# Gonadal Sex Differentiation of Hatchery-Reared Longtooth Grouper (*Epinephelus bruneus*)

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**ABSTRACT** : For the gonadal sex management of younger longtooth grouper (*Epinephelus bruneus*), this work investigated the timing and histological process of ovary differentiation and oocyte development of longtooth grouper larvae and juvenile. Specimens (from 1 to 365 DAH) were collected for gonadal histological study from June 2008 to August 2009. Rearing water temperature was ranged from 20 to 24℃. The primordial germ cells could be observed from 10 to 15 DAH, while undifferentiated gonad occurs from 20 to 50 DAH in longtooth grouper. The initial ovarian phase was 60 to 110 DAH with the formation of ovarian cavity and the increased in size of gonad. The ovarian phase started at 140 DAH with appearance of oogonia. The gonad at 365 DAH appeared to have full of oogonia and primary growth stage oocyte. Formation of ovarian cavity indicates that the ovarian differentiation beginning at 60 DAH in longtooth grouper. The gonads in longtooth grouper differentiated directly into ovaries in all fish examined.

**Key words** : Sex differentiation, Ovarian cavity, Larvae, Juvenile, Oocyte development, Longtooth grouper

## INTRODUCTION

Most grouper species are protogynous hermaphrodites, the fish first play as females and then late transform into males when they have reached a larger size and after first maturation (Sadovy & Colin, 1995; Bhandari et al., 2003). Difference in maturation age of the females and males is considered as major constraint that delays aquaculture development of these fish species. (Lee et al., 2008; Sadovy de Mitcheson & Liu, 2008). It is difficult to collect sufficient number of males from the wild or culture for artificial propagation. The testes exhibit the morphology through the sex change in most of grouper was an ovarian structure, with a lumen and sperm sinuses within gonadal walls (Sadovy & Colin, 1995; Liu & Sadovy, 2004; Alam & Nakamura, 2007; Sadovy & Liu, 2008; Liu & Sadovy, 2009; Lee et al., 2010). This indicates that male can develop directly from juvenile or female through sex change (Liu & Sadovy, 2004; Alam & Nakamura, 2007; Sadovy & Liu, 2008; Liu & Sadovy, 2009; Murata et al., 2009).

Longtooth grouper (*Epinephelus bruneus*), a coral-reef fish species, is recognized as one of the most commercially valued fish in Jeju, Korea. The artificial seed production technique was investigated since 1993 (Lee et al., 2008), and seed production has been established since 2005 (Song et al., 2005; Oh, 2006). The sex change from female to male begins at body size of 3-5 kg and larger number of males frequently was found at body size of 5-8 kg (Oh, 2006). Recent attempts have been done to induce sex maturation and produce fertilized egg, which can provide to improving artificial propagation of this species.

Differentiation and development of gonads is important approach for precisely understanding of the mechanism of sex differentiation (Nakamura et al., 1998), and provides guidance for determining the hormone sensitive period of fish (Strüssmann et al., 1996).

The aims of this study were to investigate the timing of ovary differentiation and oocyte development of longtooth grouper larvae and juvenile.

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## MATERIALS AND METHODS

### 1. Fish Rearing

Larvae from 1 to 90 days after hatching (DAH) were obtained from a private hatchery in Hamdoek, Jeju, Korea and transported to Marine and Environment Research Institute, Jeju National University. From September to November, 500 fish were reared in 3,000 L square tank for determine ovary differentiation. Filtered seawater was continuously provided in each tank. Water temperature was ranged from 20 to 24℃. During winter season (from December 2008 to March 2009), fish were reared in 500 L tanks in a recirculation system. Water temperature maintained at 22℃ using heaters, water flow rate was set at 10 L $min^{-1}$ and water was exchanged every 2 week. Fish were fed twice a day with commercial pellets at a daily ration of 3% body weight.

### 2. Fish Collection

Fish (from 1 to 365 DAH) were collected for gonadal histological study from June 2008 to August 2009. Fifteen to twenty fish were collected every day from day 1 to 10. Ten fish were collected every 5 days between day 10 to day 60, every 10 day from day 90 to day 140, and monthly from day 180 to day 365.

