelline space. The contents of the cortical vesicles modify the chorion and the perivitelline space. One of the primary functions of the cortical reaction is to establish a block to polyspermy. Although the micropyle in most fish species is not large enough to admit more than one sperm, additional blocks to polyspermy undoubtedly exist since polyspermy is not found in those species with large micropyles. This block to polyspermy clearly involves the cortical reaction and studies of *Oryzias* have shown that cortical vesicle contents form a plug in the micropylar canal that can block entry for supernumerary sperm (Hart and Donovan, 1983). The cortical vesicle contents also contain sperm-agglutinating chemicals that cause sperm to lose motility (Iwamatsu et al., 1997). In addition to these structural changes, chorion glycoproteins that are homologous to those found in the mammalian zona pellucida are altered by the astacin-like protease, alveolin, which is released from cortical alveoli during the cortical reaction (Shibata et al., 2000). It is possible that astacin modification of chorion glycoproteins reduces their previously high affinity for sperm, further reducing the possibility of polyspermy.

4. CALCIUM SIGNALING IN THE FISH OOCYTE

The membrane depolarization step is followed by a transient elevation of intracellular free calcium, an event that triggers a series of biochemical pathways that are required for the block to polyspermy, activation of metabolism, reentry into the cell cycle and execution of the developmental program. This "sperm-induced" fertilization transient is common to eggs of all species examined including organisms as primitive as the sponge and as complex as mammals (reviewed in Runft et al., 2002). Fish species examined include medaka (Gilkey, 1978; Yoshimoto, 1988; Iwamatsu, 1988a, b), goldfish (Yamamoto, 1954), and zebrafish (Lee et al., 1999). This calcium transient begins with the release of Ca^{2+} from intracellular stores in the vicinity of the micropyle and progresses as a wave of elevated $[Ca^{2+}]_i$ across the egg. Detailed analysis of this calcium wave in the zebrafish revealed a rate of propagation through the cortex of 9 μm/s and a slower rate of 69 μm/s through the central cytoplasm (Lee et al., 1999). This initial sperm-induced transient has been shown in other systems to be absolutely required for subsequent development (Runft et al., 2002).

The first indication of changing $[Ca^{2+}]_i$ in the zebrafish oocyte appears approximately 15 s post-insemination as indicated by increased calcium green fluorescence in the cortical cytoplasm in the region of the micropyle (Figure 1B arrows). This early response coincided with the early stages of chorion

elevation as cortical vesicle exocytosis begins and the egg surface contracts away from the chorion. These changes evident in micrographs were also reflected as an initial peak in total calcium green fluorescence measured by quantifying average pixel density across the entire optical section (Figure 2). Interestingly, Lee et al. (1999) reported that this initial calcium transient was also triggered, in zebrafish, by exposure to hypotonic medium such as during spawning. Thus, in zebrafish, the initial calcium transient does not require a sperm as the initiating signal, and Lee et al. (1999) have proposed that this mechanism could help ensure species specificity of fertilization since it would ensure that the oocyte would remain receptive to sperm only for a short time following spawning.

While measurement of total or cross-sectional $[Ca^{2+}]_i$ such as in Figure 2 provides a certain amount of information, detailed morphological analysis demonstrated that the initial calcium transient is not uniform throughout the oocyte. As seen in Figure 1, the increased fluorescence in the cortical cytoplasm spread across the entire egg cortex during the first 60 s with only a modest change evident in the cytoplasm. This can be presented graphically by line-scan analysis of each image as seen in Figure 4. Panels representing 30 and 60 s post-insemination demonstrate that the relative changes in calcium green fluorescence during this period were much higher at the egg cortex than in the central cytoplasm. By 2.5–5.0 mpf, the loss of cortical vesicles had left a rim of clear cortical

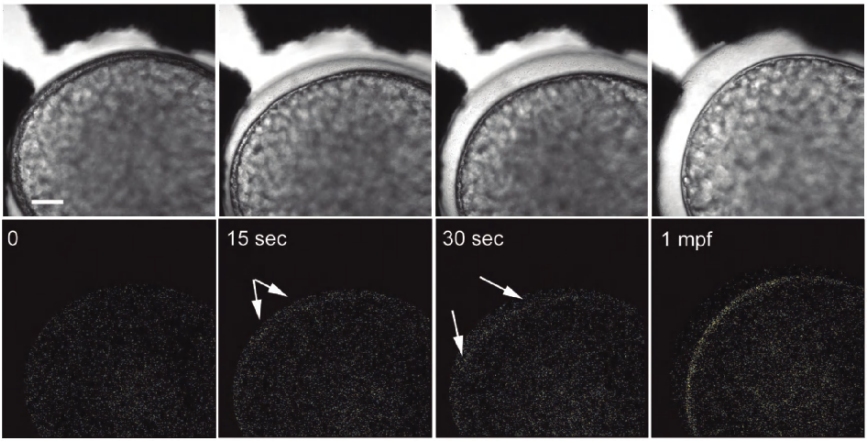

Figure 1. Changes in calcium levels at fertilization: Zebrafish oocytes were injected with calcium green-dextran (10 kDa) at an intracellular concentration of 100 nm, then imaged with a Nikon TE2000 inverted confocal microscope using a blue 488 nm laser. Fertilization was accomplished by adding a sperm suspension and aquarium water at time = 0. Differential interference contrast images are presented in the upper panels (Bar = 100 μm) and green fluorescence (presented as pseudocolor) is presented in the lower panels. (*See Color Plates*).

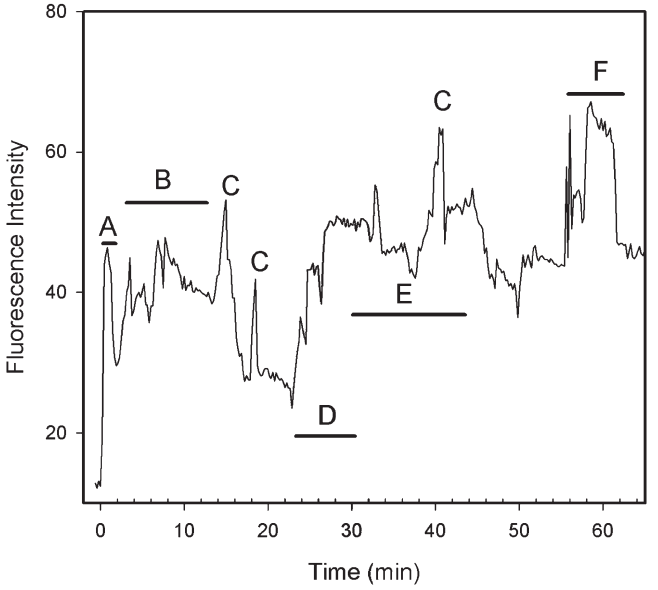

Figure 2. Quantitation of calcium green fluorescence during fertilization and egg activation. Zebrafish oocytes injected with calcium green dextran and imaged as in Figure 1 were monitored through the first cell division and images were collected at 15 s intervals. Calcium green fluorescence within a single optical section was quantified using metamorph software version 6.1 (Universal Imaging Corp, Downingtown, PA). Fluorescence intensity (*y*-axis) is expressed as average pixel intensity and time after fertilization is expressed in minutes on the *x*-axis. Specific events are labeled with letters as follows: (A) initial calcium transient involving the oocyte cortex; (B) enhanced calcium green fluorescence in the cytoplasm; (C) calcium transient corresponding to a contraction of the egg; (D) retraction of yolk from the animal pole; (E) cytoplasmic streaming and blastodisc expansion; (F) cytokinesis.

cytoplasm, which exhibited intense fluorescence (Figure 3B). This clear region was typically more pronounced in the vicinity of the micropyle. The central cytoplasm also exhibited an increase in fluorescence by 5 mpf as seen in the line scan analysis Figure 4 (bottom panel). The combined increases in calcium green fluorescence in both the egg cortex and the central cytoplasm were evident in measurement of total egg fluorescence as a second broad peak of fluorescence intensity between 2.5 and 15 mpf (Figure 2B). The intensity of both the cortical and central cytoplasmic fluorescence declined after 15 mpf as seen in Figure 2, although occasional contractions involving the entire egg correlated roughly with abrupt peaks of $[Ca^{2+}]_i$ as indicated in Figure 2C.

One consequence of the first calcium transient is a characteristic pattern of later calcium transients, which seem to perform different functions in egg activation. In mammalian oocytes, these later events take the form of repetitive oscillations (Runft et al., 2002). In zebrafish oocytes, a series of 3–4 calcium transients follow the initial transient. These do not occur with a constant frequency as in mammalian oocytes, but do involve significant increases in

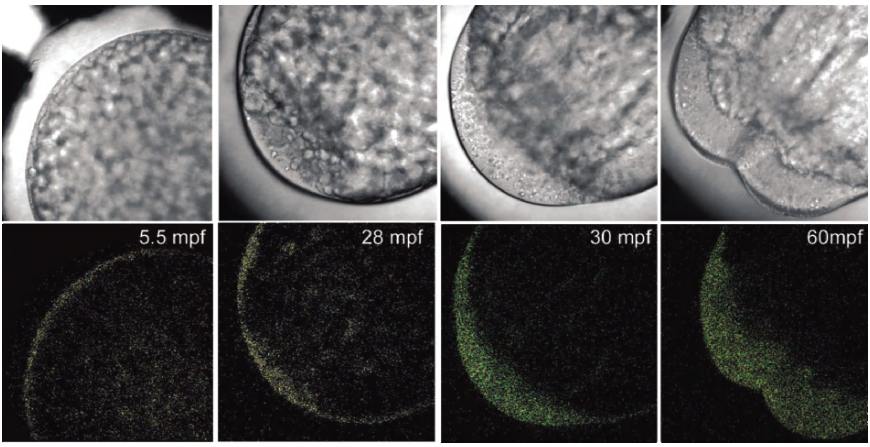

Figure 3. Changes in calcium levels leading up to mitosis. The zygote depicted in Figure 1 was monitored through the first cell division and representative time points are displayed. (*See Color Plates*).

$[Ca^{2+}]_i$ from a resting level of 60 nm to a maximal value of 90–120 nm (Creton et al., 1998). For example, the first indication of the future site of the animal pole appeared about 20–30 mpf as the yolk mass retracted from the cortical cytoplasm creating a slightly larger clear zone exhibiting intense fluorescence Figure 4A. This accumulation of cytoplasm with high $[Ca^{2+}]_i$ is evident as a progressive increase in calcium green fluorescence (Figure 2D). In addition, the distribution of calcium green fluorescence in the central cytoplasm changed by 35 mpf becoming increasingly concentrated into localized zones of intense fluorescence (Figure 3). These central zones of increased $[Ca^{2+}]_i$ eventually became organized into cytoplasmic channels or "streamers" through which cytoplasm moved through the yolk mass to accumulate at the animal pole. This cytoplasmic streaming forced additional cytoplasm into the cleared region at the animal pole and caused protrusion of the blastodisc as described thoroughly by earlier studies (Leung et al., 1998). Accumulation of highly fluorescent blastodisc cytoplasm became evident as a peak in total egg fluorescence between 35 and 50 mpf (Figure 2E). As blastodisc expansion proceeded, the continuing series of contractions become more frequent after 40 mpf. These contractions are thought to help force cytoplasm toward the blastodisc and are dependent on the availability of high levels of calcium since contractions and streaming are blocked by calcium buffers such as BAPTA (5, 5′ dibromo-1, 2-Bis(2-aminophenoxy)ethane-N, N, N, N′-tetraacetic acid) (Leung et al., 1998). In zebrafish, cytokinesis typically begins between 55 and 60 mpf with enhanced fluorescence at the site of the contractile ring (Figure 3). Cytokinesis involves the highest amplitude of calcium green fluorescence as seen in Figure 2F. Chang and Meng (1995) and Creton et al. (1998) have quantitated this mitotic transient in aequorin-injected zygotes and reported that $[Ca^{2+}]_i$ reached levels as high as 250–600 nm in localized regions near the cleavage furrow. Completion of cytokinesis triggered

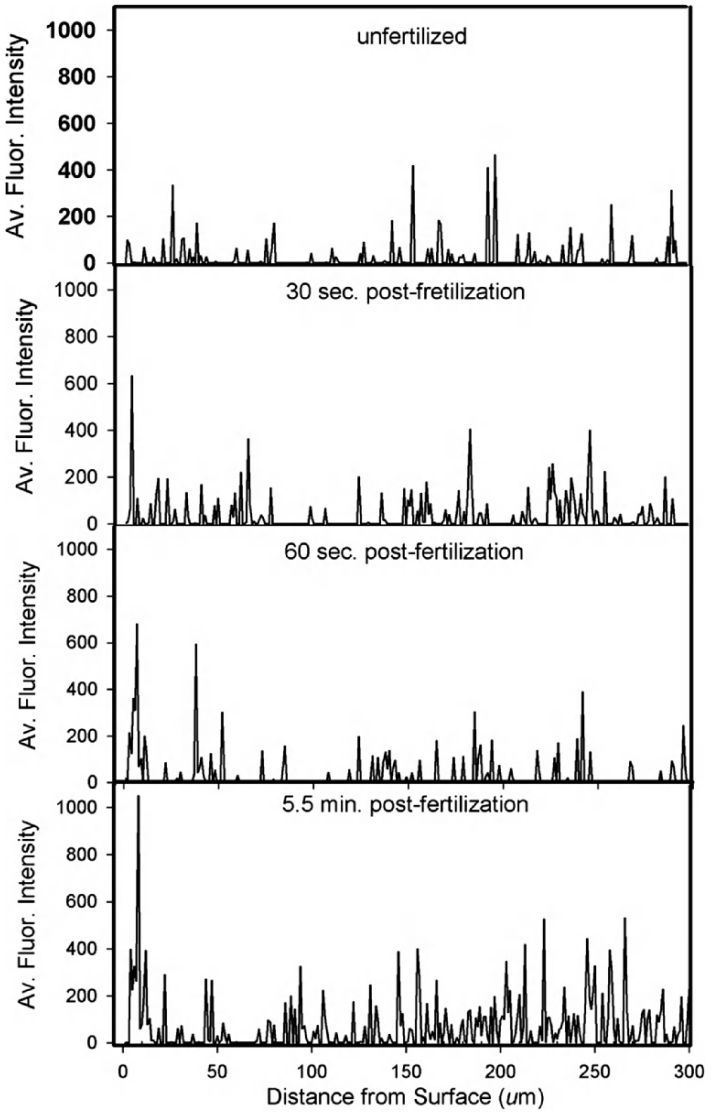

Figure 4. Line scan analysis of calcium green fluorescence. Images used for quantitation of total cross-sectional calcium green fluorescence in Figure 2 were also analyzed by Metamorph line scan analysis to demonstrate the differences in fluorescence intensity from the cell surface at the micropyle to the center of the egg. Each graph represents the average pixel density across a 10 pixel wide line beginning at the plasma membrane in the vicinity of the micropyle and ending at the center of the zygote.

resumption of cytoplasmic streaming and fluorescence returned to a level typical of the cytoplasmic streaming prior to mitosis. Subsequent mitoses were marked by similar peaks in calcium fluorescence.

5. THE EGG ACTIVATION PATHWAY

The biochemical pathway by which the sperm activates the oocyte begins with a rapid signaling event that is thought to result from the introduction of a sperm protein or other component to the ooplasm. The existence of sperm "factors" capable of triggering calcium transients in oocytes has been documented in many species (Swann, 1999), and has been documented in sperm from the fish *Oreochromis niloticus* (Coward et al., 2003). The majority of the evidence to date indicates that the critical sperm component is a phospholipase isoform (phospholipase zeta) (Cox et al., 2002; Saunders et al., 2002; Swann et al., 2004). Presumably, this phospholipase would exert its effect by producing a pool of IP3 in the vicinity of the newly incorporated sperm, although little information is available regarding the mechanism through which it would act once introduced into the egg (reviewed in Kurokawa et al., 2004). One key point that does not fit into the above model was raised in the study by Lee et al. (1999), which described the apparently normal activation of zebrafish oocytes in the absence of sperm. We also have observed this parthenogenic activation to appear normal morphologically and to exhibit normal changes in $[Ca^{2+}]_I$, cytoplasmic streaming, and blastodisc expansion. However, at the biochemical level, we were not able to detect activation of the Fyn tyrosine kinase in spontaneously activated oocytes (Wu and Kinsey, 2000). Future studies will hopefully resolve whether sperm components such as phospholipase C zeta are required for full activation and development of oocytes in fish species.

Once the initial signal has been delivered to the oocyte, a widely accepted model proposes that the sperm component triggers a phosphatase-mediated activation of Src-family protein tyrosine kinases which activate the oocyte store of phospholipase C gamma leading to accumulation of IP3 to trigger calcium release. While this model has been worked out in a number of species, it seems to apply to fertilization in fish as well. The biochemical mechanism by which changes in $[Ca^{2+}]_i$ following fertilization are regulated has been extensively studied and it is clear that oocytes from a wide variety of species can modulate cytoplasmic calcium by two pathways. The first pathway involves the action of Ins1, 4, 5, P3 (IP3) on ligand-gated calcium channels in the endoplasmic reticulum, and the second involves cyclic-ADP-ribose (cADPR) (reviewed in Coward et al., 2003). Both IP3 and cADPr can trigger calcium transients in fish oocytes or oocyte extracts (Iwamatsu, 1998; Fluck et al., 1999; Polzonetti et al., 2002). Detailed functional studies carried out in other species indicate that IP3-mediated calcium release is necessary and sufficient for the sperm-induced calcium transient that plays such a dominant role in egg activation (Runft et al., 2002). The exact role of cADPR signaling in fertilization is not clear but, given

the complexity of calcium events that occur after the initial sperm-induced calcium transient, it is possible that cADPR may play a primary role later in egg activation.

Evidence obtained from invertebrate, *Xenopus*, and zebrafish oocytes indicates that the initial, sperm-induced calcium transient requires the action of Src-family protein tyrosine kinases such as Fyn (Wu and Kinsey, 2002) or Src (Sato et al., 2002, 2003) which are though to activate phospholipase C isoforms required for the release of IP3. The activation of Fyn and possibly Src kinases at fertilization has been shown to require a plasma membrane associated phosphatase such as rPTPα and involve an SH2 displacement mechanism (Kinsey et al., 2003).

The more complicated series of calcium changes that characterize the later stages of fish oocyte activation seem to be involved primarily in moving active cytoplasm to the animal pole and preparing the oocyte for mitosis. The extent to which these localized calcium transients require IP3 has yet to be determined and the biochemical mechanism by which they are regulated remains a challenging problem for future research.

6. NUCLEAR EVENTS FOLLOWING FERTILIZATION

Most teleost oocytes contain a RNA-rich yolk nucleus, whose function is still unclear, but which might play a role in determining oocyte polarity. It has been suggested that the vegetal pole develops as a result of interaction with follicle cells near the yolk nucleus, and that the animal pole develops next, as the oocyte rotates around an axis containing the yolk and oocyte nuclei (Iwamatsu and Nakashima, 1996). As maturation-inducing hormone causes the oocyte to mature, the germinal vesicle translocates toward the micropyle near the animal pole. Later, as the result of endocrine control in the follicle, the germinal vesicle disintegrates and meiosis proceeds to metaphase II where the oocyte is arrested and awaits fertilization. The signal transduction events that regulate meiotic progression of the fertilized oocyte have been studied primarily in marine invertebrates, *Xenopus*, or in the mouse system. MAPK activity, which increases progressively prior to GVBD, is required for maintenance of MII arrest. At fertilization, Ca/calmodulin-dependent kinase is stimulated and triggers degradation of cyclins, thus overcoming the effect of MAPK in arresting the cycle at MII. The calcium transient also leads to PKC activation, which eventually suppresses MAPK activity (Kumano et al., 2001). The decline in MPF preceeds the decline in MAPK activity and it is thought that MAPK activity still has to play a role in spindle function during polar body emission (Kotani and Yamashita, 2002; Fan et al., 2004).

The sperm nuclear envelope breakdown and chromatin decondensation begin shortly after sperm incorporation, forming the male pronucleus. The male and female pronuclei move towards the animal pole with the help of microtubule networks – one near each pole. Recently a gene called futile cycle has been

shown to play an essential role in the action of the chromosomal microtubules required for pronuclear congression at the zebrafish blastodisc (Dekens et al., 2003). Once translocated to the blastodisc, the two pronuclei remain adjacent to each other during blastodisc expansion and until mitosis begins.

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CHAPTER 14

LOW-TEMPERATURE PRESERVATION OF FISH GONAD CELLS AND OOCYTES

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1. INTRODUCTION

Ensuring the retention of a record of the genetic diversity of species involves, at its simplest form, the long-term storage of material from somatic and reproductive cell lines. However, to have real practical benefit such material should be in the form of viable cellular material, ideally from the germline or reproductive cell lines. The ability to generate banks of cryopreserved sperm, eggs, and embryos that retain full viability following recovery from the frozen state would be the most powerful facilitating tool in species conservation and commercial applications such as aquaculture. The nuclear genomes of fish show a full range of sex chromosome differentiation, ranging from the all-autosomal karyotype as seen in the zebrafish to the genetically and cytogenetically differentiated sex chromosomes seen in the guppy (Traut and Winking, 2001). The preservation of haploid genomes in the viable form of male and female reproductive cells, or of viable embryos, is vital if normal populations of males and females are to be established.

Cryopreservation of fish gametes has been studied extensively in the last three decades and the successful cryopreservation of the spermatozoa from many species including salmonid, cyprinids, silurids, and acipenseridae is well documented (Magyary et al., 1996; Maisse, 1996; Rana and Gilmour, 1996; Tsvetkova et al., 1996; Lahnsteiner, 2000; Tiersch and Mazik, 2000). In the last 25 years, attempts to cryopreserve fish eggs and embryos have been conducted on over 20 fish species (Harvey, 1983; Zhang et al., 1993; Zhang and Rawson, 1996; Hagerdorn and Kleinhans, 2000; Cabrita et al., 2003a, b; Robles et al., 2003, 2005). Although eggs or embryos have been shown to survive for a short time after cooling to subzero temperatures, successful cryopreservation of fish eggs and embryos remains elusive (Harvey, 1983; Zhang et al., 1989; Zhang et al., 1993; Hagedorn et al., 1996; Robles et al., 2003, 2005). Recent reported success for flounder (*Paralichthys alivaceus*) embryos by vitrification (Chen and Tian, 2005), has been disputed (Edashige et al., 2006) as has been the reported success in cryopreservation of carp embryos (Zhang et al., 1989). There has been virtually no published information on the cryopreservation of ovary or unfertilized eggs of fish species until recently (Lubzens et al., 2003, 2004, 2005; Isayeva et al., 2004; Plachinta et al., 2004a, b; Valdez et al., 2005; Zhang et al., 2005a, b).

In this chapter we aim to discuss the importance of maternal genome banking, review the principles of cryobiology, provide information on the current status of oocyte preservation, highlight the advantages and obstacles in fish oocyte cryopreservation and discuss future prospects in maternal genome banking of fish species.

2. IMPORTANCE OF MATERNAL GENOME BANKING

Cryopreservation of spermatozoa saves only the paternal genome and is not sufficient for preservation of genetic diversity that depends also on the maternal genome. The role of the maternal products during zebrafish oogenesis and early

embryo development is well documented and discussed in Chapters 2, 3, and 6. The zygotic genome activation occurs at the midblastula transition, prior to which all processes are under the control of the maternal products. In addition to maternal mRNA that is expressed during embryonic development, mitochondrial DNA makes a vital contribution to embryo development and cellular functioning. In zebrafish (*Danio rerio*) mtDNA is a 16,596-nucleotide sequence containing 13 protein genes, and 22 tRNAs and 2 rRNAs (Broughton et al., 2001). Following zygotic expression the female products continue to contribute to essential functions along with new zygotic products, including axis formation, germ layer, and germ cell specification (Pelegri, 2003). The importance of both paternal and maternal derived germ plasm (Hagedorn and Kleinhans, 2000) has been the basis for the continuing attempts at developing methods for cryopreservation of fish embryos. However, the lack of success to-date in the cryopreservation of fish embryos has prompted alternative approaches to be investigated, using material that can be successfully cryopreserved. There are four main procedures that have been employed – (i) androgenesis using frozen sperm, (ii) chimera production exploiting the ability to cryopreserve isolated blastomeres and their subsequent insertion into recipient embryos, (iii) nuclear transplantation from embryonic cells into eggs, and (iv) primordial germ cell (PGC) transplantation.

(i) Androgenesis can provide homozygous lines for biomedical research and monostocks for commercial purposes, and improved survival rates of androgenetic individuals are being achieved. This procedure can be performed with cryopreserved spermatozoa but long-term survival is low (Babiak et al., 2002). The very nature of the procedure, the insertion of the diploidized nucleus from sperm into a denucleated oocyte, means that there is no nuclear maternal genome contribution and mitochondrial inheritance is through the maternal material of the recipient oocyte.

(ii) Chimera production from frozen blastomeres has the advantage of banking the diploid embryonic cells comprising both male and female parental genome contributions. The procedure is a highly skilled operation involving blastomere isolation and microinjection into selected embryos, and the fate of the donated cells in the developing embryo is uncertain with respect to their distribution (Takeuchi et al., 2001). The ideal distribution pattern would be for such cells to contribute totally or in part to the developing gonads. Successful cryopreservation procedures of fish blastomeres has been reported for a wide range of fish species; zebrafish (Harvey, 1983), trout (Nilsson and Cloud, 1993; Calvi and Maisse, 1998), whiting (Strussmann et al., 1999), perejerrey (Strussmann et al., 1999), medaka (Strussmann et al., 1999), chum salmon (Kusuda et al., 2002), and goldfish (Kusuda et al., 2004). Whilst blastomere cryopreservation and subsequent insertion into early stage embryos offers a route for maternal genome banking, its success rate is relatively low and it does not allow for reconstitution of the individual parental genomes.

(iii) Nuclear transplantation is an important technique used in genetic modification and cloning. In this technique, the nucleus in an unfertilized egg is exchanged with a nucleus from a donor cell. In fish, transplantation of embryonic cells or embryonic fibroblast long-term cell culture was performed into non-enucleated and enucleated unfertilized eggs (Niwa et al., 1999, 2000; reviewed in Di Berardino, 2001, Wakamatsu et al., 2001, Lee et al., 2002), with successful production of fertile diploid fish (Bubenshchikova et al., 2005). This technique could also be used with cryopreserved embryonic cells (Wakamatsu et al., 2001). Also here, maternal traits in the offspring will originate from the recipient egg and the success rate is very low.

