Fate of Methyltestosterone in the Pond Environment: Detection of MT in Pond Soil from a CRSP Site

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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 for food with younger fish 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. We have investigated the fate of MT in semi-closed systems such as ponds. In a previous study, we reported that MT in the water peaks at approximately 3.6 ng ml⁻¹ at 28 days after the onset of feeding with MT-impregnated food, decreasing to background levels by 35 days after the onset of feeding (i.e., within a week of ceasing treatment with MT-impregnated food). We have also shown that MT accumulated in sediments of model ponds, reaching 2 to 6 ng g⁻¹ at 28 days after the onset of feeding with MT-impregnated food, and remained detectable in the soil between 2.8 and 2.9 ng g⁻¹ after 84 days (eight weeks after ending treatment with MT-impregnated food), which demonstrated persistence of MT in soil for nearly three months after cessation of treatment (Contreras-Sánchez et al., 2000; Fitzpatrick et al., 2000). In this study we determined the concentration of MT in soil and water samples collected in nursery ponds from the Sagana Fish Farm (SFF), Kenya, and the Laboratory of Aquaculture at the Universidad Juárez Autónoma de Tabasco (UJAT), Mexico, after masculinizing treatment with MT (60 mg kg⁻¹) for four weeks. These two CRSP sites have different histories regarding MT usage; namely, MT has been used for several years at SFF while never used before at UJAT.

METHODS AND MATERIALS

Sagana Fish Farm, Kenya

Soil samples were collected from three points (near pier, halfway between pier and drain, and near drain) in one of the sex-reversal ponds at the hatchery. Samples were then frozen and shipped to Oregon State University (OSU) for MT analysis.

Laboratory of Aquaculture at UJAT, Mexico

Nile tilapia (Oreochromis niloticus) fry were collected from a spawning tank. Fry were counted and randomly assigned to either MT-feeding or EtOH (vehicle)–feeding, and each treatment was triplicated. Replicates were housed in 1 × 1 × 0.75 m hapas made of mosquito mesh, and hapas were placed in a 7 × 15 m earthen pond. The MT-treated replicates were located at one end of the pond and the control replicates at the other end.

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 fed the MT (60 mg kg⁻¹) or control diet for four weeks (from 15 to 43 days post-fertilization [dpf]). 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). After 28 days of dietary treatment (on 44 dpf), fry were fed with regular fish food. At 90 to 100 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.

To collect water and soil samples, seven sampling points were set along the pond as follows: points 1 and 7 were located under treatment hapas (1 = control treatment; 7 = MT treatment); all other sampling points were two meters apart from one another (points 2 to 6). Water samples (12 ml) were collected with pipettes into 15-ml polypropylene tubes and stored at \(-20^\circ C\) until analysis for MT. Soil core samples were collected with long 1.25-cm-diameter PVC pipes, placed in whirl pak bags, had excess water poured off, and were stored at \(-20^\circ C\) until shipment to OSU for analysis. Water and soil samples were taken at the onset (July 13) and end (August 9) of treatment.

**Radioimmunoassay**

For analysis of MT concentration, 1.0 ml of each water sample and 0.2 g of each soil 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 3H-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. Extraction efficiency for MT for the RIA was checked by adding a known amount of 3H-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). 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 per date) and because the goal of the study was descriptive (presence/absence).

**RESULTS**

**Sagana Fish Farm, Kenya**

Soil samples obtained from Kenya showed MT concentrations between 3,900 and 4,800 pg g\(^{-1}\) (Figure 1), having the lowest concentration near the drain and similar values near the pier and the middle point. Unfortunately, we had no samples from ponds that have not been used for sex inversion to determine background levels.

**Laboratory of Aquaculture at UJAT, Mexico**

MT concentrations in soil at the beginning of the experiment ranged between 400 and 800 pg g\(^{-1}\) with a mean value of 482 pg g\(^{-1}\). After 28 days of feeding we detected slightly higher values of MT, ranging between 320 and 900 pg g\(^{-1}\) with a mean of 691 pg g\(^{-1}\) (Figure 2). We observed no pattern related to the location of the treatments (i.e., sampling locations near MT-fed hapas did not show higher levels of MT). Detected values of MT in the water were low at both the onset and end of the experiment (Figure 3). Values ranged between 4 and 10 pg ml\(^{-1}\) of water (mean = 6.8 pg ml\(^{-1}\)) at the beginning and between 3.7 and 7 pg ml\(^{-1}\) (mean = 4.4 pg ml\(^{-1}\)) at the end of the experiment. The pond used for sex inversion at UJAT was never used before for treatments with MT.

**DISCUSSION**

Despite the widespread use of methyltestosterone for masculinizing tilapia in aquaculture facilities, little is known about the fate of this potent synthetic steroid in the pond environment. Few studies have been dedicated to detect MT or its metabolites in body tissues of the sex-inversed fish (Cravedi et al., 1989; Goudie et al. 1986a; 1986b; Curtis et al., 1991);
However, little has been done to detect if MT or its metabolites can dissociate from the impregnated food and accumulate in the pond environment. Recent studies in our laboratory have shown that MT can be detected in the water during MT treatment and that it eventually accumulates and remains in the sediments of model ponds for up to eight weeks (Fitzpatrick et al., 1999; 2000).

We found that MT levels in sediments from SFF were higher at the sediments of model ponds for up to eight weeks (Fitzpatrick et al., 1999; 2000).

Although we have no evidence that the detected levels at Farm while background levels were found at UJAT, Mexico. In the sediments of sex-inversion ponds from the Sagana Fish Farm and suppressing serum testosterone levels in male rats (Clark et al., 1997). Furthermore, it has been demonstrated that overexposure of fish to MT can result in paradoxical feminization due to a hypothesized conversion of MT into estrogen by the enzymatic action of aromatase (Piferrer and Donaldson, 1991; Piferrer et al., 1993; Eding et al., 1999; Rinchard, et al., 1999). Unfortunately, very little is known about the potential effects of residual MT in ponds where large amounts of fish are masculinized. In our laboratory, we found that reusing tanks with sediments from trials involving feeding with MT-impregnated food resulted in the appearance of few intersex fish; however, we did not address the possibility of other effects. These findings suggest that caution should be used when using steroids for aquacultural purposes and that more research is needed to understand the fate of exogenous hormones in the pond environment as well as the potential effects on the health of non-target organisms, including farm workers.

**Anticipated Benefits**

We detected the anabolic steroid 17α-methyltestosterone (MT) in the sediments of sex-inversion ponds from the Sagana Fish Farm while background levels were found at UJAT, Mexico. Although we have no evidence that the detected levels at SFF represent a health or environmental risk, these results suggest that caution should be exercised because of the risk of unintended MT exposure of pond workers, fish, and other organisms.

**Literature Cited**


