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Endocrine Control of Sex Differentiation in *Oreochromis niloticus*

Mostafa A. Mousa*, Noha A. Khalil, Mohamed F. Kora
Aquaculture Division, National Institute of Oceanography and Fisheries (NIOF), Cairo, Egypt

***Corresponding Author:** mostafa_mousa2002@yahoo.com

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ABSTRACT
Steroid hormones; androgen and estrogen are thought to be the primary drivers of the tilapia sex differentiation (male and female). Cytochrome P450 aromatase is a steroidogenic enzyme, which changes androgens into estrogens and mediates the biosynthesis of estrogens. The current study investigated the aromatase immunoreactivity in the Nile tilapia's (*Oreochromis niloticus*) gonads larvae under temperature-induced and sex differentiation that occurs naturally. When the tilapia larvae were subjected to 35°C of high temperatures, the majority of them produced significantly more males (84%) than the controls (38%) at 25°C. The immunoreactivity of the enzyme aromatase was higher during normal ovarian differentiation than during testicular differentiation. This was observed in gonads subsequent to the commencement of ovarian differentiation, which involves the increase in both stromal and germ cells. In all temperature-exposed cases, there was a decline in aromatase immunoreactivity. Temperature caused females to become more masculine to varying degrees in their offspring. Additionally, the aromatase immunoreactivity decreased in males at 35°C. This shows that, for the purpose of drive differentiation toward testis development, aromatase repression within the gonad is necessary. Aromatase immunoreactivity was detected in the cytoplasm of germ cells and, to a lesser degree, in adjacent Leydig cells, following the start of differentiation in testis. This observation suggests that the aromatase immunoreactivity could be involved in the paracrine regulation of spermatogenesis. In conclusion, the temperature endocrine control for the tilapia sex differentiation shown in this research should help produce all-male populations of this fish in a way that is environmentally friendly, safe, easy to understand, and acceptable.

INTRODUCTION

One steroidogenic enzyme that converts androgens to estrogens is called aromatase of cytochrome P450, or P450arom (**Pasmanik & Callard, 1985; Diotel *et al*., 2011; Chaube *et al*., 2023**). The production of estradiol-17 by P450arom is required for the ovaries to develop **(Li *et al*., 2019; Zhou *et al*., 2021; Wang *et al*., 2022)**. P450arom expression during sexual differentiation in various species has been reported in a number of recent reports **(Guiguen *et al*., 2010; von Schalburg *et al*., 2014; Hayashida *et al*., 2023)**. This enzyme has been demonstrated to have increased activity in the tilapia

(Guiguen *et al*., 1999). Moreover, aromatase antibodies were used to detect a positive staining in the gonads that were thought to be female **(Nakamura *et al*., 1998)**. In the tilapia (*Oreochromis niloticus*), a positive immunoreaction against P450arom is seen in differentiating ovaries but not in differentiating testes **(Chang *et al*., 1997; Sakai *et al*., 2008)**. Nevertheless, P450arom's function in the differentiation of fish sex is still unknown, especially in species where phenotypic sexual differentiation is temperature-sensitive. The use of aromatase inhibitors, which prevent the synthesis of oestrogen, partially or completely reduced ovarian development, which made several fish more masculine **(Piferrer *et al*., 1994; Guiguen *et al*., 1999; Uchida *et al*., 2004; Bhandari *et al*., 2006; Göppert *et al*., 2016)**. Taken together, these studies show that oestrogen synthesis levels in the growing gonad are crucial for ovarian development and play a significant part in fish sex differentiation.

The tilapia fish is thought to be among the most significant fish species of commercial interest because of its high reproductive and productive potential. However, some reproductive characteristics cause management challenges and lower the productive intensity of the aquaculture tilapia, such as early maturation, asynchronous gonadal development, and low prolificacy. Industry focus has been directed toward the tilapia's genetic and sexual control in an effort to minimize genetic and phenotypic variability and maximize production. Sex in *O. niloticus* is established by temperature (TSD), genetics (GSD), or interactions between genotype and temperature **(Baroiller & Clota, 1998; D'Cotta *et al*., 2001; Baroiller *et al*., 2009; Pandit *et al*., 2015)**. In addition to basic biological knowledge, the practical application of temperature regulation as a sex control measure in the commercial tilapia production forms the basis of our understanding of the tilapia TSD mechanism. Producing all-male offspring has several advantages: males grow more quickly than females, and it would prevent overcrowding caused by females' high reproductive rates. Hormones are no longer used as a means of sex control due to an increasing number of health and environmental issues, which highlights the significance of taking the temperature into consideration.

