RESEARCH ARTICLE

HISTOLOGY, HISTOCHEMISTRY, AND ULTRASTRUCTURE OF STEROIDOGENESIS DURING TESTICULAR CYCLE OF *MUGIL CEPHALUS* IN THE NATURAL HABITAT

Ferial A. El-messady[1]; Noha A. Khalil[2]; Mostafa A. Mousa[3*]

[1]Zoology Department, Faculty of Science, Menoufia University, Menoufia, Egypt
[2]Fish Reproduction Laboratory, National Institute of Oceanography and Fisheries (NIOF), Qalyubia, Egypt
[3]Fish Reproduction Laboratory, National Institute of Oceanography and Fisheries (NIOF), Alexandria, Egypt

*Correspondence:
Mostafa Mousa
Fish Reproduction Laboratory
NIOF, Alexandria, Egypt
E-mail:
mostafa_mousa2002@yahoo.com

ABSTRACT

Recently, an increasing attention has been paid to the productivity and propagation of grey mullet "*Mugil cephalus*". In view of the fact that the steroidogenic hormones play a key role in fish maturation and production of fertile ova, the present work was planned to determine and investigate the steroidogenic cells in the testes of grey mullet, and thence to determine their levels of activities during its maturing cycle, aiming at making use of the obtained data in fish breeding and culture. Mature males of *M. cephalus* were obtained from the natural habitat (Al Bardawil Lagoon). Cytochemical and ultrastructural investigations were performed for the steroidogenic cells containing 3β-hydroxysteroid dehydrogenase. Five developmental stages were quite distinguishable in *M. cephalus* males during the testicular cycle: immature stage, stimulating spermatogenesis, rapid spermatogenesis, mature or ripe, and spent stages. The electron microscopy showed that the interstitial (Leydig), lobular bounding, and Sertoli cells inspected during rapid spermatogenesis displayed prominent lipid droplets and cytoplasmic organoids typical of protein-synthesizing cells (i.e. rough endoplasmic reticulum, abundant ribosomes, and distinct Golgi apparatus). In addition, Sertoli cells, in particular, possessed certain microtubules that are known to serve in transporting metabolites. Furthermore, the testes examined after spawning (spent ones) by electron microscope have designated that Sertoli cells, as well as some Leydig cells, had been changed into phagocytic ones to get ride from the non-ejaculated sperm cells. In conclusion, the present results could be very valuable in the problems concerning fish maturation in captivity.

INTRODUCTION

Accurate knowledge of the gonads cycle and their functional mechanism in fishes is essential for good management in both natural fisheries and fish farming; the morphology, histology, ultrastructure, and

cyclical changes in the fish testes have been studied in many teleosts[1-6]. Also, the histology and histochemistry of the testes and ovaries in a large number of teleosts have been investigated by many researchers who had proved that the gonads of those fish secrete steroid hormones[2,7-9]. Although the exact distribution of the tissues secreting steroid hormones is not known, there are at least five different cellular sources that have been suspected to be implicated in androgen production: the interstitial cells, lobule boundary cells, Sertoli cells, germ cells, and the epithelial cells of testicular efferent ducts[2,8,10,11].

The sex-steroid synthesizing cells are characterized by the presence of Δ 5-3β-hydroxysteroid dehydrogenase (3β-HSD) and glucose-6-phosphate dehydrogenase (G6PD)[12-16]. These key enzymes play main roles in the metabolic pathways during the synthesis and production of sex steroids[17]. Histochemically, 3β-HSD has been localized in the interstitial cells of the testis in many of teleost fishes; *Xiphophorus maculatus*[12], *Ictalurus nebulosus*[18], *O. niloticus*[19], *Padogobius martensi*[13], *Serrasalmus spilopleura*[20] and *Carassius auratus*[16]. Also, the histochemical studies have also revealed the presence of 3β-HSD activity in the Sertoli cells of *Cymatogaster aggregate*[21], *Salmo gairdneri*[22] and *O. niloticus*[19]. In addition, 3β-HSD activity was detected in interstitial cells and lobule boundary cells of *Sarotherodon mossambicus*[23], and *O. niloticus*[19]. Furthermore, the lobule boundary cells have 3β-HSD positive reaction and misnamed Sertoli cells in the testis of *O. niloticus*[24]. The histochemical observations were confirmed by the electron microscopy that the testicular 3β-HSD and G6PD-positive cells of teleosts had ultrastructural features typical of the steroid-producing cells; e.g. *Padogobius martensi*[25], *Torpedo marmorata*[26], *Synbranchus marmoratus*[27], *Larimichthys polyactis*[28], and *Kareius bicoloratus*[29]. However, not all available electron microscopic results supply consistent support for the histochemical findings.

In spite of the biochemical investigations have appeared that the gonads of *M. cephalus* produce sex steroid hormones[7,30], no available information concerning the real steroid-secreting sites in the gonads of this fish. Although, the wide concern of the mullet, few investigations were done on the location and fine structure of the steroid-secreting cells during its gonads maturation. Thus, the real location of steroid hormones secretion in the mullet gonad is still disputable. The present research was done to determine the steroids-producing cells by both the histochemical and the ultrastructural methods in the testes of *M. cephalus*.

MATERIAL AND METHODS
Fish collection and sampling
From Al Bardawil Lagoon, 100 adult males of *M. cephalus* (with standard length larger than 28 cm) were collected alive during the testicular cycle (from September until December, 2020). Before the dissection of fishes, they were anesthetized in clove oil at a dose of 40 mg/L of water[31]. After fish collection, the total and standard lengths of fish were measured to the nearest 0.1 cm. The gonads were extirpated from the body cavity, weighed to the nearest 0.01 g and the gonadosomatic index (GSI) was calculated for each fish according to the following formula:

GSI = (Weight of the gonad/Gutted weight) × 100

Based on the gonads morphology, histological appearance, and their gonado-somatic index (GSI), five sexual maturity stages were signified as adult males stage I, stage II, stage III, stage IV (Ripe), and stage V (spent or post-spawning)[32].

Enzyme histochemistry
Immediately after dissection of fishes, the gonads were cut into fragments, which were fixed in a mixture of 1% formaldehyde (freshly prepared from paraformaldehyde) and 0.25% glutaraldehyde in a 0.1 mol phosphate buffer at pH = 7.4[33]. Fixation

lasted for 20 minutes at 4°C under constant shaking, and the fixative was renewed after 10 minutes. Also, other fragments of gonads were fixed in 4% glutaraldehyde in 0.1 mol phosphate buffer (pH = 7.4).

The incubation media were freshly prepared just before use. To prepare 10 mL of the incubation medium of the 3β-hydroxysteroid dehydrogenase, 2 mg dehydroepiandrosterone were dissolved either in a mixture of N, N-dimethyl formamide (0.3 mL) and propylene glycol (0.3 mL) or in 0.6 mL of acetone (final concentration of substrates; 0.7 mmol), which considered as medium "A". Approximately, 10 mg of nicotinamide adenine dinuleotide (NAD) and 10 mg of nitro-blue tetrazolium (NBT) were mixed in 9.4 mL of 0.1 mol phosphate buffer (at pH = 7.2) and considered as medium "B". Phenazine methosulfate (PMS), an electron carrier, was added to the mixture of the media "A+B" just before the beginning of incubation. The final concentration of PMS varied from 0.1 mmol to 10 mmol. All of this operation had to be performed in the dark because of the high photosensitivity of PMS.

The fixed tissue fragments were incubated for a period varying from 30 minutes to one hour at 37°C in the darkness in a shaking water bath. After incubation, the tissue fragments were rinsed in the phosphate buffer, dehydrated through an ascending series of ethyl alcohol, cleared, infiltrated in 1.0% celloidin in methyl benzoate (two changes, 1-5 hours for each change), and placed in two changes of benzene (15-45 minutes for each change) before finally infiltrating and embedding in paraffin wax (paraplast; milting point: 56-58°C). Transverse sections of gonads were cut at the thickness of 2-4 μm. These sections were counterstained with eosin.

