PD/A CRSP Eighteenth Annual Technical Report

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

Wilfrido M. Contreras-Sánchez and Martin S. Fitzpatrick
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 examined the persistence of 17α-methyltestosterone (MT) in the environment after its use for masculinizing Nile tilapia in nursery ponds at two CRSP sites: Sagana Fish Farm, Kenya, and the Universidad Juárez Autónoma de Tabasco (UJAT), Mexico. Fry were treated with a masculinizing dose of MT (60 mg kg^{-1}) for four weeks beginning at the initiation of feeding. Concentrations of MT were determined by radioimmunoassay, which revealed that the levels of MT in the sediments from the Sagana Fish Farm had a mean value of 4,567 pg g^{-1}. The concentration of MT slightly decreased near the drain of the pond. Concentration of MT in water and sediments from UJAT showed background levels after first-time use of MT in the pond. These results suggest that accumulation of MT may take place after masculinization of a significant number of fish.

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 $1 \times 1 \times 0.75$ m hapas made of mosquito mesh, and hapas were placed in a 7×15 m earthen pond. The MT-treated 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°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°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 ^{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
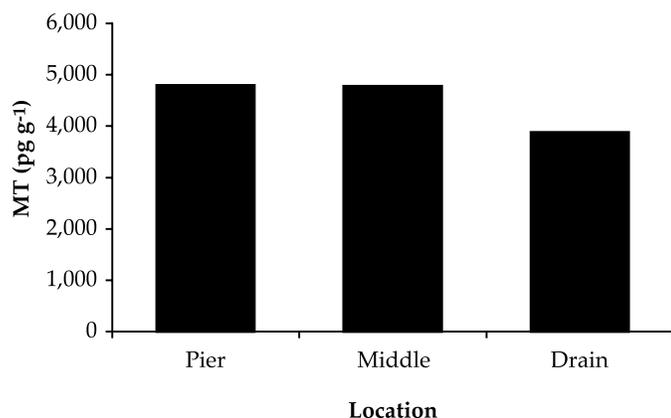
in 1 ml of phosphate-buffered saline containing gelatin, 0.5 ml was removed from each and the amount of radio-activity counted by scintillation spectroscopy (extraction efficiencies were 73.3% for water and 71.4% for soil). Concentrations of MT in water, soil, and interface at the various sample times 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);

Figure 1. Concentration of methyltestosterone in sediments from a sex-reversal pond in Sagana Fish Farm, Kenya.
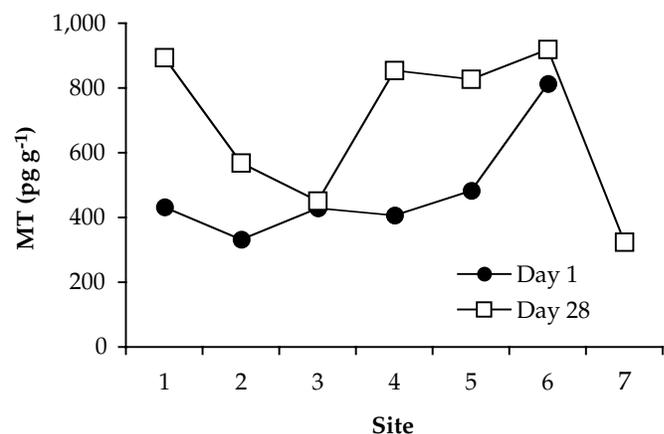

Figure 2. Concentration of methyltestosterone in sediments from UJAT, Mexico; sampling sites are distributed between control hapas (1) and MT-treated hapas (7).
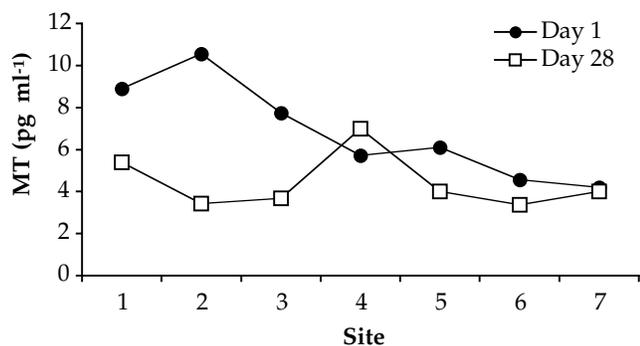
Figure 3. Concentration of methyltestosterone in water from UJAT, Mexico; sampling sites are distributed between control hapas (1) and MT-treated hapas (7).

