Zoological Studies **47**(3): 302-316 (2008)

Zoological Studies

Development of Ovarian Tissue and Female Germ Cells in the Protandrous Black Porgy, *Acanthopagrus schlegeli* (Perciformes, Sparidae)

Mong-Fong Lee[1], Jing-Duan Huang[2], and Ching-Fong Chang[1,*]

[1]*Department of Aquaculture, National Taiwan Ocean University, Keelung 202, Taiwan*
[2]*Institute of Marine Biology, National Taiwan Ocean University, Keelung 202, Taiwan*

(Accepted November 7, 2007)

Mong-Fong Lee, Jing-Duan Huang, and Ching-Fong Chang (2008) Development of ovarian tissue and female germ cells in the protandrous black porgy, *Acanthopagrus schlegeli* (Perciformes, Sparidae). *Zoological Studies* **47**(3): 302-316. The morphology of developing bisexual gonads and female germ cells in the protandrous black porgy, *Acanthopagrus schlegeli* (Sparidae), is described using light and electron microscopy. The central (ovarian) cavity and sex differentiation were observed in the differentiating gonad at 5 mo of age. Testicular tissue was the main tissue in the gonad with a small portion of ovarian tissue limited to the area around the central cavity and main blood vessel. Oogonia which were grouped as cell nests in the germinal epithelium and oocytes appeared during the period from the differentiated gonad to the 1st spawning period. Ovarian lamellae, containing oogonia and primary oocytes, developed towards the central cavity after the 1st spawning season. The testicular tissue regressed but ovarian tissue developed during the non-spawning period in 1^+- and 2^+-yr-old fish. Female germ cells were classified into oogonia, chromatin-nucleolus oocytes, perinucleolar oocytes, cortical alveolar oocytes, vitellogenic oocytes, and maturing oocytes. Only primary oocytes, the most advanced type of oocyte, could be found in the bisexual ovary before the occurrence of the sex change. Testicular tissue regressed together with the development of vitellogenic oocytes during the sex change in some 3-yr-old fish. A blood vessel reached each oocyte. There were light and dark electron-dense areas in the germinal epithelium connected by cell junctions. Oogonia and oocytes had many mitochondria and highly electron-dense structures such as cement and nuage. Granulosa and theca cells surrounded the oocytes. Organelles such as mitochondria, endoplasmic reticula, and Golgi bodies were found in the highly electron-dense area of granulosa cells at the vitellogenic oocyte stage. Microvilli extended from oocytes through the zona radiata to granulosa cells. The developmental profile of ovarian lamellae in ovarian tissue and changes in the testicular/ovarian tissue in the bisexual gonad were further summarized. These histological data provide important information for further studies on the molecular mechanism of sex differentiation and sex change in the protandrous black porgy. http://zoolstud.sinica.edu.tw/Journals/47.3/302.pdf

Key words: Gonadal development, Sex differentiation, Sex change, Fish, Oocyte development.

Oocyte and ovarian development, growth, and maturation have been described in many gonochoric fish: in the swordfish (*Xiphias gladitus*) by Arocha (2002), striped bass (*Morone saxatilis*) by Mylonas et al. (1997), carp (*Cyprinus carpio*) by Linhart et al. (1995), zebrafish (*Brachydanio rerio*) by Selman et al. (1993), pipefish (*Syngnathus scovelli*) by Begovac et al. (1988), red sea bream (*Pagrus major*) by Matsubara et al. (1988), and perch (*Perca fluviatilis*) by Treasurer et al. (1981). However, there are few such reports in hermaphrodite fish. Studies on the development of oocytes and ovarian tissue have been reported in protogynous fish: in the ricefield eel (*Monopterus albus*) by Chan et al. (1967), wrasse (*Thalassoma duperrey*) by Nakamura et al. (1989), black sea perch (*Centropristis striatus*) by Cochran et al. (1991), and damselfish (*Dascyllus albisella*) by

*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

Asoh et al. (2001). On the contrary, there is less information on the development of oocytes in protandrous fish mainly due to the difficulty in obtaining female fish.

The black porgy, *Acanthopagrus schlegeli* Bleeker, is a marine protandrous hermaphrodite. Fish are functional males for the 1st - 2nd yr of life but begin to sexually change into females during the 3rd yr (Chang and Yueh 1990). However, only about 40% of cultured black porgies change to females at 3 yr of age, while the rest remain functional males during the 3rd spawning season. The fish has bisexual gonadal tissue during the 1st - 3rd yr of age before the sex change (Huang et al. 2002). Testicular tissue undergoes intensive spermatogenesis, and testicular development occurs during the spawning season, resulting in the fish becoming male. During the spawning season at 3 yr of age, the testicular tissue regresses, together with the development of ovarian tissue and vitellogenic oocytes, resulting in a sex change in fish from male to female. The direction of development or regression of the testicular tissue seems to be very important to the occurrence of sex change to the female phase in 3-yr-old black porgy. This sex pattern provides a unique model to study the mechanism of sexual development in fish. Furthermore, studies of the ultrastructure of female germ cells and the relationship between female germ cells and somatic cells are critical to understanding the molecular mechanism of ovarian development. Histological changes in the bisexual gonad and gonadal characteristics during sex change are complicated processes, but this information is important for understanding the sex-change mechanism. However, a detailed histological description of ovarian development and its correlation with the sex change in black porgy are still lacking. Therefore, the objectives of the present study were to examine female germ cells at various stages of development by light microscopy, and also to further describe female germ cells and somatic cells by scanning and transmission electron microscopy.

MATERIALS AND METHODS

Black porgy

Black porgies at the ages of 0-3 yr were collected. The gonads were dissected out and prepared for the histological studies. The annual life cycle of the black porgy was divided into a spawning period (from Jan. to Mar.), a post-spawning period (Apr. to May), an intersex period (June to Sept.), and a pre-spawning period (Oct. to Dec.) (Huang et al., 2002).

Gonadal histology by light microscopy

The gonad was fixed in Bouin's solution for 24-72 h (according to the size of the gonad), followed by washing with 70% alcohol solution to remove any picric acid. Tissues were dehydrated in an alcohol series of 80%, 85%, 90%, 95%, and 100%, and then soaked in xylene. They were embedded in paraffin wax and sectioned at 5 μm thicknesses. Paraffin sections were stained with hematoxylin/eosin (H and E) or Weigert's iron hematoxylin/trichrome according to previous studies (Huang et al. 2002). The stained sections were sealed with Entellan (Merck, Darmstadt, Germany). The ovarian tissues and oocytes were observed under an optical microscope.

Scanning electron microscopy (SEM)

The gonad was cut cross-wise into slices (at thicknesses of 1-5 mm). Gonad tissues and oocytes were soaked in a fixative solution (0.1 M buffer solution of sodium cacodylate, 3% glutaraldehyde at pH 7.4, and 1% paraformaldehyde) at 4°C for 24 h. They were then washed with a 0.1 M buffer solution of sodium cacodylate, post-fixed with a cold 1% osmium tetroxide (OsO_4) solution for 2 h, followed by a final wash in a 0.1 M buffer solution of sodium cacodylate. The fixed tissue was dehydrated in an ethanol series. Samples were then put into a critical-point dryer (HCP-2 critical point dryer, Hitachi, Tokyo, Japan) for critical-point drying. The dried samples were pasted onto an aluminum platform and then gilded by E101 ion sputter (Hitachi). Finally, they were put into a Hitachi S-2400 scanning electron microscope for observation.

Transmission electron microscopy (TEM)

The gonad was cut into small cubes 0.5 mm long, and then fixed and dehydrated as described for the SEM sample. Spurr's solutions with different proportions of alcohol and pure Spurr's medium (3:1, 1:1, and 1:3) were prepared. Samples were soaked in these Spurr's solutions in the order given and finally placed on a Spurr's plate, filled with Spurr's medium, and placed at 70°C for 14 h for hardening. Ultrathin sections

were cut at 70-100 nm using an ultramicrotome (RMC MTX ultramicrotome, Research Manufacturing, Tucson, AZ, USA). Sections were stained with uranyl acetate and lead citrate, and examined with a Hitachi H-600 TEM.

RESULTS

Ovarian structure of the gonads

The central cavity (ovarian cavity) with primordial germ cells appeared in gonads of juvenile fish at 5 mo of age (Figs. 1A-C). Blood vessel organization was clearly observed at the side of the central cavity (Figs. 1B, C). Putative ovarian tissue developed from the inner side of the central cavity, while putative testicular tissue appeared in the distal part of the gonad (Fig. 1B). The connective tissue was at the outer side of the ovarian tissue (Figs. 1D), and it separated the ovarian and testicular tissues at a later stage of development (Fig. 1D). Before the 1st spawning period, germ cells in the germinal epithelium were mainly oogonia, which were grouped into cell nests (Fig. 2A). As the spawning period approached, the central cavity and ovarian tissue were pushed towards the edge of the gonad by the maturing testicular tissue (Fig. 2B). In the 1st and 2nd spawning periods, all fish showed male function (Fig. 2B).

During the post-spawning season and intersex period in 1^{+}-yr-old fish, the testicular tissue regressed, yellow bodies appeared (Fig. 3A), and an ovarian lamella developed in the ovarian tissue (Fig. 3B). The ovarian lamella was the basic structure of the ovarian tissue (Fig. 3B). At this early developmental stage of ovarian tissue, the ovarian lamellae (composed of the germinal epithelium, germ cells, and follicle cells) began to develop and protrude from the cell nests (Fig. 3D). Germ cells in the early ovarian lamellae were mainly oogonia with a few deeply eosin-staining oocytes (Fig. 3B). Later, many oogonia rapidly developed into primary oocytes in the ovarian lamellae, pushing the oogonia and early oocytes towards the edge of the ovarian lamella wall (Fig.

