Developmental Biology of the Supermale YY Tilapia *(Oreochromis niloticus):* Histogenesis of the Reproductive System

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ABSTRACT

Histogenesis of the reproductive system of supermale (YY) tilapia and XY tilapia reared at the Central Luzon State University was analyzed with the use of paraffin sections. In the course of development, the primordial germ cells appeared at the same age in YY and XY males, i.e., at 8 days posthatch. These cells which were larger in the YY (1.85 μm) than in the XY male (0.9 μm) later established themselves in the gonadal anlage by days 9-22. The lobules appeared earlier in the YY at day 15. Blastema of the reproductive duct appeared in the YY at day 23 and in the XY at day 27. By day 79, meiotically active cells were abundant in both groups. By day 95, the YY fish showed mature sperm cells in the fully differentiated testis while it was at day 105 in the XY fish. The supermale consistently demonstrated bigger testis, with thicker somatic tissues, more spermatogenic cells, and more advanced developmental stage than XY fish of the same age. Germ cell and nuclear size in the YY and XY fish were not statistically significantly different, although the general trend was that spermatogenic cells were bigger in the supermale tilapia. ANOVA ($\alpha = 05$) showed significant difference in size of the testis, spermatocysts, and vas deferens. The study showed that with the same rearing conditions and same age, the larger supermale tilapia has superior reproductive capacity with its larger testis and ducts, faster histogenesis and spermatogenesis, and higher gonadosomatic index (GSI).

Keywords: developmental biology, supermale Tilapia, histogenesis, reproductive system

INTRODUCTION

The Nile tilapia (*Oreochromis niloticus*), an introduced species from Israel, is an important source of protein for Filipinos. It is generally the most important tilapia species in the Philippines and has gained wide acceptance and great economic importance in freshwater aquaculture since it is highly adaptable to domestication and can tolerate a wide range of conditions. The ease of breeding, however, has been one of the major drawbacks in the widespread adoption of tilapia farming technology (Guerrero & Abella, 1976). Excessive reproduction leads to superfluous recruitment, particularly in pond populations, resulting in competition for available food and sparse resources. Consequently, up to 50% of the biomass at harvest are unmarketable recruits because of size constraints.

Several practical solutions have been proposed to solve this problem, including manual sexing, use of predators to cull fry, and prevention of spawning in cages (Mair

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et al., 1997). Presently, the most practical and effective method is the production of monosex male populations. Male tilapias are preferred for rearing since they have a faster growth rate than females, and less energy is diverted into gamete production. Thus, hormonal sex reversal by oral administration of the androgen 17aa-methyltestosterone to produce nearly all male population has been practiced in tilapia farming for more than a decade now (Mair et al., 1997).

Recently, YY technology was introduced in the Philippines to improve the production of all male tilapia population. YY technology is a joint research undertaking between the University of Wales, Swansea, UK and the Freshwater Aquaculture Center of Central Luzon State University in the Philippines. It concentrates on the genetic manipulation of sex. This is achieved through a combination of feminization and progeny testing to identify the novel YY genotype that sires only XY male progeny, referred to as Genetically Male Tilapia (GMT). Most likely, the immediate benefit of the application of this genetic manipulation technique in tilapia is the further development, adaptation, and extension of the technology for large-scale production of genetically all-male *O. niloticus*. This newly introduced technology is presently being disseminated in the Philippines, where YY males are being mass produced.

Inspite of the success of the YY technology, the exact mechanism of sex determination and differentiation in *O. niloticus* is still uncertain. The monofactorial system of sex determination in Nile tilapia does not explain the occasional deviations from expected sex ratios based on studies involving sex reversal and chromosomal set manipulation.

Dr. Graham C. Mair (*pers. comm*), the proponent and senior consultant of the Fish Genetic Project based in Freshwater Aquaculture Center in Central Luzon State University, Munoz, Nueva Ecija, (FAC-CLSU) discussed the need for histological investigation which could be of use in evaluating the presently existing sex control technologies. This study analyzes the histogenesis of the reproductive system of YY tilapia in comparison with that of the XY male.

