Elimination of Methyltestosterone (MT) from Intensive Masculinization Systems: Use of Activated Charcoal in Concrete Tanks

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Final Report

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Abstract

This study tested the hypothesis that activated charcoal can eliminate 17α-methyltestosterone (MT) from water used in intensive sex-inversion systems. Two amounts of charcoal (2.5 and 5.0 kg) placed in filters and a control group (no charcoal) were evaluated for both sex-inversion efficacy and MT persistence in the water. Fry (2,200 to 2,945 m^{-3}) were treated with a masculinizing dose of MT (60 mg kg^{-1} feed) for four weeks beginning at the initiation of feeding in concrete ponds containing 7.13 m^3 of water. Water samples were collected from the sex-inversion tank before the onset of treatment and weekly beginning on the first day of treatment. Activated charcoal used in the treatment was exposed to direct sunlight for 24 or 48 h, and samples were collected at different times for MT detection. All samples were extracted using ether, and the concentration of MT determined by radioimmunoassay at Oregon State University. Masculinization rates were not significantly different between treatments in a trial or between trials ($P > 0.05$). Mean percentage of males for treatments with 0.0, 2.5, and 5.0 kg of activated charcoal were 93.8, 93.5, and 94.0%, respectively. Controls averaged 51.7% males, which was significantly less than the MT-fed groups ($P < 0.001$). MT concentrations in the water ranged between 0.14 and 9.17 ng ml^{-1}, and the largest detected value occurred as an isolated case with all other values being below 1.0 ng ml^{-1}. Our data demonstrate that activated charcoal efficiently captures MT and that vegetal charcoal has a higher adsorption capacity than mineral charcoal. We recommend the use of activated charcoal filtration systems to eliminate excess MT and potentially increase masculinization.

Introduction

The administration of natural and synthetic steroids during early development of fish is used to induce sex inversion in several species of commercial importance. This common practice allows for the production of monosex populations and ensures increased productivity in the aquaculture industry (Contreras-Sánchez, 2001). The most commonly used steroids are 17α-methyltestosterone (MT), androstenedione, 17α-methyldihydrotestosterone, and trenbolone acetate. However, fry must be fed diets containing MT before gonadal differentiation has occurred to ensure effective masculinization, and this has been the key for the success of tilapia culture (Popma and Green, 1990). The use of MT is considered to be the best technique to obtain all-male populations of tilapia (Phelps and Popma, 2000). These populations have greater growth potential because no energy is shunted toward reproduction and there is no competition with younger fish (Green et al., 1997).

Several researchers, however, have warned about potential problems caused by the use of steroids in aquaculture (Budworth and Senger, 1993; Abucay et al., 1997; Rinchard, et al., 1999; Eding et al, 1999). We have demonstrated that MT can be detected in the water during feeding of MT-impregnated diets and that it eventually accumulates and remains in sediments of aquaria and masculinization ponds (Fitzpatrick et al.,

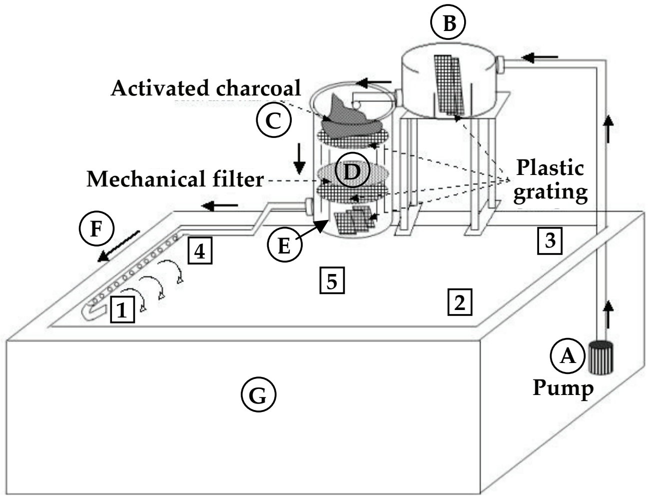

Figure 1. Intensive masculinization system adapted with a charcoal filter. Components are: A) submersible pump, B) sediment trap, C) charcoal filter section, D) mechanical filter section, E) biological filter section, F) return PVC pipe (creates a water curtain), and G) concrete tank (4.0 × 1.8 × 1.2 m). Sampling points are indicated by numbers (1–5). Details are not drawn to scale.

2000; Contreras-Sánchez, 2001; Contreras-Sánchez et al., 2001). In order to address these potential problems, our laboratory evaluated the use of filters with charcoal to eliminate MT from aquaria (Contreras-Sánchez, 2001). We found that charcoal filtration of water from systems where substrate was not present lowered the amount of MT in the water to almost background levels, and the treatment resulted in almost complete masculinization of all three broods tested (100, 98, and 100% males).

