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Optimization of dose of methyltestosterone (MT) hormone for sex reversal in tilapia (*Oreochromis niloticus* L.)

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Abstract
This paper describes the optimization of dose of methyltestosterone (MT) hormone for masculinization of tilapia (*Oreochromis niloticus*). Five treatments (i.e. T_1, T_2, T_3, T_4 and T_5) with different doses such as 0, 40, 50, 60 and 65 mg of MT hormone were mixed with per kg of feed for each treatment and fed the fry four times a day up to satiation for a period of 30 days. The stocking density was maintained 10 spawn/liter of water. The growth of fry at different treatments was recorded weekly and mortality was recorded daily. At the end of hormone feeding the fry were reared in hapas fixed in ponds for another 70 days and at the 100th day the fish were sexed by the gonad squashing and aceto-carmine staining method. The analysis of growth data did not show any significant variation in length and weight of fish among the different treatments. High mortality of fry ranging 66% to 81.6% was observed in different treatments and highest mortality was observed during the first twelve days of the experiment. The sex ratio analysis showed that T_2 (40 mg/kg) and T_5 (65 mg/kg) produced 93.33% of sex reversed male and T_3 (50 mg/kg) and T_4 (60 mg/kg) produced 96.66% sex reversed male, and these ratios were significantly ($p<0.05$) different from 1:1 male: female sex ratio. The control, T_1 (0 mg/kg) contained 43.33% male progeny. From these results it is suggested that either 50 mg/kg or 60 mg/kg of MT with a feeding period of 30 days could be considered as an optimum dose for masculinization of tilapia (*O. niloticus*).

Key words: Masculinization, Methyltestosterone, Tilapia

Introduction

Hormonal sex reversal is a technique of changing of sexes from one sex to another in fish by administering synthetic steroid hormones before and/or during the period of sexual differentiation. In this technique, the first feeding fry are treated with male hormones or androgens (i.e.17α-methyltestosterone), which develops testes and male sexual characteristics at maturity, while treatment with female hormones or estrogens (i.e.17β-estradiol) produces individuals with ovaries and female characteristics in fish (Hussain 2004). The sex reversal technique is very simple, economic, low inputs cost involving and ensures high production and high net profit which can be done by a

technician without sophisticated laboratory and equipment. A total of 35 tilapia seed production hatcheries have established that are producing 10-12 billions fry every year and a number of commercial farms have been established, which are producing roughly about 0.02 million tons of marketable size fish (Hussain 2008).

The production of tilapia for food has long been hindered by the precocious maturity and uncontrolled reproduction exhibited by these fish (Wohlfarth and Hulata 1981). These difficulties have been cited by Macintosh *et al.* (1985) as the reason for the commercial inviability of tilapia in Indian Major Carp culture systems. Female tilapias are capable of spawning every four to six weeks under ideal condition in their natural environment (Jalabert and Zohar 1982, Little *et al.* 1993). The excessive reproduction of tilapia species leads to overcrowding, competition for food and stunted the growth in aquaculture system, which resulted in low yields of harvestable size of fish. To overcome this problem and to increase the yield of tilapia several methods have been proposed and developed. These include the use of suitable predator species (Guerrero 1982), the generation of infertile fry through triploidy and the use of hybrid crosses to produce monosex broods (Pruginin *et al.* 1975, Hanson *et al.* 1983, Majumdar and McAndrew 1983). Monosex population offers several benefits in aquaculture, including faster growth and prevention of unwanted reproduction (Mair *et al.* 1991, Green *et al.* 1997). So, the culture of monosex population of tilapia is preferable.

The use of male sex steroids to induce sex inversions of genotype females into phenotypic males has proven to be one of the successful methods to produce monosex population (Hunter and Donaldson 1983). The androgen 17α-methyltestosterone (MT) (Ridha and Lone 1990) and the estrogen diethylstilbestrol (DES) are the most widely used hormones for sex inversion in tilapia. The direct masculinization of tilapias using hormone is the most common method for monosex male production (Shelton *et al.* 1978; Guerrero 1979, Guerrero and Guerrero 1988). In this process, male steroid is administered to first feeding tilapia fry so that the undifferentiated gonadal tissue of genetic females develops into testicular tissue, producing individuals that grow and function reproductively as males. However, in many cases the hatchery operators used very high or low dose of hormones and fed the fry with hormone mixed feed for shorter or longer period of time beyond the actual time needed for the sex conversion. As a result, 100% monosex population is not produced in their operation. So, optimization of hormonal dose and duration for feeding can minimize the production cost of the hatcheries and ensure to produce desired level of monosex population. For these reasons, the research was conducted to optimize the dose and duration of MT for the sex reversal of *O. niloticus*.

