Oogenesis in Teleost Fish

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Abstract
Oogenesis is a very important biological phenomenon to generate haploid reproductive cells, eggs. Basic information on endocrine control of oogenesis in fish has been accumulated by using a variety of methods, such as histological, biochemical and molecular techniques for over 50 years. This monograph describes basic information on physiological functions of ovarian follicles, as sites of steroidogenesis and growth factor production, and also their physiological roles in oogenesis. Application of basic information to artificial hormonal control of eel reproduction is also mentioned.

1. Introduction
Oogenesis is a very important biological phenomenon to generate haploid reproductive cells, eggs. Numerous studies on reproductive physiology of fish, especially female reproductive physiology, have been carried out over almost 50 years. Basic information on endocrine control of oogenesis has been accumulated by using a variety of methods, such as histological, biochemical and molecular techniques. The basic information has also provided new ideas to create techniques to control a variety of female reproductive events and makes a great contribution to aquaculture industry in improving protocols for higher efficiency of egg production. This monograph describes information on the endocrine regulation of oogenesis and its application to control sexual maturation in eel valuable for aquaculture. Almost all information in this monograph has been provided by our studies which were carried out in our laboratory for the past 30 years.

2. Ovarian follicle

2-1. Functional morphology of follicle cells
The ovary in most teleosts is a hollow sac-like organ into which numerous ovigerous lamella extend (Figs. 1, 2). The ovary consists of two particular cell types; cells forming ovarian structures (somatic cells) and germinal line cells that generate haploid reproductive cells (gametes). Somatic cells construct ovarian components, such as the ovarian capsule, interstitial tissue (supporting tissue or stroma), and ovarian follicles (Figs. 1, 2). Vascular and nervous tissues also penetrate into the ovarian stroma. There are two different ovarian types (cystovarian type and gymnovarian type) which are classified according to the pattern of ovarian capsule formation (Fig. 2). The cystovarian type ovary is surrounded by the ovarian capsule; this type occurs in many teleost fishes such as goldfish, tilapia, and yellowtail. They have an ovarian cavity and oviduct. The gymnovarian type ovary lacks a part of the ovarian capsule and therefore, ovulated eggs are discharged directly into the abdominal cavity and spawned through the genital pore; this type occurs in salmonids and eels.

Germ line cells consist of diploid oogonia which derive from primordial germ cells and oocytes which differentiate from diploid oogonia and undergo the first meiotic division after a number of mitotic cell divisions. Developing oocytes become surrounded by a continuous follicular layer (granulosa cell layer) and the distinct outer layer of the follicular envelop (theca
cell layer) which forms from the surrounding stromal connective tissue elements (Fig. 3). The granulosa cell layer is composed of morphologically single cell type, except for a highly specialized micropyle cell which occupies and forms a micropyle (Fig. 4). Theca cell layer (Fig. 3) is composed of fibroblasts, collagen fibers, blood vessels and “special theca cells” (steroid-producing cells) (Fig. 5A). More detailed information is provided by previous reviews on ovaries and ovarian follicles including gametes (Nagahama 1983; Guraya 1986; Le Menn et al. 2007).

The granulosa cells of the oocyte at a vitellogenic stage of the yellowtail (Seriola quinqueradiata) are squamous in shape and attached to the zona radiata by short cytoplasmic processes, like microvilli (Figs. 3C, 5B). A large and flattened nucleus is located at central portion of the cells. The granulosa cells in this stage develop rough endoplasmic reticulum and well-developed Golgi complex consisting of stacks of several flattened cisternae associated with many small vesicles. Round, oval or rod-shape mitochondria with lamellar cristae are seen in the cytoplasm. These ultrastructural features are common in other teleost species, such as salmonid fish (Kagawa et al. 1981; Kagawa 1985), and red seabream, Pagrus major (Matsuyama et al. 1991). The granulosa cells possessed...
the ultrastructural features suggestive of protein synthesis, i.e. abundant rough endoplasmic reticulum and well-developed Golgi complex, rather than features of typical steroid producing cells. Several remarkable changes in the fine structure of granulosa cells are observed during oocyte maturation, such as cuboidal or columnar cell shape with wide intercellular spaces and extensive dilated rough endoplasmic reticulum containing amorphous substance in their cavities (Kagawa et al. 1981). These morphological changes in the granulosa cells prompt us to estimate their functional changes during oocyte maturation. Although many progressive studies have been done for the last few decades in the role of granulosa cells, physiological meanings of these morphological changes have not yet been evident. They are supposed to be implicated in the synthesis of steroid converting enzyme and ovulatory enzyme during oocyte maturation and ovulation (see the following section for more detail).

Fig. 4. The micropyte and micropylar cells. (A) Scanning electron micrographs of rainbow trout micropyte (M) and zona radiate (Z). (B, C) Electron micrographs of goldfish micropylar cells. Micropylar cell processes, which is morphologically distinct from the granulosa cells (G), extend thorough the zona radiata to the oocyte surface. M in (C): micropylar cell process. Reprinted from Hoar, Randall and Donaldson (eds), Fish Physiology, IX(A), Nagahama. The functional morphology of teleost gonads, 223–275, © 1983, with permission from Elsevier.

Fig. 5. High magnification of a special theca cell (A) and a granulosa cell (B) of a tertiary yolk globule oocyte of the yellowtail, Seriola quinqueradiata. Special theca cell possessing characteristic cellular features of steroid-producing cell; the round or oval mitochondria (M) with tubular and well-developed tubular smooth endoplasmic reticulum (sER) throughout their cytoplasm. Granulosa cell possessing characteristic cellular features of protein synthesis; well-developed rough endoplasmic reticulum (rER) and Golgi complex (G).

Remarkable cells found in the theca cell layer are so-called “special theca cells”. They are distinguished from other fibroblasts by a relatively large amount of cytoplasm and a less electron-dense nucleus (Figs. 3C, 5A). The most characteristic cellular features of these cells are the round or oval mitochondria with tubular or sometimes flattened cristae and well-developed tubular smooth endoplasmic reticulum throughout their cytoplasm. These morphological characteristics indicate that the special theca cells are the steroid-producing cells. Similar morphological observations on the special theca cells have been reported in many other teleost fishes, such as salmonidae (van den Hurk and Peute 1979; Kagawa et al. 1981; Kagawa 1985), zebrafish (Yamamoto and Onozato 1968), tilapia (Nicholls and Maple 1972), and goldfish (Nagahama et al. 1976). These cells possessing morphological features of the steroid producing cell contain Δ^5-3β-
hydroxysteroid dehydrogenase (3β-HSD), a key enzyme known to be involved in steroid hormone biosynthesis. Histochemical analysis at light- and electron-microscopy clearly demonstrates that 3β-HSD are localized in the special theca cells (Kagawa et al. 1981). Moreover, precipitates of copper ferrocyanide indicating the localization of 3β-HSD activity were observed in contact with the outer surface of the smooth endoplasmic reticulum and also found in the lumen of mitochondrial cristae in the steroid producing interrenal cells (Fig. 6) (Kagawa and Nagahama 1980). These histochemical analyses indicate that the special theca cells possessing characteristic morphological features, such as prominent smooth endoplasmic reticulum and mitochondria with tubular cristae, are the steroid-producing cells.

From electron microscopic observations, it is certain that the special theca cells are the cellular source of steroid in the teleost ovary. Histochemical data also support this fact. In the case of granulosa cells, most histochemical studies have indicated that the cells have 3β-HSD and 17β-hydroxysteroid dehydrogenase (17β-HSD) activities in many teleost fish (see review, Nagahama 1983), thus strongly suggesting that the granulosa cells are the most likely sites of steroid production in teleost fish. However, as mentioned previ-ously, granulosa cells do not possess any ultrastructural features typical of steroid-producing cells; these cells contain organelles suggestive of protein synthesis (Hoar and Nagahama 1978; Nagahama et al. 1978). This discrepancy indicates that morphological features do not always provide conclusive evidence and we realized that we do not have any direct evidence, concerning the steroid producing site in the teleost ovarian follicle. Moreover, these morphological observations gave us an important idea to clarify mechanisms of steroid production in the teleost ovarian follicle. Roles of granulosa and theca cells on ovarian steroid production had not been elucidated until we started to study using an in vitro culture system in teleost fish (see Subsection 2.3).

2-2. Gametes (Process of oogenesis)

The process of oogenesis is divided into the following four different phases from morphological and physiological features, cell inclusion and nuclear states of gametes; the proliferation phase, primary growth phase, secondary growth phase, and maturation phase.

