Detection of MT in Aquarium Water after Treatment with MT Food

Eighth Work Plan, Reproduction Control Research 3A (RCR3A)
Final Report

Martin S. Fitzpatrick, Wilfrido M. Contreras Sánchez, Ruth H. Milston, Rik Hornick, and Grant W. Feist
Department of Fisheries and Wildlife
Oregon State University
Corvallis, Oregon, USA

Carl B. Schreck
Oregon Cooperative Fishery Research Unit
Biological Resources Division—U.S. Geological Survey
Department of Fisheries and Wildlife
Oregon State University
Corvallis, Oregon, USA

Abstract

The following study tested the hypothesis that 17α-methyltestosterone (MT) persists in the environment after its use for masculinizing Nile tilapia (*Oreochromis niloticus*). Fry were treated with a masculinizing dose of MT (60 mg kg^{-1}) for four weeks beginning at the initiation of feeding in model ponds which consisted of 3.7-l jars that contained 3 cm of soil. Water and soil samples were taken before the onset of treatment and weekly beginning on the last day of treatment (water samples were also taken weekly during the four-week treatment period). Concentrations of MT were determined by radioimmunoassay, which revealed that the levels of MT in the water peaked between approximately 1 and 2 μg l^{-1} at 14 and 21 days after the onset of feeding. Concentration of MT in water decreased to background level by 35 days after the onset of feeding (one week after the end of treatment with MT-impregnated food). In contrast, the levels in the soil were 1.4 to 1.7 μg kg^{-1} at 28 days after the onset of feeding with MT-impregnated food and remained detectable in the soil at between 0.8 and 1.6 μg kg^{-1} through 49 days (three weeks after ending treatment with MT-impregnated food). These results suggest that MT persists in sediments for at least weeks after cessation of MT treatment, which raises the possibility that unintended exposure to MT may occur.

Introduction

Production of all-male populations of tilapia through treatment of fry with 17α-methyltestosterone (MT)-impregnated food has become common throughout the world. All-male populations often have greater growth potential because no energy is shunted toward reproduction and no competition with younger fish occurs (Green et al., 1997). Despite the success of this technique, significant "leakage" of MT into the pond environment may occur from uneaten or unmetabolized food. This leakage poses a risk of unintended exposure of hatchery workers as well as fish or other non-target aquatic organisms. Furthermore, in some countries, pond sediments are dredged and sometimes used to prepare soil for crop production, thereby spreading the risk of exposure to MT to terrestrial systems and to other aquatic systems. Therefore, determining the fate of MT in semi-closed systems such as ponds will yield important information on both safety and efficacy of MT use for masculinization. To determine if MT potentially remains within the pond environment, the following study was undertaken using model pond systems.

Methods and Materials

Methods for obtaining fry were the same as those described in technical reports RCR2A (pp. 17-18) and RCR2B (pp. 19-21) of this document. Breeding families of Nile tilapia, *Oreochromis niloticus*, were placed in 200-l aquaria (one male to three females) where water temperature was maintained at 28 ± 1°C. Once breeding occurred, the other fish were removed and the brooding female was left to incubate the progeny. Model ponds were set up two days before the expected time of fry release. Each model pond consisted of a 3.8-l jar containing 3 cm (approximately 500 g) of packed soil. In Experiments 1 and 2, soil was obtained from one of the dry Soap Creek (Oregon State University) ponds located north of Corvallis. In Experiment 3, soil was obtained from a meadowed hill near the Principal Investigator's house located north of Corvallis. Each model pond contained 3 l of dechlorinated tap water. Once the female released the fry from her mouth (usually at 280 Celsius Temperature Units (CTU) post-fertilization), fry were removed from the tank and randomly assigned to the model ponds at a stocking rate of 47 fry $container^{-1}$ (1 fry [3.3 $cm]^{-2}$, which corresponds to a recommended stocking rate for masculinization of 3,000 fry m^{-2} [Popma and Green, 1990]).

