The Morphology of Gonadal Tissue and Male Germ Cells in the Protandrous Black Porgy, *Acanthopagrus schlegeli*

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Jing-Duan Huang, Mong-Fong Lee and Ching-Fong Chang (2002) The morphology of ganadal tissue and male germ cells in the protandrous black porgy, *Acanthopagrus schlegeli*. *Zoological Studies* **41**(2): 216-227. The morphology of developing bisexual gonads and male germ cells in the protandrous black porgy, *Acanthopagrus schlegeli*, is described using light and electron microscopy. Conspicuous differences in structural characteristics were observed in the lobular structure in reproductive, non-reproductive, and regressed testicular tissues. Testicular tissue showed significant alternations and disintegrated in the sex-changing gonad. Sertoli cells contained mitochondria, free ribosomes, Golgi complexes, and endoplasmic reticulum. Cytoplasmic processes of Sertoli cells formed cysts which contained synchronous germ cells (spermatogonia, spermtocytes, spermatids, and spermatozoa). Mitochondria, endoplasmic reticulum, oil droplets, and free ribosomes were observed in Leydig cells. Perinuclear cement or nuage was found in spermatogonia. Spermatogenesis and spermiogenesis are described in male germ cells. Decreased cell size, an increasing nuclear-cytoplasmic ratio, and a reduced number of mitochondria were characteristics observed during development of spermatogonia to spermatids. Intracellular movements (diplosome, mitochondria migration, loss of cytoplasm, and nuclear rotation and depression) and structural changes (chromatin, formation of flagella) appeared during spermiogenesis. A cytoplasmic bridge was found in spermatocytes and spermatids. Intercellular junctions (at least gap junctions and tight junctions) appeared in Sertoli cells which were adjacent to other Sertoli cells or germ cells. These data provide important information for the further study of ultrastructural changes in bisexual gonads during the controlled and natural sex change in the protandrous black porgy. http://www.sinica.edu.tw/zool/zoolstud/41.2/216.pdf

Key words: Gonadal development, Leydig cell, Sertoli cell, Sex change, Ultrastructure.

Black porgy, *Acanthopagrus schlegeli* (Bleeker), a marine protandrous hermaphrodite in family Sparidae of the Perciformes, is widely distributed in tropical ocean and considered a particularly interesting species in commercial aquaculture in Asia including Taiwan. It has multiple spawning patterns, and its spawning period lasts from late winter to early spring (January-March). Fish are functional males for the first 2 yr of life but begin to sexually change to females after the 3rd year (Chang and Yueh 1990). About 30%-50% of 3-yr-old black porgy will change to females. This sex pattern provides a very good model to study the mechanisms of sexual development in fish. The relative ratios of testicular and ovarian tissues were reported in previous studies (Chang et al. 1997, Lee et al. 2001).

Testicular tissue undergoes intensive spermatogenesis in the prespawning seasons and fish become functional males with a very small portion of ovarian tissue surrounded by testicular tissue at 2 yr of age (Lee et al. 2000). Then, the testicular tissue regresses and the ovarian tissue develops, and the fish become females at 3 yr of age according to observations of gross anatomy in the gonads (Lee et al. 2001). Histological characteristics during gonadal development at various sex stages have still not been studied in black porgy. The direction of the development or regression of testicular tissue in the bisexual gonad is considered to be important in the process of sex change. Furthermore, the ultrastructure of male germ cells, and the relationship between male germ cells and somatic cells are criti-

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cal to testicular development. Male germ cells, mainly focusing on spermiogenesis (from spermatids to spermatozoa), have been described to some extent in black porgy using transmission electron microscopy (Gwo and Gwo 1993). However, the ultrastructure of somatic cells, and the relationship between male germ cells and somatic cells have not been examined in black porgy. Histological changes and characteristics of gonadal sex change are complicated, but this information is important for understanding the sex change mechanisms. Therefore, we examine the histology of the gonads at various stages by light microscopy, and further describe basic information of the ultrastructural characteristics in somatic and male germ cells by scanning and transmission electron microscopy in this study. Information on the ultrastructural characteristics can be used to further investigate ultrastructural changes in gonadal tissues in the future.

MATERIALS AND METHODS

Black porgy

A two-yr-old male and 3-yr-old sex-changing female black porgy (body weight 300-700 g) were collected during different seasons of the year. Non-reproductive (May-August), early reproductive (October-December), and reproductive (January-March) seasons were selected for the present experiments. Gonads were collected and fixed for histological examination by either light microscopy or electron microscopy.

Gonadal histology for light microscopy

Gonadal tissue was fixed with Bouin's solution. A piece of each gonad was embedded in paraffin and sectioned at 5 μm. Transverse sections were stained with hematoxylin/eosin, and Weigert's iron hematoxylin/trichrome stain reagent. Weigert's iron hematoxylin was prepared by mixing 20 ml of hematoxylin (Merck, Darmstadt, Germany) with 20 ml of Weigert's iron hematoxylin B (Electron Microscopy Science, EMS, Fort Washington, PA). Trichrome stain reagent was prepared by mixing 6 g of chromotrope 2R (Sigma, St. Louis, MO), 0.3 g of fast green FCF (Sigma), 0.8 g of phosphotungstic acid (Sigma), and 1 ml of acetic acid, with water added to 100 ml.

Scanning electron microscopy (SEM)

Gonadal tissues were pre-fixed with 1% glutaraldehyde (EMS) and 3% paraformaldehyde (EMS) in a buffer of 0.1 M sodium cacodylate (pH 7.4) for 24 h and then were post-fixed with 1% osmium tetroxide (EMS) in the same buffer for 2 h. After dehydrating in an ethanol series, critical-point-drying, and coating with gold, samples were examined with a Hitachi S-2400 scanning electron microscope (Hitachi, Tokyo, Japan).

Transmission electron microscopy (TEM)

Gonadal tissues were cut into a small cube, then fixed and dehydrated as described for the SEM sample. After infiltration, they were embedded in Spurr's medium. Ultra-thin sections were obtained by using an RMC MTX ultramicrotome (Research Manufacturing Company, Tucson, AZ) and were stained with uranyl acetate and lead citrate. Sections were examined with a Hitachi H-600 transmission electron microscope.