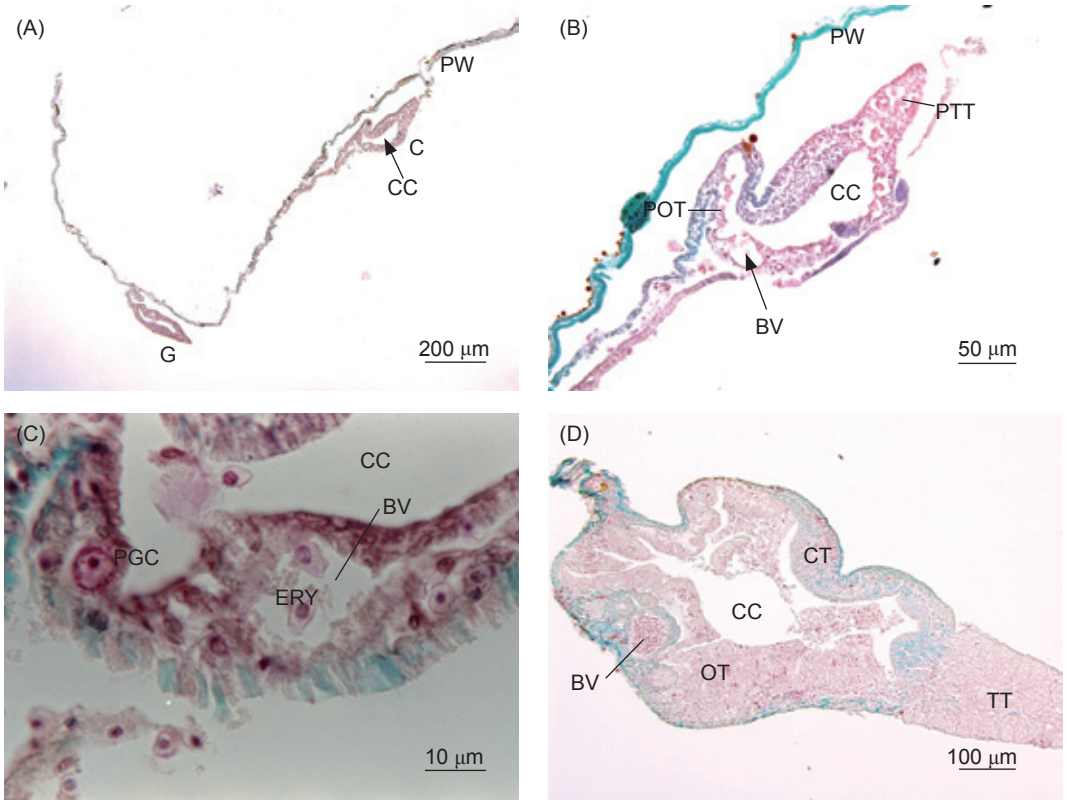

Fig. 1. Early gonadal development at the fingerling stage in 0^{+}-yr-old fish. (A) Paired gonads with central cavities in 5-mo-old fish. (B) Magnified 5-mo-old gonad with putative ovarian and testicular tissues. (C) Appearance of a primordial germ cell near the lateral side of the blood vessel on the inner wall of the central cavity. (D) Ovarian and testicular tissues separated by connective tissue in an 8-mo-old gonad.

3C). The ovarian lamellae became branched and hyperplasic (Fig. 3D) and protruded into the central cavity (Fig. 3E). Finally, the fish had a well-developed bisexual gonad (Figs. 4A, B) with more testicular tissue than ovarian tissues in the post-spawning season (Fig. 4A), and a bisexual gonad mainly with ovarian tissue and a small portion of testicular tissue in the intersex period (Fig. 4B). A prominent artery and veins also appeared in the wall of the ovary (Figs. 5A, B). Blood vessels passed through the ovarian lamellae with their branches and capillaries reaching each oocyte (Figs. 5C, D). In the 3rd yr, testicular tissue had completely regressed in some fish, resulting in a sex change and development of vitellogenic ovaries in these fish (Figs. 6A, B).

Two types of germinal epithelium were present on the inner side of the central cavity. One was a light-colored germinal epithelium with slight electron density, and the other was a dark germinal epithelium with strong electron density (Fig. 7A). The germinal epithelia were connected to each other by cell junctions (Fig. 7B). Oogonia (Fig. 7A) were localized in the germinal epithelium. Oocytes and the germinal epithelium were separated by a basal lamina (Fig. 7B). Cytoplasmic extensions of granulosa cells surrounded the primary oocytes (Fig. 7A).

Oogenesis

Based on the characteristics of female germ cells, oocytes were classified into 6 phases: an oogonia phase (Fig. 2A), primary oocytes (Fig. 9A) with a chromatin-nucleolus (Fig. 8A), a perinucleolar phase (Fig. 8B), a cortical alveolar phase (Fig. 8C), a vitellogenic phase (Fig. 8D), and a maturation phase (Fig. 8E).

Oogonia phase: Oogonia were often found in cell nests towards the inner wall of the central cavity in gonads at 6 mo old to the 1st spawning period, when the testicular tissue was the main developing tissue (Fig. 2A). Oogonia had many mitochondria. Highly electron-dense structures such as cement surrounded by mitochondria and nuage were present around the nucleus (Fig. 7A).

Chromatin-nucleolus and perinucleolar phases: After the 1st spawning season, the ovarian lamella began to develop, and oogonia rapidly grew into chromatin-nucleolus oocytes each with a large nucleolus (Fig. 8A) and perinucleolar oocytes each with small nucleoli (Fig. 8B). The cytoplasm of chromatin-nucleolus oocytes and perinuclear oocytes had basophilic activity and clear chromatin, and there were numerous nucleoli in the nucleus (Figs. 8A, B). Oocytes remained at this chromatin-nucleolus and perinucleolar stage in bisexual gonads until the sex change. Nucleoli of primary oocytes migrated out of the nucleus (Fig. 9B), and oocytes had many mitochondria (Fig. 9C) and cytoplasmic microvilli (Fig. 9D). In this phase, 2 follicle layers surrounded the oocytes, namely granulosa cells (forming the inner layer) and theca cells (forming the outer layer), separated by a basal lamina (Fig. 9D). Theca cells and granulosa cells had a flat shape and extended around the oocytes (Fig. 9D).

Cortical alveoli phase and vitellogenic phase: During the 3rd pre-spawning period, when fish were about to change sex, oocytes entered the cortical alveoli phase. Transparent particles (cortical alveoli) were observed around the nucleus of oocytes in the beginning (Fig. 8C) and later moved to the periphery of the cytoplasm (Fig.

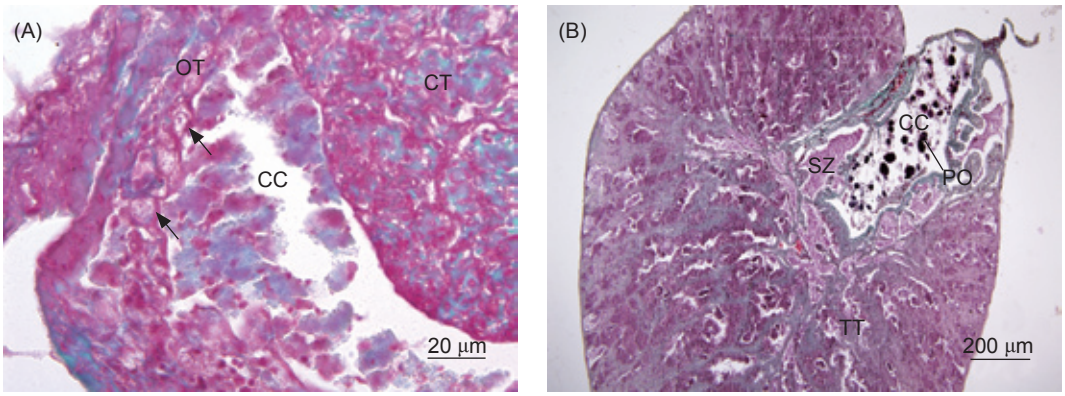

Fig. 2. Gonadal development in 7-mo- and 1-yr-old fish. (A) Oogonia grouped as cell nests (arrow) in the germinal epithelium in 7-mo-old fish. (B) The central cavity was compressed to the edge of the gonad with the development of mature testicular tissue which were filled with spermatozoa in 1-yr-old fish during the spawning period.

8C). In the vitellogenic stage, the yolk gradually accumulated and finally filled the cytoplasm (Fig. 8D). During the maturing process, small oil droplets fused together to become a large drop. Yolk particles also gradually accumulated, and the nucleus migrated to the cytoplasmic periphery (Fig. 8E).

