

STEROID IMMERSION FOR MASCULINIZATION OF TILAPIA

Eighth Work Plan, Reproduction Control Research 2 (RCR2) and 3 (RCR3)

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INTRODUCTION

The production of single sex populations offers several advantages in tilapia aquaculture, including enhanced growth and prevention of unwanted reproduction. A number of androgens have been shown to masculinize various tilapia species, including 17 α -methyltestosterone (MT; summarized by Pandian and Varadaraj, 1990 for *Oreochromis mossambicus*); mibolerone (Torrans et al., 1988 with *O. aureus*); fluoxymesterone (Phelps et al., 1992 with *O. niloticus*); norethisterone acetate (Varadaraj, 1990 with *O. mossambicus*); 17 α -ethynyltestosterone (Shelton et al., 1981 with

O. aureus); 17 α -methylandrosterone (Varadaraj and Pandian, 1987 with *O. mossambicus*), and trenbolone acetate (TBA) (Galvez et al., 1996 with *O. niloticus*).

Aquaculturists usually administer hormones to fish through the diet, but this method is prone to inefficiencies such as uneven exposure to steroid due to the establishment of feeding hierarchies or the availability of supplemental feed from pond primary productivity. Immersion of tilapia fry in steroid solutions may be one way to achieve masculinization and avoid these inefficiencies.

This technique is well-developed in salmonid aquaculture (Piferrer and Donaldson, 1989; Feist et al., 1995); however, it remains largely experimental in tilapia culture. Most of the reported studies immersed tilapia fry in androgens for periods of one to five weeks (Varadaraj and Pandian, 1987; Torrains et al., 1988). Recently, Gale et al. (1995) demonstrated that immersion for just three hours in 17α -methyl-dihydrotestosterone (MDHT) on two days resulted in masculinization of Nile tilapia. The study described below was undertaken to determine if these findings could be extended through examination of the effects of:

1. rearing density on efficacy of MDHT immersion,
2. a single immersion in MDHT, and
3. immersion in TBA.

METHODS AND MATERIALS

Breeding families of Nile tilapia (*Oreochromis niloticus*) obtained from Auburn University were placed in 200-l aquaria (one male to three females). The temperature was maintained at $28 \pm 2^\circ\text{C}$. Time of spawning was monitored every 2 hours. All spawning occurred between 1600 h and 1900 h. Once breeding occurred, the other fish were removed leaving the brooding female to incubate the progeny. At 280 Celsius Temperature Units (CTU) post-fertilization, fry were removed from the tank and randomly assigned to experimental groups (CTU are calculated by multiplying mean temperature by the number of days, e.g., ten days at $28^\circ\text{C} = 280$ CTU). Two hundred and eighty CTU was used as a reference because Gale et al. (1995) obtained 90 to 100% masculinization by immersing fry on day 10 and day 13 post-fertilization (dpf) while maintaining the brooding females at a mean temperature of 28°C . Each treatment within an experiment was replicated two or three times depending on the number of fry available and the objective of the experiment. The fry used in each experiment came from a single female. Each replicate was housed in a 3.8-l glass jar containing dechlorinated tap water maintained at $28 \pm 2^\circ\text{C}$ under constant aeration. Treatments consisted of immersions in either steroid or ethanol, which were mixed one minute before addition of fry. Steroids were obtained from Sigma Chemical Company (St. Louis, Missouri) and stored in stock solutions of ethanol (1 mg ml^{-1}). The following provides a description of each treatment:

Experiment 1: Effects of Density

Fry were immersed for two hours in $500\ \mu\text{g l}^{-1}$ of MDHT at 280 and 364 CTU (10 and 13 dpf at 28°C) using 33, 67, 100, or 200 fish l^{-1} in each replicate. Fish in the control group were immersed in $0.5\ \text{ml}^{-1}$ of ethanol vehicle (ETOH) using 33 fish l^{-1} in each replicate. Each experimental group was conducted in triplicate with the exception of the 200 fish l^{-1} density in which the number of fry in the brood permitted only one replication for this treatment.

