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Development of Gonadal Tissue and Aromatase Function in the Protogynous Orange-Spotted Grouper *Epinephelus coioides*

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Ya-Ju Tsai, Mong-Fong Lee, Chia-Yung Chen, and Ching-Fong Chang (2011) Development of gonadal tissue and aromatase function in the protogynous orange-spotted grouper *Epinephelus coioides*. *Zoological Studies* **50**(6): 693-704. The protogynous orange-spotted grouper *Epinephelus coioides* is a diandric type of hermaphrodite. We found that a paired gonad developed in a forked morphology at 1 mo of age, an ovarian cavity within the gonad was formed at 4 mo of age, and ovarian lamellae containing oogonia and primary oocytes proliferated at 6.5 mo of age. A single spermatogenic cyst (SSC) developed at the margin of the ovarian lamella containing primary oocytes and then various stages of spermatogenic cysts (VSCs) proliferated within the gonads of primary males. In contrast, an SSC was present at the margins of the ovarian lamellae containing vitellogenic oocytes and atretic oocytes of secondary males at 5.4 yr of age. Positive immunohistochemical expression of proliferating cell nuclear antigen was detected in nuclei of oogonia, cortical alveoli oocytes, vitellogenic oocytes, spermatogonia, and spermatocytes, while perinucleolar oocytes, spermatids, and spermatozoa were negatively stained. After administration of an aromatase inhibitor (AI) in juvenile fish for 2 mo, they changed sex into males. Adult fish fed diets containing the AI for 4 mo also changed sex into males. Abundant mature spermatogenic cysts became the main components within the testicular lamellae where single residual primary oocytes were scattered. Immunohistochemical signals of aromatase were present in somatic cells around the oogonia, cortical alveoli oocytes, vitellogenic oocytes, and spermatogenic cysts. The results suggest that aromatase (estrogen) plays an important role in sex differentiation and is also involved in sex change in the orange-spotted grouper. http://zoolstud.sinica.edu.tw/Journals/50.6/693.pdf

Key words: Aromatase, Fish, Gonadal differentiation, Male development, Sex change.

Sustainable seed production is important for the mariculture industry. Due to difficulties acquiring mature broodstock from nature and fishery farms to carry out artificial propagation in some gonochoristic fish and hermaphrodites, treatment with steroid hormones (estrogens or androgens) was the traditional manipulation to induce sex change in teleosts since Yamamoto (1969) proposed the concept of sex inducers. Cytochrome P450 aromatase (P450arom), one of the critical steroidogenic enzymes, aromatizes androgens to produce estrogens. Decreases and increases in plasma estrodiol-17β concentrations are respectively related to the initiation of sex change in the protogynous wrasse *Thalassoma duperrey* (Nakamura et al. 1989) and protandrous black porgy *Acanthopagrus schlegeli* (Chang et al. 1994). Therefore, this enzyme was reported to be involved in sex differentiation in fish (Guiguen et al. 2009). However, an aromatase inhibitor (AI), a non-steroid chemical, blocked the biosynthesis pathway of androgens to estrogens and was involved in a sex change of gonochoristic Japanese flounder *Paralichthys olivaceus* (Kitano et al. 2000),

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Nile tilapia *Oreochromis niloticus* (Bertollaafonso et al. 2001), protogynous wrasse *Halichoeres trimaculatus* (Nozu et al. 2009), and protandrous black porgy *A. schlegeli* (Lee et al. 2002).

The orange-spotted grouper *Epinephelus coioides* is a protogynous hermaphrodite and an important mariculture species in Asia. It was recently reported to be a diandric protogynous teleost (Liu and Sadovy 2009, Grandcourt et al. 2009). There are 2 types of males: primary males develop directly from juveniles and secondary males occur through a sex change in adult female fish. Implantation of pellets containing the exogenous androgen, 17α-methyltestosterone, into the orange-spotted grouper stimulated a sex change (Yeh et al. 2003). However, the impacts of the administration of AI on gonadal development in these fish are still unknown. Therefore, in the present study, we treated fish with an AI by feeding and implantation and examined gonadal development and characteristics of immunohistochemical expression of proliferating cell nuclear antigen (Pcna, a signal for cellular proliferation activity) and P450arom to investigate the development of gonads in the orange-spotted grouper.

MATERIALS AND METHODS

Animals

Fingerlings (2 wk old) of orange-spotted grouper *E. coioides* were obtained from the Mariculture Center, Taiwan Fisheries Research Institute (Tainan, Taiwan). Fish were cultured in seawater tanks at the Department of Aquaculture, National Taiwan Ocean Univ. (NTOU; Keelung, Taiwan) for experiments of the present study (Table 1). All experimental fish were acclimated in a pond at the university culture station in seawater with a natural light system. A recirculating system was designed for each 2.5-ton fiber-reinforced plastic (FRP) tank with a nylon filter, biofilter, and deproteinizing devices to remove organic substances and feces. Seawater conditions in the recirculation system were as follows: a salinity of 32 ppt, a water temperature of 27 ± 0.5°C, and a pH of 8.2. All procedures and investigations were carried out under regulations of the NTOU Institutional Animal Care and Use Committee and were performed in accordance with standard guidelines.

Preparation of diets and pellets containing the AI

AI (1,4,6-androstartriene-3,17-dione; Steraloid, Newport, RI, USA) was first dissolved in absolute alcohol as an AI stock solution and then in distilled water. To prepare AI pellets, AI, coconut oil, and α-cellulose were mixed in a ratio of 1: 3: 6. The AI content in the pellet was 100 μg AI/mg of pellet.

For the diet preparation, the control (without AI) and AI diets (20 mg AI/kg feed) were individually blended in a mixer, and distilled water was included to achieve a proper moisture content (10.1%). The mixed ingredients were made into pellets using an extruder (Ming Seng Machinery,

Table 1. Gonadal stages of juvenile (3 mo of age) and 18-mo-old fish fed or implanted with an aromatase inhibitor (AI). AMC, abundant mature cysts distributed in the testicular lamella; SSC, a single spermatogenic cyst located at the margin of the ovarian lamella; CA, cortical alveolar oocyte; OG, oogonium; PO, primary oocyte; VO, vitellogenic oocyte; VSC, various stages of spermatogenic cysts intermingled with oocytes within ovarian lamella

Experiment	Group	No. of fish	BL (cm) / BW (g)	Ovarian tissues				Testicular tissues		
			(mean ± SEM)	OG	PO	CA	VO	SSC	VSC	AMC
Juvenile fish fed AI	Control	5	12.44 ± 0.101 / 28.83 ± 0.639	5	0	0	0	0	0	0
	Treatment	5	12.64 ± 0.138 / 30.27 ± 0.992	3	0	0	0	0	0	2
Adult fish fed AI	Control	19	30.7 ± 0.531 / 456.82 ± 21.403	19	18	16	3	3	0	0
	Treatment	22	27.44 ± 0.324 / 286.27 ± 9.186	22	22	21	2	5	2	1
Adult fish implanted with AI	Control	9	35.53 ± 1.523 / 696.24 ± 82.706	9	9	8	4	3	1	0
	Treatment	11	40.96 ± 2.575 / 976.96 ± 136.592	11	11	6	1	2	2	0

BL, mean total length (cm); BW, mean total weight (g). Values are expressed as the mean ± standard error of the mean (SEM).

