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The Histochemistry of Steroidogenesis During the Reproductive Cycle of *Oreochromis niloticus*

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ABSTRACT

Monitoring the maturation of fish gonads requires an understanding of the locations of reproductive hormone formation both under the influence of environmental factors and under normal conditions. A crucial role in the biosynthesis of active steroid hormones is played by 3β-hydroxysteroid dehydrogenase (3β-HSD). The male and female *Oreochromis niloticus* gonads at different stages of the annual sexual cycle were analyzed for 3β-HSD using histochemical techniques to pinpoint the locations of steroid synthesis. In females, a specific cell type in the thecal layer, interstitial cells, and ovarian wall epithelial cells exhibited 3β-HSD activity. In males, only Leydig cells (interstitial cells) and lobule boundary cells exhibited 3β-HSD activity. During the stages of sexual maturation and spawning, the Leydig cells' steroidogenic activity increased. Additionally, during the maturing and spawning stages, 3β-HSD exhibited a strong reaction that increased in intensity and the apparent quantity of cells exhibiting a reaction. However, after spawning, the reaction decreased. Furthermore, during the oogenesis process, which is the development of the previtellogenic oocytes to the ripe oocytes, 3β-HSD activity was elevated in the various phases of ovarian maturation. In conclusion, it can be stated that the cells producing steroid hormones in the male and female gonads of *O. niloticus* have been sufficiently identified, and their steroid hormone-producing capacity has been demonstrated. Naturally, more research is necessary to apply this finding to the development of fish gonads during the reproduction in aquaculture settings or under situations where this teleost species is concerned to environmental treatments.

INTRODUCTION

Good management in fish farming and natural fisheries depends on having a precise knowledge of the gonad cycle and its functional mechanism. Gonadal and hypophysial hormones play a vital part in regulating the timing of reproduction in teleost fish. Although it is true that teleost gonads are recognized for steroid hormones secretion **(Chung *et al*., 2010; Ruksana *et al*., 2011; Sarkar & Upadhyay, 2014; Uribe *et al*.,**

2014; Rajakumar & Senthilkumaran, 2020), there is an ongoing debate regarding the distribution of the steroidogenic endocrine tissue. Sertoli cells, germ cells, lobule boundary cells, interstitial cells, and testicular efferent duct epithelial cells are the five cellular sources that have been identified as the site of androgen production **(Jun *et al.*, 2006; Chung, 2008; Chung *et al.*, 2010; Uribe *et al.*, 2014)**. Similarly, steroid-producing sources in the ovary have been identified as the granulose cells, some thecal cells, the corpus luteum, corpora atretica, and interstitial gland tissue **(Guraya, 1976a; Saidapur & Nadkarni, 1976; Khalil, 2001; Mousa *et al.*, 2023)**.

The fish in the genus *Oreochromis*, formerly *Sarotherodon*, have high rates of growth and reproduction and able to tolerate a variety of environmental factors, including temperature, salinity, acidity, and poor water quality. This makes them ideal for culture. Locally known as "The Nile bolti" or "The Nile tilapia," *Oreochromis niloticus* is currently among the most important economically farmed fish species in our nation. The location and arrangement of the steroidogenic endocrine tissue in *O. niloticus* gonads have received minimal study, despite the tilapia's widespread significance. Using histochemical analysis, **Yaron (1971)** proved that the *O. niloticus* ovary's granulose and theca cells both contained 3β-hydroxysteroid dehydrogenase (3β-HSD). However, only granulose cells from *Sarotherodon aureus* have demonstrated this enzyme activity **(Livni, 1971)**. Several teleost fishes have interstitial cells in their testis where 3β-HSD has been histochemically localized; *Xiphophorus maculatus* **(Schreibman *et al.*, 1982)**, *Ictalurus nebulosus* **(Rosenblum *et al.*, 1987)**; *Padogobius martensi* **(Cinquetti & Dramis, 2003)**; *Serrasalmus spilopleura* **(Nóbrega & Quagio-Grassiotto, 2007)** and *Carassius auratus* **(Sarkar & Upadhyay, 2014)**. Furthermore, only the interstitial cells and lobule boundary cells exhibited activity of 3β-HSD in the testis of *Sarotherodon mossambicus* **(Yaron, 1966)**, *L. ramada* **(Khalil, 2001)**, and *M. cephalus* **(El-messady *et al.*, 2023)**. Additionally, **Dougbag *et al.* (1988)** mislabeled the Sertoli cells in *O. niloticus* testis as lobule boundary cells, despite the cells having a positive 3β-HSD reaction.