Experiment 2: Effect of Number and Timing of Immersions

Fry were immersed either once for two hours at either 280, 310, or 364 CTU (10, 11, or 13 dpf), or twice at either 280 or 364 CTU in $500\ \mu\text{g l}^{-1}$ of MDHT at a density of 33 fish l^{-1} in each replicate. Fish in the ETOH control group were immersed at 280 and 364 CTUs. Each experimental group consisted of two replicates, whereas feed treatments consisted of three replicates.

Experiment 3: Effects of Steroid and Mode of Application

Fry were fed $60\ \text{mg kg}^{-1}$ of food for 28 days at a density of 47 fish per jar (field densities were proposed by Popma and Green, 1990). Immersion treatments consisted of 33 fish l^{-1} immersed at 292 CTU for 48 hours in either MT or TBA; at 310 CTU for two or four hours in TBA; and two immersions at 310 and 364 CTU each for two hours in either MT or TBA. All steroid immersion concentrations were $500\ \mu\text{g l}^{-1}$. Two control groups were incorporated—one used food treated with ETOH and the other involved fry immersion for four hours in water containing 0.5 ml of ETOH vehicle at 310 CTU. For each experiment, fry were collected after each immersion, jars were thoroughly cleaned, and then fish were reallocated in fresh, dechlorinated tap water. After seven days, fry were transferred to Oregon State University's Warm Water Research Laboratory, Corvallis, Oregon, and reared in 75-l fiberglass tanks with a recirculating system. Temperature and pH were monitored daily; ammonia, nitrites, dissolved oxygen, alkalinity, and hardness were checked weekly. Water temperature in the grow-out system was maintained at $28 \pm 2^\circ\text{C}$. At 60 to 70 dpf fish were weighed and sex ratios were determined. Gonads were stained with aceto-iron hematoxylin (Wittman, 1962) and examined, to determine sex, using squash (10 and 40 x magnification) preparations.

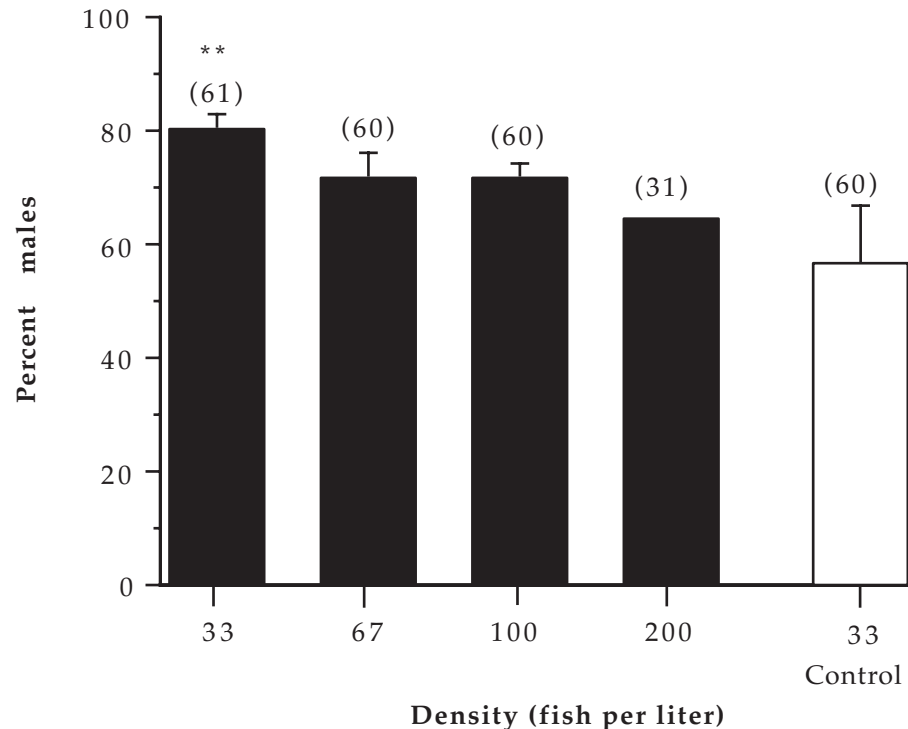


Figure 1. Masculinizing effects of double MDHT immersions on *Oreochromis niloticus* fry at different densities. Sample size is shown in parentheses. ** Indicates significant difference to control ($P < 0.01$). Error bars = SE.