RESULTS

Characteristics of gonadal histology

Testicular tissue was dominant in the gonad with a few primary oocytes around the central cavity in 2-yr-old males during the reproductive season (Fig. 1a). Ovarian and testicular tissues were separated by connective tissue. Ovarian tissue became dominant in bisexual gonads during the non-reproductive season (Fig. 1b). Testicular tissue contained lobules and cysts; Sertoli cells and germ cells were found in the cysts in the reproductive season (Fig. 2a). Lobules in the non-reproductive season also contained Sertoli cells and spermatogonia, but with a wide lumen and no clear cysts (Fig. 2b). Bisexual gonads (non-reproductive season, Fig. 3a), lobules (Fig. 3b), and cysts with various stages of germ cells were characterized in the early reproductive season (Fig. 3c) by SEM.

No clear lobular or cystic structures were observed in the regressed testicular tissue of 3-yr-old sex-changing female fish during the reproductive season (Fig. 4a, b). Yellow bodies were found in regressed testicular tissue (Fig. 4b). Only a very few intact spermatogonia (Fig. 4a,c), and many clusters of basal lamellae (Fig. 4c), but no clear somatic cells (Fig. 4a,c) were found in the disintegrating testis. Spermatogonia in regressed testicular tissue still had the perinuclear "cement" associated with mitochondria (Fig. 4c).

Ultrastructure of Sertoli and Leydig cells

Sertoli cells and spermatogonia surrounded the lumen of a lobule in the non-reproductive season (Fig. 5a). Spermatogonia with many mitochondria were associated with Sertoli cells in lobules in the early reproductive season (Fig. 5b). Masses of an electron-opaque substance were observed outside the nuclear membrane and have been named the perinuclear "nuage" in spermatogonia (Fig. 5b). Leydig cells, blood vessels, and putative pericytes with endothelial cells were found in the interlobular area (Fig. 5b). Mitochondria, rough endoplasmic reticulum, free ribosomes, and oil droplets were observed in Leydig cells in the early reproductive season (Fig. 5b, c). Gap junctions were found between spermatogonia and Sertoli cells in the early reproductive season (Fig. 5d). Sertoli cells with cytoplasmic extensions as a cystic structure encompassing spermatocytes were observed in the early reproductive season (Fig. 6a-c). Sertoli cells had a triangular nucleus, free ribosomes, and cisterna mitochondria (Fig. 6a, c).

Development of male germ cells

At different stages of male germ cells, spermatocytes, spermatids and spermatozoa were found in testicular tissue using SEM in the early reproductive and reproductive seasons (Fig. 7a-d). Mature sperm each with a flagellum were found in the lobules, and 2 mitochondria were in the midpiece
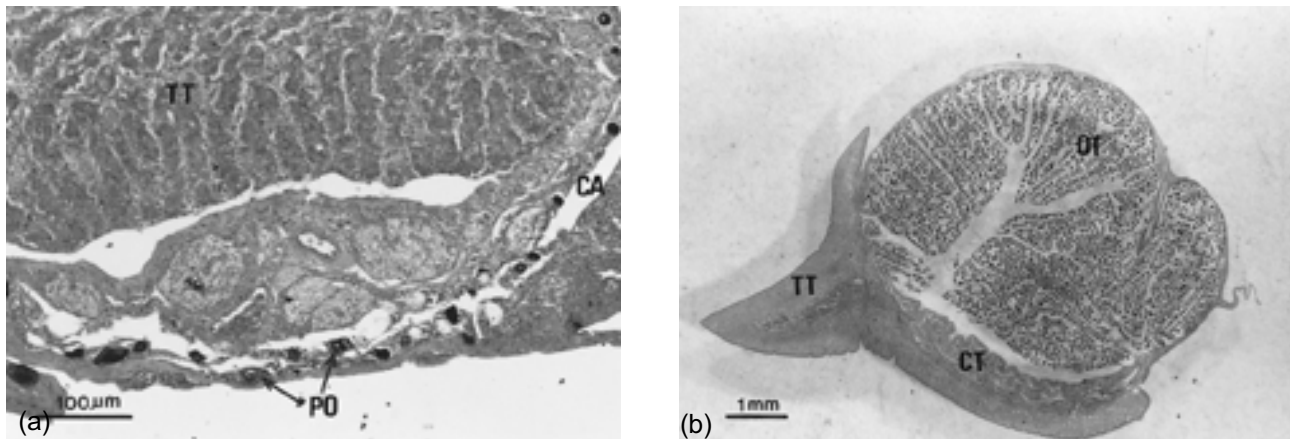

Fig. 1. (a) Bisexual gonad during the reproductive season with dominant and functional testicular tissue (TT), and a few primary oocytes (PO) around the central cavity (CA) of the ovarian tissue. (b) Bisexual gonad during the non-reproductive season with dominantly ovarian tissue at the stage of primary oocytes. Small testicular tissue is separated from ovarian tissue by the connective tissue (CT).
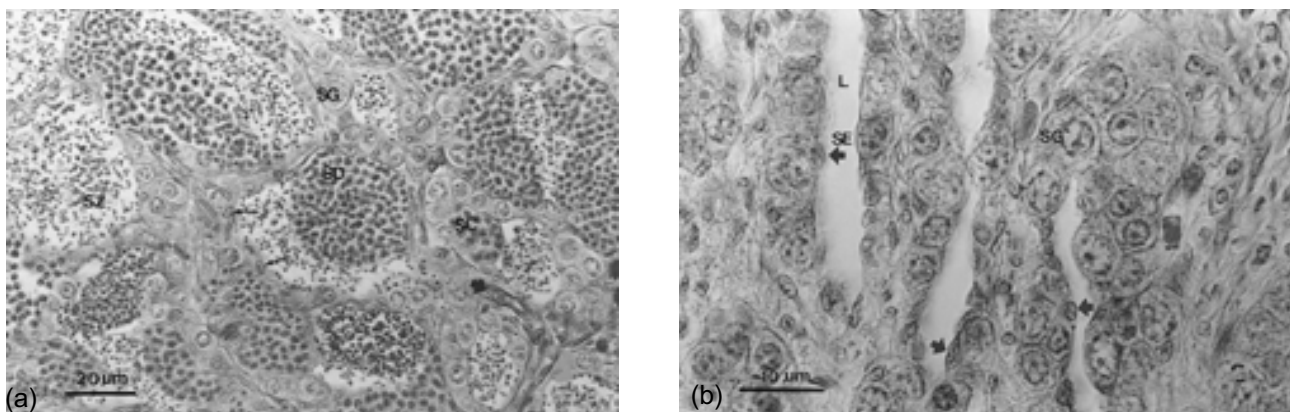