Thus, there is ongoing debate regarding the precise location of steroid hormone production within the tilapia gonad. The goal of the present investigation was to identify the steroidogenic tissues in *O. niloticus*'s testes and ovaries using histochemical techniques and to monitor steroidogenesis activity throughout the reproductive cycle.

MATERIALS AND METHODS

Animals

Living *O. niloticus* adults of both sexes were collected from the Nile River at various stages of sexual maturity during the reproductive cycle.

Gonads histology

The obtained livers from sacrificed fish were cut into small pieces and fixed immediately in 10% neutral buffered formalin. Then, they were processed for embedding in paraffin and cut at the thickness of 4- 6µm. Staining was carried out in Harris's alum

hematoxylin, following the method of **Mallory (1938)**, with an aqueous solution of eosin (1%) applied as a counter stain.

Enzyme histochemistry

According to **Berchtold (1977)**, the gonads were fixed for the demonstration of 3β-hydroxysteroid dehydrogenase (3β-HSD). The fish were anesthetized in a solution of clove oil (Sigma) at a concentration of 40mg/ l before being handled. They were then perfused via the ascending aorta with 20ml of normal saline, followed by 50ml of 1% paraformaldehyde and 0.25% glutaraldehyde in saline phosphate buffer 0.1M (pH 7.4) that contained 2.5% polyvinylpyrrolidone (PVP) and 2.5% polyvinylalcohol (PVA) at 4^{o}C. As soon as the fish was dissected, small testicular fragments were taken out and postfixed in the fixative mixture at a temperature of 4^{o}C for two to three hours. Moreover, the fixative was changed after an hour. Following multiple phosphate buffer washes, tissue fragments were left in a cryoprotective solution containing 5% sucrose at 4^{o}C for an entire night. The preserved tissue pieces were incubated in the enzyme medium for a duration that was adjusted to 1 to 2 hours at 37^{o}C in complete darkness within a vibrating water bath.

The incubation medium of 3β-HSD (Berchtold, 1977)

To prepare the incubation medium, 10ml of the solution was freshly prepared following the outlined procedure. Initially, 2 milligrams of dehydroepiandrosterone (DHA) were dissolved in 0.6 milliliters of acetone, achieving a final substrate concentration of 0.7mM. Alternatively, DHA can be dissolved in a combination of 0.3 milliliters of n,n-dimethylformamide (DMF) and 0.3 milliliters of propylene glycol. Subsequently, 9.4ml of 0.1 M phosphate buffer at pH 7.2 was prepared, containing 10mg of nitro-blue tetrazolium (NBT) and 10 mg of nicotinamide adenine dinucleotide (NAD). Finally, just before the incubation commenced, the electron carrier phenazine methosulfate (PMS) was added to the combined solution (A+B). The final concentration of PMS varied from 0.1mM to 10 mM, depending on the experimental requirements.

The tissue fragments were first rinsed in phosphate buffer, then dehydrated using an increasing series of ethyl alcohol. They were then cleared and infiltrated in 1% celloidin in methylbenzoate, and lastly they were infiltrated and incorporated into paraplast (m.p.: 56–58^{o}C). Additionally, at a thickness of 5µm, serial transverse sections were cut. The sections underwent xylene deparaffinization, graded ethanol rehydration, distilled water washing, and 1% aqueous eosin counterstaining. The sections underwent a series of procedures following the counterstaining process: tap water washing, alcohol dehydration, xylene clearing, and DPX mounting.