Ilan, Taiwan) with a 2-mm diameter and a rotation cutter. The pelleted diets were dried at 40°C for 10 h and then stored at -20°C until used for the feeding trial.

Experimental design

Juvenile and adult fish fed diets containing the AI

Juvenile fish (3 mo old) were divided into 2 equal groups (n = 10) and fed diets containing AI (20 mg/kg diet, n = 5) or the control (with no AI, n = 5) for 2 mo. Fish were sacrificed after treatment. Fish (n = 41, 18 mo old) were also divided into AI (n = 22) and control groups (n = 19) and then similarly fed a diet containing AI (20 mg/kg diet) or the control diet. The diets were fed ad libitum to fish 2 times a day (morning and afternoon). After feeding these diets for 4 mo, fish were sacrificed at 2-mo intervals.

Implantation of pellets containing the AI into the dorsal muscle of adult fish

Adult fish (n = 11, 4 yr old) were implanted with pellets containing AI (40 mg/kg body weight) into the dorsal muscle. Pellets were implanted into the fish once a month for 3 mo (3 implantations in total). Another 9 fish were implanted with pellets without AI as the control group.

Fish collection

Fish were anesthetized prior to handling in a bath of 0.5 ml/L 2-phenoxyethanol. Body weight and length were measured. The gonads were collected for histological studies.

Gonadal histology

The gonads were fixed in Bouin's solution for 24-72 h (according to the size of the tissues), followed by washing with 70% alcohol several times to remove as much of the picric acid as possible. Tissues were dehydrated in an alcohol series at concentrations of 80%, 85%, 90%, 95%, and 100% and then soaked in xylene. They were embedded in paraffin wax (Sherwood Medical, St Louis, MO, USA) and sectioned at 5-6 μm thick. Paraffin-embedded sections were treated with traditional hematoxylin and eosin (H&E) or trichrome staining according to the Gomori rapid 1-step trichrome method (Bancroft and Cook 1994) to stain connective tissue. Stained sections were sealed with Entellan (Merck, Darmstadt, Germany) and observed with a light microscope.

Immunohistochemical staining with P450arom and Pcna antisera

For immunohistochemical staining, gonads were fixed in a 4% solution of paraformaldehyde overnight at 4°C, followed by dehydration with a methanol series at concentrations of 25%, 50%, 75%, and 100%. They were also embedded in paraffin and sectioned at 5-6 μm thick. Sections were rehydrated and incubated with 0.3% hydrogen peroxide at room temperature. After rinsing sections with 0.01 M phosphate-buffered saline (PBS), they were incubated with 10% normal goat serum for 30 min and with human Pcna antiserum (#sc-7907, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or antiserum against P450arom fragments (CVLKNGNYTSRFQSKQ + CNNLSQQPVEHQQ-EADHL) of the orange-spotted grouper overnight at 4°C. This was followed by incubation with biotinylated anti-rabbit immunoglobulin G (IgG) (H+L) from Vector Laboratories (Burlingame, CA, USA). Color formation was amplified with an ABC kit (avidin-biotin; Vector Laboratories) and 3, 3'-diaminobenzidine (Sigma, St. Louis, MO, USA). All sections were counterstained with hematoxylin.

RESULTS

Development of gonads in the protogynous orange-spotted grouper

The primordial paired gonads, each lobe of which was forked, were located in the upper middle position of the mesentery linked to the peritoneum in fish at 1 mo of age (Fig. 1A). Each lobe of the paired gonads began to form an ovarian cavity at 4 mo of age (Fig. 1B). The newly budded ovarian lamella around the inside of the ovarian cavity in the gonad mainly contained oogonia and a few primary oocytes in the fish at 6.5 mo of age (Fig. 1C). The gonads were arrested at the primary oocyte stage during the non-spawning period (Fig. 1D). Cortical alveoli and vitellogenic oocytes were present within the mature ovarian lamellae (Fig. 1E). The fish did not reach the mature female stage until 2 yr of age. There was a single spermatogenic cyst (SSC) composed of spermatocytes present in the margins of the