2-2A. Proliferation phase

After transformation of primordial germ cells into oogonia, oogonia multiply by mitotic cell division forming oogonia nests in association with the pre-granulosa cell (Fig. 7). After prescribed times of mitotic cell division in a fish species, oogonia became primary oocytes when chromosomes were stopped at the diplotene stage of the first meiotic prophase (Le Menn et al. 2007). During the process of the transition from oogonia to primary oocytes, oocytes were surrounded by granulosa cells, basement membrane, and theca cells (Fig. 3). The hormonal mechanisms controlling oogonium proliferation and oocyte recruitment are obscure in any vertebrate. In teleost, fragmentary information has been reported so far. Gonadotropin (GTH), steroids (estradiol-17β (E₂) and 17,20β-dihydroxy-4-pregnen-3-one (DHP)) and growth factors may be involved in the process (see Lubzens et al. 2010). It may be certain that oogonium proliferation and oocyte recruitment from oogonium goes on continuously throughout their reproductive cycle, which is different from mammalian species where oogonium proliferation is completed during their embryonic stages. Teleost is therefore a good model for studying the regulatory mechanisms of oogonium proliferation and oocyte recruitment in future studies.

2-2B. Primary growth phase

The primary growth phase consists of two particular stages, chromat in nucleus and perinucleolus stages (Fig. 7). The chromat in nucleus can be distinguished by a conspicuous nucleolus associated with chromat thread. Multiple nucleoli become located around the periphery of the nucleus at perinucleolus stages in as-
association with the increase in cell and nucleus sizes.
During the primary growth phase, the organelles and molecules used at later stages are synthesized; the intriguing temporal expression of important genes in oogenesis and embryogenesis, occur (Le Menn et al. 2007). Since hypophysectomy does not inhibit the primary growth of oocytes until arrest at the late perinucleolus or very early cortical alveoli stage (Khoo 1979), this phase seems to be GTH-independent. However, it should be evaluated whether pituitary hormones are involved in regulating primary growth, because both follicle-stimulating hormone (FSH) \( \beta \) and luteinizing hormone (LH) \( \beta \) transcripts and proteins were found in the pituitary of gilthead seabream (Wong and Zohar 2004). Growth factors, such as transforming growth factor (TGF-\( \beta \)) family, may be involved in the regulation of primary growth of oocytes, although there has been no direct evidence available in teleost (see review, Lubzens et al. 2010).

2-2C. Secondary growth phase

The secondary growth phase is characterized by prominent oocyte growth associated with the synthesis and in corporeation of yolk materials. This phase is divided into three different stages from the appearance of yolk materials; that is, yolk vesicle (known as cortical alveoli), oil droplet, and yolk globule stages. The term vitellogenesis is generally accepted as a phase of vitellogenin (Vtg) incorporation and their processing into yolk protein (yolk globule), but need to encompass incorporation of other molecules, such as carbohydrate and lipids. From these criteria, cortical alveoli and oil drop stages are termed as a “primary vitellogenic stage” (Selman et al. 1993). However, the sequence of the appearance of three yolk materials varies with species; for example, lipid droplets appear soon after yolk vesicle formation in the rainbow trout but appear after formation of yolk globules in the smelt, Hypomesus japonicus (see review, Nagahama 1983). The yolk vesicle is a membrane-limited vesicle that stains with periodic acid-Schiff (for mucopolysaccharide or glycoprotein). The yolk vesicle is synthesized within the oocyte. As the oocyte grows, the yolk vesicles increase in number and size, are eventually displaced to the periphery of oocyte cytoplasm and called cortical alveoli. The transition of primary oocytes into secondary ones is thought to be regulated by pituitary hormones (Khoo 1979). Recent studies indicate that FSH, \( E_2 \), anti-Mullerian hormone, and gonadal soma-derived growth factor may be implicated in cortical alveoli production in the oocyte (Lubzens et al. 2010).

From the histological observation, lipid droplets mainly consist of neutral fats (triglycerides) (see Guraya 1986). Recent studies indicate that lipids are
absorbed and accumulated in the oocyte from the plasma very low-density lipoproteins and from Vtgs through receptor-mediated mechanisms. Phospholipids required for embryonic development probably originate from lipids carried by Vtg.

In vitro experiments indicated that 11-ketotestosterone and insulin-like growth factor-I (IGF-I) significantly increase lipid accumulation in the presence of the triglyceride triolein (Lokman et al. 2007). Since expression of several genes associated with lipoprotein uptake was found (Luckenbach et al. 2008), the regulatory factors involved in lipid accumulation and processing will be identified in future studies.

Main cell inclusion incorporated during the second growth phase is the yolk globule. Vtg is synthesized mainly in the liver under the control of E2 and growth hormone (GH) (see Babin et al. 2007; Lubzens et al. 2010), secreted into the blood, transferred through the vascular system, and incorporated into oocytes through receptor-mediated endocytosis, involving specific receptors in the endocytotic clathrin-coated pits of vesicles. Teleosts have at least three different vitellogenins, Vtg A, Vtg B, and Vtg C (Sawaguchi et al. 2006). They revealed that the amino acid sequences are formed by several domains; heavy chain of lipovitellin, phosphvitellin, light chain of lipovitellin and \( \beta' \)-component. Each of these domains are stored as lipoproteins, highly phosphorylated proteins, and \( \beta' \)-component in yolk globules found in the oocyte cytoplasm. Recent studies have also indicated that vitamins, such as vitamin A and E, are incorporated into oocytes during the process of oocyte growth. Since the vitamin content of an egg is related to egg quality in teleosts (Palace and Werner 2006), many studies on mechanisms of vitamin transport and incorporation into oocytes have recently been published (Labzens et al. 2010).

2-2D. Maturation and ovulation phase

After completion of oocyte growth, full-grown oocytes which possess a large nucleus (germinal vesicle) in the meiotic prophase enter maturation and ovulation phases, to terminate the meiosis and become fertilizable eggs. Detailed description of this phase can be found in Sections 3 and 4.

2-3. Plasma steroid hormones

Ovarian steroid hormones have been implicated in the control of oocyte development. Measurement of steroid hormone levels in the plasma in relation to oocyte development, provides some information about roles of steroid hormones on oocyte development. In teleosts, estrogen is known to induce the synthesis and secretion of Vtgs by the liver (see review of Babin et al. 2007). High levels of plasma estrogen have been found in relation to vitellogenesis (Schreck 1973; Wingfield and Grimm 1977). However, there had been no detailed studies on plasma progestational steroids in relation to oocyte maturation at the time when we started the measurement of plasma steroid levels. In some teleosts, evidence for involvement of progestational steroids in oocyte maturation had been obtained by using in vitro incubation methods (Jalabert 1976). We aimed to obtain detailed information of changes in steroid hormone levels during the process of oocyte development by means of steroid radioimmunoassay. We chose the salmonid fish as an experimental model. In teleosts, three ovarian types are classified according to the pattern of oocyte development (e.g. synchro-
Plasma E_2, testosterone, and DHP in two salmonids were measured to estimate steroidogenesis in the ovarian follicle (Fig. 9). Changes in plasma levels of 17\(\alpha\),20\(\beta\)-dihydroxy-4-pregnen-3-one during sexual maturation of amago salmon (*Oncorhynchus rhodurus*). Each value represents the mean ± SEM. a: number of samples. b: fish possessing oocytes at the migratory nucleus stage. c: fish possessing ovulated eggs. Reprinted from *Gen. Comp. Endocrinol.*, 51, Young et al., Plasma 17\(\alpha\),20\(\beta\)-dihydroxy-4-pregnen-3-one levels during sexual maturation of amago salmon (*Oncorhynchus rhodurus*); correlation with plasma gonadotropin and in *vitro* production by ovarian follicles, 96–105, © 1983, with permission from Elsevier.

2-4. Steroidogenesis in the ovarian follicle

From a morphological point of view, teleost ovarian follicle cells are thought to be the sites of steroidogenesis. Ultrastructurally, the special theca cells located in the theca cell layer have the characteristics of steroid-producing cells. Moreover, histochemical analysis has revealed that 3\(\beta\)-HSD activity, essential for the conversion of \(\Delta^5\) steroids to \(\Delta^4\) steroids, may be present in these steroidogenic cells. However, no detailed information on the mechanisms of ovarian steroidogenesis has been reported until recently.