RESULTS

A) Histology of the putative steroid-producing cells

Male

O. niloticus testis are paired elongated structures that are located ventral to the kidneys. Each testis is made up of many seminiferous lobules that are joined together by a connective tissue (Fig. 1a, b). A distinct testicular wall made of a thin membrane of connective tissue surrounds the entire testis. A single layer of fusiform cells, also referred to as "lobule boundry cells," with elongated nuclei, surrounded the lobules (Fig. 1a). Most of the cells in the seminiferous lobules are germ cells (Fig. 1a). In the testis lobules, sertoli cells typically occupy a periphery position. Their nuclei are prominent, and their irregular shape and size make them relatively large in comparison (Fig. 1a). The interstitial or "Leydig" cells are present between the seminiferous lobules, either individually or in clusters (Fig. 1a).

Female

O. niloticus have paired elongated ovaries. Every ovary is bounded by a unique connective tissue ovarian wall. The ovary held an interstitial tissue and oocytes, which are distributed randomly (Fig. 1c, d). There are primary and vesicles stage oocytes in *O. niloticus*'s early vitellogenic ovary (Fig. 1c, d). The formation of oil droplets in the ooplasm signifies the start of the yolk deposition, which is marked by the vesicles oocyte (Fig. 1c, d). A single layer of follicle cells encloses the primary oocytes (Fig. 1c). A layer of inner granulosa cells and an outer layer of thecal cells make up the follicle as the oocytes develop (Fig. 1e, f).

B) Histochemistry of 3β-HSD of the steroid-producing cells

The steroidogenic key enzyme 3β-HSD was located in the gonads of *O. niloticus* during the reproductive cycle using histochemical localization (Figs. 1-3).

Male

A 3β-HSD positive response was limited to the lobule boundary cells and Leydig (interstitial) cells discovered in the interlobular spaces and the fibrous tissue encircling the spermatic duct and the testicular periphery (Fig. 1a, b).

Female

In females, the primary oocytes follicle cells and a specific cell type in the thecal layer of vitellogenic oocytes showed a positive response to 3β-HSD (Fig. 1d, f). Furthermore, the interstitial cells exhibited a positive response to 3β-HSD (Fig. 1d, f).

C) Seasonal changes in in the gonads 3β-HSD activity

In the testis

In the maturing and spawning stages of testis (Fig. 2a–d), the response was strong, with an increase in intensity and a noticeable rise in the number of cells exhibiting a reaction. However, in the post-spawning testis (Fig. 2e), the reaction decreased. Following that, the 3β-HSD enzyme's activity dropped during the winter and autumn resting periods (Fig. 2f).

In the ovary

During the oogenesis process, when the primary (previtellogenic) oocytes develop into the ripe oocytes, 3β-HSD enzyme activity was altered (Fig. 3). During the spawning season, spring and summer, there was a rise in the amount and activity of steroidogenic cells, which are a particular kind of cells present in the thecal layer of vitellogenic oocytes (Figs. 3b-f).