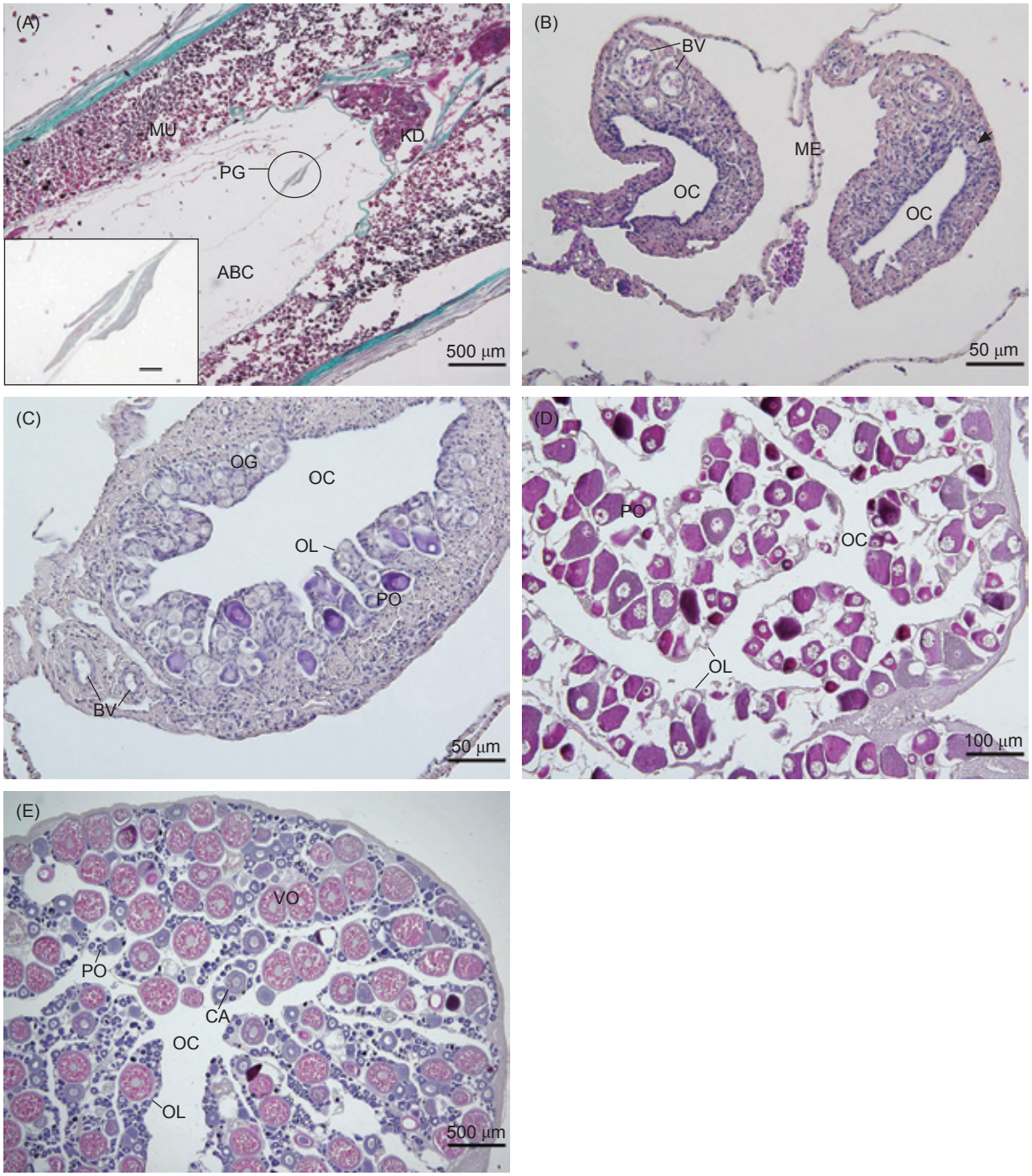

Fig. 1. Development of female gonads in the protogynous orange-spotted grouper. All sections were paraffin-embedded and stained with hematoxylin and eosin or Gomori's trichrome method. The gonadal stages of A, B, and C, were the same as steps 1, 2, and 3 in figure 7, respectively. D and E were the same as step 4' in figure 7. (A) Pairs of primordial forked gonads in the orange-spotted grouper (at 1 mo old) were located in the central upper position at the dorsal wall-linked mesentery in the abdominal cavity. Inset: A magnified view of the primordial forked gonad. Scale bar = 20 μm. (B) Each of the paired gonads in the fish (4 mo old) formed an ovarian cavity. A single putative primordial germ cell (arrow) was present beside the ovarian cavity. (C) Oogonia and a few primary oocytes were distributed within the initially proliferated ovarian lamella around the ovarian cavity of the gonads in the fish (6.5 mo old). (D) Primary oocytes (in the perinucleolar stage) were the dominant germ cells within the ovarian lamellae during the non-spawning periods in 13-25-mo-old fish. (E) Cortical alveoli and vitellogenic oocytes were present within ovarian lamellae in gonads of fish at about 25 mo of age. ABC, abdominal cavity; BV, blood vessel; CA, cortical alveoli oocyte; KD, kidney; ME, mesentery; MU, muscle; OC, ovarian cavity; OG, oogonium; OL, ovarian lamella; PG, primordial gonad; PO, primary oocyte; VO, vitellogenic oocyte.

ovarian lamellae containing primary oocytes (Fig. 2A). Under the process of the proliferation, several various stages of spermatogenic cysts (VSCs) including spermatogonia, spermatocytes, spermatids, and spermatozoa intermingled with primary oocytes within the lamellae of the gonads (Fig. 2B). An SSC containing spermatozoa was also present at the margins of the ovarian lamellae with vitellogenic oocytes and atretic oocytes (Fig. 2C) in the gonads of fish at 5.4 yr of age.

Immunohistochemical expressions of the Pcna within gonadal tissues

In order to examine the cellular proliferation activity in the gonads, Pcna staining was used. A positive signal for Pcna was shown in nuclei of oogonia within the newly formed ovarian lamella of the gonads in fish at 6.5 mo of age (Fig. 3A) and in the nuclear membrane and contents of the cortical alveoli and vitellogenic oocytes (Fig. 3B, C) in gonads of fish at 1-2 yr of age. In contrast, no Pcna signal was detected in the cytoplasm of oogonia, cortical alveoli, or vitellogenic oocytes (Fig. 3A-C). Nuclei of spermatocytes and spermatogonia/spermatids within cysts of the sex-changed gonads respectively expressed strong and weak signals for Pcna, but those of spermatozoa did not (Fig. 3D).

Oral administration of the AI

Juvenile fish received AI administration

All juvenile control fish (5 mo of age) were maintained in the female stage (Table 1) with gonads containing ovarian lamellae mainly with oogonia (Fig. 4A). Two of 5 gonads of juvenile fish (5 mo old), after treatment with feed containing the AI for 2 mo, were composed of various stages of spermatogenic cysts containing spermatozoa, spermatids, spermatocysts, and spermatogonia (Table 1, Fig. 4B).

Eighteen-month-old fish administered the AI

All control fish (18 mo of age) were maintained in the female stage (Table 1). Three of 19 fish (15.8%) developed vitellogenic oocytes (Fig. 5A), and 3 developed an SSC (Table 1, Fig. 5B). When fish were orally treated with the AI for 2 mo, their gonads, which mainly contained primary oocytes and cortical alveolar oocytes, remained in the female phase (Table 1). Although two of

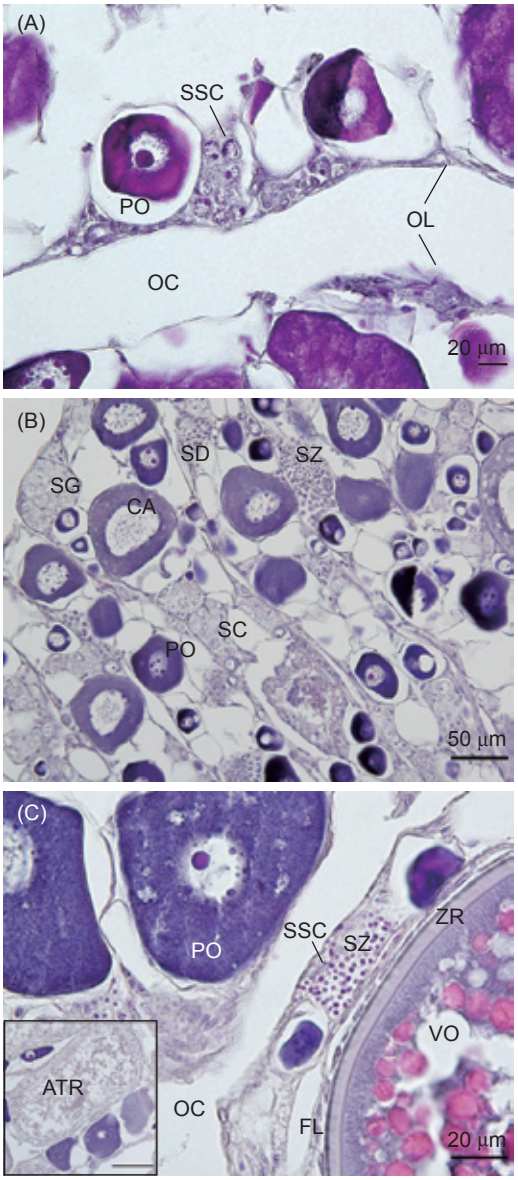

Fig. 2. Gonadal characteristics of the male phase in the protogynous orange-spotted grouper. A and B are a primary male and C is a secondary male undergoing a sex change. All sections were paraffin-embedded and stained with hematoxylin and eosin. Gonadal stages of A, B, and C are the same as steps 4, 5, and 5' in figure 7, respectively. (A) A single spermatogenic cyst containing spermatogonia was present at the margin of the ovarian lamella with primary oocytes (in the perinucleolar stage). (B) Various stages of spermatogenic cysts containing spermatogonia, spermatids, or spermatozoa within ovarian lamellae mainly containing primary oocytes and a few cortical alveolar oocytes. (C) A single spermatogenic cyst containing spermatozoa was located at the margin of the ovarian lamella and included vitellogenic oocytes and atretic oocytes. Inset: A magnified view of an atretic oocyte (ATR). Scale bar = 50 μm. CA, cortical alveoli oocyte; SSC, a single spermatogenic cyst; FL, follicular layer; OC, ovarian cavity; OL, ovarian lamella; PO, primary oocyte; SC, spermatocyte; SG, spermatogonium; SD, spermatid; SZ, spermatozoon; VO, vitellogenic oocyte; ZR, zona radiata.

