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Morphological Changes and Competence of Maturing Oocytes in the Protandrous Black Porgy, *Acanthopagrus schlegeli*

Wen-Shiun Yueh and Ching-Fong Chang*

Department of Aquaculture, National Taiwan Ocean University, Keelung, Taiwan 202, R.O.C.

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Wen-Shiun Yueh and Ching-Fong Chang (2000) Morphological changes and competence of maturing oocytes in the protandrous black porgy, *Acanthopagrus schlegeli*. *Zoological Studies* **39**(2): 114-122. The study objectives aimed to investigate the morphological changes of oocytes during final oocyte maturation and ovulation, and the maturational competence of oocytes to respond to maturation-inducing steroids in the protandrous black porgy, *Acanthopagrus schlegeli*. Females were injected with 2 successive doses of LHRH analog (10 and 50 μg per fish) on days 0 and 1. The oocytes were obtained at 6- or 3-h intervals from the genital pore after treatments. Morphological changes during oocyte maturation and ovulation were observed, and oocyte diameters in in vitro culture (with or without hormones) were measured. Oocyte maturation and ovulation occurred at 20-28 h and 36-60 h after the 2nd LHRH-A injection, respectively. It took 4-8 h to complete the process of oocyte maturation (in vivo) and 40 min for ovulation (in vitro). Hormones (17,20β-dihydroxy-4-pregnen-3-one, 17,20β,21-trihydroxy-4-pregnen-3-one, and HCG) could significantly induce oocyte maturation in oocytes collected at 9 and 6 h prior to the occurrence of germinal vesicle breakdown (GVBD). Oocyte maturation spontaneously occurred in oocytes collected at 3 h prior to GVBD. HCG showed no synergistic effects with steroids in the induction of oocyte maturation.

Key words: Black porgy, Maturation-inducing steroid, Oocyte culture, Oocyte growth, Oocyte maturation.

Fish oocyte development can be divided into oocyte growth and oocyte maturation. Vitellogenesis plays an important role in oocyte growth (from the previtellogenic oocyte to the vitellogenic oocyte up to the tertiary yolk globule stage). Germinal vesicle migration and breakdown (GVBD), coalescence of lipid droplets and yolk globules, and release of the 1st polar body are the characteristic events in the process of maturation (Nagahama et al. 1983). Plasma gonadotropin II increases during the maturation stages in fish and induces maturational competence in oocytes which react with maturation-inducing steroid (Goetz 1983, Patino and Thomas 1990, Kagawa et al. 1994).

Black porgy, *Acanthopagrus schlegeli*, is a protandrous hermaphrodite, whose sex naturally changes from male to female at the age of 3 yr or older (Chang et al. 1994 1997). It has a multiple spawning pattern, with the spawning period lasting from late winter to early spring (January to April). Vitellogenesis occurs from October until around December (Chang and Yueh 1990). The plasma sex steroids and gonadal histology during oocyte growth have been reported elsewhere (Chang and Yueh 1990, Chang et al. 1995). The oocyte maturational process is still not well known in black porgy. 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) and 17,20β, 21-trihydroxy-4-pregnen-3-one (20β-S) were shown to stimulate spermiation in male black porgy (Yueh and Chang 1997). The effects of various maturation-inducing steroids (MIS) on oocyte maturation in black porgy remain to be elucidated. Our objectives aimed to investigate the morphological changes in the process of oocyte maturation and ovulation, and the relationship of maturational competence of oocytes with the ability to react with MIS in black porgy.

*To whom correspondence and reprint requests should be addressed. Tel: 886-2-24622192 ext. 5209. Fax: 886-2-24621579. E-mail: b0044@mail.ntou.edu.tw

MATERIALS AND METHODS

Animals

One- and 2-yr-old male and bisexual black porgy, and 3-yr-old female black porgy (mean body weight, 500 g) were collected from an aquaculture farm.

Experimental design

One- and 2-yr-old males and bisexual fish, and 3-yr-old females, numbering 8, 8, and 6, respectively, were used for the measurement of oocyte diameter. Oocyte diameter (primary oocyte) was measured through the gonadal histology in 1- and 2-yr-old fish. Oocytes were collected from 3-yr-old female black porgy by a plastic tube cannulation through the genital pore and the diameters of the biggest oocytes were measured. Eight 3-yr-old female black porgy, 5 late vitellogenic/early maturing fish, and 3 mature fish were also selected to determine the distribution of oocyte size. The oocyte diameters of a total of about 3100 oocytes in each fish were measured for the experiment of oocyte distribution. Oocyte diameter and number were measured with light microscopy.

