Ovarian development and final oocyte maturation in cultured sevenband grouper *Epinephelus septemfasciatus*

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**ABSTRACT:** The histological changes in the ovary of sevenband grouper *Epinephelus septemfasciatus* were examined during vitellogenesis and final oocyte maturation. Ovaries contained oocytes at the perinucleolus stage at the start of the experiment in January. Oocytes at the primary yolk stage appeared when the gonadosomatic index (GSI) was slightly on the rise in March. After that, vitellogenesis progressed and ovaries contained oocytes at the tertiary yolk stage in May. The GSI increased drastically at the same time. These results suggest that vitellogenesis in sevenband grouper starts or is already in progress in March and is completed by May. In order to clarify the process of final oocyte maturation, luteinizing hormone-releasing hormone (LHRH) analog (des Gly10, [D-Ala6]LHRH ethylamide) was implanted in fish with oocytes at the tertiary yolk stage. The oocytes developed to the migratory nucleus stage in 18 h and reached the maturation stage in 42 h after implantation. This indicates that final oocyte maturation is completed within 42 h after stimulation of gonadotropin-releasing hormone.

**KEY WORDS:** estradiol-17β, final oocyte maturation, gonadosomatic index, LHRHa, ovarian development, sevenband grouper.

**INTRODUCTION**

Groupers (family Serranidae) are commercially important food fish in Asia. In Japan, kue *Epinephelus moara* and red spotted grouper *E. akaara* are cultured in floating net cages or tanks and their seeds are produced in artificial conditions. The sevenband grouper *E. septemfasciatus* is also a popular species for aquaculture in the south-west part of Japan. Nagasaki Prefectural Institute of Fisheries, Japan1 and Mie Prefectural Science and Technology Promotion Center, Japan2 are carrying out research on the maturation and spawning of this species to develop a technique for aquaculture. However, the technique for seed production is not well established, and yield is not stable. The available reproductive biological information on the sevenband grouper is also not sufficient; in particular, information on gonadal development such as the ovarian maturation process, reproductive cycle and hormone action on gonad is needed. Presently, the efficiency of seed production for red sea bream3 and Japanese flounder4 is greatly enhanced due to advances in the understanding of their reproductive physiology. It is therefore necessary to accumulate reproductive information to establish seed production in sevenband grouper.

In teleosts, gonadotropin (GtH) is the most important hormone in the regulation of gonadal development and maturation.5–7 Gonadotropin is synthesized in the pituitary gland and induces the synthesis of sex steroids in the gonad. In female fish, GtH induces estradiol-17β (E2), which in turn stimulates hepatic cells to synthesize vitellogenin. Vitellogenin is a precursor of yolk protein. It has also been found that GtH has an effect on final oocyte maturation via the synthesis of maturation-inducing steroid (MIS). Thus, GtH treatment is carried out in various fish species to induce maturation and ovulation in artificial seed production.8–10 Spontaneous maturation and ovulation in sevenband grouper have not been completed in artificial conditions. Treatment with GtH or gonadotropin-releasing hormone (GnRH), a hypothalamic hormone that stimulates GtH synthesis and release, is an effective method to induce maturation and ovulation in marbled grouper *E. microdon*, dusky grouper *E. marginatus*12 and Nassau grouper *E. striatus*.13–15 Effective use of this technique, however, requires information on the
reproductive cycle and gonadal development in the target species.

In the present study, the gonadal development of female sevenband grouper under rearing conditions was examined, based upon the changes in gonadosomatic index (GSI) and histological observation of oocyte development. Moreover, the process of final oocyte maturation was observed using GnRH-treated fish. In addition, the plasma E2 concentration was measured to determine the processes of endocrine changes during vitellogenesis.

MATERIALS AND METHODS

Experimental fish

Seven- and 8-year-old female sevenband grouper were produced in the Nagasaki Prefectural Institute of Fisheries. These fish were reared in a floating net cage (5 m × 5 m × 5 m) under natural photoperiod conditions at the institute. The water temperature was approximately 14°C from the middle of February to the middle of March (Fig. 1a). After that, there was a gradual rise in temperature until the middle of May (20°C). The fish were fed to satiation with moist pellets (mackerel : squid : raw krill : fish meal at a ratio of 1:1:1:3) three times a week. The total length of the female fish ranged from 53 to 70 cm, with an approximate weight of 3–6 kg.

Sampling procedure

Samples were collected every month from January to May, except for February. Six to nine female fish were anesthetized with 0.01% 2-phenoxyethanol and their total length, standard length and body weight were recorded. Blood samples were collected from the caudal vessel using a 22 G needle and heparinized syringe and transferred to a test tube. Plasma were separated by centrifugation at 1800×g for 15 min at 4°C, and stored at −30°C until use. The ovaries were removed from the body cavity and weighed, and the gonadosomatic indices (GSI = gonad weight × 100/body weight) were calculated.