Fig. 2. (a) Germ cells (spermatogonia, SG; spermatocyte, SC; spermatid, SD; spermatozoa, SZ) synchronously developing within a cyst in testicular tissue during the reproductive season. An arrow indicates a Sertoli cell. (b) Three lumens (L) appearing in non-reproductive testicular tissue. Nuclei of Sertoli cells (arrow) are aligned around the border of the lumen. Germ cells are mainly at the spermatogonia (SG) stage.
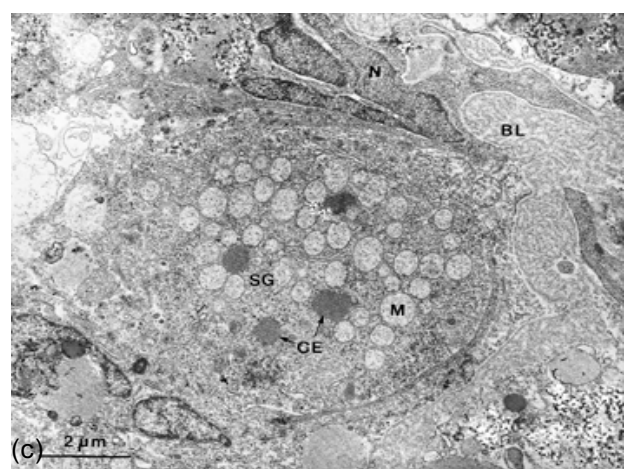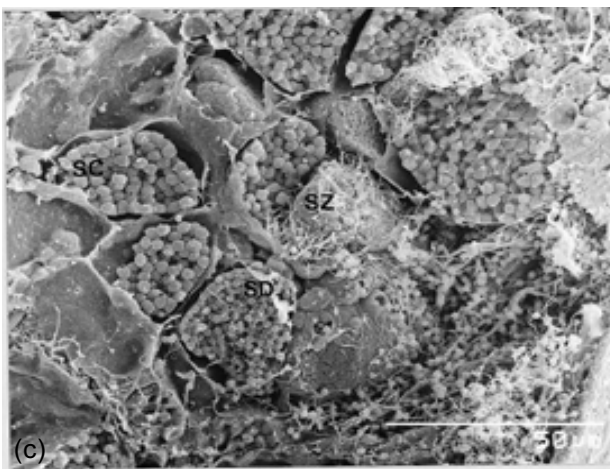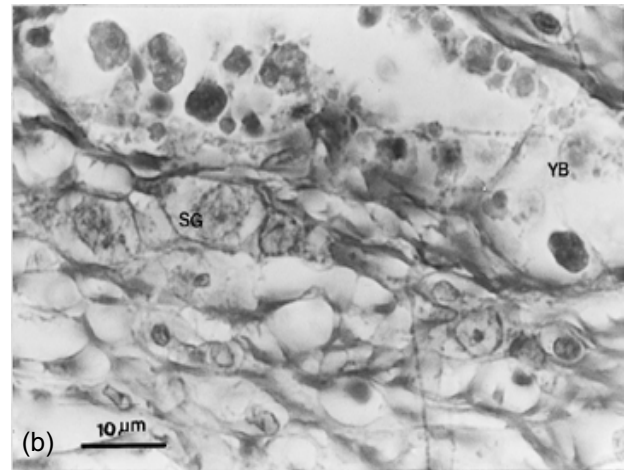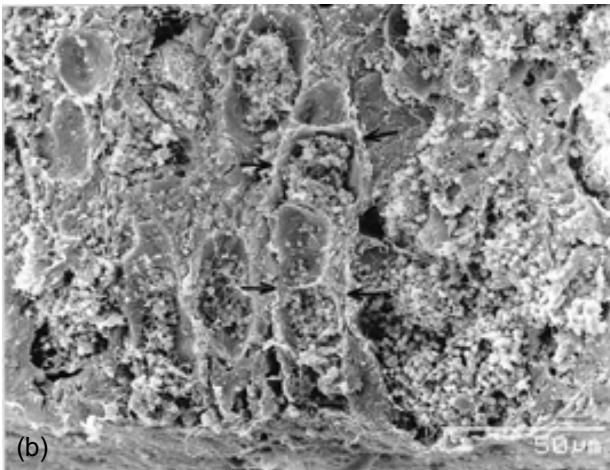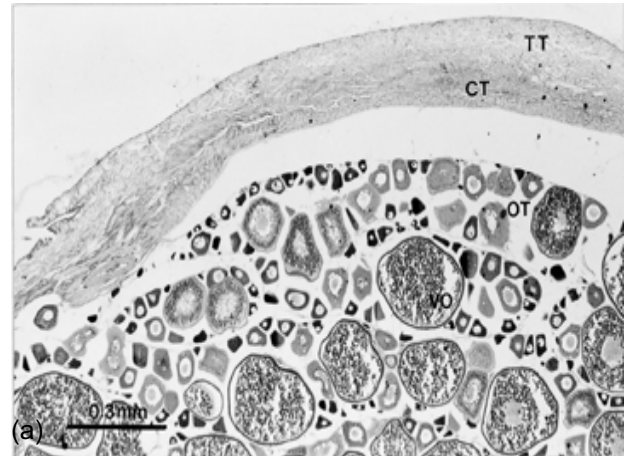
Fig. 3. (a) Bisexual gonad with testicular (TT) and ovarian tissue (OT) separated by connective tissue (CT) in the non-reproductive season by SEM. (b) Lobular structure (arrow) containing several cysts in testicular tissue in the early reproductive season. (c) Cyst with synchronous germ cells (spermatocyte, SC; spermatid, SD; spermatozoa, SZ) in the early reproductive season.