The following experimental groups were included for Experiments 1 and 2: MT-fed fish (at 60 mg kg^{-1} of food) and control fish (EtOH-treated food). An additional group (MT-fed fish with twice the amount of food as the normal MT group) was included in Experiment 3. MT food was made by spraying crushed flaked food with MT dissolved in EtOH; control food was made by spraying crushed flaked food with EtOH. In each experiment, additional jars were included that contained no fish but were subjected to the usual MT feeding regime or control diet. All treatments were triplicated. Fry were fed the treatment diets for four weeks (from 11 to 39 days post-fertilization; dpf) commencing the day after release from the female. Water temperature in the jars was maintained at 28 ± 1°C. Temperature and pH were monitored daily, while

ammonia, nitrites, and dissolved oxygen were checked weekly. Feeding rate was at 20% of calculated body weight for the first 23 days of treatment and then 10% of calculated body weight through day 28 of treatment (Popma and Green, 1990). Initially, evaporative water loss was made up twice weekly. However, degradation of water quality (high ammonia, high nitrites) required water exchange of half the water at 18, 22, and 24 days of dietary treatment in Experiment 1; in Experiments 2 and 3, half the water was exchanged routinely twice weekly throughout the period of dietary treatment.

After 28 days of dietary treatment (40 dpf), 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. Water temperature in the grow-out system was maintained at 28 ± 1°C and water quality parameters described above were monitored. At 60 to 70 dpf, sex ratios were determined by examination of gonads using squash (10 and 40X) preparations after aceto-iron hematoxylin staining (Wittman, 1962) . The weights of sampled fish were recorded at this time.

Water samples (25 to 50 ml) were collected with pipettes into 50-ml Falcon tubes and stored at -20°C until analysis for MT. Soil core samples were collected with 1.9-cm-diameter PVC pipes, placed in whirl pak bags, excess water poured off (Experiment 1) or collected into a separate 50-ml Falcon tube (Experiments 2 and 3), and the bags stored at -20°C until analysis. In Experiments 2 and 3, the excess water (called "interface" hereafter) samples were stored frozen at -20°C. For analysis of MT concentration, 1.0 ml of each water and interface sample and 0.2 g of each soil sample were extracted in 8 ml of diethyl ether, which was collected into new tubes after the aqueous phase was snap-frozen in liquid nitrogen. The extraction procedure was repeated and the ether extracts were pooled and dried down in a SpeedVac. Each dried extract was reconstituted in 1 ml of phosphate-buffered saline containing gelatin. Aliquots of the reconstituted extracts were removed and placed in 12x75-mm tubes for determination of MT concentration by radioimmunoassay (RIA). The RIA methods followed the procedure outlined in Fitzpatrick et al. (1986; 1987). Standards of a known concentration of MT were made in EtOH and used in each assay to generate a standard curve. The assay was validated by demonstration of parallelism between serial dilutions of several samples and the standard curve, and by demonstration of low cross-reactivity with testosterone and 11-ketotestosterone. Furthermore, water samples were subjected to analysis by HPLC after extraction (as above), filtering, and reconstitution in MeOH to search for possible metabolites of MT. Extraction efficiency of MT for the RIA was checked by adding a known amount of ^{3}H-MT to water, soil, and interface samples (n = 6 for each), and then extracting the samples as described above. Once each of these tubes was reconstituted in 1 ml of phosphate-buffered saline containing gelatin; 0.5 ml was removed from each and the amount of radioactivity counted by scintillation spectroscopy (extraction efficiencies were 78.4% for water, 78.2% for soil, and 66.9% for interface).

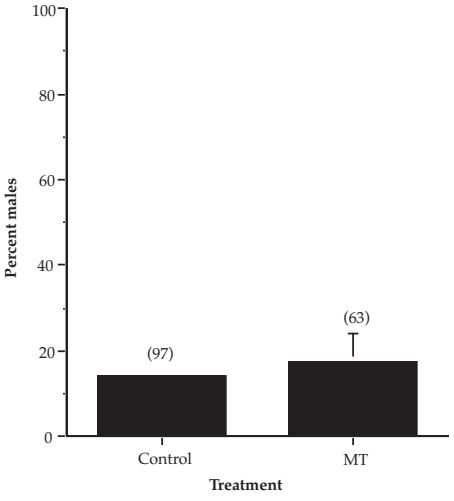

Figure 1. Effect of methyltestosterone (MT) on masculinization of *Oreochromis niloticus* fry by oral administration in Experiment 1. Fish were fed either a control diet or a diet containing 60 mg kg^{-1} of MT. Sample size for each treatment (pooled triplicates) is shown in parentheses.