Sixteen female fish (with oocyte diameters between 0.5 and 0.55 mm, and with no mature oocytes found by cannulation) were selected for the experiment. Nine fish were injected with 10 μg per fish of synthetic LHRH-A (D-Ala^6, des-Gly^{10} LHRH ethylamide analog, Sigma) at 10:00 am on day 0, followed by injection of 50 μg per fish of LHRH-A at 10:00 am on day 1 (24 h later). Nine control fish were injected with saline. After the 2nd injection, the oocytes were examined at the 2- and 4-h intervals to determine the times of oocyte maturation and ovulation, respectively.

Another experiment with the same LHRH-A treatment in females was conducted to test the in vitro oocyte culture. After the 2nd LHRH-A treatment, oocytes were obtained at 6-h intervals for 12-h and then 3-h intervals by cannulation to monitor the stage of oocyte maturation. Oocytes collected from the genital pore in 5 LHRH-A-treated fish at 3-h intervals (until the observation of GVBD) were used for the in vitro oocyte maturation experiment (with and without further hormonal treatment). For a better understanding of the characteristics of oocytes in individual fish, the time course for the occurrence of GVBD in oocytes (in vitro culture) were transformed into intervals (3, 6, 9, 12, and 15 h) before the occurrence of GVBD. Oocyte reponsiveness to the hormones employed for oocyte culture was examined in oocytes collected from the genital pore at 9, 6, and 3 h prior to GVBD in LHRH-A-treated fish.

The maturation process (from the central germinal vesicle to germinal vesicle breakdown) in black porgy is divided into 1) the central germinal vesicle (CGV), 2) the 1st stage of germinal vesicle migration (the beginning of off-centered germinal vesicle, M I), 3) the 2nd stage of germinal vesicle migration (M II, germinal vesicle having migrated at least to half-way to the animal pole), and 4) germinal vesicle breakdown (GVBD). A transparent oocyte indicates that the oocyte has reached the GVBD stage in this study.

In vitro oocyte culture

Ovarian tissues were washed and placed in DME/F12 culture medium (Dulbeco's modified Eagle's medium and Ham's nutrient mixture F-12; 1:1, v/v) containing 15 mM Hepes, 100 IU/ml penicillin, and 100 μg/ml streptomycin; the pH of the culture medium was adjusted to 7.8 with sodium bicarbonate. Around 0.05 g ovarian tissue was cultured in 24-well plastic petri dishes with 1 ml culture medium. No difference in the percentage of GVBD was found in oocytes cultured with steroids for different periods (from 1 min to 24 h); therefore, oocyte incubation for 24 h was selected in this experiment. Different hormones or combinations of 20β-S (200 ng/ml; Steraloids Inc., Wilton, NH, USA), 17,20β-P (200 ng/ml; Steraloids), human chorionic gonadotropin (HCG, 100 IU/ml), 20β-S (200 ng/ml) + HCG (100 IU/ml), and 17,20β-P (200 ng/ml) + HCG (100 IU/ml), were conducted to induce oocyte maturation. Each treatment was run in quadruplicate. 20β-S and 17,20β-P are able to induce spermiation in male black porgy (Yueh and Chang 1997) and also in vitro oocyte maturation in female black porgy (unpubl. data). Therefore, these 2 hormones were selected for the present experiment. The dosage of the treatment was expected to be highly effective for the induction of oocyte maturation according to preliminary studies. The percentage of mature oocytes as compared to the total number of oocytes (at least 0.5 mm diameter) was calculated. Morphological changes of the ooplasm during oocyte maturation and ovulation were directly observed under light microscopy. The maturing oocytes were fixed in Serr's fluid (ethanol: formalin: acetic acid = 6:3:1, v/v/v) to examine germinal vesicle position.

Statistical analysis

All data are expressed as the mean with standard

error. Comparison of significant difference ($p < 0.05$) was made with Student's *t*-test and ANOVA followed with Duncan's multiple range test.

RESULTS

The process of oocyte growth and maturation

Only oogonia and primary oocytes, 50-90 μm in diameter, were observed in the 1- to 2-yr-old fish, which were still in the bisexual stage with dominant maleness (Fig. 1). In 3-yr-old females, oocytes developed to bigger primary oocytes and the yolk vesicle stage (previtellogenic growth phase), vitellogenic phase, and tertiary yolk globule stage (0.44 mm) in the months of June to September, September to December, and December to January, respectively (Fig. 1). The accumulation of cortical alveoli in the beginning was more concentrated in the central area of the cytoplasm (Fig. 2A), then yolk globules were evenly distributed in the peripheral area of the cytoplasm (Fig. 2B). Yolk globules further concentrated in the center of the ooplasm (Fig. 2C). Finally, oocytes completed the vitellogenic process with diameters of about 0.5 mm oocyte in January (Fig. 2D). The yolk globules made the oocytes appear opaque with the center of the germinal vesicle. Then, the oocytes underwent maturation and ovulation after January (Fig. 1).