### 3. Histological Observation of Sex Differentiation

After measuring body length and weight, larvae and juvenile were fixed in Bouin's solution for 24 h, dehydrated in a graded series ethanol and embedded in paraffin. The paraffin embedded specimens were sectioned in 3 to 5 μm thickness using a rotary microtome. Slides were stained with Hansen's hematoxylin and eosin and observed on light microscope (AX70 Shop, N7COMP 370, Carl Zeiss, Germany). The germ cell and gonad diameters were measured to calculate of surface area. Gonad and germ cell surface area was calculated according to the equation described by Uguz (2008).

$$\text{Surface area } (\mu m^2) = (\text{width} \times \text{length} \times 3.14) / 4$$

### 4. Gonadal Development Phase

Gonads of fish were classified into three categories including undifferentiated-phase (UP), differentiation-phase (DP), and ovarian-phase (OP) (Table 1).

## RESULTS

### 1. Biological Parameters

During undifferentiated gonad phase (from 5 days after hatching (DAH) to 50 DAH), body weight and total length of longtooth grouper larvae ranged from 0.001 to 0.2786 g and 2.9 to 27.4 mm respectively. In initial ovarian phase (from 60 DAH to 130 DAH), body weight ranged from 0.538 to 14.908 g with the total length was 35.5 to 101.1 mm. In the ovarian gonad phase (from 140 DAH to 365 DAH), body weight and total length ranged from 16.30 to 98.47 g and 105.5 to 197.1 mm, respectively (Fig. 1).

**Table 1. Gonadal development phase in longtooth grouper**

| Gonadal phase | Gonadal development | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | PGCs | BV | GC | OC | Og | O1 | O2 | SC |
| UP | + | + | + | − | − | − | − | − |
| DP | − | + | + | + | − | − | − | − |
| Op | − | + | + | + | + | + | + | − |

For gonadal phase: UP, Undifferentiated gonad phase; DP, Differentiation phase; OP, Ovarian phase

For gonadal morphology: PGC, primordial germ cells; BV, blood vessels; GC, germ cell ; OC, ovarian cavity; Og, oogonia; O1, primary-growth stage oocyte; O2, cortical-alveolus stage oocyte; SC, spermatogenic cyst.

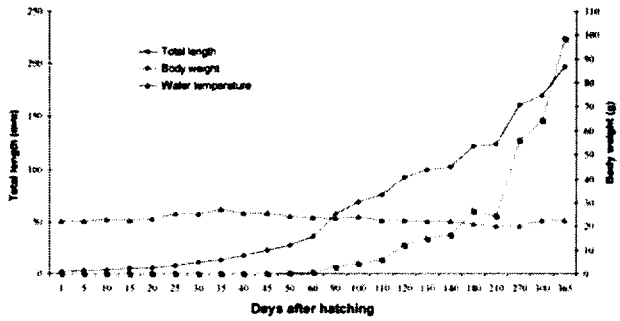


**Fig. 1. The growth of total length and body weight of longtooth grouper in indoor rearing condition.**

## 2. Gonadal Differentiation and Development

### 1) Undifferentiated Gonad Phase

The primordial germ cell (PGC) was observed in mesentery under the mesonephric duct at 10 DAH (Fig. 2A, B), in sagittal section. At 15 DAH, PGCs with a large nucleus size were located germinal epithelium line adjacent hindgut. At this stage, PGC was marked border between the cytoplasm and the nucleus, which was intensively stained by hematoxylin (Fig. 2C). The diameter of PGCs ranged from 11.7 to 25.2 $\mu m^2$ at 10 and 15 DAH. At 20 to 30