Materials and methods

Experiment site

The experiment was conducted in the hatchery of Fisheries Biology and Genetics Department, Faculty of Fisheries, Bangladesh Agricultural University (BAU),

Mymensingh. Three day-old spawns of tilapia (*O. niloticus*) were collected from a private tilapia hatchery named Reliance Aqua Farm Ltd., Bailore, Trishal, Mymensingh.

Design of the experiment

The experiment was comprised of five treatments (T_1, T_2, T_3, T_4 and T_5) of different doses (except control which was hormone free) of MT hormone. It was conducted in 5 glass aquaria of ($45\times25\times24$) cm^3 size each and contained 25 liter of water. 250 spawns were stocked in each aquarium. The average length and weight of spawns were 7.75 ± 0.05 mm and 0.006 g respectively and the stocking density was maintained 10 spawns/liter of water. The spawns were reared for 30 days.

Four diets with different doses of MT hormone i.e., 40, 50, 60 and 65 mg/kg were prepared through ethanol evaporation method (Mair and Santiago 1994). To prepare 100 g feed for each treatment, required amount of MT hormone (i.e., 4, 5, 6 and 6.5 mg MT hormone for T_2, T_3, T_4 and T_5 respectively) was diluted with 60 ml of alcohol for homogenous mixing with the feed in each treatment. Ground and sieved fish meal was used for preparing the feed. In case of control, required amount of feed was prepared by mixing ethanol only. The prepared feeds were preserved in a refrigerator at 4^0C.

Feeding and sampling of fry

The spawns were fed with hormone mixed feeds 4 times (from 7:00 am to 07:00 pm with four hour interval) a day up to satiation. The water of each aquarium was exchanged by 75% of the volume with fresh water once a day in the morning to avoid water quality deterioration due to decomposition of left over feed and feces of the spawns. In addition, the faecal out-put and wastes of feed were removed from the aquarium by siphoning at 9:00 am and 5:00 pm daily. Additional oxygen was provided to aquarium through aeration for 22 hrs everyday from two aerators and was stopped for half an hour each time during feeding.

The fish were sampled at weekly interval to determine the increase in their size (length and weight). Sampling was done in the early morning when the fish stomach was about to be empty to avoid the biasness of weight due to the presence of excessive feed. Ten fry were randomly collected from each aquarium and the weight of all fry was taken together due to their small size in an analytical balance. The length (mm) was measured by placing the fish on a petri dish having a 1 mm graph paper underneath it. Mortality of the fry was recorded daily. The experiment was continued for 30 days and at the end of the experiment the fish were transferred to hapas fixed in a pond and reared them with the normal feed until being sexed. The hormone feeding was terminated at the 30^{th} day of experiment but the fish were reared for another 70 days with normal feed for proper sexing. At the age of 100 days, the final growth and mortality (%) of fish were estimated. Water pH and temperature in each aquarium were estimated at seven days interval.

Fish sexing

The fish were sexed by gonad squashing and aceto-carmine staining method (Guerreo and Shelton 1974). The fish was killed and the viscera was removed to reveal the two thread like gonads lying along the upper surface of the body cavity on either side of the kidney. The gonads were removed and placed on a clean glass slide. A few drops of aceto-carmine stain were added and the gonads were squashed with a coverslip. The sex of the fish was identified by examining the slides under a microscope.

Statistical analysis

The length gain (mm), weight gain (g) and mortality (%) of fish of different treatments were tested using one way analysis of variance (ANOVA) followed by DMRT (Duncan's 1955) to identify differences among the means. This statistical analysis was performed with the aid of the computer software SPSS and MS excel programs.

Results

At the beginning of the experiment, the initial length and weight of 100 fry were taken. The average initial length and weight were 7.75 mm and 0.006 g respectively. Tables 1 and 2 showing the increment of length and weight of fry for the period of 30 days. During weekly sampling at 7th, 14th, 21st and 28th days of experiment, no significant variation in length and weight of fry was found although five different doses of hormone were administered. No significant growth variations were also found when the fish were sampled at the day of 100.