(iv) The novel option of cryopreservation of PGCs entails exciting future opportunities for preservation of both maternal and paternal genomes. Animal gametes are derived from sexually undifferentiated PGCs which are the progenitors of the germ cell lineage giving rise to either eggs or sperm. Methods were developed for isolating PGCs by specific cell markers such as *Gfp* gene driven by *vasa* gene promoter, followed by GFP-dependent flow cytometry (Yoshizaki et al., 2000; Takeuchi et al., 2002; Kobayashi et al., 2004). These isolated PGCs, were injected into the peritoneal cavities of newly hatched embryos and were found to migrate and colonize the genital ridge of the recipient embryos. The donor-derived PGCs proliferated and differentiated in the host embryo giving rise to mature eggs or sperm in the allogeneic gonad and later on to the production of live fry, carrying the donor-derived haplotype. The development of successful methods for cryopreservation of PGCs, provided novel approach to the preservation of maternal genome resources (Kobayashi et al., 2003, 2006). A multitude of possible scenarios could stem from application of these techniques, including for example, xenotransplantation of cryopreserved PGCs into closely related species, where the gametes produced by the surrogate parents give rise to the PGCs donor species. The wide plasticity of this system has been recently demonstrated by successful transplantation of testicular germ cells that were obtained from adult male trout, into newly hatched male or female embryos, where the testicular germ cells differentiated into spermatozoa or fully functional eggs in male or female recipients, respectively. The donor-derived spermatozoa or eggs produced normal offspring (Okutsu et al., 2006). For now, this technique has only been established for transplantation of heterologous PGCs between closely related salmonid species (Takeuchi et al., 2004). Another disadvantage lies in the need to wait until the surrogate fish reaches sexual maturity before recovering the gametes of the donor fish. This long period (more than two years), from the time of transplantation to the spawning time of the host, could be shortened if PGCs xenotransplatation could be performed into fish with shorter generations times (Takeuchi et al., 2003, 2004; Yoshizaki et al., 2005).

The problems and limitations of current approaches to preserve maternal genetic material point to the importance and urgency of the development of successful protocols for the cryopreservation of fish oocytes.

3. PRINCIPLES OF CRYOBIOLOGY

Cryopreservation is a technique by which viable cells, tissues, and even organisms can be sustained for extended periods. Effective cell conservation requires that changes in genetic material of the cells concerned are prevented, and that the required patterns of gene expression are reliably reproduced in recovered material. In conventional cryopreservation, a level of stability is imposed on the system by ultra-low temperatures (<−130°C), and storage is at, or close to, −196°C using liquid nitrogen (LN or the vapour immediately above it), as a practical and convenient cryogen. At such a temperature, normal cellular chemical reactions do not occur, as kinetic energy levels are too low to allow the necessary molecular motion (Grout et al., 1990). Although the consequences of the exposure of living cells to lowered temperatures are diverse and paradoxical, there are fundamental underlying mechanisms that determine how all biological systems respond to the lowering of temperature and the solidification of liquid water. Two notable damages can usually be classified after cells and tissues have been exposed to lowered temperatures: chilling injury, which is used to refer to the damage following exposure to low temperature without freezing, and freezing damage.

3.1. Chilling Injury

Many types of cells and tissues are damaged when cooled to temperatures close to or <0°C without freezing. The essential features of direct chilling injury have been outlined as follows (Morris, 1987): (a) all cell types may be considered sensitive to chilling provided that they are cooled rapidly enough to a sufficiently low temperature; (b) cellular viability is dependent upon the rate of cooling with more injury observed following "rapid" rather than "slow" cooling; (c) chilling injury is almost independent of the rate of warming, when this does not significantly increase the length of exposure to low temperature; (d) injury is increased as the period of isothermal incubation at the reduced temperature is extended; (e) loss of membrane permeability occurs following rapid cooling, and in some instances, may be reversed upon rewarming; and (f) the response of any cell type may be modified by the culture conditions before cooling or by the addition of specific compounds.

3.2. Freezing Injury

Liquid water is essential to the structure and function of living cells, and solidification of water by freezing is usually destructive or even lethal to cells. The stresses to which cells are exposed during freezing will mainly result from the

following three aspects (Grout and Morris, 1987): (a) the mechanical effects of extracellular ice crystals at cell surfaces, especially in tissues with cellular interconnections; (b) alterations in the physical properties of solutions outside the cell, including the concentration of solutes which result from the nucleation of a proportion of extracellular water; (c) intracellular freezing if it occurs. When the temperature is down to about −5°C, the cells and their surrounding medium remain unfrozen both because of supercooling and the depression of the freezing point by the protective solutes that are frequently present. Between about −5°C and −15°C, ice forms in the external medium either spontaneously or as a result of deliberate "seeding" of ice, but the cell content remain unfrozen and supercooled. One of the most fundamental consequences of the presence of ice in the external medium is the effect on the composition of the unfrozen fraction of the extracellular solution. Because solute concentration in the extracellular solution increases as the temperature decreases and the ice phase grows, a chemical potential imbalance between the biomaterial and the unfrozen external solution results. The supercooled water in the cells has, by definition, a higher chemical potential than that of water in the partly frozen solution outside the cell, and in response to this difference in potential, water flows out of the cell and freezes externally (Mazur, 1984; Toner, 1993). The subsequent physical events in the cell depend on the cooling rate. If cooling is sufficiently slow, the cell is able to lose water rapidly enough by exosmosis to concentrate the intracellular solutes sufficiently to eliminate supercooling and maintain the chemical potential of intracellular water in equilibrium with that of extracellular water. The result is that the cell dehydrates and does not freeze intracellularly. If the cooling is too rapid, the rate at which the chemical potential of water in the extracellular solution decreases is much faster than the rate at which water can diffuse out of the cell and the end result is intracellular ice formation (IIF). It is an implicit assumption that the formation of ice inside the cell is inevitably lethal (Mazur, 2004). However, if cooling rate is too slow, cells will experience "solution effects" during freezing, i.e. the exposure of cells to the changing hypertonic liquid environment as ice crystallizes out of solution (Mazur, 1965). During very slow cooling, cells will be exposed to the extreme hypertonic conditions in the residual liquid fraction for a sufficiently long period to experience damage before reaching the "safe" ultra-low temperatures used for storage. Whether the solution effects are due to the high salt concentration *per se*, the physical reduction in liquid space compacting the cells, the physical relationship between the cells and the surrounding ice matrix, pH fluctuations with temperature and concentration of buffers, or to some other aspect of the events, has not been satisfactorily demonstrated (Watson and Fuller, 2001).

3.3. Cryoprotectant

Cryoprotectant is any additive that when provided to cells before freezing yields a higher post-thaw recovery than can be obtained in its absence (Karow Jr.,

1969). There are two major categories of cryoprotective agents: (a) permeating cryoprotectants, e.g. methanol, dimethy sulfoxide (DMSO), glycerol and propylene glycol (PG) which are low-molecular weight chemicals and can penetrate the cell membrane; and (b) non-permeating cryoprotectants, e.g. hydroxyethyl starch, polyvinyl pyrrolidone and various sugars, which are high-molecular weight agents and cannot enter cells. Cryoprotectants of each group play different roles during cooling and thawing. The permeating cryoprotectants produce a considerable freezing point depression in addition to that due to any electrolytes present within the system, eventually leading to a ternary (cryoprotectants-saltwater) eutectic point at a low temperature (Shepard et al., 1976). In a freezing system, total solute concentration in the unfrozen phase is uniquely determined by temperature. Hence, the inclusion in the system of e.g. DMSO necessarily results in a decrease in concentration of other solutes. As the increasing salt content of the residual liquid is the main cause of "solution effects" (Mazur, 1965) both the reduction of damaging salt enrichment and its shift to lower temperatures are believed to have beneficial effects. This explanation of cryoprotective properties is also called osmotic buffering. The cryoprotective effect of non-permeating cryoprotectants is mainly based on dehydration of cells prior to cooling, which results in reduced ice crystal formation during freezing. Some high-molecular weight (>50,000) cryoprotectants such as polyvinyl pyrrolidone, polyvinyl alcohol, and hydroxyethyl starch, protect cells during freezing and thawing by altering ice crystal formation to an innocuous size and shape. Whilst cryoprotective agents can protect living cells from massive distortions of cellular and environmental geometry, a variety of cryoprotectants can themselves be damaging to cells, especially when used in high concentrations (Fahy, 1986; Arnaud and Pegg, 1988; Pegg and Arnaud, 1988). Fahy and co-workers (Fahy, 1984; Fahy et al., 1990) have amassed a convincing body of evidence that the basis for the detrimental effects of cryoprotectants is not simply osmotic, but due to direct "biochemical" injury. Injuries such as inactivation or denaturation of specific enzymes, disruption of transmembrane ionic pumps, or other related perturbations of cellular structure and function, by implication, are most likely due to the direct interaction of the cryoprotectant with proteins and biological membranes.

3.4. Controlled Slow Freezing and Vitrification

Two approaches have been used in cryopreservation of biological materials: controlled slow freezing and vitrification. Controlled slow-freezing procedures are characterized by: (1) the addition of molar concentrations of penetrating cryoprotectants such as DMSO, glycerol, or other cryoprotectants to the cell suspension; and (2) the use of a controlled freezing to the storage temperature. The basic steps required to preserve embryos by this approach are: collect and assess embryo quality; equilibrate embryos in a solution containing molar concentrations of a cryoprotective solute; freeze embryo suspension using controlled cooling to

temperatures below –130°C; low-temperature storage at –196°C; warm and thaw embryo suspension using controlled conditions; remove cryoprotective solute from embryo suspension; return embryos to normal physiological conditions (Rall, 1993). Vitrification is the solidification of a liquid brought about not by crystallization but by an extreme elevation in viscosity during cooling. During vitrification the solution is said to become a glass; molecular motions are significantly arrested, marking the effective end of biological time but without any of the changes brought about by freezing. Cells capable of being vitrified need no longer satisfy classical constraints of optimal cooling and warming rates, but instead can neatly escape both "solution effects" injury and the dangers of intracellular freezing (Fahy et al., 1984). Successful vitrification requires optimization of several steps, and the factors that influence the survival of embryos by vitrification include the concentration and composition of the vitrification solution, the procedure used to equilibrate embryos in this solution, the cooling and warming conditions, and the procedure used to dilute embryos from the vitrification solution (Rall, 1987).

4. CRYOPRESERVATION OF FISH OOCYTES

Developing methods for cryopreservation of oocytes entails determining the optimal type of cryoprotectants and its concentration, the permeability of oocyte membranes to cryoprotective agents, evaluating oocytes tolerance to chilling, the appropriate rate of cooling to cryogenic temperatures and the rate of thawing.

One of the first steps required for developing methods for cryopreservation of fish oocytes, is the need to devise tools for quantitative estimation of oocyte viability. This is required for assessing the effects of CPAs, the effect of chilling, cooling to cryogenic temperatures, post-thawing viability or any other type of oocyte manipulation. Staining of oocytes with trypan blue (TB), thiazol blue (MTT), and CFDA have been used for quick assessment of viability. A functional test based on *in vitro* incubation of mature oocytes, in presence of hormones that facilitates germinal vesicle breakdown (GVBD) has also been shown effective for late stage III or stage IV oocytes (see below). Genomic and proteomic tools (as those described in Chapters 3 and 4, respectively) offer additional options for assessing the damage incurred to oocytes during preservation procedures but they require long procedures and are costly. They can be used, however, to support or verify the suitability of specifically devised cryopreservation procedures.

4.1. Fish Oocytes Sensitivity to Cryoprotectant Toxicity

Toxicity of cryoprotectants to zebrafish (*D. rerio*) oocytes has been investigated (Plachinta et al., 2004a). Commonly used cryoprotectants DMSO, methanol, ethylene glycol (EG), PG, sucrose and glucose were studied. Stage III (vitellogenic), stage IV (maturation), and stage V (mature egg) zebrafish follicle-enclosed oocytes

were incubated in Hank's medium (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 4.2 mM $NaHCO_3$) containing different concentrations of cryoprotectants (0.25–4 M) for 30 min at room temperature. Three different tests were used to assess oocyte viability: TB staining, thiazolyl blue (MTT) staining and *in vitro* maturation followed by observation of GVBD. The study showed that the toxic effect of cryoprotectant on zebrafish oocytes generally increased with increasing concentration. MTT test was shown to be the least sensitive testing method and gave poor correlation to subsequent GVBD results (Figure 1). Sensitivity of vital tests increases in the order of MTT, TB, and GVBD. GVBD is the most reliable method for assessing oocytes viability as it assesses overall oocyte capability of development after cryoprotectants treatment. However, GVBD can only be applied to stage III oocytes as later developmental stages have already gone through maturation and GVBD process *in vivo*. The authors recognized that TB test may not be ideal as it only assesses the membrane damage as appose to whole cell physiological status. However, TB is the fastest test so far and can be used for all oocytes developmental stages which allow the comparisons to be made. The authors pointed out that new methods need to be developed, for fish oocytes viability assessment, including the use of molecular markers. In this study, GVBD test showed that cryoprotectant toxicity to stage III zebrafish oocytes increased in the order of methanol, PG, DMSO, EG, glucose, and sucrose. No observed effect concentrations (NOECs) for stage III oocytes were 2, 1, 1, 0.5, <0.25 M and <0.25 M for methanol, PG, DMSO, EG, glucose, and sucrose, respectively (Table 1). TB test also showed that the toxicity of tested cryoprotectants increased in the

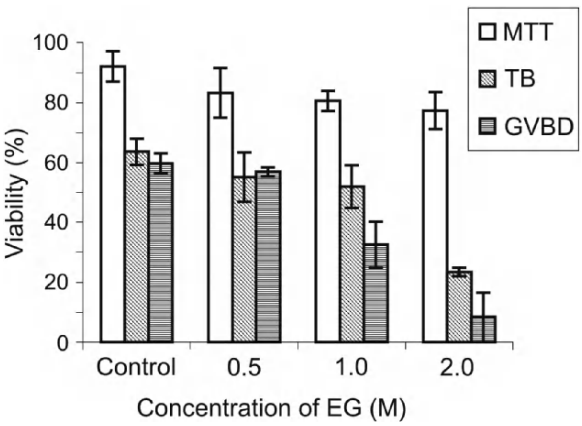

Figure 1. Comparison of the three viability tests: MTT staining, TB staining, and GVBD observation. Stage III zebrafish oocytes were exposed to different concentrations of EG for 30 min at room temperature.

Table 1. The no observed effect concentration (NOEC) of cryoprotectants to three developmental stages of oocytes obtained from different viability tests for zebrafish (A) and the gilthead sea bream (B)

A. Zebrafish oocytes

	Stage III		Stage IV	Stage V
CPA	TB	GVBD	TB	TB
DMSO	3 M	1 M	>2 M	1 M
Methanol	>4 M	2 M	>2 M	–
EG	1 M	0.5 M	1 M	1 M
Propylene glycol	>4 M	1 M	>2 M	–
Glucose	0.5 M	<0.25 M	0.5 M	–
Sucrose	0.25 M	<0.25 M	0.25 M	–

The highest concentration of CPAs used in the experiments was 4 M except TB test on stage IV oocytes, where the highest concentration used was 2 M.

B. Gilthead sea bream oocytes

	Stage IV	
CPA	MTT	GVBD
DMSO	4 M	2 M
Methanol	6 M	6 M
EG	6 M	–
Propylene glycol	4 M	–
Glucose	0.5 M	<0.25 M
Sucrose	0.25 M	<0.25 M
EG + sucrose	3 + 0.3 M	

The highest concentration of CPAs used in the experiments with stage IV oocytes, was 0.3 M for glucose, 0.5 M sucrose, 4 M for DMSO and propanediol, 8 M for methanol and EG.

same order. The sensitivity of oocytes to cryoprotectants appeared to increase with development stage with stage V oocytes being the most sensitive.

Studies performed on the oocytes of the gilthead sea bream (*Sparus aurata*; GSB), a marine species that is extensively cultured in the Mediterranean area, showed that the tolerance of stage III–IV oocytes to cryoprotectants differed from those reported for zebrafish (Table 1B) and they tolerated 6 M solutions of methanol and EG. For GSB oocytes, TB staining was not suitable as an indicator of viability and MTT was found to present more reliable results (Lubzens et al., 2003, 2004). These results indicate that oocyte tolerate permeating and non-permeating cryoprotective agents at variable concentrations. It remains to be shown, however, to what degree cryoprotective agents permeate oocytes, in order to proceed with elucidating the optimal cooling protocol that will lead to effective cryopreservation.

4.2. Fish Oocytes Membrane Permeability to Water and Cryoprotectants

Investigation into fish oocyte membrane permeability is essential for developing successful protocols for their cryopreservation. In general, two approaches can be used for assessing membrane permeability; indirect and direct measurements. Three methods have been used so far in studying membrane permeability; (a) volumetric changes in oocytes exposed to cryoprotectant solutions, (b) direct measurements by HPLC for assessing uptake or accumulation of compounds (DMSO), and (c) uptake or accumulation of radiolabelled cryoprotectant such as methanol.

The permeability of the zebrafish (*D. rerio*) oocyte membrane to water and cryoprotectants has been studied and fish oocyte membrane permeability parameters were reported for the first time by Zhang et al. (2005a). The study was conducted on stage III and stage V zebrafish oocytes. Volumetric changes of stage III oocytes in different concentrations of sucrose solutions were measured after 20 min exposure at 22°C and the osmotically inactive volume of the oocytes (V_b) was determined using the Boyle Van't Hoff relationship. Volumetric changes of oocytes during exposure to different cryoprotectant solutions were also measured. Oocytes were exposed to 2 M DMSO, PG and methanol for 40 min at 22°C. Stage III oocytes were also exposed to 2 M DMSO at 0°C. The dynamic volumetric changes of oocytes during exposure to different cryoprotectant solutions were recorded, measured and analysed using computer aided real time video microscopy. Hydraulic conductivity (L_p) and solute (cryoprotectant) permeability (P_s) were estimated using a two-parameter model. The osmotically inactive volume of stage III zebrafish oocytes was found to be 69.5%. The mean values ±SE of L_p were found to be 0.169 ± 0.02 and 0.196 ± 0.01 μm/min/atm in the presence of DMSO and PG, respectively at 22°C, assuming an internal isosmotic value for the oocyte of 272 mOsm. The P_s values were 0.000948 ± 0.00015 and 0.000933 ± 0.00005 cm/min for DMSO and PG, respectively (Table 2). The study also showed that the membrane permeability of stage III oocytes decreased significantly with temperature. No significant changes in cell volume during methanol treatment were observed. The L_p and P_s values obtained for stage III zebrafish oocytes are generally lower than those obtained from other aquatic invertebrates and higher than those obtained with immature medaka oocytes (Valdez et al., 2005) or fish embryos (Zhang and Rawson, 1998). It was not possible to estimate membrane permeability parameters for stage V oocytes using the methods employed in this study because stage V oocytes experienced the

Table 2. Stage III zebrafish oocytes membrane permeability parameters (*Lp* and *Ps*) when exposed to DMSO and PG at 22°C and 0°C

Parameters	DMSO (22°C)	DMSO (0°C)	PG (22°C)
Lp (μm/min/atm)	0.169 ± 0.02	0.070 ± 0.007	0.196 ± 0.01
Ps (cm/min)	0.000948 ± 0.00015	0.00041 ± 0.0002	0.000933 ± 0.00005

Values are means ± standards error of means.

separation of the plasma membrane of the oocyte from the zona radiata, creating a perivitelline space, during exposure to cryoprotectants.

Relatively high concentrations of DMSO (1.2–1.8 M) were assessed by HPLC in unfertilized medaka eggs after short periods of incubation. Permeation of DMSO was significantly increased by incubation of eggs (1–3 min) in 1 M trehalose followed by application of hydrostatic pressure (50 atm) (Routray et al., 2002).

Accumulation of radiolabelled methanol was studied in zebrafish and the Gilthead sea bream oocytes (Lubzens et al., 2005). In general, the concentration of methanol in gilthead sea bream was found to reach 1.16 ± 0.59 M after 15 min of incubation, while zebrafish oocytes were found to contain 0.39 ± 0.04 M after 60 min of incubation. Moreover, the concentration within sea bream oocytes was significantly higher than that found in fertilized eggs at various stages of embryonic development (Figure 2). Most of the radiolabeled methanol was recovered from the yolk compartment (Figure 3). The uptake of other radiolabelled or non-radiolabelled cryoprotectants by fish oocytes has not been studied so far.

A novel approach for increasing permeability to water and cryoprotectants has been reported recently for zebrafish embryos (Hagedorn et al., 2002; Lance et al., 2004) and medaka oocytes (Valdez et al., 2006). zebrafish embryos that were injected with cRNA encoding mammalian aquaporin-3 (AQP3) fused with GFP showed transient expression of AQP3–GFP throughout the embryos, including the cells forming the yolk syncytial layer, and consequently increased the permeability to water and PG of this membrane. Similarly, improved permeability to water, EG, PG, and DMSO was observed in AQP3 cRNA-injected

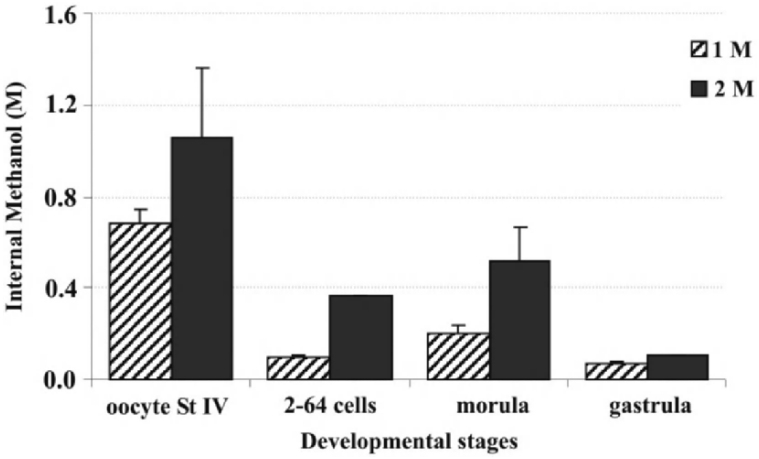

Figure 2. The internal concentration of ^{14}C methanol in Gilthead sea bream oocytes (stage IV) and fertilized eggs with embryos at 2–64 cell stage, morula and gastrula stages, after incubation for 60 min in 1 or 2 M solutions. Statistical analyses (one way ANOVA, Tukey and Duncan analyses) indicated $p < 0.00001$ between stages and that internal concentration was in the reducing order of oocytes.>morula>gastrula>2–64 cells.

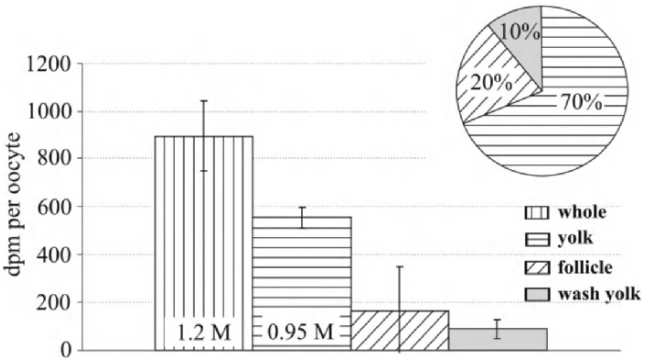

Figure 3. Distribution of radiolabeled methanol (in dpm) in Gilthead sea bream oocyte compartments after 15 min of incubation in a 4 M solution. Samples were collected from five replicate experiments and each replicate consisted of 30 oocytes. Oocytes were punctured and the yolk and membranes were collected separately. The membranes were placed in an Eppendorf vial in phosphate buffered solution and centrifuged. The pellet containing the membrane fraction and the supernatant ("wash") were counted separately. The equivalent concentration of methanol is shown for the whole oocyte and the yolk.

medaka oocytes. Artificial expression of AQP3 in mouse oocytes improved the tolerance of these oocytes to cryopreservation (Edashige et al., 2003). However, it remains to be shown whether similar success can also be achieved with fish oocytes that are 1,000 times larger than mammalian oocytes, whilst the expression of AQP3 increased permeability to water and cryoprotectants by only twofold.

4.3. Fish Oocytes Sensitivity to Chilling

Chilling sensitivity of fish oocytes was studied by Isayeva et al. (2004). Experiments were conducted with zebrafish stage III (vitellogenic) and stage V (mature) oocytes, which were chilled at 10°C, 5°C, 0°C, –5°C, or –10°C for 15 or 60 min using a low-temperature bath. Control oocytes were kept at room temperature at 22°C (Figure 4 and Figure 5). Oocyte viability was assessed using three different methods TB staining, thiazolyl blue (MTT) staining, and *in vitro* maturation followed by observation of GVBD. The results showed that zebrafish oocyte were very sensitive to chilling and their survival decreased with decreasing temperature and increasing exposure time periods at zero and subzero temperatures. Normalized survivals for stage III oocytes assessed with TB staining after exposure to 0°C, –5°C, or –10°C for 15 or 60 min were 90.1 ± 6.0%, 77.8 ± 7.6%, 71.2 ± 9.3%, and 60.2 ± 3.8%, 49.6 ± 6.7%, 30.4 ± 3.0%, respectively. The study found that the sensitivity of viability assessment methods increase in the order of MTT < TB < GVBD. Stage III oocytes were more susceptible to chilling than stage V oocytes, and that individual female had a significant influence on oocyte chilling sensitivity.

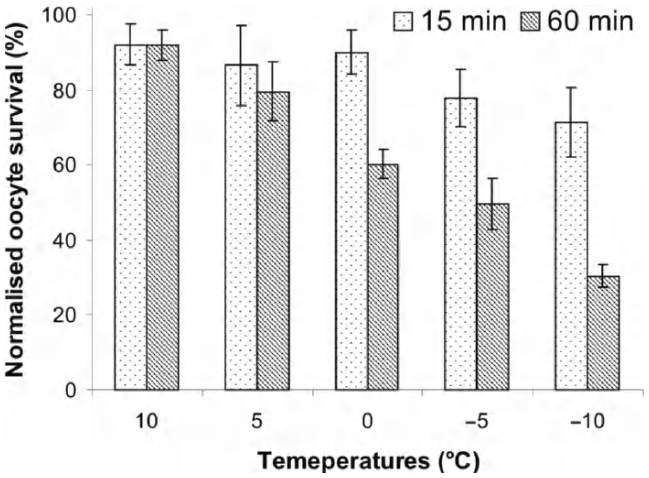

Figure 4. Survival of stage III zebrafish oocyte after exposure to different temperatures for 15 or 60 min. Oocyte viability was assessed using TB (0.2%, 3–5 min at 22°C) after they were warmed up to room temperature for 30 min. Oocyte survival was normalized with respect to room temperatures control. Values are mean ±SEM.