22 fish still contained vitellogenic oocytes, some fish proceeded to change sex (Table 1). The original ovarian lamellae of the gonads changed to develop VSCs intermingled with primary oocytes (Fig. 5C), and 1 fish did change sex into a mature male (Fig. 5D-F). Lamellae scattered with a few small residual primary oocytes were abundant with mature stages of cysts (AMCs) (Fig. 5D, E), and spermatozoa were gathered within the testicular main ducts at the margin of the testes (Fig. 5F).

Implantation of the AI into the dorsal muscle of fish

Gonadal development of adult fish (18 mo of age) in the control group and the AI implantation treatment are summarized in table 1. In the control group, four of 9 fish developed vitellogenic oocytes, 2 had an SSC, and 1 had VSCs (Table 1). In the AI-treated group, one of 11 fish developed vitellogenic oocytes, 2 had an SSC, and 2 had VSCs (Table 1).

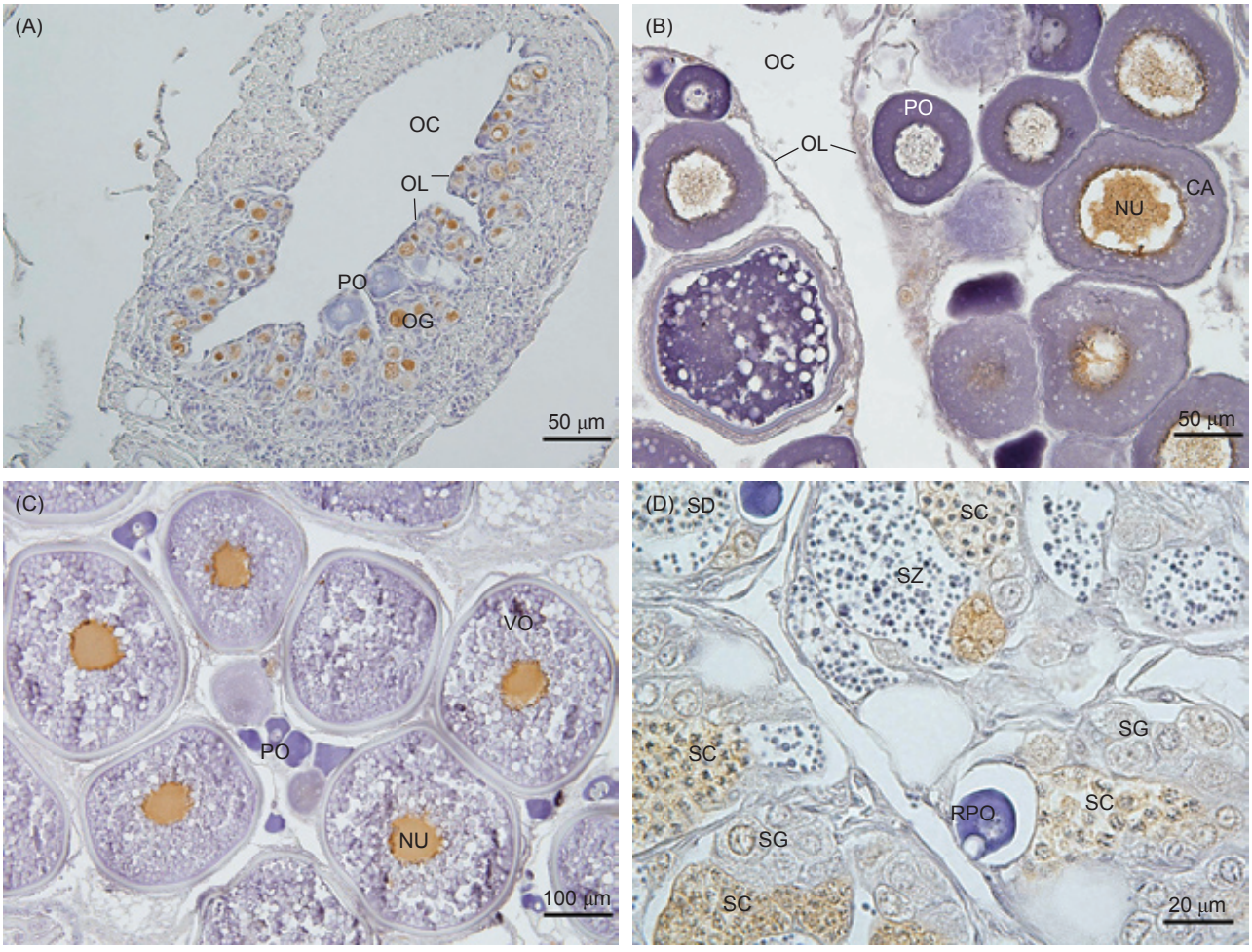

Fig. 3. Immunohistochemical expression of cellular activity in gonadal tissues of the orange-spotted grouper at different developmental stages. A-C are the female phase, and D is the male phase after treatment with an aromatase inhibitor. All sections were paraffin-embedded and stained for proliferating cell nuclear antigen (Pcna) and counterstained with hematoxylin. Gonadal stages of A, B and C, and D are the same as steps 3, 4', and 6 in figure 7, respectively. (A) Positive Pcna staining was observed in nuclei of oogonia within the newly formed lamella around the ovarian cavity of the gonad. (B) Positive Pcna staining was observed in the nuclear membrane and nuclei of cortical alveolar oocytes, while primary oocytes were negative. (C) Positive Pcna staining was found in nuclei of vitellogenic oocytes. (D) Positive Pcna staining was found in nuclei of spermatogonia and spermatocytes within cysts of sex-changed gonads, while spermatids and spermatozoa were negative. CA, cortical alveolar oocyte; NU, nucleus; OC, ovarian cavity; OG, oogonium; OL, ovarian lamella; PO, primary oocytes; RPO, a single residual primary oocyte; SC, spermatocyte; SD, spermatid; SG, spermatogonium; SZ, spermatozoon; VO, vitellogenic oocyte.

Immunohistochemical expressions of aromatase in the gonads of fish from juveniles to adults

Significant amounts of P450arom were detected in somatic cells surrounding oogonia (Fig. 6A, B) in gonads of juvenile fish (4 mo of age). The follicular layers around the vitellogenic oocytes in adult fish showed strong signals of the P450arom, and staining around cortical alveolar oocytes was weak, but that around primary oocytes was not positive (Fig. 6C). Somatic cells surrounding the cysts, spermatids within the cysts, and spermatogonia in sex-changed adult fish showed positive signals of immunoreactivity to aromatase (Fig. 6D).

