PD/A CRSP Seventeenth Annual Technical Report

Fate of Methyltestosterone in the Pond Environment: Detection of MT in Soil after Treatment with MT Food

Ninth Work Plan, Effluents and Pollution Research 2A (9ER2A)
Final Report

Martin S. Fitzpatrick and Wilfrido M. Contreras-Sánchez
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

This study examined the persistence of 17α-methyltestosterone (MT) in the environment after its use for masculinizing Nile tilapia. 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 60-l tanks that contained either 5 kg of soil, gravel, or no 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 at approximately 3.6 ng ml^{-1} at 28 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 the tanks with soil or gravel, but remained above background through 49 days in the tanks without soil. The levels in the soil were approximately 6.1 ng g^{-1} at 28 days after the onset of feeding with MT-impregnated food and remained detectable in the soil at between 2.8 and 2.9 ng g^{-1} after 84 days (eight weeks after ending treatment with MT-impregnated food). In tanks with gravel or no soil, MT was detected at higher levels in a fine sediment that formed after the end of dietary treatment. These results demonstrate that MT persists in soil for up to eight weeks after cessation of MT treatment, which raises the possibility that unintended exposure to MT may occur.

Introduction

Treatment of tilapia fry with 17α-methyltestosterone (MT)-impregnated food to produce all-male populations has become a common aquaculture practice. All-male populations are desirable because no energy is shunted toward reproduction and no competition with younger fish for food occurs (Green et al., 1997). Nevertheless, uneaten or unmetabolized food may leak significant amounts of MT into the pond environment, posing the risk of unintended MT exposure to hatchery workers as well as to aquatic and terrestrial organisms. Therefore, to increase the safety and efficacy of MT use for masculinization, we have set about to determine the fate of MT in semi-closed systems such as ponds. This study was undertaken to extend and expand earlier work (Fitzpatrick et al., 1999) which demonstrated persistence of MT in soil for nearly a month after cessation of treatment.

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 10 days post-fertilization (dpf). Fry were removed from the tank and randomly assigned initially to 3.8-l jars until 420 CTU (15 dpf) when they were placed in the model ponds at a stocking rate of 200 fry $tank^{-1}$ (1 fry per 9.0 cm^2). This value corresponds to one-third of the recommended stocking rate (by area) for masculinization of 3,000 fry m^{-2} (Popma and Green, 1990). However, the volume used in model ponds in this study was limited by the tank height, conferring a stocking rate by volume of 4 fish l^{-1} (1 fish l^{-1} more than the recommended stocking rate). Model ponds were set up two days before the expected time of fry release.

Water quality and survival data obtained from previous experiments suggested the need for modifying the original experimental design proposed for this study. The proposed design included the use of 3.8-l glass chambers; instead, 60-l aquaria were used. Two aquaria contained 5,000 g (approximately 3 cm) of packed soil, which was obtained from a meadowed hill north of Corvallis, Oregon; two other aquaria contained gravel or no soil, respectively. Each model pond contained 50 l of dechlorinated tap water. To determine the fate of MT in sediments of model ponds, the following experimental groups were included: MT-fed fish in an aquarium containing soil; control-fed fish in an aquarium containing soil; MT-fed fish in an aquarium containing gravel; and MT-fed fish in an aquarium with no soil. The latter two treatments were

included to minimize soil effects on water quality that could affect survival and/or efficacy of treatment. Because the number of fry obtained from the spawning was limited, the experiment treatments were not 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) in the jars because in this system active feeding does not initiate until about 15 dpf. Delaying treatment in this instance also 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 4 weeks (from 15 to 43 dpf). Water temperature was maintained at 28 ± 1°C in the jars and at 26.5 ± 1°C in the model ponds. Temperature was monitored daily; pH, ammonia, nitrites, and dissolved oxygen were checked weekly. 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'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 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 pipets 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 pipet and stored at -20°C until analysis. Fine sediments were precipitated by centrifugation, and a 1-ml subsample was oven-dried 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 was counted by scintillation spectroscopy (extraction efficiencies were 73.3% for water and 71.4% for soil).

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 MT in water, soil, and interface 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 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

The percentages of males in the MT-treated groups with soil (47.0%) and gravel (48.4%) were significantly higher than the control group (10.4%; $P < 0.0001$). The MT-treated group with no substrate had significantly more males (19.3%) than the control group ($P = 0.02$), but significantly less males than the treatments with substrate ($P < 0.0001$; Figure 1). Mortality values ranged from 6 to 10%, and showed no significant differences between treatments ($P = 0.52$).