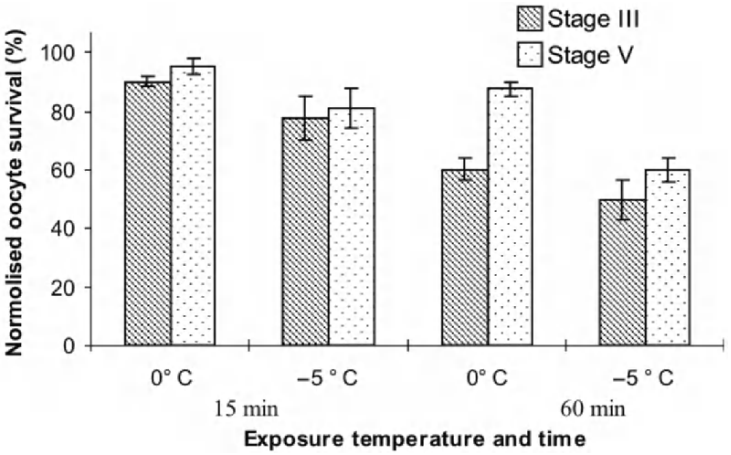

Figure 5. Survival of stage III and stage V zebrafish oocyte assessed with TB staining (0.2%, 3–5 min at 22°C) after exposure to low temperatures for 15 or 60 min. Oocyte viability was normalized with respect to room temperatures. Values are mean ±SEM.

There is only one other study on chilling sensitivity of zebrafish oocytes in the literature and the work was conducted with only two types of oocytes: with or without yolk (Pearl and Arav, 2000). In this study Perl and Arav reported that less than 30% of large oocytes survived after 15 min exposure to 0°C whilst Isayeva et al. (2004) reported a survival of 63.7% for stage III oocytes under

similar conditions. The discrepancies of the two studies may be due to the facts that in Perl and Arav's study, cFDA (5-carboxy-fluoresceindiacetate) was used for assessment of oocyte viability (which gives ambiguous results, Isayeva et al., 2004) and mixed oocytes at different developmental stages were also used. The oocytes of cold acclimated carp (*Cyprinus carpio*) were also found to be chilling sensitive and their survival decreased with decreased temperature and increased exposure time (Dinnyes et al., 1998). Chilling sensitivity in zebrafish oocytes was linked to lipid phase transition of the oocyte membranes (Pearl and Arav, 2000). Thermal stress placed on membranes during cooling may result in transitions from the liquid crystalline to gel phase. As a consequence of the transition, the cell may leak its content to the surrounding medium (Crowe et al., 1989; Drobnis et al., 1993). Pearl and Arav (2000) results show phase transition in small and large zebrafish oocytes exposed to temperature between 12°C and 22°C, indicating that chilling damage may occur at temperatures well above the water freezing temperatures and may depend of the lipid composition of membranes (Drobnis et al., 1993).

4.4. Low-Temperature Storage of Fish Gonad Cells and Oocytes

Although there was no reported information in the literature on cryopreservation of fish oocytes and storage at −196°C, several studies have been carried out on storage of fish gonad cells and oocytes at reduced temperatures. It has been reported that the development of fish gametes and eggs was affected by changes in temperature (Barrett, 1951; Marr, 1966; Poon and Johnson, 1970; Jensen and Alderdice, 1984). Combs and Burrows (1957) found that both chinook (*Oncorhynchus nerka*) and pink (*O. garbuscha*) salmon eggs could tolerate 1.7°C for long periods if they were initially stored at 5.6°C for a month. A further study of Combs (1965) defined the stage of development at which low water temperatures could be tolerated by chinook salmon eggs as being 144 h of incubation. Maddock (1974) reported that the incubation period of brown trout (*Salmo trutta*) ova could be extended for up to 4 months at a temperature of 1.4°C if ova were initially stored at a temperature of 7.6°C for 13 days. The study of Pullin and Bailey (1981) on cryopreservation of plaice (*Pleuronectes platessa*) eggs at low temperatures showed that the survival rate increased with increasing development of the eggs used. Harvey and Ashwood-Smith (1982) found that although the freezing point of isolated, unfertilized eggs of rainbow trout (*S. gairdneri*) is −1.7°C, these eggs supercool readily to temperatures around −20°C when ice is absent from the surrounding medium. They found that eggs cooled at 1°C/min in paraffin oil generally remain unfrozen to −18°C unless seeded. Harvey and Ashwood-Smith (1982) reported that unfertilized salmonid ova can be stored at −1°C for up to 20 days in artificial media and in ovarian fluid. In all cases ovarian fluid preserved fertility longer than the synthetic media.

The study of the effect of cryoprotectants on non-freezing storage of zebrafish embryos at zero and subzero temperatures by Zhang and Rawson (1995)

indicated that the presence of sucrose or trehalose slightly enhanced cooling tolerance of the embryos. Methanol proved to be an optimal cryoprotectant (when compared with DMSO or PG) and the optimal methanol concentration, when supplemented with sucrose (0.1 M), was temperature dependent, being 1 M at 0°C, 2 M at –5°C, 3 M at –10°C, and 5 M at –15°C, with embryo survival of 30.2 ± 3.5%, 28.6 ± 5.8%, 27.3 ± 12.1%, and 14.3 ± 4.2% after storage for 48, 24, 6, and 1 h, respectively. Some factors such as oxygen and bacterial infection limit the development and survival of fish eggs during storage (Pullin and Bailey, 1981). The study of Garside (1966) on the effects of oxygen in relation to temperature on the development of embryos of brook trout (*Salvelinus fontinalis*) and rainbow trout (*S. gairdneri*) showed that the velocity of development was increasingly retarded by progressively lower levels of dissolved oxygen.

4.5. Cryopreservation of Fish Oocytes Using Controlled Slow Cooling

Studies on cryopreservation of zebrafish (*D. rerio*) vitellogenic oocytes using controlled slow cooling was carried out by Plachinta et al. (2004b, 2005). Stage III (vitellogenic) oocytes were used together with cryoprotectants DMSO, methanol, EG and PG. Cryoprotectant solutions were prepared in Hank's medium. 2 M PG, DMSO, methanol, and 1 M EG were used in this study. Oocytes were incubated in CPA solutions for 30 min at room temperature before frozen with different cooling rates (1°C/min or 2°C/min) to –10°C, –15°C, –20°C, or –25°C. Two viability assessment methods were applied in this study: TB staining and GVBD observation. Results showed that oocytes viability decreased with temperature, and methanol appeared to be the most effective cryoprotectant. Normalized oocyte survivals in 2 M methanol were 74.7 ± 12.0%, 64.4 ± 27.9%, 55.4 ± 7.6%, and 47.4 ± 5.37% at –10°C, –15°C, –20°C, and –25°C, respectively when assessed with TB. At –25°C, which is the lowest temperature assessed in the study, the normalized survival of oocytes frozen in 2 M methanol, DMSO, PG, and 1 M EG were 47.4 ± 5.37%, 34.0 ± 20.6%, 32.7 ± 9.7%, and 26.8 ± 11.7%, respectively (TB). GVBD observation was more sensitive than TB staining. At –10°C normalized oocyte survivals obtained using GVBD observation after freezing in 2 M methanol, DMSO, PG, and 1 M EG were 37.8 ± 25.3%, 7.6 ± 5.3%, 3.6 ± 5.8%, and 5.8 ± 7.4%, respectively. At –15°C the highest oocytes survival obtained with GVBD observation was 7.3 ± 6.3% for all cryoprotectants used (Plachinta et al., 2004b). Plachinta et al. (2005) also studied the effect of certain supplements in 4 M methanol based cryoprotective medium on stage III zebrafish oocytes viability after controlled slow cooling to –196°. The control media used in these studies were 4 M methanol in Hank's and 4 M methanol in KCl-buffer. Polyethylene glycol-400 (PEG-400) (5%), PEG-200 (5%), poloxamer-188 (10 mg/mL); sucrose (0.1 M), glucose (0.2 M), and sucrose + glucose (0.1 and 0.1 M, respectively) were used as additives to the control media. Zebrafish oocytes were incubated in the above media for 30 min, they were then loaded into 0.5 mL plastic straws and frozen using the following

protocol: cooling from 20°C to –12.5°C at 2°C/min; manual seeding at –7.5°C and hold for 5 min; slow freezing from –12.5°C to –50°C at 0.3°C/min; samples were then plunged into LN. After 10 min in LN, samples were thawed (~200°C/min) in a 27°C water bath. Oocytes were then washed either in Hank's or KCl-buffer twice and viability was assessed using TB staining (0.2% for 5 min). Results showed that KCl buffer was more beneficial as a medium for cryopreservation of zebrafish oocytes when compared with Hank's. At –50°C, viability of oocytes frozen with 4 M Methanol in KCl buffer and Hank's were 36.2 ± 6.8% and 16.2 ± 5.2%, respectively. Oocytes survival in KCl-buffer based media following plunge into LN were: 8.7 ± 1% for 4 M Methanol; 12.3 ± 4.3% for 4 M Methanol + 5% PEG-200; 4.7 ± 1.1% for 4 M Methanol + 5% PEG-400; 7.1 ± 2.4% for 4 M Methanol + 10 mg/mL Poloxamer-188; 16.3 ± 4.2% for 4 M Methanol + 0.2 M Glucose; 11.3 ± 3.2% for 4 M Methanol + 0.1 M Sucrose, and 4.3 ± 0.5% for 4 M Methanol + 0.1 M Sucrose + 0.1 M Glucose. The results showed that the addition of low concentrations of sugars (0.1–0.2 M) to a freezing medium appeared to have some beneficial effects.

Studies on membrane integrity and cathepsin activities of zebrafish (*D. rerio*) oocytes after freezing to –196°C using controlled slow cooling were carried out by Zhang et al (2005b) using 2 M methanol and 2 M DMSO as cryopotectants. Cathepsins are involved in the breakdown of yolk proteins in oocytes during GVBD and the oocytes become translucent (Chapters 1, 2, and 12). Opaque zebrafish oocytes that were subjected to ice nucleation during slow cooling to cryogenic temperatures, turn translucent after ice nucleation (similar to Gilthead sea bream oocytes shown in Figure 7), suggesting a possible involvement of cathepsins in this event. In these experiments, stage III oocytes were exposed to 2 M methanol or 2 M DMSO (both prepared in Hank's medium) for 30 min at 22°C before they were loaded into 0.5 mL plastic straws and placed into a programmable cooler (Planer KRYO550/16). Oocytes were cooled from 20°C to –7.5°C at 2°C/min, manually seeded at –7.5°C and samples held for 5 min, then cooled from –7.5°C to –40°C at 0.5°C/min and –40°C to –80°C at 10°C/min. Samples were then plunged into LN and held for at least 10 min, and thawed by immersing straws into a 27°C water bath for 10 s. Thawed oocytes were washed twice in Hank's medium. Membrane integrity and cathepsin activities of oocytes were assessed both after cryoprotectant treatments at 22°C and after freezing in LN. Oocytes membrane integrity was assessed using TB staining. Cathepsin B and L colorimetric analyses were performed using substrates Z-Arg-ArgNNap and Z-Phe-Arg-4MβNA-HCl, respectively. 2-Naphthylamine and 4-Methoxy-2-Naphthylamine were used as standards. Cathepsin D enzymatic activity was performed by analysing the level of hydrolytic action on haemoglobin. In this study, DMSO appeared to be a better cryoprotectant than methanol. TB staining showed that 63.0 ± 11.3% and 72.7 ± 5.2% oocytes membrane stayed intact after DMSO and methanol treatment for 30 min at 22°C whilst 14.9 ± 2.6% and 1.4 ± 0.8% stayed intact after freezing in DMSO or methanol to –196°C. Analysis of cathepsin activities also showed that the activity

of cathepsin B was not affected by 2 M DMSO treatment (8.03 ± 1.02 nmol/min/mg/mL of 2-Naphthylamine compared to 8.25 ± 1 nmol/min/mg/mL 2-Naphthylamine of control). However, oocyte cathepsin B activity was lowered when treated with 2 M methanol (4.13 ± 0.3 nmol/min/mg/mL of 2-Naphthylamine). Freezing to −196°C, oocyte cathepsin B activity was decreased to 3.61 ± 0.7 and 1.5 ± 0.5 nmol/min/mg/mL of 2-Naphthylamine with 2 M DMSO and 2 M methanol, respectively. For cathepsin D, the results showed that both 2 M DMSO (0.48 ± 0.03 U/mg) and 2 M methanol (0.3 ± 0.1 U/mg) treatments decreased the activity when compared to the control (0.7 ± 0.1 U/mg). Activities were decreased by cooling to 0.2 ± 0.03 and 0.08 ± 0.01 U/mg for 2 M DMSO and 2 M methanol, respectively. Cooling reduced activity of cathepsin L to 8.4 ± 0.8 and 3.7 ± 1 μmol/min/mg/mL of 4-Methoxy-2-Naphthylamine for DMSO and methanol, respectively, compared to 12.5 ± 2.4 μmol/min/mg/mL of 4-Methoxy-2-Naphthylamine for control. The results indicate that cryoprotectant and cooling treatments altered the activities of lysosomal enzymes potentially involved in yolk mobilization in zebrafish oocytes.

One of the main obstacles during cryopreservation procedures of sea bream oocytes is related to oocyte size (Lubzens et al., 2005). The relatively small oocytes (<300 μm in diameter) were observed as viable by MTT staining for 2 h after thawing from cryogenic storage. In contrast, vitellogenic oocytes or mature oocytes (400 or >600 μm in diameter, respectively), were not viable by MTT staining (Figure 6). Ice nucleation during the freezing process seems to be one of the barriers. While the oocytes of all sizes seem viable at −16°C, only the small size oocytes remained viable after ice nucleation (Figure 6). The damaged oocytes seem more translucent, indicating some changes in the crystalline structure of yolk (Figure 7). A translucent appearance of oocytes occurs naturally during oocyte maturation in sea bream and is associated with the proteolysis of yolk as discussed in Chapters 2 and 12. It is possible that the oocyte internal compartments are damaged during freezing, releasing proteases (e.g. cathepsins) or affecting ion transport mechanisms that ultimately change the physical structure of the yolk proteins. In any event, these results lead to the conclusion that while sea bream oocytes are relatively more permeable, tolerate higher concentrations of cryoprotectant solutions than fertilized eggs, the obstacles for cryogenic storage of mature oocytes are related to their size and the damage incurred to the yolk structure, by ice nucleation. Moreover, it seems that sea bream oocytes tolerate exposure to subzero temperatures during the freezing process as the damage occurred only after ice nucleation.

5. ADVANTAGES AND OBSTACLES IN FISH OOCYTES CRYOPRESERVATION

In contrast to mammalian species where methods for cryopreservation of embryos and oocytes are well established (reviewed in Ambrosini et al., 2006), attempts to successfully cryopreserve yolk-laden fish oocytes and embryos has

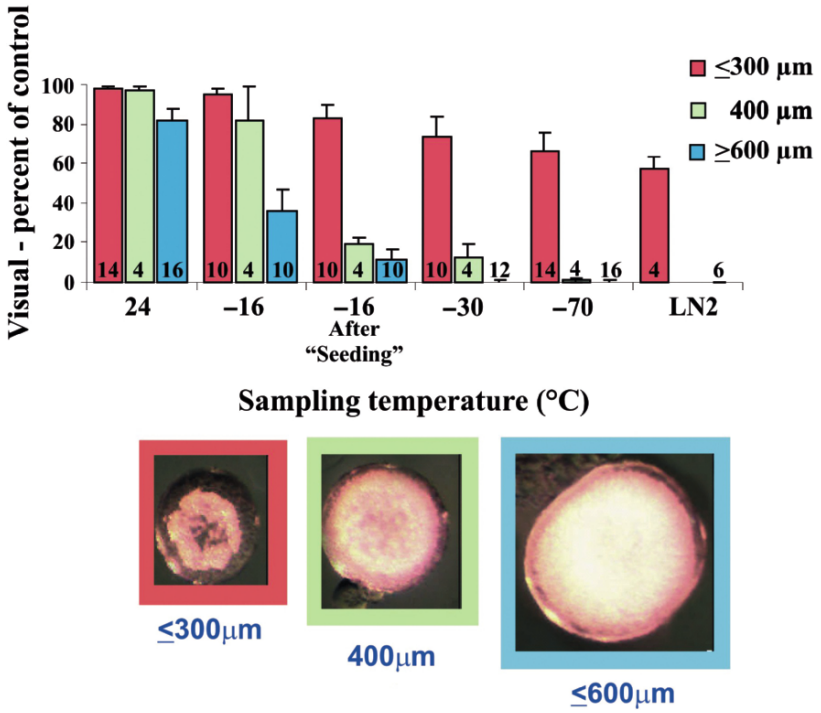

Figure 6. Percent of viable Gilthead sea bream oocytes after slow cooling to –16, –30, –70 and –196°C (LN2). Viability is shown before and after ice nucleation ("seeding") that was performed at –16°C. Three oocyte sizes were tested: ≤300, 400, and ~600 µm in diameter. Between 4 and 16 replicate vials were tested at each temperature and each replicate consisted of 30–50 oocytes. The number of replicates are shown on each bar. Viability was assessed by MTT staining and close visual examination for detection of translucent oocytes. (*See Color Plates*).

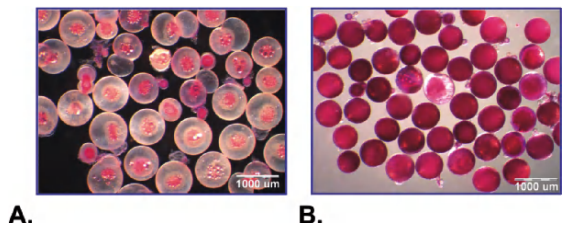

Figure 7. MTT stained translucent Gilthead sea bream oocytes (~600 µm) that were subjected to slow cooling cryogenic procedures, thawed from LN (panel A) and incubated for 1 h in 75% L-15 culture medium. Control oocytes (panel B) were incubated for the same period of time (8–10 h) in culture medium. (*See Color Plates*).

remained elusive (see below). The main obstacles that were found in cryopreservation of fish embryos include: (a) their relatively large size, resulting in a low surface to area volume ratio that could retard water and cryoprotectant efflux and influx, respectively, (b) compartments such as the blastoderm and yolk, differing in water content and permeability properties, (c) the chorion surrounding the eggs and the yolk syncytial layer, inhibiting water and cryoprotective influx and efflux, and (d) chilling injury at specific developmental stages. The high water content of pelagic floating eggs is another problematic barrier for most cultured marine fish species. Cryopreservation of fish oocytes offers several advantages, such as the smaller size of the oocytes, the absence of a fully formed chorion that may render the oocyte more permeable to solutes than the egg and the relatively lower water content in oocytes of pelagic spawners. Early oocyte developmental stages (or ovarian follicles) in fish thus represent the most suitable stage for cryopreservation. However, these oocytes will need to undergo *in vitro* maturation, ovulation, and fertilization, after cryogenic storage.

There are currently limitations for choosing the most appropriate oocyte stage for cryopreservation. In teleosts, oocytes reach their final size during vitellogenesis, which involves the uptake of hepatic precursor proteins and lipids by the oocytes, among them, the uptake of the yolk precursor protein, vitellogenin (Vtg). After the completion of vitellogenesis, the ovarian follicle is stimulated by luteinizing gonadotropins to activate the resumption of meiosis in the oocytes and subsequently the oocyte is released from the surrounding follicle layers during ovulation. In many marine teleosts, a pronounced volume increase of the oocyte due to water uptake, occurs concomitantly with oocyte maturation (see Chapter 12) and results in the production of pelagic (or floating) eggs. Development of cryopreservation procedures of previtellogenic or early vitellogenic follicles will require completion of vitellogenesis (including incorporation of Vtg) by *in vitro* techniques after cryogenic storage, and this is not possible by current technologies. Alternatively, the cryostorage of large immature oocytes that have completed the uptake of yolk protein precursors will require *in vitro* procedures for resuming oocyte maturation and ovulation, after thawing. While these procedures have been or are in progress of development for several fish species, they require additional studies to attain reliability. This also includes *in vitro* manipulation of the hydration process of oocytes of pelagic spawners discussed in Chapter 12.

Another advantage for promoting cryogenic storage of oocytes is that it leaves open the option of the spermatozoa donor, in contrast to embryos where the paternal genetic contributor has to be decided before cryogenic storage.

6. FUTURE STUDIES IN MATERNAL GENOME BANKING OF FISH SPECIES

Immense problems are encountered in attempting to succeed in cryopreserving the maternal genome. As there are no consistent successful results todate in cryopreservation of fish embryos and consequently, examining the possibility

of cryopreservation of oocytes is a reasonable and logical direction in view of studies in mammalian species. Successful methods for cryopreservation of oocytes, from a mammalian species have already been achieved in 1958 but interest was lost due to successful methods for cryopreservation of embryos. The special advantage of cryopreservation mammalian oocytes for cryobanking was identified long ago as it leaves open the paternal genome donor, until after thawing. In humans, it was introduced in situations where embryo freezing was not morally acceptable or in events of diseases in unmarried female patients. Specific cryopreservation related injury encountered during freezing and thawing procedures were found. These include alteration in the structure of the zona pellucida, premature cortical granule exocytosis, cytoskeleton, and meiotic spindle morphology (Ambrosini et al., 2006). One of the main differences in evaluating successful procedures in mammalian species vs. teleosts is that while in mammals one or a few embryos need to survive cryopreservation procedures for re-implantation into the uterus, the methods devised for fish must be suitable for production of several tens, hundreds, or thousands of live larvae that are cultured simultaneously in aquaria or tanks.

Unlike mammalian species, fish oocytes and eggs contain yolk that consists of proteins, lipids and in several species also lipid droplets. Fish oocytes are relatively smaller than their respective eggs and constitute a single compartment and do not have permeability barriers such the yolk syncytial layer found in embryos (Hagedorn et al., 1996, 1998), The initial studies showing lower permeability of oocytes vs. eggs (Prescott, 1955; Loeffer and Lovtrup, 1977; Harvey and Ashwood-Smith, 1982) and more recently Routray et al., 2002, were performed on ovulated or mature eggs. Oocytes or more correctly ovarian follicles seem to be more permeable to water and cryoprotectants than embryos (Zhang et al., 2005a; Valdez et al., 2005), with differences between freshwater species such as medaka, zebrafish, and the marine sea bream. The permeability may not be sufficiently high for cryopreservation because of the relatively large size of fish oocytes. One of the avenues under intense investigation is the artificial expression of AQP3 or other aquaglyceroporins that may act as cryoprotectant channel in fish oocytes and embryos (Hagedorn et al., 2002; Valdez et al., 2006). In AQP3-injected zebrafish embryos, permeability to PG increased by 50%, while the permeability of AQP3-injected medaka oocytes to several cryoprotectants (glycerol, EG, PG, and DMSO) and water was nearly twice higher than that of controls. It remains to be shown whether this procedure improves viability of oocytes after freezing to cryogenic temperatures.

The yolk content was shown to be implicated in chilling sensitivity of zebrafish embryos (Liu et al., 1999) and possibly contributes to the chilling sensitivity of zebrafish oocytes (Isayeva et al., 2004). However, brief exposure to low temperatures was not found as an obstacle during freezing procedures of GSB oocytes. Damage to the yolk was observed immediately after inducing ice nucleation during the cryogenic freezing procedures. This may have resulted from damage to the oocyte cytoskeleton and was observed in relatively large vitellogenic or maturing oocytes but not in small size oocytes that just embarked on

Vtg uptake. It remains to be shown whether this damage could be avoided by improving permeation of cryoprotectants in larger oocytes. Alternatively, if previtellogenic oocytes can be cryopreserved, methods for *in vitro* vitellogenesis will have to be developed.

Assessment of oocyte viability is a key issue in studies involving manipulation of oocytes. Viable dyes, such as TB or MTT, indicative of cell membrane damage and occurrence of active metabolism, respectively, are quick and simple for performing the vast array of empirical experiments. The results obtained with dyes should, however, be correlated with functional viability. As mentioned above, differences are encountered in their use in different fish species and more methods should be sought. GVBD in *in vitro* incubated oocytes could be one way of assessing functional viability but this is limited to oocytes responding to maturation inducing hormones. Genomic and proteomic tools reported in Chapters 3 and 4, could be used for evaluating a method of choice for cryopreservation of oocytes but not in routine empirical studies, due to their relatively high cost and long processing period.

Implantation of PGCs into surrogate parents with subsequent production of xenogenic, donor-derived offspring seems to offer an exciting solution to maternal genome banking in fish. The universality of this approach requires additional studies demonstrating that it can be performed between widely divergent species and that gamete production could be carried out in surrogate parents showing a short generation time (Takeuchi et al., 2004). However, the number of produced offspring in small size fish such as zebrafish, would be too small for direct cultivation in the aquaculture industry, but could serve for genome banking through production of broodstock fish. This again would prolong the period of recovery from cryogenic storage (of PGCs) to live sexually mature fish.

The vision of large-scale cryopreservation of fish oocytes or eggs, similar to that of spermatozoa cryobanking, awaits future progress in structural and functional studies. These should include better understanding of oocyte and egg compartments and transformation in the structure of yolk globules, lipids, and yolk proteins, during chilling and cryogenic procedures.

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CHAPTER 15

PROMOTING OOCYTE MATURATION, OVULATION AND SPAWNING IN FARMED FISH

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1. REPRODUCTIVE DYSFUNCTIONS OF FISH

Aquaculture, especially of marine species, is quite a new agricultural activity in relation to domestic animal production. With the exception of carp culture (family Cyprinidae) in Asia which started many centuries ago, and rainbow trout (*Oncorhynchus mykiss*) farming in Europe and North America which was

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commercialized in the last century, aquaculture as we know it is being practiced for only a few decades (Kirk, 1987). As a result, it is doubtful that a "domestic" fish species exists today, at least according to the interpretation of the word in terrestrial animal husbandry. In addition, even carp and rainbow trout, which are considered highly domesticated, do not reproduce readily in captivity.

In order to establish aquaculture as a successful and efficient agricultural activity, there is a need to control reproductive processes in fish, in order to obtain high-quality seed (i.e. eggs and sperm) and produce juveniles for grow-out without the need to obtain them from the wild. Surprising as it may appear, the aquaculture industry of species such as the freshwater eels (*Anguilla* spp.), the yellowtail and greater amberjack (*Seriola* spp.), groupers (*Epinephelus* spp.) and the bluefin tuna (*Thunnus thynnus*), is based almost exclusively on the collection of juveniles or adults from the wild (Ottolenghi et al., 2004). In some fish species, it is sometimes possible to control reproduction by manipulating environmental parameters, such as photoperiod, water temperature, tank depth and/or volume, spawning substrate, etc. Even then, the existence of the artificial environment with the associated human presence is by default an inhibiting factor on reproduction, whereas it is often impractical or even impossible in some fishes to simulate the environmental parameters accompanying reproductive maturation in the wild (i.e. spawning migration, depth, riverine hydraulics, etc.). Therefore, hormonal therapies have been employed in the past decades in order to control reproduction in cultured fishes and induce or synchronize oocyte maturation (OM), ovulation, and spawning. In some species, hormonal manipulations are the only way to produce fertilized eggs, whereas in other fishes exogenous hormones are used only as a management tool to enhance the efficiency of egg production and facilitate hatchery operations.