Layers of the zona radiata formed during the vitellogenic stage (Fig. 10A). As the oocytes grew and matured, the zona radiata layers became thick and were divided into the Z1 layer (close to the follicle cells) with an electron-transparent layer, the

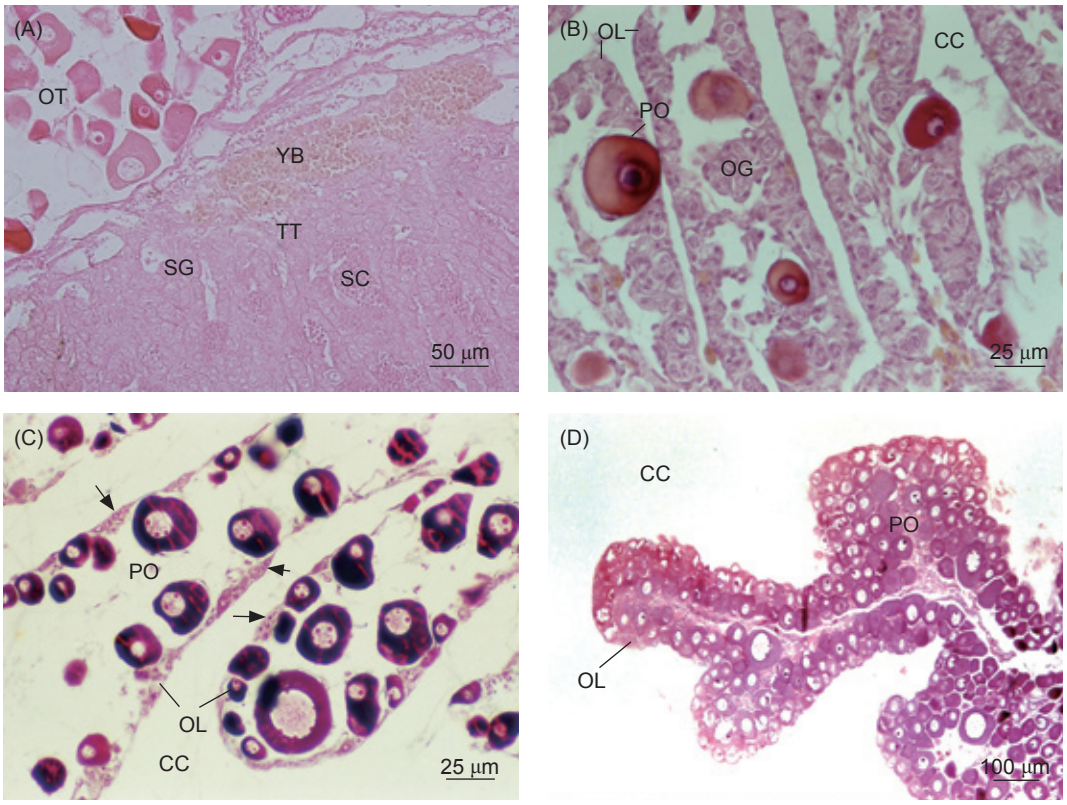

Fig. 3. Gonadal structure after the 1st spawning period in 1⁺-yr-old fish. (A) Testicular tissue regressed and yellow bodies appeared after the spawning period. (B) Ovarian lamella began to form mainly with oogonia and a few primary oocytes right after the post-spawning season. (C) Development of oogonia into primary oocytes in the ovarian lamellae during the late post-spawning period. Small oocytes and oogonia (arrow) were enclosed in the edge of the ovarian lamella wall. (D) Proliferation and elongation of ovarian lamellae (hyperplasia) during the post-spawning and intersex periods.

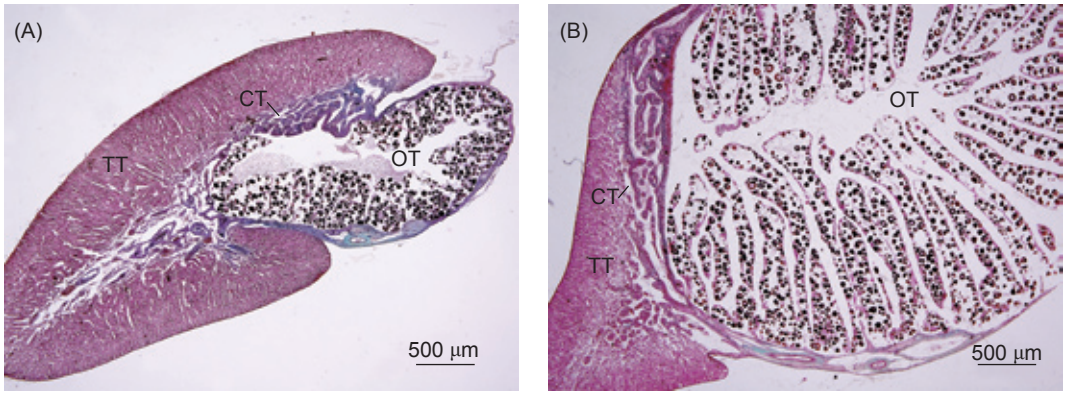

Fig. 4. Two types of bisexual gonads after the 1st spawning period in 1⁺-yr-old fish. A bisexual gonad with (A) testicular tissue as the main tissue during the early post-spawning period. (B) Ovarian tissue as the main tissue during the post-spawning and intersex periods.

Z2 layer as a long cylinder-shaped layer, and the Z3 layer (close to the oocytes) with several layers of a horizontal, overlapping, long structure (Figs. 10B, C). The Z3 layer increased in number (from 1 to 7 layers) during the growth and maturation process. The zona radiata layers were closely linked to each other and regularly arranged. Microvilli were stretched out from the cytoplasm of an oocyte, which passed through the oocyte membrane and penetrated the layer of the zona radiata, ultimately reaching granulosa cells (Figs. 10B, C).

The nuclei of granulosa cells were flat coin-shaped under SEM observation (Fig. 10D). The electron density of the cytoplasm appeared to be polarized with slight electron density on the side

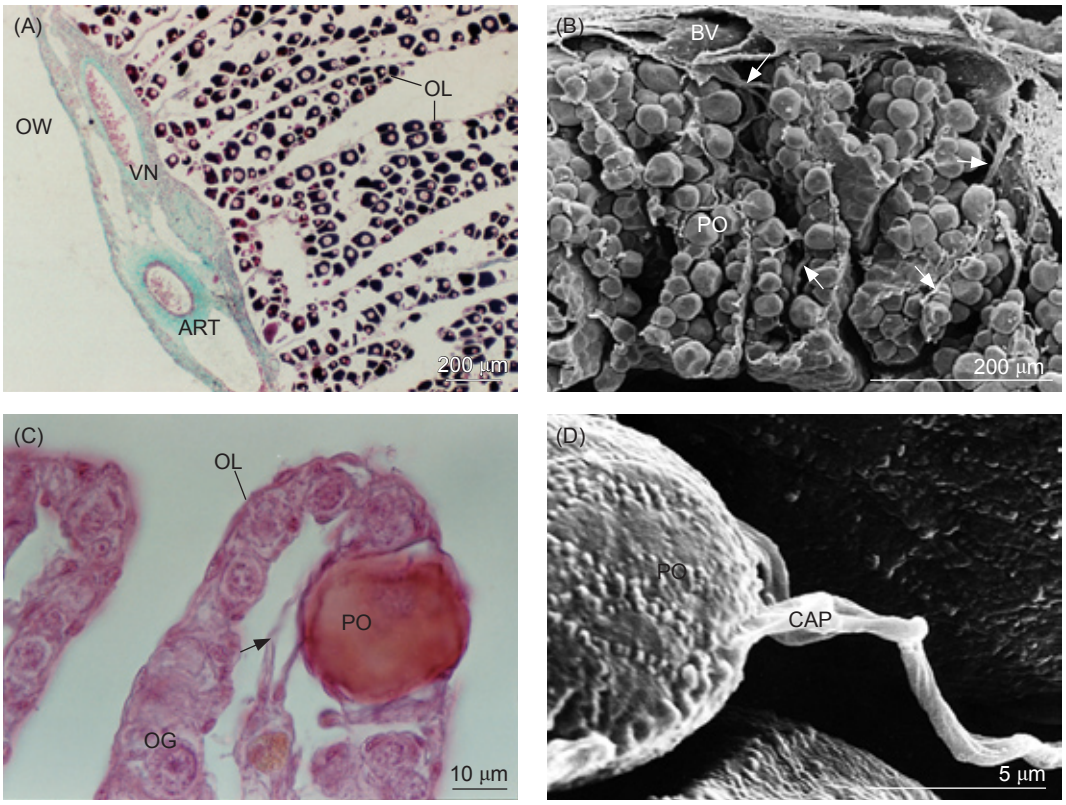

Fig. 5. Structure and distribution of the vascular system in the ovary of 1+-yr-old fish. (A) Blood vessels (an artery and vein) pass through the wall of the ovary. (B) Blood vessels (arrow) and ovarian lamellar structure under SEM. (C) Capillary branches (arrow) pass through the lumen of the ovarian lamella and reach each oocyte as revealed on the histological section stained with trichrome and (D) by SEM observation.