Water samples had a background level of MT between non-detectable (nd) and 0.02 ng ml^{-1}. During the treatment period, the levels of MT in the water varied from 0.04 to 3.61 ng ml^{-1} in

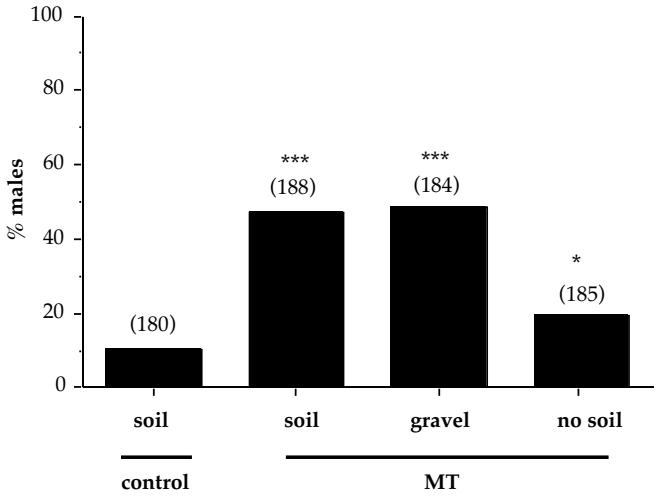

Figure 1. Effect of dietary treatment with 17α-methyltestosterone (MT) on masculinization of *Oreochromis niloticus* fry in tanks with soil, gravel, or no soil. Fish were fed either a control diet or a diet containing 60 mg kg^{-1} of MT. Graph depicts the percentage of males in each treatment with sample sizes indicated in parentheses. Statistically significant differences from controls are represented by asterisks (* $P < 0.05$; *** $P < 0.001$).

MT-treatment with soil (MT-soil); 0.02 to 0.50 ng ml^{-1} in MT treatment with gravel (MT-gravel); and from 0.05 to 4.97 ng ml^{-1} in MT-treatment with no soil (MT-no soil; Figure 2a). After removal of the fish and cessation of MT treatment, MT levels in water declined rapidly in MT-soil and MT-gravel tanks; however, MT remained about 10 times the background in water from the MT-no soil tank through week 12.

A background level of MT was detected in soil samples collected at the beginning of the experiment (mean = 0.5 ng g^{-1}). In the MT-soil tank, MT increased to 6.1 ng g^{-1} at the conclusion of dietary treatment (Figure 2b), remained near this level for two more weeks, and then decreased to about 3 ng g^{-1} through the end of the experiment (Eight weeks after the cessation of dietary treatment). Concentrations of MT in the fine sediment in the MT-gravel and MT-no soil tanks were considerably higher than MT levels in soil or water (Figure 2c). One week after cessation of feeding, the level of MT in fine sediment was 168.7 ng g^{-1} in the MT-no soil tank and remained elevated through week 12. In the MT-gravel tank, MT ranged from 22.9 to 99.2 ng g^{-1} of fine sediment throughout the eight weeks following cessation of treatment.

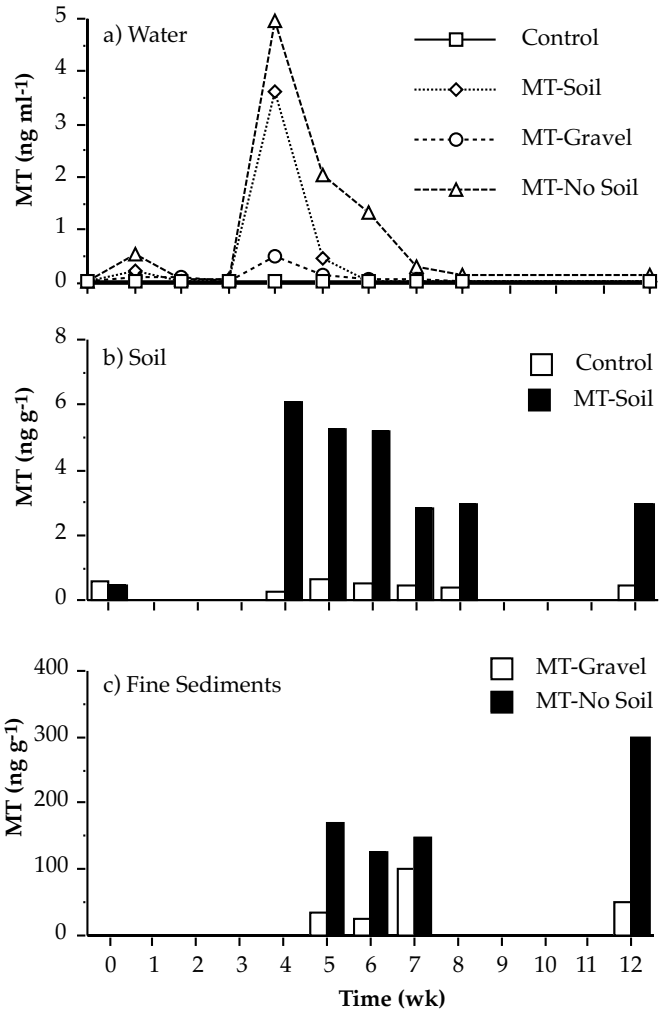

Figure 2. Levels of 17α-methyltestosterone (MT) in the environment during and after dietary treatment of *Oreochromis niloticus* fry. Fish were fed either a control diet or a diet containing 60 mg kg^{-1} of MT through week 4. Graph depicts MT levels in (a) water, (b) soil, and (c) fine sediments.