For example, in salmonids, which require insemination *in vitro* for the production of fertilized eggs, OM, and ovulation are often induced with hormones. This is to synchronize egg collection and fry production, thereby minimizing handling and stress to the fish, and reducing labor requirements (Goren et al., 1995; Haffray et al., 2005). In Pacific salmon (*Oncorhynchus* spp.), hormonal therapies can also advance ovulation by a few weeks (Goren et al., 1995), thus reducing losses due to pre-spawning mortality (Slater et al., 1995). Hormonal therapies in fishes are also employed for the collection of gametes for interspecific hybridization and chromosome set manipulation. Finally, genetic selection also requires hormonal therapies to enable proper maturation and timely collection of gametes for artificial fertilization. Therefore, hormonal therapies have an important role in broodstock management, and will continue to be a necessary tool even after fish become properly "domesticated" and reproduce spontaneously in captivity.

1.1. Failure of Vitellogenesis, Oocyte Maturation, and Ovulation

Although there is great variation among fishes, it is true that all cultured species today exhibit some degree of reproductive dysfunction, necessitating routine or occasional hormonal therapies to induce OM, ovulation, and spawning (Zohar

and Mylonas, 2001), whereas at times it is also necessary to induce gametogenesis (Miura et al., 1991a; Ohta et al., 1997). The most easy to address reproductive problem in females is observed in salmonids (*Onchorhynchus* and *Salmo* spp.). For example, whereas fish do undergo vitellogenesis, OM, and ovulation, they fail to spawn their eggs when reared in captivity (Bromage and Cumaranatunga, 1988; Zohar, 1989), presumably because they are not offered the gravel substrate necessary to build a nest for depositing their eggs (Liley and Kroon, 1995). Failure of spawning poses management problems in the hatchery, because ovulation is not synchronized among females, and it takes 6–10 weeks for all females in a broodstock to undergo ovulation. To obtain the eggs, all nonovulated females are checked manually for ovulation two or three times a week during the spawning season. Such handling is laborious and can result in stress, injury, disease, and often high mortalities, especially in Pacific and Atlantic salmon kept at sea (Slater et al., 1995).

The most common reproductive dysfunction of captive fishes is the unpredictable occurrence or failure of OM, and hence ovulation or spawning. Some examples of fishes exhibiting this type of dysfunction include various flatfishes (Berlinsky et al., 1996; Berlinsky et al., 1997; Larsson et al., 1997; Mugnier et al., 2000), members of the Serranidae family (Tucker, 1994; Watanabe et al., 1995; Watanabe et al., 1998b), the striped bass (*Morone saxatilis*) and white bass (*M. chrysops*) (Mylonas and Zohar, 2001a), the fugu (*Takifugu* spp.) (Yang and Chen, 2004; Chen, 2005), the shi drum (*Umbrina cirrosa*) (Barbaro et al., 2002; Mylonas et al., 2004c), the dusky grouper (*Epinephelus marginatus*) (Marino et al., 2003), and the bluefin tuna (Mylonas et al., 2005), to name a few. Fishes exhibiting this type of dysfunction undergo vitellogenesis, but with the onset of the spawning season the oocytes fail to undergo OM. This type of reproductive dysfunction may often diminish over the years, after many generations of fish are produced and reared in culture conditions.

The final and most severe form of reproductive dysfunction is the failure to undergo or complete vitellogenesis, which is exemplified by the freshwater eels, as well as by most captive populations of greater amberjack (*Seriola dumerili*) in the Mediterranean (Garcia et al., 2000) and the Mekong River catfish (*Pangasius bocourti*) (Donaldson, 1996). In the Japanese eel (*Anguilla japonica*), the current approach to the artificial manipulation of vitellogenesis involves weekly treatments with gonadotropin (GtH) preparations for a period of 7–17 weeks (Ohta et al., 1997). OM and ovulation is induced by a combination treatment of pituitary extracts (which contain GtHs) and the maturation inducing steroid 17, 20β-dixydroxy-progesterone (see Chapter 11), and fertilization is done *in vitro* after manual stripping of the eggs.

1.2. Endocrine Causes of Reproductive Dysfunctions

The lack of vitellogenesis, OM, and ovulation or spawning in cultured fishes is presumably due to the absence of the appropriate environmental stimuli, and the existence of stressors imposed by captivity (Schreck et al., 2001). For example,

many of the commercially important cultured fishes migrate hundreds of kilometers to reach the environmental niches where conditions are optimal for the survival of their offspring. During this migration or with the arrival at the spawning grounds, the fish may experience significant environmental changes – e.g. water salinity or chemistry, temperature, depth, or substrate. In addition, the maintenance of fish in small tanks due to space and cost considerations, in combination with the existence of unnatural stimuli (e.g. mechanical sounds and human presence) may have negative effects on the reproductive function of fish. As a result, captive broodstocks often become arrested in advanced stages of vitellogenesis, followed by follicular atresia (Zohar, 1989).

The first suggestions as to the endocrine nature of the failure of fish to undergo OM and ovulation came from the finding that pituitaries of reproductively mature fish contained a factor able to induce ovulation when injected to another mature individual (Houssay, 1930). Presumably, the GtH present in the pituitary of reproductively mature fish acted on the gonads of the recipient fish and induced OM and ovulation. These results suggested that perhaps the reason reproductively mature fish do not undergo OM, ovulation, and spawning in captivity is due to a failure of the pituitary GtH stores to be released in the circulation. Later studies in cultured gilthead sea bream (*Sparus aurata*) demonstrated that levels of luteinizing hormone (LH) in the pituitary increased during vitellogenesis and peaked with the approach of the spawning season (Zohar et al., 1988, 1995a). However, plasma levels of LH in most females remained undetectable and oocytes underwent atresia. On the other hand, in females that spawned spontaneously, OM and ovulation were preceded by a distinct surge of LH in the plasma. These data further indicated that in fish failing to ovulate, LH was produced and accumulated in the pituitary, but was not released to the bloodstream in order to trigger OM and ovulation.

More recent and conclusive evidence of the effect of captivity on the brain–pituitary–gonad axis came from a series of studies in striped bass. In these studies, the levels of various reproductive hormones were compared between wild fish captured on their spawning grounds and cultured fish during the spawning season (Mylonas et al., 1997b, 1998; Steven, 2000; Steven et al., 2000; Mylonas and Zohar, 2001a). In wild females, a plasma LH surge accompanied OM and ovulation, whereas plasma LH levels in females reared in captivity remained low and unchanged at the completion of vitellogenesis. However, levels of LH and its mRNA in the pituitary did not differ between wild and captive females (Steven, 2000), demonstrating again that LH was synthesized and stored in the pituitary during vitellogenesis, but not released into the circulation of captive fish. In addition, mRNA levels of the pituitary receptor for the major LH-releasing hormone, gonadotropin-releasing hormone (GnRH), were similar between wild and captive females. This further suggests that the disruption in LH release from the pituitaries of captive fish is not due to a dysfunction in pituitary responsiveness, but may originate in the hypothalamic control of pituitary function. In fact, differences were observed between wild and

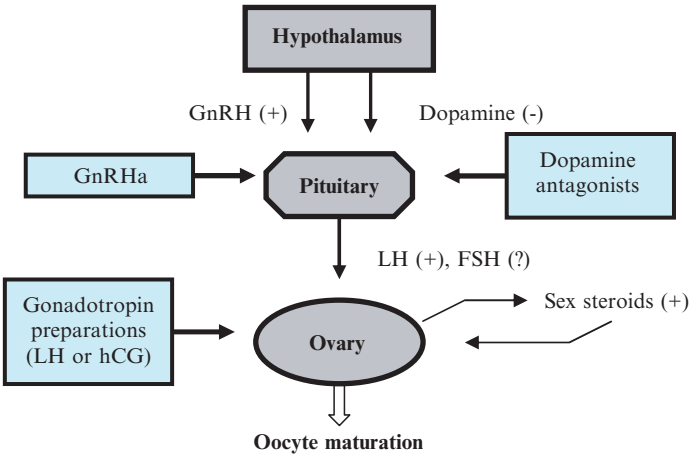

Figure 1. Schematic representation of the brain–pituitary–gonad axis and its endocrine control, along with the sites of intervention using hormonal therapies for the induction of oocyte maturation. (*See Color Plates*).

captive females undergoing OM, when comparing the pituitary content of the endogenous GnRH peptides. The GnRH mRNA levels within the brain, however, were similar between the two groups, indicating that the altered pituitary content of GnRH in captive fish may be a result of altered release from the hypothalamus, rather than deficient synthesis (Steven, 2000; Steven et al., 2000). Considered together, the above results strongly suggest that the failure of many cultured fish to undergo OM, ovulation and spawning in captivity is the result of a lack of LH release from the pituitary to the circulation, most probably due to captivity-induced altered release of the relevant and necessary GnRH(s) from the hypothalamus. This is the reason manipulations of OM, ovulation, and spawning in cultured fishes utilize either GtH preparations that act directly at the level of the gonad, or GnRH agonists (GnRHa) that act at the level of the pituitary to induce release of the endogenous LH stores (Figure 1). Endogenous LH, in turn, acts at the level of the gonad to induce steroidogenesis and the process of OM, ovulation, and spawning.

2. HORMONAL THERAPIES

Hormonal therapies to induce OM, ovulation, and spawning have been employed in fish hatcheries well before our understanding of the hormonal failure in the reproductive axis. The first applications employed the method of "hypophysation," by which ground pituitaries or pituitary extracts (containing LH) obtained from broodfish during the spawning season were injected into reproductively mature individuals of the same or different species (Von Ihering, 1937; Migita et al., 1952;

Ball, 1954). With the advent of protein isolation techniques, purified LH of piscine origin became available (Donaldson, 1973; Yaron, 1995), together with human chorionic gonadotropin (hCG) which has a high degree of structure homology with LH (Ludwig et al., 2002). In the last two decades, after the discovery of GnRH (Schally, 1978) and the synthesis of highly active agonists of GnRH (Crim and Bettles, 1997), spawning induction therapies shifted from the use of GtHs. This was partly due to the fact that by acting at a higher level of the brain–pituitary–gonad axis and stimulating the release of the fish's own LH, GnRHas may provide for a more integraded stimulation of reproductive processes and other physiological functions. Today, both GnRHas and GtHs are used extensively in spawning induction therapies, GtHs having the advantage that their effectiveness does not rely on the existence of an active or responsive pituitary.

2.1. Gonadotropin (GTH) Preparations

Hypophysation as a method to induce OM, ovulation, and spawning is still employed in freshwater aquaculture (i.e. Cyprinidae), especially in developing countries or remote areas where access to expensive purified hormones is limited. This is because pituitaries from mature fish may be readily available on-site or from local slaughterhouses, whereas purified GtHs or synthetic GnRHas are less widespread and relatively expensive (Thalathiah et al., 1988). Of more worldwide use are commercial preparations of carp pituitary extract (CPE) and purified salmon GtH (Ohta et al., 1997; Brzuska, 2004). For hypophysation, a ratio of one pituitary from a donor fish for a recipient fish of similar weight has been used for males, whereas the ratio is 1.5:1 in the case of females (Fontenele, 1955). Treatment is usually split into a small priming dose (10–20% of total) and a larger resolving dose given 12–24 h apart. Effective doses range from 2 to 10 mg of pituitary per kilogram body weight of the recipient fish (Thalathiah et al., 1988; Parauka et al., 1991; Kucharczyk et al., 1997; Chen, 2005). The major drawbacks of the use of hypophysation are (a) the potential for transmission of diseases from donor to recipient fish and (b) the variation of LH content in donor pituitaries. The latter may vary according to body weight, sex, and age of donor fish, the time of year the pituitaries were collected, and the period of time elapsed from the death of the fish to the collection and preservation of the pituitary (Yaron, 1995). Purified preparations of salmon and carp LH of standardized potency have also been available for some time (Donaldson, 1973; Yaron, 1995). Similar to hypophysation, treatment with purified LH is done in two steps, utilizing a priming and resolving dose, whereas effective doses may range between 3 and 5 mg kg^{-1} (Kucharczyk et al., 1997; Ohta et al., 1997; Brzuska, 2004; Leonardo et al., 2004). Due to species specificity of fish LHs, the commercially available preparations are usually limited to phylogenetically related fish species.

Although the problems of biosecurity and dose variation were solved by the purification of LH from fish pituitaries, availability remained restricted and the cost very high. On the other hand, hCG has been available in clinical grade, standardized preparations throughout the world. Consequently, hCG has been employed in spawning induction trials of many species in culture. Unlike GtH preparations of piscine origin, hCG is often given in a single dose, ranging between 100 and 4,000 international units (IU) kg^{-1}. The effectiveness of hCG after a single treatment is probably due to this GtH's relatively long residence time in circulation (Ohta and Tanaka, 1997). This is not related to the fact that it is a heterologous hormone, since it has been shown to have a significantly longer half-life compared to the GtHs of pituitary origin – i.e. follicle stimulating hormone (FSH) and LH – both in fish (Fontaine et al., 1984) and humans (Ludwig et al., 2002). Characteristic of the long half-life of hCG in circulation and its prolonged effect on gonadal maturation is the induction of spermatogenesis and spermiation of Japanese eel after a single injection (Miura et al., 1991a, b). However, this hormone is not as effective in inducing vitellogenesis even after multiple treatments, whereas salmon GtH is more effective (Ohta et al., 1997; Ijiri et al., 2003). After years of trials, an hCG preparation has been recently approved for commercial utilization in spawning induction therapies in fish (CHORULON, Intervet International bv, The Netherlands).

One of the main advantages of the use of GtH preparations in hormonal therapies is that they act directly at the level of the gonad and can be effective even if pituitary LH stores are low, or the pituitary gonadotrophs are not responsive to GnRHa. In such situations, GnRHa may not be effective at all or may require a long time to elicit a response. In the case of using gravid wild fish for the production of seed, or the transport of cultured broodstock from outdoor ponds/cages to indoor hatchery facilities, a long period between treatment and spawning may result in pre-spawning mortalities, due to stress induced by capture and transportation. Under these circumstances, piscine LH or hCG may be more appropriate in inducing OM, ovulation, and spawning (Hodson and Sullivan, 1993; García et al., 2001).

2.2. Gonadotropin-Releasing Hormone Agonists (GnRHa)

The use of GnRHas for spawning induction therapies has important advantages over the use of GtH preparations. For example, GnRHas are synthesized and obtained in pure form and, therefore, do not pose a disease transmission threat. Also, the use of GnRHas is generic due to the structural similarity of native GnRHs among fishes (Lethimonier et al., 2004). The most important advantage, however, is that by acting at a higher level of the brain–pituitary–gonad axis, GnRHas may provide for a more intergraded stimulation of reproductive processes by directly or indirectly stimulating the release of other hormones involved in OM, such as growth hormone (Le Gac et al., 1993), insulin-like growth factors (Negatu et al., 1998), prolactin (Weber et al., 1995), and thyroid hormones (Cyr and Eales, 1996).

	1	2	3	4	5	6	7	8	9	10	
Native peptides											
Mammal (mGnRH)	pGlu-	His-	Trp-	Ser-	Tyr-	Gly-	Leu-	Arg-	Pro-	Gly-	NH_2
Sea bream (sbGnRH)	pGlu-	His-	Trp-	Ser-	Tyr-	Gly-	Leu-	Ser-	Pro-	Gly-	NH_2
Salmon (sGnRH)	pGlu-	His-	Trp-	Ser-	Tyr-	Gly-	Trp-	Leu-	Pro-	Gly-	NH_2
Chicken II (cGnRH II)	pGlu-	His-	Trp-	Ser-	His-	Gly-	Trp-	Gln-	Pro-	Gly-	NH_2
Synthetic agonists											
Mammal (mGnRHa)	pGlu-	His-	Trp-	Ser-	Tyr-	DAla-	Leu-	Arg-	Pro-	NEt	
Salmon (sGnRHa)	pGlu-	His-	Trp-	Ser-	Tyr-	DArg-	Trp-	Leu-	Pro-	NEt	

Figure 2. Amino acid sequences of common native gonadotropin-releasing hormone (GnRH) variants in fish, and two synthetic agonists used for hormonal therapies in fish. Variants have been named after the organism from which they were first identified. Differences in the primary structure of native variants compared to the mammalian form are underlined. Agonists are synthesized by a D-amino acid substitution at position 6 and an ethylamide (ET) substitution at position 10.

Agonists of GnRH instead of the native peptides are employed in hormonal therapies, because native GnRHs are degraded quickly in circulation by endopeptidases located in the pituitary, liver, and kidney (Zohar et al., 1990). Substitutions of the GnRH decapeptide at position 6 with a dextrorotatory (D) amino acid and at position 10 with an ethylamide group (Figure 2), produce superactive agonists which are resistant to enzymatic degradation (Goren et al., 1990; Weil et al., 1992; Ulloa-Aguirre and Timossi, 2000), thus being cleared from circulation much slower than the native GnRHs (Gothilf and Zohar, 1991; Forniés et al., 2003; Haffray et al., 2005). As a result, GnRHas remain in the circulation much longer and stimulate a stronger release of LH from the pituitary. Furthermore, due to their modified polarity and tertiary structure, some of these GnRHas also exhibit increased binding affinity to the pituitary GnRH receptors (De Leeuw et al., 1988; Habibi et al., 1989; Pagelson and Zohar, 1992). Increased resistance to enzymatic cleavage and higher receptor binding affinity results in GnRHas which are 30–100 times more potent than the native GnRHs in inducing LH release (Peter et al., 1988; Zohar et al., 1989; Crim and Bettles, 1997). Recently, the GnRHa Azagly-nafarelin (GONAZON, Intervet International bv, The Netherlands) has been approved for use in hormonal therapies in aquaculture fish, and its efficacy has been so far documented in salmonids (Haffray et al., 2005), finally making GnRHa officially available to the aquaculture industry.

2.3. Other Pharmacological Therapies

Primarily in freshwater fishes, dopamine antagonists (DA) have often been used in combination with GnRHas in hormonal therapies (Peter et al., 1993). In these species, dopamine inhibits the basal release of LH and reduces or inhibits

GnRH-induced LH release (Peter and Yu, 1997). Administration of DA (e.g. domperidone, pimozide, reserpine, or metoclopramide) prior to the injection of GnRHa removes the inhibition on the gonadotrophs and enhances the stimulatory effect of GnRHa on LH release. In salmonids, catfishes, and cyprinids (Saligaut et al., 1999; Silverstein et al., 1999; Yaron et al., 2003) the inhibiting role of dopamine is well documented, but with the exception of the mullets (*Mugil* spp.) (Glubokov et al., 1994; Aizen et al., 2005), it appears to be absent in most commercially important marine fishes (Copeland and Thomas, 1989; King et al., 1994; Zohar et al., 1995a; Prat et al., 2001; Kumakura et al., 2003b). Currently, hormonal manipulations of reproduction using a combined GnRHa/DA treatment are used mostly in cyprinids (Yaron, 1995; Mikolajczyk et al., 2003; Mikolajczyk et al., 2004), catfishes (Silverstein et al., 1999; Brzuska, 2001; Wen and Lin, 2004), and mullets (Glubokov et al., 1994; Aizen et al., 2005).

In search of the optimal hormonal therapy for the induction of spawning, some researchers have also examined the efficacy of combinations of GtHs together with GnRHas, though the results do not seem to be better compared to treatments using only one of the hormones (Wen and Lin, 2004).

2.4. Tailoring the Therapy: Acute Injections vs. Sustained-Release Delivery

The use of GtHs and, especially, GnRHas in aquaculture has indeed revolutionized broodstock management, as predicted almost three decades ago (E.M. Donaldson, FAO World Conference on Aquaculture, Kyoto, Japan, 1976; unpublished), and with the recent approval of commercial hCG and GnRHa preparations for use in the aquaculture industry, it is sure that hormonal therapies will become established as practical and often indispensable tools for hatchery managers. Administration of these hormones has been done mostly via injections in saline, but it was recognized even from the first hypophysation experiments that administration of a hormone would be more efficacious in the form of a sustained-release treatment (Fontenele, 1955; Aida et al., 1978). Also, some work has been carried out examining the potential of oral delivery of GnRHa (Thomas and Boyd, 1989; Solar et al., 1990; McLean et al., 1991; Sukumasavin et al., 1992; Schep et al., 1999; Roelants et al., 2000; Mikolajczyk et al., 2002), but this method has not progressed adequately. On the contrary, more effort has been invested in the past two decades in the development and application of injectable or implantable sustained-release delivery systems for GnRHas, for the control of reproductive processes in cultured fishes (see reviews by Zohar, 1996; Mylonas and Zohar, 2001b). Much less work has been done in developing GtH-delivery systems, partly due to the high cost and species specificity of highly purified piscine LHs (Sato et al., 1995; Sato et al., 1997; Zohar and Mylonas, 2001).

The interest in developing GnRHa-delivery systems stems from both the reproductive physiology of fish and the need to optimize broodstock management practices. For example, although GnRHas resist enzymatic degradation in

the blood circulation compared to the native peptides, their maximum half-life does not exceed 23 min *in vivo* (Gothilf and Zohar, 1991). Plasma GnRHa levels after a single injection are elevated for a period of a few hours to a few days, depending on the specific GnRHa, initial dose, fish species, and water temperature (Crim et al., 1988; Harmin and Crim, 1993; Zohar et al., 1995a; Mylonas and Zohar, 2001b). The brief residence time of GnRHa in circulation is probably the reason why a single injection of GnRHa does not always result in 100% OM and ovulation (Mikolajczyk et al., 2003; Kaminski et al., 2004), and in species with asynchronous ovarian development and daily spawning rhythms, long-term ovulation is effected in only a small percentage of the broodstock (Zohar et al., 1995a; Zohar and Mylonas, 2001). Therefore, GnRHa-based hormonal therapies for many fishes involve multiple injections of GnRHa, given over the course of hours or days (Pankhurst et al., 1986; Mylonas et al., 1992; Dabrowski et al., 1994; Slater et al., 1995). Also, in species like the European sea bass (*Dicentrarchus labrax*), the barramundi (*Lates calcarifer*), and the silver perch (*Bidyanus bidyanus*), which have a multiple-batch group-synchronous ovarian development, one or two GnRHa injections were necessary to induce ovulation of the first batch of oocytes (Almendras et al., 1988; Carrillo et al., 1995; Levavi-Sivan et al., 2004), while further ovulations could only be obtained with additional injections (Mylonas et al., 2003). Repetitive handling of broodstock requires substantial labor, time, and monitoring. Especially in situations where the broodfish are kept outdoors – in ponds or cages, it is very time-consuming and labor intensive to crowd, capture, anaesthetize, and inject the fish with hormones. Furthermore, repetitive handling is stressful to the fish and can often result in injury, disease, and prespawning mortalities, or at the very least it can adversely affect the progression of OM (Pankhurst and Van Der Kraak, 1997).

The striped bass, a species which completes vitellogenesis but rarely undergoes spontaneous OM in captivity (Mylonas and Zohar, 2001a), exemplifies the ineffectiveness of even multiple GnRHa injections to induce ovulation. Treatment of post-vitellogenic females under ambient water temperature (7–18°C) with two GnRHa injections resulted in elevations of plasma GnRHa for at least 7 days (Figure 3). The resulting increases in plasma LH, 17β-estradiol (E_2), and testosterone (T) followed the profile of plasma GnRHa, and once GnRHa was cleared from the circulation LH decreased significantly and plasma E_2 and T returned to pre-treatment levels. There was no plasma elevation of 17, 20β, 21-trixydroxy-progesterone – the maturation inducing steroid, and although 60% of the females initiated the early stages of OM (germinal vesicle migration) (Mylonas et al., 1997a), none progressed further than the peripheral germinal vesicle stage. As a result, no ovulation was observed.

Obviously, more than two consecutive GnRHa injections are necessary to support a slow OM in striped bass. Sustained-release GnRHa-delivery systems were used to provide the long-term elevation of GnRHa, required to stimulate the necessary changes in the profile of all reproductive hormones

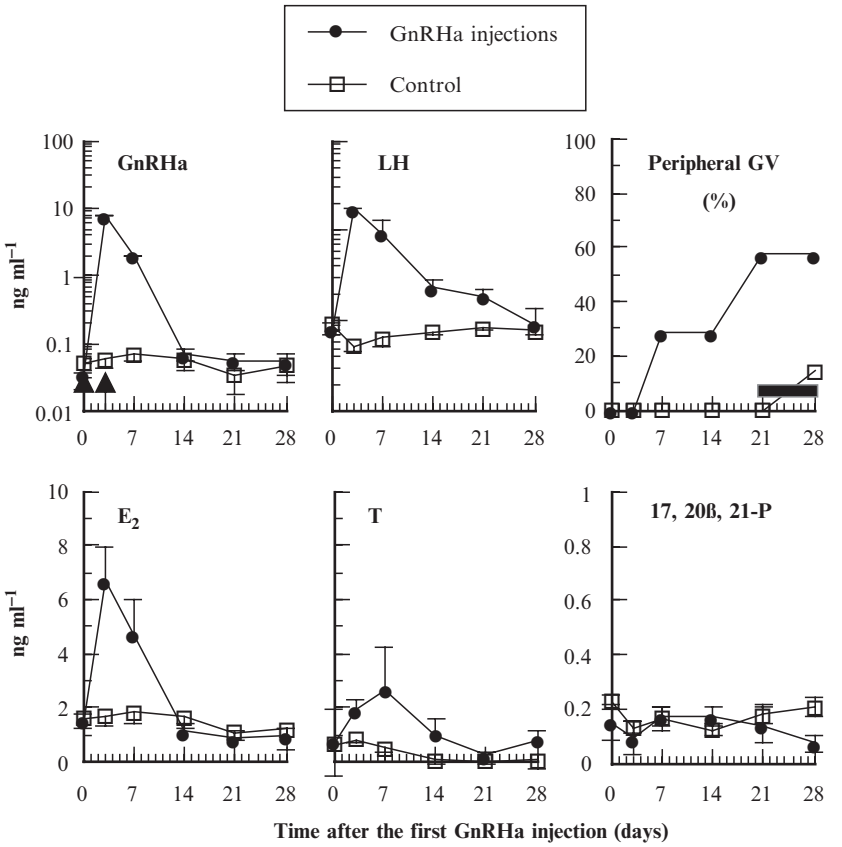

Figure 3. Mean (±SEM) plasma hormone levels of cultured striped bass ($n = 7$) and cumulative percentage of females having oocytes at the peripheral germinal vesicle (GV) stage after two injections (arrows) of GnRHa (15 μg kg^{-1}) during the spawning season (April). With the exception of the 17, 20β, 21-trixydroxy-progesterone, all plasma hormone profiles (LH, E_2, and T) were significantly changed by the GnRHa treatment (ANOVA, $P \leq 0.05$). The horizontal bar indicates the time when oocytes in atresia were observed. None of the females in the study completed OM and no ovulation was observed. (From Mylonas and Zohar, 2001b.)

for the appropriate duration, in order to result in complete OM and successful ovulation (Figure 4).