GVBD and ovulation occurred at 20-28 h and 36-60 h after the 2nd LHRH-A injection (in vivo), respectively (Fig. 3). Control fish (without LHRH-A treatment) showed no sign of oocyte maturation, and oocyte size remained at 0.5 mm over the 4-d experimental period. The diameters of mature oocytes were 0.71 mm or even larger. It took at least 4-8 h to complete the processes of oocyte maturation (from the 1st stage of germinal vesicle migration to germinal vesicle breakdown) in vivo. Oocytes had a 0.57-mm diameter with larger yolk globules (due to coalescence) and a less dense appearance during the first 1-2 h of the maturational process (Fig. 4A) as compared to the vitellogenic oocytes (Fig. 2D). The germinal vesicle became slightly off-center at this stage (Fig. 4B). Oocyte diameters increased to 0.6-0.64 mm in 2-4 h, ooplasm became partially transparent, and the germinal vesicle migrated toward the animal pole. At 4-6 h, oocytes reached 0.62-0.65 mm in diameter, and became transparent with an oil droplet (Fig. 4C); the germinal vesicle continued to migrate further toward the animal pole (Fig. 4D). At the 6- or 8-h stage, oocytes had diameters of 0.64-0.80 mm and a single oil droplet with transparent ooplasm (Fig. 4E), while the germinal vesicle had broken down and disappeared (Fig. 4F). Larger mature oocytes (1 mm diameter) could also be ob-

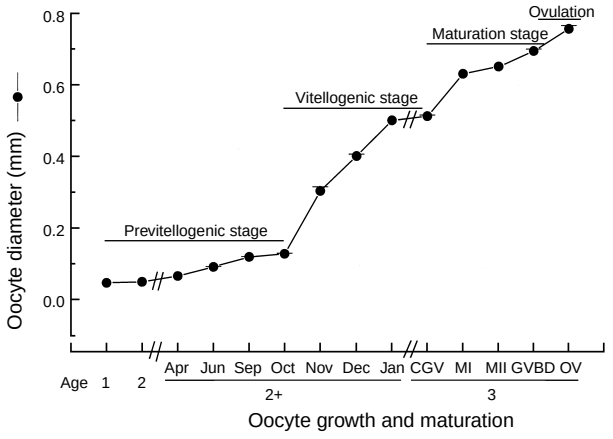

Fig. 1. Changes of oocyte diameter (mean ± standard error; 6-8 fish per value, 10 oocytes per fish) in the processes of oocyte growth and maturation in black porgy. CGV (central germinal vesicle), M I (the 1st stage of germinal vesicle migration), M II (the 2nd stage of germinal vesicle migration), GVBD (germinal vesicle breakdown), OV (ovulation). Values are expressed as the mean ± SE.

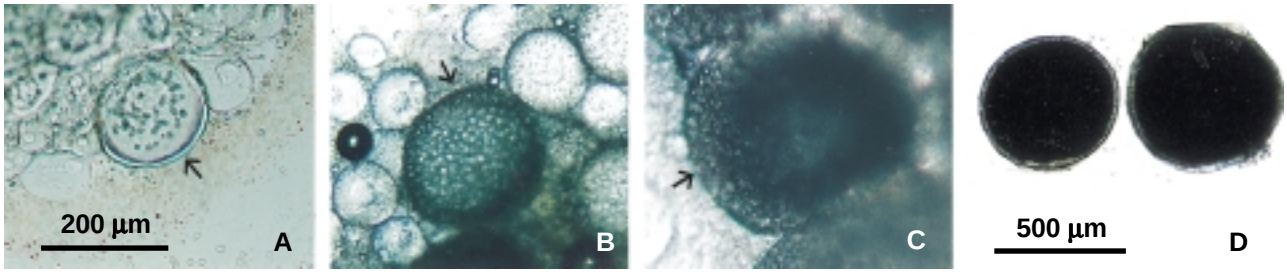

Fig. 2. Morphological changes of developing oocytes in 3-yr-old black porgy. In the beginning, more cortical alveoli have accumulated in the perinucleus area (A), then yolk globules are evenly distributed in the ooplasma (B). Further, yolk globules are more concentrated in the center of the ooplasma (C), and finally oocytes complete vitellogenesis (D). The scale of oocytes in A, B, and C is the same.

served (Fig. 5). The distribution of oocyte diameters differed between late vitellogenic/early maturing fish and mature fish as shown in figure 5.