REVIEW OF RELATED LITERATURE

YY Male

The existence of YY male fish was first shown by Yamamoto as early as 1958 in medaka, *Oryzias latipes* using the technique of hormonal sex reversal and selective breeding. Subsequently, YY males were also produced in guppy, *Poecilia reticulata* (Yamamoto 1963), in goldfish, *Carassius auratus* (Yamamoto 1975), and in tilapia, *Oreochromis niloticus* and *O. mossambicus* (Varadaraj & Pandian, 1989).

There have been several published reports on YY males in fish. However, the survival of YY male fish was very low. In *Salmo gairdneri*, the viability of YY individuals was observed only until the 'eyestage' of development (Johnstone et al., 1979). Thus, the viability of these genotype males in salmonids is still in question (Calhoun & Shelton, 1983). Pandian & Sheela (1995) concluded that the presence of more than one Y chromosome rendered the male or female fish less viable. However, Mair et al. (1997) reported that the YY male genotype of Nile tilapia *(O. niloticus)* was viable and fertile like the normal (XY) males. Mair (1997) further demonstrated the feasibility of large scale production of all male tilapia through genetic manipulation of sexual phenotype. He elaborately described the distinct steps in the production of the novel genotype 'YY' (supermale) Nile tilapia.

YY Male Technology

The development of YY male technology involves a series of stages of feminization and progeny testing. The model for the breeding program in the production of YY males by genetic manipulation of sex in *O. niloticus* was proposed based on the extensive research on the genetics of sex determination in tilapia species carried out at the University College of Wales, Swansea, UK. Studies revealed that this species exhibits predominantly monofactorial genotypic system with male heterogamety (XY) and female homogamety (XX) similar to that of humans (Penman et al., 1987; Mair et al., 1990.

It was proposed that by sex reversal (male to female), followed by progeny testing (to determine the genotype of a fish by the sex ratio of its progeny), it would be possible to identify sex- reversed female (^EEEE) with male genotypes (XY). These ^EEEE could then be crossed with normal males (effectively crossing two males). The progeny of this cross would contain approximately ¼ of the novel genotype YY. This can be identified by the all-male sex ratio of their progeny in crosses with normal females (YY and XX gives only XY male progeny). Further crosses of YY males with ^EEEE followed by a second generation with sex reversal could then be crossed with YY males for the production of all-male producing YY male broodstock.

This simple monofactorial hypothesis, however, failed to explain some deviations from predicted sex ratios. It was hypothesized that these arise through environmental influences, such as temperature (Mair et al., 1990; Trombka & Avtalion, 1993). The temperature effect on sex determination, however, still does not explain the occurrence of the small percentage of males in putative monosex female progeny reared at ambient temperature. These deviations were also suspected to be due to autosomal or polyfactorial genetic effects.

The technique is now widely adopted in Philippine aquaculture, particularly in the FAC-CLSU where the Fish Genetic Research Project, in collaboration with the University of Wales, Swansea, is based. Continuous research and development is being undertaken to further improve the sex control methodologies practiced in tilapia fish farming.

MATERIALS AND METHODS

The study was conducted in collaboration with the Fish Genetics Breeding Program-Genetically Male Tilapia of the FAC-CLSU, and the Natural Sciences Research Institute (NSRI), University of the Philippines, Diliman, Quezon City.

The Egypt-Swansea strain of *Oreochromis niloticus* produced by the Fish Genetic Research Program was used in the study. The fry were secured and reared at the breeding facilities of FAC-CLSU. The tissues for histological study were processed at the NSRI, U.P. Diliman, Quezon City.

Experimental set-up

Two groups of undifferentiated fry, the XY males, which served as the control, and the YY males, were grown for approximately 5 months.

Six hundred fry in each group were collected from the down-welling artificial incubator and transferred into well-aerated 30L- rectangular glass aquaria maintained at ambient temperature 30±4°C. Two (2) glass aquaria were used in each group with a stocking density of 300 fry per aquarium (10 fry per liter). The fry used were of the same age at the first feeding stage, following completion of yolk sac absorption (<10 mm). Cleaning of waste by siphoning was done daily. Deep well water was replaced every two days.