In mammals, synthesis and release of LH is regulated by the frequency and amplitude of the GnRH pulses released into the hypothalamus–pituitary portal system (Gharib et al., 1990). In fish, it has not been possible to document the natural mode and rate of GnRH release, since unlike all other vertebrates most fishes do not possess a hypothalamus–pituitary portal system, and hypothalamic GnRH is secreted directly in neuronal synapses at the pituitary gonadotrophs. The presence of an episodic release of LH in rainbow trout

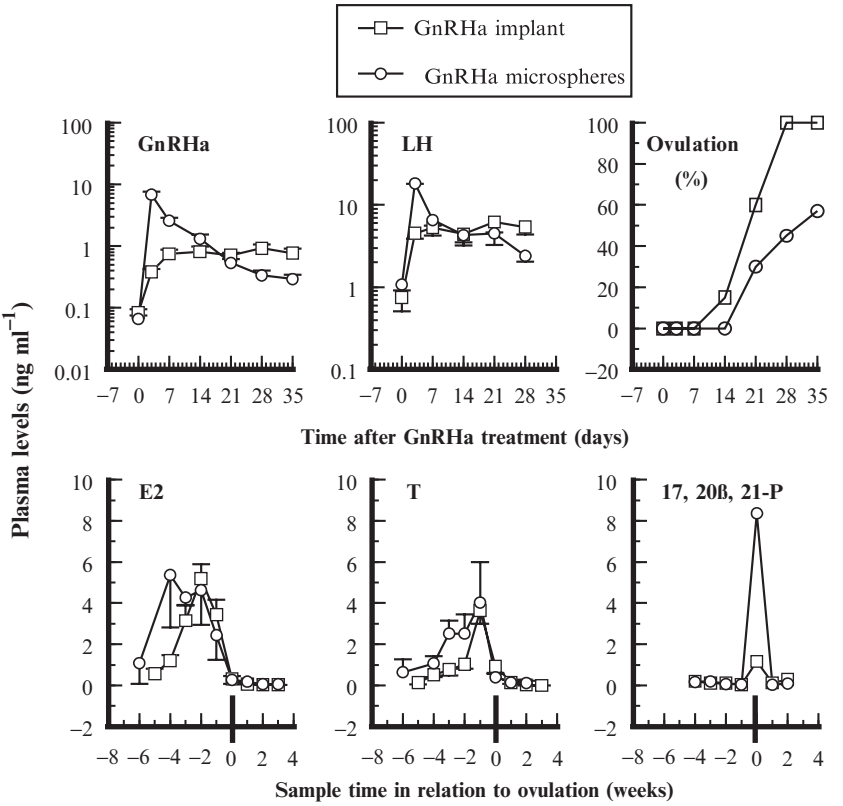

Figure 4. Mean (±SEM) plasma hormone levels and cumulative ovulation percentage of cultured striped bass (n = 7) after treatment with different GnRHa-delivery systems (15–20 µg GnRHa kg^{-1}) during the spawning season (April). Plasma data for the steroid hormones (n = 1–7) were plotted vs. the sampling time when the fish were found to have already ovulated (Ov). Changes in plasma 17, 20β, 21-trixydroxy-progesterone were observed in two females only, which happened to be sampled 12 h before ovulation. (From Mylonas and Zohar, 2001b.)

(Zohar et al., 1986) suggests that a pulsatile GnRH release may also be functional in some fishes. However, contrary to the situation in mammals (Ulloa-Aguirre and Timossi, 2000), treatment with GnRHa-delivery systems in all fish species tested so far does not result in pituitary desensitization and inhibition of LH release, but a sustained elevation of LH for many days or weeks (Weil and Crim, 1983; Sokolowska et al., 1984; Breton et al., 1990; Mylonas et al., 1997c, 1998). So, it appears that in fish, unlike the situation in mammals, birds, and reptiles (Chang and Jobin, 1994), sustained GnRHa treatment does not produce anti-gonadotropic effects.

In some fishes, nevertheless, GnRHa injections in single or multiple applications, may be an equally appropriate or even better approach compared to GnRHa-delivery systems. For example, in the group-synchronous multiple-spawning shi drum, although GnRHa-delivery systems induced ovulation or spawning in 95% of the treated females compared to 69% of females given a single injection (Barbaro et al., 2002), multiple spawnings were not induced (Mylonas et al., 2000; Barbaro et al., 2002). Multiple cycles of OM, ovulation, and spawning in this species could be induced only with multiple GnRHa injections (Mylonas et al., 2004c). In the also group-synchronous multiple-spawning European sea bass, although different types of GnRHa-delivery systems induced multiple spawns, spawning was not synchronous among females and egg quality was low compared to other studies (Forniés et al., 2001), suggesting that constantly high plasma LH levels may not induce the appropriate maturational events for multiple spawning of viable eggs. On the other hand, multiple injections spaced 7–14 days apart induced up to four consecutive spawns (Figure 5), producing eggs of high fecundity, as well as percentage fertilization, hatching, and larval survival (Mylonas et al., 2003). So, the advantage of

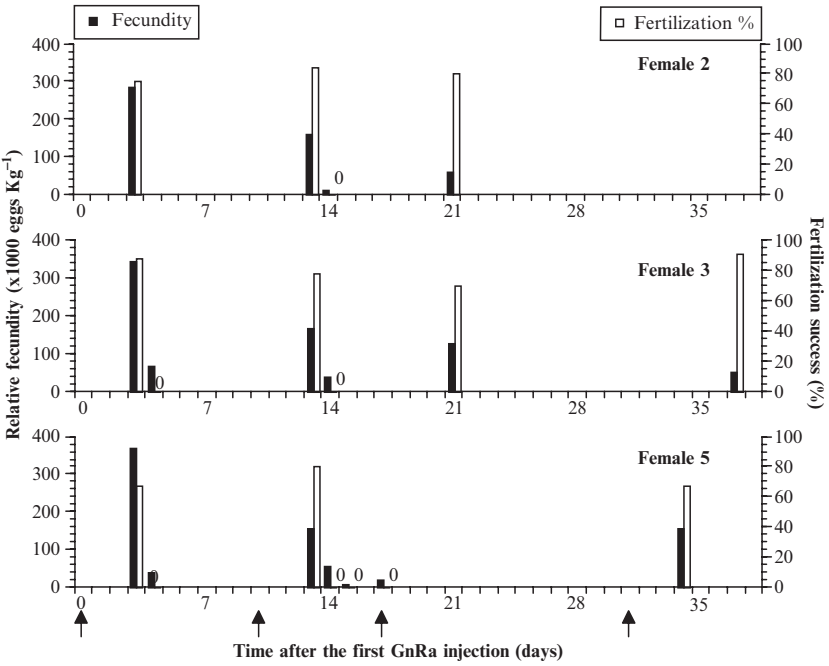

Figure 5. Relative fecundity and fertilization success of individual European sea bass spawns after treatment with multiple injections (arrows) of GnRHa (10 μg kg^{-1}). The zeros (0) refer to fertilization percentages of residual eggs released the days immediately after the major spawning events. (From Mylonas et al., 2003.)

GnRHa-delivery systems in this species is only the increased reliability in inducing OM of the most advanced batch of post-vitellogenic oocytes, but not the stimulation of multiple spawnings.

It is unclear, why the European sea bass and shi drum do not respond as well to sustained GnRHa treatment compared to other fishes with multiple-batch group-synchronous or asynchronous patterns of OM (reviewed by Mylonas and Zohar, 2001b). One explanation may be the greater interval between spawning events observed in these species. For example, the ovulation/spawning interval is 1 day for the white bass (Mylonas et al., 1997c), barramundi (Almendras et al., 1988), summer flounder (*Paralichthys dentatus*) (Berlinsky et al., 1997), and the gilthead sea bream (Barbaro et al., 1997); 2 days for turbot (*Scophthalmus maximus*) (Mugnier et al., 2000) and the dusky grouper (*E. marginatus*) (Marino et al., 2003); and 2–4 days for the striped trumpeter (*Latris lineata*) (Morehead et al., 1998) and American shad (*Alosa sapidissima*) (Mylonas et al., 1995). In the case of the European sea bass and shi drum, the spawning interval appears to be about 7 days (Asturiano et al., 2002; Mylonas et al., 2003, 2004c). Completion of vitellogenesis and recruitment of subsequent batches of oocytes to undergo OM between spawning events may require a period of relatively low plasma LH in these species, while OM and ovulation requires acute increases in plasma LH, as described in other fishes (Peter and Yu, 1997).

3. APPLICATIONS OF HORMONAL THERAPIES

The hormonal therapies employed in female broodfish can be categorized according to the reproductive process that they attempt to induce or facilitate, as well as according to the mode of ovarian development of the fish. Reproduction is separated into the stages of oocyte proliferation and growth (i.e. vitellogenesis) (see Chapters 1 and 2), and OM and ovulation (see Chapters 11 and 12). Ovulation/spawning can occur in a single or multiple times. The stage of reproduction to be induced, as well as the mode of ovulation and spawning may influence the choice of hormonal therapy.

3.1. Vitellogenesis

Hormonal therapies for the stimulation of vitellogenesis are relatively rare, especially using GnRHa, since most fishes undergo vitellogenesis in captivity. Also, the effectiveness of GnRHa is usually limited to individuals well undergoing vitellogenesis; therefore, the process may be enhanced but not initiated. For example, gonadal development was not stimulated in the Pacific herring (*Clupea harengus pallasi*) when fish were treated with GnRHa-delivery systems in September during early vitellogenesis (Carolsfeld et al., 1988). In the ayu (*Plecoglossus altivelis*) (Aida, 1983) and winter flounder (*Pleuronectes americanus*) (Harmin et al., 1995), GnRHa treatment of immature fish prior to the onset of vitellogenesis was ineffective in inducing gonadal development. Finally,

treatment of pubertal striped bass with GnRHa-delivery systems did not induce vitellogenesis (Holland et al., 1998a). On the other hand, GnRH emulsified in Freund's adjuvant induced vitellogenesis in the ayu, in individuals that were at the early stages of oogenesis and were maintained under photoperiodic conditions inhibiting to gonadal development (Aida, 1983). Also, treatment of winter flounder with a GnRHa-delivery system during vitellogenesis induced elevations in plasma T and E_2 levels, and increased oocyte diameter and gonadosomatic index (Harmin et al., 1995). Finally, in the milkfish (*Chanos chanos*), multiple treatments with GnRHa-delivery systems enhanced vitellogenesis and advanced spawning up to 2 months (Lee et al., 1986a), and in the grey mullet (*Mugil cephalus*) a combination of sustained-release delivery systems for both GnRHa and T enhanced vitellogenesis to the stage that females could be induced to spawn (de Monbrison et al., 1997). In the latter species, it was also recently shown that use of a DA alone or together with a sustained-release GnRHa treatment was effective in enhancing vitellogenesis to the point that females could be induced to spawn with another hormonal therapy (Aizen et al., 2005).

The only exception to the almost universal failure of GnRHa in inducing (i.e. initiating and promoting) vitellogenesis is the red sea bream (*Pagrus major*), where treatment of sexually mature, but reproductively inactive females (Matsuyama et al., 1995; Gen et al., 2001), as well as sexually immature, prepubertal females (Kumakura et al., 2003b) with a GnRHa-delivery system induced vitellogenesis, followed by OM, ovulation, and spawning within 3 weeks. These studies showed that a single treatment with a GnRHa-delivery system induces elevations in pituitary GnRH-receptor mRNA, FSHβ- and LHβ-mRNA, and plasma LH, E_2 and T, resulting in induction of vitellogenesis, as well as OM and ovulation.

Surprisingly, little is known today regarding the general inability of GnRHa treatments to induce vitellogenesis. This is related to the limited knowledge of the endocrine function of the brain and pituitary during this part of the reproductive cycle, compared to their better known function during OM and ovulation. Similar to other vertebrates, it has been well demonstrated in fish, that the pituitary synthesizes and releases both FSH and LH (see review by Yaron et al., 2003). In females, FSH is thought to be responsible for the regulation of vitellogenesis, while LH is responsible for OM and ovulation. Unfortunately, with the exception of salmonids, there are no assays available to measure FSH, due to difficulties in producing anti-FSH antibodies from the very small FSH amounts contained in fish pituitaries. As a result, almost all studies in non-salmonid fishes investigating the role of FSH during vitellogenesis are based only on measurements of FSHβ-mRNA levels in the pituitary, and not of the released protein in the blood.

Based on the available research, some speculation is warranted on the reason GnRHa treatments do not usually stimulate vitellogenesis, whereas they are quite successful in inducing OM, ovulation, and spawning (section 3.2). For example, GnRH may not be strongly involved in the regulation of this stage of

the reproductive cycle or, perhaps, FSH is not stimulated by the administration of GnRHa at this stage (Mateos et al., 2002; Kumakura et al., 2004). Also, LH release may be under a strong dopaminergic inhibition, thus preventing the response to exogenous GnRHa (Aizen et al., 2005). A very likely reason could also be that the pituitary may be unresponsive to the administered GnRHa at this stage, and as a result FSH and/or LH is not released (Crim and Evans, 1983; Breton et al., 1998). For example, in the Southern flounder (*P. lethostigma*) (Berlinsky et al., 1996) and summer flounder (Berlinsky et al., 1997), although GnRHa-delivery systems were ineffective in inducing OM in females in early or mid-vitellogenesis, daily injections of CPE induced vitellogenesis followed by OM. Synthesis and release of LH, as well as gametogenesis may be achieved in some fishes in response to GnRHa, after priming with T (Crim et al., 1988), or after co-treatment of GnRHa with T (Lee et al., 1986b; de Monbrison et al., 1997; Henry et al., 1998), suggesting that the pituitary must first be exposed to gonadal steroids before GnRHa can stimulate release of LH. Presumably, combined GnRHa and T treatments are more effective than GnRHa alone, because T stimulates pituitary synthesis of LH, which is then released in response to the GnRHa treatment (Crim et al., 1988). In striped bass, however, although GnRHa and T stimulated LH release, the gonads were unresponsive and vitellogenesis was not induced (Holland et al., 1998a). Finally, hormones other than FSH or LH may be involved in the initiation and progression of vitellogenesis, and these hormones may not be under the control of GnRHa.

GtH preparations have been much more successful in inducing vitellogenesis, the best example being that of the freshwater eels, which do not undergo any gonadal development in captivity. In the Japanese eel, for example, treatment with purified salmon pituitary extract (SPE) which contains LH, can induce complete gametogenesis (Ohta et al., 1997; Suetake et al., 2002; Saito et al., 2003). However, for the SPE treatment to be effective, the therapy must be given on a weekly basis over the course of many months. The same long-term therapy with SPE (11–29 weekly injections) was required for the induction of gametogenesis in the more recent attempts to control reproduction in the European eel (*A. anguilla*) (Pedersen, 2003, 2004). Similarly in the Mekong catfish (*P. bocourti*), 2–10 daily injections of 500 IU kg^{-1} hCG were necessary to stimulate oocyte growth to the point that a resolving treatment with two injections of hCG spaced 8–10 h could induce OM and ovulation (Cacot et al., 2002). Finally in the grey mullet, vitellogenesis was induced, but not completed, with pregnant mare's serum gonadotropin (PMSG) using three injections per week (Kuo, 1995). These procedures are obviously cumbersome, labor intensive, and potentially injurious to the fish, and a better approach using a GtH-delivery system has been investigated in order to reduce handling and treatment of the fish (Sato et al., 1996; Sato et al., 1997). Such a delivery system has been developed using an emulsion of lipophilized gelatin (LG), loaded with purified salmon LH (sLH) and was shown in the Japanese eel to produce a sustained elevation of plasma sLH for 24 days after treatment. Compared to immature females treated

weekly with sLH in saline, females given the LG emulsion attained a higher gonadosomatic index after 9 weeks and developed gonads with oocytes at the germinal vesicle migration stage (Sato et al., 1997).

A unique and very promising way to induce gametogenesis in the European eel through the elevation of the endogenous FSH and LH is currently being developed (van den Thillard and Spaink, unpublished data). The method utilizes genetically engineered eel cell lines, which contain the genes of eel LH and FSH, controlled by a constitutive promoter. Once implanted subcutaneously to immature eels, such cells are expected to produce continuously the two endogenous eel GtHs, thus stimulating gonadogenesis. Preliminary work has showed encouraging results.

3.2. Oocyte Maturation, Ovulation, and Spawning

The most common reproductive dysfunction in cultured fish is the failure of females to undergo OM, ovulation, and spawning after the completion of vitellogenesis. Hence, most of the applications of hormonal therapies have focused on this aspect of reproduction control. Three modes of ovarian development have been identified in fish: synchronous, group-synchronous, and asynchronous (Wallace and Selman, 1981; Tyler and Sumpter, 1996). Synchronous ovarian development is characteristic of semelparous species like the Pacific salmons and freshwater eels, which reproduce only once in their lifetime. Group-synchronous fish are further separated into single-batch and multiple-batch spawners. In these fishes, distinct populations of oocytes at different stages of development are present in the ovary. There is a population of primary oocytes and one or more populations of developing oocytes. During the annual spawning season, females with single-batch group-synchronous ovarian development undergo OM and ovulate only once (e.g. striped bass and rainbow trout). Species like the European sea bass, shi drum and greater amberjack have a multiple-batch group-synchronous ovarian development and undergo multiple ovulations within the course of a few weeks. Finally, species with asynchronous ovarian development, such as the gilthead sea bream (Zohar et al., 1995a), red porgy (*P. pagrus*) (Mylonas et al., 2004a), and red sea bream (Watanabe and Kiron, 1995) have a population of primary oocytes and a heterogeneous population of vitellogenic oocytes, from which several batches are recruited and undergo OM, ovulation and spawning during the annual spawning season, in daily or almost-daily intervals. For the purpose of hormonal therapy applications, fish are separated into two classifications: single-time spawners (synchronous and single-batch group-synchronous) and multiple spawners (multiple-batch group-synchronous and asynchronous).

3.2.1. Single-time spawners

As mentioned earlier, GtH preparations are still widely used for the manipulation of reproduction in cultured fishes. Specific information on doses and treatment

protocols using LH of piscine origin or hCG can be found in earlier extensive reviews (Donaldson, 1973; Lam, 1982; Donaldson and Hunter, 1983; Zohar and Mylonas, 2001). Since then, many more species or studies have been added to the list. For example in the European catfish (*Silurus glanis*), carp pituitary homogenate (4 mg kg^{-1}) was successful in inducing ovulation, though in a smaller percentage of females compared to a combined GnRHa/DA treatment (Brzuska, 2001). In the Japanese catfish (*S. asotus*), a single injection of hCG (10,000 IU kg^{-1}) was effective in inducing OM and ovulation in yearling females for an extended period of time from June to September (Kumakura et al., 2003a). In yet another catfish species, the Brazilian "cachara" (*Pseudoplatystoma fasciatum*), CPE and hCG were both effective in inducing ovulation, whereas a combined CPE/hCG treatment was ineffective (Leonardo et al., 2004). Also, a single injection of hCG at 1,000 or 2,000 IU kg^{-1} was effective in inducing ovulation with similar fertilization and hatching percentages in the Korean spotted sea bass (*Lateolabrax maculatus*) (Lee and Yang, 2002). Finally, in wild-caught ocellated puffer (*Takifugu ocellatus*), both single- and double-injections of extracted pituitaries (6 mg kg^{-1}) or hCG (2500 IU kg^{-1}) were 100% effective in inducing OM and ovulation, resulting in high fertilization success (Chen, 2005), while in photoperiod-manipulated pikeperch (*Sander lucioperca*), single or multiple injections of hCG (200 IU kg^{-1} per injection) were effective in inducing ovulation after 66–71 h (Zakes and Szczepkowski, 2004).

One of the first applications of GnRHa, either in the form of injections or sustained-release delivery systems, has been for the synchronization of ovulation in salmonids (Donaldson et al., 1981; Crim and Glebe, 1984; Breton et al., 1990). Traditionally, synchronization of ovulation using GnRHa injections (10–100 μg kg^{-1}) involved two injections spaced 3 days apart, given around 2 weeks before the expected date of maturation of the broodstock. If the treatment is given too early, a third injection may be required to successfully complete OM and ovulation in all females. Given at the expected date of maturation, a single GnRHa injection may also induce ovulation in 80–90% of the females within 2–4 weeks (Breton et al., 1990; Haraldsson et al., 1993; Pankhurst and Thomas, 1998; Haffray et al., 2005), whereas the two-injection protocol induces 100% ovulation (Van Der Kraak et al., 1985; Sullivan et al., 1989; Mylonas et al., 1992).

Single or multiple injections of GnRHa are also widely employed in other single-time spawning fishes. As in salmonids, when a two-injection protocol is used, GnRHa is given in a priming (5–10%) and resolving dose (95–90%), and if a DA is also employed it is given with the priming dose. For example, in the ocellated puffer both a single and double injections of GnRHa (50 μg kg^{-1}) were effective in inducing OM and ovulation, without any difference in egg fertilization success (Chen, 2005), while similar results were obtained using 2–4 injections of GnRHa (total of 40–300 μg kg^{-1}) in the obscure puffer (*T. obscurus*) (Yang and Chen, 2004) and the bullseye puffer (*Spoeroides annulatus*) (Duncan et al., 2003). In the grey mullet, two injections of GnRHa (30 μg kg^{-1}) spaced 22.5 h apart, along with the DA metoclopramide (15 mg kg^{-1}) were very effective in

inducing spawning within 24 h (Aizen et al., 2005). Similarly, two injections of GnRHa (20 μg kg^{-1}) spaced 6 or 12 h apart, together with the DA pimozide (5 mg kg^{-1}) induced ovulation in 95% of treated common carp (*Cyprinus carpio*) (Mikolajczyk et al., 2004). In the same fish, two injections of GnRHa (80 μg kg^{-1}) spaced 6 h apart, without a DA, induced ovulation of only 45% of treated females (Mikolajczyk et al., 2003). Two injections of GnRHa in combination with a DA have been used successfully also in the koi carp (*C. carpio*) (Arabaci et al., 2004), lake mullet (*Chalcalburnus tarichi*) (Arabaci and Sari, 2004), and wild catfish (*S. asorus*) (Wen and Lin, 2004). Finally, a single injection of GnRHa (20 μg kg^{-1}) induced ovulation in 1-year-old tench (*Tinca tinca*) (Rodríguez et al., 2004). GnRHas in the form of injections will continue to be used widely in aquaculture, partly due to their greater availability and lower cost compared to the GnRHa-delivery systems, which are not widely available commercially and/or are not yet approved for use in aquaculture applications.

The use of GnRHa-delivery systems begun in salmonids and has been extensive, both in research and commercial situations (under experimental licenses), as they offer considerable advantages compared to the multiple injection method. Similar to a well-timed two-injection protocol, a single application of a GnRHa-delivery system (10–50 μg kg^{-1}) may stimulate OM and ovulation of 100% of treated females within 2 weeks (Breton et al., 1990). More importantly, however, due to the sustained-release of GnRHa, the timing of application does not have to be as precisely determined and applications can be initiated up to 6 weeks prior to the expected onset of ovulation (Crim et al., 1983; Crim and Glebe, 1984; Goren et al., 1995). Advancement of ovulation by a few weeks is important in preventing pre-spawning mortalities in Pacific salmon, especially if fish are kept at sea.

The superiority of GnRH-delivery systems over the use of injections has been demonstrated also in various other species with single-batch group-synchronous ovarian development. For example, emulsified GnRH induced ovulation in Japanese plaice (*Limanda yokohamae*) and goby (*Acanthogobius flavimatus*), whereas the same dose given as an injection was ineffective (Aida et al., 1978). In the yaqui catfish (*Ictalurus pricei*), a GnRHa-delivery system was the only hormonal preparation able to induce spawning, whereas hCG, catfish pituitary extract, and combined sGnRHa/DA treatments were ineffective (Mylonas and Zohar, 2001b). In the tiger puffer (*T. rubripes*), GnRHa-delivery systems (400 μg kg^{-1}) induced ovulation in 18 and 10 days in fish with mean oocyte diameter of 800–900 μm and 900–1,000 μm, respectively (Matsuyama et al., 1997). This latter result underscores a very important advantage of GnRHa-delivery systems compared to injections, which is the flexibility in the timing of application. Whereas a single GnRHa injection would fail in females which have not fully completed vitellogenesis, a GnRHa-delivery system is able to induce OM and ovulation in fish of lesser maturity, albeit requiring a longer period of time. An extensive review of applications of GnRHa-delivery systems in inducing OM, ovulation, and spawning in single-batch group-synchronous species has been

published recently (Mylonas and Zohar, 2001b), and since then more species have been added to the list, including the bullseye puffer (Duncan et al., 2003), cobia (*Rachycentron canadum*) (Kilduff et al., 2002), devil stinger (*Inimicus japonicus*) (Takushima et al., 2003), and common carp (Brzuska and Bialowas, 2002).

3.2.2. Multiple spawners

As for single spawners, single or multiple injections of GnRHa are also employed in the induction of OM, ovulation, and spawning of multiple spawning fish. For example, it was already mentioned earlier (section 2.4) that multiple injections are more appropriate than delivery systems for the induction of multiple spawning in European sea bass (Mylonas et al., 2003), whereas GnRHa injections (Mylonas et al., 2004c) and GnRHa-delivery systems (Mylonas et al., 2000; Barbaro et al., 2002) were equally effective in the shi drum. In three other members of the Sciaenidae family, the red drum (*Sciaenops ocellatus*), spotted seatrout (*Cynoscion nebulosus*), and orangemouth corvina (*C. xanthulus*), a single injection of 100 μg GnRHa kg^{-1} induced OM, ovulation, and spawning within 30–35 h, whereas a second injection 3 weeks later induced further spawnings in the spotted seatrout (Thomas and Boyd, 1988). Also, a single injection of GnRHa was successful in inducing OM and tank spawning in the silver perch (Levavi-Sivan et al., 2004), while multiple GnRHa injections induced ovulation in the Reeves shad (*Tenualosa reevesii*) (Wang et al., 2003), though only a single spawning event was observed in both species.