In the current work, we examined the immunolocalization of Cytochrome P450 aromatase in the Nile tilapia, *O. niloticus*, during both temperature-induced and normal sex differentiation. This was explained by the observation of gonad immunoreactivity for aromatase during sex differentiation. A detailed histological explanation of gonad differentiation was correlated with this analysis. The findings of this study could help us comprehend how much the right temperature influences the aromatase enzyme's activity and how this helps differentiate and determine the Nile tilapia sex.

MATERIALS AND METHODS

Study site

From the first of January 2022 to the 30th of July 2023, the present investigation was conducted at El-Matareyya Research Station.

Spawning and larval production

The males and females broodfish were kept apart in two separate ponds since January in preparation for spawning. A 40% protein diet was provided to them on a daily basis to guarantee a good quality and quantity of eggs. We used 250– 500g of the medium-sized tilapia broodfish. On May 1st, semi-natural spawning occurred in hapas with a temperature between 23- 25°C. At a ratio of two males and four females per hapa, brood *O. niloticus* were stocked into thirty fine meshes, one-meter-square spawning hapas. Every day, the breeding activity was observed. Following the process of breeding, the fertilized eggs were gathered, placed into plastic funnels for hatching, and given access to a running water current.

The treatments and the experimental design

Larvae were sorted into 3 groups at seven days post fertilization (dpf), with three aquariums in every group. Every aquarium has roughly 500 larvae, or 10 larvae per liter. Fry from a single brood were used to control for the time elapsed after fertilization (and consequently, developmental stage), as the sensitivity of fish to differentiate sexually by temperature depends on the developmental stage. The first set of aquariums (control) was maintained at 25ºC and was not given any treatment. Temperatures of either 30 or 35°C (masculinizing treatment) were administered to the other groups. According to **Baroiller and D'Cotta (2001)**, the estimated labile period for sexual differentiation (14– 24 days after fertilization) was covered by the 25-day temperature regimen. Daily partial water exchange (70%) kept the aquaria's water quality stable. Pressurized air was utilized to perform a gentle aeration. Additionally, an ambient photoperiod was maintained in all aquaria. Additionally, fresh plankton gathered from a pond previously fertilized using plankton net provided the larvae with a natural diet. Upon finalization of the investigations, cool water was added gradually to return the temperature levels to normal. Following their removal from aquariums and counting, the fish were stocked into $1m^3$ fine mesh cages that were reared in outdoor ponds to grow on for sexing.

Larval sampling and handling

Six distinct ages of the tilapia larvae were sampled at various points after hatching. Random samples of twenty larvae were collected at days 10, 21, 28, 42, 70, and 98 after hatching (ph). After being given anesthesia, the larvae were weighed and measured individually.

The larvae had been anesthetized in a solution (40mg/ l) of clove oil (Sigma) for histological study. The larvae were then fixed in Bouin's fluid, of whole fry, at early stages (from 10- 42 days ph) and later (from 42- 98 days ph) on either trunk or gonads, at an ambient temperature for 48 hours. After fixation, the specimens were moved to 70% alcohol and dehydrated using a series of graded ethanol solutions. Then, they were cleaned in xylene and embedded in paraplast (M.P. 56–58˚C). Serial transverse and longitudinal sections, each measuring 5µm in thickness, were then cut and placed on glass slides. **According to Conn (1953)**, certain sections were stained with Harris' alum hematoxylin for microscopic inspection, and a 1% aqueous solution of eosin was utilized as a counter-stain.

Immunocytochemical procedures

As previously mentioned by **Mousa and Mousa (1999)**, immunohistochemical staining was typically carried out using a vectastain ABC (Avidin-biotin peroxidase complex) Kit (Vector Laboratories). Xylene deparaffinization, rehydration using graded

ethanol and two 10-minute washings in phosphate-buffered saline (PBS; pH 7.4) were applied to sections. Every incubation was conducted at 4°C, and each step was followed by washing in PBS. The primary antibody, rabbit anti-aromatase cytochrome P-450 antiserum (R-10-2) (provided by Dr. Yoshio Osawa, Endocrine Biochemistry Department, Hauptman-Woodward Medical Research Institute, Inc., Buffalo, New York, USA), was diluted with a ratio of 1:1000 and incubated on the sections at 4°C for an overnight period. The sections were then incubated for one hour with the biotinylated secondary antibody (Vector Laboratories) and for forty-five minutes with avidin-biotin-conjugated peroxidase. After that, the sections were cleaned and stained for three to five minutes using 3, 3-diaminobenzidine tetrahydrochloride (DAB) (Sigma) with 0.01 percent H_2O_2 in 0.05 M Tris-buffered saline (pH 7.6). The sections underwent a series of procedures following the enzyme reaction: tap water washing, alcohol dehydration, xylene clearing, and DPX mounting.

