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Effect of Treatment Timing and Dose on Masculinization with Trenbolone Acetate

Ninth Work Plan, Reproduction Control Research 5A (9RCR5A)
Final Report

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Abstract

Preliminary studies in our laboratory showed that the synthetic androgen trenbolone acetate (TA) is a good candidate for masculinizing Nile tilapia fry using short immersions. In this study, we investigated the effects of treatment timing and treatment dose on the masculinizing potential of TA. Our results suggest that maximum masculinization can be achieved by short-term immersion on 13 and 14 days post-fertilization. Immersion prior to and after these days resulted in less or no masculinization. We tested the effects of dosage by using the traditional single factor experiment as well as a novel approach: the fractional factorial experiment. In one experiment, immersion in all doses (500, 750, and 1,000 μg l^{-1}) of TA resulted in significant masculinization with no differences observed between doses. In a subsequent experiment with fry from a different brood, none of the doses resulted in significant masculinization. The fractional factorial experiment was designed to simultaneously examine the effects of treatment dose, treatment duration, and density of fish. Significant masculinization occurred in some treatments; however, no clear pattern of interaction emerged among these factors. Nevertheless, this experimental approach holds great promise for gaining rapid screening results which will be useful in designing follow-up experiments.

Introduction

Masculinization of tilapia for the production of male-biased populations continues to be an important tool for aquaculturists to prevent unwanted reproduction (which shunts energy away from growth towards gamete production) and to produce the sex with the larger growth potential (Green et al., 1997). Previous work in our laboratory has shown that short-term immersion in androgenic steroids can result in masculinization of Nile tilapia (Gale et al., 1995, 1999; Contreras-Sánchez et al., 1997). These studies showed that immersion in androgen has the potential to be an alternative to dietary treatment with steroids for the masculinization of tilapia. A variety of androgens—especially synthetic androgens—are effective masculinizing agents (Hunter and Donaldson, 1983); however, there may be differences in their potency. We have shown that a single immersion in the non-aromatizable synthetic androgen 17α-methyldihydrotestosterone (MDHT) on 13 days post-fertilization (dpf) at 28°C, which corresponds to 364 Celsius Temperature Units (CTU), is as effective as two immersions in MDHT at 280 and 364 CTU (Fitzpatrick et al., 1999a). Both treatments resulted in significantly more males being produced than in the controls.

Trenbolone acetate (TA) has been reported to be an effective masculinizing agent when fed to tilapia (Galvez et al., 1996). Because we have had variable success masculinizing tilapia by immersion in 17α-methyltestosterone (MT) or MDHT, we decided to examine the efficacy of immersion in TA. Trenbolone acetate has been widely used in the cattle industry for growth enhancement and is considered a potent androgenic and masculinizing agent (Galvez et al., 1996). To determine the best treatment conditions for masculinization by immersion, studies must be conducted on the factors that are believed to play a critical role in determining efficacy. These major factors are: type of hormone, timing of treatment (relative to fish development), hormone dosage, duration of exposure, and density of fish during immersion. We have found that the best density for achieving significant masculinization by single immersion is 33 fish l^{-1} (Contreras-Sánchez et al., 1997; Fitzpatrick et al., 1999b). Previous studies in our laboratory showed that significant masculinization can be achieved by single immersion in MDHT if the fry are exposed to the hormone at 364 CTU (13 dpf). In the present study we determined if the results obtained with MDHT could be improved by using the synthetic steroid TA.

Fish density, hormone dosage, and length of exposure are factors that may interact to influence the degree of masculinization; therefore, a factorial design is needed to establish the minimum dosage, maximum density, and the shortest exposure needed for successful treatment. One approach is to conduct a single experiment in which all factors are examined simultaneously at different levels; however, this would require large numbers of tanks and more tilapia fry than can be produced from a single spawning. Therefore, our approach up

to this time has been to examine one factor at a time while holding all others constant. We will describe our studies on the effects of day of exposure and hormone dose, which were based on the best treatment conditions obtained thus far. However, this approach limits the amount of information that can be gained on the interactions among the various factors. To investigate these interactions, we carried out a fractional factorial design (Kuehl, 1994) to examine the influence of multiple factors (hormone dosage, exposure duration, and fish density) on the efficacy of TA. This design allows information to be obtained on factors of interest in the early stages of experimentation when the number of treatments exceeds the resources (Kuehl, 1994). Results of fractional factorial experiments can then be utilized in designing follow-up studies.