Data were pooled from replicate tanks because there was no evidence of tank effects within treatments (Fisher's test or ANOVA). Sex ratio and mortality data were analyzed using Fisher's exact test with exact p-values (a more conservative test than the chi-square test for small sample sizes) estimated in GraphPad Prism™. The mean final weights of sampled fish from experiments two and three were analyzed for differences between groups using one-way ANOVA; mortality was included as a possible confounding variable. For all analyses differences were considered statistically significant when the p-value (P) was less than 0.05.

RESULTS

Experiment 1: Effects of Density

Treatment with MDHT resulted in masculinization of tilapia (Figure 1). The percentage of males in the 33 fish l^{-1} treatment (80.3%) was significantly higher than the percentage of males in the control group (56.7%); ($P = 0.004$); whereas the percentages of males in the 67 and 100 fish l^{-1} treatments (both treatments produced 71.7% males) were

not significantly different from controls ($P = 0.06$). The proportion of males in the only replicate of the treatment with 200 fish l^{-1} (64.5%) was not significantly different from the control group.

Experiment 2: Effect of Number and Timing of Immersions

Single immersion in MDHT at 364 CTU resulted in 79.3% males (Figure 2), which was not significantly different from the two immersions at 280 and 364 CTU (82.9% males). Each of these treatments had a significantly higher proportion of males than the ETOH control group (56.6% males; $P < 0.001$). No significant masculinization effects were observed in groups immersed in MDHT at either 280 or 310 CTU.

Experiment 3: MT Fed, TBA, and MT Immersions

The control fry used for the MT fed and TBA immersion treatments in this experiment showed female-biased sex ratios (15.6 and 13.2% males, respectively) (Figure 3). No significant differences were found between control groups and MT-fed fish (14.1% males); or between control and tilapia