Adjacent sections were stained in accordance with the above-described protocol, but incubation in the primary antiserum was skipped to verify the specificity of the immunoreactive procedures. Furthermore, primary antiserum was substituted with normal bovine serum. In these sections, no positive cells or structures were detected.

RESULTS

Normal sex differentiation

After 4 days of fertilization, the tilapia fry hatched upon raising at the standard temperature of 25ºC. They (or nearly) finished reabsorption of yolk-sac by the seventh day post-hatching (ph). When the experiments began, two tubular gonads were present in fry that were 10 dph old. A mesogonium appeared to be closely attached to the dorsal peritoneal wall of the coelomic cavity (Fig. 1a). Moreover, the primordial germ cells were dispersed throughout the stroma of the sexually undifferentiated gonads. Precursory germ cells (PGC) were easily identifiable by their large nucleus, bright cytoplasm, and spherical or oval shape (Fig. 1a).

The fry began to show signs of dimorphism by day 21ph, with some (16± 0.54mm; 110± 6.64mg) demonstrating a growth in the quantity of germ cells within their gonads. Between day 21 and day 28, the ph was observed to cause an increase in the quantity of germ cells. These cells were actively proliferating between days 21 and 28 of the experiment (Fig. 1a). Concurrently, a narrow indentation was noted on the laterally facing surfaces of these gonads. A classic ovarian cavity eventually developed from this groove (Fig. 2a). As a result, the presence of auxocytes indicated that the female gonads met both the somatic and germinal criteria for differentiation (Figs. 3a, 4a).

From a mitotic perspective, the gonads of the remaining fry (50 percent of the individuals) remained quiescent, with an early and gradual increase in PGC number (Fig. 2c). The somatic and germinal components both underwent active proliferation after 28 days post hatching. At 42 days ph, these somatic components diffused inside the gonads to gradually form lobules, which led to the lobular configuration characteristic of testicular differentiation (Fig. 3c). Following 42 days post-fertilization, the quiescent

germ cells underwent meiotic prophase (Fig. 3c). Spermatogenesis was initiated in a particular area of the gonad during the next 28 days, and at 70 days ph, the fish's entire testes developed progressively (Fig. 4c). The testes showed an active spermatogenesis at 98 days ph (Fig. 4c), while the most developed oocytes in the ovaries were still in the previtellogenic stage (Fig. 4e).

Endocrine control of sex differentiation

All larvae reared at control temperature exhibited a strong aromatase immunoreactivity in their undifferentiated gonads (Fig. 1b, d). During normal ovarian differentiation, aromatase enzyme immunoreactivity was stronger than during testicular differentiation (Fig. 2b, d). After ovarian differentiation began, stromal and germ cell proliferation was seen in the gonads (Figs. 3b, 4b, f). Aromatase immunoreactivity was detected in Leydig cells and, to a lesser degree, in the cytoplasm of neighboring germ cells following the start of testicular differentiation (Figs. 3d, 4h).

The sexual differentiation of the tilapia larvae was influenced in a masculinizing way by temperatures above 35°C. The percentage of males increased to 84% due to the high temperature (Table 1 & Fig. 3g). But in the 30°C treatment, there were variations in the degree of masculinization, indicating various aspects of intersexuality. Fig. (3e) and Table (1) show that male gonads in some cases (43%) surrounded regions free of germ cells or contained oocytes scattered among sperm mother cells. Furthermore, 40% of gonads at 30°C had the testis-typical lobular structure.

Temperature caused genetic females to develop into more masculine to varying degrees in their offspring. In all cases, there was a decline in aromatase immunoreactivity (Figs. 1f, h, 2f, h, 3f, h, 4d). Males at 35°C showed a further decrease in aromatase immunoreactivity, with the differences between the control and treated groups being more noticeable (Figs. 2h, 3h, 4d).