Electron microscopy technique
Immediately after dissection of the fish, the gonads were cut into several parts. Those assigned for the ultrastructural studies were fixed in 4% glutaraldehyde in 0.1 mol cacodylate buffer (pH = 7.2-7.4) at 4°C for 2-3 hours. Thereafter, washing in 5% sucrose in 0.05 mol cacodylate buffer (pH = 7.2-7.4) was performed three times each one 15 minutes. The specimens could be left in the fourth change overnight at 4°C. The post-fixation was performed in 1% osmium tetroxide in 0.2 mol cacodylate buffer (pH = 7.2-7.4) for 2 hours at 4°C. Thereafter, rinsing in 0.1 mol cacodylate buffer (pH = 7.2-7.4) was done three times and washing in distilled water at 4°C.

The tissues were dehydrated in an ascending series of ethanol and embedded in epoxy resin. The processes of dehydration, infiltration, and embedding were done as indicated in the Durcupan ACM set (D0166, Sigma-Aldrich Chemie GmbH, Taufkirchen, Munich, Germany).

For light microscopy, semi-thin sections were cut on a JUM-7 ultramicrotome (Jeol, Tokyo, Japan) with glass knives and stained with 1.0% toluidine blue in 1.0% sodium borate. The ultra-thin sections were cut on a JUM-7 ultramicrotome with a glass knife, and placed on 200-mesh copper grids. The sections were then stained with uranyl acetate (saturated in 70% ethyl alcohol) followed by lead citrate[34] and examined with a JEM = 10 CX transmission electron microscope (JEOL Ltd., Akishima, Tokyo, Japan) at 80 kV.

All used chemicals in the present study were of high purity and purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Munich, Germany).

RESULTS
Histology of the putative steroids-secreting cells during testis maturation in *M. cephalus*
The putative (suspected) steroid-secreting cells in the testis of *M. cephalus* are presumably the interstitial cells, lobules boundary cells, as well as the Sertoli cells as illustrated in Figure "1". With the seasonal changes in the testicular activity (i.e. testicular recrudescence) of male *M. cephalus*, the presumably steroid-producing
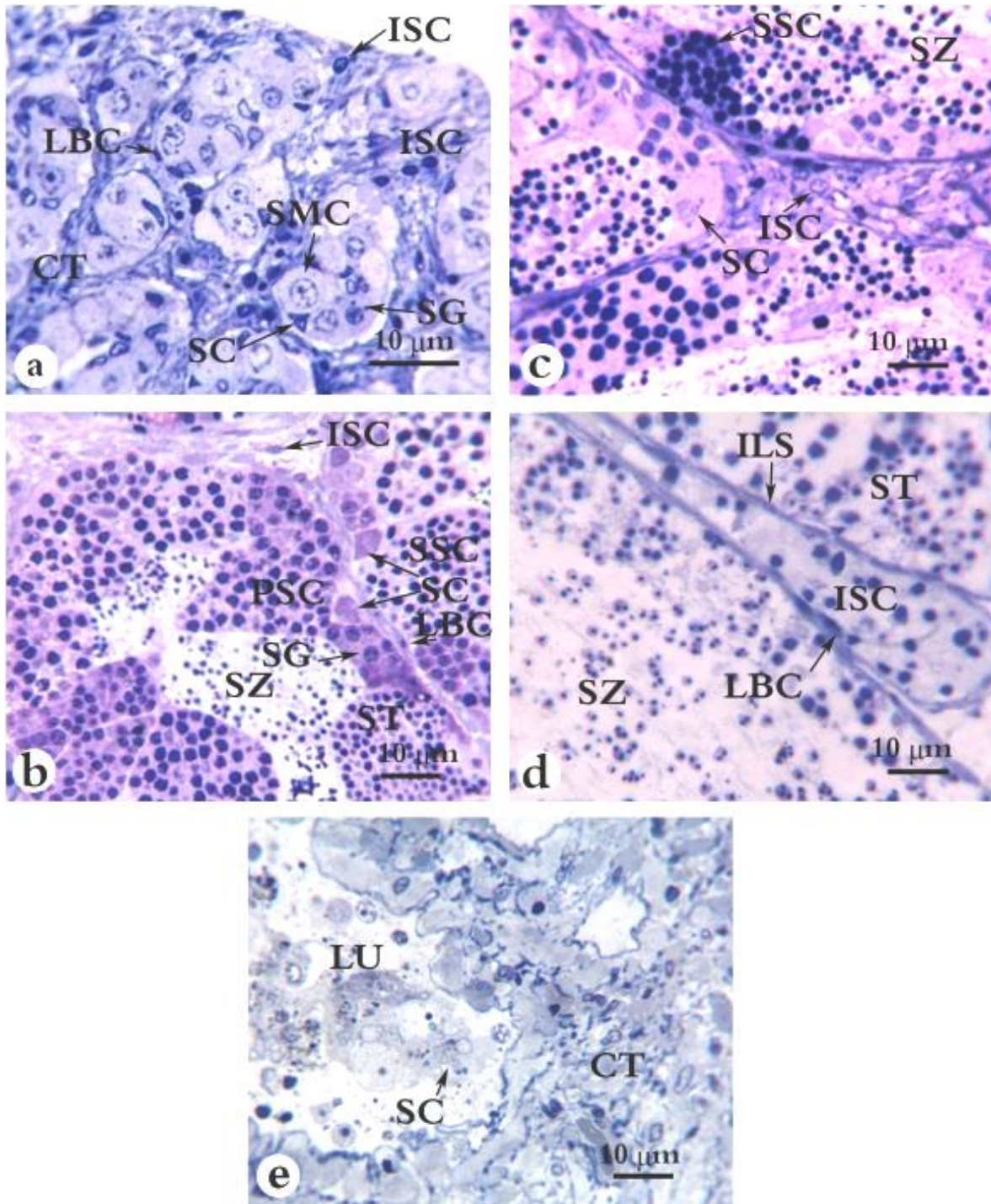
Figure 1: Semi-thin section of *M. cephalus* showing: (**a**) Immature testis, demonstrating the interstitial cells (ISC) with oval nuclei, lobule boundary cells (LBC) with elongated nuclei, and Sertoli cells (SC) with irregular nuclei. Besides, sperm mother cells (SMC), spermatogonia (SG), and interlobular connective tissue (CT) were present. (**b**) Stimulating spermatogenesis, designating the abundant interstitial cells (ISC) in the interlobular spaces, lobule boundary cells (LBC), and the Sertoli cells (SC) inside the lobules. Also, the spermatogonia (SG), primary spermatocytes (PSC), secondary spermatocytes (SSC), spermatids (ST), and spermatozoa (SZ) were illustrated. (**c**) Rapid spermatogenesis stage, marking abundant large interstitial cells (ISC) in the interlobular spaces, small-sized lobule boundary cells (LBC), and large Sertoli cells (SC). Also, the secondary spermatocytes (SSC), spermatids (ST), and spermatozoa (SZ) were demonstrated. (**d**) Ripe testis, displaying abundant interstitial cells (ISC) in the connective tissue of interlobular septum (ILS), lobule boundary cells (LBC), spermatids (ST) and spermatozoa (SZ). (**e**) Spent testis, perceiving the phagocytic Sertoli cells (SC) inside the lumens of seminiferous lobules (LU), beside the interstitial cells (ISC), remnants of germ cells, spermatozoa (SZ), and the thick interlobular connective tissue (CT).

cells have manifested certain quantitative and qualitative alterations. In the stage I (immature stage), the testis contained small sized lobules and a relatively large interlobular spaces filled with dense stroma consisting of connective tissue and blood capillaries (Figure 1a). The GSI was 0.35±0.15 (Table 1). The main components of the lobules comprised sperm mother cells and spermatogonia. The interstitial cells were occurring either singly or in small groups at both the periphery of the testis and in the interstices. These cells had comparatively large sizes with oval nuclei stained darkly with toluidine. The lobule boundary cells of the immature stage exhibited elongated shapes with also elongated nuclei. Sertoli cells commonly existed peripherally in the testis lobules being in close proximity to the germ cells (sperm mother cells and spermatogonia). They had comparatively large sizes and irregular shapes with prominent nuclei (Figure 1a).