Fig. 4. (a) Regressed testicular tissue in a vitellogenic ovary in a sex-changing female (connective tissue, CT; ovarian tissue, OT; testicular tissue, TT; vitellogenic oocyte VO). (b) Disintegrated and regressed testicular tissue with yellow bodies (YB), a few spermatogonia (SG), and no intact lobular structures in a sex-changing female fish. (c) Clusters of basal laminae (BL) and nuclei (N), and an intact spermatogonia (SG) with perinuclear cement (CE) and mitochondria (M) in the disintegrated testicular tissue by TEM.
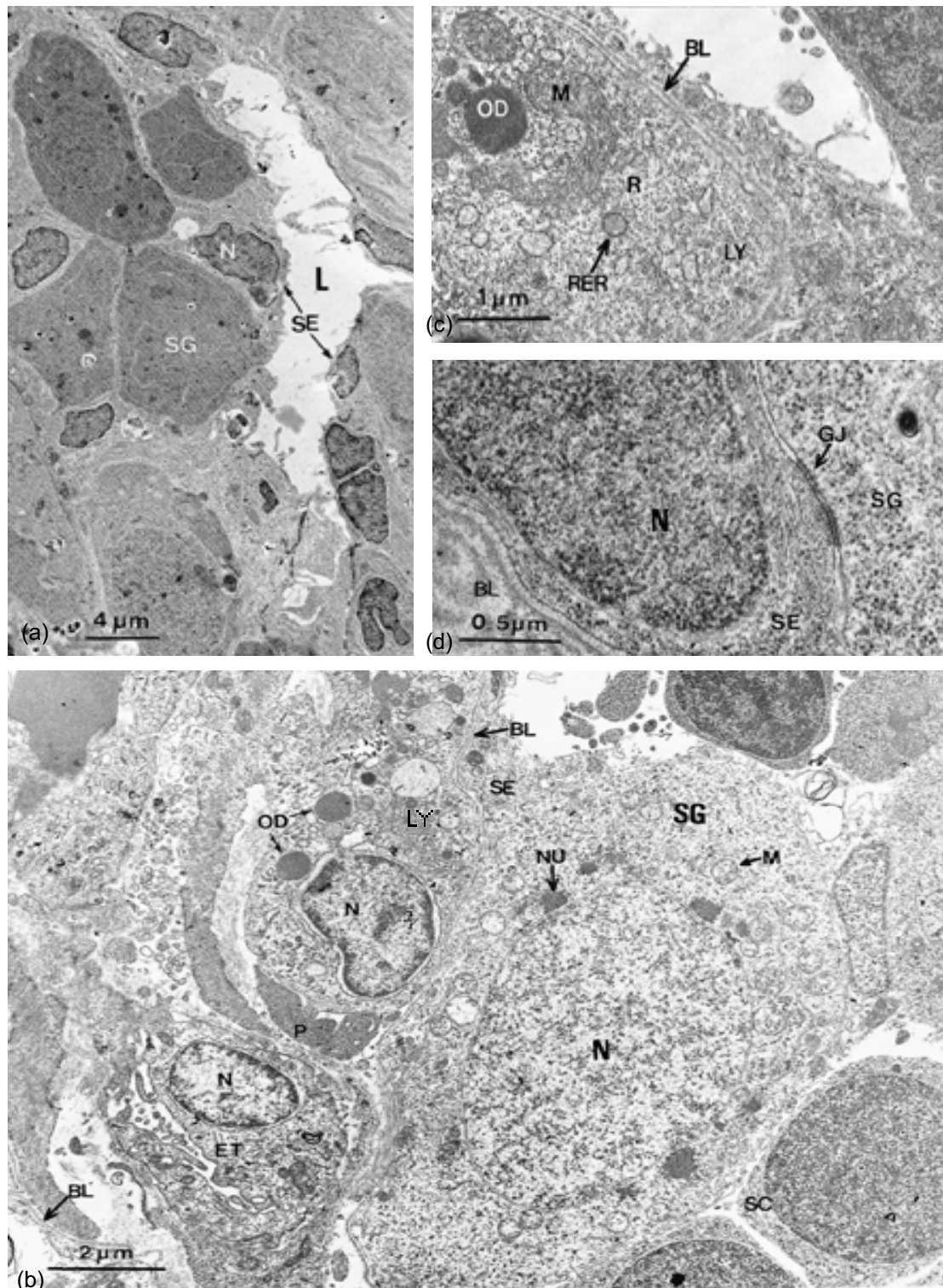
Fig. 5. (a) Lumen (L), Sertoli cell (SE; its nucleus, N), and spermatogonia (SG) in testicular tissue during the non-reproductive season. (b) Leydig cell (LY; its oil droplet,OD), pericyte (P), and capillary composed of endothelial cells (ET) located in the interstitium separating the spermatogonia (SG) from the basal lamina (BL) in the early reproductive season. Mitochondria (M), the nucleus (N), and perinuclear "nuage" (NU) can be observed in spermatogonia in the early reproductive season. (c) Cisternal mitochondria (M), rough endoplasmic reticulum (RER), free ribosomes (R), and an oil droplet (OD) found in a Leydig cell (LY) in the early reproductive season. (d) Sertoli cell (SE; its nucleas, N) with gap junctions (GJ) adjacent to spermatogonia (SG) in the early reproductive season.

of the sperm in the reproductive season (Fig. 7c, d). A cytoplasmic bridge was found in spermatocytes with homogenous nuclear chromatin in the early reproductive season (Fig. 8a). Synaptonemal complexes, diplosomes, and heterogenous nuclear chromatin were observed at the pachytene stage of prophase I in primary spermatocytes in the early reproductive season (Fig. 8b). Secondary spermatocytes were found to have condensed chromatin in the early reproductive season (Fig. 8c).

The flagellum developed from the distal centriole of a cytoplasmic diplosome in spermatids with heterogenous chromatin in the reproductive season (Fig. 9a). Spermatids were still connected by a cytoplasmic bridge in the reproductive season (Fig. 9b). Then, the nucleus rotated, and the flagellum was located at the nuclear fossa of a spermatid with homogenous charomatin in the reproductive season (Fig. 9c). Formation of a cytoplasmic canal, loss of the cytoplasmic bridge, and movement of cytoplasmic organelles with homogenous chromatin were found in spermatozoa (Fig. 9c). A translucent but small volume of cytoplasm as well as lamellar and tubular structures were present in the spermatozoa in the reproductive season (Fig. 10a). Two mitochondria and a flagellum containing an axoneme with a typical 9+2 structure were observed in the spermatozoa in reproductive season (Fig. 10b).