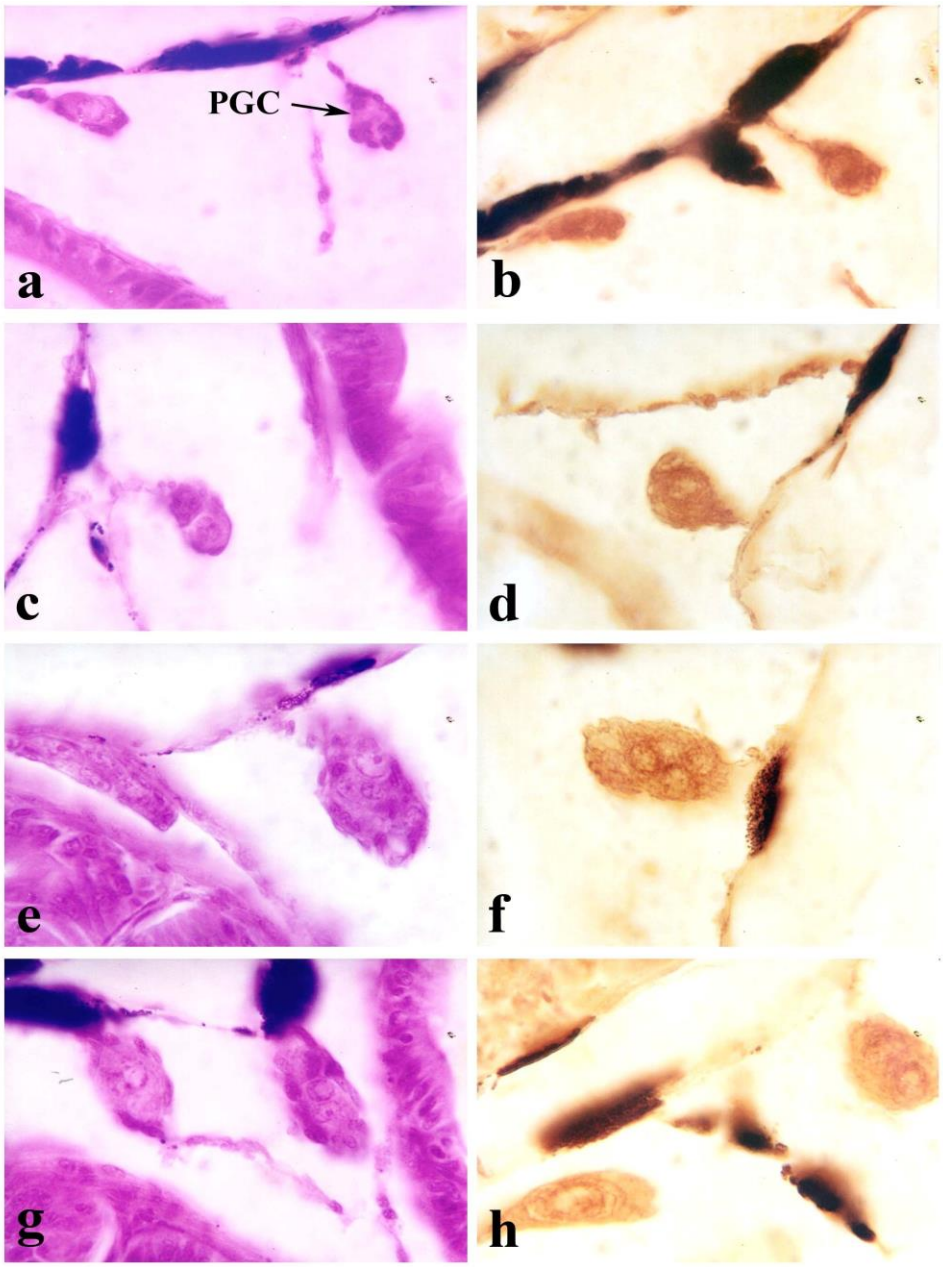

Fig. 1. Cross sections of *O. niloticus* larvae. X 500. **(a, c, e and g)** stained with Harris' hematoxylin and eosin; **(b, d, f and h)** immunostained with aromatase antibody. **a)** Undifferentiated gonads of *O. niloticus* fry at 10 days posthatching (10 dph) showing primordial germ cell (PGC). **b)** Primordial gonads at 10 dph indicating strong immunoreactivity. **c)** Gonad at 21 dph from control group showing division of PGC. **d)** Gonad as in **(c)** exhibiting strong aromatase immunoreactivity. **e)** Gonad at 21 dph from 30ºC group showing elongation of gonads and multiplication of germ cells. **f)** Gonad as in **(e)** exhibiting moderate aromatase immunoreactivity. **g)** Gonads at 21 dph from 35ºC group showing elongation of gonads and no multiplication of germ cells. **h)** Gonads as in **(g)** exhibiting weak aromatase immunoreactivity

