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Masculinization of Tilapia by Immersion in Trenbolone Acetate: Detection of Trenbolone Acetate after Treatment

Ninth Work Plan, Reproduction Control Research 5C (9RCR5C)
Progress Report

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Abstract

In previous experiments we have found that two 3-hour immersions in trenbolone acetate (TA) can successfully masculinize Nile tilapia fry. In this study we are investigating how the concentration of TA in the immersion water changes before and after treatment to determine the amount of hormone uptake and estimate the potential for reuse of the treatment water. Nile tilapia fry were subjected to two 3-hour immersions at 11 and 13 days post-fertilization (dpf) in water containing 500 µl of TA. Surprisingly, we have found that the concentration of TA before and after treatment is highly variable and below the expected levels. We are currently assessing whether TA comes out of solution and forms precipitates or binds to the jar glass.

Introduction

One of the major criticisms to masculinization by immersion in TA hormone solutions is that the concentration of hormone used is higher than the amount of 17α-methyltestosterone (MT) used in feeding trials. Although the TA immersion protocol presents advantages over MT feeding treatment for hormone control and disposal, as well as fewer risks of environmental contamination, it might not be welcomed by producers because of the potential increased costs in comparison to feeding treatment. In previous studies, we have shown that single and double immersions in the non-aromatizable synthetic androgens 17α-methyldihydrotestosterone (MDHT) and trenbolone acetate (TA) during early development are effective masculinizing treatments (Contreras-Sánchez et al., 1997, 1999, 2000; Gale, 1999). However, little is known about hormone uptake by the fry and the potential for reuse of treatment water in subsequent masculinization treatments.

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). These factors make TA a very good candidate for fish masculinization and facilitate its acceptance among farmers and administrators involved in the regulatory process. In order to determine if the water used for the immersions contains TA in concentrations sufficient for reuse, we examined the fate of TA after three-hour immersions of Nile tilapia fry by analyzing water samples before and after immersions.

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. Spawning occurred between 1600 and 1900 h. Once breeding occurred, the other fish were removed and the brooding female was left to incubate the progeny. At 10 days post-fertilization (dpf; 280 CTU), fry were removed from the tank and randomly assigned to experimental groups. Development of the fry was expressed in CTUs (mean water temperature in °C × the number of days since fertilization). The fry used in the 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.

Experimental Design

Nile tilapia fry were immersed twice for three hours in either steroid (TA) or ethanol vehicle (EtOH), which were mixed before the addition of fry; one immersion took place at 11 dpf (308 CTU) and the other at 13 dpf (354 CTU). An additional treatment consisted of adding hormone solution to the water, but no fish were placed in it. Fry were collected after each immersion, jars were thoroughly cleaned, and then fish were reallocated in fresh dechlorinated tap water. 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.

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. In all systems temperature and pH were monitored daily; ammonia, nitrites, and dissolved oxygen were checked weekly. 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.

TA Detection

Water samples were collected two times on day 0 (before and after treatment) and once on days 1, 2, 4, 7, and 14. From each sample 2.0 ml were extracted in 8 ml of diethyl ether. The organic phase of each sample was collected in a new tube after the aqueous phase was snap-frozen in liquid nitrogen. The extraction procedure was repeated and the ether extracts were pooled for each sample and dried down in a SpeedVac. Each dried extract was reconstituted in 1 ml of methanol. Aliquots of the reconstituted extracts were removed to 150 µl glass inserts for determination of TA concentration by High Performance Liquid Chromatography (HPLC). The HPLC methods followed the procedure outlined in Huang et al. (1983) and modified by Feist et al. (1990). The HPLC analysis was performed using a Waters System consisting of a 600 controller, 717 autosampler, 996 photodiode array detector, a Dell Dimension V400c computer, Millenium PDA software, and a reverse phase C18 column (flow rate 0.4 ml min^{-1}). We used an isocratic mobile phase of water: methanol:acetonitrile:isopropanol (62:28:5:5) followed by a linear gradient (3.3% min^{-1}) of water:methanol:butanol (35:45:20) for 30 minutes monitored at a wide variety of wavelengths but specifically analyzed at 254, 280, and 340 nm. This system allows for the separation of 19 steroid standards with detection limits of 3 ng for each steroid. Each sample was analyzed once.