DISCUSSION

This is the 1st report to examine bisexual gonadal tissue and testicular development using light, scanning, and transmission micrscopy together in a hermaphroditic fish species-protandrous black porgy. Only a few studies in other hermaphroditic fish have investigated the gonadal characteristics by light microscopy (Pollock 1985, Yeung and Chan 1987) and by transmission electron microscopy (Yeung et al. 1985, Bruslé-Sicard et al. 1992, Besseau and Bruslé-Sicard 1995, Bruslé-Sicard and Fourcault 1997). The testicular tissue of black porgy belongs to the unrestricted spermatogonial testis type (Grier 1981, Pudney 1993). The histology of bisexual tissue separated by connecting tissue was demonstrated in black porgy using light microscopy and SEM. Ovarian tissue comprised a very small portion and only appeared in the central cavity in functional males during the spawning season. In contrast, ovarian tissue became the major tissue in the gonads during the non-spawning (post-spawning and non-reproductive) season. Primary oocytes at the perinucleolus stage were the only oocytes found in the bisexual ovary. These findings are consistent with previous data (mainly from the early pre-reproductive to reproductive season, September-March of the next year) (Chang et al. 1995a, b, Lee et al. 2000). Our findings also further broaden the histological data especially during the non-reproductive season in black porgy. The ratio of testicular and ovarian tissues varied, and it was season-dependent (Chang et al. 1997, Lee et al. 2001).

Testicular tissue regressed in the sex-changing fish. Cysts and lobules disintegrated. No clear boundary layers between lobular and interlobular tissues were observed. Plasma membranes and cytoplasmic organelles decomposed, and only the nucleus could be observed using TEM. The presence of lysosomes (data not shown), an absence of clear somatic cells, but very few intact germ cells were found in regressed testicular tissues. Therefore, the data indicate that the ultrastructure of somatic and germ cells does not appear in regressing testicular tissues in sex-changing black porgy. Degeneration of mitochondria to lysosomes was
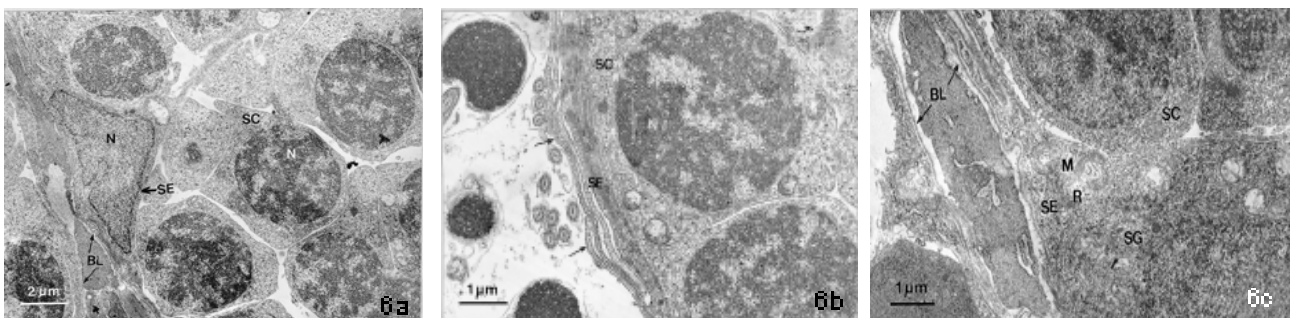

Fig. 6. (a) Sertoli cell (SE; its nucleus, N) appearing around spermatocytes (SC) in the early reproductive season. (b) Finger-shaped cytoplasmic process (arrow) of a Sertoli cell (SE) encompassing a spermatocyte (SC) in the early reproductive season. (c) Spermatogonia (SG) and spermatocyte (SC) separated by a Sertoli cell (SE; its mitochondria, M; free ribosome, R; basal lamella, BL) in the early reproductive season.

found in thecal and granulosa cells of sex-changing protogynous *Monopterus albus* (Yeung et al. 1985). A disrupted membrane, degenerating nucleus, and the presence of lysosomes were also observed in spermatogonia and Sertoli cells of the protandrous *Sparus aurata* (Bruslé-Sicard and Fourcault 1997). The temporal relationship between germ cells and somatic cells in terms of disintegration in the process of sex change would be interesting to study further in black porgy.