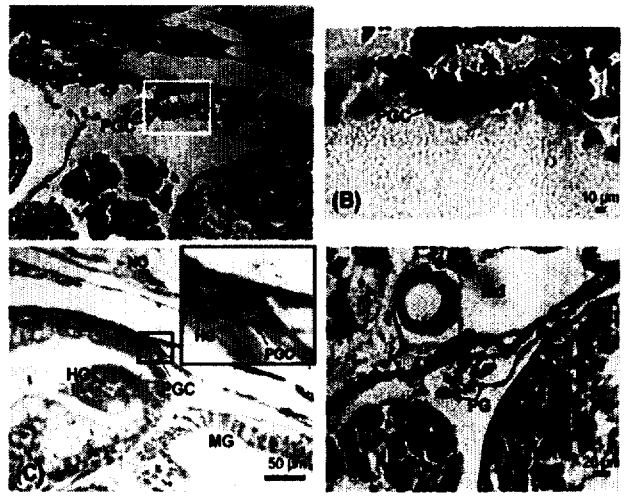


**Fig. 2. Gonads of larvae longtooth grouper at 10 to 20 DAH. (A).** Sagittal section show primordial germ cell located in mesentery between mesonephric duct and intestine at 10 DAH; (B). High magnification of primordial germ cell of picture A; (C). Sagittal section at 15 DAH showed primordial germ cell located germinal epithelium line at anterior part of the body cavity; (D). Cross section gonad at 20 DAH showed pair primordial gonad with a few somatic cell below mesonephric duct. G, gut; Gd, gut duct; HG, hindgut; MG, midgut; Md, mesonephric duct; NO, notochord; Pa, pancreatic; PG, primordial gonad; PGCs, primordial germ cells.

**Table 2. Summary of observation on gonad development during sex differentiation of longtooth grouper at 15 to 60 DAH**

| Age (DAH) | Body length (mm) | Sex | | | | Histological characteristics of gonads |
|---|---|---|---|---|---|---|
| | | U | D | O | | |
| | | | | Og | O1 | |
| 10 | 3.7 ± 0.5 | 10 | - | - | - | PGCs were located above hindgut with a large nucleus size. PGC was marked border between the cytoplasm and the nucleus, which was intensively stained by hematoxylin. |
| 15 | 4.9 ± 0.3 | 10 | - | - | - | |
| 20 | 5.7 ± 1.0 | 10 | - | - | - | The pair primordial gonads are present in the posterior region of the abdominal cavity, immediately below the mesonephric duct with a few somatic cells and the PG increased the size with age. The blood vessel was first occurred in primordial gonad at thirty five days after hatching. |
| 25 | 8.0 ± 0.7 | 10 | - | - | - | |
| 30 | 11.6 ± 1.0 | 10 | - | - | - | |
| 35 | 13.3 ± 1.4 | 10 | - | - | - | |
| 40 | 17.7 ± 2.0 | 10 | - | - | - | The germ cells were appeared with a large nuclei, which were stained by hematoxylin. |
| 45 | 22.3 ± 1.9 | 10 | - | - | - | |
| 50 | 27.4 ± 2.4 | 10 | - | - | - | |
| 60 | 35.5 ± 3.1 | 6 | 4 | - | - | The initial ovarian cavity formation which was indicated by the presence of two elongated aggregations of somatic cells in the gonad. |

Note: U, undifferentiated gonad; D, differentiation gonad; O, ovarian; Og, oogonia; O1, primary growth stage oocyte.

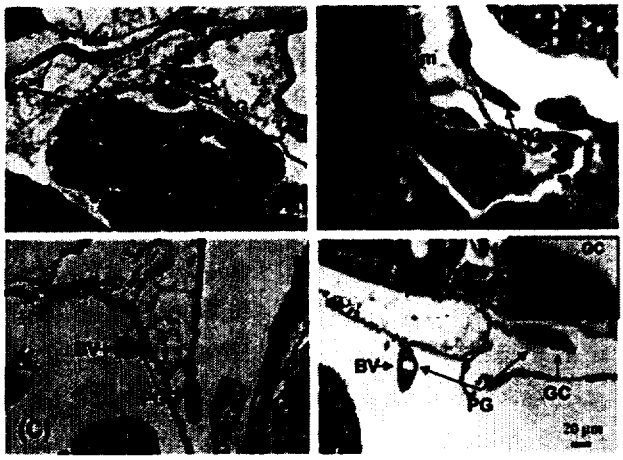


**Fig. 3. Gonadal development of longtooth grouper at 25 to 40 DAH (A).** Cross section gonad at 25 DAH showed primordial gonad with somatic cells located under mesospheric duct (pancreas); (B). Cross section primordial gonad at 30 DAH located between liver and intestine (pancreas); (C). Cross section primordial gonad at 35 DAH located adjacent gut with appeared of blood vessel; (D). Cross section gonad at 40 DAH showed primordial gonad with germ cells surround by somatic cells. BV, blood vessel; G, gut; GC, germ cell; m, mesentery; Md, mesospheric duct; PG, primordial gonad; Pa, pancreatic.