In the stage II (stimulating spermatogenesis), the seminiferous lobules appeared relatively large in size (GSI of 0.8±0.25; Table 1) surrounded by lobule boundary cells and containing germ cells at various stages of maturation (i.e., spermatogonia, spermatocytes, spermatids, and spermatozoa; Figure 1b). Besides, Sertoli cells were seen in those lobules. Figure "1b" also showed the relatively small interlobular spaces occupied with connective tissue elements embodying interstitial cells. The interstitial cells exhibited a gradual increase in number. They had small-sized nuclei together with a sparse amount of cytoplasm. The lobule boundary cells being lesser in number and smaller in size and with smaller nuclei, than before, but the Sertoli cells had apparently increased in size and contained faintly stained nuclei. In addition, the testicular lobules contained spermetogonia, spermatocytes, spermatids, and spermatozoa (Figure 1b).

Table 1: Gonadosomatic index (GSI, %) of adult males *M. cephalus* at different stages of maturation during testicular cycle in natural habitat.

Testis Stage	Gonadosomatic index GSI%	
	Mean±SD	Number of fish
I. Immature stage	0.35±0.15	20
II. Stimulating spermatogenesis	0.80±0.25	20
III. Rapid spermatogenesis	3.10±0.38	20
IV. Mature (ripe) testis	10.55±0.65	20
V. Spent testis	1.25±0.35	20

Data were expressed as mean ± standard deviation (SD).

In the stage III (rapid spermatogenesis), the seminiferous lobules of the testis at this stage are revealing the predominance of spermatids and spermatozoa, but the number of spermatocytes was relatively low (Figure 1c). In this stage, the GSI was 3.1±0.38 (Table 1). The interstitial cells, during this period of development, have exhibited rapid multiplication with large sizes. The lobule-boundary cells were also apparently increased in both number and size. Besides, Sertoli cells of the testis were comparatively large in size in these stages, embodying relatively large-nuclei, and stained faintly with toluidine blue (Figure 1c).

In the stage IV (ripe testis), the seminiferous lobules appeared fully packed with mature spermatozoa. The GSI was 10.55±0.65 (Table 1). However, spermatids were still visible, though in small numbers (Figure 1d). The interstitial cells were

quite numerous in the interstitial septum of connective tissue, exhibiting large sizes and small nuclei and stained darkly with toluidine blue. On the other hand, Sertoli cells were clearly decreased in both size and number. Also, the lobule boundary cells were increased in size, but were conversely reduced in number However, spermatids were still visible, though in small numbers (Figure 1d).

In the stage V (spent testis), the seminiferous lobules of the testes examined after spermiation had seemingly diminished in size, and their walls were acquiring a thickened appearance owing to the contraction of their contents of elastic fibers (Figure 1e). In this stage, the GSI was 1.25±0.35 (Table 1). The interstitial cells of the spent testes were irregular in shape, large in size, and had large nuclei ranging from oval to round shapes. It is also clear that Sertoli cells of the spent testis had turned to be active phagocytes, being migrated to the lumen of the seminiferous lobules playing a conspicuous role in phagocytizing the residual sperms, as well as other degenerative germ cells (Figure 1e).

The histochemical localization of the steroid dehydrogenases (3β-HSD) in the testis of *M. cephalus*
The histochemical localization of 3β-HSD was examined in mature (or ripe) and in spent stages. In the ripe testis, the histochemical localization of 3β-HSD was detected in the interstitial and sertoli cells (Figure 2a). In the spent testis, obtained after spawning, which was also incubated in the steroid dehydrogenases media, gave a positive reaction for 3β-HSD (Figure 2b). The reaction was mainly restricted to the phagocytic Sertoli cells, which were thence located inside the lumen of the seminiferous lobules.

Ultrastructure of the steroid-producing cells during testis maturation in *M. cephalus*
In the immature stages of spermato-genesis, the interstitial cells were obviously situated within the interstitium either singly or in clusters (Figure 3a). They contained an extensive amount of rough endoplasmic reticulum, as well as oval or round mitochondria with tubular cristae embedded in a relatively dense matrix. Besides, these cells possessed moderately-sized nuclei containing aggregates of heterochromatin; occasionally, spherical lipid droplets, and free ribosomes and collagen fibers were present in the cytoplasm. The lobule boundary cells of the immature testes characterized by having large elongated nuclei with prominent nucleoli and a small amount of cytoplasm containing collagen fibers (Figure 3a).

Accompanying spermatogenesis of *M. cephalus*, during the stimulating spermato-genic stage, the secretory activity of the interstitial cells had exhibited an obvious increase (Figure 3b). They were noticeably manifesting almost the same characteristic features of the steroid-producing cells, namely extensive smooth endoplasmic reticulum, with dilated vesicular cisternae, numerous mitochondria with tubular cristae, large sized lipid droplets, as well as a few collagen fibers. In addition, they embodied large elongated nuclei with dense heterochromatin material. Both steroido-genic organelles characteristic for the steroid-producing cells, as well as the protein-synthesizing organelles, were quite detectable in the Sertoli cells (Figure 3b). Sertoli cells also exhibited large lipid droplets, dilated cisternae of smooth endoplasmic reticulum, microtubules and free ribosomes (Figure 3c). Also, they had small nuclei with irregular outlines and containing heterogeneous chromatin material (Figure 3c).