For all experiments, sex ratio data were pooled from replicate tanks because there was no evidence of tank effects within treatments (Fisher's test or ANOVA). Intersex fish were counted as females for the purposes of analysis in order to be conservative. 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™. Concentrations of MT in water, soil, and interface 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 (presence/absence). 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.

RESULTS

For Experiments 1 and 2, no significant effect of MT-treatment on sex ratio was observed. In Experiment 1, the percentage of males in the MT-treated group was 15.9%, which was not significantly different from the control group (17.3%; Figure 1). In Experiment 2, the MT-treated group had 86.7% males compared with 84.3% males in the control group (Figure 2). In Experiment 3 (Figure 3), the fish treated with MT at the normal ration had significantly more males (63.0%) than the controls (51.4%; P = 0.0146) and the fish treated with MT at twice the normal ration (45.7%; P = 0.0038). For all the experiments, the level of mortality was randomly distributed among treatments, with significant variation between replicates (data not shown).

The levels of MT were determined only for samples taken in Experiments 1 and 2. For jars that contained fish, the levels of MT in the water increased in Experiment 1 to an average of 1.2 µg l^{-1} after one week of feeding MT-impregnated food (Figure 4a). Concentrations of MT in the water remained elevated throughout the feeding treatment and declined to pretreatment levels by one week after the cessation of dietary treatment. There was a higher background level of MT for the soil; nevertheless, MT in the soil increased to a mean 1.7 µg g^{-1} of soil at the conclusion of dietary treatment with MT (Figure 4b) and remained elevated through the end of the experiment (three weeks after cessation of dietary treatment). In Experiment 2, similar results were found. Levels of MT in the water increased to 0.8 µg l^{-1} after one week and decreased to background levels within a week of cessation of dietary

treatment (Figure 5a). Concentrations of MT in the soil increased to a mean of 1.4 µg g^{-1} of soil at the end of four weeks of dietary treatment with MT (Figure 5b) and remained well above background levels through the end of the experiment. The average interface level of MT was 0.2 µg ml^{-1} at the end of four weeks of dietary treatment and remained at this level through the end of the experiment. Variation in the levels of MT measured in the soil samples in both experiments was considerably higher than that in the water samples.

In jars that contained no fish but received the same amount of MT (data not shown), the average level of MT in the water was 2.3 µg l^{-1} at the end of week 1, 3.1 µg l^{-1} at the end of week 4 in Experiment 1, and at pretreatment levels by week 5. Mean level of MT in the soil for this experiment was 13.1 µg g^{-1} of soil at the end of week 4 and 8.9 µg g^{-1} of soil at the end of week 7. In Experiment 2, average MT concentration in the water was 2.7 µg l^{-1} at the end of week 1, 2.3 µg l^{-1} at the end of week 4, and at pretreatment level by the end of week 5. Mean soil MT was 5.1 µg g^{-1} of soil at the end of week 4 and 8.8 µg g^{-1} of soil at the end of week 7.

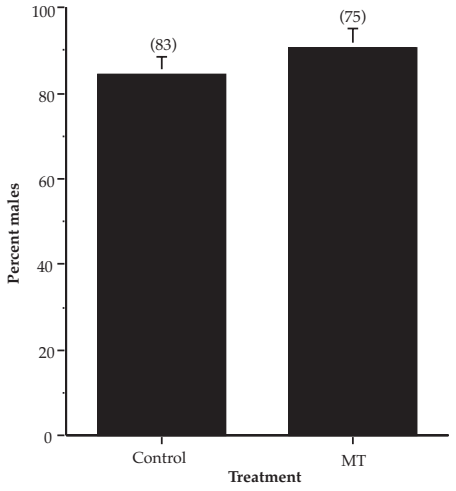

Figure 2. Effect of methyltestosterone (MT) on masculinization of *Oreochromis niloticus* fry by oral administration in Experiment 2. Fish were fed either a control diet or a diet containing 60 mg kg^{-1} of MT. Sample size for each treatment (pooled triplicates) is shown in parentheses.