The fry were fed with commercially available fry mash (Tatch feed with 33% crude protein) during the first two months. Before feeding fry mash was sieved with a 1 mm-mesh size screen to remove particles that were too large to be ingested by the fish. Feeding was done four times a day (0800, 1100, 1400, and 1700 hrs) at the rate of 20% of the body weight for the first seven days, 15% for the second week, and 10% for the succeeding weeks of the experimental period. Adjustment of the diet ration was calculated by collecting and weighing ten percent (10%) of the total population of the fry every week.

The fingerlings (about 100) were then transferred to fine-meshed hapas, 1m x 2m x 1m in dimension, suspended in a fertilized earthen pond, and fed with Tatch feeds (31% crude protein). The fish were harvested after 5 months, during the onset of gonadal maturation, and again during the appearance of fully formed sperm cells.

Histological preparation

Preparation of Juvenile and Adult Reproductive Organs

Histological processing was done on specimens from the time of hatching until the attainment of full sexual differentiation. Starting from the time of hatching, 10 fry in each group were collected and fixed in Bouin's fluid everyday for 20 days, then roughly every week for the succeeding weeks until gonadal maturation.

The region between the pronephros and the anus (approximate location of the gonads of the immature or juvenile fish) and the testis of the maturing and mature tilapia were processed for light microscopy.

Light microscopy

Samples were fixed in Bouin's fluid, dehydrated in a series of graded ethyl alcohol (10%, 20%, 30%,40%, 50%,60%,70%,85%,95% and 100%), and cleared in cedarwood oil for at least 6 hours and in xylene for 1- 2 hours. The samples were then infiltrated in soft-hard paraffin at a ratio of 2:1, 1:1, and 1:2, respectively, after which, tissues were embedded in pure hard paraffin, trimmed and cut using a rotary microtome. A series of transverse sections (7-8 μm) of the region between the pronephros and the anus (for the immature until day 53), and the central portion of the maturing and mature testis were made and stained with Hematoxylin-Eosin and mounted with entellan mounting medium. Prepared slides were then examined and selected parts were analyzed and photodocumented.

RESULTS AND DISCUSSION

Results of light microscopy studies showed differences in the timing and pattern of differentiation and maturation of the testis of the normal male (XY) and YY male Nile Tilapia. Generally, the testis of the YY males, differentiated and matured earlier, and had bigger components and cells compared to the XY (GMT) males.

Table 1 summarizes the development of the testis and gonadosomatic index (GSI) (GSI=wt. of testis/weight of fish x 100) of YY and XY fish at different ages of development based on significant histological changes in the testes.

Hatching to 22 days posthatch

Gonads of the fry of both XY and YY, about 8 days posthatching, showed the primordial germ cell (PGC) lying in the genital ridge. The PGCs are distinguishable from somatic cells based on shape and size. PGCs are large cells, of round to oval shape, with round and prominent nuclei, and lightly staining cytoplasm. They are much larger compared to somatic cells. The PGCs

Table 1. Summary of testicular development and gonadosomatic index (GSI) of YY and XY male tilapias at different developmental ages

Developmental Age (days post hatch)	YY	XY
8	• PGC in early genital ridge • Bipotential gonad • GSI cannot be measured	• smaller PGCs in genital ridge • smaller (0.9-1.0 μm) • GSI cannot be measured
15	• early lobule formation • GSI cannot be measured	• no lobules yet • GSI cannot be measured
23	• Efferent duct primodium appears • GSI = 0.2	• No efferent duct yet • GSI = 0.2
27	• bigger duct • GSI = 1.2	• efferent duct anlage • GSI = 1.2
54	• substantial number of seminiferous tubules • GSI = 1.9	• few seminiferous tubules • GSI = 1.1
80	• Substantial number of spermatocysts • GSI = 2.1	• Fewer spermatocysts • GSI = 1.5
95	• Sperm ready for spermiation • GSI = 2.9	• Few sperm • GSI = 1.6
105	• Abundant sperm • GSI = 3.6	• sperm ready for spermiation • GSI = 2.6

observed in this study and their localization were similar to the PGCs observed by Boco (1977) in *Tilapia mossambica* and by Herrera (1984) in *Tilapia nilotica.* YY gonads were larger in diameter (1.8-2.0 μm) than those of the XY (0.9-1.0 μm, see Table 2). YY PGCs appeared generally bigger (1.85μm) than those of the XY (0.9), although statistical analysis showed no significant difference. The formation of the paired gonadal primordia at 8 days is parallel to the findings of Eckstein & Spira (1965) in *T. aurea* and Nakamura & Takahashi (1973) in *O. mossambica*. The gonad anlage consisted of primordial germ cells enveloped by somatic cells.