DISCUSSION

These experiments confirm previous studies using smaller containers (Fitzpatrick et al., 1999) which 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 at the end of dietary treatment and consistently returned to background levels in tanks with substrate. However, when no substrate was present, MT levels remained above 1 ng ml^{-1} for two weeks after the end of treatment. The accumulation and persistence of MT in soil was demonstrated to extend through eight weeks after the end of dietary treatment. Our results suggest that sediments act as a trap for MT while in tanks with no substrate, MT remains in suspension for longer periods of time until finally binding to fine particles to form a film at the bottom of the tank.

A significant level of masculinization was obtained from dietary treatments with MT tanks with substrate; however these values are below what has been reported by other researchers (see Green et al., 1997). Interestingly, the fish fed with MT but kept in tanks with no substrate had significantly lower masculinization values than those with either soil or gravel. MT in the water and in the fine sediments was high in the MT-no soil tank; therefore, the fish may have been exposed to higher doses of MT. Since MT can be converted to estrogenic compounds by the enzyme aromatase, the low level of masculinization in the MT-no soil group may be the result of paradoxical feminization. Previous results showed that lower levels of masculinization are obtained when tilapia fry are fed twice the recommended dose of MT (Fitzpatrick et al., 1999).

The inability to obtain 95 to 100% masculinization is troubling but is not uncommon in production facilities around the world. The concentrations of MT were measured in the diets (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. One possible explanation is that because water filtration was not provided during treatment, water quality deteriorated within the tanks to limit the effectiveness of MT by stressing the fish.

These data confirm previous results (Fitzpatrick et al., 1999) showing that treatment of tilapia with MT results in leakage of this anabolic steroid into the environment. This study extends previous work by showing that MT may persist in the soil for at least two months after cessation of treatment with no indication that the levels will decline thereafter. Thus, MT persistence may pose an exposure risk to humans and other organisms.

ANTICIPATED BENEFITS

The anabolic steroid 17α-methyltestosterone (MT) can be detected in the sediments of model ponds for up to two months after its use for masculinizing tilapia fry. MT dissipates from water a few days after fish are taken out of the treatment tanks. Removal of fish from the system allows suspended material to precipitate carrying with it MT and/or its metabolites. However, MT is persistent in sediments for up to eight weeks after the end of treatment. 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 organisms. Further research is needed to determine the nature of the background levels of MT detected in the radio-

immunoassay in soil samples, as well as possible metabolites produced after MT treatment.

LITERATURE CITED

Fitzpatrick, M.S., G. Van Der Kraak, and C.B. Schreck, 1986. Profiles of plasma sex steroids and gonadotropin in coho salmon, *Oncorhynchus kisutch*, during final maturation. Gen. Compar. Endocrin., 62:437–451.

Fitzpatrick, M.S., J.M. Redding, F.D. Ratti, and C.B. Schreck, 1987. Plasma testosterone concentration predicts the ovulatory response of coho salmon (*Oncorhynchus kisutch*) to gonadotropin-releasing hormone analog. Can. J. Fish. Aquat. Sci., 44:1351–1357.

Fitzpatrick, M.S., W.M. Contreras-Sánchez, R.H. Milston, R. Hornick, G.WFeist, and C.B. Schreck, 1999. Detection of MT in aquarium water after treatment with MT food. In: K. McElwee, D. Burke, M. Niles, and H. Egna (Editors), Sixteenth Annual Technical Report. Pond Dynamics/ Aquaculture CRSP, Oregon State University, Corvallis, Oregon, pp. 81–84.

Green, B.W., K.L. Veverica, and M.S. Fitzpatrick, 1997. Fry and fingerling production. In: H. Egna and C. Boyd (Editors). Dynamics of Pond Aquaculture. CRC Press, Boca Raton/New York, pp. 215–243.

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

Popma, T.J. and B.W. Green, 1990. Sex Reversal of Tilapia in Earthen Ponds. International Center for Aquaculture and Aquatic Environments Research and Development Series No. 35, Auburn University, Alabama, 15 pp.

Cite as: [Author(s), 2000. Title.] In: K. McElwee, D. Burke, M. Niles, X. Cummings, and H. Egna (Editors), Seventeenth Annual Technical Report. Pond Dynamics/Aquaculture CRSP, Oregon State University, Corvallis, Oregon, [pp. ___.]