Plasma E_2, testosterone, and DHP in two salmonids (white-spotted char, *Salvelinus leucomaenis*, and amago salmon *Oncorhynchus rhodurus*) were measured by radioimmunoassay (Kagawa et al. 1981, 1983; Young et al. 1983a). E_2 levels gradually increased during vitellogenesis (June to September), reached a peak in September, and rapidly decreased in mature and ovulated fish in October (spawning season) (Fig. 8). The seasonal pattern of plasma testosterone levels lagged behind and followed those of E_2 during vitellogenesis, but levels remained high in mature and ovulated fish. E_2 levels and the gonadosomatic index (GSI) values correlated well during vitellogenesis: GSI values showed a linear increase, and reached a peak in October and suddenly decreased to low levels in ovulated fish. There was a good correlation between plasma E_2 levels and GSI values during the vitellogenic period. Since the ovaries contain ovarian follicles at the same developmental stages in white-spotted char and amago salmon, the relationship between plasma steroid levels and oocyte development is easily estimated. It is suggested that E_2 is implicated in the control of the oocyte growth by the synthesis of Vtg in the liver. E_2 is synthesized in vitellogenic ovarian follicles and the production decreases at the time of oocyte maturation. Testosterone is not the major androgen secreted by male teleosts, since higher plasma levels of 11-ketotestosterone produced by the testis is the physiologically functional androgen in teleosts (Idler et al. 1961; Schulz et al. 2010). The maximally high plasma testosterone levels in mature and ovulated females corresponded with the enhanced capacity of preovulatory follicle to produce testosterone, could conceivably be related to the decrease of aromatase activity which converts testosterone to E_2. Thus, testosterone in female fish is used as a precursor of E_2. *In vitro* experiments for synthesis of E_2 in the ovarian follicles show direct evidence to demonstrate the hypothesis (see the following section). Plasma DHP levels (Fig. 9) were low in vitellogenic females (June to September) and in those with full-grown oocytes, and were elevated in mature and ovulated female amago salmon (Young et al. 1983a) and masu salmon, *Oncorhynchus masou*. *In vitro* studies have shown that this steroid is the most potent maturation-inducing steroid (MIS) in other salmonids and teleosts (Nagahama 1987b; Nagahama 1997). After obtaining more precise information from the experiments of *in vitro* steroid production (see the following section), plasma steroid levels of mature and ovulated females can be estimated to measure the roles of these steroid hormones on ovarian and testicular development in other teleost fish, including fish possessing the group synchronous or the asynchronous ovaries. Over all literatures published until recently, roles of steroid hormones on ovarian development have been discussed from our primary observation obtained from salmonoids and have come to the same conclusion.

2-4. Steroidogenesis in the ovarian follicle

Fig. 9. Changes in plasma levels of 17\(\alpha\),20\(\beta\)-dihydroxy-4-pregnen-3-one during sexual maturation of amago salmon (*Oncorhynchus rhodurus*). Each value represents the mean ± SEM. a: number of samples. b: fish possessing oocytes at the migratory nucleus stage. c: fish possessing ovulated eggs. Reprinted from *Gen. Comp. Endocrinol.*, 51, Young et al., Plasma 17\(\alpha\),20\(\beta\)-dihydroxy-4-pregnen-3-one levels during sexual maturation of amago salmon (*Oncorhynchus rhodurus*); correlation with plasma gonadotropin and in *vitro* production by ovarian follicles, 96–105, © 1983, with permission from Elsevier.
Follicle layers were developed. In combination with steroid radioimmunoassay, we obtained the data on steroid hormone levels in incubation media in a short time.

The oocytes of teleosts are surrounded by a complex multilayer follicle which varies in structure between and within different groups of teleosts. Common to all teleost ovarian follicles, salmonid ovarian follicles consist of an inner granulosa cell layer separated by a relatively thick basement membrane from an outer theca cell layer (Figs. 3, 10). These anatomical features and the large size of ovarian follicle gave rise to the development of a simple separation technique for the two layers. Moreover, as described previously, we easily obtained the ovarian follicle at the same developmental stage. Their ovaries are quickly removed and kept in ice-cold HEPES-NaOH buffered (pH 7.5) trout balanced salt solution (TBSS) (Kagawa et al. 1982, 1983). Oocytes with the follicle layer are isolated from ovaries with large forceps. Thereafter, under a dissecting microscope, a small puncture is made in the oocyte with a pair of fine watch makers’ forceps and then the follicle layer (thecal cell layer) can be grasped on either side of the puncture using the forceps, and peeled away from the remaining oocyte with the granulosa layer. The two different cell layers (especially the granulosa layer) are then washed with TBSS to remove the yolk. The purity of each follicular preparation assessed by light microscopy reveals no contamination of the granulosa layer with theca cells and less than 10% contamination of the theca cell layer with granulosa cells (Fig. 10). After separating the fol-

Fig. 10. Light and scanning electron micrographs of follicular preparations of amago salmon oocytes. (A) Intact follicle, showing thecal layer (T), granulosa layer (G), and zona radiata (Z). (B) Thecal layer separated from a follicle, showing a small patch of granulosa cells (G) attached to the basement membrane (arrowhead). (C) Follicle from which the thecal layer was removed, showing the uniform granulosa layer (G) and the zona radiata (Z). (D) Scanning electron micrograph of a granulosa layer preparation, consisting purely of granulosa cells. Reprinted from Gen. Comp. Endocrinol., 47, Kagawa et al., Estradiol-17β production in amago salmon (Oncorhynchus rhodurus) ovarian follicles: Role of the thecal and granulosa cells, 440–448, © 1982, with permission from Elsevier.
licle layers, each preparation is incubated in plastic tissue culture dishes containing 1 ml TBSS (10 follicular preparations/well) with or without gonadotropic hormones, such as partially purified chinook salmon gonadotropin (SG-G-100, 0.01–1 µg/ml) (open bars) for 18 hr. Each value represents the mean ± SEM of the three replicates. Reprinted from Gen. Comp. Endocrinol., 47, Kagawa et al., Estradiol-17β production in amago salmon (Oncorhynchus rhodurus) ovarian follicles: Role of the thecal and granulosa cells, 440–448, © 1982, with permission from Elsevier.

Fig. 11. Effects of SG-G100 on estradiol-17β and testosterone secretion by amago salmon follicles. Follicles were incubated in Ringer alone (R, shaded bars) or Ringer with various doses of chinook salmon gonadotropin (SG-G-100, 0.01–1 µg/ml) (open bars) for 18 hr. Each value represents the mean ± SEM of the three replicates. Reprinted from Gen. Comp. Endocrinol., 47, Kagawa et al., Estradiol-17β production in amago salmon (Oncorhynchus rhodurus) ovarian follicles: Role of the thecal and granulosa cells, 440–448, © 1982, with permission from Elsevier.

G100. These results indicate that GTH directly controls E2 production in amago salmon follicles. By using similar incubation methods, many studies on in vitro steroid production have been done in many other teleost fish thereafter.

Using follicles from the vitellogenic amago salmon, we prepared four different follicular preparations (Fig. 1): (1) intact follicles (oocytes with complete follicle layers), (2) thecal cell layer with a small proportion of granulosa cells attached, (3) granulosa cells, (4) co-culture of theca and granulosa cell layers preparations (Kagawa et al. 1982). Each preparation was incubated in plastic tissue culture dishes containing TBSS with different doses of SG-G100. Incubations were carried out as shown previously. E2 in the incubation media was measured by radioimmunoassay (Kagawa et al. 1981). SG-G100 enhanced E2 production in both intact follicles and co-culture of theca and granulosa cell layers in a dose dependent manner (Fig. 11). Media from the thecal cell layer group contained small amounts of E2 after incubation with SG-G100 while media from the granulosa cell layer group contained no detectable amounts of E2. The small amounts of E2 produced by the thecal cell layer group maybe due to contamination of the preparation by granulosa cells. Out in vitro data presented here clearly show that in the amago salmon, both the thecal cell layer and granulosa cell layer are necessary for the stimulation of in vitro estrogen production by GTH. From these in vitro data we proposed for the first time in teleost "two-cell type model" for production of E2 in the teleost ovarian follicle (Fig. 12). The measurement of concentration of testosterone in media from the same experiment showed that SG-G100 enhanced testosterone production by thecal layers (Fig. 11). Testosterone production was also enhanced by SG-G100 in the intact follicle and co-culture incubations, but the relative increase compared to hormone-free control levels was much
less. These results suggest that, as in mammals (Hamberger et al. 1978), the thecal layer contributes to E2 synthesis by providing testosterone to the granulosa layer for aromatization. This hypothesis is supported by the ability of the granulosa layer to produce E2 in the presence of exogenous testosterone. The two-cell type model is also applicable to the synthesis of MIS in amago salmon (Young et al. 2005) (Fig. 12).