In XY, PGCs with large nuclei were observed at 15 days posthatching. At this time, an early lobule was already observed in YY. Gonads at this stage were still sexually indifferent histologically.

23 to 32 days posthatch

From day 23 to 32 posthatching, gonads increased in size. Both somatic cells and germ cells increased in number. Initial testicular differentiation was observed to occur at this stage both for XY and YY males, although YY gonads differentiated earlier than XY gonads. A slit-like space, the lumina primordium of the efferent ducts, was observed in the stromal tissue in the central region of the XY gonad at day 27 and at day 23 in YY. The initial appearance of the blastema of the efferent duct was the primary criterion set by Nakamura & Takahashi (1973) for testicular differentiation in *T. mossambica*. The findings in this study are similar to the result obtained by Nakamura & Takahashi (1983) in the gonadal differentiation of the normal XY male in Nile tilapia. Herrera (1984, 1987, 1996) observed onset of testis differentiation in younger (16-20 days old) fish probably due to earlier transfer of fry to larger cement ponds instead of aquaria.

33 to 79 days posthatch

At 33 to 46 days, clusters of germ cells and somatic cells in the stroma of the gonad (testis) were observed. There was a significant change in the size and shape of the gonads. YY gonad was bigger and had larger cells than XY gonad. Seminiferous tubules can be seen distinctly at day 54, both in XY and YY. Within the tubules were large PGCs, spermatogenic and somatic cells. The YY gonad was observed to be bigger, with more tubules and gonial cells than in XY. The testis of both XY and YY were in a state of very active spermatogenesis at this stage. Although the general trend was bigger spermatogenic nuclear size in YY than in XY, statistical analysis showed no significant difference.

80 to 145 days posthatch

Spermatogenic cells increased rapidly in number in the testes by 80 day posthatching. More gonial cells and cysts were observed in YY than in XY. Active spermatogenesis was observed and spermatogenic cells were at various stages, in the numerous large tubules both in XY and YY testes. The testis at this stage

Table 2. Comparison of the reproductive system of YY and XY *O. niloticus* L.

Structure	YY		XY	
	Range	Mean± standard deviation	Range	Mean± standard deviation
Testes length (mm)	45.0 - 52.0	49.23 ± 2.34	35.0 - 37.0	36.47 ± 0.76
Testes diameter (mm)	1.8 - 2.5	2.07 ± 0.23	1.6 - 2.0	1.78 ± 0.16
Seminiferous tubule diameter (μm)	2.2 - 11.0	5.25 ± 0.55	2.2 - 11.0	4.00 ± 0.29
Spermatocyst diameter (μm)	1.3 - 10.0	3.73 ± 0.47	1.0 - 7.0	2.88 ± 0.26
Spermatogonia nucleus diameter (μm)	0.3 - 1.1	0.67 ± 0.05	0.3 - 1.3	0.66 ± 0.18
Primary spermatocyte nucleus diameter (μm)	0.3 - 0.8	0.41 ± 0.14	0.3 - 0.8	0.44 ± 0.03
Secondary spermatocyte diameter (μm)	0.3 - 0.6	0.40 ± 0.03	0.3 - 0.6	0.35 ± 0.0.03
Spermatid diameter (μm)	0.1 - 0.2	0.17 ± 0.01	0.1 - 0.2	0.16 ± 0.00
Vas efferens diameter (μm)	375.0 - 720.0	512.34 ± 22.51	245.0 - 405.0	323.15 ± 23.47