METHODS AND MATERIALS

Breeding families of Nile tilapia, *Oreochromis niloticus*, were placed in 200-l aquaria (one male to three females). The temperature was maintained at 28 ± 1°C. Time of spawning was monitored every two hours. All spawning occurred between 1600 and 1900. Once breeding occurred, the other fish were removed and the brooding female was left to incubate the progeny. At 10 dpf (280 CTU), fry were removed from the tank and randomly assigned to experimental groups. Development of the fry was expressed in CTU (mean water temperature in °C x the number of days since fertilization). The fry used in each experiment came from an individual female. Each replicate was housed in a 3.8-l glass jar with dechlorinated tap water. The water in all treatments was maintained at 28 ± 1°C under constant aeration. Treatments consisted of immersions in either steroid or ethanol, which were mixed 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}) at 4 ± 1°C.

The number of treatments and replicates included in each experiment was based on the number of fry obtained from a single spawning. Fry were collected after each immersion, jars were thoroughly cleaned, and then fish were reallocated in fresh dechlorinated tap water. Seven days after the final immersion, fry were transferred to Oregon State University's Warm Water Research Laboratory, Corvallis, Oregon, and reared in 75-l fiberglass tanks in a recirculating system. For the fractional factorial experiment, fish were reared in 25-l plexiglas chambers in a recirculating system. In all systems temperature and pH were monitored daily; ammonia, nitrites, and dissolved oxygen were checked weekly (Table 1). Water temperature in the grow-out system was maintained at 28 ± 1°C. At 70 to 80 dpf, sex ratios were determined by microscopic examination (10 and 40X) of gonads using squash preparations in Wright's stain (Humason, 1972). The weights of sampled fish were recorded at this time.

Effect of Treatment Timing on Masculinization with TA

Experiment 1
Fry were immersed for three hours at 336, 364, or 392 CTU (12, 13, or 14 dpf) in 500 μg l^{-1} of TA at densities of 33 fish l^{-1} in each replicate. Fish in the EtOH control group were immersed at 364 CTU at a density of 33 fish l^{-1}. Each experimental group was triplicated.

Experiment 2
Fry were immersed for three hours at 308, 336, 364, 392, 420, or 448 CTU (11, 12, 13, 14, 15, or 16 dpf) in 500 μg l^{-1} of TA at densities of 33 fish l^{-1} in each replicate. Fish in the control groups were immersed in EtOH at either 364 or 420 CTU at a density of 33 fish l^{-1}. Each experimental group was triplicated.

Experiment 3
Fry were immersed for three hours at 336, 364, 392, or 420 CTU (12, 13, 14, or 15 dpf) in 500 μg l^{-1} of TA at densities of 33 fish l^{-1} in each replicate. Fish in the control group were immersed in EtOH at 364 CTU at a density of 33 fish l^{-1}. Each experimental group was triplicated.

Effect of Dose on Masculinization with TA

Experiment 4
Based on results obtained from the previous experiments, fry were immersed for three hours at 364 and 392 CTU (13 and 14 dpf) in 500, 750, or 1,000 μg l^{-1} of TA; 500, or 1,000 μg l^{-1} of MDHT; or EtOH vehicle (control group) at densities of 33 fish l^{-1}. Because of brood size, treatments were not replicated.

Experiment 5
Fry were immersed for three hours at 364 and 392 CTU (13 and 14 dpf) in 500, 750, or 1,000 μg l^{-1} of TA or in EtOH vehicle (control group) at densities of 33 fish l^{-1}. Because of brood size, treatments were not replicated.