Histological observation

Pieces of the ovaries were fixed in Bouin’s solution for 24–48 h, dehydrated through a series of graded alcohol concentrations and embedded in paraffin. Sections were cut at a thickness of 6–8 µm and were stained with Mayer’s hematoxylin and eosin. The cross-sections of ovaries were observed under a microscope (Olympus BX50, Tokyo, Japan).

Induction of ovulation by LHRHa for observation of the process of final oocyte maturation

Nine females at late vitellogenic stage (oocyte diameter >420 µm) were selected for implantation with luteinizing hormone-releasing hormone (LHRH) analog (des Gly10, [D-Ala6] LHRH ethylamide, LHRHa). The LHRHa (Sigma Chemical, MO, USA) was administered at a dose of 50 µg/kg body weight by implantation of a cholesterol pellet. Cholesterol pellet was prepared according to the method of Lee et al. The pellet weighed approximately 30 mg with an average length of 6 mm and a diameter of 2 mm. At 0 h, 18 h, 24 h and 42 h after implantation, each female fish was anesthetized with 0.01% 2-phenoxyethanol and intraovarian oocytes were sampled using medical tubing (SH No. 2, Kaneka Medix, Japan) with an inside
The diameter of 2 mm and an outside diameter of 3 mm. The tubing, connected with a 5 mL syringe, was inserted into the oviduct through the genital pore. Samples were sucked out through this tubing and fixed in Bouin’s solution for later histological examination.

**Measurement of plasma E\textsubscript{2} concentration**

Plasma concentrations of E\textsubscript{2} were measured by enzyme-linked immunosorbent assay according to the method of Asahina et al.\textsuperscript{17} The horseradish peroxidase-conjugated antigen and the antibody against E\textsubscript{2} were purchased from Cosmo Bio (Tokyo, Japan).

**Statistics**

All data are presented as mean ± standard errors (SEM). The data were analyzed by one-way factorial analysis of variance (ANOVA) followed by Fisher’s protected least significant difference (PLSD) using STATVIEW 5.0 for Macintosh (Abacus Concepts, Berkeley, CA, USA).

**RESULTS**

**Changes in gonadosomatic index**

Seasonal changes in GSI are shown in Fig. 1(b). The GSI was 0.2 ± 0.1 in January. Although the GSI tended to increase slightly in March, it remained low until April. A rapid increase in GSI was observed in May (5.2 ± 1.1) and the mean was significantly higher than that in other months ($P < 0.001$).

**Changes in plasma E\textsubscript{2} concentration**

No significant difference was observed in plasma E\textsubscript{2} concentrations in female fish during the experiment (Fig. 2). However, plasma E\textsubscript{2} tended to increase from January to March, then remained at a level of approximately 1.0–1.5 ng/mL until May.

**Changes in oocyte maturity**

According to the histological classification of oocytes, the process of oocyte development in female sevenband grouper is divided into seven stages: perinucleolus (pn), yolk vesicle (yv), primary yolk (py), secondary yolk (sy), tertiary yolk (ty), migratory nucleus (mn) and maturation (m).

Monthly changes in ovaries are shown in Fig. 3. The ovaries in January contained oocytes at the perinucleolus stage only (Fig. 3a). In March, oocytes at the yolk vesicle stage appeared in ovaries (Fig. 3b). Additionally, oocytes at the primary yolk stage were observed in some fish. By the end of April, ovaries of all female fish contained oocytes at the primary and the secondary yolk stages (Fig. 3c). The ovaries in the middle of May contained oocytes at the tertiary yolk stage, just prior to the migratory stage (Fig. 3d).

**Morphological changes in final oocyte maturation induced by LHRHa**

Oocytes at the migratory nucleus stage and the maturation stage were observed in the ovary of fish treated with LHRHa. The morphological changes in oocytes at 0 h, 18 h, 24 h and 42 h after LHRHa treatment are shown in Fig. 4. Ovulation occurred in all treated fish after 42 h of LHRHa implantation. Oocytes at the tertiary yolk stage at 0 h (initial time) did not change at 6 h after LHRHa treatment (data not shown). The oocytes gradually moved into final oocyte maturation and oocytes at the migratory nucleus stage were observed at 18 h and 24 h after the treatment (Fig. 4b,c). After 42 h, the oocytes reached the maturation stage, when the yolk globule became a complete single mass and the oil droplets aggregated (Fig. 4d). Oocytes at this stage underwent breakdown of germinal vesicle (GVBD).
DISCUSSION