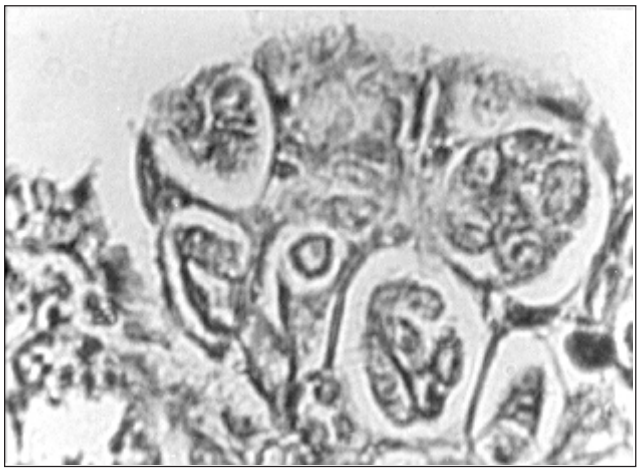

Fig. 1. XY testis at 105 days posthatching (430X) had less sperm than YY testis

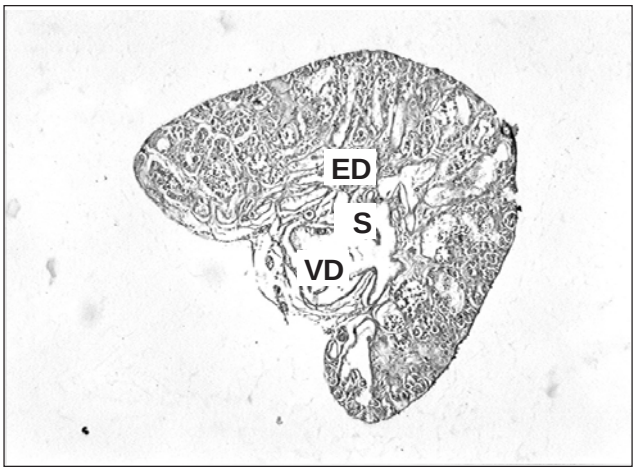

Fig. 3. XY testis at 145 days posthatching (100X) shows the efferent ducts (ED), vas deferens (VD), and sperm (S)

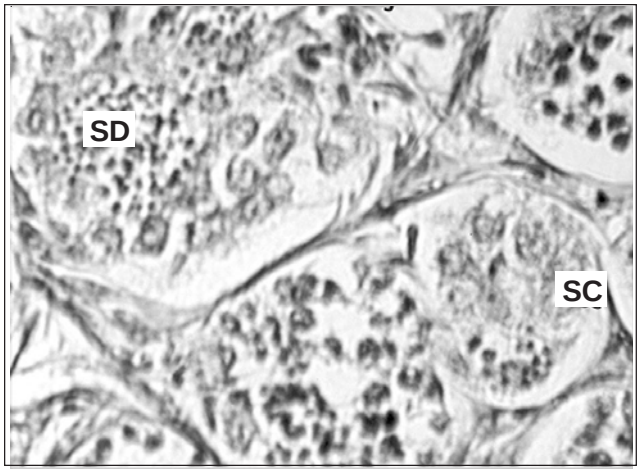

Fig. 2. YY testis at 105 days posthatching (430X) show the seminiferous tubules, gonial cells, spermatyocytes (SC) and spermatids (SD) undergoing spermatogenesis

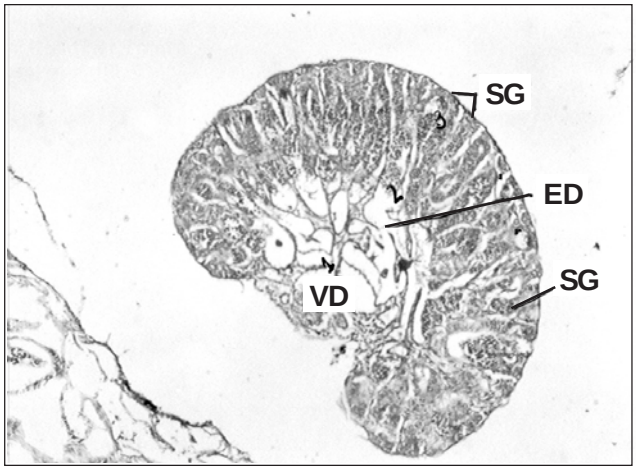

Fig. 4. YY testis at 145 days posthatching (100X) shows the spermatogenic cells (SG), efferent ducts (ED), and vas deferens (VD)

contained numerous large tubules filled with cells in varying stages of spermatogenesis. In YY testis, abundant sperms filled the efferent ducts and main ducts ready for spermiation at day 95. The sperms were later observed in XY testis at day 105 (Fig. 1). More sperms were found in YY testis than in XY testis (Fig. 2).