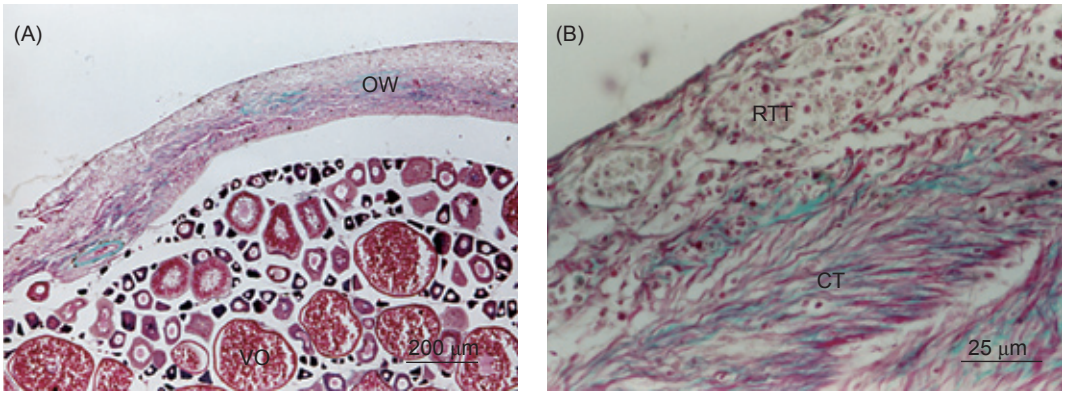

Fig. 6. Connective tissue in the ovarian wall of 3-yr-old fish. (A) Connective tissue separating the ovarian and testicular tissues becomes a part of the ovarian wall when a male fish changes sex to a female. (B) Regressed testicular tissue found in the ovarian wall.

close to the zona radiata layer and high electron density on the side close to the basal lamina and theca cells (Fig. 10B). Microvilli, extending from the oocytes, reached the highly electron-dense area of granulosa cells (Fig. 10B). Organelles, such as mitochondria, endoplasmic reticula, and Golgi bodies, were unevenly localized in the highly electron-dense area of granulosa cells (Fig. 10B).

Maturation and ovulation phase: After ovulation, the surface of the zona radiata had become the outer surface of the ovum (i.e., the vitellin envelope or chorion). A lattice with sunken stripes appeared, which was densely covered with membrane apertures (Figs. 11A, B). Below the surface layer of the ovum were rope-shaped interlocking apophyses on the inner wall of the

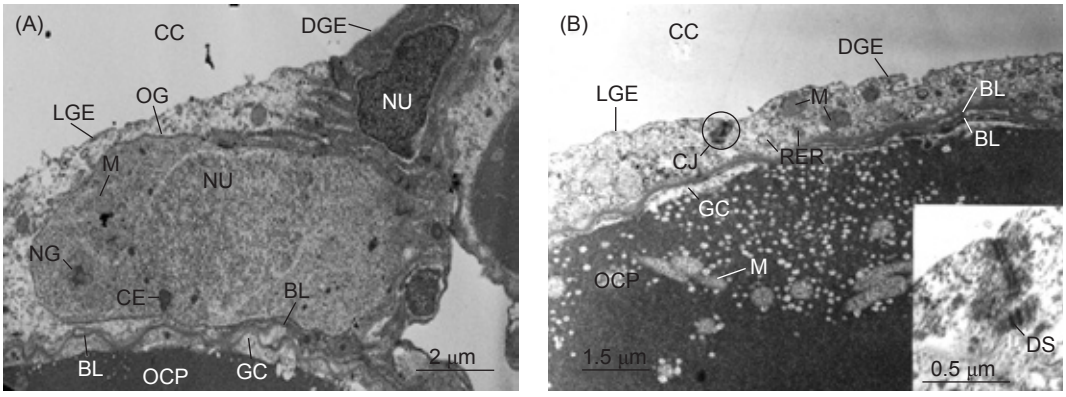

Fig. 7. Ultrastructure of the germinal epithelium by TEM in 1+-yr-old fish. (A) The germinal epithelium as a part of the wall of the ovarian lamella. Two types of germinal epithelia were found in the ovarian lamella: dark germinal epithelium and light germinal epithelium. Oogonia were localized in the germinal epithelium. Oogonia possessed a nuage structure, which was surrounded by mitochondria and had a highly electron-dense area, and a cement structure, which also had high electron density and was localized around the nucleus. (B) Cell junction (circle) joining the germinal epithelia. Oocytes, surrounded by the follicular layer, were separated from the germinal epithelium by the basal lamina. Insert, Magnified view of a cell junction (desmosome).

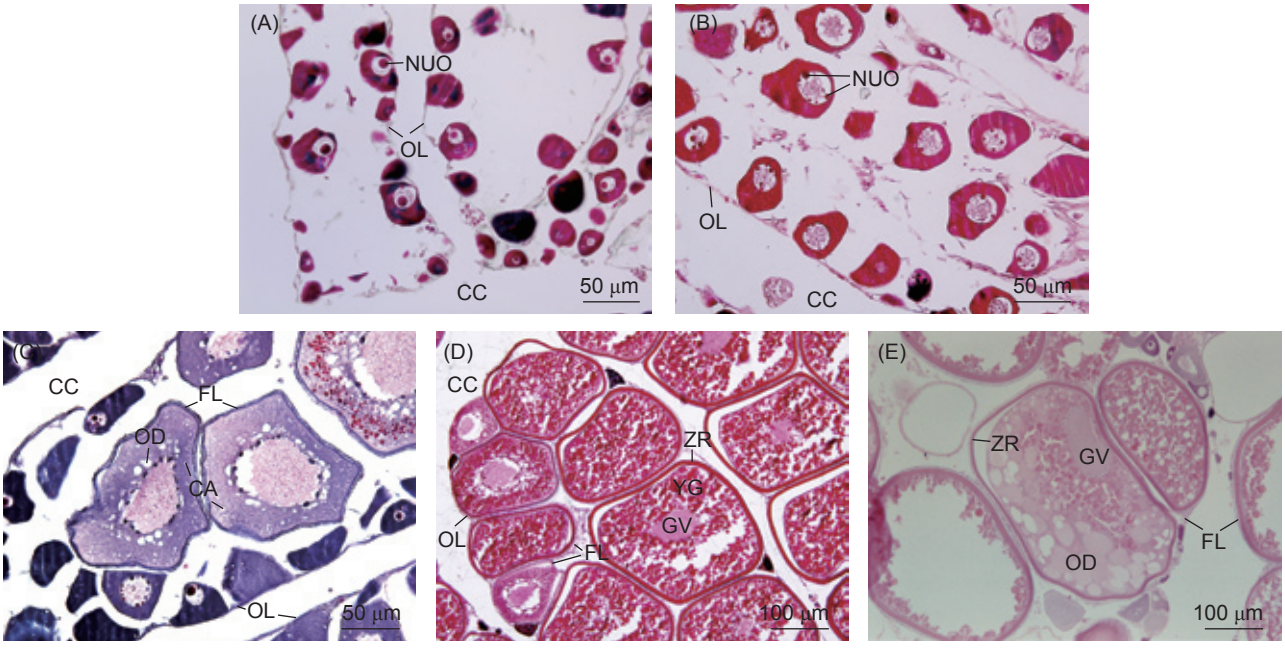

Fig. 8. Differentiation and development of oocytes in 1+-3-yr-old fish. (A) Oocytes in the chromatin-nucleolus phase exhibiting a large nucleolus in the nucleus. (B) Oocyte in the perinucleolar phase possessing chromatin and a small nucleolus. (C) Oocyte in the cortical alveoli phase with cortical alveoli in the cytoplasm and around the nucleus. (D) Vitellogenic phase with yolk granules in the cytoplasm. (E) The germinal vesicle had moved to the periphery of the maturing oocytes and oil droplets had begun to fuse.

zona radiata (Fig. 11C). On the surface of the ovum, there were corresponding hollow stripes and many small apertures (Fig. 11D). Each oocyte had a spiral funnel-shaped micropyle (Figs. 12A, B). After fertilization, the spiral funnel-shaped structure of the micropyle extended upward and closed (Fig. 12C).

Regression of the ovary

In the 3rd post-spawning period, the ovulated ovary still had both the ovarian lamellae and follicle residues (Figs. 13A, B). In the ovulated ovarian lamellae, there were few oocytes in either the perinucleolar or cortical alveoli phase. On the contrary, ovarian lamellae in the non-ovulated ovary still had perinucleolar and vitellogenic oocytes which had undergone decomposition (Fig. 13C). The zona radiata layer of the non-ovulated ovary had also decomposed into fragments (Fig. 13D).

Development of the germinal epithelia and ovarian lamellae

The development of the ovarian lamellae is summarized in figure 14. Oogonia first appeared in the germinal epithelium, which then moved to the space between the ovarian wall and germinal epithelium, and grew to become primary oocytes. The oogonia remained at the edge of the germinal epithelium. Advanced germ cells (such as primary oocytes) with blood vessels and germinal epithelium (containing oogonia) extended towards the central cavity forming the ovarian lamellae.