It is well known that one of the major problems in aquaculture is the elimination of culture wastes from water. The amount and type of residues will depend on the species cultured, the stage of development, and the feeds used (Wheaton, 1982). To lower the environmental impacts caused by aquacultural practices, different technologies have been developed to preserve water quality and reduce residue levels during fish culture. These systems are known as Recirculation Aquaculture Systems (RAS; Timmons et al., 2001) and are widely used because they allow for efficient disposal of wastes in aquaculture.

In order to eliminate MT from aquaculture effluents, we proposed the use of a RAS in an intensive system for masculinizing tilapia fry using MT-impregnated food on a large scale. In this system the excess MT can be eliminated from the water and the substrate by means of continuous filtration through activated charcoal filters.

METHODS AND MATERIALS

Experiment A: Elimination of MT from the Water of Intensive Sex-Inversion Systems

This experiment was conducted at the Laboratory of Aquaculture, UJAT, and consisted of four treatments:

1) Fry fed MT at 60 mg kg^{-1} food for 28 d; water filtered through a 0.0 kg activated charcoal bed
2) Fry fed MT at 60 mg kg^{-1} food for 28 d; water filtered through a 2.5 kg activated charcoal bed
3) Fry fed MT at 60 mg kg^{-1} food for 28 d; water filtered through a 5.0 kg activated charcoal bed
4) Fry fed control food for 28 d in grow-out pond

Because of tank availability, each treatment had a single experimental unit and the entire experiment was repeated four times (trials). Three concrete tanks (4.0 × 1.8 × 1.2 m) were used as experimental units for the MT elimination trials and 1 m^3 hapas were used for the control group. Each concrete tank was adapted with a 250-l chemical filter divided into three sections—a sediment trap, a granulated-charcoal bed (vegetal or mineral depending on availability), and a biological filter (Figure 1). Each recirculation system had a 1/6 horsepower (hp) submersible pump creating a flow of 60 l per minute and a return PVC pipe with several holes that formed a water curtain while water returned to the concrete tank.

Nile tilapia (*Oreochromis niloticus*) fry were obtained from spawning ponds from both the state hatchery, José Narciso Rovirosa, and the Laboratory of Aquaculture at UJAT. Fry were selected by grading with a 3-mm mesh (Popma and Green, 1990), counted, and randomly assigned to each of the experimental units. The target density was 3,000 fry m^{-3}; however, the number of fish used in each trial varied because of fry availability at the time of the trial.

MT-impregnated diets were made by spraying crushed food (API-Tilapia 1™, 40% protein) with MT dissolved in ethanol, and control food was made by spraying crushed food with ethanol. Fry were fed the MT (60 mg kg^{-1}) or control diet for four weeks. Feeding rate was 20% of calculated body weight until fish reached 15 mm (approximately 15 d) and then 10% of calculated body weight through the rest of the treatment (Popma and Green, 1990). After 28 d of dietary treatment, fry were counted to estimate survival, moved to a grow-out pond, and fed with regular fish food. At 90 to 100 d post-fertilization, 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 from the sex-inversion tank on days 0, 7, 14, 21, 28, and 35. Five sites were established for each tank (one for each corner and one at the center of the tank). Samples were obtained each day at 0730 h (before first feeding), frozen (–20°C), and preserved until processed for MT detection at Oregon State University (OSU). From the filters that contained charcoal, charcoal samples were taken at days 0, 7, 14, 21, and 28. At the ends of trials 2 and 3, charcoal was exposed to sunlight and samples were collected randomly at 12, 24, and 48 h to determine if sunlight eliminated MT from the charcoal. Charcoal samples were stored in Whirl-Pak® bags, frozen (–20°C), and preserved until processed for MT detection at OSU.

In each of the experimental units, dissolved oxygen (DO), pH, and temperature were measured daily. Ammonia, nitrates, and nitrites were measured twice a week. To maintain water quality, water exchange (25%) was performed twice a week.