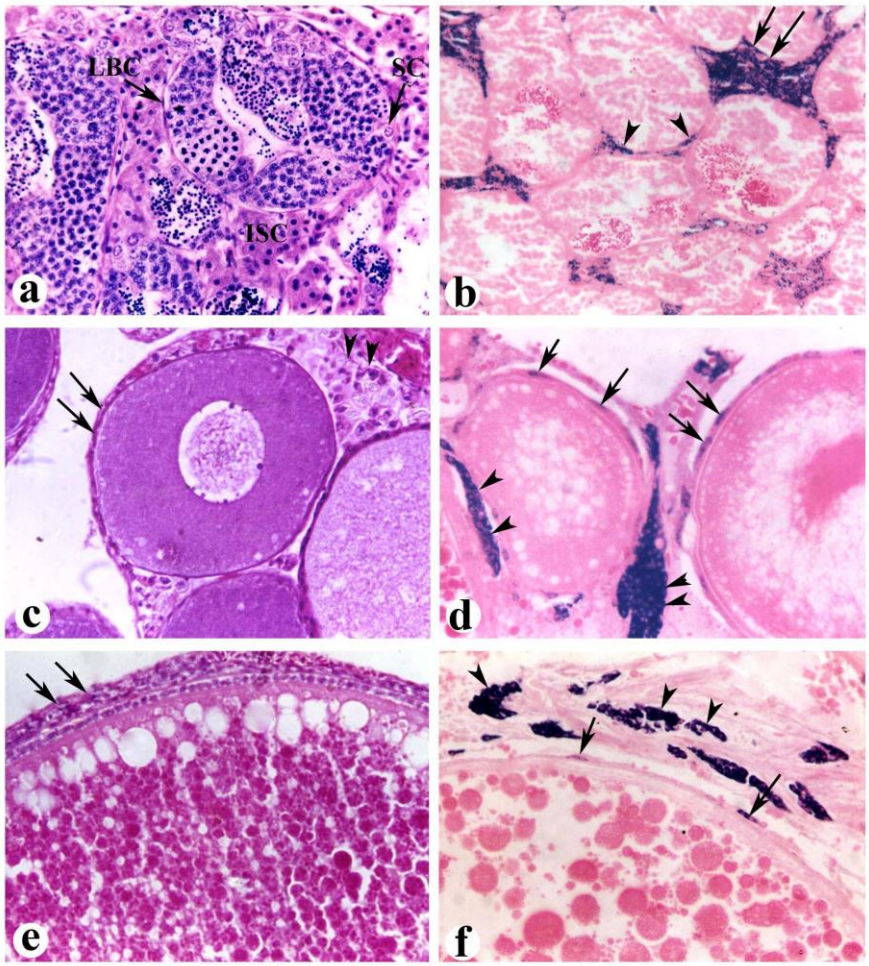

Fig. 1. Transverse sections of *O. niloticus* gonads, at different maturity stages, stained with Harris's hematoxylin and eosin (a, c and e) or incubated with the substrate of 3β-HSD enzyme: a) Testis, obtained during the period of rapid spermatogenesis,showing seminiferous lobules containing Sertoli cells (SC) and germ cells in different maturity stages, and bounded by lobule boundary cells (LBC). The interstitial cells (ISC) are present in between the seminiferous lobules. (X400). (b) Parts of transverse sections of *O. niloticus* testes, showing positive reaction of 3β-HSD in both the interstitial cells (ISC) (arrows) and lobule boundary cells (LBC) (arrow heads). (X400). c) A part of *O. niloticus* ovary, obtained during the stage of early vitellogenesis, showing vesicle oocytes (arrows), and interstitial tissue (arrow heads). (X100). d) Parts of transverse sections of *O. niloticus* ovaries, showing: positive reaction of 3β-HSD in the exterior layer of vitellogenic oocyte follicle (arrows) and interstitial cells (arrow heads). (X100). e) Part of transverse section of vitellogenic *O. niloticus* ovary, displaying the follicle layers of vitellogenic oocyte (arrows). (X400). f) A magnified portion of *O. niloticus* vitellogenic ovary, showing positive reaction of 3β-HSD in a special cell type in the thecal layer of vitellogenic oocyte (arrows) and interstitial cells (arrow heads). (X400)