Ovulation

After completion of maturation, oocytes underwent ovulation. It took about 40 min (in vitro) for the entire process of ovulation (from rupture of the follicle to extrusion of the oocyte). A slit appeared on the opposite site of the junction between the follicle and ovarian stroma (Fig. 6A). The oocyte was partially expelled when the slit expanded during the process of ovulation (Fig. 6B, C). The follicle layer ruptured, and the ovulated oocyte was barely attached to the ruptured follicle layer and stroma (Fig. 6D). Finally, the oocyte completely separated and was released from the ovarian tissue. The oocyte diameter did not vary during the course of ovulation (Fig. 6A-D).

Oocyte responsiveness to hormones

After in vitro culture (without hormonal treatment), oocyte transparency occurred in oocytes collected 3 h prior to GVBD in 2 fish (Fig. 7). Other fish (n = 3) had transparent oocytes collected as early as 6-9 h prior to GVBD, but the number of transparent oocytes significantly increased in oocytes collected 3 h before GVBD (Fig. 7).

17,20β-P, 20β-S, or HCG could not induce GVBD in vitro when the oocytes were collected 12 h prior to the occurrence of GVBD in fish. All the hormonal treatments except (20β-S + HCG) could significantly induce in vitro oocyte maturation at 9 h prior to the occurrence of GVBD in fish as compared to the control group (Fig. 8). All hormone-treated groups significantly responded to the respective hormone in vitro in oocytes collected 6 and 3 h prior to the occurrence of germinal vesicle breakdown in fish

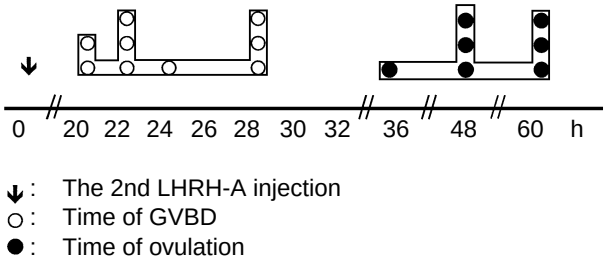

Fig. 3. Time of occurrence of oocyte maturation (germinal vesicle breakdown, GVBD (○) and ovulation (●) in black porgy after 2 injections of LHRH analog (10:00 am on days 0 and 1). Of 9 fish in the LHRH analog group, but 2 fish died. Therefore, ovulation data could not be collected.

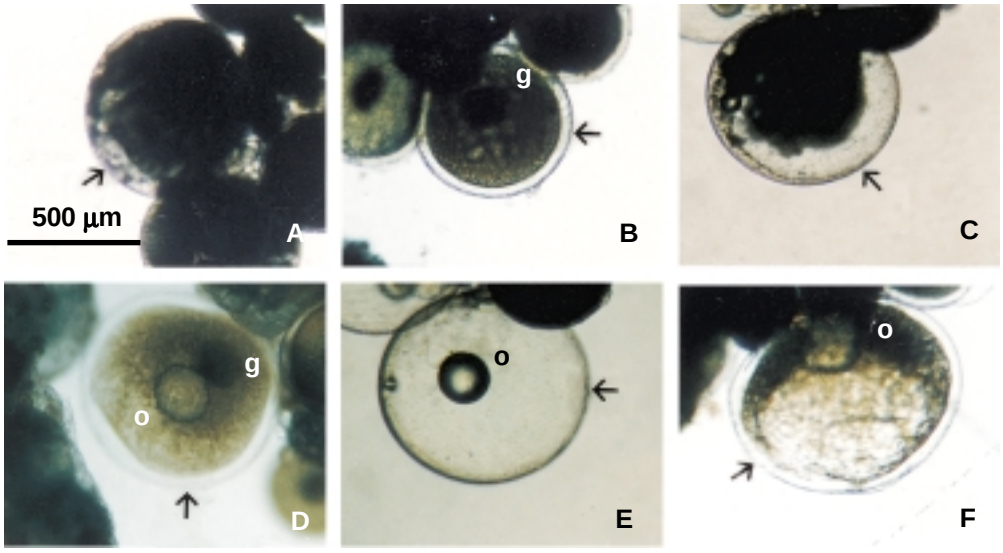

Fig. 4. Morphological changes of maturing oocytes in black porgy. 1) Larger yolk globules with less dense appearance in the cytoplasm during the first 1-2 h were observed in early maturing oocytes (A). The slightly off-center germinal vesicle was observed after the early maturing oocyte was fixed (B). 2) At the 4-6-h stage, the oocyte had transparent ooplasm, a clear oil droplet (C), and a germinal vesicle migrating toward the periphery (D). 3) At the 6-8-h stage, mature oocytes were noted with a single oil droplet (E) and the disappearance of germinal vesicles (F). The symbols, "g" and "o", represent germinal vesicle and oil droplet, respectively. The scale of oocytes in A-F is the same.