Effect of Dose, Exposure Time, and Fish Density on Masculinization with TA

Experiment 6: Fractional Factorial
Fry were immersed at 308, 336, 364, 392, and 420 CTU (11, 12, 13, 14, and 15 dpf) in TA or EtOH. Fry densities were 10, 19, 38, 75, or 150 fish l^{-1}; hormone dosages were 62.5, 125, 250, 500, or 1,000 μg l^{-1}; exposure duration was 0.75, 1.5, 3, 6, or 12 hours. Because we decided to use a fractional factorial design, only certain combinations of treatment conditions were used (Table 2a). To choose the combination of treatment factors to be used, a model was generated using Statistical Analysis Systems for Windows, release 6.10 (SAS Institute Inc., Cary, NC). Under this model, only replication around the middle treatment level for each factor is recommended (38 fish l^{-1}; 250 μg l^{-1}; 3 h). The

Table 1. Mean ± SE values for temperature, pH, dissolved oxygen (DO), nitrites, hardness, alkalinity, and ammonia for all immersion experiments.

Experiment	Temperature	pH	DO	Nitrites	Hardness	Alkalinity	Ammonia
1 (Time)	28.85 ± 0.14	7.79 ± 0.06	6.28 ± 0.08	0.01 ± 0.08	159.40 ± 15.04	89.00 ± 5.66	0.17 ± 0.05
3 (Time)	26.80 ± 0.20	6.56 ± 0.36	6.24 ± 0.06	0.07 ± 0.03	160.40 ± 12.26	55.20 ± 4.80	0.70 ± 0.46
4 & 5 (Dose)	28.72 ± 0.14	7.02 ± 0.98	6.58 ± 0.07	0.02 ± 0.004	n.a.	n.a.	0.23 ± 0.05
6 (Frac. Fact.)	28.25 ± 0.16	7.83 ± 0.84	6.30 ± 0.23	0.04 ± 0.09	n.a.	n.a.	0.13 ± 0.35

Table 2a. The combinations of factors tested for effects on masculinization of Nile tilapia are shown for Experiment 6: Fractional Factorial.

Experimental Design	Density (fish l^{-1})	Dosage ($\mu g\ l^{-1}$)					
		0	*62.5*	*125*	*250*	*500*	*1,000*
0.375 H							
	10						
	19						
	38				1		
	75						
	150						
0.75 H							
	10						
	19			1		1	
	38						
	75			1		1	
	150						
1.5 H							
	10				1		
	19						
	38	1	1		6		1
	75						
	150				1		
3 H							
	10						
	19			1		1	
	38						
	75			1		1	
	150						
6 H							
	10						
	19						
	38	1			1		
	75						
	150						

fractional factorial design is effective in screening studies to check on many factors, under the assumption that only a few effects are important. However, the fractional factorial design carries the caveat that follow-up experiments must be conducted using suitable replication once the levels for the various factors are chosen.

Statistical Analysis

For experiments 1 and 3, data were pooled from replicate tanks, because there was no evidence of tank effects within treatments (Chi-square test). Pairwise comparisons for 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 were analyzed for differences between groups using one-way ANOVA including mortality as a possible confounding variable. For all analyses, differences were considered statistically significant when the p-value (P) was less than 0.05. For experiment 2, the data were not analyzed statistically because of the sex ratio bias (see Results). Experiments 4 and 5 had only one experimental unit (no replication); therefore, pairwise comparisons were performed for sex ratio and mortality data using Fisher's exact test. We performed a response surface analysis for the data from the fractional factorial experiment. In this analysis, linear and quadratic equations primarily form contours that may show how the response increases or decreases based on the interactions of the factors tested, as well as the trends along levels of the factors.

RESULTS

Effects of Treatment Timing

Experiment 1

Significant masculinization of tilapia fry was obtained with single immersions in TA (Figure 1). The percentage of males in the groups immersed for 3 h on 12, 13, and 14 dpf was significantly higher than that in the control group (46.5, 72.3, and 69.3% versus 18.1%, respectively; $P \leq 0.0001$). The percentage of males in the 13 and 14 dpf treatments were not significantly different from each other ($P = 0.26$). The proportion of males in the 12 dpf immersion treatment was significantly higher than the control, but significantly lower than the 13 and 14 dpf treatments ($P < 0.001$ in all cases).