The testis consisted of lobules lined by Sertoli cells and germinal cysts and filled with germ cells. The lobular lumen was wider in the intersex stage than in the reproductive season. Sertoli cells and spermatogonia were located in the peri-lobular area. Lobules were separated by basal laminae, and cap-illaries contained endothelial cells; Leydig cells were found in the area of the interlobular tissue. Other types of interstitial cells (myoid cells, fibroblasts, and macrophages) were indicated in trout testis (*Oncorhynchus mykiss*) (Cauty and Loir 1995). We also first identified in fish gonads that putative peri-cyte surrounded the blood vessel (Fig. 5b). The peri-capcyte processes extend from the cell body of a pericyte along the capillary, usually parallel to the long axis of the capillary or extending to other capil-laries (Tilton 1991). Sertoli cells had an irregularly shaped nucleus and were smaller than Leydig cells and spermatogonia. We also found mitochondria (Fig. 6c) and Golgi complexes (data not shown), but there were no clear endoplasmic reticulum in Sertoli cells. Gap junctions (Fig. 5d) were found between
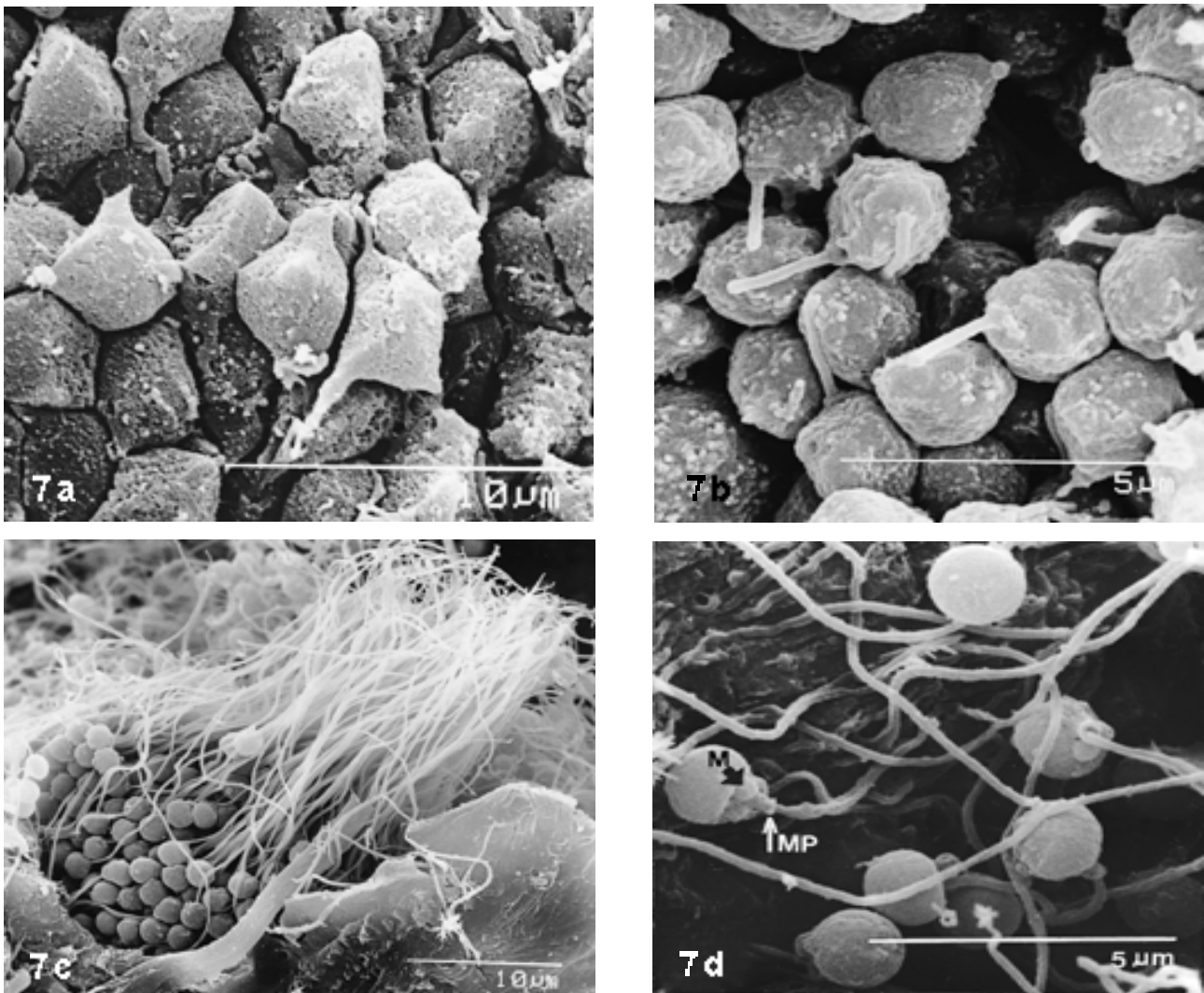

Fig. 7. (a) Spermatocyte (SC) in the early reproductive season. (b) Spermatid (SD) with a flagellum (F) in the early reproductive season. (c). Spermatozoa (SZ) in the reproductive season. (d) Mitochondria (M) in the midpiece (MP) of a mature sperm in the reproductive season by SEM.

cells (Sertoli cells and spermatogonia). Gap junctions are specialized plasma membrane structures that provide cytoplasmic continuity between cells; their function may be to allow the direct intercellular exchange of nutrients and factors controlling the development of germ cells by Sertoli cells (Enders 1993). Tight junctions were also found between adjacent Sertoli cells (data not shown). Tight junctions may form a Sertoli cell barrier (blood-testis barrier) (Byers et al. 1993, Pudney 1993). Leydig cells were observed in these studies to contain rough endoplasmic reticulum, free ribosomes, oil droplets, Golgi complexes (data not shown), and mitochondria. Sertoli cells contained mitochondria, free ribosomes, Golgi
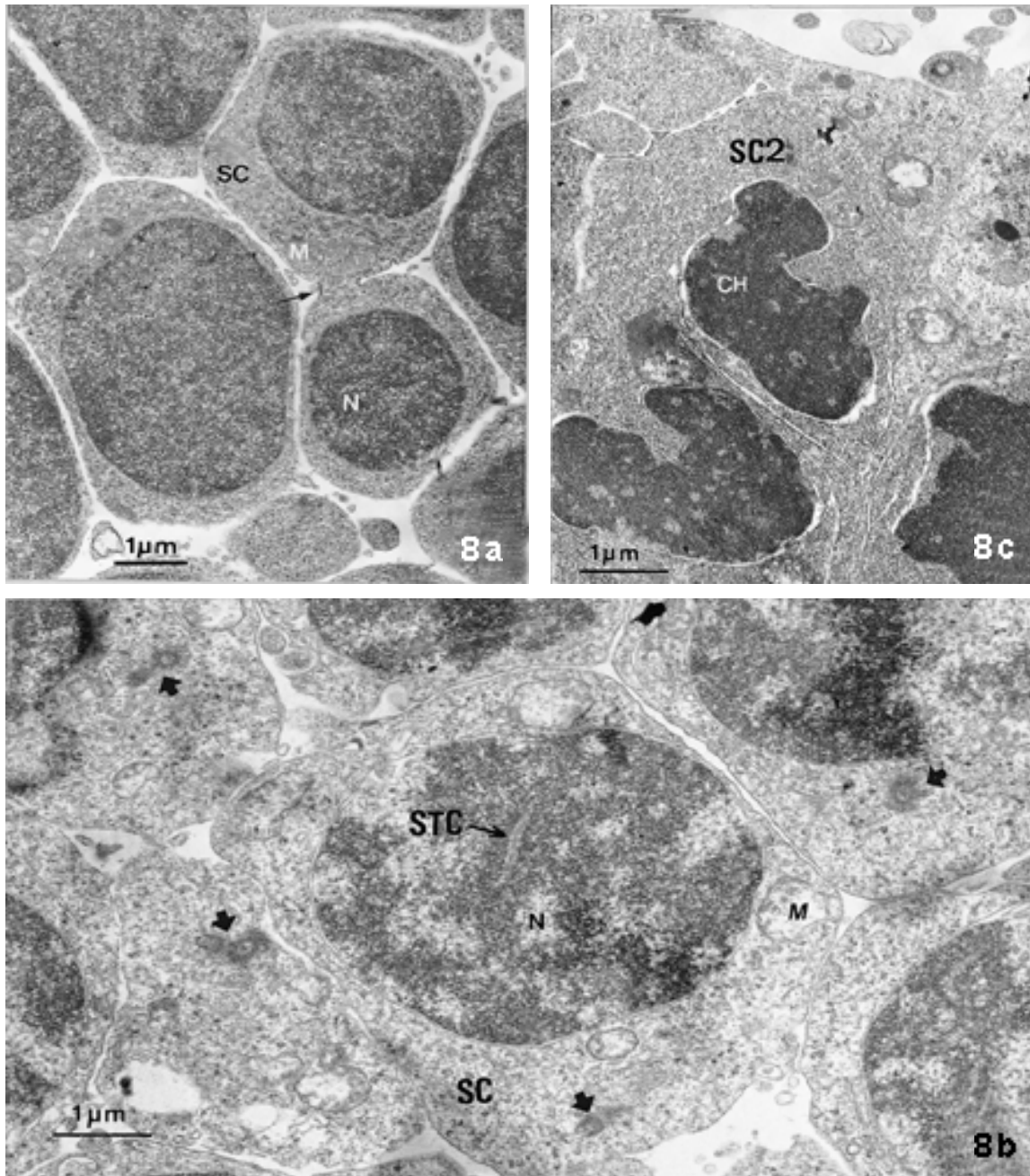

