# Fate of Methyltestosterone in the Pond Environment: Impact of MT-Contaminated Soil on Tilapia Sex Differentiation

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*Final Report*

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## Abstract

The following study examined the effect of environmentally persistent 17α-methyltestosterone (MT) on sex differentiation of Nile tilapia (*Oreochromis niloticus*). Three different broods of fry were treated one after the other 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 60-l tanks that contained 5 kg of soil. Four weeks after the last MT treatment, two different broods of tilapia fry were fed control feed while being maintained in the tanks that had contained the MT-treatment groups. Water and soil samples were taken before the onset of treatment and on the last day of treatment during each treatment cycle. Concentrations of MT were determined by radioimmunoassay, which showed that the levels of MT in the water were elevated between about 200 and 1,250 pg $ml^{-1}$ during the three cycles of MT and then returned to background levels during the remaining cycles of control diet feeding. Analysis of water samples taken shortly after adding the MT-impregnated food to the tanks revealed that MT leaks into the water within a minute of treatment. The levels of MT in the soil were elevated to about 2,000 pg $g^{-1}$ after one feeding cycle and remained elevated between 1,400 and 3,300 pg $g^{-1}$ through three months after the conclusion of the last MT feeding cycle, including the time during which the control-fed fry were raised in these tanks. The sex ratios of the groups fed control food while being maintained in the tanks that had contained the MT-treatment groups were not different from control fish; however, several individuals in the former groups had intersexual gonads, suggesting some impact on development.

## Introduction

Treatment of tilapia fry with methyltestosterone (MT)-impregnated food for producing all-male populations is a common practice throughout the world. All-male populations offer the production advantage of enhanced growth potential (Green et al., 1997). However, 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 used to "prepare soil" for crop production, thereby spreading the risk of MT exposure to terrestrial organisms.

Previously, we conducted studies on the fate of MT in small 3.7-l model ponds that indicated that MT leaked into the water during the feeding treatment period but decreased to background levels once the treatment ended. However, MT was found in the soil of these model ponds at the end of the treatment period and persisted in the soil for at least three weeks after treatment (through the end of the study). Thus, MT persists in the soil for a considerable time after treatment of tilapia fry, suggesting the possibility that if the sediments were subsequently disturbed by tilapia during nest building or searching for food, then MT might be resuspended into the water, which could cause further exposure of fry.

The following study was undertaken to determine if MT contamination of the soil could affect sex differentiation of subsequent broods of fry that were placed in the same aquaria.

## Methods and Materials

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. The female was forced to release the fry from her mouth at 280 Celsius Temperature Units (CTU) or ten days post-fertilization (dpf). Fry were removed from the tank and randomly assigned to model ponds (60-l aquaria) at a stocking rate of 175 fry $tank^{-1}$ (1 fry per 7.9 $cm^2$). This value corresponds to 1/3 of the recommended stocking rate (by area) for masculinization of 3,000 fry $m^{-2}$ (Popma and Green, 1990). However, the volume used in our model ponds was limited by the tank height, conferring a stocking rate by volume of 3.5 fish $l^{-1}$ (0.5 fish $l^{-1}$ more than the recommended stocking rate). Model ponds were set up two days before the expected time of fry release. Each aquarium contained 5,000 g (approximately 3 cm) of packed soil, which was obtained from a meadowed hill near the Principal Investigator's house located north of Corvallis, Oregon. Each model pond contained 50 l of dechlorinated tap water.

To determine the effect of MT contamination in sediments on sex differentiation of tilapia, three separate broods of fry were treated with MT-impregnated food in sequence (with 32 and 11 days, respectively, elapsing between the end of one treatment and the beginning of the subsequent treatment). Following MT treatment, two separate broods of fry were fed a control diet in sequence in the MT-treatment tanks (11 and 36 days, respectively, between the end of one 4-wk feeding and the beginning of the next). At the same time as all these treatment groups, different groups of fish from the same brood were fed a control diet. All experiment treatments were replicated. MT-impregnated food was made by spraying crushed flaked food with MT dissolved in EtOH; control food was made by spraying crushed flaked food with EtOH. Fry were initially fed with Hatchfry Encapsulon™ (Argent Chemical Laboratories) until 15 dpf. Delaying treatment allowed the fry to reach the initial size proposed by Popma and Green (1990) for treatment. Fry were fed MT (60 mg $kg^{-1}$) or control diet for four weeks (from 15 to 43 dpf). Water temperature in the model ponds was maintained at 28 to 30°C, except for the first week of the study when water temperatures were 24 to 25°C. Temperature was monitored daily; pH, ammonia, nitrites, and dissolved oxygen were checked weekly. The feeding rate was at 20% per calculated body weight for the first 23 days of treatment and then 10% per calculated body weight through day 28 of treatment (Popma and Green, 1990). Water lost by evaporation from the model ponds was restored twice weekly.

After 28 days of dietary treatment (on 44 dpf), fry were transferred to the Oregon State University 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 also checked. At 80 to 90 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.

