Full Length Article

PRODUCTION OF HORMONE-FREE MONOSEX POPULATION OF NILE TILAPIA, *OREOCHROMIS NILOTICUS* (LINNAEUS, 1758)

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ABSTRACT

The possibility of production of hormone-free all male Nile tilapia (Oreochromis niloticus) in F_2 generation, through the production of YY super males in F_1 generation, was investigated in the present study. Feminization of Nile tilapia was carried out using the hormone, 17 beta estradiol at 100, 200 and 300 mg/kg concentrations, orally through feed. The hormone enhanced the growth significantly ($p<0.05$) both in terms of length (55.39±0.67 cm) and weight (55.39±0.67 g) at 300 mg/kg feed when compared to other treatments and control. With the increasing concentrations (300 mg/kg) of the hormone, the survival rate decreased significantly ($p<0.05$) to 62±1.53%. The feminization rates increased significantly ($p<0.05$) with increasing concentrations of the hormone, with the highest feminization percentage of 98.27±0.87% observed at 300 mg/kg concentration of the hormone. Two fish from F_1 generation with the PIT tag numbers 81 and 88 produced all male progeny which was confirmed through morphological examination. The genotype of these two YY supermales was re-confirmed through molecular genotyping with the help of primers $amhX_{+36}$ and $amh\delta Y_{+5}$ DNA markers, specific to X and Y chromosomes, respectively using RAPD-PCR.

Key Words: Nile tilapia, Sex reversal, 17 beta estradiol, YY super male, RAPD-PCR

Received : 13.03.2023 Revised : 02.05.2023 Accepted : 02.05.2023

INTRODUCTION

Tilapia belongs to the family Cichlidae under order Perciformes and is native to Africa and Middle East, and has emerged from mere obscurity to one of the most productive and internationally traded food fish in the world. The farming of tilapia, especially of the Nile tilapia (*Oreochromis niloticus*) is believed to have originated more than 4000 years ago from Egypt. Aquaculture of tilapia on the

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scientific lines was recorded in Kenya in 1924 and soon spread to many parts of the world. The Nile tilapia is the predominantly cultured species among several species of tilapia which are cultured commercially worldwide (Ridha, 2006). Tilapias are the second most important group of edible fishes in the world, next only to the carps.

The Nile tilapia, *Oreochromis niloticus* production was nearly 2.54 million tons in 2009 (FAO, 2011), exceeding the harvest of Atlantic salmon, *Salmo salar* (Maclean *et al*., 2002). Although, native to Africa, tilapias are cultured widely in Asia and the Far East (Maclean *et al*., 2002).

Nile tilapia is a tropical species that prefers to live in shallow water. It is an omnivorous grazer that feeds on phytoplankton, periphyton, aquatic plants, small invertebrates, benthic fauna, detritus and bacterial films associated with detritus. Sexual maturity in ponds is reached at an age of 5-6 months. Spawning begins when the water temperature reaches 24°C. The female incubates the eggs in her mouth and broods the fry after hatching until the yolk sac is absorbed. A female weighing 600-1000 g can produce 1000 to 1500 eggs. The Nile tilapia can live longer than 10 years and reach a weight exceeding 5 kg. The Nile tilapia, like any other species of tilapia, attains maturity in 3-4 months and is a prolific, continuous breeder leading to heavy losses in aquaculture industry due to pond overcrowding with small, under-market size fish (Baroiller and Jalabert, 1989; Beardmore *et al*., 2001).

In Nile tilapia farming, males grow bigger and faster than the females. Hence, an all-male population of Nile tilapia is preferred which also avoids unwanted spawning (Dan and Little, 2000; Carandang, 2007; Mateen, 2007). Presently the production of all male mono-sex population of the Nile tilapia utilizes the treatment of fish fry with androgenic steroid hormones or their synthetic analogues. But due to various environmental concerns related to hormone use like possible hazardous effect of hormone residues on water quality, biodiversity, health of consumers amidst growing concerns for food security, a different approach to produce all male mono-sex tilapia population for culture in a non-hazardous, consumer-friendly and environment-friendly way is inevitable. Also, sale of treated fish is illegal in many countries like the European Union (White *et al*., 2006).