Sustained treatment with GnRHa has been used effectively in a variety of multiple-batch group-synchronous fish, which either do not undergo OM and ovulation in captivity or do so unpredictably. For example, GnRHa-delivery systems induced two consecutive spawns within 3 days in white bass (Mylonas et al., 1997c) and greater amberjack (Mylonas et al., 2004b), five spawns within 7 days in the barramundi (Almendras et al., 1988), five ovulations within 2 weeks in striped trumpeter (Morehead et al., 1998), one to four ovulations within 7 days in the black sea bass (*Centropristis striata*) (Watanabe et al., 2003), and seven ovulations within 10 days in the dusky grouper (Marino et al., 2003). Similar results have been also obtained in other fishes with a similar mode of ovarian development (see Mylonas and Zohar, 2001b; Shein et al., 2004; Berlinsky et al., 2005).

The greatest potential, however, of sustained-release GnRHa-delivery systems is in the induction of OM, ovulation, and spawning in multiple spawning females with a daily or almost daily ovulation/spawning frequency. For example, the red porgy, red sea bream, and gilthead sea bream (Sparidae family) have an asynchronous mode of ovarian development and are capable of undergoing OM and spawning on a 24 h cycle for periods up to 4 months (Watanabe and Kiron, 1995; Zohar et al., 1995a; Mylonas et al., 2004a). In the early days of the aqauculture of the gilthead sea bream, when spontaneous spawning occurred rarely, a single GnRHa injection at the onset of the spawning season induced spawning in the majority of females, but only 20% continued spawning on a

daily basis. On the contrary, >70% of the females given a GnRHa-delivery system continued spawning on a daily basis. Similar results have been obtained with the other two sparids (Matsuyama et al., 1995; Zohar and Mylonas, 2001). In this way, GnRHa-delivery systems result in a significant increase in the total number of eggs produced in a hatchery, by increasing the number of broodfish undergoing OM, and the number of ovulations per spawning season (Barbaro et al., 2002). Also, a GnRHa-delivery system has been examined recently in Atlantic bluefin tuna, a species posing a great challenge to the aquaculture industry (Ottolenghi et al., 2004). A GnRHa-delivery system (40–80 μg kg^{-1}) was administered underwater to untranquilized fish swimming in a rearing cage, and was shown to induce OM, ovulation, and spawning, with production of viable larvae both after spontaneous spawning and after *in vitro* fertilization (Mylonas et al., 2006).

Members from various flatfish families also present significant difficulties in undergoing OM in captivity, and application of GnRHa-delivery systems has proven very successful in inducing multiple spawnings, often of improved quality compared to the few naturally spawning females. For example, in captive-reared Southern flounder a GnRHa-delivery system induced up to three ovulations in all females with mean oocyte diameter of >500 μm (Berlinsky et al., 1996). In wild-caught summer flounder, the same treatment induced daily ovulations for 8 days (Berlinsky et al., 1997), whereas in fish maintained for more than a year in captivity, the same treatment induced not only ovulation, but also tank spawning (Watanabe et al., 1998a). Similarly in turbot, treatment with a GnRHa-delivery system induced multiple ovulations in 100% of treated fish compared to 50% of controls, and it reduced the interovulation period (Mugnier et al., 2000). Also, in the yellowtail flounder, two different GnRHa-delivery systems induced an average of eight consecutive ovulations, compared to three in control fish, resulting in the production of twice as many eggs and of higher fertilization and hatching percentage than control females (Larsson et al., 1997). The same two GnRHa-delivery systems have also induced daily spawnings for up to 2 weeks in the Senegal sole (*Solea senegalensis*), though with very limited fertilization success (Agulleiro et al., 2006; Howell et al., 2006). Finally, GnRHa-delivery systems were shown to stimulate daily ovulations in the greenback flounder (*Rhombosolea tapirina*) (Poortenaar and Pankhurst, 2000).

The use of GtH preparations for the induction of multiple spawning in fish is somewhat limited, compared to single spawning species. Nevertheless, in the greenback flounder a single injection of hCG produced daily ovulations, in a similar fashion to a GnRHa-delivery system (Poortenaar and Pankhurst, 2000). In the whitemouth croaker (*Micropogonias furnieri*) a single injection of hCG was effective in inducing the first ovulation within 2 days after treatment, but it was not reported if the females continued ovulating (Berois et al., 2004). Similarly, in chub mackerel (*Scomber japonicus*) a single injection of hCG induced ovulation 36 h later, but since the fish were sacrificed for histological evaluations it was not known if more cycles of OM and ovulation could have

been induced by the treatment (Shiraishi et al., 2005). Also in the yellowtail (*S. quinqueradiata*), both a single and a double-injection protocol of hCG was effective in inducing spawning, though only for a single time (Chuda et al., 2001; Chuda et al., 2002). Finally, in the tropical catfish (*Heterobranchus longfilis*), a single injection of hCG induced 100% ovulation and the same treatment could be repeated successfully after 6–8 weeks (Nguenga et al., 2004).

4. EGG QUALITY

Egg quality is an important parameter for commercial fish hatcheries, as it can limit the number, as well as the quality of fry produced from a broostock. However, our knowledge of either the intrinsic factors or the environmental parameters influencing egg quality is limited (Kjørsvik et al., 1990; Bromage, 1995; Brooks et al., 1997). Recent studies have made significant advances towards this direction (Lahnsteiner, 2000; Nocillado et al., 2000; Lahnsteiner et al., 2001; Lahnsteiner and Patarnello, 2003; Shin et al., 2003). Still, the criteria for the assessment of good quality eggs used in commercial hatcheries are limited to egg buoyancy (Lahnsteiner and Patarnello, 2003), number of oil droplets and blastomere morhphology (Shields et al., 1997), or even simply fertilization and hatching success (Mylonas et al., 1992). The purpose of this section is not to review the current knowledge of the factors that determine egg quality in general, but rather to point out some considerations one should have in mind when employing hormonal therapies to induce OM, ovulation, and spawning.

In the case of species which do not spawn spontaneously after ovulation in captivity and fertilization is achieved artificially after stripping, it has been shown that the amount of time during which the eggs remain in the ovarian or abdominal cavity after ovulation and before stripping is directly related to loss of egg quality, a process referred to as "overripening" (Bromage et al., 1994). This period varies among fishes from many days in salmonids (Lahnsteiner, 2000); to <6 h in chub mackerel (Shiraishi et al., 2005), 4–6 h in Atlantic halibut (*Hippoglossus hippoglossus*) (Bromage et al., 1994), <3 h in snapper (*P. auratus*) (Hobby and Panhurst, 1997), 2 h in tiger puffer (Chuda et al., 1998), and 1 h in tilapia (*Oreochromis niloticus*) (Bromage et al., 1994); to only 30 min in the white bass (Mylonas et al., 1996). Therefore, in order to ensure high egg quality and fertilization success, the time of ovulation after hormonal therapy (i.e. latency period) must be predicted to a high degree of accuracy in most species (Chen, 2005), in order to limit the interval between ovulation and stripping (i.e. overripening). When hormonal therapies for the induction of OM and ovulation are tried for the first time in a species, an overripening period of 1–2 h may be considered a good starting point, subject to further evaluation. The latency period depends on both intrinsic (Wendling et al., 2000) and environmental parameters (Brzuska, 1999), some of which include species, water temperature (Yaron, 1995), type of hormone and dose (Wen and Lin, 2004), as well as the history of

fish in the preceding period (e.g. low vs. high temperatures) (Van Der Kraak and Pankhurst, 1996; Tveiten et al., 2001) and the stage of ovarian maturity at the time of the hormone treatment (Matsuyama et al., 1997). The stage of ovarian maturation is usually determined after obtaining an ovarian biopsy and (a) measuring the mean or maximum oocyte diameter (Garcia, 1989; Mylonas et al., 2004b; Shiraishi et al., 2005), (b) determining the position of the germinal vesicle (Lutes et al., 1987; Billard et al., 1995; Mylonas et al., 1995; Yaron, 1995), or (c) identifying the onset of coalescence of the lipid droplets (Mylonas et al., 1997a, 2003; Fauvel et al., 1999). A more sophisticate method for verifying that females have reached the appropriate developmental stage at which hormonal induction should be applied, has been developed recently in the striped bass on the basis of the *in vitro* OM after hormonal stimulation of biopsied oocytes from prospective females (Weber et al., 2000). Finally, the occurrence of >5–10% full-grown oocytes undergoing atresia, in some fishes it is an indication that a female may not respond successfully to a hormonal therapy (Cerdá et al., 1997). Specific guidelines must be developed for each particular species and hormonal therapy, with modifications made according to the specific stock and hatchery conditions.

One question often posed in regards to hormonal therapies is their effect on egg quality, compared to naturally ovulating or spawning broodfish (Slater et al., 1995). It is assumed that hormonal therapies are used only if a broodstock is not reproducing normally in captivity, or for management purposes, such as to increase synchronization of maturation, or to implement interspecific hybridization or genetic selection programs. In that respect, it makes little practical difference if the resulting egg quality is slightly, yet significant statistically, reduced compared to naturally spawning populations. Nevertheless, appropriate hormonal therapies do not usually have a negative effect on egg quality (Gillet et al., 1996; Barbaro et al., 1997; Mugnier et al., 2000; Duncan et al., 2003; Haffray et al., 2005), whereas at times they can even improve egg fecundity (Barbaro et al., 1997; Morehead et al., 1998; Mikolajczyk et al., 2004) and quality (Larsson et al., 1997; Mylonas et al., 2003) compared to spontaneously maturing populations.

Fertilization success may be sometimes reduced if very high doses of a hormone are used (Billard et al., 1984; Mylonas et al., 1992), though the mechanisms for such an effect are not clear. One explanation may be found in the slight asynchrony of the processes of OM and ovulation, and the effect an exogenous hormone stimulation may over-impose. Hormonal therapies induce OM via synthesis of the maturation inducing steroids, whereas ovulation is stimulated via synthesis of prostaglandins (see Chapter 12). One of the events that take place during OM is the resumption of meiosis, and its progression from the first meiotic prophase to the second meiotic anaphase at the end of OM, at which time the oocyte is considered fertilizable. It has been suggested that meiotic maturation may not be completed in synchrony with ovulation (Goetz, 1983; Nagahama, 1983). Therefore, some eggs may not be ready to be fertilized

immediately after ovulation, even if ovulation occurs naturally. Completion of meiotic maturation may take place soon after ovulation – depending on water temperature, while the eggs remain in the ovarian or abdominal cavity. This hypothesis is supported by experiments in salmonids, which reported a lower fertilization success of eggs stripped immediately after ovulation, compared to eggs striped a short time after ovulation (Hirose et al., 1977; Springate et al., 1984). Similarly, based on biochemical evaluations of gilthead sea bream eggs, it was recently suggested that some eggs do not reach full maturation prior to ovulation, which results in their failure to be fertilized (Lahnsteiner and Patarnello, 2003). It is possible that due to a very strong, rapid and unnatural stimulation provided by an excessive dose of a hormonal therapy, the processes of OM and ovulation become greatly out of phase, resulting in the ovulation of unfertilizable eggs, which can not complete meiotic or biochemical maturation in the ovarian or abdominal cavity. It is important, therefore, to establish empirically the appropriate hormone dose in each species of interest.

Although at times differences in egg quality between hormonal treatments have been reported in the same species, there is no established trend between GtH and GnRHa preparations, or between injection and sustained-release treatments. For example in the European catfish, though both treatments were effective in inducing ovulation, fish treated with a combination of GnRHa/DA produced eggs of higher fertilization and hatching success than those obtained from females treated with CPE (Brzuska, 2001). On the contrary in carp, although a double-injection therapy with CPE and a single injection of GnRHa were equally effective in inducing ovulation and producing fertilized eggs, 2-day larval survival was higher for the CPE treatment (Brzuska, 2004). Finally, in the ocellated puffer there were no differences in egg quality between females induced to ovulate with pituitary extract, hCG, or GnRHa in a single or double-injection protocol (Chen, 2005). Obviously, the effect of a hormonal treatment on egg quality may vary and it is difficult to make general recommendations in favor of one treatment over another based on the produced egg quality. Nevertheless, one criterion to chose one hormonal therapy over another would be the degree of synchronization of the response time to ovulation or spawning among different broodfish, both because greater synchrony – i.e. less variation – has significant management advantages for the hatchery, and because it may also indicate a physiologically more appropriate response of the females (Wen and Lin, 2004).

5. FUTURE DIRECTIONS

As described in this chapter, GnRHa-based spawning induction technologies have been successfully applied to numerous commercially important farmed species. It is also clear, that recent progress in understanding the GnRH system in farmed fish will lead to more efficient and cost-effective approaches.

The current GnRHa-based spawning induction therapies were developed before it was discovered that many commercially important Perciform fishes

(such as sea breams, freshwater and seawater basses, groupers, etc.) have three forms of GnRHs in their brain (Powell et al., 1994; Gothilf et al., 1995; Alok and Zohar, 2005). While two of these forms, salmon (s) and chicken (c) GnRH-II, were previously found in many species, including salmonids and cyprinids, the third form named sea bream (sb) GnRH was demonstrated to be a novel GnRH peptide present in more evolved fishes. Further studies led to the conclusion that sbGnRH is the principle endogenous LH releaser and the most physiologically relevant form to the completion of OM, ovulation, and spawning in gilthead sea bream and other perciform species (Gothilf et al., 1997; Holland et al., 1998b; Senthilkumaran et al., 1999; Rodríguez et al., 2000; Okuzawa et al., 2003). This suggests that in these species, sbGnRH is the GnRH form that should be targeted in future studies on the effects of confinement on the GnRH system and the failure to spawn in captivity. In addition, cGnRH-II has been shown to be the most potent LH releaser in gilthead sea bream and other fishes (Zohar et al., 1995a, b; Bosman et al., 2000). Since both the sbGnRH and cGnRH-II forms are present in the pituitary of fish undergoing final gonadal development (Holland et al., 1998b), and a peak in their synthesis is observed 8 h before ovulation in gilthead sea bream (Gothilf et al., 1997), a combined administration of sbGnRH and cGnRH II agonists should also be considered for spawning induction. The recent cloning and studies of the pituitary GnRH receptors in a number of farmed fishes (Alok et al., 2000; Lethimonier et al., 2004) will help in identifying the most potent GnRHa combination(s) and in determining their doses and mode of application to optimize physiologically tailored GnRHa administration, and in turn achieve optimal ovulation and spawning (Alok and Zohar, 2005).

As described in section 1.1.2, it is clear that the failure of captive fish to undergo OM, ovulation, and spawning is the result of the lack of LH secretion from the pituitary. Consequently, most spawning induction related research and development efforts have focused on the use of exogenous GnRHs to trigger the release of LH and the chain of events leading to final gonadal development. However, it is also evident that the endocrine system upstream from the pituitary is impaired in captive fish, which adversely affects the normal functioning of the endogenous GnRH system in captive broodstocks. The next generation of spawning induction technologies will thus be based on understanding the nature of the captivity-induced alterations in the GnRH system, and on developing strategies to correct them. Studies in the striped bass demonstrated that captivity results in altered secretion patterns of the relevant sbGnRH from the brain to its final destination in the pituitary (Steven et al., 2000). Understanding the mechanisms involved in regulating the synthesis and secretion of the relevant GnRHs will no doubt lead to the development of new strategies to simulate in captive broodstock the natural patterns of GnRH synthesis and release occurring in wild fish. The recent cloning and characterization of multiple GnRH genes and their cDNAs from a variety of fish species (Sherwood et al., 1997; Alok and Zohar, 2005) paves the way for a more complete elucidation of

the regulation of GnRH gene expression and synthesis, and for a better understanding of the effect of confinement on the function of the GnRH system. Moreover, the recent development of transgenic zebrafish overexpressing a GnRH promoter that drives a green fluorescent reporter gene (Abraham, Du, Knight, and Zohar, unpublished data) introduces a very powerful tool for unveiling the environmental and endocrine mechanisms involved in stimulating, inhibiting, or altering GnRH production. Those recently developed tools and emerging studies may lead in the future to simple strategies to manipulate the expression of specific GnRH genes and/or their release, thereby overcoming the adverse effect of confinement on the GnRH system and leading to successful completion of oogenesis, OM, and ovulation.

Finally, optimizing the success of spawning induction therapies and the quality of the eggs produced requires a better understanding of the recently discovered complex hormonal processes occurring inside the gonad. In addition to the well-documented patterns of gonadal steroidogenesis, a number of additional endocrine factors such as growth factors and catecholamines have also been shown to play a critical role in the acquisition of ovarian maturational competence (Patiño et al., 2001; Patiño and Sullivan, 2002; Vidal et al., 2004; Dufour et al., 2005). The recent discovery of the existence of a full GnRH–GtH–Steroid axis in the ovaries of the gilthead sea bream (Wong and Zohar, 2004) establishes a new paradigm in the endocrine regulation of reproduction in fish, which may have implications in the manipulation of ovulation and spawning in aquaculture. Studying the functional significance and patterns of the local gonadal endocrine system is important in determining the timing of spawning induction and in tailoring the GnRH-based induction therapy, in order to ensure optimal spawning success and quality of eggs.

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COLOR PLATES

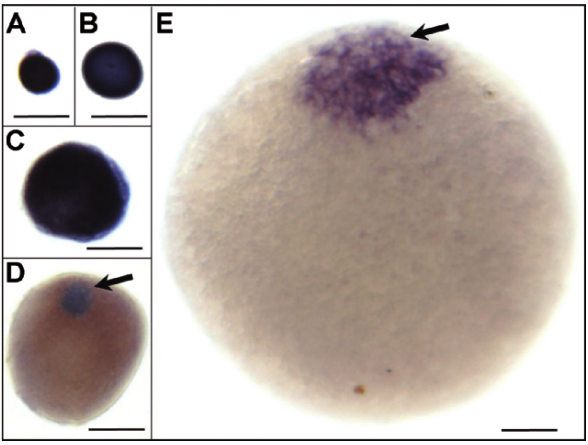

Figure 3.1 Localization of metallothionein 2 (*mt2*) transcripts in zebra fish fully grown follicles as revealed by whole-mount *in situ* hybridization. Stage-specific polarized distribution of *mt2* short transcript isoform in stage IB (A, B), II (C), early stage III (D), and stage IV (E) follicles. The *met2* hybridization signal detected at early stages was widely distributed in the ooplasma, whereas it concentrated at the animal pole from early stage III to the end of vitellogenesis. For stages III and IV, the animal pole, oriented toward top of page, is indicated by an arrow. Scale bar = 100 μm.

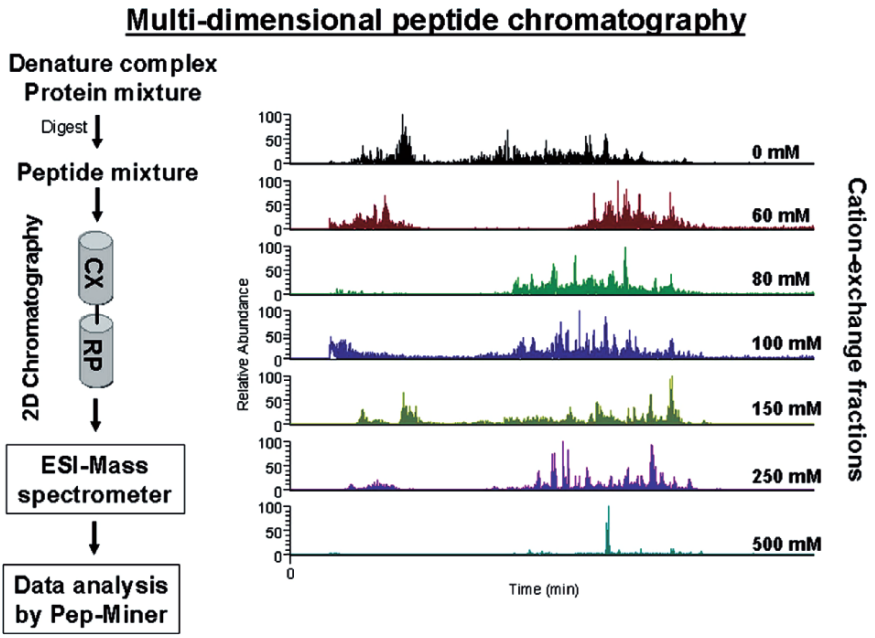

Figure 4.1 Two-dimensional chromatography of tryptic peptides of oocytes proteins. Total protein extracts of zebra fish oocytes were proteolyzed with trypsin and the peptides were separated in the first dimension on a disposable strong cation exchange columns and on the second dimension on uRP-HPLC connected to MS/MS. Each chromatography displays the total-ion-current chromatogram (TIC) for each of the salt elutions of the peptides.

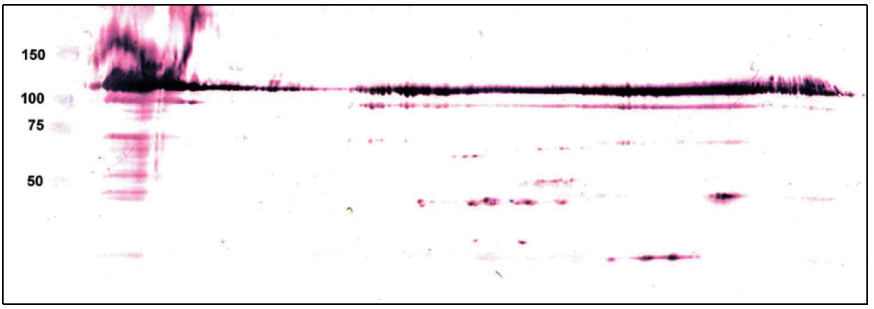

Figure 4.4 2D-PAGE and Western analysis of zebra fish oocytes proteins. The total protein extract of stage IV of the zebra fish oocytes were resolved by 2D-PAGE and transfer to nitrocellulose membrane. The membrane was developed with rabbit anti-Vtg polyclonal antibodies.

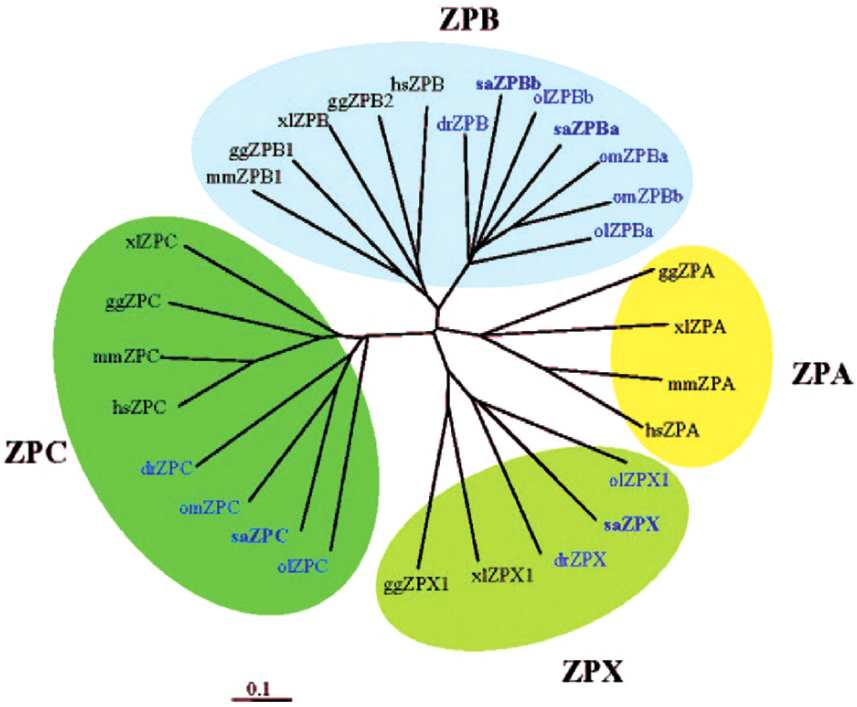

Figure 5.2 ZP protein sequence similarity analysis displayed in a phylogenetic tree. Clades containing ZPA, ZPB, ZPC, and ZPX are circled. Mouse (*Mus musculus*) mmZPA (nm_011775), mmZPB1 (nm_009580), mmZPC (x14376); human (*Homo sapiens*) hsZPA (m90366), hsZPB (nm_021186), hsZPC (nm_007155); chicken (*Gallus gallus*) ggZPA (xm_424608), ggZPB1 (aj289697), ggZPB2 (ab025428), ggZPC (ab031033), ggZPX1 (aj698915), African clawed frog (*Xenopus laevis*) xlZPA (af038151), xlZPB (u449950), xlZPC (u44952), xlZPX1 (af225906); rainbow trout (*Oncorhynchus mykiss*) omZPBa (af231706), omZPBb (af231707), omZPC (af231708); zebra fish (*Danio rerio*) drZPB (af331968), drZPC (af095457); Japanese medaka (*Oryzias latipes*) olZPBa (af128808), olZPBb(d89609), olZPC (af128813), olZPX1 (af128807); gilthead sea bream (*Sparus aurata*) saZPBa (ay928800), saZPBb (ay928798), saZPC (x93306), saZPX (ay928799).

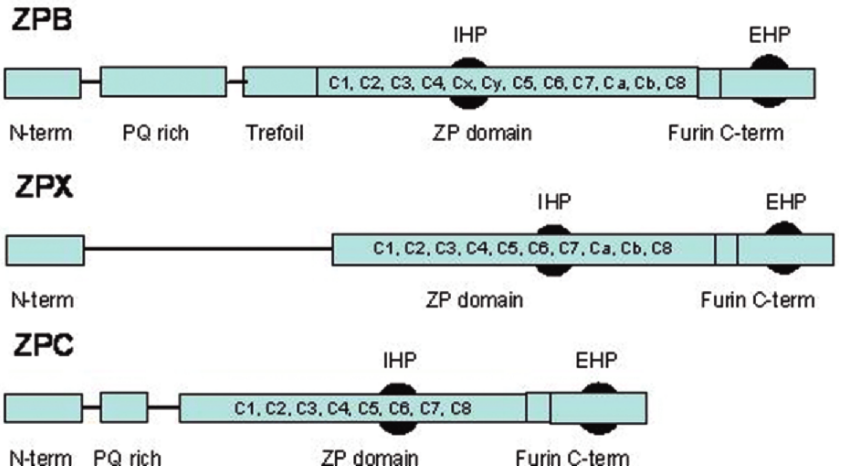

Figure 5.3 Schematic representation of the teleost ZP protein domains. All ZP proteins contain an N-terminal signal sequence that is cleaved of in the mature protein. There is a PQ rich domain in ZPB and ZPC proteins that appear to be lacking in ZPX proteins. The ZP domain contains 8–12 conserved cysteins. All ZP contain C1–8 while ZPB and ZPX contain an additional 2 cysteins Ca and Cb, and these are in common with mammalian ZP proteins. In the teleost ZPB proteins there are 2 additional cysteins (Cx and Cy) that are not present in mammalian ZPA or ZPB or in teleost ZPX. The cysteins create intramolecular sulphydryl bridges. Downstream of the ZP domain, there is a furin-like cleavage site in all ZP proteins followed by a C-terminal domain that is cleaved off in the mature protein. In addition to this there are two hydrophobic patches, one internal (IHP) and one located in the C-terminal domain (external; EHP). The IHP sequence has been proposed to participate in polymerization of ZP proteins.