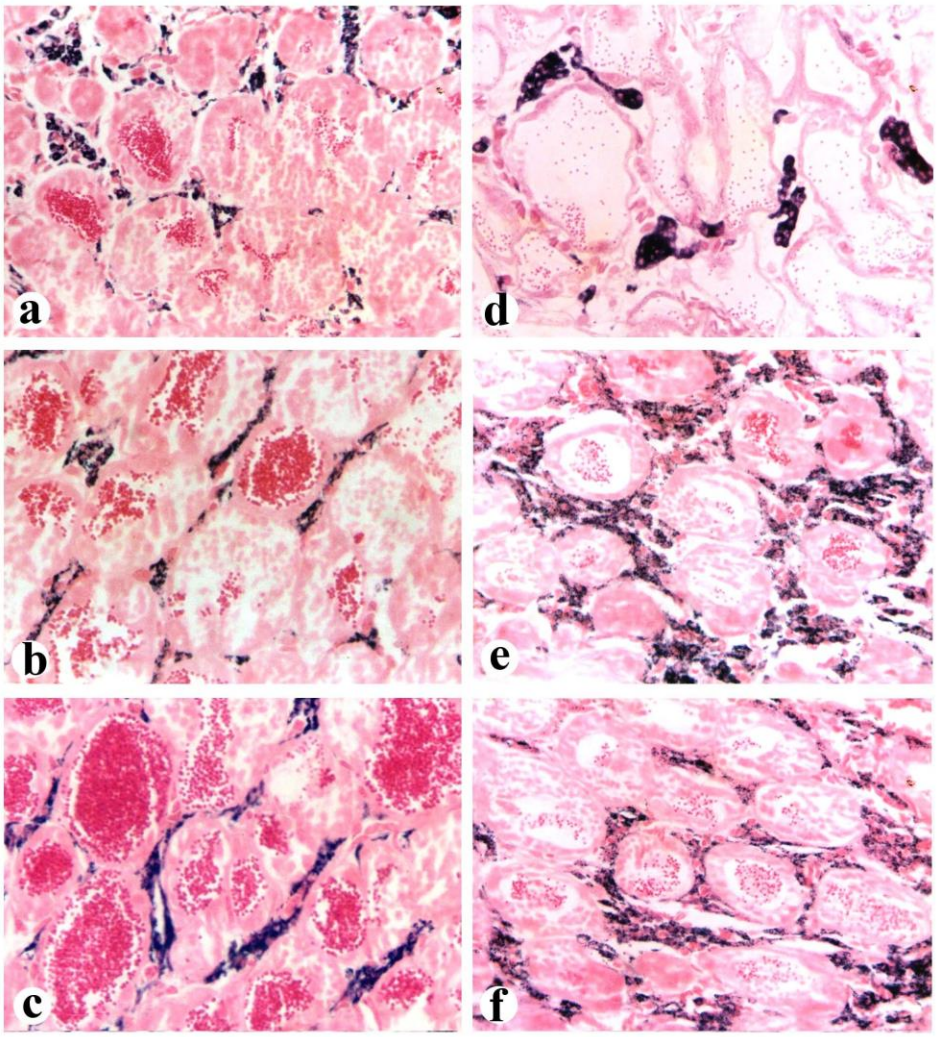

Fig. 2. Parts of transverse sections of testes of *O. niloticus*, at different maturity stages, incubated with the substrate of 3β-HSD enzyme, showing positive staining in the cells producing steroids of the different testicular stages (X400): a) Stimulating spermatogenesis. b) Rapid spermatogenesis. c) Ripe stage. d) Spawning (running) stage. e) Postspawning stage. f) Resting stage. Note the strong reaction of 3β-HSD in the steroid synthetic cells during maturing and spawning stages (spring and summer), and moderate reaction in postspawning testis. Also, a weak reaction of 3β-HSD in the steroidogenic cells was obtained during the resting period (autumn and winter)