In this model, the thecal cell layer produces 17α-hydroxyprogesterone that is converted to DHP by the granulosa cell layer where GTH acts to enhance the activity of 20β-hydroxysteroid dehydrogenase (20β-HSD), the key enzyme involved in the conversion of 17α-hydroxyprogesterone to DHP (Young et al. 1986; Nagahama 1987a, b).

The capacity of intact follicles to produce E2 in response to GTH stimulation increases during oocyte growth, but rapidly decreases in association with the ability of the oocyte to mature in response to GTH (Kagawa et al. 1983). Testosterone production by the thecal layer preparations in response to GTH also increases during the course of oocyte growth and peaks during the postvitellogenic period. Aromatase activity in granulosa cell layers increases during vitellogenesis and decreases rapidly in association with the ability of the oocyte to mature in response to GTH (Young et al. 1983b; Kanamori et al. 1988). This decrease in aromatase activity appears to be coincident with the decreased ability of intact follicles to produce E2 in response to GTH. Immediately prior to oocyte maturation, intact ovarian follicles of salmonid fish acquire an increased ability to produce DHP in response to GTH. A decrease in C17-20 lyase and/or 17β-HSD activity in thecal cells and an increase in 20β-HSD in granulosa cells appear to be the major factors responsible for the rapid increase in DHP production by follicles during oocyte maturation. Molecular approaches indeed signified the changes steroidogenic enzyme genes such as aromatase and 20β-HSD and their related transcription factors in ayu and tilapia, to complement our research done earlier related to the site of production of steroids during vitellogenesis and oocyte maturation (Senthilkumar et al. 2002; Tanaka et al. 2002; Zhou et al. 2007; Nagahama and Yamashita 2008; Senthilkumar 2011).

Numerous studies have reported that FSH and LH stimulate E2 production by vitellogenic teleost ovarian follicles in vitro (Young et al. 2005). Several recent papers show that the GTH stimulate the increase in expression of one or more of the genes encoding ovarian steroidogenic proteins, including StAR, 3β-HSD, and P450 aromatase (Young et al. 2002; Kagawa et al. 2003a).

### 2-5. Sites of IGF-I production and its physiological roles

Substantial evidence supports the view that ovarian follicles are the site of IGFs production, reception, and...
action in mammals (Adashi 1993). However, in teleosts, IGF-I is known as a factor, which is synthesized in the liver by GH to regulate development and somatic growth (Duan et al. 1994) and precocious maturation (Moriyama et al. 1997). Since mRNA of IGF-I in the ovary of coho salmon (Duan et al. 1993) and existence of IGF-I receptors in the ovary of carp (Niu et al. 1993) have been demonstrated, it is expected that IGF-I may be produced in the ovary and may participate in the physiological regulation of the teleost ovary. We first examined the presence and cellular distribution of IGF-I in the ovarian follicles of red seabream during oocyte growth and final maturation of oocytes by immunocytochemistry (Kagawa et al. 1995a). Immunoreaction of IGF-I is present in the ovary, mainly in the granulosa cell layer, of the red seabream (Fig. 13). These results concur with previous studies that showed the expression of IGF-I mRNA in the ovary of coho salmon (Duan et al. 1993), indicating that granulosa cells of the ovary is one of the IGF-I-producing sites in teleosts. In the next step, we examined effects of hCG, cAMP, and inhibitors of RNA (actinomycin D) and protein (cycloheximide) synthesis on in vitro IGF-I production by the ovarian follicles of red seabream (Kagawa et al. 1999). Specific radioimmunooassay for IGF-I showed that IGF-I was secreted into the incubation medium without hCG treatment. Dibutyryl cAMP slightly increased IGF-I production but actinomycin D and cycloheximide totally inhibited secretion of IGF-I from the ovarian follicles (Fig. 14). These results directly show that the ovarian follicle of red seabream can produce IGF-I. Moreover, similar to the mammals (Hacey et al. 1992), the stimulation of IGF-I gene transcription is required for the production of IGF-I in the ovarian follicles of red seabream. Although endocrine factors involved in IGF-I production in the ovarian follicle of red seabream have not yet been identified, FSH, LH, E2, GH, and other growth factors are thought to be candidates for IGF-I production, as suggested in mammalian species (Hsu and Hammond 1987; Mondschein and Hammond 1988).

Immunoreactivity of IGF-I in the granulosa cell layers changed during development of the oocytes. Immunoreactivity first appeared in the granulosa cell layers of the lipid stage oocytes, became intense at the primary yolk globule stage, and decreased with the progress of oocyte growth (Fig. 13). Heterogeneity of IGF-I gene expression occurs in the developing ovarian follicles of the rat (Zhou et al. 1991). This mRNA expression correlated with the distribution of mitotically active granulosa cells, suggesting that IGF-I acts in an autocrine and paracrine manners to promote granulosa cell replication. IGF-I may thus function as an autocrine mitogen for granulosa cells in the red seabream, as shown in male dogfish, *Squalus acantias*, in which IGF-I is involved in the proliferation of Sertoli cells and spermatogonia (Dubois and
Callard 1993). Strong immunoreactivity was present in the granulosa cell layer of primary yolk globule stage oocytes (Fig. 13). Moreover, the follicles at the primary yolk globule stage synthesize in vitro E2 in response to LH (Kagawa et al. 2003a). Thus, it is expected that IGF-I has a physiological role on steroidogenesis in the ovarian follicle. To clarify the involvement of IGF-I in E2 production in the ovarian follicle of red seabream, in vitro effects of IGF-I on aromatase activity and P450 aromatase gene expression in the ovarian follicles of red seabream, Pagrus major, 1562–1568, © 2003, with permission from Society for the Study of Reproduction.

Physiological roles of growth factors, such as epidermal growth factor, TGF-β, activin and IGF-I, on oocyte maturation have been reported in mammalian species (see Kagawa et al. 1994a). Moreover, possible involvement of IGF-I and insulin in oocyte maturation has also been reported in lower vertebrates, such as Xenopus laevis and goldfish (Maller and Koontz 1981; Lessman 1985). Therefore, we examined the effects of various growth factors on in vitro oocyte maturation germinal vesicle breakdown (GVBD) in oocytes of the red seabream. IGF-I affects on oocyte maturation and maturational competence (ability in response to MIS) are described in the following section (see Subsection 3.1).

3. Oocyte maturation and ovulation

Two temporally and mechanistically distinct stages of GTH-dependent oocyte maturation have been described in several teleost fishes. The ovarian follicle acquires the ability to produce MIS and the oocyte acquires to respond to MIS during the first stage of maturation, whereas in the second stage the follicle produces MIS and, consequently, the oocyte is released from meiotic arrest. Depending on the species, morphological indices such as onset of lipid coalescence, germinal vesicle migration or hydration of oocytes also occur during the process of oocyte maturation and ovulation. General and detailed information on maturational competence (Patiño et al. 2001) and oocyte maturation (Nagahama and Yamashita 2008; Lubzens et al. 2010) and ovulation (Goetz and Garczynski 1997) are provided in other reviews. In this section, hormonal mechanisms of maturational competence, nuclear maturation and cytoplasmic maturational competence are described by data obtained from valuable fish species for aquaculture in Japan, such as the red seabream and the Japanese eel, Anguilla japonica.

3-1. Maturational competence and GVBD

GTH's action on final oocyte maturation has been studied in many fish species using in vitro incubation techniques (Kagawa 1994). GTH acts primarily on the follicle layers of the oocytes to produce MIS. Nagahama and Adachi (1985) conclusively identified
DHP as the MIS of amago salmon and this steroid has been recognized as one of the most potent MIS in many other fish species. Afterwards, 17α,20β,21-trihydroxy-4-pregnen-3-one (DHP, 10 ng/ml). Each value represents the mean ± SEM of three replicates. The different letters represent significant difference at p < 0.05. Reprinted from Gen. Comp. Endocrinol., 112, Kagawa et al., GTH II but not GTH I induces final maturation and the development of maturational competence of oocytes of red seabream for in vitro, 80–88, © 1998, with permission from Elsevier.

hCG and DHP which were related to the time of day when oocytes were isolated (Kagawa et al. 1994b). Oocytes isolated 18 hr before spawning (oocyte at migratory nucleus stage) have the ability to respond to DHP and oocytes isolated 9–13 hr before spawning underwent GVBD (Fig. 17). Incubation of DHP-insensitive oocytes isolated 36 hr before spawning (oocyte at the tertiary yolk globule stage) with hCG or red seabream LH resulted in oocytes undergoing GVBD when incubated with DHP, but red seabream FSH was ineffective (Kagawa et al. 1994b, 1998b) (Fig. 18). These experiments indicate that LH induces maturational competence of oocytes of red seabream but FSH does not. hCG acts on inducer of maturational competence, as similar to LH. Actinomycin D (transcriptional inhibitor) and cycloheximide (a translational inhibitor) totally inhibited LH-induction of GVBD. These results indicate that production of new proteins through a mechanism of RNA synthesis by LH is necessary for the development of the maturational competence. The MIS receptor is one of the proteins synthesized in response to LH, since inhibitors of RNA and protein synthesis blocked GTH-dependent increase of MIS receptor concentration (Thomas et al. 2002).