(Fig. 8). In the oocytes collected 9 h prior to the occurrence of GVBD, the effective order is HCG > 17,20β-P > 20β-S > 17,20β-P + HCG > 20β-S + HCG > control, in which the effectiveness of the first 4 treatments in inducing GVBD did not significantly differ at the 5% level. In oocytes collected 6 h prior to the occurrence of GVBD, the effective order is 17,20β-P > 17,20β-P + HCG > 20β-S + HCG > HCG > 20β-S > control, in which the effectiveness of the first 5 treatments did not significantly differ at the 5% level; in the 3-h oocytes, the order is 17,20β-P > 20β-S + HCG > 17,20β-P + HCG > 20β-S > HCG > control, in which the effectiveness of the first 4 treatments did not significantly differ at the 5% level (Fig. 8). The control oocytes collected from the 3-h period could undergo maturation with no further hormonal treatment (Fig. 8). Oocyte maturation in vitro could not be observed in the control (without hormonal treatment) oocytes collected 9 and 6 h prior to GVBD (Fig. 8).

According to the responsiveness to hormones, oocyte maturation could be classified into the following 3 stages (Fig. 9). Oocytes able to undergo maturation with no hormonal treatment are defined as the spontaneous stage (Fig. 9). Oocytes able to undergo maturation with exogenous steroid are defined as the steroid-sensitive stage (Fig. 9). The period in which exogenous gonadotropin failed to induce oocyte maturation in vitro was designated as "?" in figure 9.

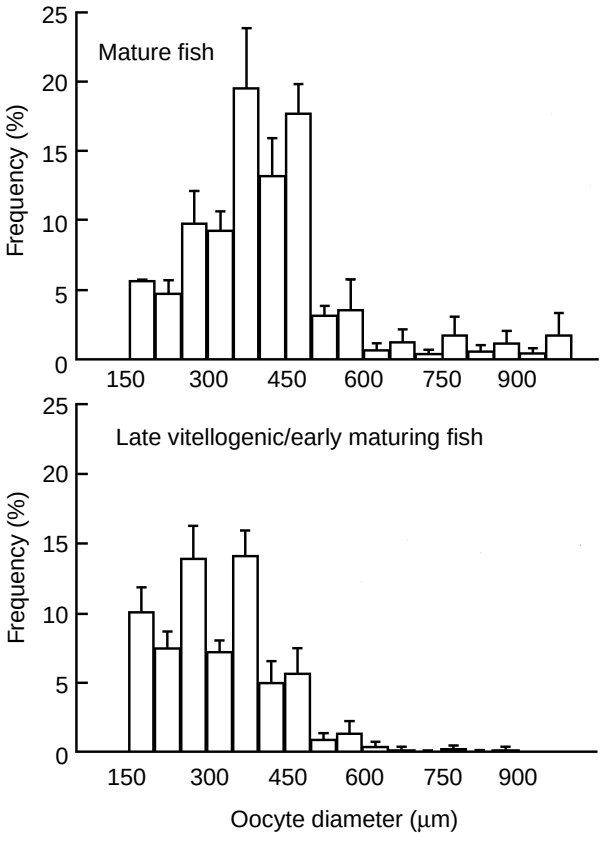

Fig. 5. Distribution frequency (%) of oocyte diameters in mature (n = 3) and late vitellogenic/early maturing (n = 5) 3-yr-old female black porgy. Oocyte diameters of a total of 3100 oocytes in each fish were measured. Oocytes smaller than 150 μm were not shown. Each histogram represents the percentage (mean ± SE) of oocyte diameter at 50-μm intervals.