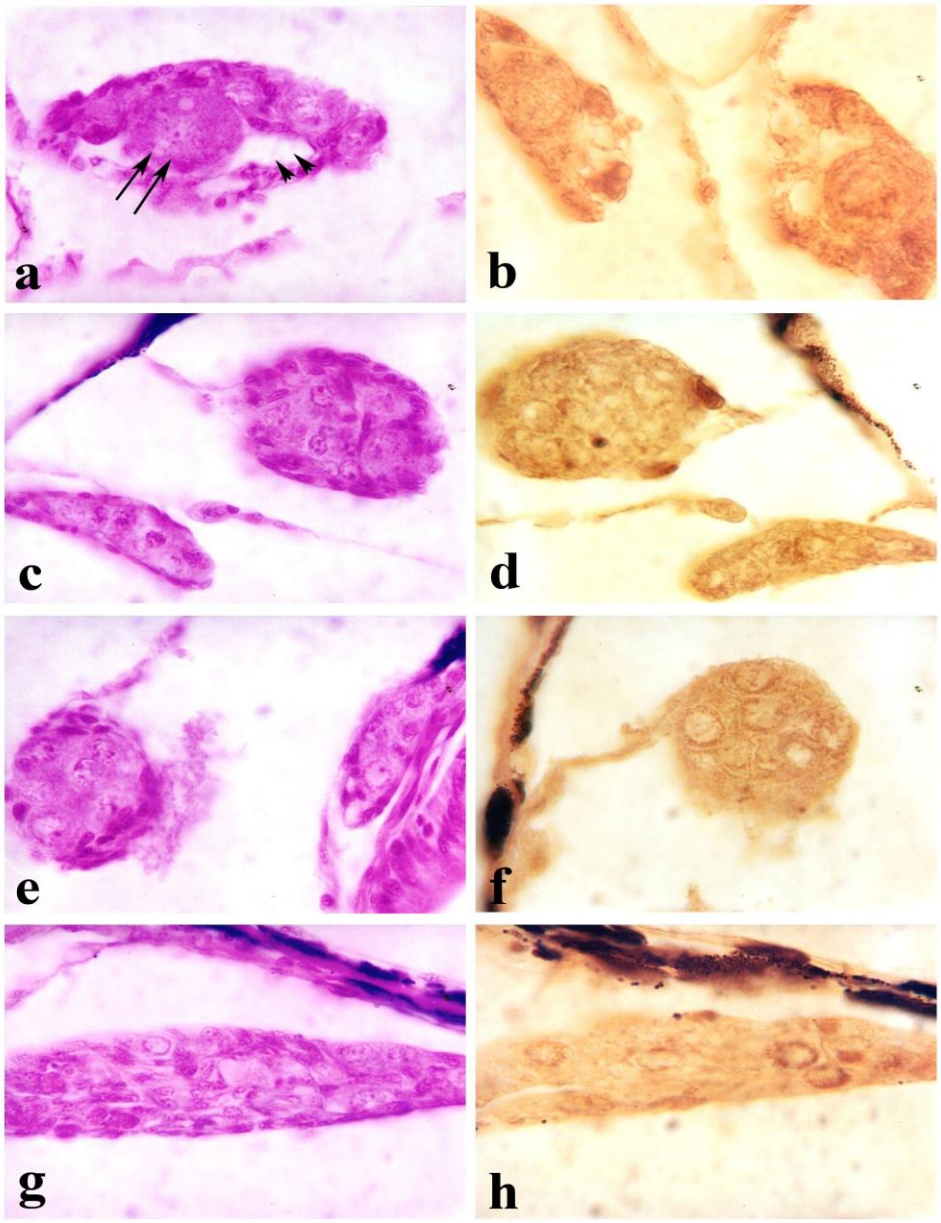

Fig. 2. Cross sections of *O. niloticus* larvae. X 500. **(a, c, e and g)** stained with Harris' hematoxylin and eosin; **(b, d, f and h)** immunostained with antiserum against aromatase. **a)** Gonad at 28 dph from control group beginning the oocyte previtellogenitic stage (arrows) and showing the ovarian cavity (arrowheads). **b)** Gonads as in **(a)** exhibiting strong aromatase immunoreactivity. **c)** Gonads at 28 dph from control group showing primordial testis. **d)** Gonads as in **(c)** exhibiting weak aromatase immunoreactivity. **e)** Gonads at 28 dph from 30ºC group representing primordial testis. **f)** Gonad as in **(e)** exhibiting weak aromatase immunoreactivity. **g)** Gonad at 28 dph from 35ºC group showing testis with less number of germ cells. **h)** Gonad as in **(g)** exhibiting weak aromatase immunoreactivity