The following period, identified as the rapid spermatogenic stage (Figure 3d,e). The steroid-producing cells (interstitial cells, lobule boundary cells, and Sertoli cells) have manifested a high level of secretory activity. Concerning the interstitial cells, they contained distinct rough and smooth endoplasmic reticulum, mitochondria with tubular or lamellar cristae, free ribosomes,
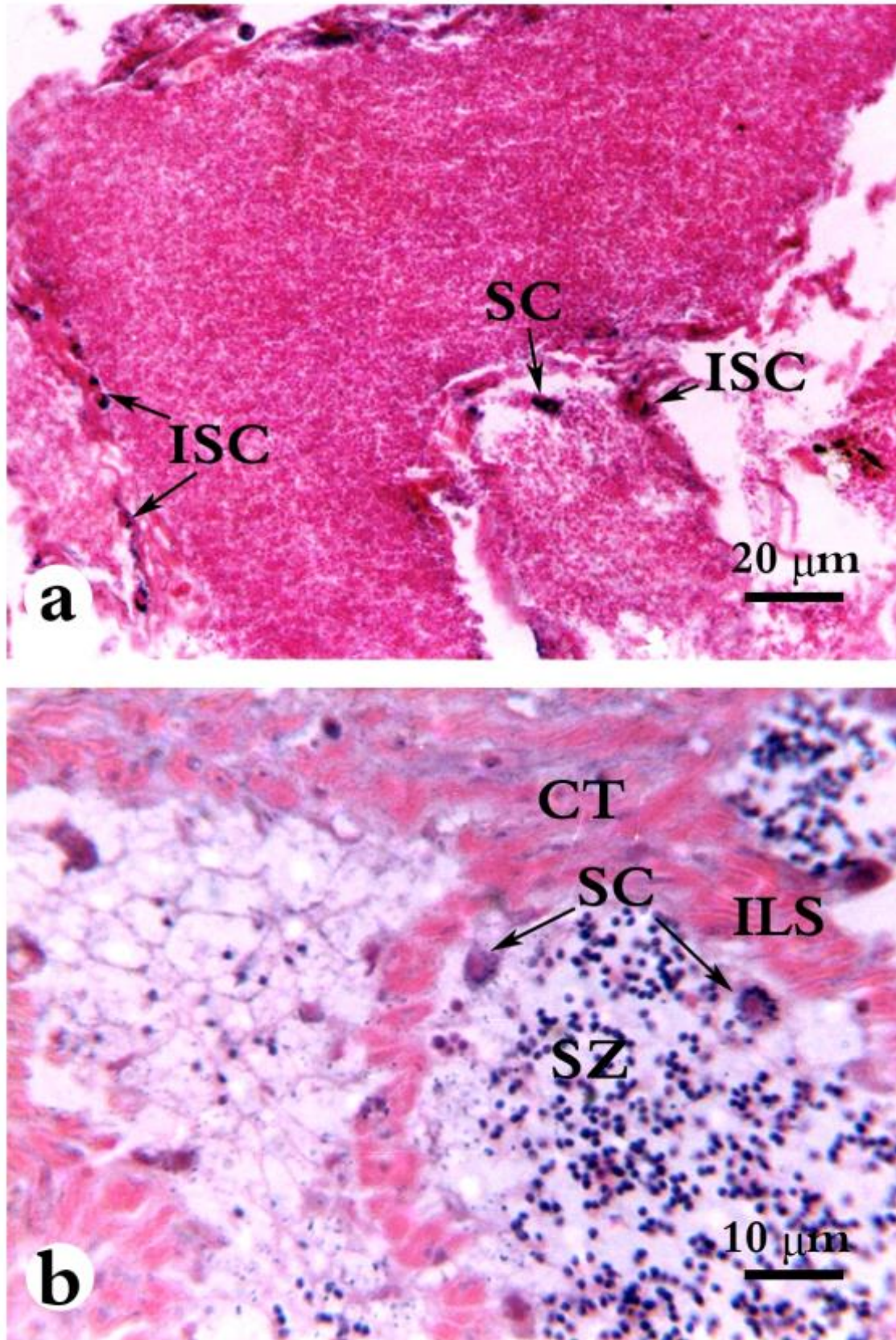
Figure 2: (**a**) Ripe testis of *M. cephalus* illustrating the positively reacting interstitial cells (ISC) and Sertoli cells (SC) with the substrate of 3β-HSD enzyme. (**b**) Spent testis of *M. cephalus*, showing the positive reaction of Sertoli cells (SC), located inside the seminiferous lobules, with the substrates of 3β-HSD enzyme. Beside remnants of germ cells, spermatozoa (SZ), and the thick interlobular septum (ILS) contained connective tissue (CT). 3β-HSD: 3β-hydroxysteroid dehydrogenase.
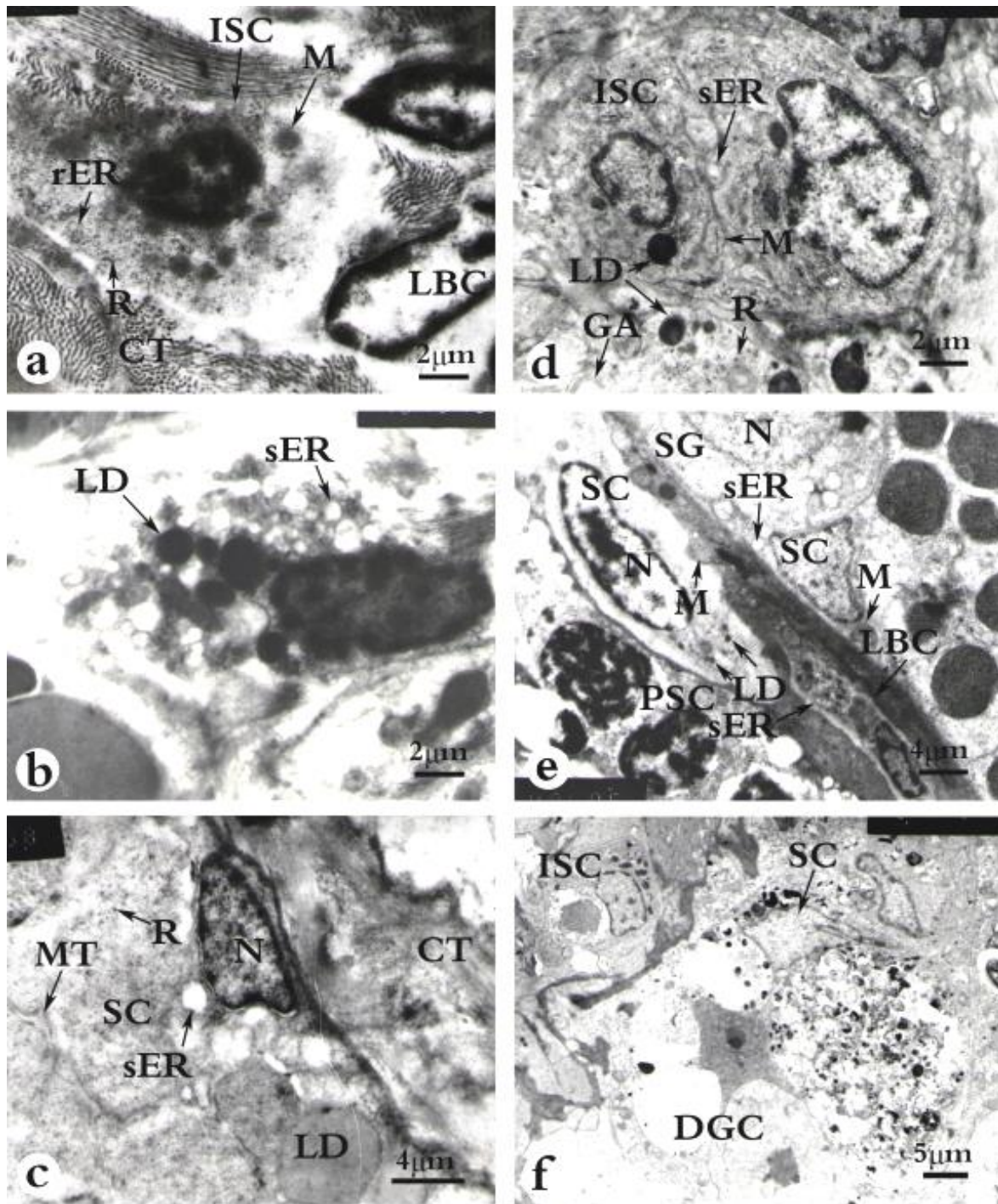
Figure 3: Ultrathin section of the testis of *M. cephalus* showing: (**a**) Immature testis, designating the interstitial cells (ISC), containing mitochondria (M), rough endoplasmic reticulum (rER), lipid droplets (LD), free ribosomes (R), lobule boundary cells (LBC), and collagen fibers (CF). (**b**) A stimulating spermatogenesis stage, illustrating interstitial cells with many elements of extensive smooth endoplasmic reticulum (sER), large lipid droplets (LD), beside the nucleus (N) containing dense heterochromatin. (**c**) A stimulating spermatogenesis stage, showing Sertoli cell (SC) with large lipid droplets (LD), large cisternae of smooth endoplasmic reticulum (sER), microtubules (MT), free ribosomes (R), the nuclei (N), collagen fibers (CF). (**d**) A rapid spermatogenesis stage, revealing interstitial cells (ISC) with large lipid droplets (LD), mitochondria (M), rough endoplasmic reticulum (rER), and smooth endoplasmic reticulum (sER). Besides, ribosomes (R), Golgi apparatus (GA), microtubules (MT), and nuclei (N) were illustrated. (**e**) A rapid spermatogenesis stage, showing lobule boundary cells (LBC) with lipid droplets (LD), mitochondria (M) and smooth endoplasmic reticulum (sER), and Sertoli cells (SC) containing mitochondria (M) with dense matrix and smooth endoplasmic reticulum (sER) with dilated cistemae. Beside spermatogonia (SG), primary spermatocytes (PSC), and nuclei (N) were present. (**f**) Spent testis, showing the phagocytic interstitial (ISC), Sertoli cells (SC), and degenerated germ cells (DGC).

and lipid droplets (Figure 3d). Besides, these cells contained large prominent nuclei with dense chromatin material. The Sertoli cells of the testis at this spermatogenic stage had lipid droplets, mitochondria, rough endoplasmic reticulum, distended Golgi apparatus, microtubules, ribosomes, and polysomes (Figure 3d). The lobule boundary cells of the rapid spermatogenic testis were also characterized by exhibiting lipid droplets with different sizes numerous mitochondria, endoplasmic reticulum (smooth and rough ones) ones, and free ribosomes (Figure 3d,e). Their nuclei were elongated with prominent nucleoli and a dense chromatin material (Figure 3e).