Development of ovarian and testicular tissues in the bisexual gonad

The development of ovarian and testicular tissues in the bisexual gonad is summarized in figure 15. In the early differentiated gonad, early female germ cells first appeared in the putative ovarian tissue which was close to the ovarian cavity and main blood vessel. The putative

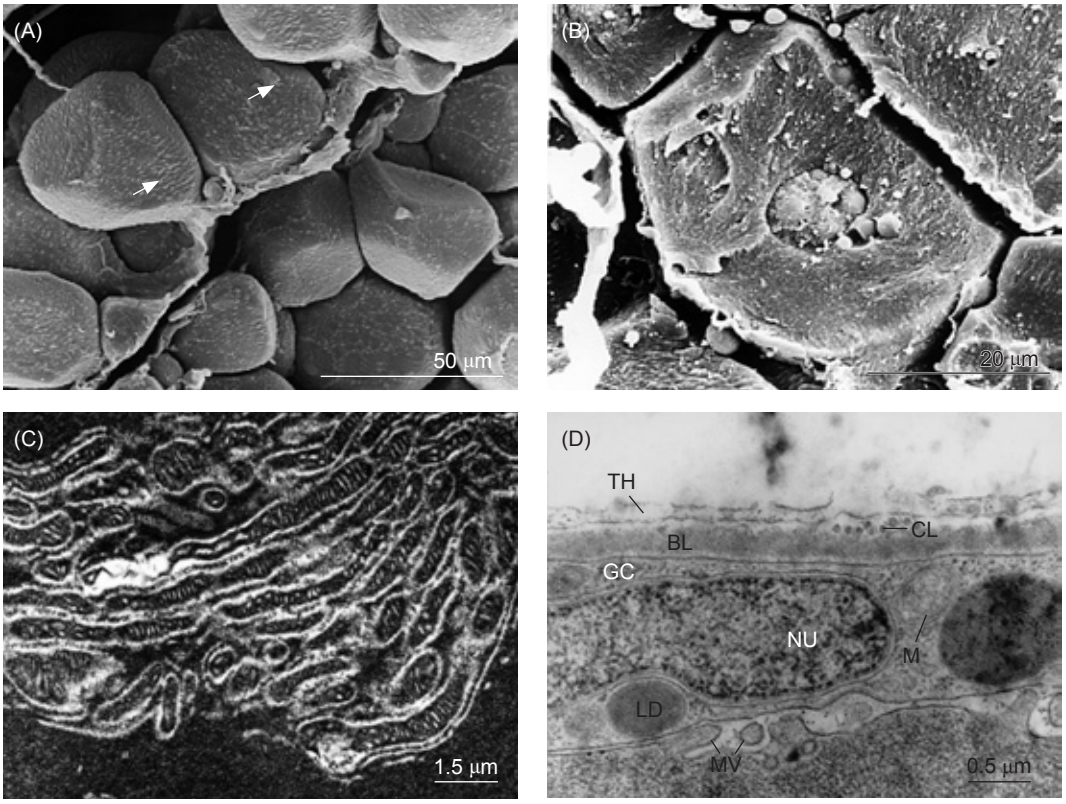

Fig. 9. Ultrastructure of primary oocytes under SEM and TEM in 2^{+}-yr-old fish. (A) Oocytes with small warts (arrow) on the surface and a blood vessel under SEM. (B) The nucleolus had moved from the nucleus to the cytoplasm. (C) The presence of abundant mitochondria in the oocyte cytoplasm. (D) Formation of microvilli by projecting cytoplasm of the oocyte. Granulosa cells were localized close to the oocyte, while theca cells were at the outer edge of the follicular layer. The basal lamina separated granulosa cells from theca cells. Collagen fibers were present between theca cells and the basal lamina.

testicular tissue was in the distal area of the gonad. The ovarian tissue developed from the central cavity and was separated from the testicular tissue by connective tissue. During the non-spawning season in 1^+- and 2^+-yr-old fish, the ovarian tissue became the dominant tissue. Testicular tissue became the dominant tissue in the bisexual gonad during the 1st and 2nd spawning seasons. In 3rd-yr fish, testicular tissue again developed, and fish remained functional males (Fig. 15; step 9A), or fish had changed sex and become females when the testicular tissue completely regressed. This was followed by development of ovarian tissue with vitellogenic oocytes in some 3-yr-old fish (Fig. 15; step 9B).

DISCUSSION

This is the 1st report to investigate the early development and ovarian growth of ovarian tissue in the bisexual gonad in relation to sex change in the protandrous black porgy using light, scanning, and transmission electron microscopy. The earliest period at which the central cavity was first observed in juveniles was at 5 mo of age. The central cavity is the most important characteristic of gonadal sex differentiation in fish (Nakamura et al. 1998). We also observed differentiation of ovarian tissue as well as testicular tissue in concomitance with formation of the central cavity at the age of 5 mo. These characteristics did not appear in the gonad of 4-mo-old fish (data not shown). Our observation suggests that gonadal sex differentiation occurs at the age of 4-5 mo in the black porgy. Sex differentiation occurs much earlier in most gonochoristic fish species: 23-26 d in tilapia (*Oreochromis niloticus*) (Nakamura and Nagahama 1989); 15-20 d in females and 40-45 d in males of the round goby (*Neogobius melanostomus*) (Moiseeva 1984); 28 d in females and 42 d in males of the African catfish (*Clarias gariepinus*) (van den Hurk et al. 1989); 45-55 d in rainbow trout (*Salmo gairdneri*) (van den Hurk and Slof 1981); 65-75 d in females and 75-85 d in males of coho salmon (*Oncorhynchus kisutch*)

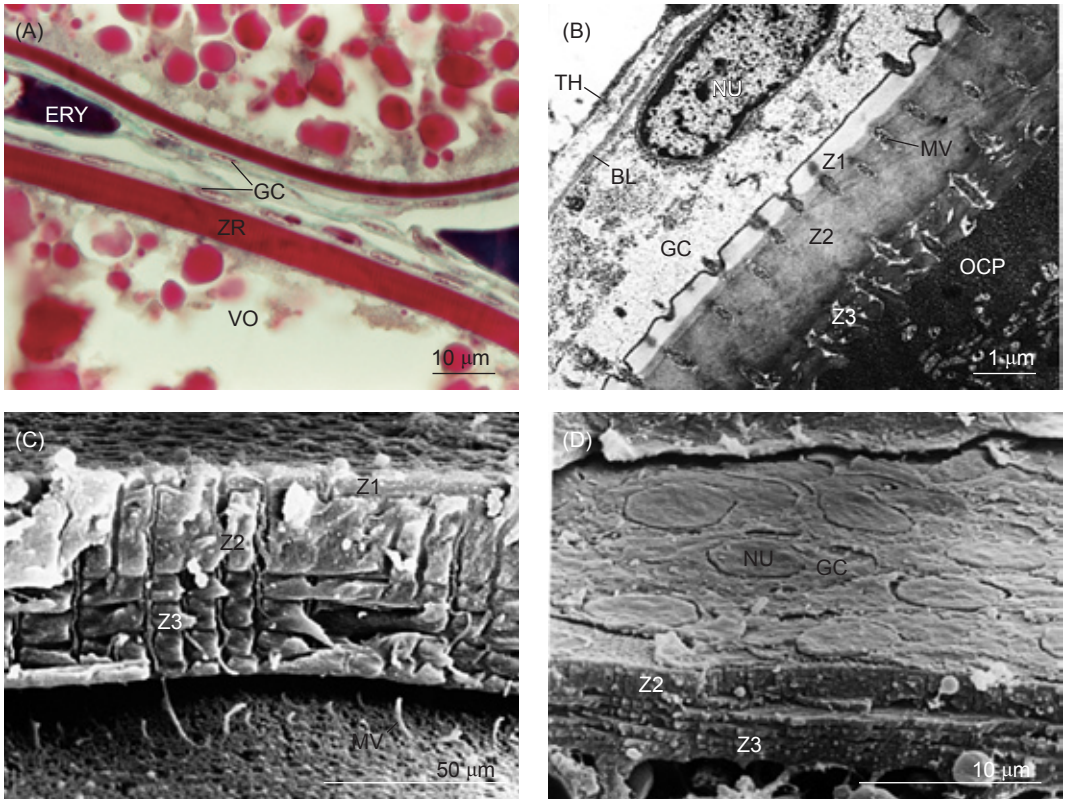

Fig. 10. Zona radiata structure in a vitellogenic oocyte in a 3-yr-old fish. (A) Vitellogenic oocytes with the zona radiata and granulosa cells. (B) The zona radiata became thicker and multilayered as the oocytes grew. Z1, The layer close to granulosa cells; Z2, the middle part which appeared columnar in shape; Z3, horizontal, overlapping, cuboidal layers. (C) Highly magnified image of the zona radiata with Z1, Z2, and Z3 layers under SEM. Many microvilli extend from the oocytes to the zona radiata. (D) Nuclei of granulosa cells had a coin shape under SEM.

(Feist et al. 1990); and 17 wk post-fertilization in the common carp (*C. carpio*), (Parmentier and Timmermans 1985), according to the histological characteristics of the gonads. Because connective tissue separates the testicular and ovarian tissues, the bisexual gonad in the black porgy is the delimited type. The structure of the black porgy ovary belongs to the cystovarian type, with a hollow organ and numerous projections of ovigerous lamellae (Dodd 1977).

Early female germ cells (oogonia) were found only in the germinal epithelium facing the central cavity, not in the connective tissue area of the ovarian wall. These histological data suggest that the source of female germ cells is the germinal epithelium. Our observation is consistent with the findings in common snook (*Centropomus undecimalis*) (Grier 2000). In contrast, the source of oocytes was considered to be the underlying ovarian stroma in *Fundulus heteroclitus* (Brumment et al. 1982).