We have described the histological changes in oocyte development and maturation of sevenband grouper in the present study. The ovaries comprised only oocytes at the perinucleolus stages in January, and the GSI was low. Vitellogenic oocytes appeared in the ovaries in March, and they developed rapidly from April to May, suggesting that vitellogenesis started in March and progressed actively between April and May. The GSI was high, reaching more than 5 in May, when ova-
ries contained oocytes at the tertiary yolk stage. These results indicate that spawning of sevenband grouper under rearing conditions could begin in May. Histological changes in the ovary of several species of groupers have been shown to correspond well with the changes in GSI. The GSI of red grouper *E. morio* collected from the central west Florida shelf, increased rapidly in March, and maturing oocytes appeared at the same time. Moreover, the GSI of honeycomb grouper *E. merra* increased from April through June, synchronizing with oocyte development. Although the vitellogenic and spawning seasons of groupers are different from species to species, the pattern of gonadal development and the composition of oocytes are similar in all species. The ovary of sevenband grouper contained perinucleolus-, yolk vesicle- and yolk-stage oocytes in the spawning season in May, suggesting that several batches of oocytes may be released during the spawning period. Our study demonstrates that oocyte development in sevenband grouper is group synchronous. This trend is also observed in red grouper, honeycomb grouper, and white grouper *E. aeneus*.

It has been accepted that the cyclical environmental changes such as photoperiod and water temperature mediate the annual cycle of gonadal development in teleosts. The initiation of gonadal development and maturation is strongly associated with water temperature in spring–summer-spawning fishes. In red grouper and honeycomb grouper, and golden rabbitfish *Siganus guttatus*, GSI increases and gonadal development starts when the water temperature is on the rise. In addition, there is evidence that the initiation of vitellogenesis in mudskipper *Periophthalmus modestus* is induced in artificial conditions by increasing environmental temperature. The vitellogenesis of sevenband grouper started in early spring, and proceeded during the spring, when the water temperature increased gradually. It is assumed that the increasing temperature has an important role in the initiation of vitellogenesis and oocyte development.

The importance of ovarian estrogens in the regulation of vitellogenesis has been demonstrated in many teleosts. Annual changes in plasma steroids were investigated in red grouper by Johnson *et al.* The levels of E2 (and testosterone, which is a precursor steroid of E2), increased rapidly in female fish having gonads with both yolk vesicle-stage oocytes and vitellogenic oocytes, suggesting that E2 has a critical role in the initiation and maintenance of vitellogenesis. In the present study, E2 levels tended to increase in female fish with both vitellogenic oocytes and yolk vesicle-stage oocytes just prior to the period of vitellogenic growth. It can readily be deduced that the synthesis of vitellogenin is induced in hepatic cells by E2, as in other teleosts. Utarabhand and Bunlipatanon reported that plasma vitellogenin levels are enhanced by E2 treatment in *E. malabaricus*. Our results suggest that E2 supports the onset of vitellogenesis and the development of oocytes in sevenband grouper.

The female fish of some serranid species do not readily spawn in captivity. In the present study, spontaneous oocyte maturation, ovulation and spawning did not occur completely, although oocytes in sevenband grouper developed to the tertiary yolk stage in the floating net cage. Thus, we induced the final oocyte maturation and ovulation by LHRHa treatment to observe these processes. According to histological observation, the migration of nucleus was confirmed at 18 h after the hormone treatment, and GVBD and aggregation of yolk globule and oil droplets were completed by 42 h after LHRHa treatment. Marino *et al.* reported that the ovulation of captive-reared dusky grouper can be induced using gonadotropin-releasing hormone analog. The morphological changes in oocytes during the final maturation agree with our results. These changes observed in groupers are almost the same as the general condition in marine teleosts. The response to GnRH and the mechanism of the final oocyte maturation seem to be common in serranid species. Further studies are required to elucidate in detail the effects of LHRHAs implantation on reproduction in sevenband grouper.

It is well known that GnRH induces production of MIS in ovarian follicles via secretion of GtH in female fish that have reached the final stages of vitellogenesis. *17α,20β*-dihydroxy-4-pregnen-3-one (17α,20β-DHP) and *17α,20β*,21-trihydroxy-4-pregnen-3-one (20β-S) are known as MIS in teleosts. Unfortunately, the MIS has not been identified in serranid species. In red grouper, *17α,20β*-DHP and 20β-S were measured in female fish at various stages of oocyte development. Although no significant increase in plasma levels of either MIS was observed in female fish during the phase of final oocyte maturation, our results suggest that MIS synthesis in ovarian follicles and the expression of its action in oocytes were induced in sevenband grouper within 42 h after the LHRHa treatment.

In summary, vitellogenesis of sevenband grouper starts in early spring and the spawning season begins in May in the south-west part of Japan. In addition, LHRHa administration is effective in inducing the final oocyte maturation and ovulation in female sevenband grouper with tertiary yolk-stage oocytes. These results provide important information for the development of artificial seed
production in this species reared in floating net cages.

ACKNOWLEDGMENTS

We thank all the members of the Nagasaki Prefectural Institute of Fisheries for their kind cooperation with this research. This work was supported in part by a grant from the Prefectural Collaboration of Regional Entities for the Advancement of Technological Excellence, JST.

REFERENCES