Fully mature XY and YY testis at day 145 are shown in Figs. 3 and 4. By 145 days, the average length of the testis of the XY fish was 36.47±0.76 mm while the YY fish was 49.23±2.34 mm. Statistical analysis using ANOVA (aa=0.05) proved that in supermale, the seminiferous tubules (mean diameter 5.25±0.55 μm), and spermatocysts (mean diameter 3.73±0.47 μm) were significantly larger than in the XY male. Comparison of the data on the size of the nuclei of various spermatogonia cell types showed no significant difference using ANOVA. Nuclei of spermatogonia of the supermale and XY male had mean diameters of 0.67±0.05 μm and 0.66±0.18 μm, respectively. Primary spermatocyte nuclei of YY measured 0.41±0.14 μm while XY had 0.44±0.03 μm. The secondary spermatocytes, spermatids, and sperm of the YY showed no significant difference in the statistical analysis of the sizes, although a general trend was larger sizes for the YY male (Table 2).

In the whole process of differentiation of the reproductive system of YY and XY tilapia, a positive correlation was observed between testis size and degree of maturation. In YY and XY fish of the same age, the gonadal maturation and testis size increase were faster in YY than in XY. No gross morphological, as well as histological abnormalities were observed in the YY male. Since testis size was bigger in the YY fish, there would also be more tubules, increased surface area of the seminiferous epithelium, and more spermatogenic cells. The presence of a bigger vas deferens in the YY could be necessary for the bigger spermiation bulk during spawning.

The lack of significant differences between the sizes of the spermatogenic cells may mean the absence of a negative effect of the lack of an X chromosome, or the presence of 2 Y chromosomes. Instead, such condition of 2 Ys and no X chromosome may be advantageous, as evidenced by the bigger size of the YY fish, its testis and ducts, and the formation of more spermatogenic cells.

In human beings, X chromosome carries several genes necessary for life (Hickman et al., 1993) and absence of this is fatal (Umaly & Roderos, 1985). Scott et al. (1989) suggested that the X and Y sex chromosomes of tilapia are poorly differentiated, which may explain why YY males are viable. Ohno (1970) noted that X and Y are nearly identical in genetic composition in many fishes and amphibians. Wolforth & Hulata (1981) observed no case of heteromorphic chromosomes that could be regarded as sex chromosomes in many teleost species. The double dose of Y or lack of X chromosome may give the fish advantage as shown in this study. Investigation on the gene composition of the tilapia chromosomes is would be very enlightening.

CONCLUSIONS

The study showed that using the same rearing conditions and comparing same age specimens, the larger supermale tilapia has superior reproductive capacity with its larger testis and ducts, higher gonadosomatic index, and faster histogenesis, testicular differentiation, and spermatogenesis. Since XY males with 1 Y chromosome have superior growth over XX females with no Y chromosome, there is the possibility that 2 Y chromosomes may effect the growth superiority observed in supermales. More investigations are needed to clarify the role of X and Y chromosomes in tilapia, possibly using the tools of molecular biology and biotechnology.

RECOMMENDATIONS

Electron microscope analysis of the spermatogenic cells of YY and XY fish is recommended to elucidate on possible differences.

Analysis of the gene composition, as well as gene expression of the X and Y chromosomes would be of invaluable help in making conclusive statements about the definitive advantage of having 2 Y chromosomes and no X chromosome. Factors affecting sex determination and differentiation should also be studied to properly evaluate the seemingly advantageous presence of 2 Y chromosomes. Capacity of the supermale sperm in fertilization should be investigated to determine the reproductive superiority of YY males over the XY males.

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