DAH, primordial gonad with a few somatic cell appeared below the mesonephric duct at either side of the mesentery in the posterior portion of the body cavity (Fig. 2D; 3A, B). The cross section surface area of primordial gonads at this stage ranged from 87.0 to 176.6 $\mu m^2$ (Fig. 8). The blood vessel was observed in the primordial gonads at 35 DAH (Fig. 3C). The average of primordial gonad surface at this stage was 202.4 ± 31.4 $\mu m^2$. At 40 DAH, the germ cells were distinguished from the somatic cells by their larger size. Nuclei of the germ cells were stained by hematoxylin more intensively than the cytoplasm (Fig. 3D). The primordial gonad increased in size with surface area of 357.5 ± 123.1 $\mu m^2$ (Fig. 8). At 45 DAH, the germ cells showed nuclei containing dispersed chromatin in the form of an irregular meshwork, which numerous small chromatin masses were suspended (Fig. 4A). One individual showed relatively large gonads with the surface area of 588.8 and 785.0 $\mu m^2$ while the average size was 543.8

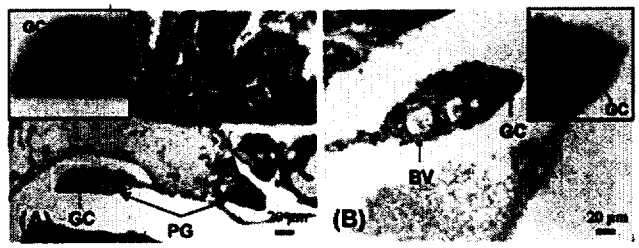


**Fig. 4. Gonadal development of longtooth grouper at 45 to 50 DAH.** (A). Cross section with undifferentiated gonad at 45 DAH showed primordial gonad with somatic cells; (B). Undifferentiated gonad at 50 DAH was composed of germ cell, somatic cell and blood vessel. BV, blood vessel; GC, germ cell; G, gut; PG, primordial gonad.

$\mu m^2$. At 50 DAH, the larvae were completed metamorphosis to become juvenile fish with average body weight and total length was 0.279 ± 0.061 g and 27.4 ± 2.4 mm, respectively. The primordial gonads were pear-shape consisting germ cells surround by somatic cells, which characteristic were similar to observed in 40 and 45 DAH (Fig. 4B). The surface area of gonad was 744.1 ± 227.0 $\mu m^2$ (Fig. 8).

### 2) Differentiation Phase

At 60 DAH, out of ten fish examined only four individual appeared to have initial ovarian cavity which was indicated by the presence of two elongated aggregations of somatic cells in the gonad (Fig. 5A). The number of somatic cells was increased and the blood vessel was immigrated in the central of gonad. The surface area of primordial gonad was 956.1 ± 510.3 $\mu m^2$ (Fig. 8). At 90 DAH, the two elongation sheets of somatic cells more developed upward and downwards to forming ovarian cavity (Fig. 5B). The gonad increased size with average of surface area was 10,126.5 ± 3,958.3 $\mu m^2$ (Fig. 8). The ovarian cavity was clearly observed with a large space in the central part of the gonad at 110 DAH (Fig. 5C). The gonad was rapidly increased in size compared with the gonads at 90 DAH which indicated by an average gonad surface area was 27,867.5 ± 7,274.4 $\mu m^2$ (Fig. 8). At 120 DAH to 130 the somatic cells continued mitosis to increase size of gonad (Fig. 8). At this stage, the gonads were

