Fate of Methyltestosterone in the Pond Environment: Use of MT in Earthen Ponds with No Record of Hormone Usage

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Abstract

The following study examined the persistence of 17α-methyltestosterone (MT) in the environment after its use for masculinizing Nile tilapia in nursery ponds located in the Universidad Juárez Autónoma de Tabasco, Mexico. Fry harvested from spawning ponds were treated with a masculinizing dose of MT (60 mg kg^{-1}) for four weeks. Concentrations of MT were determined by radioimmunoassay. MT was not detectable in the water at any time. In the sediments, MT was not detectable during the first 10 days of treatment. Afterwards MT was detectable in all sampling points (mean = 146.7 pg g^{-1}; SE = 21.3). MT values varied from not detectable to 368.9 pg g^{-1}. Masculinizing efficiency was low in the first trial (87.4% males) but increased significantly afterwards, reaching 92.6% males in the second trial and 98.7% in the third trial.

Another outcome of this investigation is a manual on tilapia masculinization using synthetic steroids. This manual is intended to reach fry producers, extension agents and technicians; it contains a general description of the biology of the tilapias, traditional culture practices, masculinization methods, and a detailed section on safe handling of steroids.

Introduction

In tilapia culture the production of all-male populations through treatment of fry with 17α-methyltestosterone (MT)–impregnated food has become the most popular procedure. All-male populations have greater growth potential because no energy is shunted toward reproduction and no competition with younger fish occurs (Green et al., 1997). Contreras-Sánchez (2001) demonstrated that significant "leakage" of MT to water and sediments occurs in small, closed systems, probably 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 to anabolic steroids if MT persists in the environment after treatment of tilapia fry.

Some researchers have warned about unintended effects of steroid administration, such as fish-to-fish transfer of steroids (Budworth and Senger, 1993), biased sex ratios in untargeted organisms (Abucay et al., 1997), and paradoxical feminization (Rinchard et al., 1999; Eding et al., 1999). If MT is being added to the food in amounts that efficiently masculinize fish despite steroid loss to the environment, then 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 is detectable within the pond environment, the following study was undertaken.

Methods and Materials

Laboratory of Aquaculture at UJAT

Nile tilapia, *Oreochromis niloticus*, fry were collected daily from a spawning tank. Fry were selected by grading with a 3-mm mesh (Popma and Green, 1990), counted, and randomly assigned to either MT-feeding or ethanol (EtOH; vehicle)–feeding treatments. Fry were housed in $1 \times 1 \times 0.75$ m hapas made of mosquito mesh, and hapas were placed in a 7×15 m earthen pond. The MT-treated experimental units were located at one end of the pond and the control units at the other end. Three masculinization trials were established at different dates throughout the experiment. A total of seven hapas were assigned to each treatment: three for trial 1 (density = 3,000 fry $hapa^{-1}$), two for trial 2 (density = 2,000 fry $hapa^{-1}$), and two for trial 3 (density = 5,000 fry $hapa^{-1}$). The number of fry per treatment was established based on fry availability.

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 MT (60 mg kg^{-1}) or control diet for four weeks. Feeding rate was at 20% per calculated body weight for the first 23 days of treatment and then 10% per calculated body weight through 28 days of treatment (Popma and Green, 1990). After 28 days of dietary treatment, fry were moved

to a grow-out pond and 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, three sampling points were set along the pond as follows: points 1 and 3 were located under treatment hapas (1 = control treatment; 3 = MT treatment); the other sampling point was located in the middle of the pond. 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 long 1.25-cm-diameter PVC pipes, placed in Whirl-Pak bags, excess water poured off, and stored at –20°C until shipment to Oregon State University (OSU) for analysis. All water and soil samples were collected early in the mornings, before initiation of feeding. Samples were taken weekly starting five days before the onset of the experiment (23 February).

Radioimmunoassay

For analysis of MT concentration, 1.0 ml of each water sample and 1.0 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 Animal Pharm Services, 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. 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 64.3% for water and 61.6% for soil). Concentrations of MT in water and soil 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

Detection of MT in the Pond Environment

MT was not detectable in water at any sampling point throughout the entire experiment. MT concentrations in soil at day 10 of treatment were below the detectable limit (Figure 1). At day 17, MT was detectable at all sampling points; values ranged between 113.0 pg g^{-1} at the sampling point located under the MT treatment hapas to 186.2 pg g^{-1} at the sampling point underneath the EtOH-fed cages. After this sampling date, values of MT varied significantly in both ends of the pond (under the MT and the control hapas), while at the middle of the pond MT concentrations remained nearly constant. 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). The pond used for sex inversion at UJAT had not been used for six months for treatments with MT.

Masculinizing Efficiency of MT

The efficacy of MT for masculinizing Nile tilapia fry was low during the first trial (87.4% males); however, these values increased thereafter and remained elevated throughout trials 2 (92.6% males) and 3 (98.7%). These results were significantly higher than those of the control groups maintained in the masculinizing pond (40.5, 36.4, and 53.3% males, respectively) and the control groups raised in a grow-out pond that received no MT treatment (46.2, 35.0, and 52.5% males, respectively). No significant differences were found between control groups.

Manual on Tilapia Masculinization and Safe Handling of Steroids

A manual on tilapia masculinization in Spanish was developed that contains a general description of the biology of the tilapias, traditional cultural practices, masculinization methods, and a detailed section on safe handling of steroids. This manual is intended to reach farms that are currently producing fry (some of which are already using masculinizing steroids), extension agents who provide training to farmers, technicians, and high school and undergraduate students in fields related to aquaculture. We consider the section on safe handling of steroids a very important part of the manual since most farmers are not aware of the potential detrimental effects of mishandling steroids. The manual will be published at UJAT and will be available through the Internet via UJAT and CRSP websites.