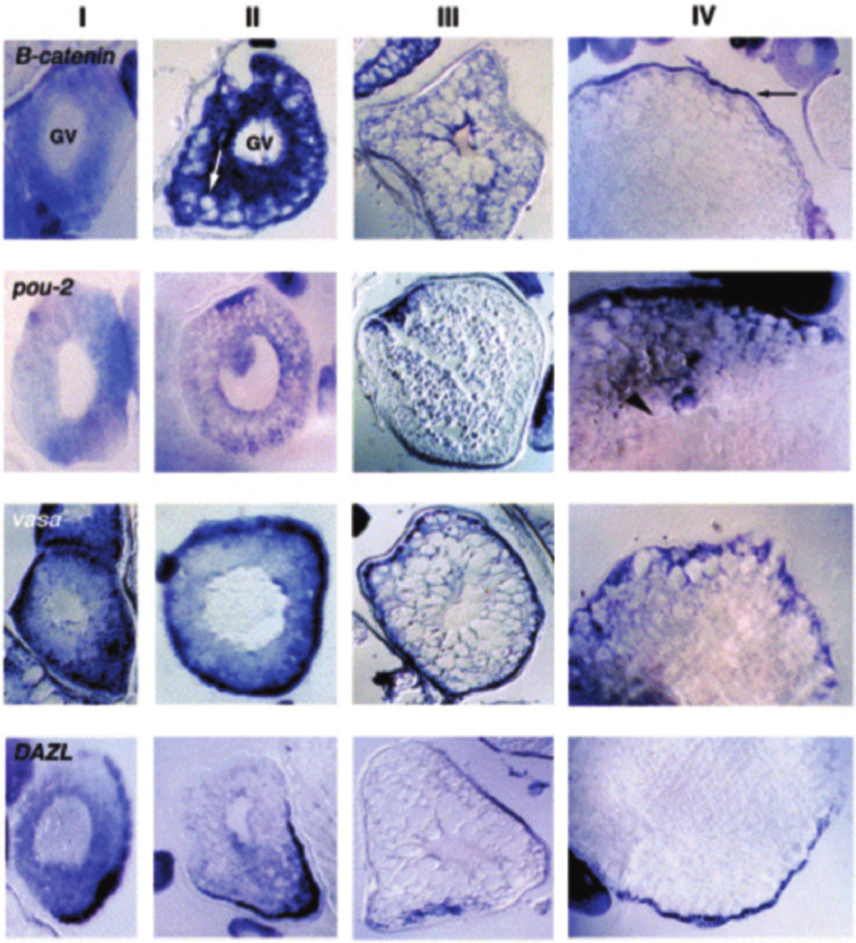

Figure 6.2 Patterns of mRNA localization at during oogenesis (stages I–IV). mRNAs represent the various classes of observed patterns: ubiquitous (*β-catenin*) and localized to the animal region (*pou-2*), the entire cortex (*vasa*) or the vegetal cortex (*DAZL*). (Adapted from C. Howley and R.K. Ho with permission from Elsevier.)

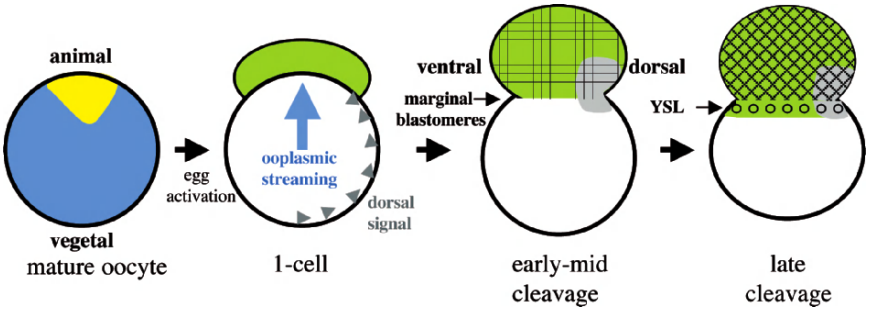

Figure 6.3 Redistribution of maternal factors during egg activation. Ooplasmic streaming through axial streamers within the forming yolk cell allows ubiquitously distributed factors (blue) to accumulate in the forming cell at the animal pole. Cortical translocation of a dorsal signal originally localized to the vegetal pole of the oocyte results in its movement (arrowheads) to the animal pole along one side of the embryo. This side becomes the prospective dorsal region during the blastula stage (grey). Factors already localized at the animal region during oogenesis (yellow) remain in that region after activation. The yolk syncytial layer (YSL) forms from the most marginal blastomeres, thus allowing a direct connection of nuclei in this layer to the yolk cell. See text for details.

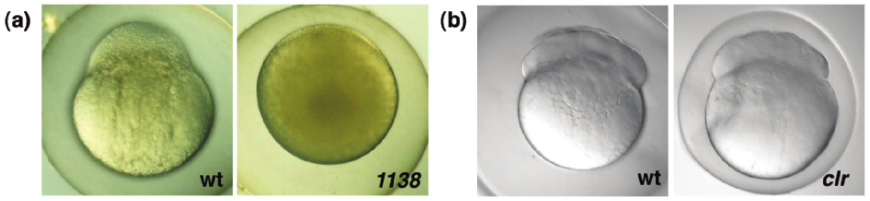

Figure 6.4 Mutations affecting egg activation. A mutation in the line 1138 exemplifies defects in cell lifting, presumably due to a defect in ooplasmic streaming. In the case of the mutation *claustro (clr)*, ooplasmic streaming is normal but chorion expansion is reduced. (Adapted from F. Pelegri et al., with permission from Wiley-Liss, Inc.)

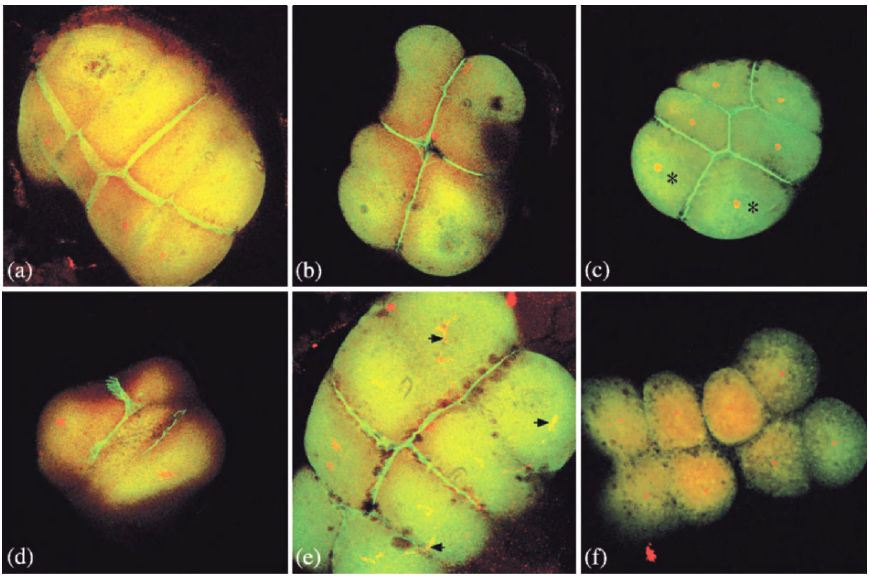

Figure 6.5 Mutations affecting early cellular processes. Confocal images of 8-cell stage embryos, labeled with an antibody against β-catenin protein, which labels cell adhesion junctions at the newly formed membranes (green) and the DNA dye propidium iodide (red). The embryo in (a) shows the normal appearance of these markers. A mutation in *futile cycle* results in defects in pronuclear fusion (b). A fraction of cells (asterisks) in *cobblestone* mutants do not initiate mitosis (c, note larger size of affected cells). A mutation in *barrette* affects nuclear division and the pattern of membrane deposition (d). *golden gate* mutants show abnormal DNA bridges (arrowheads) indicative of defects in chromosome segregation during mitosis (e). A mutation in *aura* results in defects in the deposition of adhesive membrane at the furrow (f).

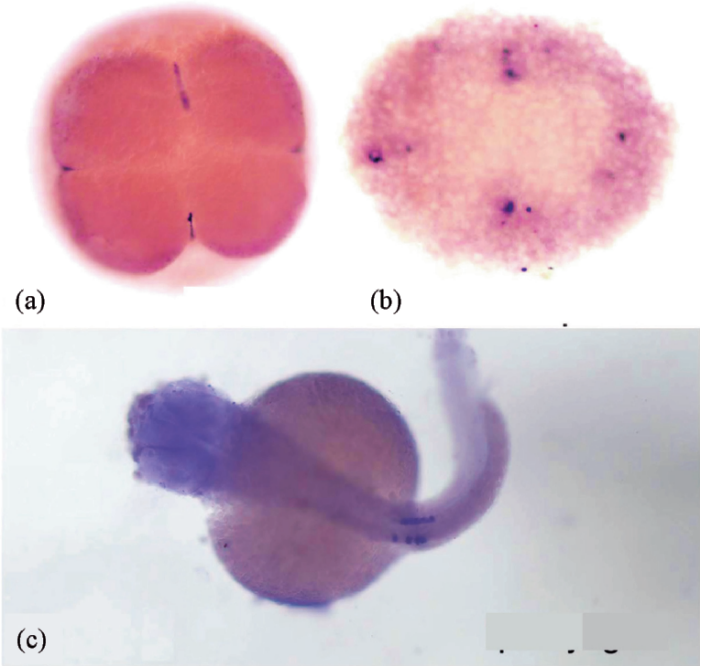

Figure 6.7 mRNA localization pattern of the *vasa* RNA in the germplasm and primordial germ cells. (a) *vasa* mRNA, together with other components (see text), localizes to the furrow of the first and second cell cleavages, allowing to visualize the zebra fish germ plasm. Cells that inherit the germplasm become the primordial germ cells, here visualized as four clusters at the late blastula stage (b) and in the prospective gonadal region in a 24 h embryo (c).

Figure 6.9 MZ contribution of the *one-eyed pinhead (oep)* gene. (a) wild-type embryo. (b) Zygotically mutant *oep* embryos derived from heterozygous mutant mothers (*Zoep* phenotype), showing cyclopia and a general curvature of the embryo. (c) Zygotically mutant *oep* embryos derived from homozygous *oep* mutant mothers (*MZoep* phenotype), showing a lack of mesendodermal derivatives along the trunk (e.g. v-shaped somites indicated in (A) and (B) with arrowheads). Side views of 24 h embryos with anterior to the left and dorsal up.

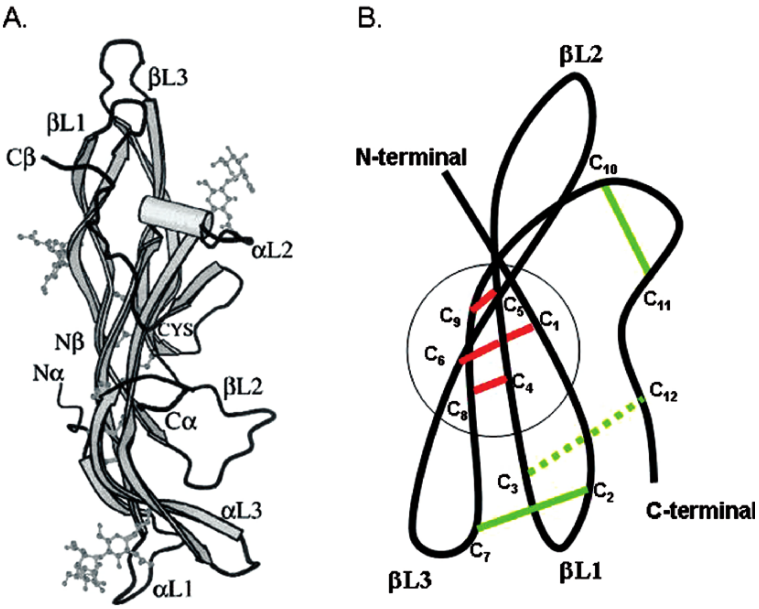

Figure 7.1 (A) Ribbon diagram showing the crystal structure of human FSH. The α-subunit (light gray ribbon) and the β-subunit (dark gray ribbon) demonstrate the typical fold of "cystine-knot" cytokines. Subunit N- and C-termini, as well as main loops (e.g. αL1, αL2, αL3, βL1, βL2, βL3) are identified. The carbohydrate and the disulfide bonds in both subunits are represented by ball-and-stick models. (B) Schematic structure of human FSH β-subunit showing disulfide bond pairings. The "cystine knot" motif is circled, and the "seatbelt" configuration that distinguishes the β-subunit from the α-subunit is marked with dashed line. The figure was prepared by using the program MOLSCRIPT. (Kraulis, 1991.)

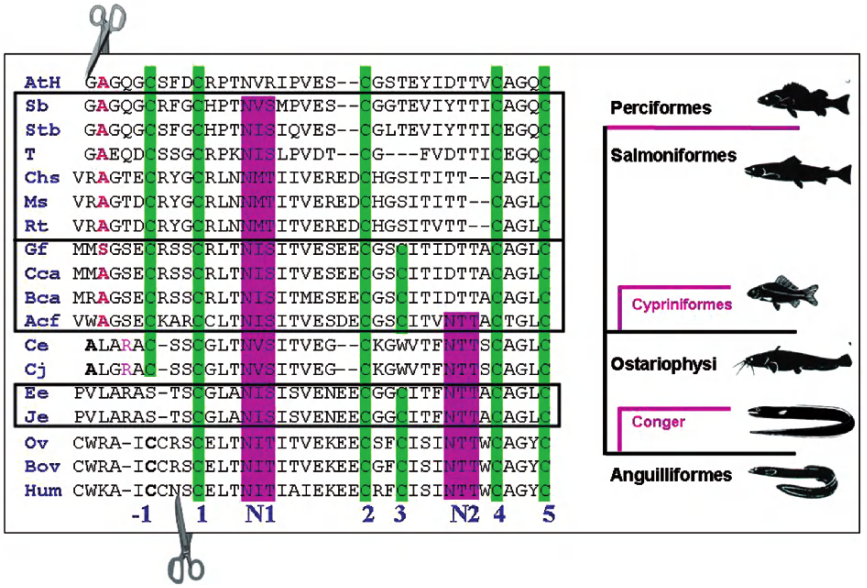

Figure 7.2 (Right panel) Evolutionary model for the FSHβ molecules in teleosts. The pink branches represent the lineages exhibiting semiparallelism. (Left panel) Multiple sequence alignment of the FSHβ N-terminal. The sequences include a small portion of the signal peptide enclosing the tentative cleavage site (indicated by scissors). Cysteine residues and putative N-linked glycosylation sites are highlighted with green and pink background, respectively.

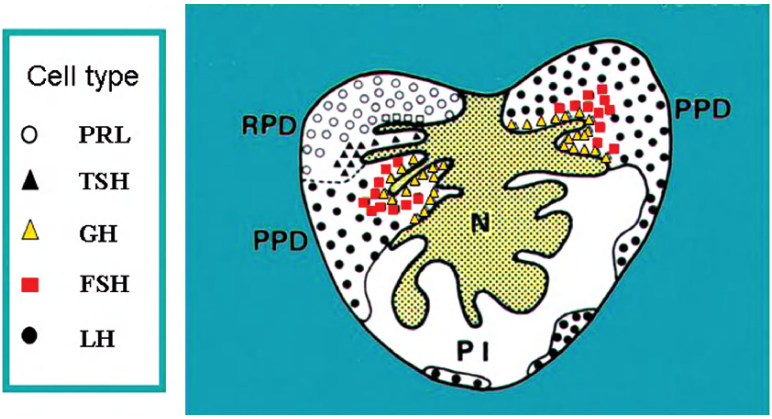

Figure 7.3 Cartoon representing a typical teleost adenohypophysis, with relative localization of the gonadotrophs, LH and FSH producing cells. *Abbreviations*: GH – growth hormone; PRL – prolactin; TSH – thyroid-stimulating hormone; N – neurohypophysis; PI – pars intermidia; PPD – proximal pars distalis; RPD – rostral pars distalis.

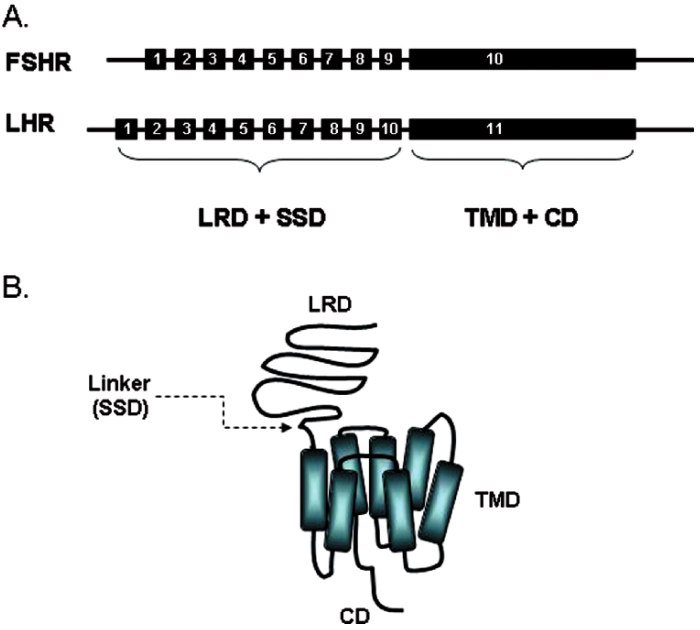

Figure 7.5 Structural characteristics of the GtHR genes (A) and proteins (B). The gene xons are represented by black boxes and are numbered (10 and 11 exons in the genes encoding for FSHR and LHR, respectively). *Abbreviations*: CD – cytoplasmic domain; LRD – leucine-rich repeat domain; SSD-signaling-specificity domain; TMD – transmembrane domain.

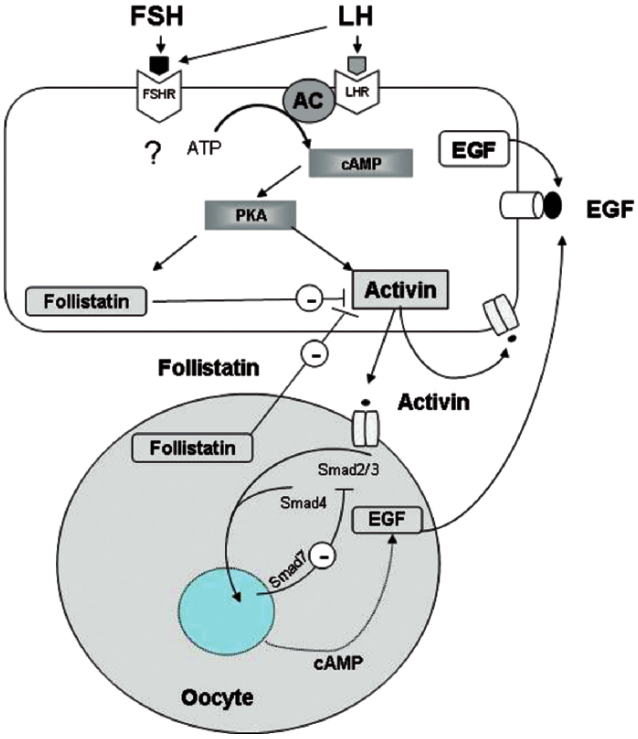

Figure 7.7 Tentative regulatory interplay between follicle cells and the oocyte. Data obtained so far in zebra fish, suggest that activin mainly produced in the follicle cells, affects the oocyte, which in turn produces follistatin and EGF. The latter feedback-regulate follicle cells function. (Ge, 2005.)

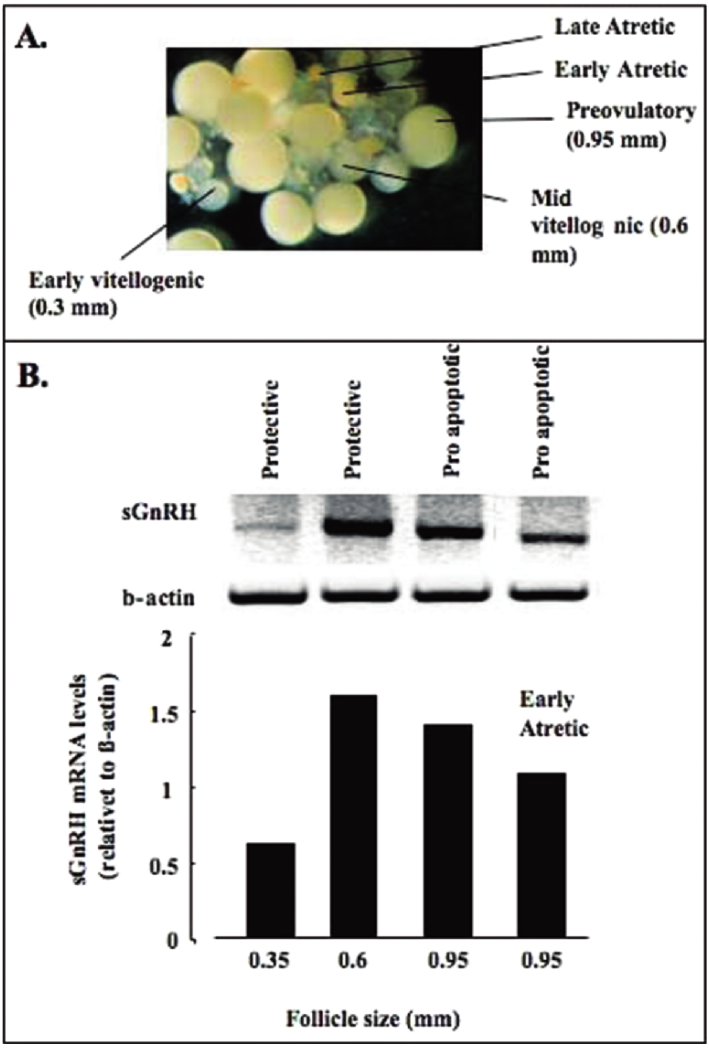

Figure 9.1 Morphological appearance and expression of salmon GnRH transcript in goldfish ovarian follicles at different stages of maturity; 0.35 mm: early vitellogenic; 0.6 mm: mid-vitellogenic; 0.95 mm: mature, preovulatory. (B) Values are expressed relative to b-actin levels to correct for total amount of RNA. The quantified values represent the mean of two observations. Early atretic follicles were obtained from the ovaries that failed to ovulate and showed signs of atresia (appearance of orange pigment and signs of degeneration (A).

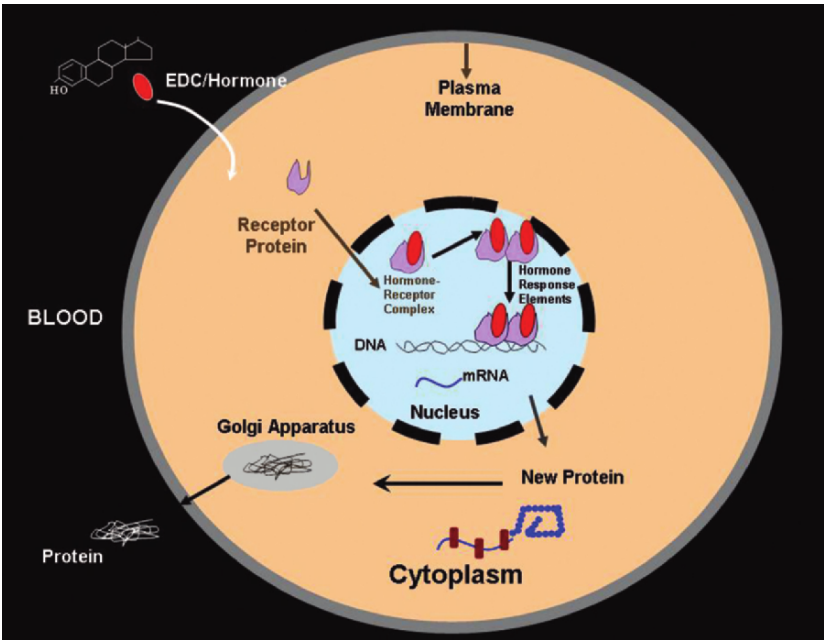

Figure 10.1 Endocrine disrupting chemicals (EDCs) can exert their effects via receptor-mediated mechanisms. In this example, an EDC interacts with cellular receptors to elicit effects.

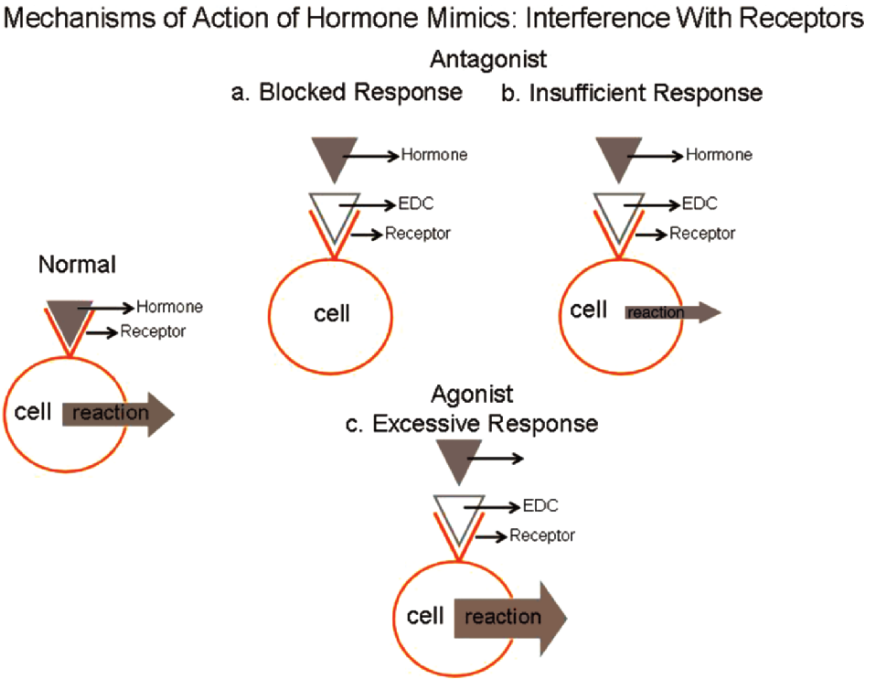

Figure 10.2 Mechanisms of action of endocrine disrupting chemicals (EDCs) acting on steroid hormone receptors. Upon interaction of EDCs with cellular receptors, two outcomes are possible: (1) EDC can act as an antagonist and induce a total cessation or a reduction of response (a) and (b); or (2) it can act as an agonist eliciting an enhanced response (c).