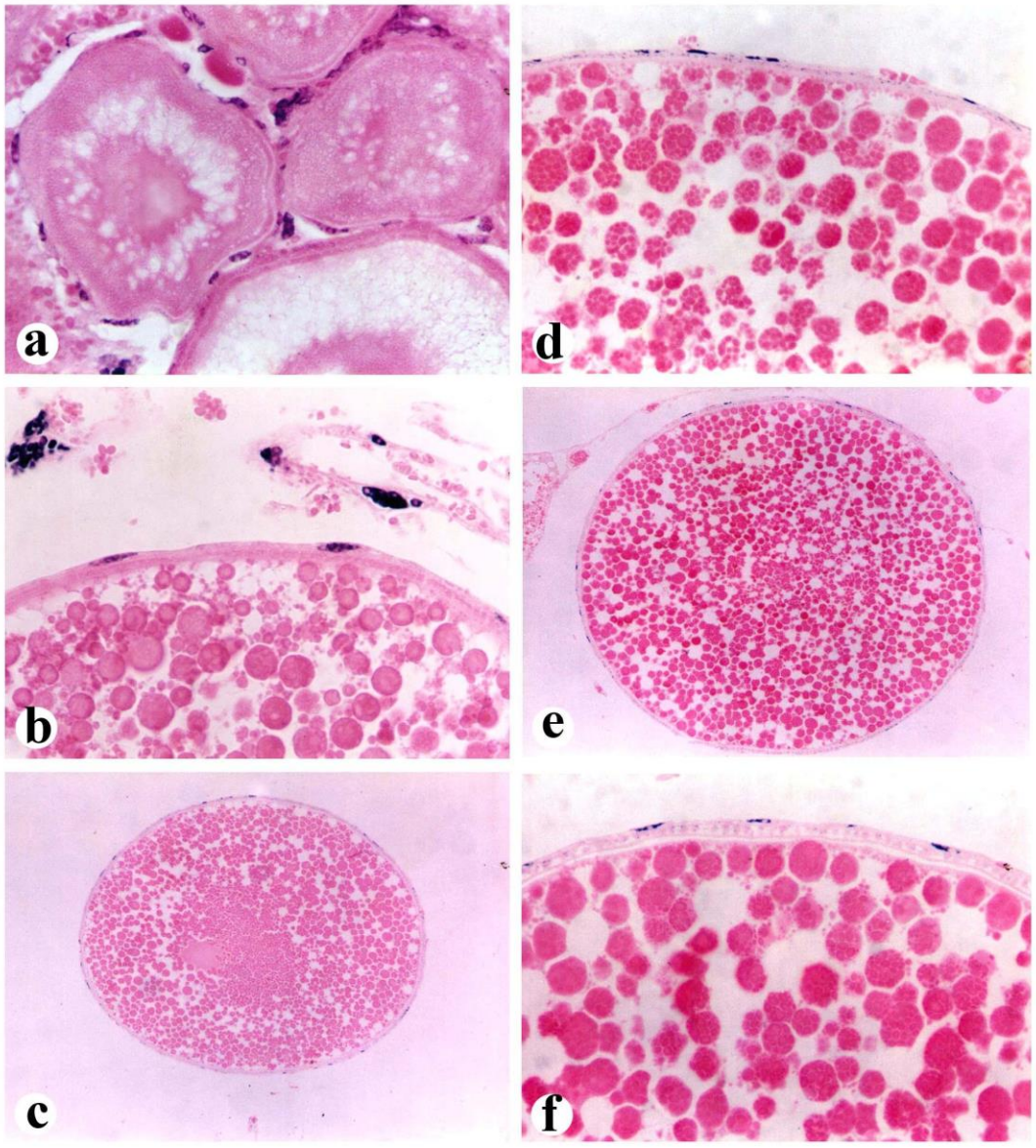

Fig. 3. Parts of transverse ovaries sections of *O. niloticus*, at different maturity stages, incubated with the substrate of 3β-HSD enzyme, displaying the positive reaction of the special cells in the theca of vitellogenic oocyte. (a, b, d and f; X400), (c and e; X100): (a) Vesicle oocytes. (b) Primary yolk oocyte. (c) and (d) secondary yolk oocyte. (e) and (f) Tertiary yolk oocyte. The number, size and 3β-HSD enzyme activity of the special thecal cells increased during oocyte maturation

DISCUSSION

The present research reveals the specific anatomical location of the steroidogenic cells during *O. niloticus* testicular recrudescence. The histological structure of the putative (suspected) steroidogenic cells was one of the practical techniques actually employed in this regard. Furthermore, 3β-HSD histochemical confirmation has been

used. One feature of cells capable of synthesizing sex steroids is the existence of 3β-hydroxysteroid dehydrogenase (3β-HSD). This enzyme plays a crucial part in important metabolic pathways that result in the synthesis of estrogens and androgens. Considering the current histochemical findings, only Leydig cells (interstitial cells) and lobule boundary cells in male *O. niloticus* exhibited 3β-HSD activity. Likewise, **Yaron (1966)** noted 3β-HSD positive reaction in the interlobular and interstitial cells of *Sarotherodon mossambicus* testis. Similarly, Leydig cells (interstitial cells) and lobule boundary cells in *L. ramada* showed 3β-HSD activity **(Khalil, 2001)**. Nevertheless, 3β-HSD activity was only discovered in the interstitial cells of the testis of some teleosts, including *Oryzias latipes, Poecilia reticulata* **(Takahashi & Iwasaki, 1973a, b)**, *Xiphophorus maculatus* **(Schreibman *et al*., 1982)** and *Ictalurus nebulosus* **(Rosenblum *et al*., 1987)**. However, the lobule boundary cells exhibit a positive 3β-HSD reaction, and **Dougbag *et al*. (1988)** misidentified them as Sertoli cells in *Tilapia niloticus* testis. But both Sertoli and Leydig cells exhibited 3β-HSD activity in male *M. cephalus* **(El-messady *et al*., 2023)**.