Statistical Analysis

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™. Intersex fish were counted as females for the purposes of analysis in order to be conservative. Concentrations of TA in water at the various sample times were not compared statistically because of the limited sample size (n = 3 per date) and because the goal of the study was descriptive (i.e., to detect presence/absence of steroid).

RESULTS

TA concentrations were highly variable at all times. At time 0, immediately after mixing the steroid stock solution, the expected concentration of hormone was 500 µg l^{-1}. Surprisingly, at this time (before addition of fry) levels of TA in the water were lower than the expected value (mean = 178.4 µg l^{-1}; SD = 134.1). Initial values of hormone concentration range from 27.2 to 386.7 µg l^{-1} (coefficient of variation (CV) = 75.2). Concentration values of TA after the fish were removed from the jars also varied, showing no consistent pattern (mean = 162.4 µg l^{-1}; SD = 134.3; CV = 82.7). In some cases TA values were higher after removing fish than before their introduction into the jars (Figures 1a and 1b). In jars containing TA but no fish, two of the three replicates had similar levels of TA through the first 24 hours; however, one replicate had very low values throughout most of the experiment, showing an increase in hormone concentration towards the last two sampling times (Figure 1c). Most of the jars with or without fish showed a depletion of TA after seven days, reaching non-detectable levels at day 14.

No significant masculinization was observed by TA immersions on 11 and 13 dpf (54% males compared with 49% males in the EtOH-immersed controls). We also observed substantial mortality in both TA and EtOH treatments (38 and 63%, respectively).

DISCUSSION

Our findings indicate that the target dose for TA immersion is rarely achieved, perhaps because TA precipitates out of

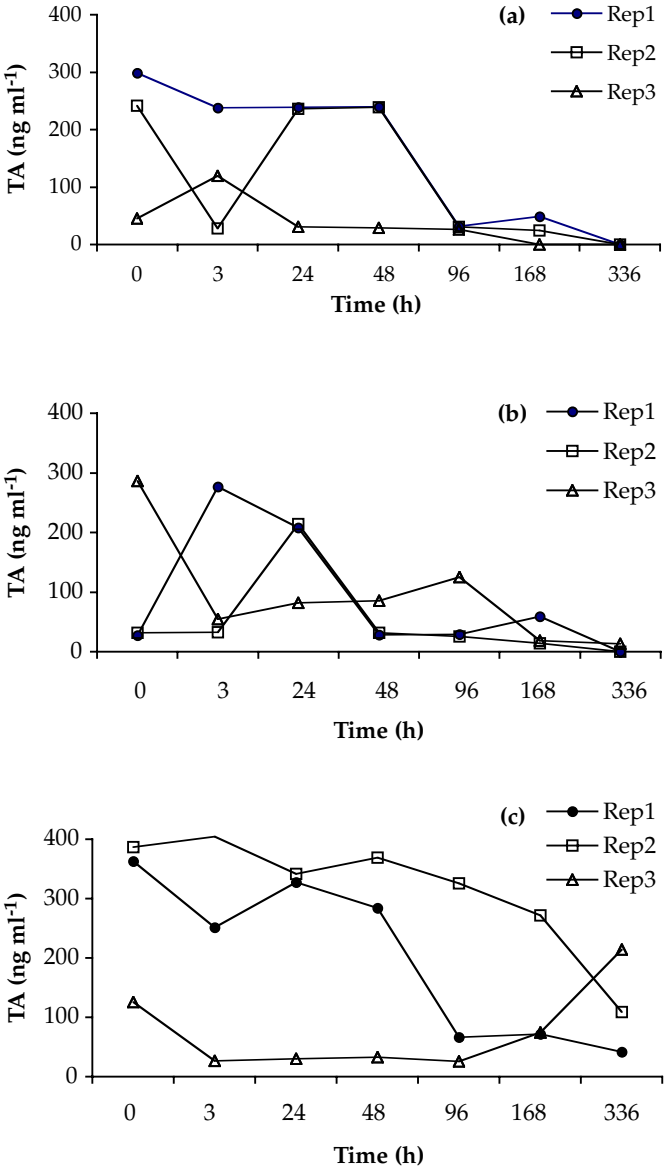

Figure 1. Trenbolone acetate (TA) levels in water through time in experimental jars containing either 33 fish l^{-1} that were immersed in TA for the first time on day 13 post-fertilization (a), or 33 fish l^{-1} immersed for the second time on day 13 post-fertilization (b), or jars containing hormone but no fish (c). Values in control jars with EtOH-vehicle were non-detectable.