In the spent stage (after-spawning), the steroid-producing Sertoli cells and some interstitial cells have been obviously transformed into phagocytic cells to get rid of the remnants of the spermatozoa and some decayed germ cells (Figure 3f). The Sertoli cells of the spent testes have obviously represented nearly the same organelles characteristic for the phagocytic cells; comprising membrane-bound hetero-phagic lysosomes containing degenerated mitochondria and endoplasmic reticulum, in addition to parts of deteriorated germ cells (Figure 4a). The phagocytic Sertoli cell was with large pinocytotic vesicles containing portions of decayed germ cells (Figure 4a). They also contained many mitochondria with lamellar cristae beside some lysosomes. The nuclei of those Sertoli cells had irregular shapes with less dense chromatin material relative to the normal corresponding cells (Figure 4a,b). The phagocytic interstitial cells of the spent testis were characterized by the presence of extensive rough endoplasmic reticulum, distinct lysosomes, degenerated germ cells, and mitochondrial elements manifesting certain degenerating signs (Figure 4c). In addition, most of those cells have exhibited almost the same characteristic features of the steroid-producing cells, such as the picture of the endoplasmic reticulum (smooth and rough), mitochondria with different sizes and lamellar cristae, and lipid droplets

(Figure 4d,e). They also had prominent nuclei with condensed peripherally located chromatin adjacent to the nuclear envelopes. The lobule boundary cells of the spent testes were characterized by the presence of endoplasmic reticulum (smooth and rough), free ribosomes, and mitochondria with different sizes and lamellar cristae (Figure 4d).

DISCUSSION

Our findings give a particular anatomical disposition of the steroidogenic cells during testicular recrudescence of *M. cephalus* by using different practical techniques including the histological structure of the putative (suspected) steroidogenic cells, the histochemical confirmation of the 3β-HSD that is fundamental for modulating the production of sex steroids, and the electron microscopy to give the accurate locating of the steroid synthesis cells in the gonads of this fish. In *M. cephalus*, inhabiting natural habitat, five developmental stages were distinguished during testicular cycle (immature, stimulating and rapid spermatogenesis, ripe, and spent stages). The presently encountered findings have indicated the presence of three types of putative steroid-producing cells (the interstitial cells, lobule boundary cells, and Sertoli cells) in the testis of the inspected fish "*M. cephalus*". These observations have received confirmation from those presented previously for other teleost fishes[35,36]. It is worthy of mentioning that in some teleost species, Sertoli cells have been misnamed "lobule boundary cells". On the other hand, the lobule boundary cells have been misnamed "Sertoli cells" in *Oreochromis niloticus*[24]. However, the testes of *Oncorhynchus kisutch* and *Oncorhynchus gorbuscha* were reported to contain only interstitial cells and lobule boundary cells[37].

The present findings have designated that Sertoli cells are present in the lobule wall, being separated from the interlobular space by a thin, but distinct, basal lamina. However, these cells were often noticed to occur in close proximity to the developing germ cells. These observations are in
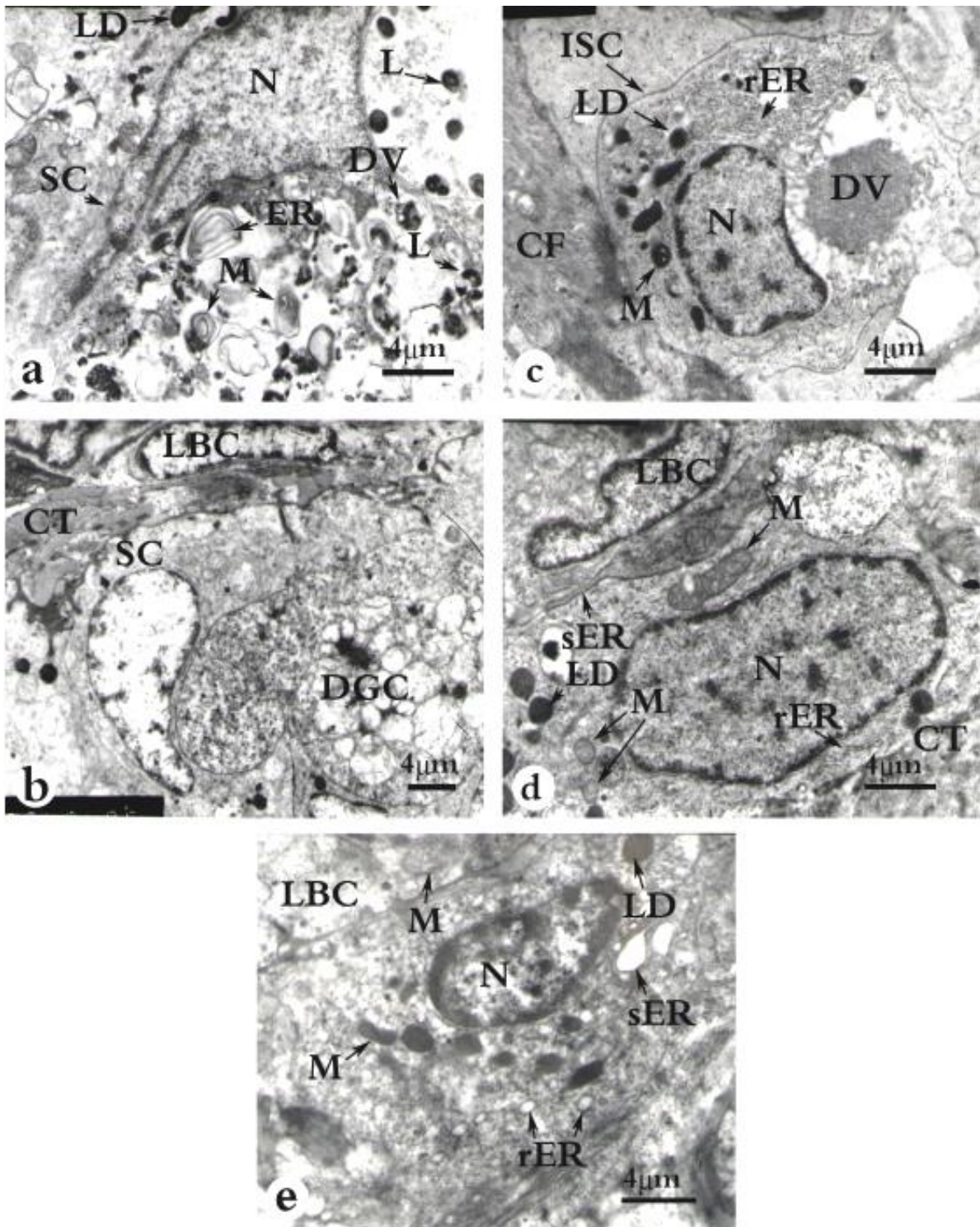
Figure 4: Ultrathin section of the testis of *M. cephalus* showing: (**a**) Spent testis, representing the phagocytic Sertoli cell (SC) with lipid droplets (LD), membrane bound digestive vacuole (DV) containing degenerating mitochondria (M), endoplasmic reticulum (ER) and lysosomes, and a nucleus (N). (**b**) Spent testis, illustrating the phagocytic Sertoli cells (SC) with pinocytotic vesicles containing a portion of degenerating germ cell (DGC), and mitochondria, beside lobule boundary cell (LBC) and connective tissue (CT). (**c**) Spent testis, showing the phagocytic interstitial cell (ISC), rough endoplasmic reticulum (rER), mitochondria (M), lipid droplets, (LD) and digestive vacuole (DV), beside nucleus (N) and collagen fibers (CF). (**d**) Spent testis illustrating lobule boundary cell (LBC) and interstitial cell (ISC) in a secretory phase, lipid droplets (LD), mitochondria (M), and both of smooth (sER) and rough endoplasmic reticulum (rER), beside nuclei (N) and connective tissue (CT). (**e**) Spent testis, designating interstitial cell (ISC), cisternae of both rough (rER) and smooth (SER) endoplasmic reticulum, lipid droplets (LD), mitochondria (M), and nucleus (N).