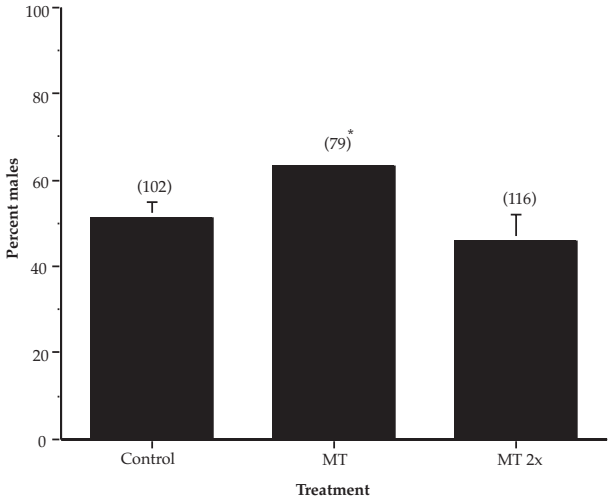

Figure 3. Effect of methyltestosterone (MT) on masculinization of *Oreochromis niloticus* fry by oral administration in Experiment 3. Fish were fed either a control diet, a diet containing 60 mg kg^{-1} of MT, or the MT diet at twice the ration (2X). Sample size for each treatment (pooled triplicates) is shown in parentheses. Statistically significant difference from control in the proportion of males is represented by an asterisk ($P < 0.05$).

DISCUSSION

These experiments demonstrate that considerable amounts of MT leak into the environment during dietary treatment. Although the levels of MT in the water peaked between approximately 1 and 2 µg l^{-1} at 14 and 21 days after the onset of feeding, the concentration decreased to background level by 35 days after the onset of feeding (one week after the end of treatment with MT-impregnated food). In contrast, the levels in the soil were 1.4 to 1.7 µg kg^{-1} at 28 days after the onset of feeding with MT-impregnated food and remained detectable in the soil at between 0.8 and 1.6 µg kg^{-1} through 49 days (three weeks after ending treatment with MT-impregnated food). Interface levels of MT were higher than background, but lower than peak levels found in the soil or water.

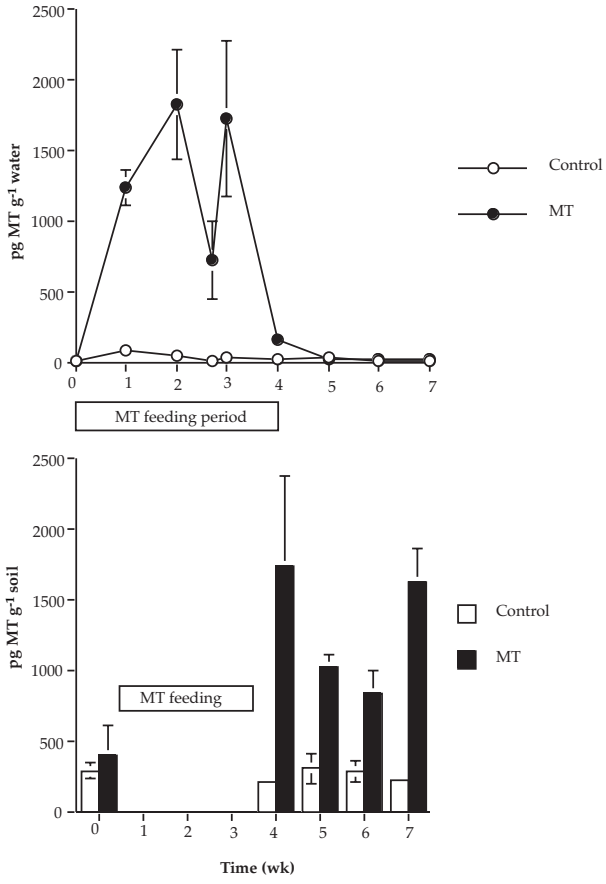

Figure 4. Levels of methyltestosterone (MT) in the environment during and after dietary treatment of *Oreochromis niloticus* fry in Experiment 1. Fish were fed either a control diet or a diet containing 60 mg kg^{-1} of MT. Means (± SE) of MT in water (A) and soil (B) are depicted (n = 3).