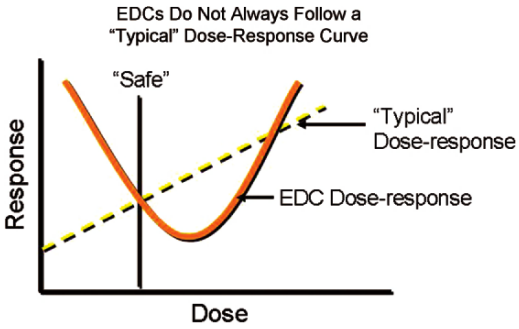

Figure 10.3 Endocrine disrupting chemicals (EDCs) do not always follow a typical dose–response curve, and U-shaped curves are common after exposure to these chemicals or to endogenous hormones. An explanation for this type of dose–response curve is that there are different modes of action at the low and high concentrations, for example, feedback inhibition of endocrine function occurs at high concentrations.

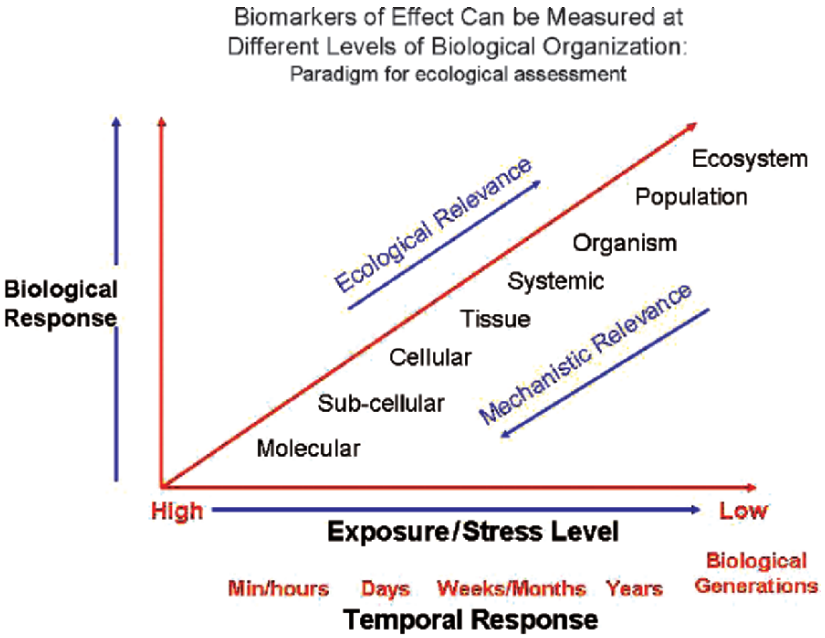

Figure 10.4 Endocrine disrupting compounds (EDCs) can exert effects at different levels of biological organization, ranging from the molecular to the ecosystem levels. Effects at low levels of biological organization have high mechanistic significance, whereas effects at higher levels of biological organization have high ecological relevance. The figure also illustrates the time needed for the different responses to occur, ranging from minutes to days for effects measured at the subcellular levels, to several generations in the case of changes at the ecosystem level.

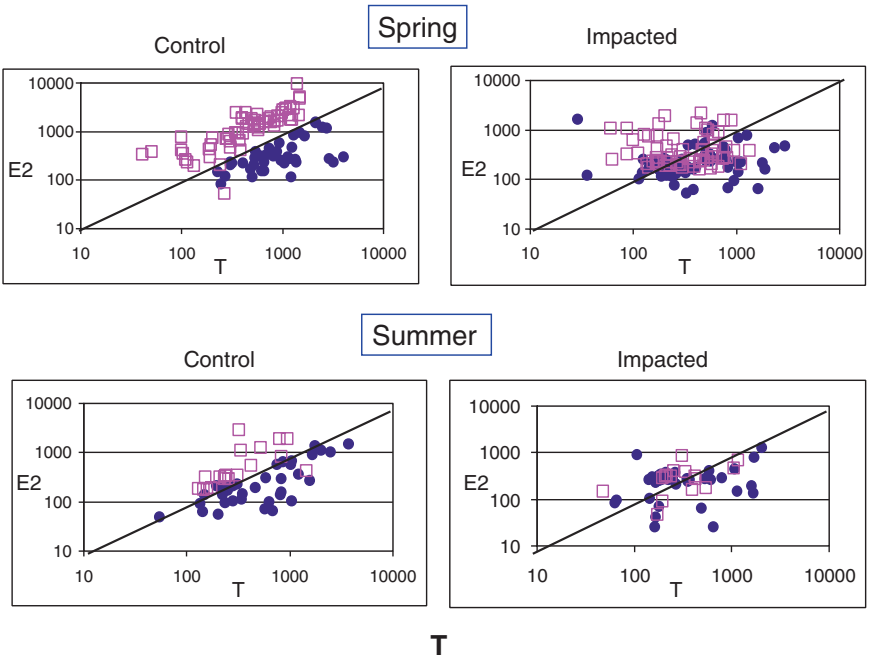

Figure 10.5 Measurement of circulating hormone levels in largemouth bass during the spring at the height of the reproductive season and in the summer when hormone levels are at their lowest. The graphs plot the concentrations of E_2 and T for each fish for samples collected at a control site and a site that is highly contaminated with p,p′-DDE, dieldrin, chlordanes, and toxaphene.

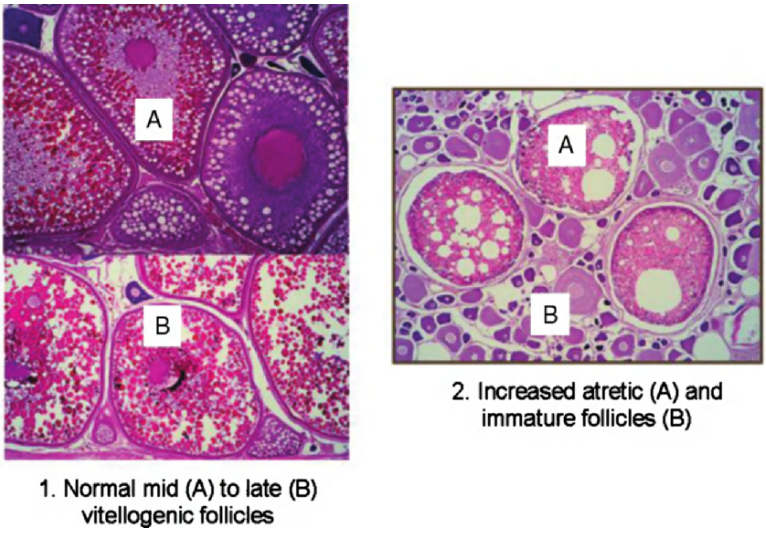

Figure 10.6 Histological changes observed in ovaries of female largemouth bass exposed chronically (56 days) to paper mill effluents (>40%). Note the presence of atretic follicles and overall depression of follicular development in ovaries from exposed females (2) compared to controls (1).

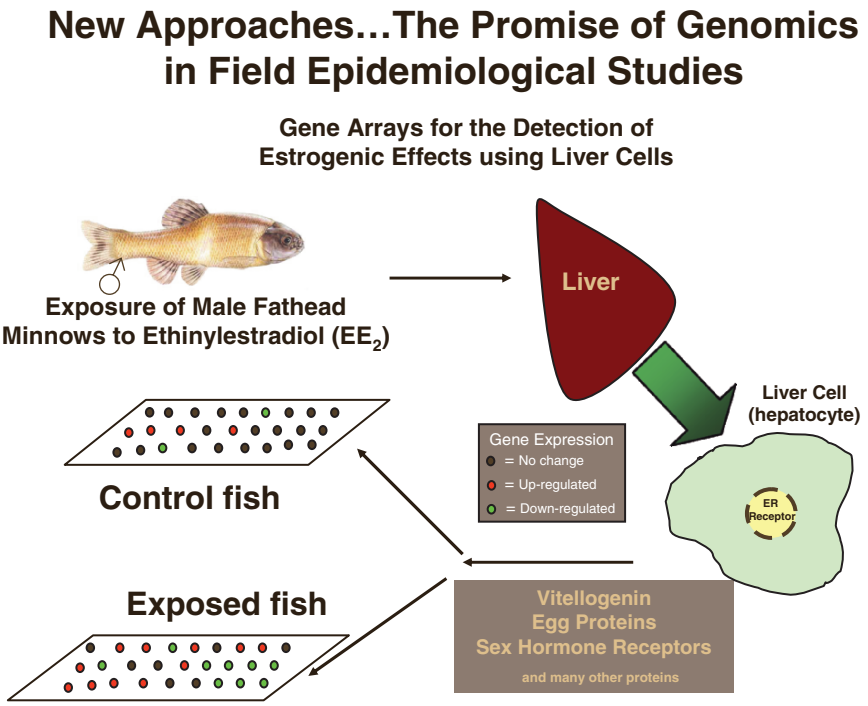

Figure 10.7 Gene arrays made on glass slides with probes for fish genes (either cDNAs or oligonucleotides) determine the relative expression levels of multiple genes following an exposure to a contaminant. In the example shown, the signal comes from total RNA extracted from liver of control fish and fish exposed to EE_2, copied into cDNA, labeled with fluorescent dyes, and then hybridized to the arrays. The level of expression for each gene is proportional to the intensity of the fluorescent dye. Fish treated with EE_2 exhibit upregulation of Vtg and other estrogen-regulated genes.

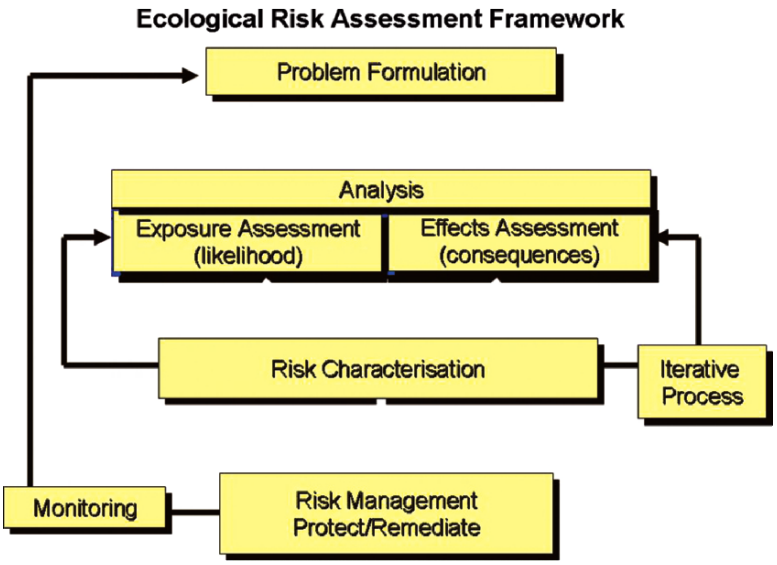

Figure 10.8 Diagram summarizing the most important steps in ecological risk assesment (ERA).

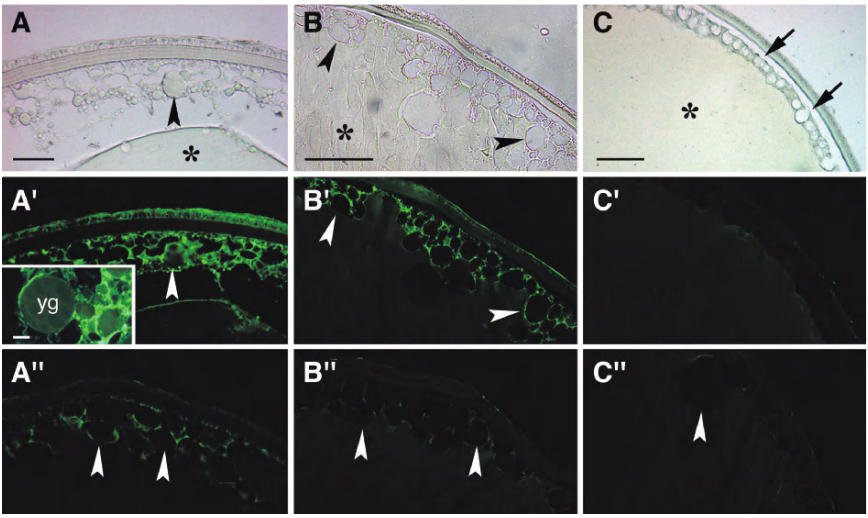

Figure 12.9 Light microscopy (**A–C**) and fluorescent images of *in vitro* maturing *F. heteroclitus* oocytes in the absence (**A′-C′**) or presence (**A″–C″**) of 100 nM bafilomycin A_1 (BA_1), labelled with the cell-permeable acidic pH probe 3-((2, 4-dinitrophenyl)-amino)propyl)-N-(3-aminopropyl)-methylamine, dihydrochloride) (DAMP). (**A′–A″**) Prematuration oocytes show acidic compartments surrounding the yolk globules (inset), and this acidification is reduced by BA_1 treatment. (**B′–B″**) Oocytes undergoing oocyte maturation show BA_1-sensitive acidic compartments located in a more peripheric area than in immature oocytes, where the fusion of the yolk globules into the central mass of fluid yolk takes place. (**C′–C″**) Yolk globule acidification is no longer visible in mature oocytes, when the fusion of yolk inclusions and proteolysis of yolk proteins finishes. Arrowheads point yolk globules (yg), and the asterisk indicates the central mass of fluid yolk. In C, the arrows point cortical alveoli at the periphery of mature oocytes. Scale bars, 50 μm (A), 20 μm (A′, inset), 100 μm (B and C). (Data from Raldúa et al., 2006.)

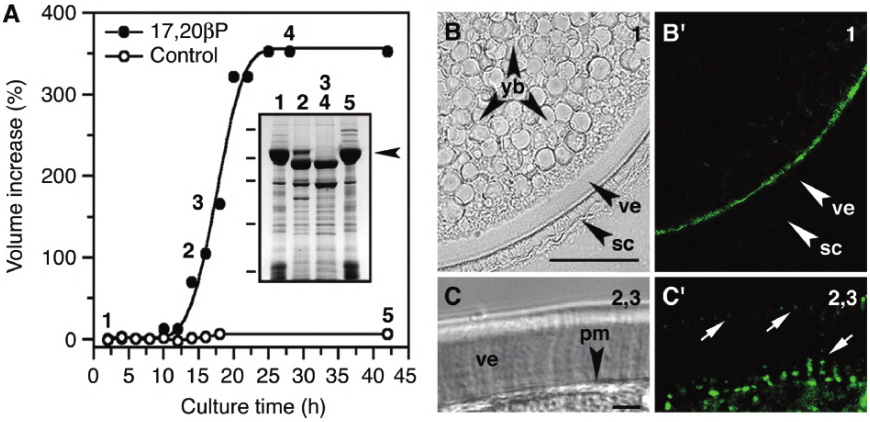

Figure 12.12 Time-course of oocyte hydration, proteolysis of major yolk proteins, and immunolocalization of SaAQP1o in the oocyte of gilthead sea bream during MIS-activated meiotic maturation *in vitro*. (**A**) Representative volume changes over time of a single oocyte treated with 0.1 mg/mL of MIS (17, 20βP) or ethanol vehicle (control). The inset shows an SDS-PAGE of follicle protein extracts (5 µg/lane) at specific hydration stage (named 1–5). The position (bars) of molecular weight markers is indicated on the left (from top to bottom: 200, 116, 97, 66, and 45 kDa). The arrowhead indicates degradation of lipovitellin of ~100 kDa. (**B–C**) Corresponding immunolocalization of SaAQP1o in oocytes at stage 1 and 2–3. Phase-contrast (**B′–C′**) and immunofluorescencen (**B′–C′**) images. In stage 1 (**B′–B′**), SaAQP1o is located within a thin layer below the oocyte plasma membrane, while during oocyte maturation and hydration, stage 2–3 (**C′–C′**), when proteolysis of the major yolk proteins is complete, SaAQP1o staining is observed within the oocyte microvilli crossing the vitelline envelope (arrows). *Abbreviations*: yb – yolk bodies; gv – germinal vesicle; ve – vitelline envelope; sc – somatic cells; pm, plasma membrane. Scale bars, 50 µm (1), 5 µm (2, 3). (Adapted from Fabra et al., 2005.)

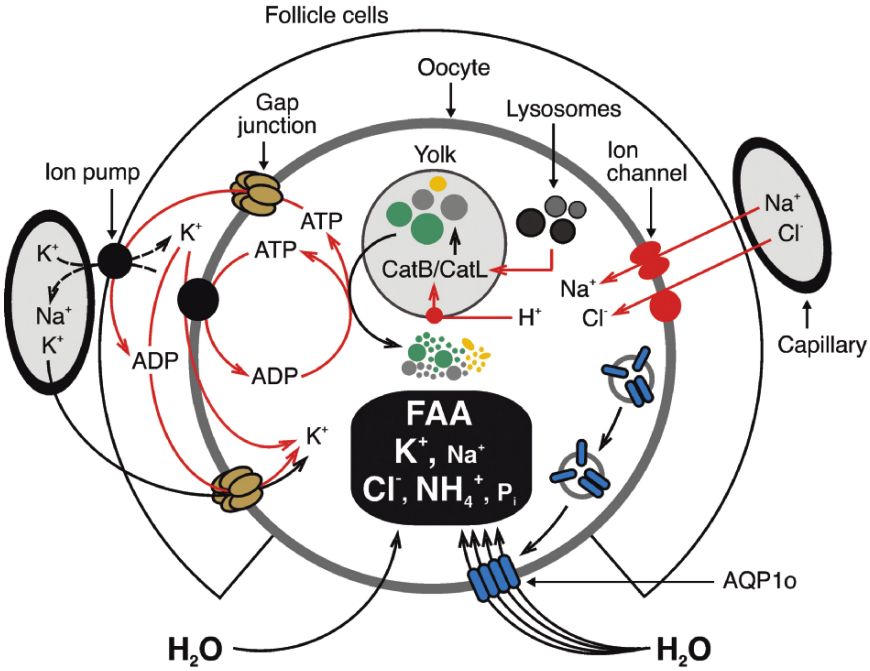

Figure. 12.14 Integrated model of molecular and cellular mechanisms involved in solute generation and water uptake during oocyte hydration in pelagophil and benthophil teleosts. In red are indicated postulated mechanims based on experimental evidences in fish oocytes or obtained from other biological systems. The major yolk components, lipovitellin, phosvitins, and β′-component, are indicated in green, grey, and yellow. *Abbreviations*: ATP – adenosine triphosphate; ADP – adenosine diphosphate; AQP1o – aquaporin-1o; CatB – cathepsin B; CatL – cathepsin L; FAA – free amino acids.

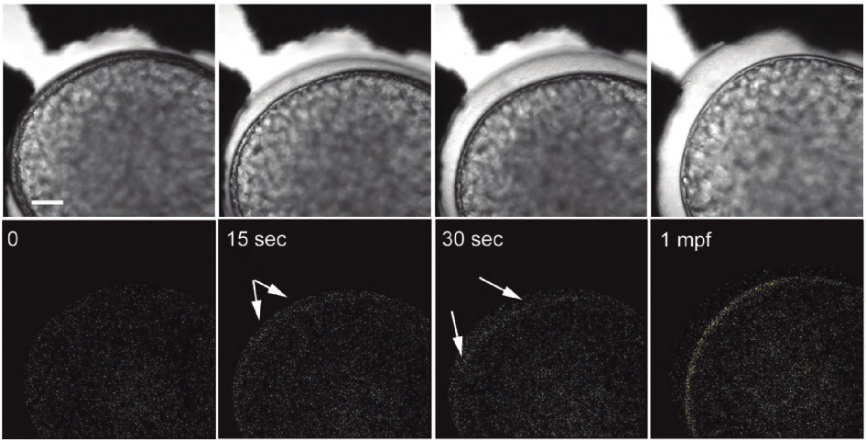

Figure 13.1 Changes in calcium levels at fertilization: Zebra fish oocytes were injected with calcium green-dextran (10 kDa) at an intracellular concentration of 100 nm, then imaged with a Nikon TE2000 inverted confocal microscope using a blue 488 nm laser. Fertilization was accomplished by adding a sperm suspension and aquarium water at time = 0. Differential interference contrast images are presented in the upper panels (Bar = 100 μm) and green fluorescence (presented as pseudocolor) is presented in the lower panels.

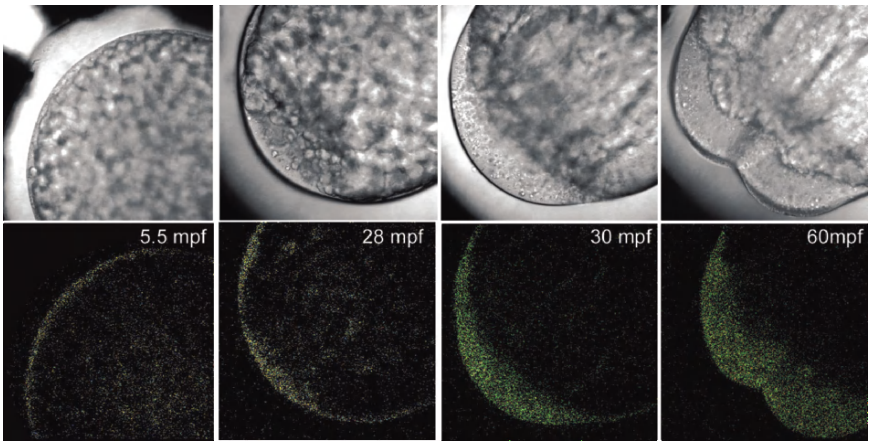

Figure 13.3 Changes in calcium levels leading up to mitosis. The zygote depicted in Figure 1 was monitored through the first cell division and representative time points are displayed.

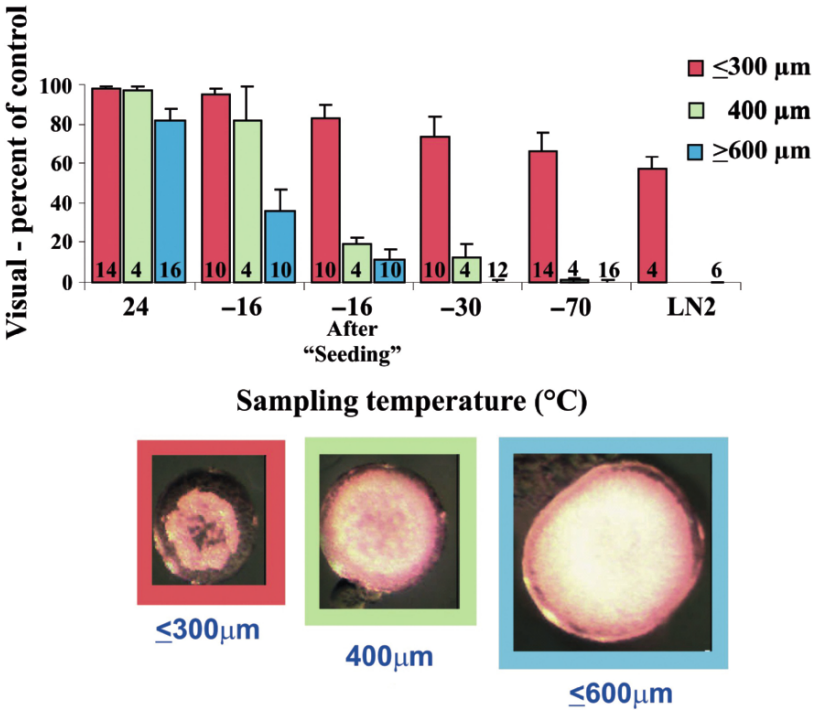

Figure 14.6 Percent of viable Gilthead sea bream oocytes after slow cooling to −16, −30, −70 and −196°C (LN2). Viability is shown before and after ice nucleation ("seeding") that was performed at −16°C. Three oocyte sizes were tested: ≤300, 400, and ≥600 µm in diameter. Between 4 and 16 replicate vials were tested at each temperature and each replicate consisted of 30–50 oocytes. The number of replicates are shown on each bar. Viability was assessed by MTT staining and close visual examination for detection of translucent oocytes.

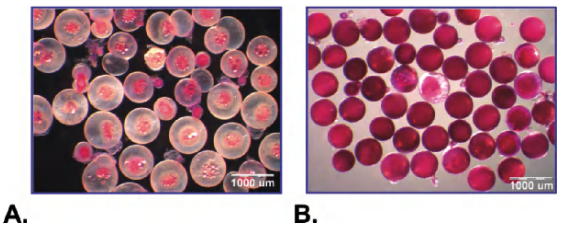

Figure 14.7 MTT stained translucent Gilthead sea bream oocytes (≥600 µm) that were subjected to slow cooling cryogenic procedures, thawed from LN (panel A) and incubated for 1 h in 75% L-15 culture medium. Control oocytes (panel B) were incubated for the same period of time (8–10 h) in culture medium.

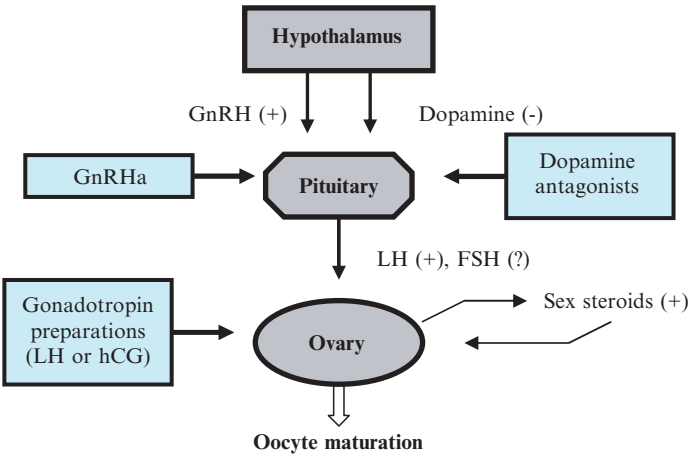

Figure 15.1 Schematic representation of the brain–pituitary–gonad axis and its endocrine control, along with the sites of intervention using hormonal therapies for the induction of oocyte maturation.