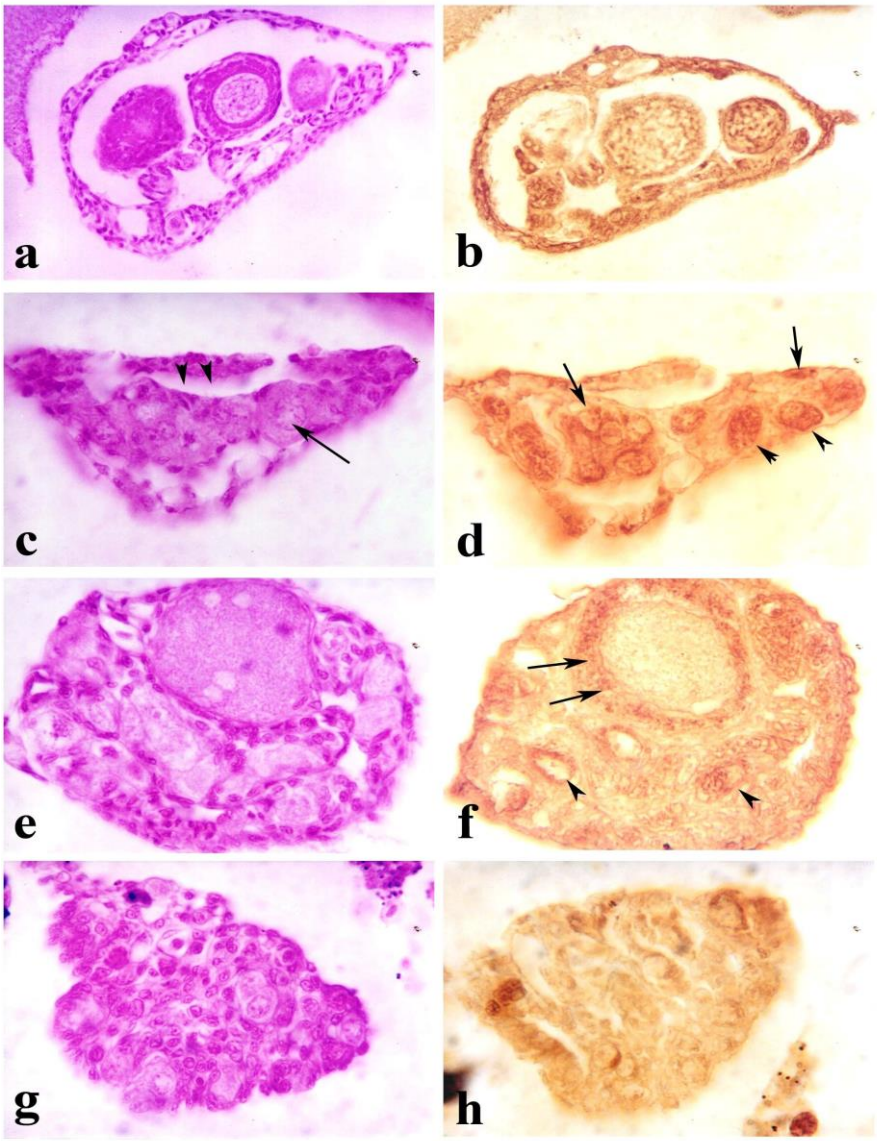

Fig. 3. Cross sections of *O. niloticus* larvae. **(a, c, e and g)** stained with Harris' hematoxylin and eosin; **(b, d, f and h)** immunostained with aromatase antibody. **(a-b)** at a magnification of X 400 and **(c-h)** at a magnification of X 500. **a)** Differentiated ovary at 42 dph from control group showing previtellogenitic oocytes. **b)** Gonad as in **(a)** exhibiting strong aromatase immunoreactivity. **c)** Differentiated testis at 42 dph from control group showing the spermatogonia (arrow) and the development of efferent duct (arrowheads) as a slit in the stromal tissue. **d)** Gonad as in **(c)** exhibiting strong aromatase immunoreactivity not only in Leydig cells (arrow) but also in spermatogonia (arrowheads). **e)** The intersex fish at 42 dph from 30ºC group showing oocyte in the testicular tissue. **f)** Gonad as in **(e)** exhibiting strong aromatase immunoreactivity not only in oocyte (arrows) but also in spermatogonia (arrowheads). **g)** Gonad at 42 dph from 35ºC group showing testis with less number of germ cells. **h)** Gonad as in **(g)** exhibiting weak aromatase immunoreactivity