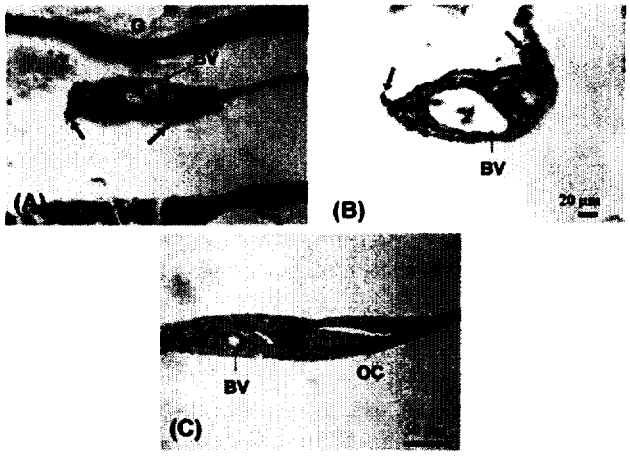


**Fig. 5. Gonadal development of longtooth grouper at 60 to 100 DAH.** (A). Cross section gonad at 60 DAH showing two somatic elongations; (B). Cross section gonad at 90 DAH showing two somatic elongations which more develop to forming the initial ovarian cavity were indicated by the thick arrows. Cross section gonad at 110 DAH show completed form of ovarian cavity; (C). BV, blood vessel; G, gut; OC, ovarian cavity.

exhibited morphological characteristic resembling in gonad at 110 DAH.

### 3) Ovarian Gonads Phase

The oogonia were first observed in the gonads of 3 individuals at 140 DAH, which indicate that gonad was entered to oogenesis. The average size of oogonia was 13.3 ± 2.0 μm in diameter which were migrated at the edge of ovarian cavity (Fig. 6A). The number of oogonia in the ovary increased at 180 DAH and size of oogonia was 16.8 ± 4.5 μm in diameter. The oogonia were observed in six individuals out of 9 fish (Table 3). The oogonia were enclosed by somatic cells and distributed between somatic tissues along the ovarian cavity. Some oogonia had already entered into mitosis division (Fig. 6B). At 210 DAH, seven individuals had ovaries containing oogonia which distributed along the inner periphery of the ovarian cavity (Fig. 6C). The oogonia at this stage were increased in number and diameter range from 10.0 to 22.5 μm. At 270 DAH, a few primary-growth stage oocytes were observed in one individual (Table 3). The primary-growth stage oocytes were resided in different lobules along of ovarian cavity (Fig. 6D). The diameter of primary-growth stage oocytes was 12.5 ± 4.7 μm. However, the primary-growth

**Table 3. Summary of observation on gonad development during sex differentiation of longtooth grouper at 90 to 365 DAH**

| Age (DAH) | Body length (mm) | Sex | | | | Histological characteristics of gonads |
|---|---|---|---|---|---|---|
| | | U | D | O | | |
| | | | | Og | Ol | |
| 90 | 57.3 ± 6.7 | 2 | 4 | - | | Two elongation sheets of somatic cells more developed upward and downwards to forming ovarian cavity. |
| 100 | 69.9 ± 9.0 | - | 8 | - | - | Ovarian cavity was completed form. Germ cell and somatic cells underwent through mitosis to increase in size of gonad. |
| 110 | 77.9 ± 11.6 | - | 8 | - | - | |
| 120 | 94.2 ± 12.5 | - | 9 | - | - | |
| 130 | 101.1 ± 8.4 | - | 7 | | - | |
| 140 | 105.3 ± 12.1 | - | 4 | 3 | - | The present of oogonia migrate at edge of ovarian cavity indicated that the gonad was entered to oogenesis. |
| 180 | 125.1 ± 12.5 | - | 3 | 6 | - | The oogonia had entered into mitosis division. |
| 210 | 125.7 ± 12.3 | - | 1 | 7 | - | The ovary was composed to the abundant of oogonia which distributed along the inner periphery of the ovarian cavity. |
| 270 | 160.6 ± 11.8 | - | - | 7 | 1 | The ovary appeared to have a few of primary growth stage oocyte. |
| 300 | 170.0 ± 19.1 | - | - | 5 | 3 | The ovary was composed to primary growth stage oocyte. |
| 360 | 197.9 ± 19.8 | - | - | 4 | 6 | |

Note: U, undifferentiated gonad; D, differentiation gonad; O, ovarian; Og, oogonia; Ol, primary growth stage oocyte.