Table 1. Growth in length (mm) of tilapia fry (*O. niloticus*) during the hormonal (MT hormone) and non-hormonal (normal feed) feeding period

Treatments / Days	Av. length day 0	Av. length day 7	Av. length day 14	Av. length day 21	Av. length day 28	*Av. length day 100
T_1 (0 mg/kg, control)	7.75 ±0.05	9.10 ± 0.09	9.85 ± 0.21	10.50 ± 0.21	11.20 ± 0.13	66.00 ± 0.68
T_2 (40 mg/kg)		8.85 ± 0.08	9.35 ± 0.15	9.90 ± 0.17	10.30 ± 0.06	65.50 ± 1.03
T_3 (50 mg/kg)		9.25 ± 0.13	9.85 ± 0.18	10.30 ± 0.18	10.65 ± 0.05	65.00 ± 0.59
T_4 (60 mg/kg)		9.65 ± 0.15	9.80 ± 0.12	10.80 ± 0.16	11.35 ± 0.07	67.00 ± 0.93
T_5 (65 mg/kg)		9.85 ± 0.18	10.35 ± 0.19	11.10 ± 0.13	11.60 ± 0.08	70.50 ± 0.78

*The fry were reared up to 100 days with normal feeding after completion of hormonal feeding at different treatments.

Table 2. Growth in weight (g) of tilapia fry (*O. niloticus*) during the hormonal (MT) and non-hormonal (normal feed) feeding period

Treatments / Days	Av. wt. day 0	Av. wt. day 7*	Av. wt. day 14*	Av. wt. day 21*	Av. wt. day 28*	Av. wt. day 100**
T_1(0 mg/kg, control)	0.006	0.017	0.023	0.033	0.043	16.17±0.41
T_2 (40 mg/kg)		0.016	0.022	0.035	0.044	16.50±0.33
T_3 (50 mg/kg)		0.015	0.021	0.036	0.042	15.70±0.45
T_4 (60 mg/kg)		0.016	0.021	0.035	0.041	16.25±0.37
T_5 (65 mg/kg)		0.017	0.022	0.037	0.043	16.60±0.62

* During weekly sampling the weight of 10 fry was taken together due to its small size, therefore, it was not possible to calculate the standard error.

**The fry were reared up to 100 days with normal feeding after completion of hormonal feeding at different treatments.

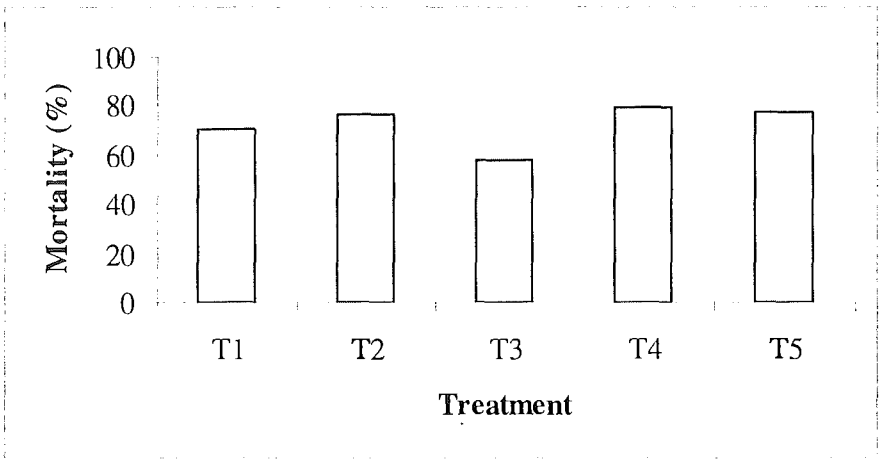

Fig 1. Mortality of *O. niloticus* fry fed with hormone (MT) – mixed feed at different treatments.

Fig. 1 shows the mortality of fry during the 30 days experiment. A high rate of cumulative mortality was observed in all treatments ranging from 58.00% to 79.60% and comparatively more mortality was observed in higher doses of hormone in first 12 days of experiment. The T_4 (60 mg/kg) showed the highest mortality (79.60%) while the T_5 (65 mg/kg) occupied the second highest position (77.20%). The mortality of T_1 (0 mg/kg, control) and T_2 (40 mg/kg) was 70.40% and 76.80% respectively which was not significantly different from T_4 and T_5. T_3 (50 mg/kg) demonstrated the lowest mortality (58.00%) and it was significantly ($p>0.01$) different from other treatments.

Fish from five treatments i.e. fish fed with 0, 40 50, 60 and 65 mg MT hormone/kg of feed were sexed at the age of 100 days and the result of sex ratios in different treatments were given in Table 3. T_3 and T_4 showed 96.66% male sex while T_2 and T_5 showed 93.33% male sex. All the treatments were significantly ($p<0.05$) different from 1:1 female: male sex ratio. The control group T_1 (0 mg/kg) contained 43.33% male sex

which was not significantly different from 1:1 sex ratio. The weekly measurement of pH and water temperature did not show any big fluctuation and both the parameters were in suitable range for tilapia fry. The ranges of pH and water temperature in different treatments were 6.75-7.50 and 26-27^{0}C respectively.