Oreochromis niloticus has primarily an XY sex-determining system, with occasional influence of autosomal loci and masculinizing effect of high temperature (Mair *et al*., 1991; D'Cotta *et al*., 2001; Griffin *et al*., 2002). It has been possible to produce genetically "all-male populations" through the development of YY "super males" (Baroiller and Jalabert, 1989; Scott *et al*., 1989) and YY individuals are viable and fertile (Carrasco *et al*., 1999). A viable alternative on a commercial scale is

the YY technology derived from males that possess a novel homogametic YY genotype, which allows for the production of genetically male tilapia based on crosses between YY males and XX females (Cruz *et al*., 1996; Mair *et al.*, 1997; Vazquez *et al*., 2014). The first step in the production of super male (YY) technology requires feminization of XY fry during their sexually undifferentiated stage and the identification of these newly created "sex-reversed females" (XY females) through a progeny test (Cruz *et al*., 1996; Mair *et al.*, 1997). These XY females will be used to produce 25% of YY males, when crossbred with normal males (XY) (Mair *et al.*, 1997; Vazquez *et al*., 2014). These YY super males when crossbred with normal females (XX) produce all male population which are free from any hormone treatment and are eco-friendly and fully safe for the consumers.

The present work was carried out with the objective of production of hormone-free all male Nile tilapia for distribution to the farmers through the production of YY super males of *O. niloticus.*

MATERIALS AND METHODS

Procurement of brood stock

The brood stock of *O. niloticus* were obtained from Manchinabele dam, Magadi Taluk, Ramanagara District, Karnataka State, India, in the month of March 2020. The collected broodstock was acclimatized to the broodstock pond conditions at Fisheries Research and Information Center (FRIC), Hesaraghatta and fed twice a day at 2% body weight, with commercial floating pellet feed, 4 mm pellet size with 28% protein, for 30-45 days and then subjected to breeding trials to produce fry to be used for feminization trials.

Fry production

The acclimatized *O. niloticus* brooders were stocked at a male: female ratio of 1:3 in breeding hapas in the ponds. The mouth of the females were periodically checked once a week for presence of eggs. The fertilized eggs were collected from the mouth of the females and hatched in a glass jar hatchery. The hatchlings were reared in nursery tanks for 15 days (Fig. 1).

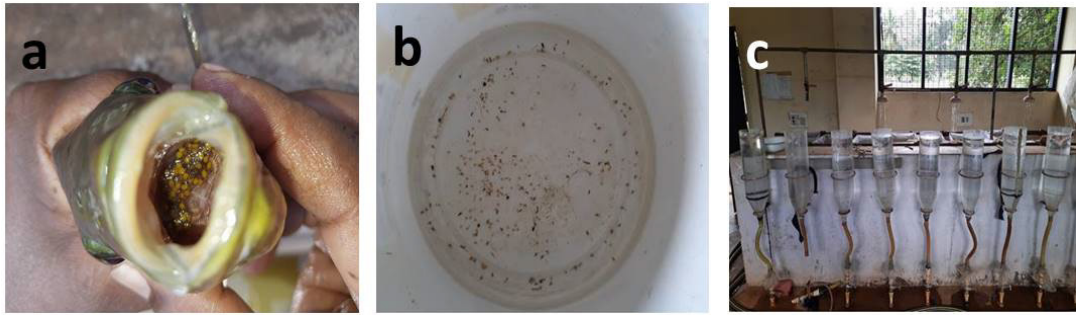

Figure 1. a. Fertilized eggs in the mouth of female Nile tilapia; b. Fertilized eggs obtained from the mouth of female Nile tilapia; c. Eggs kept for hatching in a glass jar hatchery

Preparation of feed with hormone

The hormone 17 beta-estradiol (E2), (HiMedia Laboratories, Mumbai, India) was used at three different concentrations of 100 mg/kg (T1), 200 mg/kg (T2) and 300 mg/kg (T3) of the feed. The hormone solutions at these concentrations were prepared separately by dissolving in 100 mL of 95% ethanol. The commercially available floating pellet feed was pulverized into a fine powder and used. Each of the hormone preparations was finely sprayed onto powdered fish feed, mixed several times and maintained at room temperature overnight to allow the alcohol to evaporate using the alcohol evaporation method of Guerrero and Shelton (1974). The feed for the control group was treated in exactly the same manner with the exclusion of the hormone. After drying, all the three treatment group feeds and the control were properly labeled and stored in air tight plastic containers at 4°C until further use.