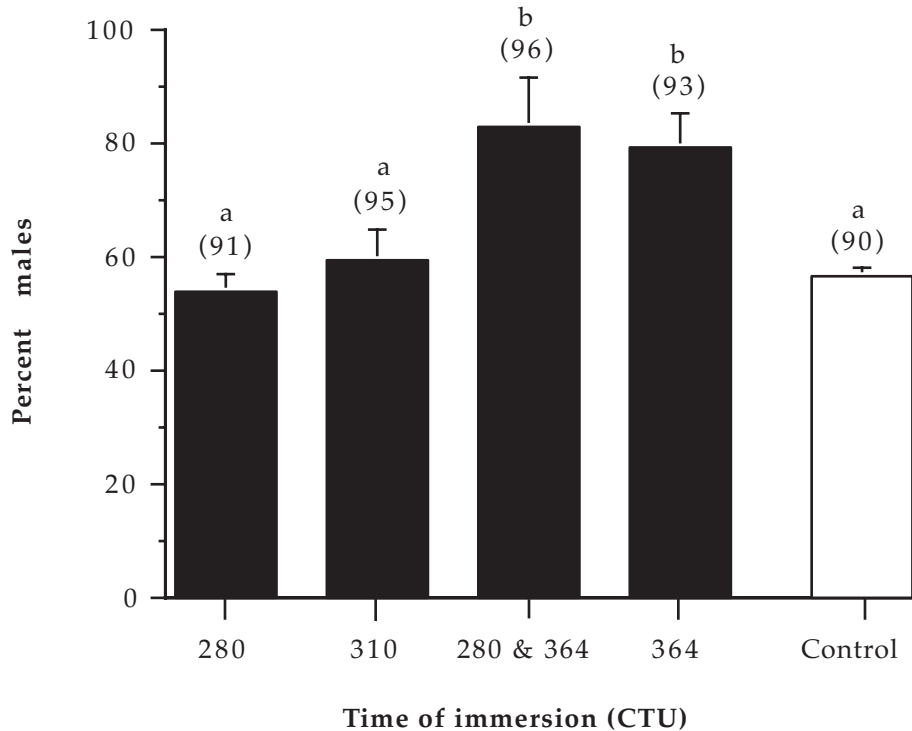


Figure 2. Masculinizing effects of single and double MDHT immersions on *Oreochromis niloticus* fry. Sample sizes are shown in parentheses. Treatments annotated with the same letter (a or b) are not significantly different. Error bars = SE.

immersed in TBA for two hours at 310 CTU (20.0% males), or between control and tilapia immersed in MT for two hours at 310 and 364 CTU (20.0% males). Single immersions in MT (26.7% males produced) and TBA (37.3% males produced) for 48 hours at 292 CTU resulted in higher proportions of males ($P = 0.049$ and 0.002 , respectively). Immersions in TBA for four hours at 310 CTU and two hours at 310 and 364 CTU produced significantly higher proportions of males (64.4 and 91.9%, respectively) ($P < 0.001$).

In all experiments mortality and final weight data were not significantly different among treatment groups. Water quality in rearing tanks was maintained close to the optimal values for tilapia culture (data not shown).

DISCUSSION

We have demonstrated that short-term steroid immersion can result in masculinization of Nile tilapia as reported by Gale et al. (1995). A single immersion in MDHT at 364 CTU (13 dpf at 28°C) was as effective as two immersions at 280 and

364 CTU. Our experiments did not result in the level of masculinization (> 93%) that Gale et al. (1995) achieved; however, the Gale et al. (1995) study used two 3-hour immersions, whereas we used two 2-hour immersions in this study. The increased effectiveness of longer duration single exposures was further demonstrated in the experiment utilizing TBA. A two hour immersion in TBA did not cause significant masculinization in the female-biased brood, but a four hour immersion did result in more males than in the controls.

The ratios of males produced by MDHT immersion at the 67 and 100 fish l⁻¹ stocking densities were nearly significantly different to controls, which suggests that stocking density may affect masculinization. At a stocking density of 33 fish l⁻¹, nearly five times the stocking density reported by Torrans et al. (1988) in a study in which *O. aureus* were masculinized by immersion for five weeks in mibolerone, MDHT caused significant masculinization with either one or two immersions. The lack of significant masculinization of tilapia exposed to MDHT for two hours at 280 or 310 CTU suggests that the period of sensitivity to steroid-induced masculinization is several days after the