DISCUSSION

The morphological changes of oocytes of black porgy during maturation are similar to those of other teleost fish (Adachi et al. 1988). Vitellogenic oocytes proceed through final maturation with coalescence of yolk globules and oil droplets. The oocyte diameter gradually increases and reaches the biggest size when a single oil droplet is located in the center and follicle layers just rupture. Hydration is considered to mainly cause the increase of oocyte diameter in other teleost fish (Wallace and Selman 1981, Oshiro and Hibiya 1981b 1982). Several events can result in increased osmotic pressure, thus inducing hydration, and therefore ovulation. Na^+/K^+-ATPase causes an increase of intra-cellular sodium levels (Greeley et al. 1991, LaFleur and Thomas 1991). Higher levels of free amino acid by proteolysis occur

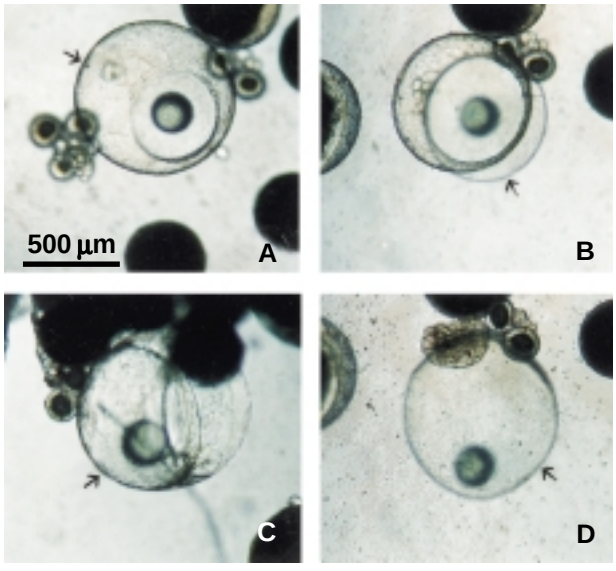

Fig. 6. Morphological changes of an ovulating oocyte in black porgy. A slit appears in the ovulating oocyte (A). Then the oocyte is partially expelled (B and C) and becomes fully ovulated with the attachment of the ruptured follicle layer (D). The scale of oocytes in A-D is the same.

during final oocyte maturation and contribute to the increase of osmotic pressure (Wallace and Selman 1985, Greeley et al. 1986, Matsubara et al. 1995). Coalescence of yolk globules also causes an increase of osmotic pressure (Oshiro and Hibya 1981a, Thorsen and Fyhn 1991). The coalescence of yolk globules and oil droplets first appears in the periphery of oocytes of black porgy, a process similar to that of red seabream *Pagrus major* (Adachi et al. 1988). But it first appears at the center of ooplasm in striped bass, *Morone saxatilis* (Mylonas et al. 1997).

Sizes of oocytes are evenly distributed in the range of oocyte diameters from 0 to 400 μm in the late vitellogenic black porgy. These profiles of oocyte distribution reflect the characteristcs of a multiple spawning pattern (as an asynchronous development) in black porgy. Oocytes of 350-500 μm in diameter become the major portion of oocytes in mature black porgy. In mature black porgy, only a small portion of vitellogenic oocytes is recruited and develop to maturation.

It took about 4-8 h to finish the process of oocyte maturation (from the 1st stage of germinal vesicle migration to germinal vesicle breakdown) in vivo on the basis of experimental observations. However, GVBD was not observed until 20-28 h after LHRH analog injection. It probably takes about 12-24 h to observe the responses of gonadotropins, MIS, and MIS receptors (the oocytes become competent) in black porgy prior to the occurrence of maturation. We also observed that it took about 40 min for the process of ovulation in vitro in black porgy. It seems that hydration and increase of oocyte size cause the elevation of intrafollicular pressure. The follicle layers were twisted because of the intrafollicular pressure, and eventually the follicle layers ruptured. Ovulation occurred 36-60 h after the 2nd LHRH-A injection. The time of ovulation might differ according to the maturity of fish and the dose of LHRH-A.