Experiment 2

All control groups (immersed in EtOH) and treatment groups contained 100% males. Therefore, the data were not analyzed further.

Experiment 3

Significant masculinization of tilapia fry was obtained with single immersions in TA (Figure 2; $P = 0.008$). No significant differences were found between the two control treatments (EtOH immersions on 12 or 15 dpf; $P = 0.76$). The percentages of males in the groups immersed for 3 h on 13 or 14 dpf were significantly higher than that in the control group (41.5 and 41.9 versus 22.6%, respectively; $P \leq 0.01$). The percentages of

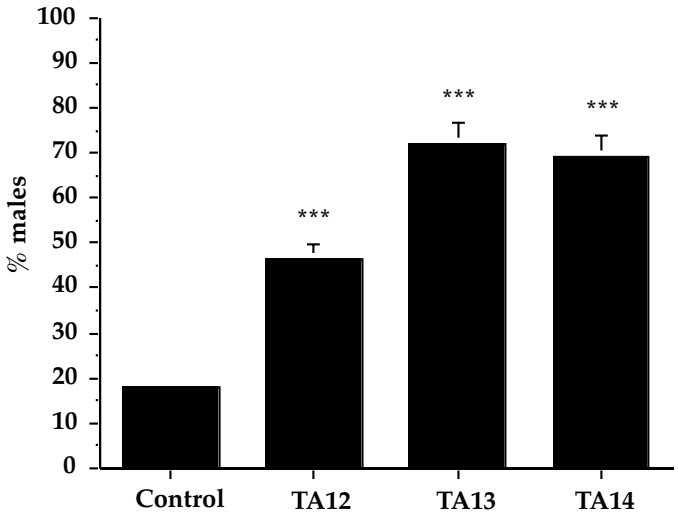

Figure 1. Effect on masculinization of a single immersion of *Oreochromis niloticus* fry in trenbolone acetate (TA) on 12, 13, or 14 days post-fertilization. Figure depicts mean + SE percentage of males in each treatment with n = 99–101 per treatment (pooled triplicates). Fish were immersed for 3 h at a density of 33 fish l^{-1}. Statistically significant differences from controls are represented by asterisks (*** $P < 0.001$).

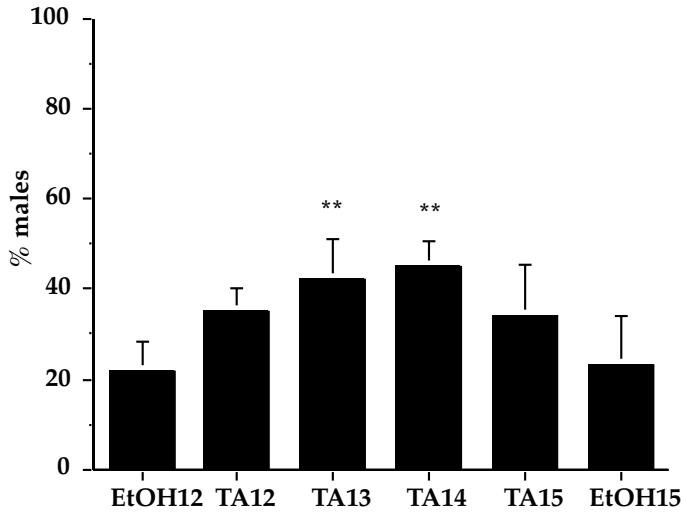

Figure 2. Effect of single immersion in trenbolone acetate (TA) on 12, 13, 14, or 15 days post-fertilization on masculinization of *Oreochromis niloticus* fry. Figure depicts mean + SE percentage of males in each treatment with n = 18–98 per treatment (pooled triplicates). Fish were immersed for 3 h at a density of 33 fish l^{-1}. Statistically significant differences from controls are represented by asterisks (** $P < 0.01$).

males in the 12 and 15 dpf treatments were not significantly different from the control groups nor between each other ($P = 0.08$, 0.09, and 0.89, respectively).