agreement with those presented in other teleost species[38-40]. Also, the presently obtained data have clarified that the seminiferous lobules of the testis of *M. cephalus* are completely lined by distinct flat lobule boundary cellular layer with elongated nuclei. These observations confirmed those declared in some other teleosts[37,41].

The interstitial cells of *M. cephalus*, as in most fishes (*Oreochromis niloticus*[24], *Oncorhynchus gorbiuscha* and *Oncorhynchus kisutch*[37], and *Ictalurus nebulosus*[18,40]), were distributed either singly or in small groups in the interstices between the seminiferous lobules. However, in *Poecilia latipinna* such cells were mentioned to be located around the sperm duct and at the periphery of the testis, but were not detected between the testis lobules[42]. Besides, the obtained results have revealed that the putative steroid-producing interstitial cells have manifested both quantitative and qualitative variations in concurrence with the successive developmental stages of the testis of *M. cephalus*. These cells were easily detected in all stages of maturity, but they appeared to attain their maximum development during ripeness and just before spermiation. Their signs of maximal activity were reflected by the remarkable increase in their numbers and their sizes. Our observations confirmed those obtained by other researchers concerning some other teleosts[29,39,40,43]. However, in general, the present histological observations concerning the steroidogenic activity of the interstitial cells of *M. cephalus* received good support from the findings presented by in *Oreochromis niloticlus*[44] and *Padogobius martensi*[25], which showed a marked correlation between the intensity of interstitial cells and the circulating levels of testosterone.

The activity of 3β-HSD was designated in the gonads of all *M. cephalus* examined by both light and electron microscopy. The presence of this enzyme is a characteristic feature of cells engaged with sex-steroid synthesizing activity. The 3β-HSD is known to occupy a key position in the metabolic pathways that lead to the formation of androgens and estrogens[13,17,25]. The occurrence of G6PD activity in the cells containing 3β-HSD enzyme provided sounding evidence of the steroidogenic potentiality of these cells, since G6PD is known to provide NADPH that is needed for hydroxylation during steroidogenelis[45,46]. The present mode of 3β-HSD occurrence is consistent with the localization of this enzyme in other teleosts such as *Torpedo marmorata*[14], *Anguilla anguilla*[15], *Cymatogaster aggregate*[21], and *Salmo gairdneri*[22]. Worthwhile is that 3β-HSD activity was detected only in the interstitial cells of the testis of a number of teleosts (e.g. *Ictalurus nebulosus*[18], *Serrasalmus spilopleura*[20], *Padogobius martensi*[13,25], and *Synbranchus marmoratus*[27]). In addition, to interstitial cells, lobule boundary cells were suspected to serve as possible sites of steroidogenesis in some teleosts such as *Tilapia mossambica*[23] and *Tilapia nilotica*[24].

As previously notified by certain researchers[25,47,48], the steroid-producing cells are believed to be characterized by certain ultrastructural features such as mitochondria with tubular cristae, smooth endoplasmic reticulum, and lipid droplets. The steroid-synthesizing cells are recognizable at the electron microscopic level by the presence of smooth endoplasmic reticulum as well as mitochondria, with tubular cristae, as already indicated previously[49]. The present ultrastructural examination of *M. cephalus* testis has revealed that the Leydig cells stand as essential sites of steroid production in the testis. These findings are also in good agreement with previous reports offered, concerning the corresponding cells in the testes of other teleosts including *Pampus argenteus*[8], *Kareius bicoloratus*[10], *Serrasalmus spilopleura*[20], *Boleophthalmus pectinirostris*[11], *Larimichthys polyactis*[28], and *Kareius bicoloratus*[29].

In addition to Leydig cells, Sertoli cells or lobule boundary cells were also considered as possible sites of steroidogenesis in teleosts[50]. This assumption was strengthened by the present ultrastructural findings of *M. cephaluis* testes, at different maturity stages, indicating a steroidogenic potency of these cells particularly at the end of the maturation phase of germ cells. Similarly, Sertoli cells or lobule boundary cells of other species have been reported to contain some ultrastructural features commonly accepted as characteristics of steroid-producing cells, comprising mitochondria with tubular cristae and a granular endoplasmic reticulum, although much less developed than those of the interstitial cells, beside many lipid droplets[22,26,37,38].

Worthwhile is that the presently gathered ultrastructural data have also displayed that the steroid synthesizing cells in the testes of *M. cephalus*, especially in the period of rapid spermatogenesis, possess certain features suggestive of protein synthesis ability including rough endoplasmic reticulum, as well as a distinct Golgi apparatus and free ribosomes. In addition, Sertoli cells have exhibited some ultrastructural features indicating their involvement in transporting metabolites (e.g. microtubules). This postulation supported the previous reports for *Poecilia latipinna*, *Carassius aurata*, *Oncorhynchus kisutch*, and *O. gorbuscha*, and *Pampus argenteus*[8,51,52].

After spawning of *M. cephalus*, Sertoli cells and some of the interstitial cells of spent testes had manifested certain features presented in the form of organelles, which are common characteristics for the phagocytic cells. These comprised a membrane-bound heterophagic lysosomes, pinocytotic vesicles containing portions of degenerating germ cells, deteriorated mitochondria, and injured endoplasmic reticulum. These findings reinforce the phagocytic role played by both Sertoli cells and some of the interstitial cells in the resorption of non-ejaculated sperm cells. A similar function assigned to Sertoli cells was reported in *Pampus argenteus*[8], *Kareius bicoloratus*[10], *Boleophthalmus pectinirostris*[11], *Padogobius martensi*[13], *Larimichthys polyactis*[28], *Kareius bicoloratus*[29], and *Synbranchus marmoratus*[53].

In conclusion, our investigation showed that during mullet spermatogenesis five developmental stages were distinguished and different cell types, i.e. Sertoli, Leydig, and lobule boundary cells, are involved in testosterone production, whose prominent role in spermatogenesis seems to be fundamental in meiotic phase.

COMPLIANCE WITH ETHICAL STANDARDS

FUNDING SOURCE DISCLOSURE

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

[1]   Chakrabarti, P. and Bose, S. (2014). Cyclical rhythms in the cytomorphology of testis of brackish water grey Mullet *Liza parsia* (Hamilton, 1822) inhabiting South-Eastern coast of India. J Entomol Zool Stud, 2: 110-118.

[2]   Uribe, M. C.; Grier, H. J. and Mejía-Roa, V. (2014). Comparative testicular structure and spermatogenesis in bony fishes. Spermatogenesis, 4(3): e983400 (DOI: 10.4161/21565562.2014.983400).

[3]   Kumar, P.; Saranya, V.; Natarajan, M. *et al.* (2015). Ultrastructure of grey mullet (*Mugil cephalus*, Linnaeus, 1758) spermatozoa as revealed from

light, scanning and transmission electron microscopy. J Appl Ichthyol, 31(6): 1113-1119.