Oogonia appeared in the germinal epithelium

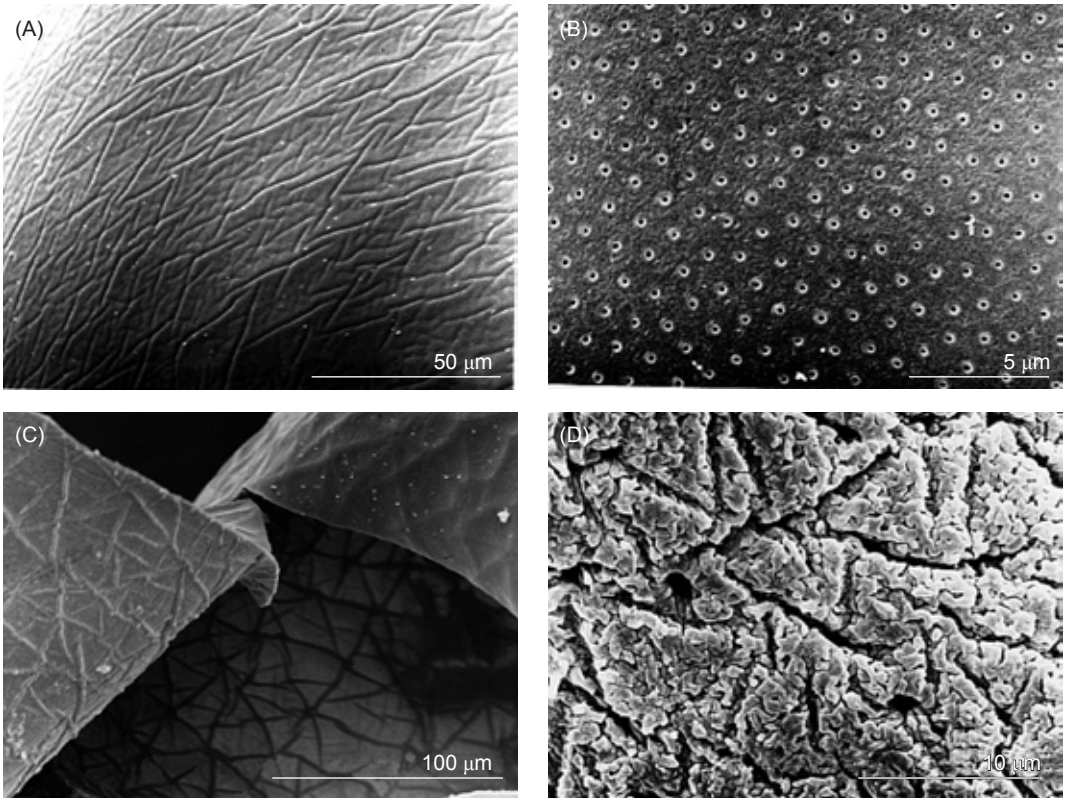

Fig. 11. Ultrastructure of the outer surface of an ovulated egg under SEM. (A) A lattice of sunken stripes appeared on the outer surface of an ovulated egg under SEM at low magnification. (B) Membrane pore structures on the surface of an ovulated egg under SEM at high magnification. (C) Rope-shaped interlocking structure on the inner wall of an ovulated egg. (D) A hollow vein and small apertures on the surface of the ooplasm after the egg membrane had been removed.

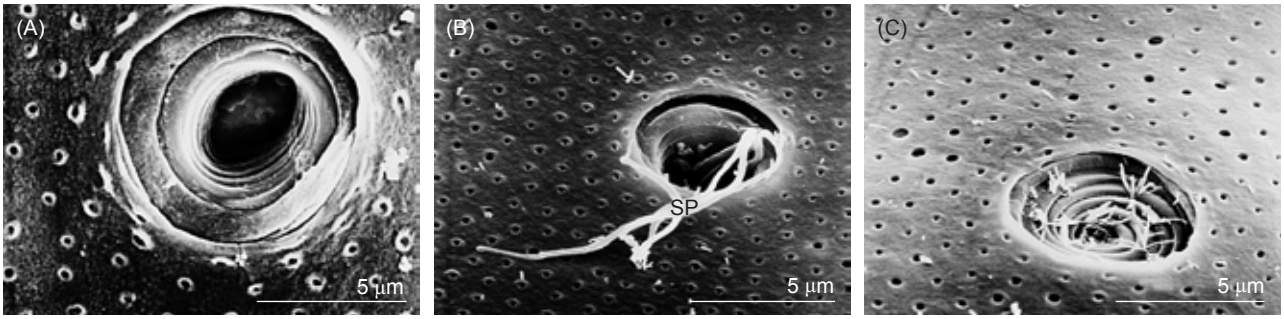

Fig. 12. Micropyle structure in an ovulated egg. (A) A spiral-funnel shaped structure placed deeply inward before fertilization. (B) Fertilized egg. (C) Spiral-funnel structure extended upward after fertilization.

on the side associated with the blood vessel which developed into ovarian tissue during early development. Cell nests with a cluster of mainly oogonia appeared in the germinal epithelium. Ovarian tissues (mainly with oogonia and a few oocytes) comprised only a small portion of the bisexual gonad during the period from sex differentiation to the 1st spawning period, when the ovarian lamella had not yet developed. During the post-spawning period after the 1st spawning period, we observed significant growth in ovarian tissue in concomitance with regression of the testicular tissue. Ovarian lamellae formed from the germinal epithelium during this period and extended towards the central cavity. This is the 1st time the ovarian lamellae have clearly been described during ovarian tissue development. Primary oocytes (at the chromatin and perinucleolus stage) were the most advanced oocytes found in the ovarian lamellae of ovarian tissue before the regression of testicular tissue in the bisexual gonad of the 2^+-yr-old fish. Vitellogenic oocytes appeared in the ovarian tissue only after the complete regression of the bisexual testicular tissue. The development of vitellogenic oocytes is considered to be a histological indicator of sex change in the protandrous black porgy (Chang et al. 1994, Du et al. 2005).

During the 1st and 2nd spawning periods (the functional male phase, mainly with testicular tissue) the ovarian tissue regressed, became a very small portion of the bisexual gonad, and was limited to the central cavity. In contrast, ovarian tissue grew to become the major tissue in the gonad during the non-spawning period with only a small portion of testicular tissue (in 1^+- and 2^+-yr-old fish). This thus suggests that a strong interaction between ovarian tissue and testicular tissue exists. Testicular tissue may play an inhibitory role in the development and growth of ovarian tissue and vitellogenic oocytes. Also, some factors may be lacking or insufficient in ovarian tissue during the non-spawning season which blocked further development of the ovarian tissue and inhibited the formation of vitellogenic oocytes in all 1^+-yr-old fish and some 2^+-yr-old fish. Plasma estradiol (E2) concentrations and gonadal aromatase activity in the black porgy were associated with natural sex change (Chang

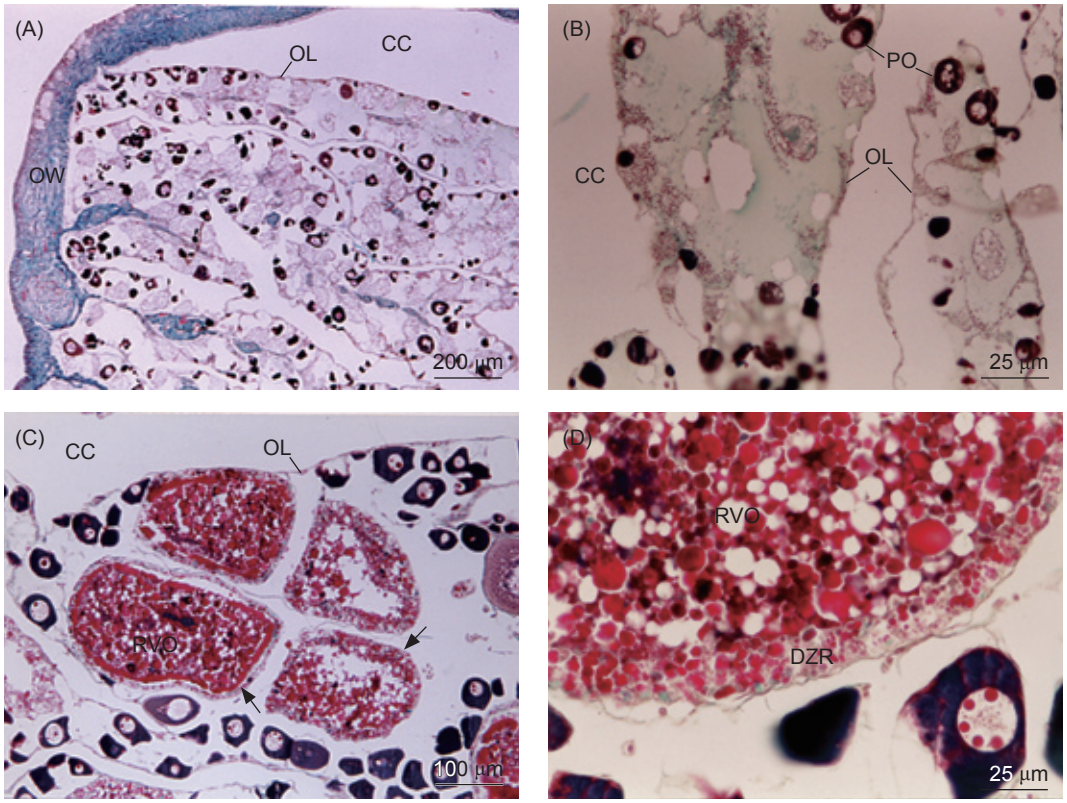

Fig. 13. Degeneration of an ovary after the spawning period in 3^+-yr-old fish. (A) An ovulated ovary during the post-spawning period. (B) Ovarian lamellae almost emptied in an ovulated ovary with very few small oocytes. (C) Decomposition (as shown by the arrow) of the zona radiata in non-ovulated vitellogenic oocytes. (D) Fragmentations of the zona radiata in a non-ovulated vitellogenic oocyte.

et al. 1994, Lee et al. 2001 2002). Exogenous E2 can induce the regression of testicular tissue and development of ovarian tissue, ultimately resulting in sex change in this species (Chang et al. 1995). The detailed mechanism of the natural sex change in the black porgy is still unknown. Our histological data provide interesting concepts for further studies on the molecular mechanism of sex change in this protandrous species.