Insulin, IGF-I, and IGF-II induced GVBD, although other growth factors, such as inhibin A, activin A, epidermal growth factor, were ineffective (Kagawa et al. 1994a). These results suggest, for the first time in teleost, that IGFs, especially IGF-I which was the most potent inducer of GVBD, are involved in the induction of GVBD of oocytes. In this study, actinomycin D did not block IGF-I-induced GVBD, a result that dif-
fers from that of hCG, where actinomycin D inhibited GVBD induced by hCG alone or in combination with DHP (Fig. 19). IGF-I thus may not mimic the role of GTH on GVBD in the red seabream. GTH is generally accepted to act on the follicular layers to produce MIS which in turn directly induces oocyte maturation. As reported in our previous paper (Kagawa et al. 1994b), data obtained in the amago salmon (Nagahama and Adachi 1985; Nagahama 1987b) indicated that actinomycin D and cycloheximide abolished GTH-induced MIS production by the ovarian follicles of the red seabream oocytes and inhibited GVBD. IGF-I-induced GVBD thus may not be mediated through MIS production in the follicular layer of the red seabream. IGF-I may act directly on oocytes of red seabream through IGF-I receptors, not via MIS production in follicle layers, as suggested in *Xenopus* oocytes (Hainaut et al. 1991). Other physiological roles of IGF-I in oocyte maturation were also indicated from our in vitro experiments of red seabream ovarian follicles (Kagawa et al., 1994b). Oocytes underwent GVBD in response to DHP when oocytes were incubated with IGF-I. Moreover, preincubation of DHP-insensitive oocytes with IGF-I resulted in oocytes undergoing GVBD in response to DHP. IGF-I can thus induce maturational competence (acquisition of ability of oocyte to respond to MIS) of oocytes of the red seabream (Patiño and Kagawa 1999; Patiño et al. 2001). In vitro incubation of incompetent oocytes (oocytes do not have the ability to respond to MIS) with IGF-I significantly increased number of heterologous (granulosa cell-oocyte) and homologous (granulosa cell-granulosa cell) gap junction, resulting that oocytes became maturational competent (Patiño and Kagawa 1999) (Fig. 20). From these data we propose that increased gap junction is an event that is functionally associated with the acquisition of oocyte maturational competence in full-grown ovarian follicles of teleost fishes. Also, this study documented for the first time in any species, that IGF-I is a potent stimulator of heterologous and homologous gap junction in ovarian follicles. The specific functions of enhanced gap junction coupling during the acquisition of oocyte maturational competence and the induction of GVBD remain unresolved.

### 3-2. Cytoplasmic maturation

In teleosts, after a relatively long period of growth (the vitellogenic phase), oocytes undergo maturation, accompanied by several maturational processes in the nucleus (such as GVBD) and cytoplasm (such as hydration, lipid coalescence, and clearing of the ooplasm; Wallace and Selman 1980, 1981). In particular, marine teleost spawning buoyant eggs in seawater, oocytes undergo a significant increase in size because of rapid water uptake during the maturational process before ovulation (Fig. 21). During these processes, the oocytes become buoyant, which is essential for their oceanic...
survival and dispersal as well as for the initiation of early embryogenesis. Therefore, the process of cytoplasmic maturation was important for fish to spawn eggs of good quality. Freshwater eels of the genus Anguilla are distributed worldwide and have unique characteristics such as a catadromous life history. The Japanese eel is believed to migrate from rivers and lakes into the ocean and spawn pelagic eggs in a particular area in the western North Pacific (west of the Mariana Islands; Tsukamoto et al. 2010). They are sexually immature and never mature under rearing conditions. Therefore, to maintain the natural eel resources and to obtain reliable supplies of glass eel (seeds) for aquaculture, development of an artificially induced breeding procedure for eels, especially induction of oocyte maturation and ovulation, has long been attempted for obtaining good quality eggs (see Section 4). However, limited information is available on the mechanisms of oocyte hydration, lipid coalescence, and clearing of the ooplasm as well as on the role of these physiologically important processes in the subsequent survival and development of the eggs and embryos of this species. Therefore, to elucidate the mechanisms of oocyte hydration in the Japanese eel, we first examined the in vivo and in vitro morphological changes and hydration process occurring during oocyte maturation and ovulation.

Cultivated female eels are sexually immature. To induce sexual maturation and obtain matured oocytes and ovulated eggs, female eels are injected with salmon pituitary extract (SPE) and the MIS, DHP (see more detailed information on induction of sexual maturation in Section 4). Full-grown oocytes (completion of vitellogenesis) and oocytes in the migratory nucleus stage were retrieved from the genital pore of fully matured eels with a polyethylene cannula and kept in ice-cold Leibovits culture medium supplemented with 2.5 g Hepes, 0.1 g streptomycin, and 100,000 IU penicillin per liter. Matured oocytes and ovulated eggs were also obtained from eels processed according to a previously described method (Ohba et al. 1996a; Kagawa 2003). Briefly, eels that had oocytes at the migratory nucleus stage (over 750 µm in diameter) were injected with SPE as a priming dose, followed 24 hr later by an intraperitoneal injection of DHP (2 ug/g body weight).
Ovulated eggs were then obtained by gently squeezing the abdomen about 12–18 hr after the DHP injection. The oocyte hydration process was quantified by determining the wet and dry weights of the follicle-enclosed oocytes of various diameters collected from different females (Fig. 21). The wet weight of these oocytes gradually increased with the increase in oocyte diameter, although the dry weight essentially remained constant throughout this change in diameter. These data indicate that water accumulation during oocyte maturation is the major factor contributing to the increase in follicular diameter. During hydration process, the cytoplasm appears translucent, and oil droplets fuse and are clearly visible (Fig. 21). These results are identical to the previous data obtained for teleosts that spawn buoyant eggs, such as the red seabream (Adachi et al. 1988), black sea bass (Selman et al. 2001) and gilthead seabream (Fabra et al. 2006).

These in vivo hydration processes replicate in vitro. In vitro experiments revealed that GTHs (hCG, SPE, recombinant eel LH) and DHP stimulated an increase in the diameter of follicle-enclosed oocytes at the migratory nucleus stage (Kagawa et al. 2009a). Addition of HgCl₂ to the incubation media inhibited the hCG- and DHP-induced increase in the follicular diameter in a dose-dependent manner (Fig. 22). Moreover, this inhibition was reversed by the addition of β-mercaptopethanol (1 mM). Previous studies have shown that treatment of maturing sea bream follicles with HgCl₂ (Fabra et al. 2005) or the ion channel blocker tetraethylammonium (TEA) (Fabra et al. 2006) inhibits the oocyte volume increase. Moreover, the pattern of water permeability of maturing sea bream oocytes in response to HgCl₂ and TEA strongly resembles that of *Xenopus laevis* oocytes expressing gilthead seabream AQP1b (SaAQP1b, recently termed AQP1b). The antibody was raised against a synthetic peptide corresponding to the underlined sequence. The numbers on the right correspond to amino acid positions in the protein sequences. Reprinted from Reprod. Biol. Endocrinol., 9, Kagawa et al., Expression and localization of aquaporin 1b during oocyte development in the Japanese eel (Anguilla japonica), 71, © 2011, with permission from BioMed Central Ltd.