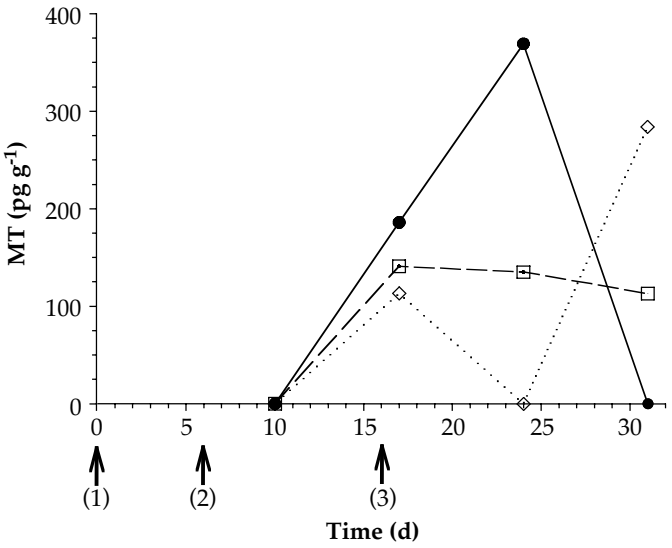

Figure 1. Concentration of 17α-methyltestosterone (MT) (pg g^{-1}) in sediments from an earthen pond used for tilapia fry masculinization. Arrows indicate initiation of feeding trials. Graph depicts MT concentration under MT-fed hapas (filled circles), at the middle of the pond (open diamonds), and under EtOH-fed hapas (open squares).

DISCUSSION

Recent studies in our laboratory have shown that MT can be detected in the water during MT treatment and eventually accumulates and remains in the sediments of model ponds for up to eight weeks (Fitzpatrick et al., 1999; Contreras-Sánchez, 2001). In the current study, we determined that during the masculinization of 23,000 tilapia fry, MT levels in sediments were lower than during a previous experiment conducted last year in the same facility (undetectable to 368.9 vs. 400 to 800 pg g^{-1}; Contreras-Sánchez et al., 2001). In this experiment, as well as in the previous one, MT values showed no trend with location of the treated hapas in the pond.

MT was not detectable in sediments for the first ten days of treatment, suggesting a lack of background levels. This is the first time we have been able to detect no background in our sediment samples. These results may be explained because we are using a new antibody and the limit for detectable values for the RIA was established at 10 pg $tube^{-1}$. Levels of MT in the pond water were below detectable limits, suggesting that the MT that leaks out to the environment may be precipitating to the sediments.

The large amount of variability detected in this experiment shows a similar pattern to that detected in previous studies (Fitzpatrick et al., 1999; Contreras-Sánchez, 2001). These patterns may be related to active bacterial degradation of the steroid (discussed in Contreras-Sánchez, 2001) or to a patchy distribution of the steroid in the pond due to dominant winds or uneaten food deposition or both.

The need to avoid exposure of untargeted organisms when steroids are administered in aquatic systems should be of great importance. Recent studies have reported that exposure of untargeted organisms to MT can result in biased sex ratios. Significant masculinization of common carps (*Cyprinus carpio*) exposed to water used in MT-impregnated feeding trials was reported by Gomelsky et al. (1994). Their findings suggested that MT (or its metabolites) can persist in the water at concentrations capable of causing sex inversion. Abucay and Mair (1997) and Abucay et al. (1997) reported incidental sex inversion in tilapias kept in aquaria and concrete tanks. These authors reported that sex ratios were significantly biased when nontarget fish were housed in the same tank where groups of fish are fed with MT.

Paradoxical feminization has been identified as a potential problem during steroid treatment. This process is thought to be caused by the enzymatic aromatization of testosterone to estradiol, and it has been documented that potent synthetic androgens (such as MT) are aromatizable (LaMorte et al., 1994). However, the mechanism of paradoxical feminization by MT has not been elucidated.

The problems with contamination of water and sediments are not only related to the immediate contact of the animal with the contaminated media; many effects are related to bioaccumulation and the transfer of the contaminants and their metabolites through the food web (Kime, 1998). Therefore, it is important to evaluate if the use of MT in aquacultural facilities requires preventive measurements such as filtration or biodegradation of the hormone and its metabolites in water and sediments.

CONCLUSIONS

Masculinization of tilapia fry using MT-impregnated food results in accumulation of small amounts of the steroid. Despite large variability in the data, it can be concluded that MT does not remain in the water but deposits in the sediments. More research is needed to determine if the concentration of MT increases when large numbers of fish are masculinized. There is no evidence in the literature that indicates if the detected levels of the steroid measured in the UJAT pond represent a health or environmental risk. However, we suggest caution when masculinizing tilapia fry because of the risk of unintended MT exposure of workers and other organisms. It is important to take measures now, either by demonstrating that the hormone and its metabolites are not a health hazard for humans or the environment or by removing these compounds from the farm effluents.

ANTICIPATED BENEFITS

We detected the anabolic steroid 17α-methyltestosterone (MT) in the sediments of sex-inversion ponds from UJAT, Mexico. The new antibody used in our latest RIA runs appears to produce no background. This antibody will allow more accurate detection of low levels of MT.

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