In female *O. niloticus*, there was 3β-HSD activity in the epithelial cell types found in the ovarian wall, interstitial cells, and the thecal layer. However, the literature that is currently available shows that there is a notable species difference in the cellular location of this enzyme activity among the fish species that have been investigated thus far. The thecal cells of *Carassius auratus* **(Nagahama *et al*., 1976)**, *Salvelinus leucomaenis* **(Kagawa *et al*., 1981)**, and *Ictalurus nebulosus* **(Rosenblum *et al*., 1987)** have been documented to contain the enzyme 3β-HSD. However, *Mugil capito, Tilapia aurea* **(Blanc-Livni, 1971)**, *Monopterus albus* **(Tang *et al*., 1974)**, and *Perca fluviatilis* **(Lang, 1981)** granulosa cells were reported to contain this enzyme. However, in *Tilapia niloticus* **(Yaron, 1971)**, *Trachurus mediterraneus* **(Bara, 1974)** and *Salmo gairdneri* **(van den Hurk & Peute, 1979)**, 3β-HSD activity was discovered in both theca and granulosa cells. Furthermore, stroma cells, also known as interstitial cells and special theca cells, were discovered to exhibit activity of 3β-HSD in the following species: *Mystus cavasius* **(Saidapur & Nadkarni, 1976)**, *Clarias lazera* **(van den Hurk & Richter, 1980)**, *L. ramada* **(Khalil, 2001)**, and *M. cephalus* **(Mousa *et al*., 2023)**.

According to earlier researches **(Christensen & Gillim, 1969; Kurosumi & Fujita, 1974)**, certain ultrastructural characteristics, including mitochondria with tubular cristae, endoplasmic reticulums, and lipid droplets, are thought to distinguish steroid-producing cells. *Oncorhynchus kisutch*, *O. gorbuscha*, and *Carassius auratus* were found to have steroid-producing cells with similar characteristics to a unique type of cells shown in the layer of oocyte theca **(Nagahama *et al*., 1976, 1978)**. Furthermore, the oocytes of various teleosts, including *Salmo gairdneri* **(van den Hurk and Peute, 1979)**, *Salvelinus leucomaenis* **(Kagawa *et al*., 1981)**, *Oncorhynchus rhodurus* **(Kagawa, 1985)**, *Pagrus major* **(Matsuyama *et al*., 1991)**, *Hippocampus barbouri* **(Senarat *et al*., 2021)**, and *M. cephalus* **(Mousa *et al*., 2023)** were found to possess the diagnostic characteristics of the steroidogenic cells. The deep involvement of theca cells in steroid production is

demonstrated by the findings of **Kagawa *et al*. (1982, 1983)** and **Nagahama (1987)** in their studies using the amago salmon, *Oncorhynchus rhodurus*. They concluded that the process of testosterone production by follicular thecal cells served as a precursor for estradiol-17β production in the granulose layer under the influence of gonadotropins.

During sexual maturation and spawning, the Leydig cells' steroidogenic activity increased. Strong reactions were observed in the maturing and spawning stages, which were succeeded by a decline in the post-spawning testis (autumn and winter) in terms of both intensity and the apparent number of cells exhibiting a reaction. Furthermore, during oogenesis-the process by which primary (previtellogenic) oocytes develop into ripe oocytes-the activity of 3β-HSD was altered. Similar changes in 3β-HSD activity were obtained during the reproductive cycle of *L.ramada* **(Khalil, 2001)**. Additionally, seasonal changes in 11β-HSD activity during gonadal maturation in *Oncorhynchus mykiss* rainbow trout were obtained by **Kusakabe *et al*. (2003)**.

In conclusion, knowledge of the endocrine processes involved in *O. niloticus* reproduction could be beneficial for the commercial aquaculture of both this valuable species and the related cichlid tilapia. Furthermore, the current findings have highlighted and stimulated the need for additional research to elucidate the impacts of environmental cues and water quality on endocrine gland secretion, which in turn affects teleost reproduction.

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Conflict of Interest

There are no conflicts of interest, according to the authors.

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