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**Fig. 23.** Comparison of the deduced amino acid sequence of Japanese eel AQP1b with those of European eel, Senegal sole, gilthead seabream and zebrafish. The six transmembrane (TM) domains and connecting loops (A–E) are indicated by brackets and horizontal arrows, respectively. Two Asn-Pro-Ala (NPA) motifs are emboldened. The antibody was raised against a synthetic peptide corresponding to the underlined sequence. The numbers on the right correspond to amino acid positions in the protein sequences. Reprinted from Reprod. Biol. Endocrinol., 9, Kagawa et al., Expression and localization of aquaporin 1b during oocyte development in the Japanese eel (Anguilla japonica), 71, © 2011, with permission from BioMed Central Ltd.
SaAQP1b; Tingaud-Sequeira et al. 2008). Consequently, it appears that SaAQP1b is essential for the mechanisms controlling water uptake by seabream oocytes. Therefore, aquaporin (AQP) could also facilitate water uptake by acting as a water channel during oocyte maturation in Japanese eels. This hypothesis is supported by our recent molecular findings (Kagawa et al. 2011).

We have isolated and characterized a Japanese eel aqp1ab cDNA derived from the ovary. The predicted amino acid sequences of the cloned Japanese eel ovary-derived aqp1ab shared 99% overall sequence identity with that of the AQP1 previously reported in the European eel, Anguilla anguilla (Martinez et al. 2005) termed AQP1dup (Fig. 23). The Japanese eel AQP1ab contains three functional domains; an N-terminal extracellular domain, a large transmembrane domain, and a C-terminal cytoplasmic domain. In particular, six potential transmembrane domains and two NPA motifs are conserved. Moreover, amino acids known to be essential for the pore-forming region in human AQP1 (i.e. Phe56, His180, and Arg195 Sui et al. 2001) were present in an analogous position in Japanese eel AQP1ab. Therefore, these amino acids in Japanese eel AQP1ab may be involved in water selective pore formation. Also, a Cys residue located N terminal to the second NPA motif, which may be involved in inhibition of water permeability by mercurial compounds (Preston et al. 1993), was identical in Japanese eel AQP1ab.

**Fig. 24.** Localization of aqp1b gene transcripts in Japanese eel oocytes by in situ hybridization. Oocyte at the perinucleolus stage (A), (B), (C), the oil stage (D), the primary yolk globule stage (E), and the secondary yolk globule stage (F). Arrowheads indicate intense aqp1b signals observed in oocytes. Reprinted from Reprod. Biol. Endocrinol., 9, Kagawa et al., Expression and localization of aquaporin 1b during oocyte development in the Japanese eel (Anguilla japonica), 71, © 2011, with permission from BioMed Central Ltd.
as conger eel *Conger myriaster* and pike eel *Muraenesox cinereus*, may substantiate this contention.

Yolk precursor Vtgs are incorporated and processed into yolk proteins, such as lipovitellin, phosvitins, and β'-component during the growth period (Matsubara et al. 1999; Hiramatsu et al. 2002; Sawaguchi et al. 2005, 2006). During oocyte maturation, the yolk globules fuse and, concomitantly, pronounced proteolysis of the yolk proteins occurs, generating an increase in free amino acids; the resulting increase in small peptides in the oocytes provides the driving force for the water influx into the oocytes (Matsubara et al. 1999; Selman et al. 2001; Fabra et al. 2006). In our study using Japanese eel, bafilomycin A1, a specific inhibitor of vacuolar proton-ATPase and acidification of yolk inclusion (Raldu’a et al. 2006), prevented the hCG- and DHP-induced oocyte hydration in a dose-dependent manner. Previous studies indicate that bafilomycin A1 prevents yolk protein hydrolysis and the generation of free amino acids (Selman et al. 2001). Therefore, in the Japanese eel, acidification of the yolk compartments is necessary for appropriate protein hydrolysis, increasing small peptides (such as free amino acids) in the oocytes and providing the driving force for the water influx into the oocytes, as suggested previously (Matsubara et al. 1999; Selman et al. 2001; Fabra et al. 2006).

### 3-3. Ovulation

Ovulation in fishes is defined as the release of mature ova from the surrounding follicular cells. The ease of manipulating fish eggs as well as ovarian fragments (follicle) in vitro makes fish an excellent experimental model for investigating regulatory mechanisms of ovulation. The release of the mature oocytes involves several processes: disruption of the connections between microvilli of granulosa cells and oocytes, thinning and formation of a rupture or hole in the follicle wall and an active process involving contraction of the smooth muscle. Several studies pertaining to fish ovulation identified the involvement of arachidonic acid and its metabolites, including PGs, in ovulation in fish (see Goetz et al. 1991). In the eel, DHP can induce in vitro both final oocyte maturation and ovulation. To estimate the DHP-induced ovulatory mechanism in the Japanese eel, we examined the in vitro effects of PGs and indomethacin (a prostaglandin (PG) endoperoxide synthase inhibitor) on in vitro ovulation in the Japanese eel.

After the ovarian pieces had been dissected into small pieces in culture medium, oocytes with follicular layers (approximately 850–900 µm in diameter) at the migratory nucleus stage were dispersed by pipetting the ovarian pieces. At this stage, oocytes have a transparent peripheral cytoplasm, and the nucleus progressively displaced towards the periphery of the oocyte and can be observed under a binocular microscope (Fig. 21). DHP-induced GVBD was completed by 15 hr af-
ter incubation, and ovulation rates increased rapidly at 18 hr and were close to maximum 24 hr after incubation (Fig. 25) (Kagawa et al. 2003b). Various doses of PGs were added into the incubation medium 15 hr after incubation with DHP. PGs examined, PGE₂, PGF₂α, and PGF₁α, but not PGE₁ induced in vitro ovulation in the oocytes of the Japanese eel. Similar to previous papers (Kagawa and Nagahama 1981; Goetz et al. 1991), PGF₂α was the most effective in inducing in vitro ovulation of the Japanese eel. Indomethacin blocked DHP-induced in vitro ovulation. Moreover, the indomethacin-inhibited ovulation is reversed by PGF₂α (Fig. 26). As indomethacin inhibits PGF synthesis in the ovarian follicles of fish, DHP-induced ovulation is mediated by the production of PGF in the follicles of the Japanese eel. Actinomycin D and cycloheximide inhibited DHP-induced in vitro ovulation, suggesting that DHP-induced ovulation requires mRNA and protein synthesis, and signal transduction pathways, including G-proteins, inositol phosphate turnover, protein kinase C and transmembrane calcium movement, are involved in mediation follicular PGF production in fish follicles (Goetz et al. 1991; Hsu and Goetz 1991). However, it can not be ruled out that both inhibitors act on preoteolytic enzymes, such as plasminogen activators, collageneolytic enzymes and neutral proteases, which are believed to play an integral role in the mechanism of degradation of the follicle wall and the formation of the rupture. However, in teleost fish, very little work has been done concerning the role of proteolytic enzymes (Berndtson et al. 1989; Goetz et al. 1991).

Furthermore, depth analysis revealed that oocyte maturation is regulated by non-genomic action of MIS, while ovulation is regulated by genomic mechanisms such as transcriptional activity accompanied by new mRNA synthesis (Theofan and Goetz 1981). One of the noteworthy points in a piscine ovulation study was the identification of membrane receptor for progestins (Tokumoto et al. 2012), which paved the way to identify the non-genomic action of steroids. However, the knowledge in the field of research pertaining to piscine ovulation is still limited. Further studies using advanced methods such as differential display, subtraction cDNA library and cDNA microarray may provide a way to identify new genes and factors that play a pivotal role in piscine ovulation. Few comprehensive studies revealed important roles for hydrolytic enzymes and metalloproteinasises in follicular rupture leading to ovulation (Ogiwara et al. 2005, 2012).

4. Artificial induction of oogenesis

Control of reproductive function in captivity is essential for the sustainability of commercial aquaculture production. Many of the commercially important fish species, such as popular freshwater eels (Anguilla spp.), the Japanese yellowtail and greater amberjack (Seriola spp.), some groupers (Epinephelus spp.) and the bluefin tuna (Thunnus spp.) do not spawn spontaneously in captivity (Ottolenghi et al. 2004). Reproduction of fish in captivity can be controlled by photoperiod, water temperature or spawning substrate. However, in some cases, it is impractical to undertake environmental manipulations, if the fish require environmental parameters (for example depth and spawning migration) for natural reproductive performance. In most case, the reproductive failure occurs in the female. In mullet (Mugil cephalus), barfin flounder (Verasper moseri), yellow tail (Seriola quinquergiradiata), oocytes develop to full-grown stage (completion of vitellogenesis) but oocyte maturation and ovulation does not occur. There have been many trials of hormonal treatments, which effectively induce oocyte maturation and ovulation in this type of fish (see reviews Zohar and Mylonas 2001; Mylonas et al. 2010).