Ovarian tissue was clearly found on the side close to the blood vessel during early development. At a later stage of development, the connective tissue separated the central cavity/ovarian tissue from the testicular tissue. This developmental characteristic may be an early feature of the delimited type of hermaphroditic gonad. Each individual oocyte was connected with capillaries which may supply nutrients for oocyte development. Oogenesis was classified into 6 stages in the present study: oogonia, chromatin-nucleolus and perinucleolar, cortical alveolar, vitellogenic, and maturation phases. According to the characteristics of the nucleolus of nuclei and cytoplasmic vitellogenesis, the development and maturation of oocytes in the ovary are classified into 8 phases in rainbow trout (*S. gairdneri*) (Yamamoto 1965) and 9 phases in red sea bream (*P. major*) (Matsuyama et al. 1988).

Perinuclear "cement" or "nuage" was found in female germ cells such as oogonia in the present study and in male germ cells in a previous study (Huang et al. 2002). Cement and nuage are generally considered to be associated with germ cells (Hogan 1978). They are the aggregates of masses of an electron-opaque substance which is (nuage) or is not (cement) associated with mitochondria. Primary oocytes had already developed the cytoplasmic projections (microvilli) to the cellular membrane. The follicle layer with granulosa cells and theca cells surrounded the oocyte as early as the stage of primary oocytes. The electron densities of the cytoplasm of

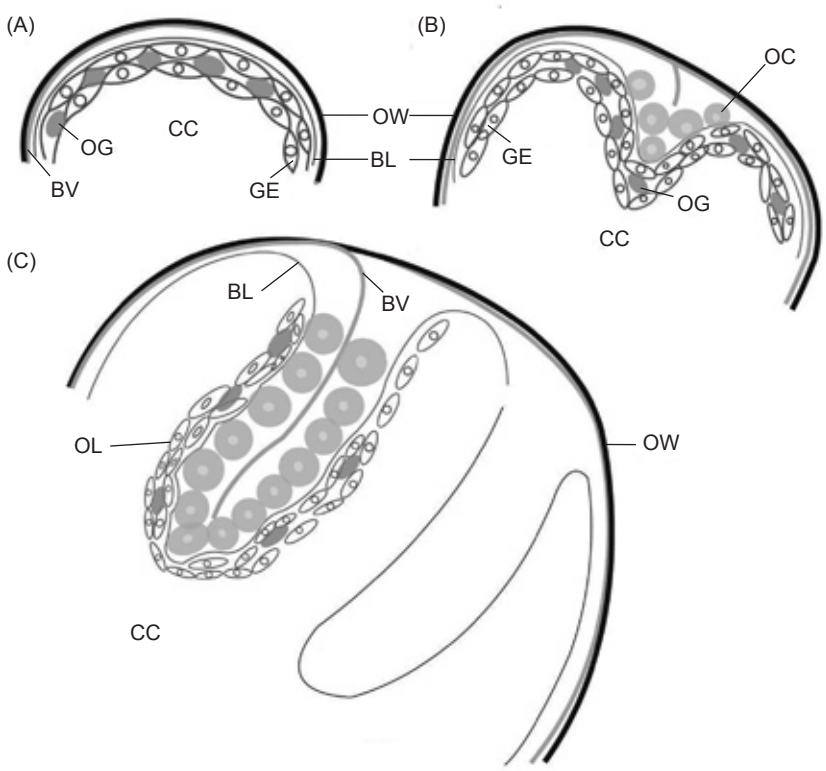

Fig. 14. Development of ovarian lamellae from 0^{+}-1^{+}-yr-old fish. (A) From the formation of the central cavity to the 1st spawning period. Oogonia first appeared in the germinal epithelium (the inner side of the central cavity which is close to the blood vessel). Oogonia grouped as cell nests in the germinal epithelium from the period of sex differentiation to the 1st spawning period. The basal lamina was present between the germinal epithelium and connective tissue. (B) After the 1st spawning period, the ovarian lamella began to develop and oogonia grew into oocytes. These growing oocytes were separated from the germinal epithelium by the basal lamina. Blood vessels elongated from the ovarian wall, entered the lumen of ovarian lamellae, and were distributed to each oocyte. (C) Ovarian lamella proliferated and protruded into the central cavity. Oogonia were enclosed by the germinal epithelium which was a part of the ovarian lamella.

granulosa cells at the vitellogenic oocyte stage appeared polarized, with low and high electron densities, under TEM observation. Mitochondria, endoplasmic reticula, and Golgi bodies were all unevenly concentrated in the region of high electron density in granulosa cells. Microvilli stretched out from the cytoplasm of vitellogenic oocytes which passed through the oocyte membrane and zona radiata layers, ultimately projecting to the cytoplasm (the region with high electron density by TEM) of granulosa cells in the follicle layer. These histological data confirm the close relationship between oocyte development and follicle cells. The follicle layer may play an important role in oocyte development (Stehr et al. 1983, Matsuyama et al. 1991, Scott et al. 1993).

In conclusion, the histological and ultrastructural details of gonadal tissue and female germ

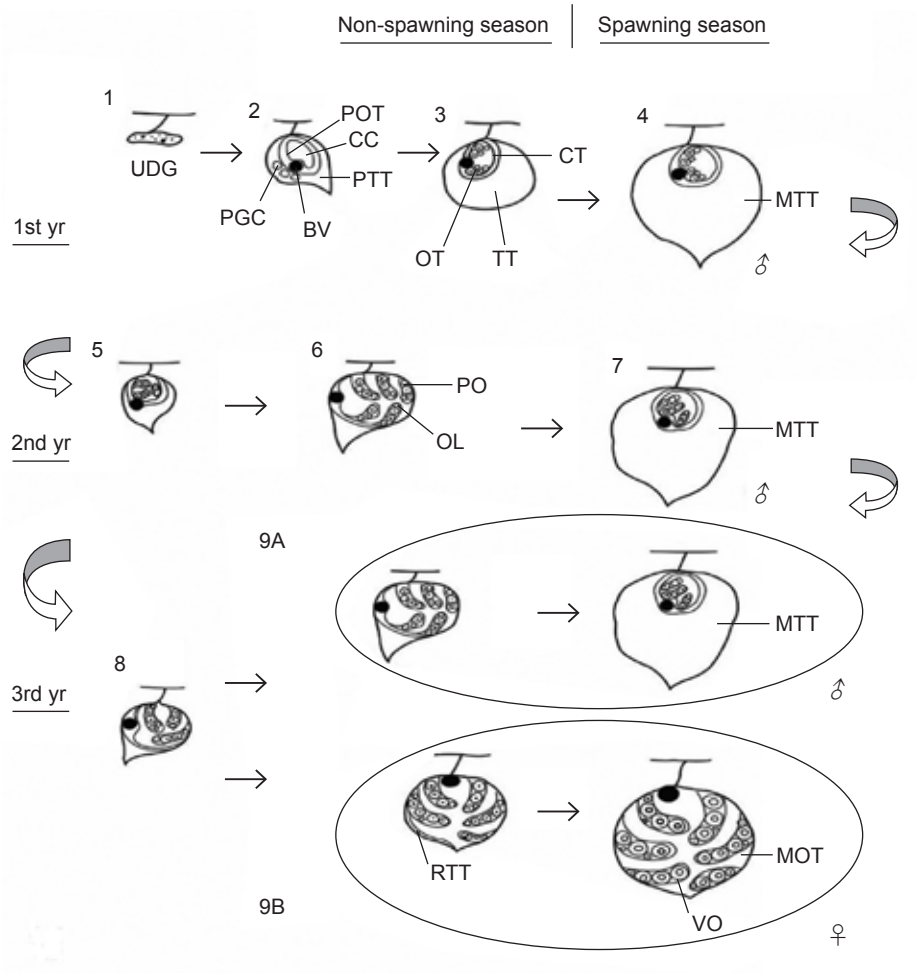

Fig. 15. Profiles of gonadal development in protandrous 0^{+}-3-yr-old black porgy. The development can be explained by the following 9 steps. (1) The gonad was undifferentiated before 5 mo of age. (2) After 5 mo of age, the gonad differentiated into a bisexual structure. Putative ovarian tissue was present towards the inner side of the central cavity, and putative testicular tissue was found at the distal end of the gonad and far from the central cavity. Primordial germ cells were near the blood vessel (black dot). (3) When a fish entered the pre-spawning period, ovarian tissue appeared as cell nests inside the central cavity and testicular tissue proliferated. (4) During the 1st spawning period, the central cavity was compressed by mature testicular tissue. (5) Testicular tissue degenerated and regressed while the ovarian lamella budded toward the central cavity during the post-spawning period. Oogonia grew into oocytes. (6) Ovarian tissue proliferated in the intersex period, but female germ cells were still at the primary oocyte stage. (7) Testicular tissue proliferated again, ovarian tissue regressed in the 2nd spawning period, and fish became functional males. (8) Testicular tissue regressed again and ovarian tissue grew after the 2nd spawning period. (9A) The fish remained as functional males (no sex change) when testicular tissue grew and became the functional testis during the 3rd spawning period. (9B) In the processes of natural sex change, testicular tissue further regressed and became residual during the intersex period. Primary oocytes proceeded to vitellogenesis during the pre-spawning period and developed into vitellogenic oocytes. These fish proceeded to change sex (i.e., become females) during the 3rd spawning period.

cells were characterized in the protandrous black porgy by light microscopy, and scanning and transmission electron microscopy. Early female germ cells appeared in the germinal epithelium during sexual differentiation and then formed cell nests. Ovarian lamellae with primary oocytes developed after the 1st spawning period. The ovarian tissue and testicular tissue increased (grew) and decreased (regressed) in area in a reciprocal pattern. Vitellogenic oocytes only appeared in the ovary after the bisexual testes had completely regressed in 2^{+}-yr-old fish if the fish was destined for a sex change. These histological data provide important, fundamental information for further studies on the mechanism of sex differentiation and sex change in the protandrous black porgy.