Fig. 8. (a) Cytoplasmic bridge (arrow) between spermatocytes (SC) (mitochondria, M; nuclcus, N) in the early reproductive season. (b) Primary spermatocyte (SC) with a synatonemal complex (STC) in the nucleus, and mitochondria (M) and a diplosome structure (arrow) in the cytoplasm in the early reproductive season. (c) Condensed nuclear chromatin (CH) in a secondary spermatocyte (SC2) in the early reproductive season.
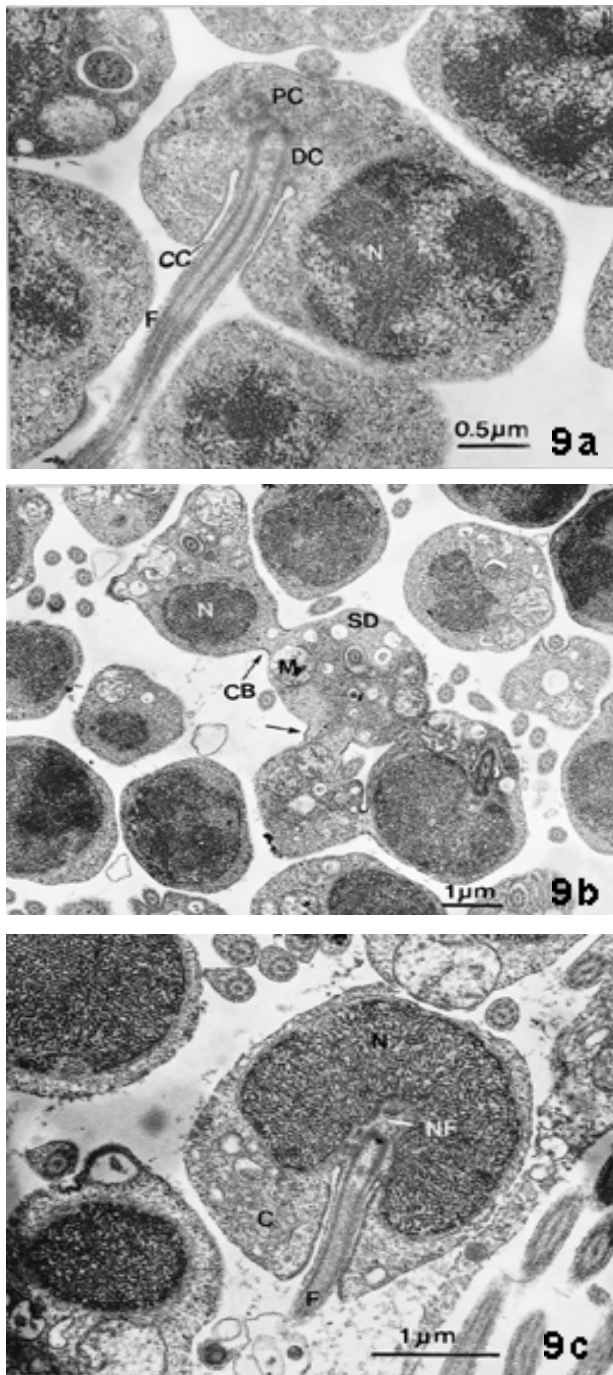
complexes, and endoplasmic reticulum. Two types of oil droplets (pale and dark) were found in the Leydig cells in black porgy. Oil droplets in Leydig cells may be related to the storage of cholesterol and steroids; they have been related to steroid biosynthesis (Loir 1990a, b). Leydig cells in the testicular interstitium are the main source of gonadal steroids (Nagahama 1986, Loir 1990a, b). On the basis of cytoplasmic features, no evidence of steroidogenesis was found in Sertoli cells, but they perform a phagocytic function in *Tilapia rendalli* (Van Vuren and Soley 1990).
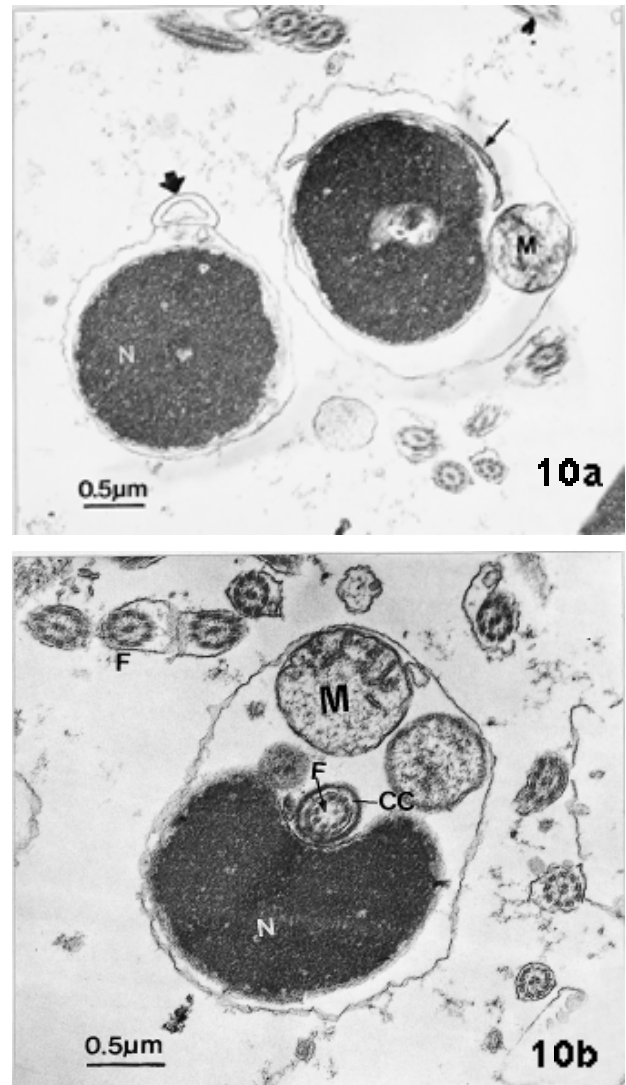