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 (Fitz-patrick et al., 1999; 2000).

We found that MT levels in sediments from SFF were higher at all three sampling points than any background levels that we have detected in previous studies (e.g., 200 to 400 pg g^{-1} at Oregon State University (Fitzpatrick et al., 1999); 700 to 900 pg g^{-1} at Auburn University (Phelps et al., 1999); and 400 to 800 pg g^{-1} at UJAT), suggesting that the usage history of MT at SFF ponds might have allowed an accumulation of MT in the sediments. Since we were not able to analyze soil samples from SFF ponds without a history of MT treatment, we cannot rule out the possibility that these high levels resulted from background levels of compounds that cross-react with the antibody used to measure concentrations. Nevertheless, these high levels from SFF soil samples point to the need for further monitoring. The values obtained from water and sediments from UJAT are low and show no trend regarding location of the treated hapas in the pond. These results suggest that MT levels are still near background values. Another possibility to explain these results is that the number of fish treated with MT is very low (n = 1500) and, considering the dimensions of the pond, the amount of MT used is too low to cause detectable accumulation.

In an effort to determine the fate of steroids used in experiments involving live fish and recirculating systems, Budworth and Senger (1993) reported that testosterone injected into rainbow trout (*Oncorhynchus mykiss*) leaked out of the fish body and eventually reached other fish present in the system. These authors highlighted the need to avoid exposure of untargeted organisms when steroids are administered in aquatic systems. Recent studies have reported that exposure of untargeted organisms to MT can result in biased sex ratios. Gomelsky et al. (1994) found significant masculinization of common carps (*Cyprinus carpio*) exposed to water used in MT-impregnated feeding trials. They also reported that the masculinizing effects of MT were stronger in recirculating systems than in tanks with flow-through water. These observations suggest that MT (and/or its metabolites) can persist in the water at concentrations capable of causing sex inversion. Incidental sex reversal in tilapias has been reported recently (Abucay and Mair, 1997; Abucay et al., 1997). These

authors indicated that in aquaria and concrete tanks, sex ratios are significantly biased when non-target fish are housed in the same tank where groups of fish are fed with MT.

The potential risks posed by unintentional exposure to MT in aquacultural facilities are not confined to the androgenic effects of the steroid (e.g., masculinization of genital tract, gonads, or secondary sexual characteristics). In laboratory studies, it has been demonstrated that long-term exposure to MT can act as a weak hepatocarcinogen in male and female mice (Taylor et al., 1984). It has also been reported that long-term exposure to MT eliminated male sexual behavior and suppressed 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

Abucay J.S. and G.C. Mair, 1997. Hormonal sex reversal of tilapias: Implications of hormone treatment application in closed water systems. Aquacult. Res., 28:841–845.

Abucay J.S., G.C. Mair, D.O.F. Skibinski, and J.A. Beardmore, 1997. The occurrence of incidental sex reversal in *Oreochromis niloticus* L. In: K. Fitzsimmons (Editor), Proceedings from the Fourth International Symposium on Tilapia in Aquaculture. Natural Resource, Agriculture, and Engineering Service (NRAES), Cooperative Extension, Ithaca, New York, pp. 729–738.

Budworth, P.R. and P.L. Senger, 1993. Fish to fish testosterone transfer in a recirculating system. Prog. Fish-Cult., 55:250–254.

Clark, A.S., E.V. Harrold, and A.S. Fast, 1997. Anabolic-androgenic steroid effects on the sexual behavior of intact male rats. Horm. Behav., 31:35–46.