Acknowledgments: We thank the electron microscopy center (National Taiwan Ocean University, Keelung, Taiwan) for providing the facilities for these experiments. We are grateful to Dr. Sherly Tomy (National Taiwan Ocean University) for suggestions and English editing. The study was partially supported by the National Science Council (Taipei, Taiwan).

ABBREVIATIONS SHOWN ON FIGURES

ART, artery; BL, basal lamina; BV, blood vessel; CA, cortical alveoli; CAP, capillary; CC, central cavity; CE, cement; CJ, cell junction; CL, collagen fiber; CT, connective tissue; DGE, dark germinal epithelium; DS, desmosome; DZR, decomposed zona radiata; ERY, erythrocyte; FL, follicle layer; G, gonad; GC, granulosa cell; GE, germinal epithelium; GV, germinal vesicle; LD, lipid droplet; LGE, light germinal epithelium; M, mitochondrion; MOT, mature ovarian tissue; MTT, mature testicular tissue; MV, microvilli; NG, nuage; NU, nucleus; NUO, nucleolus; OC, oocyte; OCP, oocyte cytoplasm; OD, oil droplet; OG, oogonium; OL, ovarian lamella; OT, ovarian tissue; OW, ovarian wall; PGC, primordial germ cell; PO, primary oocyte; POT, putative ovarian tissue; PTT, putative testicular tissue; PW, peritoneal wall; RER, rough endoplasmic reticulum; RTT, regressed testicular tissue; RVO, regressed vitellogenic oocyte; SC, spermatocyte; SG, spermatogonia; SP, sperm; SZ, spermatozoa; TH, theca cell; TT, testicular tissue; UDG, undifferentiated gonad; VN, vein; VO, vitellogenic oocyte; YB, yellow body; YG, yolk granule; ZR, zona radiata; Z1, the layer close to the granulosa cells in the zona radiata; Z2, the part which appeared columnar in shape between Z1 and Z3 in the zona radiata; Z3, the horizontal, overlapping, cuboidal layers in the zona radiata.

REFERENCES

Arocha F. 2002. Oocyte development and maturity classification of swordfish from the north-western Atlantic. J. Fish Biol. **60:** 13-27.

Asoh K, T Yoshikawa, M Kasuya. 2001. Gonadal development and non-functional protogyny in a coral-reef damselfish, *Dascyllus albisella* Gill. J. Fish Biol. **58:** 1601-1616.

Begovac PC, RA Wallace. 1988. Stages of oocyte development in the pipefish, *Syngnathus scovelli*. J. Morphol. **197:** 353-369.

Chan STH, JG Phillips. 1967. The structure of gonad during natural sex reversal in *Monopterus albus* (Pisces: Teleostei). J. Zool. Lond. **151:** 129-141.

Chang CF, EF Lau, BY Lin. 1995. Estradiol-17β suppresses testicular development and stimulates sex change in protandrous black porgy, *Acanthopagrus schlegeli*. Fish Physiol. Biochem. **14:** 481-488.

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.

Chang CF, WS Yueh. 1990. Annual cycle of gonadal histology and steroid profiles in the juvenile males and females of the protandrous black porgy, *Acanthopagrus schlegeli*. Aquaculture **91:** 179-196.

Cochran RC, HJ Grier. 1991. Regulation of sexual succession in the protogynous black sea bass, *Centropristis striatus* (Osteichthyes: Serranidae). Gen. Comp. Endocrinol. **82:** 69-77.

Dodd JM. 1977. The structure of the ovary of nonmammalian vertebrates. *In* S Zuckerman, B Weir, eds. The ovary. Vol. I. New York: Academic Press, pp. 219-263.

Du JL, YH Lee, WS Yueh, CF Chang. 2005. Seasonal profiles of brain and pituitary GnRH and plasma LH in relation to sex change of protandrous black porgy, *Acanthopagrus schlegeli*. Biol. Reprod. **72:** 922-931.

Feist G, CB Schreck, MS Fitzpatrick, JM Redding. 1980. Sex steroid profiles of coho salmon (*Oncorhynchus kisutch*) during early development and sexual differentiation. Gen. Comp. Endocrinol. **80:** 299-313.

Grier H. 2000. Ovarian germinal epithelium and folliculogenesis in the common snook, *Centropomus undecimalis* (Teleostei: Centropomidae). J. Morphol. **243:** 265-281.

Hogan JC. 1978. An ultrastructural analysis of "cytoplasmic markers" in germ cells of *Oryzas latipes*. J. Ultrastruct. Res. **62:** 237-250.

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

Lee YH, JL Du, WS Yueh, BY Lin, JD Huang, CY Lee, MF Lee, EL Lau, FY Lee, C Morrey, Y Nagahama, CF Chang. 2001. Sex change in the protandrous black porgy, *Acanthopagrus schlegeli*: a review in gonadal development, estradiol, estrogen receptor, aromatase activity and gonadotropin. J. Exp. Zool. **290:** 715-726.

Lee YH, WS Yueh, JL Du, LT Sun, CF Chang. 2002.

Aromatase inhibitors block natural sex change and induce male function in the protandrous black porgy, *Acanthopagrus schlegeli* Bleeker: possible mechanism of natural sex change. Biol. Reprod. **66:** 1749-1754.

Linhart O, S Kudo, R Billard, V Slechta, EV Mikodina. 1995. Morphology, composition and fertilization of carp eggs: a review. Aquaculture **192:** 75-93.

Matsuyama M, RT Lara, S Matsuura. 1988. Juvenile bisexuality in the red sea bream, *Pagrus major*. Environ. Biol. Fish. **21:** 27-36.

Matsuyama M, Y Nagahama, S Matsuura. 1991. Observations on ovarian follicle ultrastructure in the marine teleost, *Pagrus major*, during vitellogenesis and oocyte maturation. Aquaculture **92:** 67-82.

Moiseeva EB. 1984. Gonadal development during the indifferent period and after hatching the sex differentiation in round goby, *Neogobius melanostomus* (Gobiidae). J. Ichthyol. **24:** 83-95.

Mylonas CC, LC Woods, P Thomas, Y Zohar. 1998. Endocrine profiles of female striped bass (*Morone saxatilis*) in captivity, during postvitellogenesis and induction of final oocyte maturation via controlled-released GnRHa-delivery system. Gen. Comp. Endocrinol. **110:** 276-289.

Nakamura M, TF Hourigan, K Yamauchi, Y Nagahama, EG Grau. 1989. Histological and ultrastructural evidence for the role of gonadal steroid hormones in sex change in the protogynous wrasse *Thalassoma duperrey*. Environ. Biol. Fish. **24:** 117-136.

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

Parmentier HK, LPM Timmermans. 1985. The differentiation of germ cells and gonads during development of carp (*C. carpio*). A study with anti-carp sperm monoclonal antibodies. J. Embryol. Exp. Morphol. **90:** 13-32.

Selman K, RA Wallace, A Sarka, X Qi. 1993. Stages of oocyte development in the zebrafish, *Brachydanio rerio*. J. Morphol. **218:** 203-224.

Stehr CM, JW Hawkes. 1983. The development of the hexagonally structured egg envelope of the C-O sole (*Pleuronichthys coenosus*). J. Morphol. **178:** 267-284.

Treasurer JW, FGT Holliday. 1981. Some aspects of the reproductive biology of perch *Perca fluviatilis* L. A histological description of the reproductive cycle. J. Fish Biol. **18:** 359-376.

Van den Hurk R, CJJ Richter, J Janssen-Dommerholt. 1989. Effects of 17β-methyltestosterone and 11β-hydroxyandro stenedione on gonad differentiation in the African catfish, *Clarias gariepinus*. Aquaculture **83:** 179-191.

Van den Hurk R, GA Slof. 1981. A morphological and experimental study of gonadal sex differentiation in the rainbow trout, *Salmo gairdneri*. Cell Tissue Res. **218:** 487-497.

Wallace RA, K Selman. 1990. Ultrastructural aspects of oogenesis and oocyte growth in fish and amphibians. J. Electr. Microsc. Tech. **16:** 175-201.

Yamamoto K, I Oota, K Takano, T Ishikawa. 1965. Studies on the maturing process of the rainbow trout, *Salmo gairdneri*. 1. Maturation of the ovary of a 1 year old fish. Bull. Jpn. Soc. Fish. **31:** 123-132.

York WY, R Patino, P Thomas. 1993. Ultrastructural changes in follicle cell-oocyte association during development and maturation of the ovarian follicle in Atlantic croaker. Gen. Comp. Endocrinol. **92:** 402-418.