The total lack of masculinization resulting from dietary treatment with MT in Experiments 1 and 2 and limited masculinization in Experiment 3 are troubling but are not uncommon in production facilities around the world. The concentrations of MT in the diets were measured (data not shown) and established to be at the target dosage of 60 mg kg^{-1} of food. Therefore, the lack of masculinization was not due to improper diet preparation. Furthermore, treatment of jars without fish with the MT diet resulted in higher levels of MT in the water (during the treatment period) and soil (after the treatment period) than that found in jars containing fish, suggesting that the fish were exposed and were taking up MT. Individual broods of fish may be more or less susceptible to masculinization; however, in Experiment 1, fish from the same brood were successfully masculinized by immersion in trenbolone acetate (Contreras et al., 1997). Therefore, insensitivity to treatment does not explain the lack of masculinization. One possible explanation is that the deterioration of water quality within the jars may have limited the effectiveness of MT by stressing the fish; however, we have no other evidence to support this hypothesis at this time.

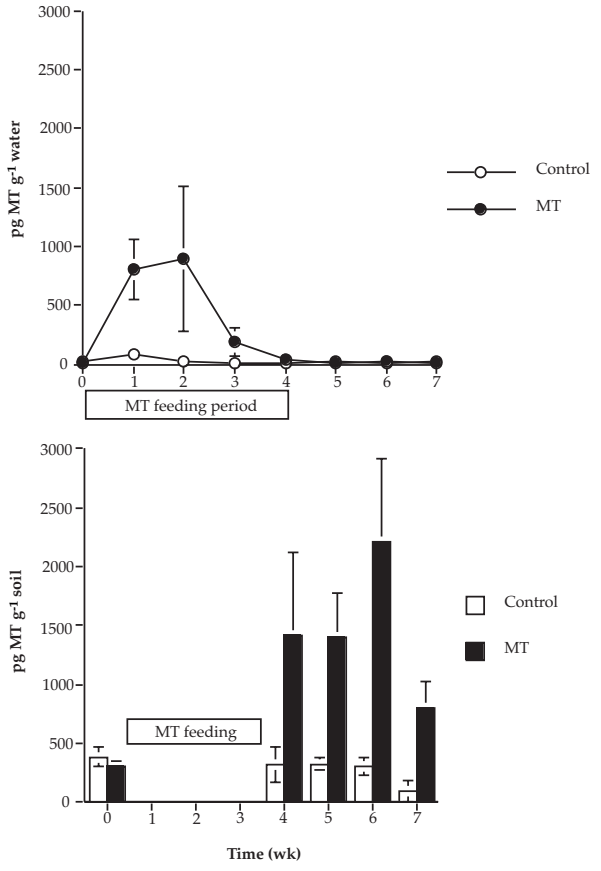

Figure 5. Levels of methyltestosterone (MT) in the environment during and after dietary treatment of *Oreochromis niloticus* fry in Experiment 2. Fish were fed either a control diet or a diet containing 60 mg kg^{-1} of MT. Means (± SE) of MT in water (A) and soil (B) are depicted (n = 3).

Nevertheless, we have established that in these model "ponds," treatment of tilapia with MT results in considerable leakage of the hormone into the environment. It rapidly disappears from the water, but remains in the soil for up to three weeks after the cessation of treatment. Thus, MT persists in the sediments for a considerable time after the end of treatment, posing a potential health risk to workers and an exposure risk to non-target fish and other organisms. Tilapia may disturb sediments when they build nests or search for food, leading to resuspension of MT from the soil into the water column. Thus, "rotating" the pond use from fry production to rearing or breeding will not reduce the risk of re-exposure.

ANTICIPATED BENEFITS

We have successfully demonstrated that the anabolic steroid 17α-methyltestosterone (MT) persists in the sediments of model ponds for at least several weeks after its use for masculinizing tilapia. This is the first time to our knowledge that residual MT has been shown to be stable in the pond environment. These findings raise concerns about the potential impact of such residual MT with regard to unintended exposure of pond workers as well as other fish and animals. These results suggest that should MT be used for masculinizing tilapia, extra care should be taken to protect workers and other biological components of the pond ecosystem from unintended exposure.

ACKNOWLEDGMENTS

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