Feminization trials

At the end of the nursery period, the sexually undifferentiated 15 dph (days post hatch) old fry were collected and used for feminization trials (Juarez *et al*., 2017) in 1 m^3 FRP tanks. Triplicates were maintained for all the three treatments with a control. One hundred fish fry were stocked into each of the tanks and fed with hormone treated feed @ 10% body weight for 30 days (Vazquez *et al.,* 2015). The tanks were cleaned daily by siphoning off the left-over feed, faecal matter and dead fry. Adequate aeration and water exchange were done on daily basis.

Evaluation of growth, survival and feminization

After the completion of the hormone treatment for 30 days, the fingerlings were stocked in grow-out ponds and fed @ 2% body weight with commercial floating pellet feed for a period of 3 months. Random samples of 30 fish per replicate were taken. The sex of the fish was determined by external examination based on differences of the papilla structure. Feminization was ascertained by gross examination of the gonads and by microscopy using acetocarmine squash method (Guerrero and Shelton, 1974). The random samples of 30 fish were taken once in 15 days for the determination of growth in terms of length and weight. At the end of post-treatment growth trials of 3 months, the fish were counted in each replicate for the determination of survival.

Production of YY super males

The feminized fish were reared to maturity and all the males of the population were culled. The females were PIT tagged and recorded. The PIT tagged females were then subjected to individual pair mating with the normal males in hapas which were labeled with the PIT tag number. PIT tagged females with more than 100 g body weight were selected for mating. A total of 20 individual pair matings were done. The hapas were checked once in a week for the signs of breeding for a period of one month.

The F_1 generation from individual pair matings were reared separately in tagged hapas to maturity for a period of 4 months. All

the females of the F_1 generation were culled and a random sample of five male fish were taken from each hapa and were PIT tagged and recorded. The PIT tagged males of F_1 generation were mated with normal females in individual pair mating manner in tagged hapas. The hapas were checked once in a week for any signs of breeding for a period of one month. The F_2 offspring were reared to maturity in separate, tagged hapas for a period of 4 months and their sex was evaluated.

Molecular genetic analysis of YY super males

Molecular genetic analysis to cross verify the genotype of the F1 males was carried out using the DNA markers amhX_{+36} and amhδY_{+5} (Triay *et al.,* 2020) (Table 1). The first marker, amhX+36 which amplifies a 1000 base pair (bp) fragment present on the promoter region of the amh gene located on the X chromosome and the second marker, amhδY+5 amplifies a 1500 bp fragment that corresponds to a 5 bp insertion in exon6 that is present in the amhδY gene, located on the Y chromosome (Triay *et al.,* 2020).

The fin clippings from the dorsal fin were collected in eppendorf tubes labelled with corresponding PIT tag numbers in absolute alcohol and stored in refrigerator till use. Genomic DNA (gDNA) was extracted as per the method of Triay *et al*. (2020) using dorsal fin clippings and the quality was checked with a spectrophotometer and on a 0.8% agarose gel. Finally, the gDNA was diluted to 50 ng/L. The genomic DNA was subjected to RAPD-PCR using the primers (Table 1).

The results obtained were grouped and subjected to statistical analysis by two-way ANOVA using SPSS – version 15.0 statistical package.

Table 1. Sequence of the primers for the DNA markers amhX_{+36} and amhδY_{+5} of the Nile Tilapia

S. No.	DNA Marker	Primer Sequence
1	amhX_{+36}	Forward- GTTTGCAATAGTTAGGGTGCTGCTG
		Reverse- GGAAATGCAGCCATTCCTGAG
2	amhδY_{+5}	Forward- AAACCTCCTTCCTTTGTGAATGTC
		Reverse- CTAGCGGCATCCACACTCCCTCAC

RESULTS AND DISCUSSION

In the present study, sexually undifferentiated 15 dph (days post hatch) old fry of the Nile Tilapia were used for feminisation. Many researchers have also used 15 dph sexually undifferenetiated Nile Tilapia in their feminisation trials (Juarez *et al.,* 2017; Vazquez, 2018). On the contrary, Gennotte *et al.* (2014) used fertilised eggs for feminisation of the Nile Tilapia. In the present study, feminisation was done using the hormone orally, through the feed. Many researchers have also reported feminisation of the Nile Tilpia by administration of the hormone through feed (Vazquez *et al.,* 2015; Juarez *et al.,* 2017; Vazquez, 2018). On the contrary, many researchers used immersion bath as a technique for administration of the hormone during feminsiation of the Nile Tilapia (Bombardelli and Hayashi, 2005; Gennotte *et al.,* 2014). In the present study, the hormone 17 beta estradiol, at three different concentrations of 100, 200 and 300 mg/kg feed was used for feminization of the Nile Tilpia. Vazquez *et al.* (2015) also used 17 beta estradiol for feminisation of the Nile tilapia. Juarez *et al.* (2017) also used 17α-Ethinyl Estradiol (EE2) at the same concentrations with an addition of 400 mg /kg feed. On the contrary, Vazquez, (2018) used a combination of estrogens, 17 beta estradiol (E2), Diethyl Stilbestrol (DES) and 17 alpha Ethinyl Estradiol (EE2).