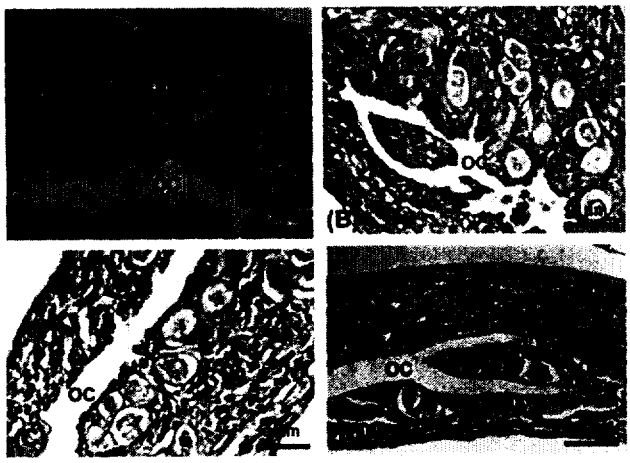


**Fig. 6. Gonad development (oogenesis) of longtooth grouper at 140 to 270 DAH.** (A). Cross section gonad at 140 DAH show appearance of oogonia; (B). Cross section gonad at 180 DAH show oogonia were entered mitosis division; (C). Cross section gonads at 210 DAH showed oogonia immigration a long ovarian caviy; (D). Cross section gonad at 270 DAH showed a few primary-growth stage oocyte immigrate a long ovarian cavity. OC, ovarian cavity; Og, oogonia; O1, primary growth stage oocyte.

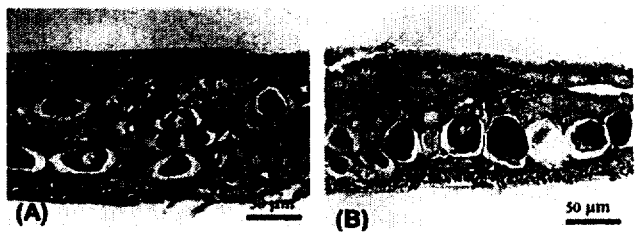


**Fig. 7. Gonad development of longtooth grouper at 300 to 365 DAH.** (A). Cross section gonad at 300 DAH show a large number of oocytes; (B). Cross section gonad at 365 DAH showed increased size of primary-growth stage oocytes; Og, Ogonia; O1, primary growth stage oocyte.

stage oocyte could not observe at this stage at all the specimen (Table 3). At 300 DAH, three individuals with ovaries containing primary-growth stage oocytes were observed (Table 3). The number of oogonia and primary-growth stage oocytes increased gradually. The diameter of oogonia was 16.3 ± 3.2 μm. At this stage, the proliferation of oogonia was obvious that the ovaries entered mitosis division (Fig. 7A). At 365 DAH, six individuals had ovaries contained primary-growth stage oocyte which increased of size to average 22.1 ± 4.9 μm in diameter (Fig. 7B).

## DISCUSSION

Sex differentiation occur at different post hatching times in most gonochoristic teleost fish (Parmentier et al., 1985; Lee et al., 1996; Braat et al., 1999; Arezo et al., 2007) and protogynous hermaphrodite (Lim, 2000; Devlin & Nagahama, 2002. Murata et al., 2009). The primordial germ cells (PGCs) arise to the primordial gonad and increase in number through mitosis during specific developmental phase (Parmentier et al., 1985; Braat et al., 1999). In this study, the observation showed that in 10 DAH longtooth grouper (*E. bruneus*) larvae, the primordial germ cells were locate between mesonephric duct and gut. In 15 DAH larvae, the PGCs were increased in number through mitotic. In teleosts fish PGCs could be observed during the embryonic development, shortly after hatching or a few weeks after hatching (Braat et al., 1999). During the period from the hatching in viviparous *Ditrema temmincki,* the PGCs were first observed in the fibrous mesenchymal tissue located between the early alimentary tract and the dorsal body wall (Lee & Lee, 1996). In other case, PGCs could be found at 15 DAH of *Vimba vimba* fish (Hliwa et al., 2003) and *Squalius cephalus* was 35 DAH (Hliwa et al., 2009). In protogynous hermaphrodite malabar grouper *Epinephelus malabaricus*, PGCs were immigrated in the primordial gonad tissue located below the mesonephric duct at the