Contreras-Sánchez, W.M., M.S. Fitzpatrick, R.H. Milston, and C.B. Schreck, 2000. Masculinization of Nile tilapia with steroids: Alternative treatments and environmental effects. In: B. Norberg, O.S. Kjesbu, G.L. Taranger, E. Andersson, and S.O. Stefansson (Editors), Proceedings of the Sixth International Symposium on Reproductive Physiology of Fish. John Grieg, A.S., Bergen, Norway, pp. 250–252.

Cravedi, J.P., G. Delous, and D. Rao, 1989. Disposition and elimination routes of 17α-methyltestosterone in rainbow trout (*Salmo gairdneri*). Can. J. Fish. Aquat. Sci., 46:159–165.

Curtis, L.R., F.T. Diren, M.D. Hurley, W.K. Seim, and R.A. Tubb, 1991. Disposition and elimination of 17α-methyltestosterone in Nile tilapia (*Oreochromis niloticus*). Aquaculture, 99:193–201.

Eding, E.H., A. Bouwmans, and J. Komen, 1999. Effects of methyl-testosterone and 11-keto-androstenedione on sex differentiation in African catfish, *Clarias gariepinus*. In: B. Norberg, O.S. Kjesbu, G.L. Taranger, E. Andersson, and S.O. Stefansson (Editors). Proceedings of the Sixth International Symposium on Reproductive Physiology of Fish. John Grieg, A.S. Bergen, Norway, p. 258.

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. Comp. Endocrinol., 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. Aqua. Sci., 44:1351–1357.

Fitzpatrick, M.S., W.M. Contreras-Sánchez, R.H. Milston, R. Hornick, G.W. Feist, and C.B. Schreck, 1999. Detection of MT in aquarium 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.

Fitzpatrick, M.S., W.M. Contreras-Sánchez, and C.B. Schreck, 2000. Methyltestosterone persists in the environment after use for masculinizing Nile tilapia. In: B. Hart and B. Brown (Co-Chairs), Book of Abstracts, Aquaculture America 2000. World Aquaculture Society, New Orleans, Louisiana, p. 112.

Gomelsky, B., N.B. Cherfas, Y. Peretz, N. Ben-Dom, and G. Hulata, 1994. Hormonal sex inversion in the common carp (*Cyprinus carpio* L.). Aquaculture, 126:265–270.

Goudie, C.A., W.L. Shelton, and N.C. Parker, 1986a. Tissue distribution and elimination of radiolabelled methyltestosterone fed to sexually undifferentiated blue tilapia. Aquaculture, 58:215–226.

Goudie, C.A., W.L. Shelton, and N.C. Parker, 1986b. Tissue distribution and elimination of radiolabelled methyltestosterone fed to adult blue tilapia. Aquaculture, 58:227–240.

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, Florida, pp. 215–243.

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

Phelps, R.P., M.S. Fitzpatrick, W.M. Contreras-Sánchez, R.L. Warrington, and J.T. Arndt, 1999. Detection of MT in pond water after treatment with MT food. In: K. McElwee, D. Burke, M. Niles, and H. Egna (Editors), Seventeenth Annual Technical Report. Pond Dynamics/Aquaculture CRSP, Oregon State University, Corvallis, Oregon, pp. 57–59.

Piferrer, F. and E.M. Donaldson, 1991. Dosage-dependent differences in the effect of aromatizable and nonaromatizable androgens on the resulting phenotype of coho salmon (*Oncorhynchus kisutch*). Fish Physiol. Biochem., 9:145–150.

Piferrer, F., I.J. Baker, and E.M. Donaldson, 1993. Effects of natural, synthetic, aromatizable, and nonaromatizable androgens in inducing male sex differentiation in genotypic female chinook salmon (*Oncorhynchus tshawytscha*). Gen. Comp. Endocrinol., 91:59–65.

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

Rinchard, J., K. Dabrowski, M.A. García-Abiado, and J. Ottobre, 1999. Uptake and depletion of plasma 17α-methyltestosterone during induction of masculinization in muskellunge, *Esox masquinongy*: Effect on plasma steroids and sex reversal. Steroids, 64:518–525.

Taylor, W., S. Snowball, and M. Lesna, 1984. The effects of long-term administration of methyltestosterone on the development of liver lesions in BALB/c mice. J. Pathol., 143:211–218.