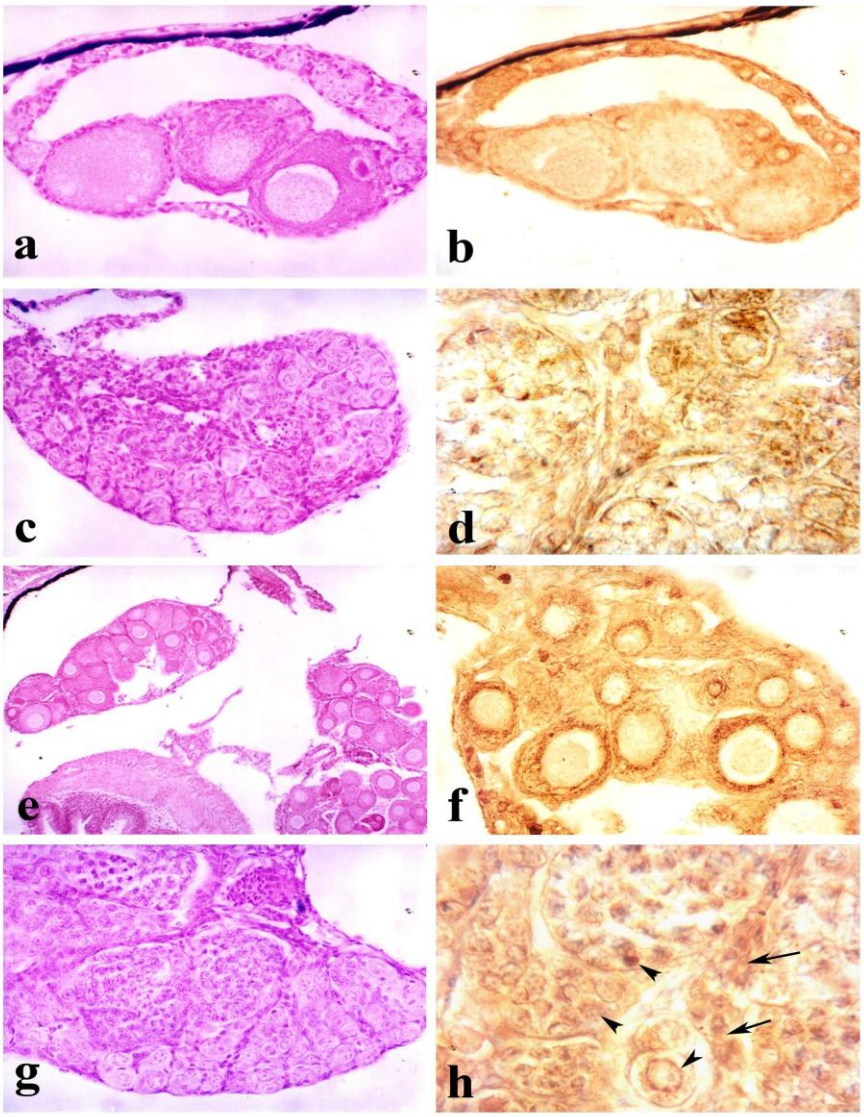

Fig. 4. Cross sections of *O. niloticus* larvae. **(a, c, e and g)** stained with Harris' hematoxylin and eosin; **(b, d, f and h)** immunostained with aromatase antiserum. **a)** Normal ovary at 70 dph from control group showing previtellogenitic oocytes. X 400. **b)** Gonad as in **(a)** exhibiting moderate aromatase immunoreactivity. X 100. **c)** Testis at 70 dph from 35ºC-treated group containing spermatogonia, spermatocytes and a large amount of connective tissue. X 400. **d)** Gonad as in **(c)** exhibiting moderate aromatase immunoreactivity. X 400. **e)** Normal ovary at 98 dph from control group filled with oocytes at previtellogenitic stage. X 100. **f)** Ovary as in **(e)** exhibiting strong aromatase immunoreactivity. X 100. **g)** Normal testis at 98 dph from control group showing an active spermatogenesis. X400. **h)** Gonad as in **(g)** exhibiting weak aromatase immunoreactivity in Leydig cells (arrows) and in the surrounding germ cells (arrowheads). X400

Table 1. The effect of temperature on sex ratio of *O. niloticus* larvae

Treatment	Sexed fish	Male	Female	Intersex	Male percent (%)
Control (25°C)	100	38	62	0	38
30°C	100	40	17	43	40
35°C	100	84	16	0	84

DISCUSSION

Light microscopy was utilized to provide a more precise description of the differentiation period in a mixed-sex population of *O. niloticus* that was raised at the standard temperature of 25ºC. Correlations with other experiments could then be conducted using these features. For O. *niloticus* tilapia, sex can be determined genetically (GSD) by temperature (TSD) or through interactions between temperature and genotype **(Baroiller & Clota, 1998; D'Cotta *et al*., 2001; Baroiller *et al*., 2009; Pandit *et al*., 2015)**. The findings presented here demonstrate that temperature has a substantial impact on *O. niloticus* sex differentiation and sex ratio, supporting findings from earlier research on this and other tilapia species **(Alvarenga & França, 2009; de Alba *et al*., 2023; Fagbémi *et al*., 2024)**.

According to the current immunohistochemical data, at 10 days after hatching, *O. niloticus* larvae's undifferentiated gonads showed strong aromatase immunoreactivity. As a result, aromatase must be activated earlier by the regulatory factor. The initial application of hormone or temperature-based sex inversion treatments for the tilapia must occur within a limited timeframe, estimated to be between 12 and 14 days post-fertilization **(Contreras-Sanchez *et al*., 2000)**. Therapy is ineffective and does not cause a reversal of sex beyond this point. Thus, sex differentiation may be triggered before or possibly on day 12 or 14 following fertilization. According to **Baroiller *et al*. (2009)**, the critical sex differentiation period in *O. niloticus* appears to span from 9 to 25 dpf. The climbing perch's (*Anabas testudineus*) gonads, however, begin to differentiate between 18–21 days post hatching **(Hidayat *et al*., 2024)**.