There was a significant difference ($p<0.05$) between T3 and the control group. Also, T1 and T2 exhibited significant difference ($p<0.05$) from the control group. However, there was no significant difference between the T1 and T2 groups. Significantly higher ($p<0.05$) length of 55.39±0.67 cm was observed in the 9th sampling post-treatment in the T3 group followed by T1 group (22.44±0.51cm), Control group (21.63±0.5 cm) and the T2 group (18.44±0.29) (Fig.2). It was observed in the present study that there was a growth suppression effect in T1 and T2 when compared to the control group. However, higher concentration of the hormone (300 mg/kg feed) resulted in significantly higher ($p<0.05$) growth in terms of length in the T3 group.

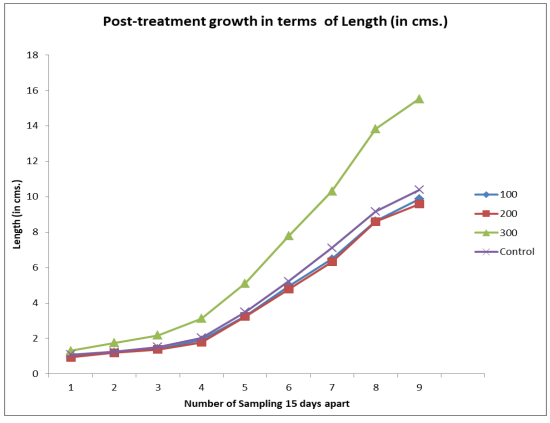

Figure 2. Post treatment evaluation of growth in terms of length (in cm)

It was observed in the present study that, at lower concentrations of 100 mg/kg feed, 17 beta estradiol did not have any significant effect ($p<0.05$) on the growth in terms of weight (22.44±0.51 g) when compared to the control (21.63±0.5 g) (Fig.3). But at a concentration of 200 mg/kg feed, the hormone suppressed the growth (18.44±0.29 g) when

compared to the control. However, at a higher concentration of 300 mg/kg feed, the hormone significantly enhanced the growth in terms of weight (55.39±0.67 g) when compared to the treatments T1, T2 and control ($p<0.05$). This may be due to growth enhancing activity of 17 beta estradiol at higher concentrations.

It was observed from the results of the present study that, high concentrations of the 17 beta estradiol hormone (300 mg/kg) significantly improved ($p<0.05$) the growth rates in terms of length and weight 55.39±0.67 cm and 55.39±0.67 g, respectively (Fig. 2 and 3) in the Nile Tilapia. Juarez *et al.* (2017) also observed that, at higher concentrations (400 mg/kg feed), EE2 had significant growth promoting activity. It was also observed from the results of the present study that, at moderate concentrations (200 mg/kg), 17 beta estradiol exhibited significantly lower growth rates ($p<0.05$) (Fig. 2 and 3) in the Nile Tilapia. Vazquez *et al.* (2015) also observed that lower concentration of 17 beta estradiol (60 mg/kg feed) had growth suppression effect.

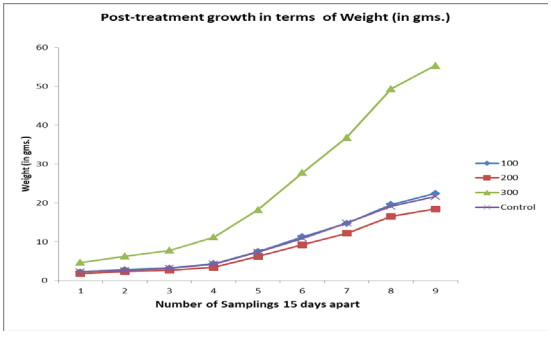

Figure 3. Post treatment evaluation of growth in terms of weight (in g)

Significantly lower percentage of survival was observed in T3 (62 ±1.53) followed by T2 (70.33 ±1.20) and T1 (72.33 ±0.88) while the control recorded highest percentage of survival (83 ±1.53) ($p<0.05$) (Fig. 4). From the results of the present study, it can be deduced that, as the concentration of 17 beta estradiol increases, the survival rate of the Nile tilapia decreases.