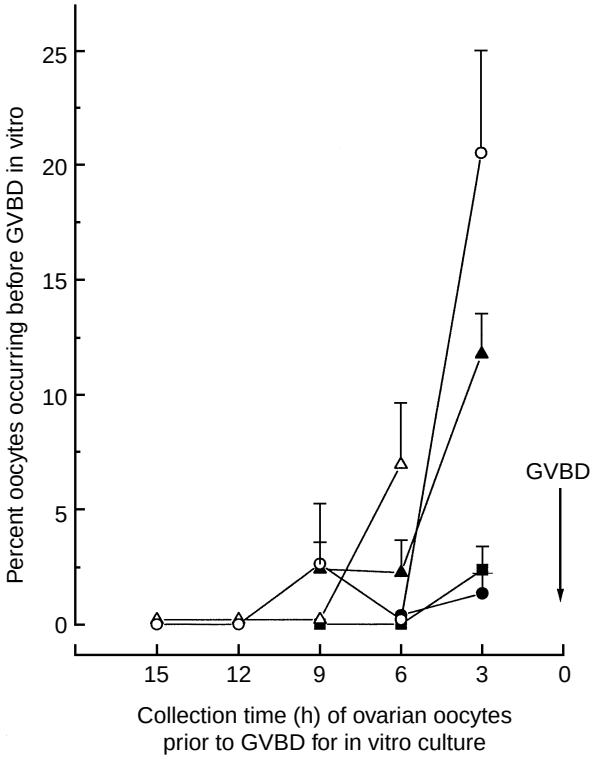

Fig. 7. Percentage of transparent oocytes (GVBD) in cultured oocytes (mean ± SE, n = 4) collected at 3-h intervals after LHRH analog treatment in black porgy (n = 5, in vivo). Symbols (○, △, ●, ▲, and ■) represent individual fish. The collected oocytes were cultured in F-12 medium with no hormones for 24 h (in vitro). Fish are indicated as being in the GVBD stage when a transparent oocyte was observed in the collected ovarian tissue. For comparison of various fish, the interval (h) before the occurrence of GVBD ("0" hour) is expressed on the X-axis.

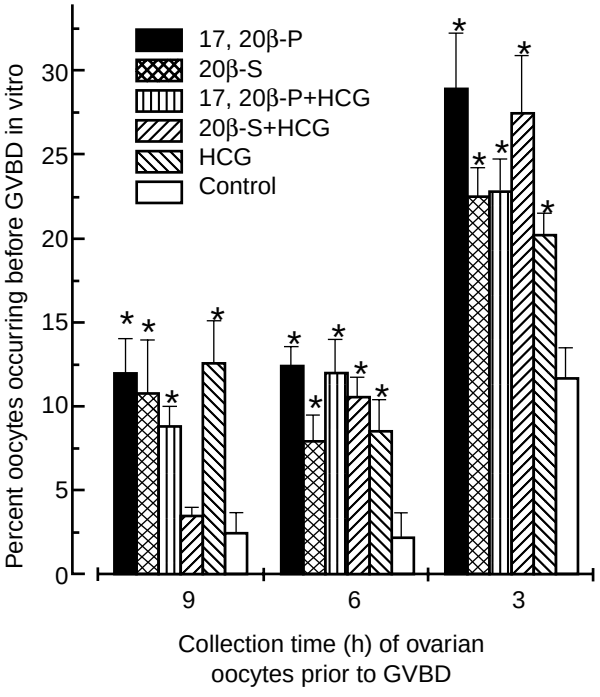

Fig. 8. Responsiveness (mean ± SE, n = 4 per value; the percentage of transparent oocytes, GVBD) of oocytes to different hormones in cultured oocytes (in vitro) collected with different time courses after LHRH analog treatment in black porgy. Fish are indicated as being in the GVBD stage when a transparent oocyte was observed in the collected ovarian tissue. An asterisk (*) indicates a significant difference as compared to the control group ($p < 0.05$). For comparison of various fish, intervals (3, 6, and 9 h) before the occurrence of GVBD ("0" hour) are expressed on the X-axis.

Oocytes could react to 17,20β-P, 20β-S, or to HCG in vitro during the period 9 h prior to GVBD. This indicates that the oocytes had already developed competence to begin final maturation during this period. The induction of MIS receptors by gonadotropin stimulation is considered to be an important process of maturation competence. However, 17, 20β-P, 20β-S, or HCG could not induce final oocyte maturation in vitro when the oocytes were collected from black porgy within 12 h after the 2nd LHRH-A treatment. This suggests that MIS receptors had not yet developed in the oocytes collected from this period. Oocyte maturation in other teleost fish can be divided into 2 phases: 1) MIS-insensitive phase, in which oocyte maturation can be induced by gonadotropin but not by MIS; and 2) MIS-sensitive phase, in which MIS alone can induce oocyte maturation. In black porgy, final maturation can be divided into 3 phases: 1) hormone-insensitive phase (0-12 h after the final injection of LHRH-A), in which oocytes do not respond to hormonal treatment (MIS or gonadotropin) for in vitro maturation; 2) MIS-sensitive phase (12 h after the 2nd injection of LHRH-A, or 3-9 h prior to GVBD), in which oocyte maturation can be induced by exogenous 17,20β-P or 20β-S; and 3) a spontaneous phase (3 h prior to GVBD), in which oocytes can undergo in vitro maturation even without hormonal treatment (Fig. 9). An MIS-insensitive phase (in which oocyte maturation can be induced by gonadotropin alone but not by MIS) in black porgy could not be identified in this experiment. The reason for failure to find a response to gonadotropin (MIS-insensitive phase) in vitro in black porgy is still not clear.