Table 3. Sex ratio of fish at different treatments. Fish were sexed at the age of 100 days

Treatments	No. of fish dissected	No. of female	No. of male	% of male
T_1(0 mg/kg, control)	30	17	13	43.33
T_2(40 mg/kg)	30	02	28	93.33*
T_3(50 mg/kg)	30	01	29	96.66*
T_4(60 mg/kg)	30	01	29	96.66*
T_5(65 mg/kg)	30	02	28	93.33*

*Significantly ($p<0.05$) different from 1:1 sex ratio.

Discussion

In the present study, treatments T_3 (50 mg/kg) and T_4 (60 mg/kg) produced 96.66%, and treatments T_2 (40 mg/kg) and T_5 (65 mg/kg) produced 93.33% sex reversed males from 30 days of masculinization experiment. The success of masculinization was quite high in all the treatments while the non-hormonal control group contained 43.33% male sex. Bombardelli *et al.* (2007) obtained 73.02% of masculinized Nile tilapia (*O. niloticus*) from 36 hours MT immersion bath. Gale *et al.* (1999) produced 83±3% of males when Nile tilapia fry were immersed in 100 μg/liter MT hormones for 13 days after fertilization. Mainardes-Pinto *et al.* (2000) compared the efficiency of 2 diets: 1(NUTRAVIT) and 2 (IP), both with 40% of crude protein, containing the synthetic androgen hormone MT and analyzed the most effective dose of this hormone on the sex reversal of Nile tilapia *O. niloticus*. A total of 9600 Nile tilapia fry at 7 days post hatching received the following treatments for 45 days: (A) 30 mg MT/kg diet 1: (B) 60 mg MT/kg diet 1: (C) 30 mg MT/kg diet 2: (D) 60 mg MT/kg diet 2 and two control groups E and F with diets 1 and 2 hormone free respectively. They found that the number of males in A, B, C and D treatments were higher than the controls groups and the dose of 60 mg MT/kg of diet as for the diets 1 and 2, was more efficient resulting in 98% of males during the experimental period.

High rate of masculinization in tilapia can be influenced by some important factors like hormone concentration, treatment duration, age and size of fry, availability of natural feed, stocking density and feeding frequency (Mair and Little 1991). In case of stocking density different studies used different stocking rates, for example, 3.6 fry/liter (Mair and Santiago 1994), 2.6 fry/liter (Shelton *et al.*1981), 1.5 to 7.75 fry/liter (Rosenstein and Hulata 1993) but the recommended stocking density for optimum masculinization of tilapia was 12 fry/liter (Mair and Little 1991). Low stocking densities can encourage the establishment of hierarchies among the treatment population where dominant fish preventing submissive fish from feeding, thus reducing the quality of

hormone ingested by the later (Mair and Santiago 1994). Considering the above important factors for sex reversal, the moderate stocking density (10 fry/liter) along with four times feeding regime, absence of natural feed and 30 days feeding durations had resulted high rate of masculinization in the present experiment and it could be a reflection of proper maintenance of the above factors.

In case of masculinization, fish size at the end of treatment period could be another factor. Dunhum (1990) cited that masculinization of *O. niloticus* fry could be unsuccessful if the fry failed to attain a standard length of 12 mm by the end of hormone treatment. All the fry in this experiment attained more or less 12 mm at the end of hormone feeding, might be one of the reasons for successful sex reversal.

The initial mortality was found high in all the treatments but mortality was decreased with the advancement of experimental time. At the end of the experiment (30 days), comparatively more fish was survived in the T_3 than those of all other treatments. In the T_1 and T_2 high mortality occurred due to electricity failure but the cause of high mortality in T_4 and T_5 could not be understood. T_3 had the lowest mortality (58.00%) and was significantly different ($p<0.01$) from other treatments. Mair and Santiago (1994) reported high mortality in both hormone and non hormone treatments. Therefore, it is difficult to predict any harmful effect of hormone on fish survival.

Although four hormonal doses as four treatments were applied to optimize the dose for masculinization, 50 mg/kg and 60 mg/kg doses of MT produced the highest percentage (96.66%) of male sex, therefore either 50 mg/kg or 60 mg/kg of MT could be recommended as optimal dose for masculinization of tilapia in hatcheries.

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