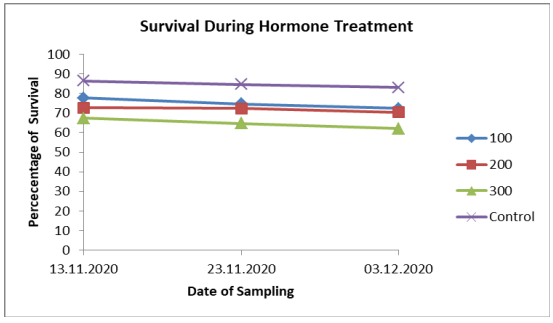

Figure 4. Percentage of survival of fry during hormone treatment

The findings of the present study revealed that at higher concentrations (300mg/kg), 17 beta estradiol exhibited significantly lower ($p<0.05$) (Fig. 4) survival rate of 62 ±1.53%. On the contrary, Vazquez *et al.* (2015) observed in their study that, survival rate was not influenced by different concentrations of the hormone in different treatments and was not significantly different from that of the control. The findings of the present study are in agreement with those of Juarez *et al.* (2017), who observed significantly lower survival percentages in the treatment groups when compared to the control during their feminization trials using 17 alpha ethinyl estradiol (EE2).

A significantly higher feminization percentage (98.27 ±0.87) was observed in T3 group, followed by T2 (92.23 ±0.88) and T1 group (87.92 ±0.83) ($p<0.05$) (Fig. 5). It was observed from the results of the present study that higher the concentrations of 17 beta estradiol higher the feminization percentages.

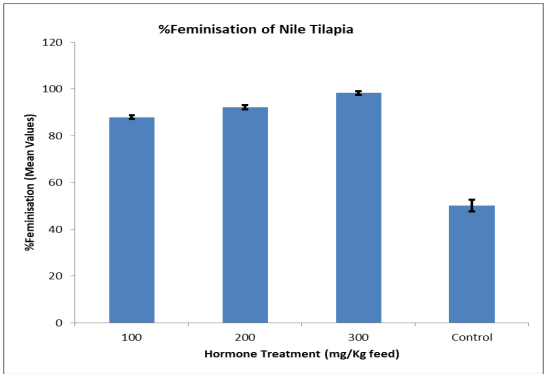

Figure 5. Feminsation percentage after the hormone treatment

It was observed in the present study that, higher concentrations of the hormone 17 beta estradiol @ 300 mg/kg resulted in significantly higher ($p <0.05$) feminization percentages of 98.27 ±0.87% (Fig. 5). The findings of the present study are in agreement with those of Vazquez *et al.* (2015) who also observed that significantly higher percentage of feminisation (69.8%) was achieved with higher concentration of the hormone when compared to lower concentration (60.2%). Juarez *et al.* (2017) also observed higher feminization rates (84% and 90%) with 300 mg/kg and 400 mg/kg concentrations of 17 alpha Ethinyl Estradiol. The results of the present study (87.92±0.83 @ 100 mg/kg) are in agreement with those of Vazquez *et al.* (2015) who reported high feminization rate (88.5± 2.1%) with 120 mg/kg of 17 beta estradiol.

In the breeding sets, to get F_1 progeny, only five pairs successfully bred with the female tag numbers, 900215000487417, 900215000487406, 900215000487255, 900215000487254 and 900215000487256. Out of the five males of F_1 generation, only two males with PIT tag numbers 900215000486988 and 900215000486981 yielded all male population. The results of the agarose gel electrophoresis of the products of the RAPD-PCR for the DNA markers amhδY_{+5} and amhX_{+36} using the respective primers revealed amplification of a 1000bp DNA product in all the eight samples with the PIT tag numbers 81, 83, 86, 87, 88, 90, 94 and 98 and amplification of a 1100 bp DNA product in six out of eight samples without any amplification of the samples with PIT tag numbers 81 and 88, respectively (Fig. 6 and Fig. 7).