[4] Senarata, S.; Jiraungkoorskulb, W. and Kettratad, J. (2018). Testicular structure and spermatogenesis of short mackerel, *Rastrelliger brachysoma* (Bleeker, 1851) in Upper Gulf of Thailand. AsPac J Mol Biol Biotechnol, 26(2): 30-43.

[5] Alix, M.; Kjesbu, O. S. and Anderson, K. C. (2020). From gametogenesis to spawning: how climate-driven warming affects teleost reproductive biology. J Fish Biol, 97(3): 607-632.

[6] Blawut, B.; Wolfe, B.; Premanandan, C. *et al.* (2021). Spatial and temporal changes in testis morphology and sperm ultrastructure of the sportfish sauger (*Sander canadensis*). *Acta Zoologica* (DOI: 10.1111/azo.12399).

[7] Suzuki, K.; Asahina, K.; Tamaru, C. S. *et al.* (1991). Biosynthesis of 17α, 20β-dihydroxy-4-pregnen-3-one in the ovaries of grey mullet (*Mugil cephalus*) during induced ovulation by carp pituitary homogenates and an LHRH analogue. Gen Comp Endocrinol, 84(2): 215-221.

[8] Chung, E.-Y.; Yang, Y.-C.; Kang, H.-W. *et al.* (2010). Ultrastructure of germ cells and the functions of Leydig cells and Sertoli cells associated with spermatogenesis in *Pampus argenteus* (Teleostei: Perciformes: Stromateidae). Zool Stud, 49: 39-50.

[9] Rajakumar, A. and Senthilkumaran, B. (2020). Steroidogenesis and its regulation in teleost-a review. Fish Physiol Biochem, 46(3): 803-818.

[10] Jun, J.-C.; Chung, E.-Y. and Yang, Y. C. (2006). Ultrastructure of germ cells, cyst epithelial cells and interstitial cells during spermatogenesis of the stone flounder, *Kareius bicoloratus*. Korean J Ichthyol, 18 (4): 311-318.

[11] Chung, E.-Y. (2008). Ultrastructure of germ cells, the Leydig cells, and sertoli cells during spermatogenesis in *Boleophthalmus pectinirostris* (Teleostei, Perciformes, Gobiidae). Tissue Cell, 40(3): 195-205.

[12] Schreibman, M. P.; Berkowitz, E. J. and van den Hurk, R. (1982). Histology and histochemistry of the testis and ovary of the platy fish, *Xiphophorus maculatus*, from birth to sexual maturity. Cell Tissue Res, 224: 81-87.

[13] Cinquetti, R. and Dramis, L. (2003). Histological, histochemical, enzyme histochemical and ultrastructural investigations of the testis of *Padogobius martensi* between annual breeding seasons. J Fish Biol, 63(6): 1402-1428.

[14] Prisco, M.; Liguoro, A.; Ricchiari, L. *et al.* (2008). Immunolocalization of 3β-HSD and 17β-HSD in the testis of the spotted ray *Torpedo marmorata*. Gen Comp Endocrinol, 155: 157-163.

[15] Grandi, G.; Chicca, M.; Tagliati, E. *et al.* (2010): Immunolocalization of steroidogenic enzymes in gonads of European eel, *Anguilla anguilla* (L.). J Appl Ichthyol, 26: 40-48.

[16] Sarkar, A. and Upadhyay, B. (2014). Histochemical detection of steroid synthesizing cells in the testes of goldfish, *Carassius auratus*, during the annual reproductive cycle. Biotech and Histochem, 89(2): 114-120.

[17] Savard, K.; Marsh, J. M.; and Howell, D. S. (1963). Progesterone biosynthesis in luteal tissue: role of nicotinamide adenine dinucleotide phosphate and NADP-linked de-hydrogenases. Endocrinol, 73(5): 554-563.

[18] Rosenblum, P. M.; Pudney, J. and Callard, L. P. (1987). Gonadal morphology, enzyme, histochemistry and palsma steroid levels during the annual reproductive cycle of male and female brown bulhead catfish, *Ictalurus nebulosus* Lesueur. J Fish Biol, 31(3): 325-341.

[19] Mousa, M. A. (1998). Immunocyto-chemical and histochemical studies on the reproductive endocrine glands of the Nile tilapia, *Oreochromis niloticus* (Teleostei, Cichlidae). J Egypt Ger Soc Zool, 27: 109-134.

[20] Nóbrega, R. H. and Quagio-Grassiotto, I. (2007). Morpho-functional changes in Leydig cells throughout the continuous spermato-genesis of the freshwater teleost fish, *Serrasalmus spilopleura* (Characiformes, Characidae): an ultra-structural and enzyme study. Cell Tissue Res, 329(2): 339-349.

[21] Wiebe, J. P. (1969). Steroid dehydro-genases and steroids in gonads of the seaperch *Cymatogaster aggregate* Gibbons. Gen Comp Endocrinol, 12: 256-266.

[22] Van den Hurk, R.; Peute, J. and Vermeij, J. A. J. (1978). Morpho-logical and enzyme cytochemical aspects of the testis and vas deferens of the rainbow trout, *Salmo gairdneri*. Cell Tiss Res, 186: 309-325.

[23] Yaron, Z. (1966). Demonstration of 3β-hydroxysteroid dehydrogenase in the testis of *Tilapia mossambica* (Cichlidae, Teleostei). J Endocrinol, 34: 127-128.

[24] Dougbag, A.; El-Gazzawy, E.; Kassem, A. et al. (1988). Histological studies on the testis of *Tilapia nilotica*. 1.- Basic structures. Alex J Vet Sci, 4(2): 49-57.

[25] Cinquetti, R. (1994). Localization of steroidogenesis in the testis of *Padogobius martensi* (Pisces, Gobiidae): histological, histochemical and ultrastructural investigation. Ital J Zool, 61(4): 309-315.

[26] Prisco, M.; Liguoro, A.; D'onghia, B. *et al.* (2002). Fine structure of Leydig and Sertoli cells in the testis of immature and mature spotted ray *Torpedo marmorata*. Mol Reprod Dev, 63(2): 192–201.

[27] Lo Nostro, F. L.; Antonellin, F. N.; Quagio-Grassiotto, I. *et al.* (2004). Testicular interstitial cells, and steroid-genic detection in the protogynous fish, *Synbranchus marmoratus* (Teleostei, Synbranchidae). Tissue Cell, 36(4): 221-231.

[28] Kang, H. W.; Chung, E.-Y.; Chung, J. S. *et al.* (2013). Ultrastructural studies of spermatogenesis and the functions of Leydig cells and Sertoli cells associated with spermatogenesis in *Larimichthys polyactis* (Teleostei, Perciformes, Sciaenidae). Anim Cells Syst, 17(4): 250-258.

[29] Kang, H.-W.; Kim, S. H. and Chung, J. S. (2016). Ultrastructural studies of germ cell development and the functions of Leydig cells and Sertoli cells associated with spermatogenesis in *Kareius bicoloratus* (Teleostei, Pleuronectiformes, Pleuronectidae). Dev Reprod, 20: 11-22.

[30] Azoury, R. and Eckstein, B. (1980). Steroid production in the ovary of the gray mullet (*Mugil cephalus*) during stages of egg ripening. Gen Comp Endocrinol, 42(2): 244- 250.

[31] Mousa, M. A. (2004). The efficacy of clove oil as an anaesthetic during the induction of spawning of thin-lipped grey mullet, *Liza ramada* (Risso). J Egypt Ger Soc Zool, 45 (A): 515-535.

[32] Mousa, M. A. and Mousa, S. A. (2000). Immunocytochemical studies of the gonadotropic cells in the pituitary gland of male mullet, *Mugil cephalus* during the annual reproductive cycle in both natural habitat and captivity. J Appl Ichthyol, 16: 98-103.