17,20β-P and 20β-S could induce oocyte maturation, but HCG did not have an additive effect on the steroids. 17,20β-P also had better stimulatory effects than did 20β-S. It is not clear whether either 17,20β-P or 20β-S is the physiological MIS in black porgy. Both steroids stimulated spermiation in 1-yr-old male black porgy during the spawning season (Yueh and Chang 1997). However, only 17,20β-P stimulated spermiation in male common carp (Yueh and Chang 1997). 17,20β-P was found to act as a MIS in salmonids and cyprinids (reviewed by Nagahama 1987, Scott and Canario 1987). 20β-S has been identified as an MIS in Atlantic croaker (*Microponias undulatus*) and spotted seatrout (*Cynoscion nebulosus*) (Trant et al. 1986, Trant and Thomas 1989a, b, Patino and Thomas 1990), belonging to the perciform family, Sciaenidae. Other studies also indicate that both 17,20β-P and 20β-S are effective in inducing final oocyte maturation in perciform species (striped bass, *Moxone saxatilis*; white perch, *M. americana*; white bass, *M. chrysops*; King et al. 1994a, b 1995). 17,20β-P and 20β-S were also proposed to be MIS factors in a multiple-spawning marine fish, tbinumeri-dragonet (*Repomucenus beniteguri*) (Asahina et al. 1991). 17,20β-P also induced final oocyte maturation in red seabream (*Pagrus major*) (Adachi et al. 1988, Kagawa et al. 1991). 20β-S is the likely MIS in gilthead seabream (*Sparus aurata*) (Canario et al. 1995). The present data show that 17,20β-P and 20β-S can stimulate final oocyte maturation in black porgy. Black porgy is classified as a Perciform, suborder Percoidei, family Sparidae. The physiological roles of either 20β-S or 17,20β-P in final oocyte maturation in black porgy are presently under investigation.

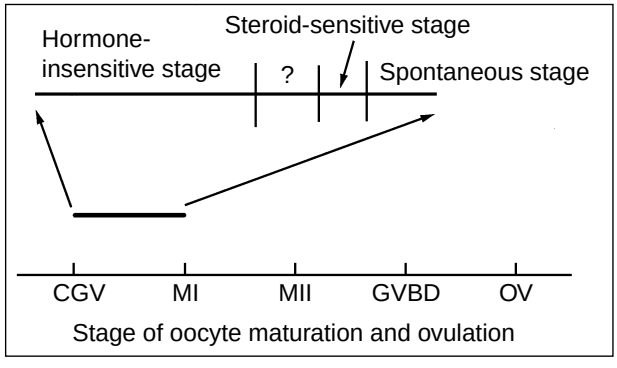

Fig. 9. Stages of oocyte maturation and ovulation in black porgy. Central germinal vesicle, CGV; the 1st stage of germinal vesicle migration, M I; the 2nd stage of germinal vesicle migration, M II; germinal vesicle breakdown, GVBD; ovulation, OV.

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黑鯛卵細胞發育及成熟之形態與反應特性

岳文勛[1]　張清風[1]

本研究之目的在探討黑鯛卵細胞發育與成熟之形態特徵及對促進成熟之類固醇(maturation-inducing steroid)之反應性。卵細胞發育可分為卵黃堆積前之成長期、卵黃堆積期、最後成熟與排卵，卵徑可由不到 0.1 mm (一年齡與二年齡) 發育至接近 1 mm。在卵黃堆積期之黑鯛卵巢所含有之卵徑均勻分布於 0-400 μm 之間，而剛進入成熟期之卵細胞卵徑主要是分布在 350-500 μm，另外有少部分卵的卵徑大於 600 μm。黑鯛在經過促性腺激素釋放素刺激後，約 20-28 小時可達卵細胞成熟， 36-60 小時可達排卵階段。以卵細胞培養條件下，約需 4-8 小時可完成卵細胞成熟過程，約需 40 分鐘完成排卵過程。兩種類固醇激素(17, 20β-dihydroxy-4-pregnen-3-one 與 17,20β,21-trihydroxy-4-pregnen-3-one) 皆可促進黑鯛卵細胞成熟。依卵細胞對激素的反應特性，可將黑鯛卵細胞成熟期區分為“對激素不感應期”、“對類固醇感應期”與“卵細胞可自然成熟期”。

關鍵詞：黑鯛，促進成熟之類固醇，卵細胞培養，卵細胞成長，卵細胞成熟。

[1] 國立台灣海洋大學水產養殖學系