The eel has long been esteemed not only in Japan but also in European countries as an important food fish. The aquacultural production of Japanese eel in Japan is about 20,000 tons a year in recent years. Japan also imports a total of 80,000 tons of eel mainly from China and Taiwan, and other countries. Seedlings for eel aquaculture are totally dependent on glass eels, natural juveniles of eel which have been captured in estuaries. However, in both East Asia and Europe, the catches of glass eels differ greatly from year to year, and have been decreasing especially in the past 25 years, resulting in a sharp rise in their price. Therefore, to maintain the natural glass eel resources and to obtain reliable supplies of glass eels for aquaculture, development of an artificially induced breeding procedure for eels has been eagerly desired.

Techniques for artificial breeding of the Japanese eel have been studied intensively since the 1960s. Yamamoto and Yamauchi (1974) first succeeded in obtaining fertilized eggs and larvae of the Japanese eel by hormone treatment, and preleptocephalus larvae were reared for 2 weeks (Yamauchi et al. 1976). Thereafter, many researchers have succeeded in obtaining eel larvae, but preleptocephalus larvae could not survive beyond the depletion of their yolk and oil droplet stores. Failures of production of the glass eel may be caused by incomplete techniques for inducing sexual maturation of female Japanese eels and incomplete rearing techniques of larvae. As mentioned in previous sections, basic information on oogenesis of female eels has remarkably progressed. Therefore, in this section, our recent researches on glass eel production, mainly on induction of sexual maturation, are mentioned.

4-1. Induction of vitellogenesis

Cultivated female eels are sexually immature and their GSI slightly increase around 1–2% in fall. Even
in female silver eels migrating down rivers for spawning in fall, GSI are 1–2% (Yamamoto et al. 1974a) and they have ovaries containing oocytes at the oil stage or oocytes at the primary yolk globule stage (Yamamoto et al. 1974b). New Zealand longfinned eel, Anguilla dieffenbachii, have ovaries containing oocytes at the mid-vitellogenic stage and their GSIs are about 7% (Lokman et al. 1998). However, cultivated and wild silver eels do not mature and ovulate under normal culture conditions (Yamamoto et al. 1974a; Dufour et al. 1988). Moreover, as most cultivated eels are male (Chiba et al. 1993), it is difficult to obtain a large number of females for experimental purposes. Feminization using estrogen may be of considerable advantage to promote research on induced maturation. Oral administration of E2 at a concentration of 10 mg/kg diet for 4 months during the juvenile stage (from 0.95 to 43.8 g in body weight) successfully induced feminization in most fish used. After cultivation for 2 and a half years, these cultivated females can be used as experimental animals for induction of maturation. Initially, they have ovaries containing oocytes at the oil stage. We examined the effects of rearing in seawater on induction of maturation in cultivated female eels (Kagawa et al. 1998a). GSI (1.5%) and oocyte diameter (approximately 200 µm) increased after 3 months in seawater. GSI values are similar to those observed in the silver forms of wild females (Yamamoto et al. 1974b). Moreover, some of them have oocytes at the primary yolk globule in their ovaries. These results suggest that rearing in seawater for 3 months induces vitellogenesis. However, vitellogenesis did not progress under prolonged rearing condition. Therefore, artificial hormonal treatments are required to induce sexual maturation of the eel.

Cultured female Japanese eels weighing approximately 300–500 g were obtained from a fish farm. After acclimation to seawater, they were kept without
feeding in 400 L indoor circulating tanks under a natural photoperiod at a water temperature of 20°C. To induce sexual maturation, they were intraperitoneally injected with SPE (20–30 mg/kg body weight) once a week. SPE was prepared by homogenizing salmon pituitary powder with 0.9% NaCl solution, followed by centrifugation at 9,700 g. The saline control group received a single osmotic pump containing only 0.9% NaCl. The implantation of osmotic pumps loaded with SPE induced vitellogenesis and increased GSI at 39–110 days. In comparison, pumps loaded with hCG inconsistently induced early vitellogenesis, while those loaded with GnRHa did not exhibit any stimulatory effect. Further, hCG induces spermatogenesis and spermiation in male eels (Ohta et al. 1996b). However, weekly injection of hormones, such as SPE and hCG, requires repetitive handling of the bloodstock and substantial labor, time and monitoring, resulting in stress and increased mortality of the fish.

In recent studies on drug delivery systems in fish, a variety of gonadotropin-releasing hormone analogue (GnRHa) delivery systems have been developed for sustained hormonal release. These include cholesterol pellets (Zohar and Mylonas 2001), microspheres prepared using copolymers of lactic and glycolic acids (Mylonas et al. 1993; Zohar et al. 1995), and nondegradable copolymers of ethylene and vinyl acetate (Mylonas et al. 1998; Zohar 1996), as well as other copolymer pellets (Hirose 1992; Matsuyama et al. 1995). These hormone delivery systems effectively induce oocyte maturation and ovulation or spermatogenesis in reproductively dysfunctional cultured fish (Zohar and Mylonas 2001). However, no reports have investigated the effects of long-term (over 1 month) sustained-release delivery systems of GTHs on sexual maturation in such fish. An osmotic pump is a delivery device for long-term administration of drugs and hormones. Osmotic pump (Osmotic Pump Type 2002; Alzet Osmotic Pumps Co., Cupertino, CA; diameter = 7 mm, length = 30 mm, reservoir volume = approximately 200 µl) can release a constant amount of hormones for a long period (Kagawa et al. 2009b). According to the manufacturer’s instruction manual, osmotic pumps can release 5 µl of a solution per day for approximately 45–50 days when the fish are maintained at a water temperature of 20°C. An osmotic pump was implanted into the peritoneal cavity of each eel after cutting open the abdomen with a fine scalpel, approximately 8 mm. The wound was not sutured, but it healed naturally within 2 weeks.

We first found that implantation of a single hCG-loaded osmotic pump induced vitellogenesis and increased GSI at 35–42 days post-implantation (Fig. 27). Thereafter, we examined the efficacy of osmotic pumps for inducing sexual maturation in female eels.

Female eels (mean body weight = 490 g, range = 405–665 g) were implanted with a single osmotic pump loaded with SPE (1.5 mg/day/fish) or hCG (75 IU/day/fish) or GnRHa (5.25 µg/day/fish). The saline control group received a single osmotic pump containing only 0.9% NaCl. The implantation of osmotic pumps loaded with SPE induced vitellogenesis and increased GSI at 39–110 days. In comparison, pumps loaded with hCG inconsistently induced early vitellogenesis, while those loaded with GnRHa did not exhibit any stimulatory effect (Figs. 28, 29). This study (Kagawa et al. 2013) demonstrates for the first time that implantation of osmotic pumps loaded with SPE stimulates vitellogenesis in sexually immature female Japanese eels, suggesting that implantation of these osmotic pumps loaded with protein hormones, instead of repeated injections of hormones, is a reliable sustained-release delivery system for inducing sexual maturation in fish.

4-2. Induction of oocyte maturation and ovulation

After oocytes complete vitellogenesis, oocyte maturation occurs before ovulation and is a prerequisite for successful fertilization: this consists of GVBD, resumption of meiosis, and oocyte cytoplasmic maturation (see Section 3). Most female eels complete vitellogenesis after repeated injection of SPE or implantation of SPE-loaded osmotic pumps, but their oocytes do not undergo final maturation and become overripe with...
oocyte cytoplasmic degeneration in response to further injections of SPE (Kagawa 2003).

The MIS (DHP) of a salmonid fish, amago salmon (Oncorhynchus rhodurus), was isolated for the first time in vertebrate species (Nagahama and Adachi 1985) and thereafter DHP has been found to be the most effective steroid in inducing oocyte maturation of several fish species, including Japanese eel (Yamauchi 1990).

In vitro administration of 17α-hydroxyprogesterone (precursor of DHP) or DHP into the incubation medium induced oocyte maturation of Japanese eel (Yamauchi and Yamamoto 1982). Thin layer chromatography also showed that 14C-labeled pregnenolone could be metabolized to 17α-hydroxyprogesterone and DHP in the follicles of eel (Yamauchi 1990) (see Section 3). These results suggest that DHP can be produced in the ovary and is therefore a candidate as the MIS in Japanese eel. Moreover, an injection of DHP (1 µg/g BW) to the post-vitellogenic females induced oocyte maturation and ovulation (Yamauchi 1990). These results prompted us to develop techniques for induction of maturation and ovulation in Japanese eel by using DHP.