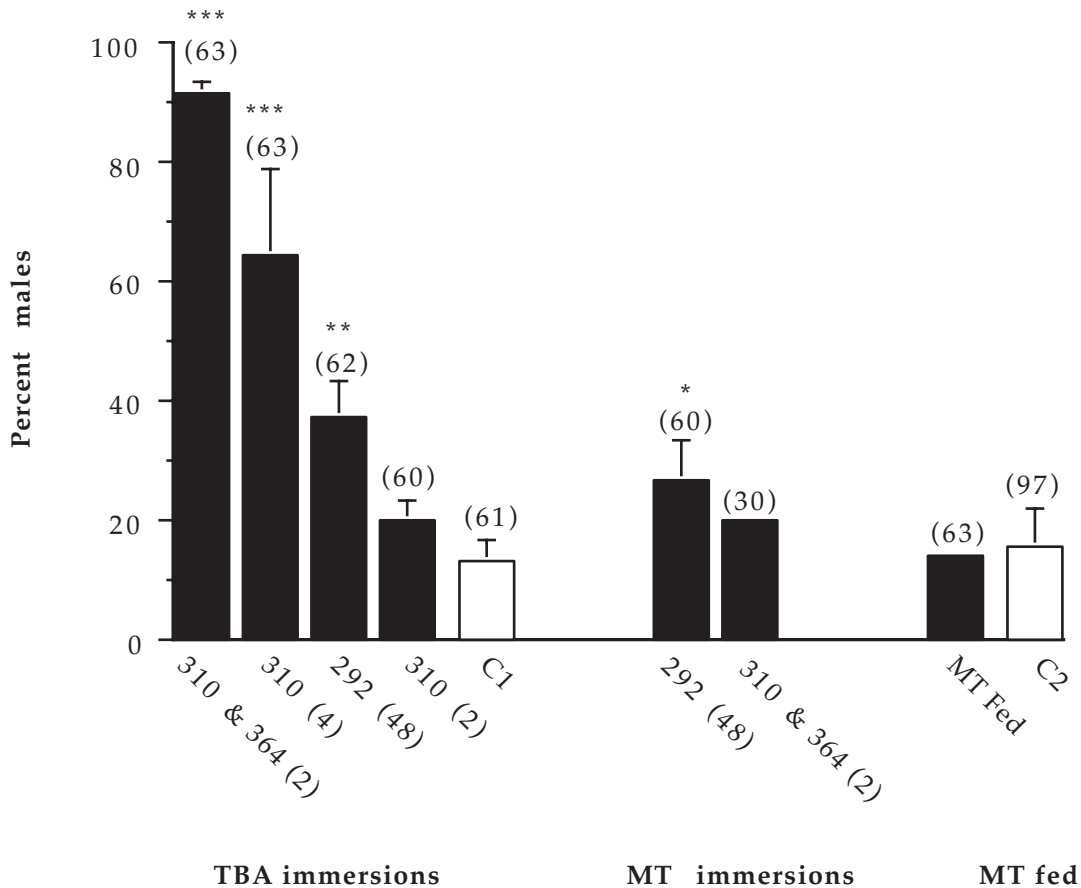


Figure 3. Masculinizing effects of TBA, by immersion, and of MT, by immersions and feeding, on *Oreochromis niloticus* fry. Treatment names are given as CTU at immersion with duration of immersion (h) in parentheses. Sample sizes are shown in parentheses at the top of each bar. Statistically significant differences are represented by asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Immersion treatments were compared with ETOH immersion control (C1) and the MT-fed treatment was compared with the ETOH-fed control (C2). Error bars = SE.

onset of feeding. However, immersion for 48-hours in TBA or MT, commencing at 292 CTU skewed the sex ratios toward males in the female-biased brood. In contrast with tilapia, salmonids must be immersed as yolk-sac fry (Piferrer and Donaldson 1989; Feist et al. 1995).

Two 2-hour immersions in TBA produced over 90% males in the female-biased brood in comparison with 20% males produced in two 2-hour immersions in MT. Interestingly, this brood of fish did not demonstrate any masculinization despite four weeks of feeding with MT diet. This result is rarely reported in the literature, but based on anecdotal information. This may be a common phenomenon, thereby pointing

to the need for further research of immersion treatment as an alternative to dietary treatment for masculinization.

ANTICIPATED BENEFITS

The use of all-male populations of tilapia for culture offers several important advantages, including enhanced growth (males grow faster and larger) and prevention of unwanted reproduction (which diverts energy away from somatic growth). Treatment with methyltestosterone-impregnated food has been shown to be an effective means of producing all-male tilapia populations. However significant

“leakage” of steroids such as MT into the pond environment may occur from uneaten or unmetabolized food and thus pose a risk of unintended exposure of hatchery workers or non-target organisms. Development of immersion in steroid as an alternative treatment for masculinizing tilapia will minimize treatment time and potentially increase the efficiency of exposure and safety in handling masculinizing steroids.

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