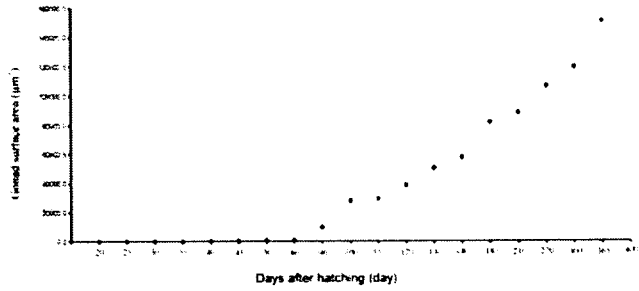


**Fig. 8. Change of gonad surface area of longtooth grouper during the experimental period.**

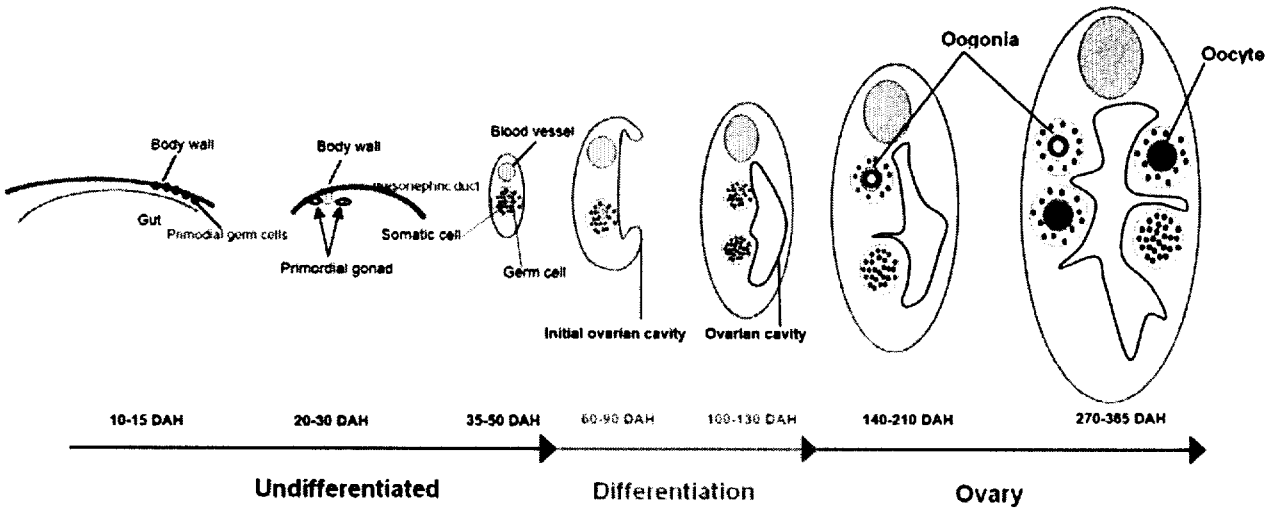


**Fig. 9. Schematic figure of ovarian differentiation of longtooth grouper in indoor rearing condition.**

dorsal side of the intestine at 11 DAH (Murata et al., 2009). Migration and development of PGCs have been described in several fish species including: rockfish *Sebastes schlegeli* (Lee et al., 1996), seabream *Pagrus major* (Lim, 2000), cichlid fish *Cichlasoma dimerus* (Meijide et al., 2005), bluegill sunfish *Lepomis macrochirus* (Gao et al., 2009). Devlin and Nagahama (2002) reported that prior to gonad sex differentiation, all somatic cells appear which was derived from a cortex epithelial layer. In this study, primordial gonads with a few somatic cells were first observed in longtooth grouper (*E. bruneus*) larvae at 20 DAH and numbers of somatic cells were increased by age of fish. The primordial gonad during early gonad development composed of a few somatic cells was observed at 3 weeks after hatching orange spotted grouper *Epinephelus coioides* larvae (Liu & Sadovy, 2009). The undifferentiated gonads were consisted of two kinds of somatic cells including the cortex and the medulla. During ovarian differentiation phase, the cortex was developed and the medulla was degenerated (Nakamura et al., 1998). In olive flounder *Paralichthys olivaceus*, four kinds of somatic cells were distributed and appeared according to the differentiated development of the testis and ovary in primitive gonad (Lee, 1990). The primordial gonad component germ cells become evident in longtooth grouper (*E. bruneus*) larvae from 40 to 50 DAH. In malabar grouper (*E. malabaricus*), the germ cells were first observed in undifferentiated gonad at 39 DAH (Murata et al., 2009). But Liu and Sadovy (2009) did not identify germ cells in primordial gonad of orange spotted grouper (*E. coioides*) at 7 weeks after hatching. This suggests that the appearance of germ cells in undifferentiated gonad depend upon fish species.

In gonochoristic teleosts fish, the formation of ovarian cavity indicates that the gonad functions as an ovary. The ovarian cavity can take one of several different forms depending different fish species (Nakamura et al., 1998). There are few studies on gonad development during sexual differentiation of protogynous hermaphrodite. In malabar grouper (*E. malabaricus*), the initial ovarian cavity information were first observed at 47 