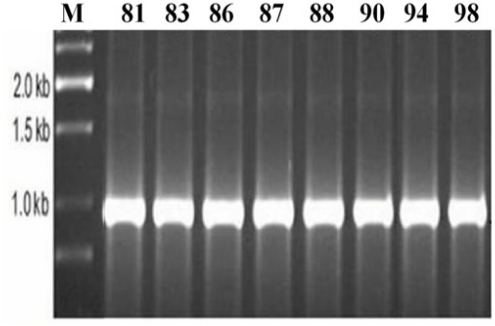

Figure 6. Electrophoresis of the RAPD-PCR products using the primers for the DNA marker amhδY_{+5}

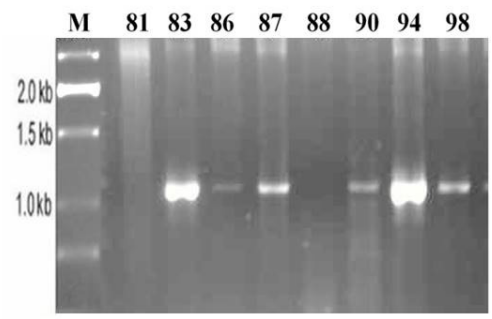

Figure 7. Electrophoresis of the RAPD-PCR products using the primers for the DNA marker amhX$_{+36}$

The results of the present study, revealed amplification of a 1000bp DNA product in all the eight samples with the PIT tag numbers 81, 83, 86, 87, 88, 90, 94 and 98 for the DNA marker amhδY$_{+5}$ (Fig. 6) implying that all the eight fish samples were males, since the primers for the marker amhδY$_{+5}$ specifically amplify portions of the Y-chromosome.

The results of the present study also revealed the amplification of a 1100 bp DNA product in six out of eight samples without any amplification of the samples with PIT tag numbers 81 and 88 for the DNA marker amhX$_{+36}$ (Fig. 7) implying that the fish with PIT tag numbers 81 and 88 are the super males with YY chromosomes, since the primers for the marker amhX$_{+36}$ amplify only the portions of X chromosome. The results of the present study are in agreement with those of Triay *et al*. (2020), who also reported that the primers for amhδY$_{+5}$ specifically amplify portions of the Y-chromosome and the primers for amhX$_{+36}$ only amplify the portions of X chromosome. However, Triay *et al.* (2020) observed that the primers for amhδY$_{+5}$ specifically amplify 1500 bp fragment of the Y-chromosome which is contradictory to the findings of the present study where 1000 bp fragment of the Y-chromosome got amplified. Also, Triay *et al.* (2020) observed that the primers for amhX$_{+36}$ specifically amplify 1000 bp fragment of the X-chromosome which is contradictory to the findings of the present study where 1100 bp fragment of the X-chromosome got amplified. Many workers have reported that the marker DNA, amhδY$_{+5}$ is specific for the Y chromosome and the marker DNA amhX$_{+36}$ is specific for the X chromosome in the Nile Tilapia (Eshel *et al*., 2014; Li *et al.*, 2020; Triay *et al.,* 2020). In the present study, the morphological identification of the all male F_2 generation of the PIT tagged F_1 super males with tag numbers 81 and 88 satisfactorily matched with the molecular identification of YY supermales.

CONCLUSION

The present study was carried out to investigate the possible production of hormone-free all male Nile tilapia in F_2 generation, through the production of YY super males in F_1 generation. The parent population was feminized using the hormone 17 beta estradiol at 100, 200 and 300 mg/kg concentrations with feed through the oral route. The results of the present study indicate that higher concentrations of the hormone 17 beta estradiol, had significantly ($p<0.05$) higher growth promoting activity both in terms of length and weight of the Nile tilapia ($p<0.05$),

while the survival rate decreased significantly as the concentration of the hormone increased ($p<0.05$). It was also observed from the results of the present study that the feminization rates also increased significantly ($p<0.05$) with increasing concentrations of the hormone 17 beta estradiol. It was observed from the present study that two YY super males of Nile tilapia produced from F_1 generation with the PIT tag numbers 81 and 88 produced all male progeny. The YY supermale genotypes were confirmed through molecular genotyping with the help of $amhX_{+36}$ and $amh\delta Y_{+5}$ DNA markers specific for X and Y chromosomes, respectively using RAPD-PCR. It is recommended from the encouraging results of the present study that further research work on the same lines can be conducted using GIFT (Genetically Improved Farmed) tilapia broodstock.

ACKNOWLEDGEMENT

The author immensely thanks the Karnataka Veterinary, Animal and Fisheries Sciences University for providing the financial assistance and necessary facilities to carry out the present research.

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