Water samples (12 ml) were collected with pipettes into 15-ml polypropylene tubes and stored at –20°C until analysis for MT. Soil core samples were collected with 0.5-cm-diameter plastic pipes, placed in whirl pak bags, excess water poured off, and stored at –20°C until analysis. In tanks with gravel and no substrate, a film of fine sediments was formed. This material was collected with a pipette and stored at –20°C until analysis. Fine sediments were precipitated by centrifugation, and then a 1-ml subsample was dried in an oven at 50°C. For analysis of MT concentration, 1.0 ml of each water sample, 0.2 g of each soil sample, and 0.2 g of each fine sediment sample 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 phosphate-buffered saline containing gelatin. Aliquots of the reconstituted extracts were removed to 12 × 75 mm tubes for determination of MT concentration by radioimmunoassay (RIA). The RIA methods followed the procedure outlined in Fitzpatrick et al. (1986; 1987). Antisera specific to MT were purchased from UCB-Bioproducts SA, and $^3$H-MT (Amersham) was generously donated by Dr. Gordon Grau of the Hawaii Institute of Marine Biology. Standards of 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, soil 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 for MT for the RIA was checked by adding a known amount of $^3$H-MT to water and soil, (n = 5 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 73.3% for water and 71.4% for soil).

Sex ratios 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 MT in water and soil at the various sample times were not compared statistically because of the limited sample size (n = 1 or 2 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 a 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

Each cycle of MT treatment of tilapia fry resulted in significant masculinization; however, the efficacy of treatment differed between cycles of MT treatment (Figure 1). In the first cycle,

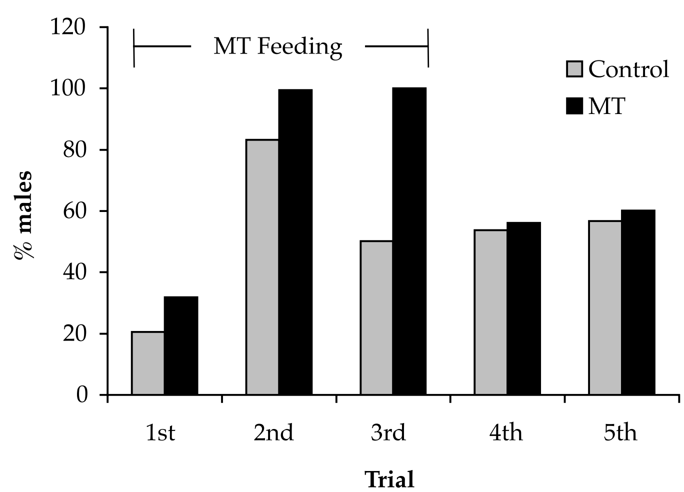


Figure 1. Masculinizing effects of MT in model ponds under consecutive feeding trials (first through third) and residual effects of MT in control-fed fish (fourth through fifth trials). Each treatment was run in duplicates.

the MT-treatment groups had 31.8% males compared with 20.5% males in the control groups; in the second cycle, the MT-treatment groups had 99.5% males compared with 83.2% males in the controls; and in the third cycle, the MT-treatment groups had 100% males compared with 50.2% males in the controls.

In the two subsequent cycles of MT treatment of tilapia fry all fed a control diet, no difference in sex ratios was found between groups in tanks that had been exposed to MT treatment compared to groups held in the control tanks (Figure 1).

The use of MT-impregnated food resulted in rapid leakage of MT into the water and subsequent deposition into the sediments. Within one minute of adding MT-impregnated food to the water, the levels of MT in the water jumped to nearly 100 pg ml$^{-1}$; within 15 minutes of feeding, levels of MT in the water were around 160 pg ml$^{-1}$ (Figure 2). Mean levels of MT in the water were elevated for the first 60 days of the experiment, ranging between about 234 and 1,179 pg ml$^{-1}$ (Figure 3), with levels dropping to background and remaining near background levels through the remaining days of the experiment. Mean levels of MT in the soil were elevated to about 15,976 pg g$^{-1}$ after one feeding cycle; levels decreased to 2,332 after 58 days of treatment and remained elevated thereafter between 1,265 and 3,193 pg g$^{-1}$ through three months after the conclusion of the last MT feeding cycle (Figure 4), including the time during which the control-fed fry were raised in these tanks. Four months after cessation of MT administration, an estimated 6.3 g of MT were still present in the soil.

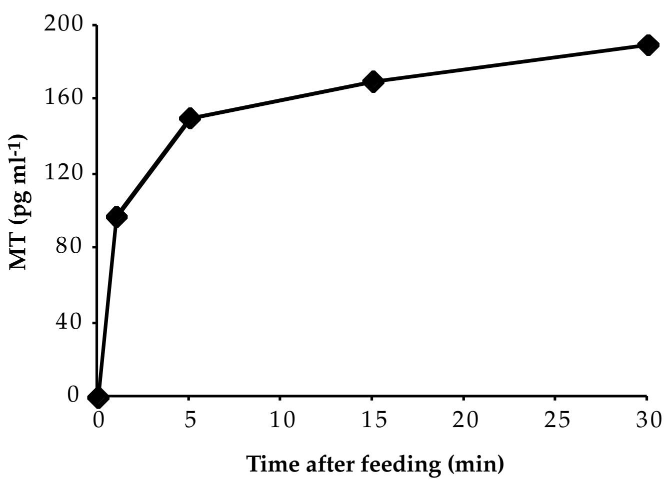


Figure 2. Detection of MT in water from an MT-fed model pond immediately after feeding.