solution after mixing with water. The surprisingly low levels of TA before the immersion of tilapia fry has forced us to validate these results by conducting further experiments. We have repeated the experiment using 500 µl l^{-1} of TA delivered using either ethanol or dimethyl sulphoxide (DMSO) as vehicle. Samples were obtained at 0 h (before fry immersion) and at 3 h (after fry immersion). We are currently processing these samples. In another experiment, we are comparing levels of TA and testosterone (T) as a way to determine if TA, which has lower solubility in water than T, precipitates out of solution at a higher rate than T.

The concentrations of TA found in the treatment water before the fish immersion may explain the lack of masculinization for this particular experiment. We suspect that a combination of factors influenced not only survival but treatment efficacy as well. These factors may include recent high levels of copper in our dechlorinated water, as well as the solution used to dechlorinate and eliminate copper (NovAqua®). We are presently analyzing these potential confounding factors by using well water in our treatments.

ANTICIPATED BENEFITS

We have found that masculinizing Nile tilapia fry by immersion can be a good alternative to feeding the fry with hormone-impregnated food, posing fewer risks to hatchery workers and the environment. However, this technique requires refinement and more consistent results. The reusage of treatment water for consecutive sex inversion can be a significant improvement to this technique.

LITERATURE CITED

Contreras-Sánchez W.M., M.S. Fitzpatrick, R.H. Milston, and C.B. Schreck, 1997. Masculinization of Nile tilapia (*Oreochromis niloticus*) by single immersion in 17α-methyltestosterone and trenbolone acetate. In: K. Fitzsimmons (Editor), Proceedings of the Fourth International Symposium on Tilapia Aquaculture. Orlando, Florida, pp. 783–790.

Contreras-Sánchez, W.M., M.S. Fitzpatrick, R.H. Milston, and C.B. Schreck, 1999. Masculinization of Nile tilapia (*Oreochromis niloticus*) by immersion in synthetic androgens: Timing and efficacy. In: B.W. Green, H.C. Clifford, M. McNamara, and G. Montaño-Moctezuma (Editors), Proceedings of the Fifth Central American Symposium on Aquaculture. San Pedro Sula, Honduras, pp. 246–248.

Contreras-Sánchez, W.M., M.S. Fitzpatrick, R.H. Milston, and C.B. Schreck, 2000. Masculinization of Nile tilapia with steroids: Alternative treatments and environmental effects. In: B. Norberg, O.S. Kjesbu, G.L. Taranger, E. Andersson, and S.O. Stefansson (Editors), Proceedings of the Sixth International Symposium on Reproductive Physiology of Fish. John Grieg, A.S., Bergen, Norway, pp. 250–252.

Feist, G., C.B. Schreck, M.S. Fitzpatrick, and J.M. Redding, 1990. Sex steroid profiles of coho salmon (*Oncorhynchus kisutch*) during early development and sexual differentiation. Gen. Comp. Endocrinol., 80:299–313.

Gale, W.L., M.S. Fitzpatrick, M. Lucero, W.M. Contreras-Sánchez, and C.B. Schreck, 1999. Production of all-male populations of Nile tilapia (*Oreochromis niloticus*) by immersion in androgens. Aquaculture, 178:349–357.

Galvez, J.I., J.R. Morrison, and R.P. Phelps, 1996. Efficacy of trenbolone acetate in sex inversion of the blue tilapia *Oreochromis aureus*. J. World Aquacult. Soc., 27:483–486.

Huang, F.L., F.C. Ke, J.J. Hwang, and T.B. Lo, 1983. High-pressure liquid chromatography separation of a mixture of corticosteroids, androgens, and progestins. Arch. Biochem. Biophys., 225:512–517.

Humason, G.L., 1972. Animal Tissue Techniques. W.H. Freeman and Company, San Francisco, 641 pp.

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