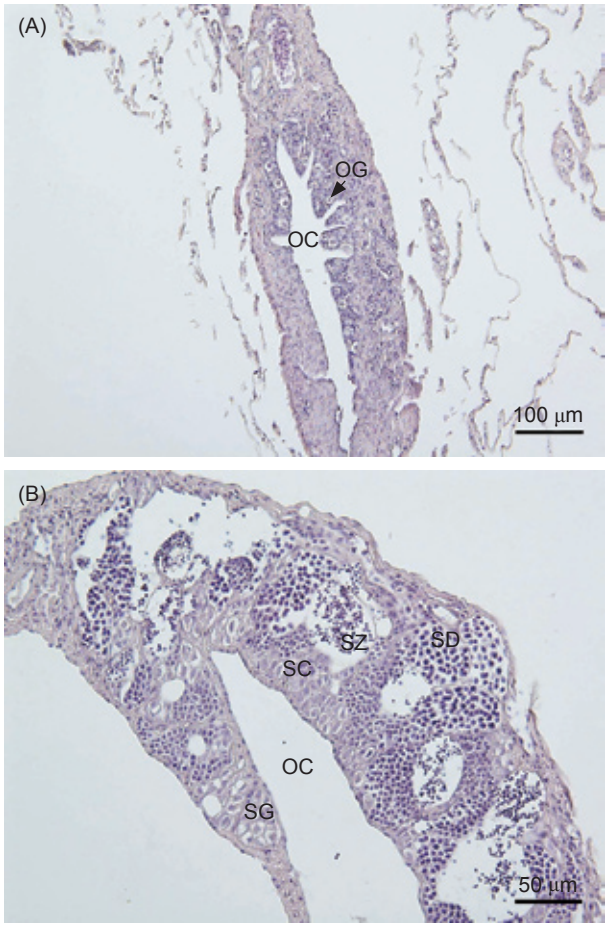

Fig. 4. Gonadal development in the orange-spotted grouper (at 5 mo old) fed diets containing no chemical (control group) and an aromatase inhibitor (AI) for 2 mo. All sections were paraffin-embedded and stained with hematoxylin and eosin. (A) In the control group, gonads of juvenile fish (at 5 mo old) were all the female type containing ovarian lamellae with oogonia. (B) Male germ cells developed, and paired gonads changed into male after AI feeding for 2 mo. Various stages of cysts including spermatogonia, spermatocytes, spermatids, and spermatozoa were present in gonads of juvenile fish. OC, ovarian cavity; OG, oogonium; SC, spermatocyte; SD, spermatid; SG, spermatogonium; SZ, spermatozoon.

Schematic illustration of gonadal development in primary and secondary males

Based on development of male germ cells (SSC/VSCs) and female germ cells (oogonia, primary oocytes, cortical alveoli, and vitellogenic oocytes), we propose for the 1st time a pathway for the development of primary and secondary males in grouper (Fig. 7).

DISCUSSION

In the present study, we applied light microscopy to examine histological characteristics of the gonads of orange-spotted grouper during development and after administration of the AI. In addition, cell activities and P450arom in the gonads were also both investigated by immunohistochemical staining. Documentary histological investigations of gonadal development in grouper indicated that the fish belongs to the diandric type; i.e., a male can be derived from a mature female through a sex change or directly from a juvenile male (Grandcourt 2009, Liu and Sadovy 2009). Our results were consistent with their findings. However, their studies did not clearly point out the critical characteristics distinguishing primary from secondary males based on histological observations. In the present study, for the 1st time, we provide precise characteristics of gonadal development between primary and secondary males in the orange-spotted grouper (Fig. 7).

Each of the paired primordial gonads in the fish (at 1 mo of age), contained a major and a minor strand of gonad. These 2 strands connect to form the ovarian cavity. Similar patterns (formation of the ovarian cavity) as an early characteristic of female differentiation were also found in other protogynous hermaphrodites (Murata et al. 2009), gonochoristic fish (Asoh and Kasuya 2002), and protandrous hermaphrodites (Besseau and Bruslé-Sicard 1995, Huang et al. 2002, Lee et al. 2008 2011), while other species were first recognized by the presence of meiotic division of oogonia (Nakamura et al. 1998). Furthermore, early morphological changes (elongation of somatic cells in the gonads facing the gonad lateral walls, an early sign of initiation of ovarian cavity formation)