## Discussion

These experiments confirm previous studies (Fitzpatrick et al., 1999; Contreras-Sánchez et al., 2000) that demonstrated that considerable amounts of MT can be found in the pond environment during and after dietary treatment with MT. MT levels in water peaked during the three cycles of dietary treatment and returned to background levels thereafter. Soil MT reached high levels after the first cycle of MT treatment and remained elevated months after the conclusion of MT treatment.

A significant level of masculinization was obtained from dietary treatments with MT tanks; however, the level of masculinization achieved after the first cycle of treatment was below what has been reported by other researchers (Green et al., 1997). This confirms our earlier results (Fitzpatrick et al., 2000), which also had suboptimal masculinization (in these studies soil was not reused, so it is comparable to the first cycle of treatment). We had previously ruled out the possibility of improper diet preparation as an explanation for the lack of masculinization achieved in the first cycle. Once one cycle of MT treatment was complete, nearly complete masculinization was achieved in subsequent cycles of MT treatment. Therefore, some "conditioning" of the soil with MT may be necessary to optimize masculinization.

The subsequent groups of fish fed a control diet in the MT-treatment tanks did not show any statistically significant effect on sex ratios; however, instances of intersex fish were observed in each of the subsequent cycles of control diet feeding. No intersex characteristics were observed in any fish from the control diet tanks. This suggests that the level of MT contamination in the soil was not sufficient to alter sex ratios;

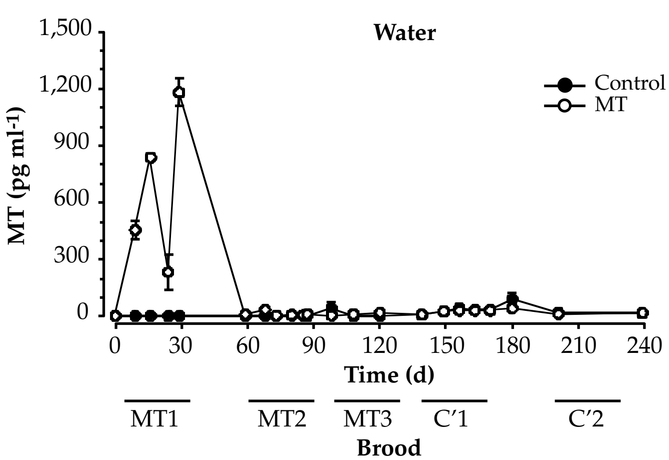


Figure 3. Mean values of MT (± SD) in water from control and MT-fed model ponds. Samples were taken at initiation and ending of each feeding trial.

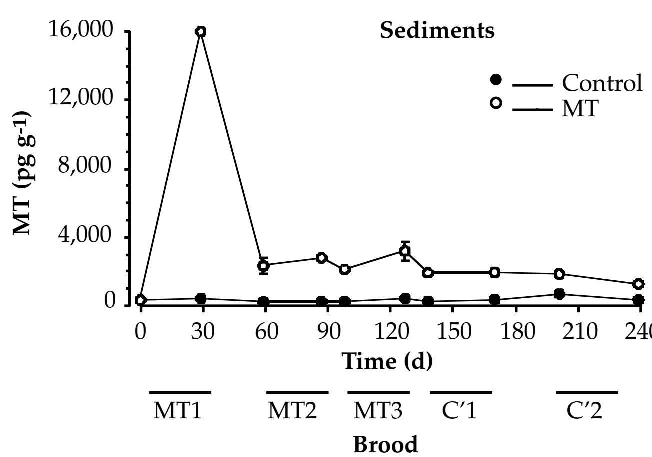


Figure 4. Mean values of MT (± SD) in sediments from control and MT-fed model ponds. Samples were taken at initiation and ending of each feeding trial.

nevertheless, some biological effects may have occurred. We have confirmed previous results (Fitzpatrick et al., 1999) showing that treatment of tilapia with MT results in leakage of this anabolic steroid into the environment. We have extended our previous work by showing that three cycles of MT feeding leads to contamination of the soil but does not alter the sex ratios of fish subsequently fed control diet.

## ANTICIPATED BENEFITS

The anabolic steroid 17α-methyltestosterone (MT) remains in the sediments of model ponds for up to three months after its use for masculinizing tilapia fry. The contaminated sediment is not associated with significant alterations in sex ratios of fry fed a control diet; nevertheless, the instances of intersexual individuals in these groups suggest that MT contamination may have a biological effect. These results will be useful in persuading those involved in MT use (e.g., farmers, researchers, and government workers) to exercise caution because of the risk of unintended MT exposure of pond workers, fish, and other organisms.

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