[33] Berchtold, J.-P. (1977). Ultracyto-chemical demonstration and probable localization of 3β-hydroxysteroid de-hydrogenase activity with a ferri-cyanide technique. Histochem, 50: 175-190.

[34] Reynolds, E. S. (1963). The use of lead acetate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol, 17: 208-212.

[35] Bisht, J. S. (1974) Seasonal histological changes in the testes of a hill-stream teleost, *Schizothorax richardsonii* (Gray/Hard). Acta Anat (Basel), 88(3): 398-410.

[36] Shrestha, T. K. and Kanna, S. S. (1978). Seasonal changes in the testes of a hill-stream teleost, *Garra gotyla* (Gray). Acta Anat (Basel), 100(2): 210-220.

[37] Nagahama, Y.; Clarke, W. C. and Hoar, W. S. (1978). Ultrastructure of putative steroid-producing cells in the gonads of coho (*Oncorhynchus kisutch*) and pink salmon (*Oncorhynchus gorbuscha*). Can J Zool, 56(12): 2508-2519.

[38] Van den Hurk, R.; Meek, J. and Peute, J. (1974). Ultrastructural study of the testis of the black molly (*Mollienisia latipinna*). II. Sertoli cells and Leydig cells. Proc Kon Ned Akad Wet Ser C, 77: 470-476.

[39] Grier, H. J. (1981). Cellular organization of the testis and spermatogenesis in fishes. Amer Zool, 21: 345-357.

[40] Burke, M. G. and Leatherland, J. F. (1984). Seasonal changes in testicular histology of brown bullheads, *Ictalurus Nebulosus* Lesueur. Can J Zool, 62(6): 1185-1194.

[41] O'Halloran, M. J. and Idler, D. R. (1970). Identification and distribution of the Leydig cell homolog in the testis of sexually mature Atlantic salmon (*Salmo salar*). Gen Comp Endocrinol, 15(2): 361-364.

[42] Van den Hurk, R. (1973). The localization of steroidogenesis in the testis of oviparous and viviparous teleosts. Proc Kon Ned Akad Wet Ser C, 76(3): 270-279.

[43] Guraya, S. S. (1976). Recent advances in the morphology, histochemistry, and biochemistry of steroid-synthesizing cellular sites in the testes of nonmammalion

vertebrates. Int Rev Cytol, 47: 99-136.

[44] Nakamura, M. and Nagahama, Y. (1989). Differentiation and development of Leydig cells, and changes of testosterone levels during testicular differentiation in tilapia *Oreochromis niloticus*. Fish Physiol Biochem, 7(1-6): 211-219.

[45] Samuels, L. T., and Eik-Nes, K. B. (1968). Metabolism of Steroid Hormones. In Metabolic Pathways (Greenberg, D. M., ed.), pp. 169-200. Academic Press, New York, NY, USA.

[46] Wiest, W. G. and Kidwell, W. R. (1969). The Regulation of Progesterone Secretion by Ovarian Dehydrogenase. In The Gonads (Mckerns, K. W., ed.), pp. 295-325. Appleton-Century Crofts, New York, NY, USA.

[47] Kurosumi, K. and Fujita, H. (1974). An Atlas of Electron Micrographs: Functional Morphology of Endocrine glands. Lgaku Shoin Ltd, Tokyo, Japan.

[48] Tenugu, S.; Pranoty, A.; Mamta, S.-K. *et al.* (2021). Development and organisation of gonadal steroidogenesis in bony fishes - a review. Aquac Fish, 6(3): 223-246.

[49] Christensen, A. K. (1975). Leydig cells. In: Handbook of Physiology (Hamilton, D. W. and Greep, R. O., ed.), pp. 57-94. Williams and Wilkins, Baltimore, MD, USA.

[50] Lofts, B. and Bern, H. A. (1972). The Functional Morphology of Steroidogenic Tissues. In Steroids in Nonmammalian Vertebrates (Idler, D. R., ed.), pp. 37-125. Academic Press, New York, NY, USA.

[51] Grier, H. J. (1975). Aspects of germinal cyst and sperm development in *Poecilia latipinna* (Teleostei: poeciliidae). J Morphol, 146(2): 229-250.

[52] Hoar, W. S. and Nagahama, Y. (1978). The cellular sources of sex steroids in

teleost gonads. Ann Biol Anim Bioch Biophys, 18(4): 893-898.

[53] Lo Nostro, F. L., Grier, H., Meijide, F. J. *et al*. (2003). Ultrastructure of the testis in *Synbranchus marmoratus* (Teleostei, Synbranchidae): the germinal compartment. Tissue Cell, 35(2): 121-132.

التركيب النسيجي، وكيمياء الأنسجة، والتركيب الدقيق لتكوين الستيرويد أثناء دورة الخصية لسمك البوري"Mugil cephalus" فى البيئة الطبيعية

فريال أحمد المسدي[1]، نهى عبد الحميد خليل[2]، مصطفى عبد الوهاب موسى[3]

[1]قسم علم الحيوان، كلية العلوم، جامعة المنوفية، المنوفية، مصر

[2]معمل تناسل وتفريخ الأسماك، المعهد القومي لعلوم البحار والمصايد، القليوبية، مصر

[3]معمل تناسل وتفريخ الأسماك، المعهد القومي لعلوم البحار والمصايد، الإسكندرية، مصر

ازداد الاهتمام مؤخرًا بتكاثر وإنتاج أسماك البورى "Mugil cephalus". ونظرًا للدور الرئيسي الذى تلعبه الهرمونات الستيرويدية في نضوج الأسماك وإنتاج البويضات المخصبة، فقد تم التخطيط للعمل الحالي لتحديد وفحص الخلايا الستيرويدية في خُصَى أسمك البوري، ومن ثم تحديد مستويات نشاطاتها خلال دورة نُضجها بهدف الاستفادة من النتائج في تكاثر واستزراع الأسماك. وتم الحصول على الذكور الناضجة لأسماك البورى من البيئة الطبيعية (بحيرة البردويل). وتم إجراء الفحوصات الكيميائية الخلوية ودراسة التركيب الدقيق للخلايا الستيرويدية التي تحتوي على إنزيم 3-بيتا هيدروكسى إستيرويد ديهيدروجينيز. وقد أمكن تمييز خمس مراحل من التغيرات أثناء دورة الخصية فى ذكور أسماك البورى: المرحلة غير الناضجة، مرحلة تحفيز تكوين الحيوانات المنوية، المرحلة السريعة لتكوين الحيوانات المنوية، مرحلة النضج، ومرحلة مابعد التفريخ. وأظهر الفحص بالمجهر الإلكتروني أن الخلايا البينية (خلايا ليدج)، والخلايا المحيطة بالفصيصات الخصوية، وخلايا سيرتولي التي تم فحصها أثناء المرحلة السريعة لتكوين الحيوانات المنوية بها قطرات دهنية بارزة وعضيات سيتوبلازمية نموذجية للخلايا المصنعة للبروتين (مثل الشبكة الإندوبلازمية الخشنة والريبوسومات الوفيرة وجهاز جولجي المتميز). بالإضافة لذلك، تمتلك خلايا سيرتولي، على وجه الخصوص، أنيبيبات دقيقة معينة معروفة بأنها تعمل في نقل النواتج الأيضية. علاوة على ذلك، فإن فحص الخُصَى فى مرحلة ما بعد التفريخ بالمجهر الإلكتروني أوضح أن خلايا سيرتولي وكذلك بعض الخلايا البينية قد تحولت إلى خلايا بلعمية للتخلص من خلايا الحيوانات المنوية المتبقية بعد التفريخ. الخلاصة، يمكن أن تكون النتائج الحالية ذات قيمة كبيرة في المشاكل المتعلقة بنضج الأسماك عند الاستزراع.