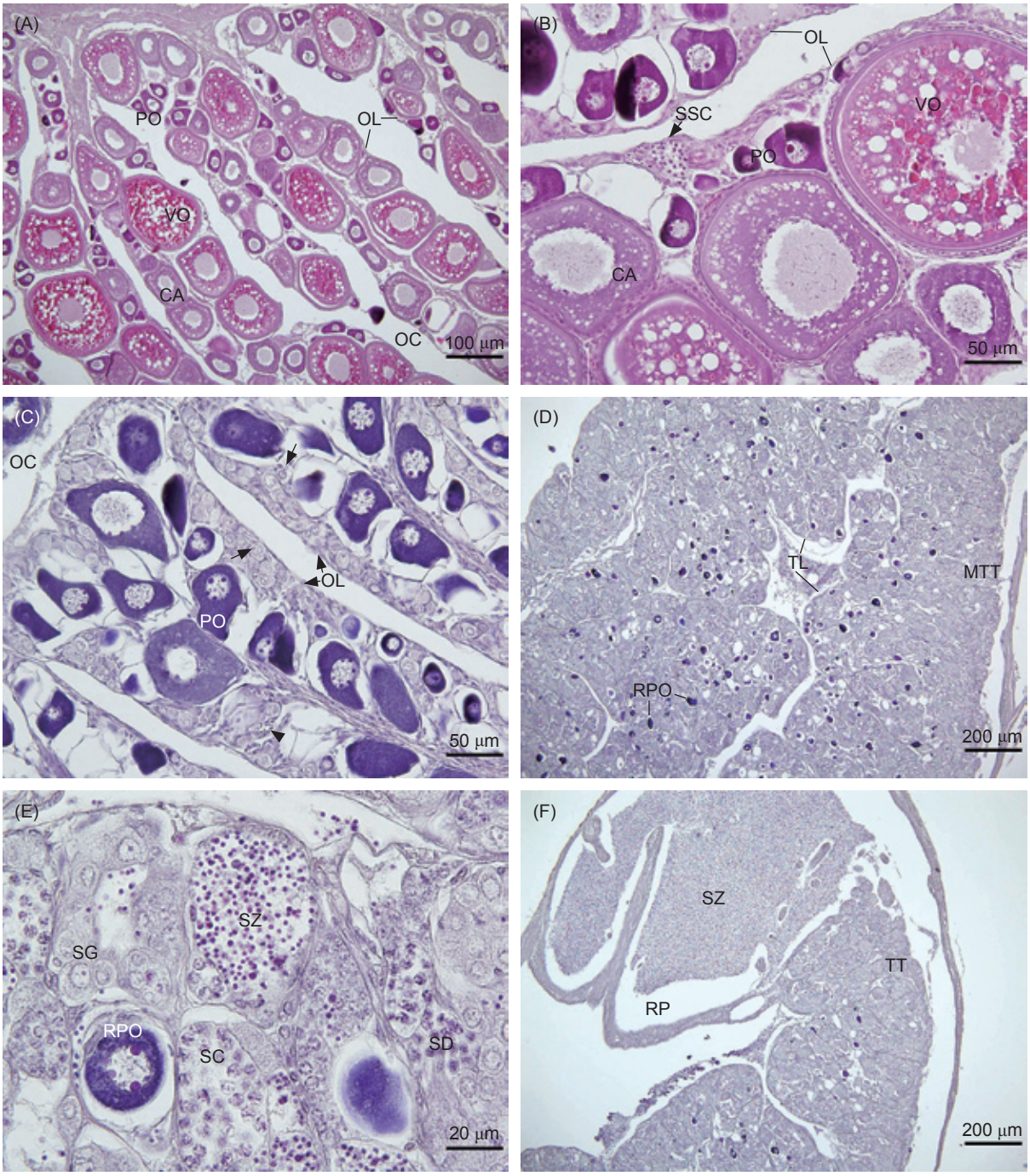

Fig. 5. Gonadal development in 18-mo-old orange-spotted grouper fed diets containing no chemical (control group) and an aromatase inhibitor (AI) for 4 mo. A and B are the control group, and C-F are the AI group. All sections were paraffin-embedded and stained with hematoxylin and eosin. (A) In the control group, all fish were in the female phase, and 3 of 19 fish developed vitellogenic oocytes. There were another 3 of 19 fish with a few single spermatogenic cysts (arrow) distributed at the margin of the ovarian lamella (B). (C) Various stages of spermatogenic cysts including spermatogonia (arrows) and spermatozoa (arrowhead) were intermingled with primary oocytes in ovarian lamellae of gonads after feeding the AI. (D) A sex-changed gonad with a few single residual primary oocytes scattered within the mature testicular lamellae appeared in one of the AI-treated fish. (E) A partially magnified view of D indicating abundant mature cysts mixed with 2 single residual primary oocytes. (F) The reproductive passage within the sex-changed gonad contained spermatozoa after treatment with the AI. SSC, a single spermatogenic cyst; CA, cortical alveolar oocyte; MTT, mature testicular tissue; OC, ovarian cavity; OL, ovarian lamella; PO, primary oocyte; RP, reproductive passage; RPO, a single residual primary oocyte; SC, spermatocyte; SD, spermatid; SG, spermatogonium; SZ, spermatozoon; TL, testicular lamella; TT, testicular tissue; VO, vitellogenic oocyte.

were observed by around 47 d post-hatching in Malabar grouper *E. malabaricus* (Murata et al. 2009). In juvenile males (primary males), the SSC first appeared at the margin of ovarian lamellae with primary oocytes (Fig. 2A). When the male gonads began to develop at the juvenile stage, VSCs were intermingled with primary oocytes within the lamellae (Fig. 2B). These processes were destined to develop a primary male (steps 3 to 6 in Fig. 7). The bisexual construction of the gonads in the orange-spotted grouper belongs to the undelimited type according to the definition of Sadovy and Shapiro (1987). Female germ cells were mixed with male germ cysts within the same lamella.

Although we did not use natural mature male fish for the control group in the present study, AI-fed treatment of adult fish did stimulate orange-spotted grouper to change sex into mature males (Fig. 5D-F). In addition, the SSC also appeared at the margin of ovarian lamellae containing vitellogenic oocytes and atretic oocytes in ovaries of female fish (5 yr old) (Fig. 2C). This indicated that the fish changed sex into males from mature females. These findings suggest that SSC/VSCs mixed with primary oocytes or vitellogenic oocytes