For all single immersion experiments, mortality and final weights were not significantly different among treatment groups. Water quality in rearing tanks was maintained close to the optimal values for tilapia culture (Table 1).

Effects of Dose

Experiment 4

Immersion of tilapia fry in 500, 750, and 1,000 µg l^{-1} of TA and 1,000 µg l^{-1} of MDHT resulted in significant masculinization of tilapia fry (Figure 3a; $P \leq 0.0001$). Control fish were 45% males. Fry immersed in 1,000 µg l^{-1} of TA showed the highest percentage of males (94.0%), followed by those in 500 µg l^{-1} of TA (80%), 1,000 µg l^{-1} of MDHT (76%), and 750 µg l^{-1} of TA (70%); however, the group treated with 500 µg l^{-1} of MDHT (47%) did not show significant masculinization. All significant differences had $P < 0.05$. High mortality was observed in treatments groups immersed in 1,000 µg l^{-1} of MDHT and in 500 and 1,000 µg l^{-1} of TA.

Experiment 5

All treatments in this experiment had similar percentages of males (46 to 54%; Figure 3b) which did not differ from the control group (49%).

Some fish in experiments 4 and 5 showed malformed heads and mouths, incomplete development of the operculum, and segmented dorsal fins.

Effect of Dose, Exposure Time, and Fish Density on Masculinization with TA

Experiment 6: Fractional Factorial

Significant masculinization was achieved by immersion of tilapia fry in TA. The best results obtained ranged between 72.1

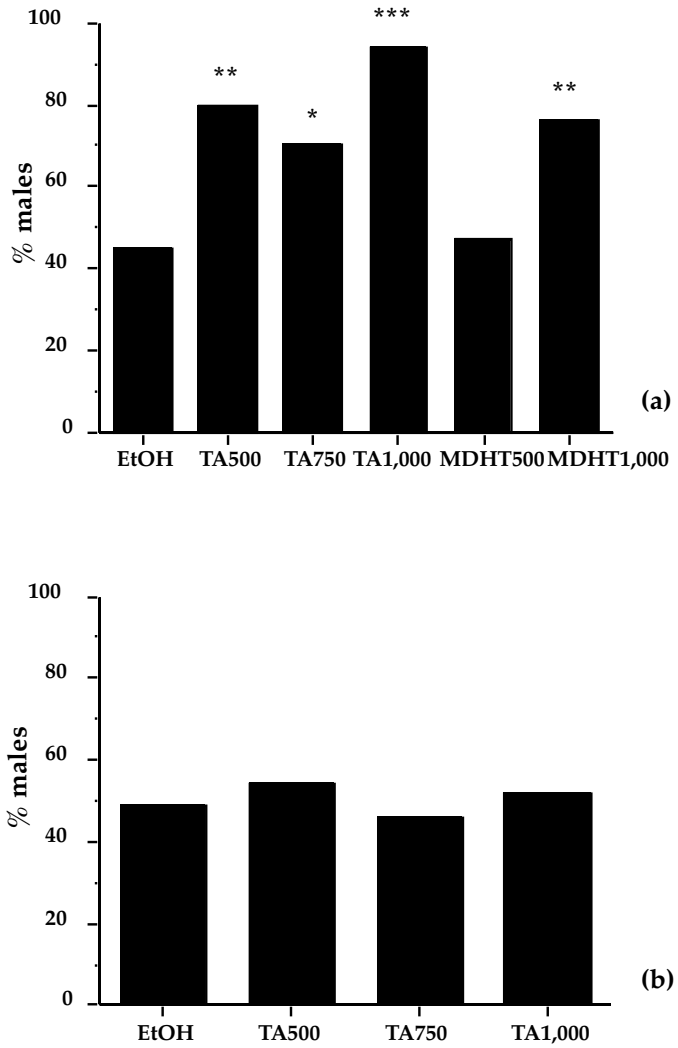

Figure 3. Effect of dose of trenbolone acetate (TA) and 17α-methyldihydrotestosterone (MDHT) on masculinization of *Oreochromis niloticus* fry. Fish were immersed for 3 h at a density of 33 fish l^{-1} on 13 and 14 days post-fertilization. Graph depicts the percentage of males in each treatment with samples sizes of 15 to 49 for (a) and 45 and 48 for (b). Statistically significant differences from controls are represented by asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