In order to estimate the critical developmental stage of oocytes to induce oocyte maturation and ovulation with the injection of steroids, the effects of 17α-hydroxyprogesterone and DHP on oocyte maturation at various developmental stages were assessed by means of in vitro incubation techniques (Kagawa et al. 1995b). Oocytes undergo GVBD in response to both 17α-hydroxyprogesterone and DHP when oocytes reach over 700 µm in diameter and become increasingly sensitive to DHP over 800 µm in diameter, which have characteristics of the migratory nucleus (Table 1). The peripheral area of oocytes becomes transparent when oocytes reach over 700 µm in diameter. These results suggest that oocytes at the migratory nucleus stage acquire the ability to respond to MIS (maturational competence, see Section 3). In eels, maturational competence and ability to produce MIS may be acquired by the injection of SPE when oocytes become larger than about 700 µm in diameter. However, the process of production of MIS is lacking in the ovarian follicle of the eel, since DHP can be produced if precursor steroids were added to the incubation medium (Yamauchi 1990). Therefore, further studies are necessary to clarify the mechanism of the precursor synthesis in the ovarian follicle of the eel.

Fig. 30. Photograph of an ovarian biopsy from a genital pore with polyethylene cannula. After anesthetizing the female eel, ovarian pieces were sacked using polyethylene cannula.

Fig. 31. Changes in ovulation rates after incubation with DHP in the Japanese eel. Dotted line: oocytes below 850 µm in diameter. Solid line: oocytes over 850 µm in diameter.

Table 1. Effects of 17,20β-dihydroxy-4-pregnen-3-one on germinal vesicle breakdown in oocytes of the Japanese eel. Reprinted with permission from Fish. Sci., 61, Kagawa et al., In vitro effects of 17α-hydroxyprogesterone and 17α,20β-dihydroxy-4-pregnen-3-one on final maturation of oocytes at various developmental stages in artificially matured Japanese eel Anguilla japonica, 1012–1015, Table 3, © 1995, The Japanese Society of Fisheries Science.

<table>
<thead>
<tr>
<th>Oocyte diameter (µm)</th>
<th>Percentage of GVBD: Concentration of steroid (ng/ml)</th>
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<tbody>
<tr>
<td>600–700</td>
<td>0: 0 100: 10 1: 1 0</td>
</tr>
<tr>
<td>700–800</td>
<td>84.0 ± 11.6: 68.1 ± 11.6 0: 0</td>
</tr>
<tr>
<td>800–900</td>
<td>100: 100 100: 30.4 ± 8.9 0</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SE.
eter and the developmental stage of oocyte can be utilized as a reliable indicator of the ability to induce maturation by injection of DHP. Using these parameters, we verified the effects of DHP injection on inducing maturation and ovulation in vivo (Ohta et al. 1996a). Oocyte diameter and maturity stage were determined by taking oocytes from the genital pore with a polyethylene cannula from the females (Fig. 30). Females that possessed oocytes of over 750 µm in diameter at the migratory nucleus stage were injected with SPE (20 mg/fish). Thereafter, female eels that possessed oocytes of over 850 µm in diameter were injected with SPE as a priming dose followed 24 hr later by injection of DHP (2 µg/g body weight) intraperitonially. Injection of DHP successfully induced oocyte maturation and ovulation in almost all females used in the experiments (Ohta et al. 1996a, 1997). The ovulation rates obtained in our experiments are much higher than those reported in the previous studies (Satoh et al. 1992). Therefore, DHP injection after SPE is a very useful method for induction of the last phase of oocyte maturation and ovulation in Japanese eel.

The advantage of the method for induced maturation by using DHP is that the ovulation occurs in a short but well-defined period of time (Kagawa et al. 1997). Ovulation occurs 15 to 21 hrs after DHP injection.

Table 2. Ovulation time of the Japanese eel injected with 17,20β-dihydroxy-4-pregnen-3-one at 9:00 or 18:00. Reprinted with permission from Fish. Sci., 63, Kagawa et al., Induced ovulation by injection of 17,20β-dihydroxy-4-pregnen-3-one in the artificially matured Japanese eel, with special reference to ovulation time, 365–367, Table 1, © 1997, The Japanese Society of Fisheries Science.

<table>
<thead>
<tr>
<th>Injection time</th>
<th>No. of fish used</th>
<th>Ovulation time (Time after injection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9:00</td>
<td>15</td>
<td>0  10  5*</td>
</tr>
<tr>
<td>18:00</td>
<td>18</td>
<td>0  0  4  12  2</td>
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</tbody>
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*Number of fish ovulated.
About 60% females ovulated 18 hrs and the remaining females ovulated 15 or 21 hrs after DHP injection (Table 2). Moreover, even if the time of DHP injection was changed from 9:00 to 18:00, a majority of the females ovulated 18 hrs after DHP injection. Therefore, a shift in the time of DHP injection can change the time of ovulation, indicating that the possibility that ovulation can be induced at a desired time by correctly timing of DHP injection.

Fertilization and hatching rates of our methods (Kagawa et al. 1997) are relatively high. Fertility and hatching rates of females ovulating 18 hrs after DHP injection are about 60% and 50%, respectively, although those of females ovulating 18 or 21 hrs after DHP injection are fewer than 30 and 20%, respectively. The reason for high fertility and hatching rates in the females ovulating earlier was not clear at the time. We thought that further studies must be carried out to develop the methods for obtaining females, which consistently ovulated in the shortest possible time after DHP injection, since shortening the time of ovulation may improve the quality of eggs. Further experiments showed that DHP-induced in vitro ovulation occurred earlier in oocytes over 850 µm in diameter than in smaller oocytes below 850 µm in diameter (Fig. 31). Moreover, relatively high incubation temperature accelerated DHP-induced in vitro ovulation in our experiments. From these data, we improved the methods for induction of oocyte maturation and ovulation as shown below.

In addition to ovulation, we have newly obtained evidence about maturational competence of oocytes and DHP production in the ovarian follicles. During oocyte maturation phase, fully grown oocytes acquire ability to respond to the MIS (maturational competence). SPE (or DHP) did not induce GVBD and ovulation in vitro in fully grown oocytes at the tertiary yolk globule stage below 700 µm in diameter. One day after SPE injection, oocytes at the migratory nucleus stage, approximately 800 µm in diameter, underwent GVBD and ovulation in vitro in response to SPE and DHP (Kumamaru and Kagawa 2008). Oocytes became increasingly sensitive to SPE in association with the increase of oocyte diameter when fish were injected with SPE every day, but oocytes obtained from female eels treated with single SPE-injection became less sensitive to SPE and DHP later on. Moreover, pre-incubation with SPE and subsequent incubation with DHP induced GVBD. These results indicate that oocytes acquire the ability to respond to DHP at the migratory nucleus stage over 800 µm in diameter and, furthermore, SPE has an essential role for initiation and maintenance of the ability of oocytes to respond to GTH and DHP.

After acquisition of maturational competence, oocytes become sensitive to SPE or DHP in association with the continuous increase of oocyte diameter by hydration. Recently, we found that SPE did not stimulate in vitro DHP production of ovarian follicles at the tertiary yolk globule stage between 600–700 µm in diameter. However, in vitro DHP production was stimulated by SPE in ovarian follicles over 800 µm in diameter and even the production became higher in ovarian follicles with 900 µm in diameter (unpublished data). These results indicate that ovarian follicles acquire the potency to produce MIS (DHP) at the migratory nucleus stage over 800 µm, more specifically at 900 µm in diameter.

New methods for artificial induction of spawning have been developed from the basic information obtained by in vitro and in vivo experiments described above. Proposed methods are shown in Fig. 32. Eels having ovaries containing full-grown oocytes (approximately 700–750 µm in diameter) were injected with SPE to induce oocyte hydration and maturational competence. Eels possessing competent oocytes (850–900 µm) were injected again (a priming dose) with SPE to induce and maintain maturational competence and ability to respond to GTH. Final treatments of GnRHa in combination with SPE given 24 hr after SPE-priming dose to female eels (900–950 µm in diameter) succeeded in induction of spawning in a rearing tank with spermiated male eels injected with hCG. Average fertility and hatchability are approximately 80 and 70%, respectively.

5. Future direction of studies on oogenesis

As mentioned in this monograph, many lines of information have been provided since we started a series of studies. However, we are still far from full understanding of the dynamic process associated with the oogenesis, from oogonia proliferation to ovulation. Recently, most of the research efforts were focused on endocrine regulation and optimizing rearing protocols to obtain good quality eggs. Since teleosts are good experimental animals for studying the regulatory mechanisms of oogenesis, further studies on oocyte growth and oocyte maturation will provide valuable information contributing to development of techniques for obtaining good quality eggs.

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