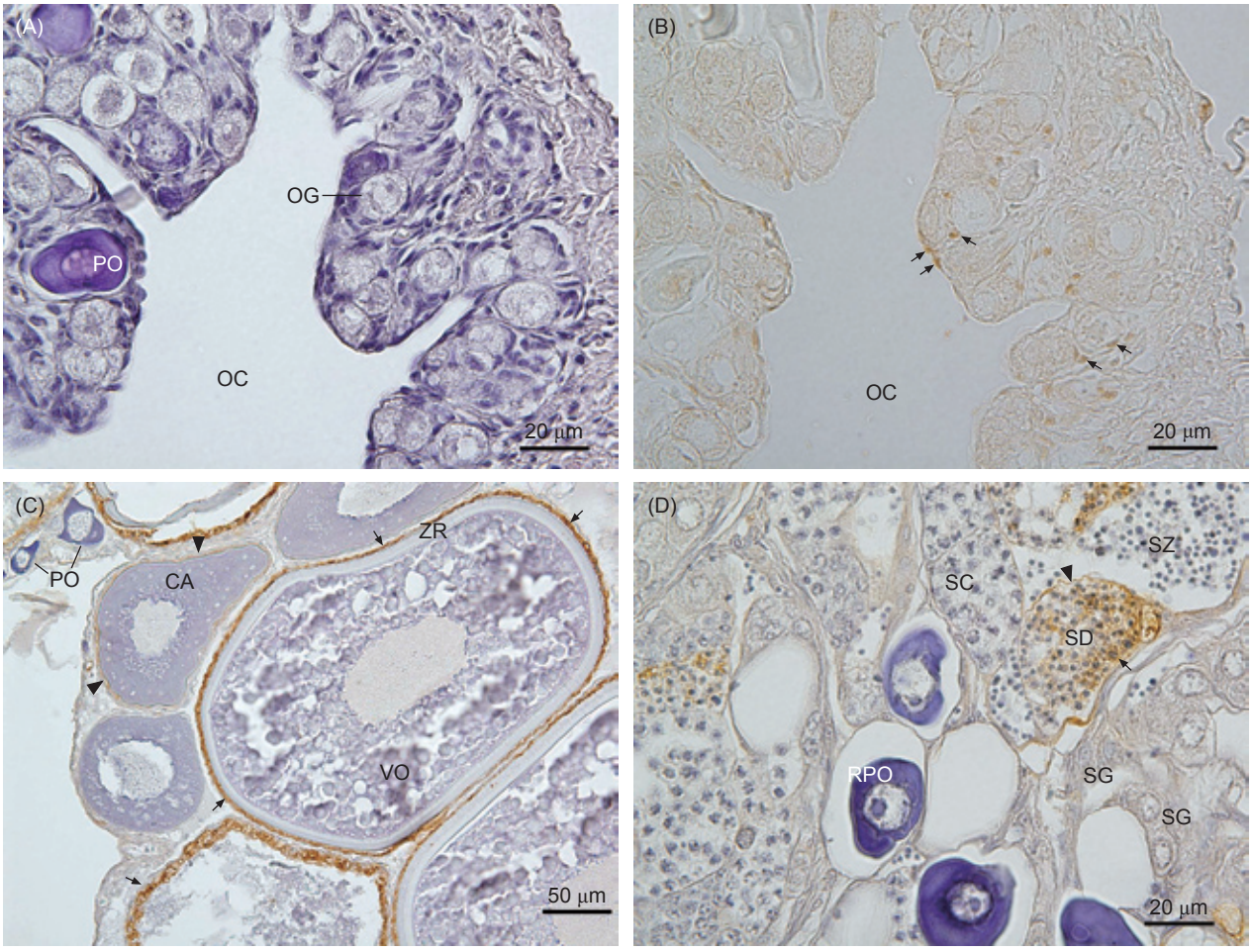

Fig. 6. Immunohistochemical expression of aromatase in gonads of the protogynous orange-spotted grouper. All sections were paraffin-embedded, stained for cytochrome P450 aromatase (P450arom), and counterstained with hematoxylin. A-C are the control group, and D is the aromatase inhibitor (AI)-treated group. (A) Oogonia stained with hematoxylin and eosin. (B) Positive reactions of P450arom were detected in somatic cells (arrows) surrounding oogonia around the ovarian cavity. (C) Follicular layers surrounding cortical alveolar oocytes (arrowheads) and vitellogenic oocytes (arrows) (but not primary oocytes) were positively stained for P450arom with faint and strong signals, respectively. (D) Positive signals of P450arom are shown in somatic cells surrounding cysts (arrowhead) and spermatids within cyst cells (arrows) in sex-changed adult fish. Spermatogonia also had faintly positive P450arom staining. Cysts containing spermatocytes and spermatozoa could not be clearly stained. CA, cortical alveoli oocyte; OC, ovarian cavity; RPO, a single residual primary oocyte; PO, primary oocyte; SC, spermatocyte; SD, spermatid; SG, spermatogonium; SZ, spermatozoon; VO, vitellogenic oocyte; ZR, zona radiata.

are the 2 ontogenetic characteristics of gonadal development that distinguishes primary from secondary males in the orange-spotted grouper (Fig. 7). Their coexistence with primary oocytes within the ovarian lamellae or with vitellogenic oocytes and atretic oocytes represents primary or secondary males, respectively (Fig. 7). Nevertheless, for proper growth and proliferation of germ cells, somatic cells around germ cells should coordinate and communicate with germ cells. However, how somatic cells differentiated and developed in correspondence with sex change of germ cells are still not known.

Cellular activity was indicated by immunohistochemical staining of Pcna in the present study. Nuclei of oogonia, cortical alveoli, and vitellogenic oocytes but not primary oocytes were active in gonads of the control group (juvenile fish to mature females) and in AI-treated male fish. Positive Pcna staining was also found in spermatogonia and spermatocytes. Ovarian tissue with the appearance of cortical alveoli and vitellogenic oocytes was considered the active stage and in the direction of ovarian growth. Negative Pcna staining of primary oocytes is consistent with the arrest stage of oocyte meiosis and also an inactive stage of ovarian tissue.

In the present study, gonadal tissues of two of 5 fish in AI-treated juvenile fish completely changed sex into various stages with male germ cysts (Fig. 4B). The AI was involved in depleting estrogens and caused early sex change in the protogynous honeycomb grouper *E. merra* (Bhandari et al. 2004). Therefore, it can be inferred that estrogens

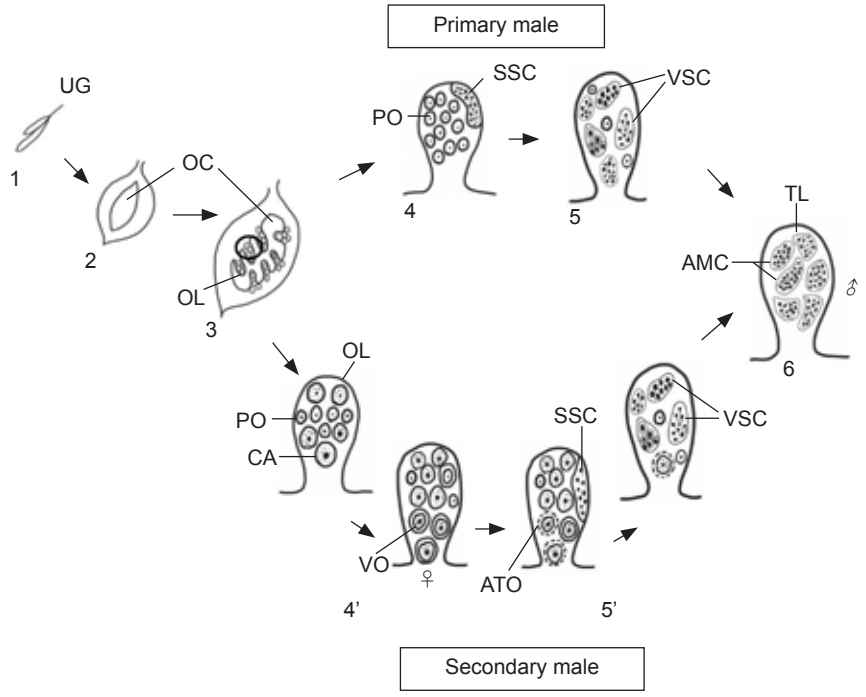

Fig. 7. Schematic illustrations of gonadal characteristics corresponding to development of the protogynous orange-spotted grouper *Epinephelus coioides*. The developmental processes can be illustrated by the following 6 steps. (1) An undifferentiated and forked gonad is present in the fish at about 1 mo of age. (2) Gonads in fish at about 4-5 mo of age formed an ovarian cavity, while the ovarian lamella had not yet developed. (3) Ovarian lamellae including oogonia and primary oocytes developed within the ovarian cavity of gonads of fish at about 7 mo of age. Primary and secondary males developed separately after this step. For brief illustrations, a single ovarian lamella (circle) was chosen to represent the status of the gonad in the following. (4) For development of primary males, a single spermatogenic cyst (SSC) containing spermatogonia, spermatocytes, spermatids, and spermatozoa was present at the margin of the ovarian lamella with primary oocytes. (4') For development of secondary males, the ovarian lamellae only developed different stages of oocytes and had no spermatogenic cysts yet. At the end, vitellogenic oocytes proliferated within the ovarian lamellae. (5) Various stages of spermatogenic cysts (VSCs) containing spermatogonia, spermatocytes, spermatids, and spermatozoa were intermingled with primary oocytes. (5') An SSC appeared at the margin of the ovarian lamellae and contained vitellogenic oocytes and atretic oocytes. Then a sex change occurred, and VSCs became the dominant tissue within the testicular lamellae. (6) Abundant mature cysts proliferated in the testicular lamellae constituting the mature testicular tissue in gonads of sex-changed fish. AMC, abundant mature cysts distributed in the testicular lamellae; ATO, atretic oocyte; CA, cortical alveolar oocyte; OC, ovarian cavity; OL, ovarian lamella; PO, primary oocyte; TL, testicular lamella; UG, undifferentiated gonad; VO, vitellogenic oocyte.

may also play an important role in female sex differentiation during the period of about 3-5 mo after hatching in the orange-spotted grouper. Furthermore, due to the difficulty of implanting the AI into 3-mo-old juvenile fish, we just applied the AI orally to juvenile fish. For 1-2-yr-old fish, both oral administration and implantation were conducted. However, it seemed that there was no significant difference in gonadal development between these 2 manipulations (Table 1). Owing to difficulties in differentiating primary from second males according to their outer body information at the beginning of the experiment, the experimental fish showed variations in the gonadal status. That was probably the reason why we could not obtain significant differences in sex change between the control and AI-treated groups.