Fig. 9. (a) A proximal centriole (PC) perpendicular to a distal centriole (DC). A flagellum (F) growing from the distal centriole and formation of a cytoplasmic canal (CC) can be observed in a spermatid with heterogenous nuclear chromatin in the reproductive season. The diplosome is still in the cytoplasm and has not rotated into the nucleus (N). (b) Cytoplasmic bridge (CB) and mitochondria (M) still appearing in spermatids (SD) and mitochondria (M) in the reproductive season. (c) A diplosome having rotated and invaded the nucleus (N) with homogenous chromatin in the reproductive season. The proximal centriole is located within the nuclear fossa (NF), and cytoplasmic organelles (C) have become polarized.

Fig. 10. (a) Membranes (thick arrow) and tubular structures (thin arrow) with translucent cytoplasm and discarded cytoplasmic structures appearing in the spermatids in the maturation to spermatozoa stage of the reproductive season. (b) Two mitochondria (M) found in the spermatozoa with a cytoplasmic canal (CC) and the 9+2 structure of the flagellum (F), respectively, in the reproductive season.

Many cysts were found in the lobules. Germ cells in a cyst developed synchronously as shown by light microscopy and SEM. This is similar to what occurs in other gonochoristic species (reviewed by Grier 1981). Characteristics of different stages of the male germ cell are described in the present study. Two types of nuclear chromatin, heterogenous and homogenous, were found in spermatocytes and spermatids. Spermatocytes having either heterogenous or homogenous nuclear chromatin has been clearly observed before. This may be related to the transformation of basic proteins in the nucleus (Grier 1981). There were fewer spermatocytes with homogenous nuclear chromatin than with heterogenous chromatin. Formation of a synaptonemal complex is one of the characteristics of primary spermatocytes. Spermiogenesis in black porgy belongs to type 1 which is consistent with previous findings (Gwo and Gwo 1993). Two types of spermiogenesis have been described in teleosts (Mattei 1970). In type 1, rotation of the nucleus occurs, the diplosome enters the nuclear fossa, and the flagellum is symmetrically located. In type 2, there is no nuclear rotation, the diplosome remains outside the fossa, and the flagellum is asymmetrically located. Movement of cytoplasmic organelles and a decrease in the cytoplasmic volume were found in the stages of spermatocyte and spermiogenesis. Long tubular structures were found in the cytoplasm of spermatocytes and spermatids. These structures may be related to the decreased cytoplasmic volume during spermiogenesis. Sprando and Russell (1988) indicated 3 possible ways to decrease cytoplasm in fish: 1) formation of residual bodies and phagocytization by Sertoli cells, 2) formation of tubular complexes and then disintegration, and 3) dehydration and chromatin condensation.

The distinctive changes observed from spermatogonia to spermtocytes are as follows: decreased size, increased nuclear-cytoplasmic ratio, and a reduced number of mitochondria. These characteristics of germ cells are similar to findings in other species (Bruslé and Bruslé 1978). Perinuclear ¨cement¨ or ¨nuage¨ is generally considered to be associated with germ cells (Hogan 1978). We found perinuclear ¨cement¨ (Fig. 4c) and ¨nuage¨ (Fig. 5b), i.e., aggregates of masses of an electron-opaque substance being associated with (¨nuage¨) or without (¨cement¨) mitochondria in black porgy. The origin and function of the perinuclear ¨nuage¨ or ¨cement¨ in male germ cells are not clear. Balbiani bodies (analogous to perinuclear nuage or cement) were also found in developing oocytes and are considered a natural protein

in oocytes (reviewed by Nagahama 1983). Formation of a cytoplasmic canal and diplosome-flagellum, elongation of the nucleus, and nuclear rotation were similarly found in spermatids as shown in the present study and in many other species (Bruslé 1981, Gwo and Gwo 1983). Cytoplasmic bridges were observed in spermatocytes and spermatids but not in spermatozoa. Spermiogenesis is achieved with the rupture of the cytoplamic bridge (Bruslé 1981). Tubular structures surrounding the nucleus and the discarded vesicle were observed in spermatozoa.

In conclusion, the histology and ultrastructure of gonadal tissue and male germ cells were characterized in the protandrous black porgy by light microscopy and both scanning and transmission electron microscopy. Cellular characteristics in black porgy are similar to other fish species. Testicular tissue underwent significant changes and disintegrated in sex-changing gonads. These data provide important and fundamental information for the further study of ultrastructural changes in bisexual gonads in controlled and natural sex change.

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黑鯛生殖腺與雄性生殖細胞組織與微細構造特性之研究

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　　本研究利用光學與電顯技術探討黑鯛生殖腺組織構造之特性。在黑鯛的不同生殖階段（生殖季節、非生殖節與性轉變），精巢小葉構造有顯著的不同，在性轉變之萎縮精巢之小葉構造形成崩解的狀況。賽氏細胞（Sertoli　cell)及其細胞質延伸體構成包囊，包囊內之生殖細胞發育具有同步化，萊氏細胞（Leydig　cell)與賽氏細胞具有各種重要的胞器，同時賽氏細胞與生殖細胞形成間隙接合。本實驗並觀察雄性生殖細胞發育特徵，包括由精原細胞、精母細胞、精細胞至精子，並包括精子變態期。本研究對於進一步瞭解黑鯛人為控制或自然性轉變之生殖腺之變化提供至為重要之基礎資料。

關鍵字：生殖腺發育，賽氏細胞，萊氏細胞，性轉變，超顯微結構。

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