DAH by appearance two elongations of somatic tissues developed both upward and downward and more developed at 74 DAH. But these ovarian cavities were not connected to each other. The ovarian cavity formation was accomplished at 144 DAH (Murata et al., 2009). In orange spotted grouper (*E. coioides*) the formation of ovarian cavity were signal of gonadal wall protruded dorsally and ventrally from the area of blood vessels at 26 weeks after hatching, whereas was

observed in humpback grouper *Cromileptes altivelis* at 17 weeks after hatching (Liu & Sadovy, 2009). To similar in studied of Liu and Sadovy (2009) and Murata et al. (2009), in this study showed the initial information of ovarian cavity were first observed at 60 days after hatching with a presence of two elongated aggregations of somatic cells in the gonad and increased side at 90 DAH. At 110 DAH, the formation of an ovarian cavity appeared at the central of gonad. This result suggests that in longtooth grouper (*E. bruneus*) the ovarian cavity was formed early (110 DAH) than malabar grouper (*E. malabaricus*) (144 DAH), orange spotted grouper (*E. coioides*) (22 weeks after hatching) and humpback grouper (*C. altivelis*) (30 weeks after hatching).

In teleosts fish, ovarian development in female is first detectable with the proliferation of somatic cells and oogonia and early oocyte differentiation (Nakamura et al., 1998; Devlin & Nagahama, 2002). The ovarian ontogeny is varied by germ cell meiosis before or after the formation of the ovarian cavity (Nakamura et al., 1998). For *Vimba vimba* (L. 1758), cichlid fish *Cichlasoma dimerus* and fathead minnows *Pemephales promelas,* the oogonia were entered meiosis division and appeared of primary stage oocyte prior to the formation of ovarian cavity (Hliwa et al., 2003; Meijide et al., 2005; Uguz, 2008). In the contrary, several studies have reported that the meiosis division of oogonia occurs after the formation of the ovarian cavity in gonochoristic parrot fish *Oplegnathus fasciatus*, protogynous hermaphrodite: including seabream *Pagrus major*, malabar grouper (*E. malabaricus*), orange spotted grouper (*E. coioides*) and humpback grouper (*C. altivelis*) (Kim, 1998; Lim, 2000; Murata et al., 2009; Liu & Sadovy, 2009). Like other protogynous hermaphrodite fish species, longtooth grouper (*E. bruneus*) appeared to undergo through meiotic division of oogonia and development of primary growth stage oocyte after the formation of ovarian cavity.

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