Immunohistochemical staining of P450arom appeared in follicular layers of oogonia, cortical alveolar oocytes, and vitellogenic oocytes but not in primary oocytes. These data indicated that aromatase plays an important role in the development of female germ cells, especially early oogonia and growing oocytes in grouper. In contrast, follicular layers around primary oocytes in the estrogen-fed and AI-fed juvenile protandrous black porgy *Acanthopagrus schelegeli* exhibited immunoreactivity to P450arom (Wu et al. 2008). It was interesting to observe that testicular tissues in the sex-changing male grouper (such as cysts, spermatids, and spermatogonia) showed positive aromatase staining. Somatic cells surrounding spermatogenic germ cells also expressed positive signals in gonads of the juvenile protandrous black porgy (Wu et al. 2008), and these are consistent with the results of the present study in the AI-treated sex-changed grouper. Low levels of estrogens facilitated testicular development (spermatogenesis and androgen production) in the protandrous black porgy (Chang et al. 1995, Wu et al. 2008). Estrogen was suggested to play important roles in the early spermatogenetic cycle in the male Japanese eel *Anguilla japonica* (Miura et al. 1999). However, the immunoreactivity of P450arom in the testis in any phase was negative in the hermaphrodite gobiid fish *Trimma okinawae* (Sunobe 2005). Therefore, the roles of P450arom in gonads of male fish are interesting and probably dependent on specific reproductive and endocrinological situations.

In conclusion, in the present study, we propose the histological characteristics distinguishing primary from secondary males in gonadal development of the diandric type in the protogynous orange-spotted grouper *E. coioides*. Estrogens are involved in sex differentiation and sex change in the orange-spotted grouper. Immunohistochemical staining of cell activities and P450arom was also determined in gonadal development.

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REFERENCES

Asoh K, M Kasuya. 2002. Gonadal development and mode of sexuality in a coral-reef dameselfish, *Dascyllus trimaculatus*. J. Zool. Lond. **256:** 301-309.

Bancroft JD, HC Cook. 1994. Clinical Cytology. Manual of Histological Techniques and their Diagnostic Application. Edinburgh: Churchill Livingstone, pp. 323-342.

Besseau L, S Bruslé-Sicard. 1995. Plasticity of gonad development in hermaphroditic sparids: ovotestis ontogeny in a protandric species, *Lithognathus mormyrus*. Environ. Biol. Fish. **43:** 255-297.

Bhandari RK, M Higa, S Nakamura, M Nakamura. 2004. Aromatase inhibitor induces complete sex change in the protogynous honeycomb grouper, *Epinephelus merra*. Mol. Reprod. Develop. **67:** 303-307.

Chang CF, EL Lau, BY Lin. 1995. Stimulation of spermatogenesis or of sex reversal according to the dose of exogenous estradiol-17β in juvenile males of protandrous black porgy, *Acanthopagrus schlegeli*. Gen. Comp. Endocrinol. **100:** 355-367.

Chang CF, MF Lee, GR Chen. 1994. Estradiol-17β associated with the sex reversal in protandrous black porgy, *Acanthopagrus schlegeli*. J. Exp. Zool. **268:** 53-58.

Grandcourt EM, TZ Al Abdessalaam, F Francis, AT Al Shamsi, SA Hartmann. 2009. Reproductive biology and implications for management of the orange-spotted grouper *Epinephelus coioides* in the southern Arabian Gulf. J. Fish Biol. **74:** 820-841.

Guiguen Y, A Fostier, F Piferrer, CF Chang. 2009. Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. Gen. Comp. Endocrinol. **165:** 352-366.

Huang JD, MF Lee, CF Chang. 2002. The morphology of gonadal tissue and male germ cell in the protandrous black porgy, *Acanthopagrus schlegeli*. Zool. Stud. **41:** 216-227.

Kitano T, K Takamune, Y Nagahama, SI Abe. 2000. Aromatase inhibitor and 17α-methyltestosterone cause sex-reversal from genetical females to phenotypic males and suppression of P450 aromatase gene expression in Japanese flounder (*Paralichthys olivaceus*). Mol. Reprod. Develop. **56:** 1-5.

Lee MF, JD Huang, CF Chang. 2008. The development of ovarian tissue and female germ cells in the protandrous black porgy fish, *Acanthopagrus schlegeli*. Zool. Stud. **47:** 302-316.

Lee MF, JD Huang, CF Chang. 2011. Development of the genital duct system in the protandrous black porgy, *Acanthopagrus schlegeli*. Anat. Rec. **294:** 494-545.

Liu M, Y Sadovy. 2009. Gonad development during sexual differentiation in hatchery-produced orange-spotted grouper (*Epinephelus coioides*) and humpback grouper (*Cromileptes altivelis*) (Pisces: Serranidae, Epinephelinae). Aquaculture **287:** 191-202.

Miura T, C Miura, T Ohta, MR Nader, T Todo, K Yamauchi. 1999. Estradiol-17β stimulates the renewal of spermatogonial stem cells in males. Biochem. Biophys. Res. Comm. **264:** 230-234.

Murata R, H Karimata, MA Alam, M Nakamura. 2009. Gonadal sex differentiation in the Malabar grouper, *Epinephelus malabaricus.* Aquaculture **293:** 286-289.

Nakamura M, T Kobayashi, XT Chang, Y Nagahama. 1998. Gonadal sex differentiation in teleost fish. J. Exp. Zool. **281:** 362-372.

Nozu R, Y Kojima, M Nakamura. 2009. Short term treatment with aromatase inhibitor induces sex change in the protogynous wrasse, *Halichoeres trimaculatus*. Gen. Comp. Endocrinol. **161:** 360-364.

Sadovy Y, DY Shapiro. 1987. Criteria for the diagnosis of hermaphroditism in fishes. Copeia **1:** 136-156.

Sunobe T, M Nakamura, Y Kobayashi, T Kobayashi, Y Nagahama. 2005. Aromatase immunoreactivity and the role of enzymes in steroid pathways for inducing sex change in the hermaphrodite gobiid fish *Trimma okinawae*. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. **141:** 54-59.

Wu GC, S Tomy, M Nakamura, CF Chang. 2008. Dual roles of cyp19a1a in gonadal sex differentiation and development in the protandrous black porgy, *Acanthopagrus schlegeli*. Biol. Reprod. **79:** 1111-1120.

Yamamoto T. 1969. Sex differentiation. *In* WS Hoar, DJ Randall, eds. Fish Physiology. Vol. 3. New York: Academic Press, pp.117-175.

Yeh SL, CM Kuo, YY Ting, CF Chang. 2003. Androgens stimulate sex change in protogynous grouper, *Epinephelus coioides*: spawning performance in sex-changed males. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. **135:** 375-382.