and 83.3% males in comparison to 51.1% in controls. These results included a wide variety of values for dosage, density, and duration of exposure. No clear pattern of interaction between the three factors tested was apparent (Table 2b).

Some fish in this experiment showed white spots in their eyes as well as malformed heads and mouths.

DISCUSSION

Masculinization of tilapia fry can be achieved through short-term immersion in TA. Our results indicate that significant masculinization occurs when fish are immersed in 500 µg l^{-1} of TA for three hours at 420 or 448 CTU (13 or 14 dpf) at a density of 33 fish l^{-1}. In an earlier study, we obtained 92% males in fry immersed in 500 µg l^{-1} of TA for two hours on 11 and 13 dpf, while the controls had only 14% males (Contreras-Sánchez et al., 1997). This suggests that the window of sensitivity for masculinizing tilapia is short and may vary between broods, which in turn supports the idea that there are other major

factors that influence susceptibility to androgen-induced masculinization. These differences among broods may be due to differences in embryo developmental stages, sensitivity to the steroid used, and/or inbreeding.

The single factor dose experiments suggest that increases in the percentage of males produced cannot be achieved through minor increases in concentration of hormone (e.g., 500 μg l^{-1} versus 1,000 μg l^{-1}). However, due to small brood sizes and high mortality rates, our results are still inconclusive. Since water quality in our experiments has been carefully monitored and shown to be excellent (Table 1), we speculate that the mortality and malformations observed may indicate inbreeding problems. These trials will need to be repeated with outbred populations in order to draw more substantive conclusions on dose effects.

The fractional factorial design presented an opportunity to explore the influence of several major factors and their interactions. However, despite significant masculinization results, this technique needs refinement and perhaps larger experimental units. We feel confident that a larger sample size will provide better results for this design. Once again, the use of new broodstock will help to clarify this.

Table 2b. The percentage of males in the treatment groups is for Experiment 6: Fractional Factorial. Statistically significant differences from controls are depicted with * ($P < 0.05$).

Results	Density (fish l^{-1})	Dosage (μg l^{-1})					
		0	*62.5*	*125*	*250*	*500*	*1,000*
0.375 H							
	10						
	19						
	38				52.4		
	75						
	150						
0.75 H							
	10						
	19			73.7		53.3	
	38						
	75			72.1*		69.6	
	150						
1.5 H							
	10				81.8		
	19						
	38	52.2	58.6		58.7 ± 4.1		55.2
	75						
	150				66.7		
3 H							
	10						
	19			83.3*		60.0	
	38						
	75			62.2		77.8*	
	150						
6 H							
	10						
	19						
	38	50.0			73.7		
	75						
	150						

Sex ratios deviating substantially from the theoretical 1:1 were obtained in the control groups of some of the experiments. Progeny sex ratios have ranged from 15 to 100% males and seem to be linked to specific adult crosses. We have documented this result previously (Fitzpatrick et al., 1999c) and it has also been reported in Shelton et al. (1983).

ANTICIPATED BENEFITS

We have successfully refined the technique for masculinizing Nile tilapia by immersion in masculinizing steroid. This latest development defines the times at which the highest masculinization can be achieved. Trenbolone acetate is a good candidate for successful masculinization of tilapia. The use of fractional factorial design and larger sample sizes will allow us to refine this technique to increase effectiveness and consistency. The optimization of this technique may enable farmers to masculinize tilapia with androgens while minimizing the risk of pond contamination with MT.

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