We found that gonial multiplication starts at roughly day 21 ph, which implies that sex differentiation is already under way at that point. Temperatures above 35°C had a masculinizing effect on the sexual differentiation of the tilapia larvae. This high temperature caused the percentage of males to rise to 84%. However, various aspects of intersexuality were indicated by differences in the degree of masculinization in the 30°C treatment. In certain instances, (43%) male gonads encircled areas devoid of germ cells or held oocytes dispersed among sperm mother cells. Additionally, at 30°C, 40% of gonads exhibited the lobular structure typical of testicles. Besides, during the thermo-sensitive period (i.e., 11–23 dpf), *O. niloticus* larvae were exposed to a high temperature of 36°C at

various times of the day for a peiod of 12 days **(De Alba *et al*., 2024)**. All of the temperature-treated larvae exhibited up-regulated gene expression for testicular differentiation and down-regulated gene expression for ovarian differentiation, according to the results, which led to a higher proportion of males **(Kobayashi *et al*., 2004b; De Alba *et al*., 2024)**. In addition, high temperature (37°C) treatment can induce permanent sterility in the female Nile tilapia (*O. niloticus*, Chitralada) through a complete loss of germ cells **(Pandit *et al*., 2015)**. Additionally, a three-month study by **Nduku *et al*. (2022)** examined the efficacy of temperature and hormone sex in reversing *O. niloticus* fry and influencing their growth in hapa nets. Temperature shock and 17-α-methyltestosterone hormone were found to have sex reversal efficacy rates of 92.86 and 91.18%, respectively. According to **Nduku *et al.* (2022)**, the findings suggest that temperature shock may serve as a viable substitute for the tilapia sex reversal without adversely affecting growth.

In females, strong aromatase enzyme immunoreactivity was seen early in differentiation, before ovarian meiosis, and later, at a post-differentiating stage, immunoreactivity increased in tandem with ovarian growth. Comparable findings were found in *Rana rugosa* **(Kato *et al*., 2004)** and *Trimma okinawae* **(Kobayashi *et al*., 2004a)**. The gonads of the all-male tilapia also showed evidence of aromatase immunolocalization. Nevertheless, compared to the female fish, the immunoreactivity was significantly lower. The testes appear quiescent and undifferentiated at these stages, with few somatic cells and fewer germ cells. A connection between the high aromatase expression found in genetic females and elevated oestradiol-17β levels measured in gonads from the same stage of ovarian differentiation was demonstrated by **D'Cotta *et al*. (2001)**. In contrast, reduced amounts of oestradiol-17β were exhibited by males but were consistent with the low levels of aromatase mRNAs **(D'Cotta *et al*., 2001)**. Additionally, the results of the immunohistochemical analysis showed that aromatase immunoreactivity was strongly seen in Leydig cells and, to a lesser extent, in the cytoplasm of nearby germ cells following the onset of testicular differentiation. This might possess a paracrine effect on the control of spermatogenesis. Similar aromatase immunolocalization was noted in stallion testes **(Sipahutar *et al*., 2003)**.

In the present study, albeit to a different extent, temperature resulted in genetically female progenies becoming more masculine. We found that a decrease in aromatase immunoreactivity is induced in these phenotypic males by temperature. Further reduction in immunoreactivity caused by temperature had an impact on the aromatase immunolocalization of genetic males. As in the case of the tilapia, the Japanese flounder *Paralichthys olivaceus* has been shown to undergo high temperature masculinization, exhibiting a suppression of aromatase expression after sex differentiation began to occur **(Kitano *et al*., 1999)**. In this species suppression of aromatase was suggested in view of the gradual decrease exhibited in phenotypic males. Oestradiol-17β levels throughout the body were measured, and they were only found to

be significant in females when aromatase expression changed to a female-specific level **(Kitano *et al*., 1999)**.

The current study demonstrates that during female differentiation, at a point when ovarian differentiation commences, the gonad acquires high aromatase immunoreactivity. One possible explanation is that aromatase driving ovarian differentiation. Since a decrease in immunoreactivity to aromatase was noted in both genetically and the phenotypically male tilapia, temperature may be responsible for a similar mechanism to that of natural male differentiation in the tilapia. We suggest that temperature and genetic regulatory factors suppress aromatase expression, preventing ovarian differentiation and promoting testicular development.

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Conflict of Interest

The authors state that there are no conflicts of interest.

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