Radioimmunoassay

All MT analyses were performed at the Oregon Cooperative

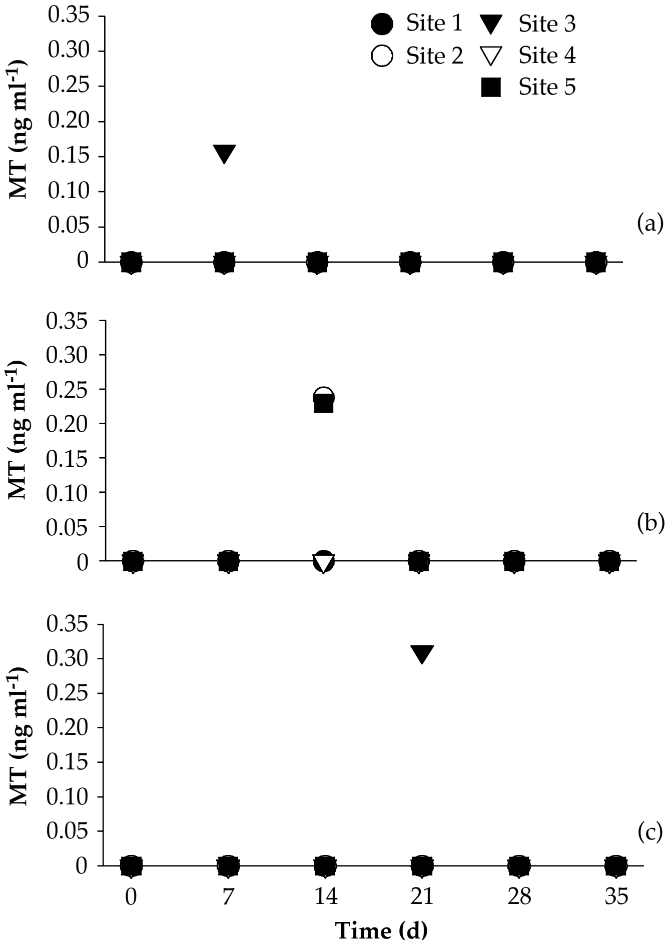

Figure 2. Concentration of 17α-methyltestosterone (MT) in water from five sampling points (sites 1–5) measured at different times during masculinization Trial 1. Treatments were: (a) no activated charcoal (EU-1), (b) 2.5 kg of activated charcoal (EU-2), and (c) 5.0 kg of activated charcoal (EU-3). For this trial new vegetal charcoal was used in the filtration systems.

Fish and Wildlife Research Unit laboratory, Department of Fisheries and Wildlife, OSU. For analysis, 0.5 ml of each water sample and 0.2 g of each charcoal sample were extracted with 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 0.5 ml of phosphate-buffered saline containing gelatin (PBSG). 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) and Fitzpatrick et al. (1987). Antiserum specific to MT was purchased from Animal Pharm Services, and ^{3}H-MT (Amersham) was generously donated by Gordon Grau of the Hawaii Institute of Marine Biology. Standards of known concentration of MT were made in ethanol 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 charcoal (n = 5 for each) and then extracting the samples as described above. Once each of these tubes was reconstituted in 1 ml of PBSG, 0.5 ml was removed from each, and the amount of radioactivity was determined by scintillation spectroscopy. Extraction efficiencies were 85.6% for water and 29.6% for charcoal. Concentrations of MT in water and charcoal 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 experiment was descriptive (presence/absence).

Study B: Implementation of Safe Intensive Sex-Inversion System in Local Tilapia Farms

This study was conducted at two sites: the *ejido* Rio Playa and the state hatchery, José Narciso Rovirosa. A third site (Centro de Producción y Fomento Piscicola "Lacandona," Chiapas) was originally considered, but the lack of electricity at the site restricted our work to two locations.

Ejido Rio Playa

Nile tilapia fry were obtained from spawning ponds. Thirty-two thousand fry were selected by grading with a 3-mm mesh, counted, and stocked in 2 m^2 mosquito mesh hapas at a density of 3,000 fry m^{-2}. The hapas were placed in a 40 m^2 concrete pond adapted with two 250-l chemical filters. Both filters simultaneously received water pumped from a single external pump. Based on the results from the first experiment, we decided to use 3.0 kg of activated charcoal in each of the filters. To determine the efficiency of the masculinization treatment, a control group of 3,000 fry was placed in a hapa and fed in a grow-out pond.

State-Hatchery, José Narciso Rovirosa

The procedure used in this hatchery was similar to the one used in Rio Playa; however, the dimensions of the ponds and hapas, as well as the density used, differed. Eighty thousand fry were obtained from spawning ponds, selected by grading with a 3-mm mesh, counted, and stocked in 4 m^2 mosquito mesh hapas at a density of 5,000 fry m^{-2}. The hapas were placed in a 110 m^2 concrete pond adapted with the chemical filters (same as described above).

In both sites, fish treated for masculinization were fed with commercial MT-impregnated food, El Pedregal (Silver Cup™, Toluca, Mexico), containing 60 mg of MT kg^{-1} of food. The same feed, containing no MT, was fed to control fish. Feeding rates, survival, and sex ratios were determined as described above. To maintain water quality, water exchange (20%) was performed weekly in both farms and for both treated and untreated fish.

Water (12 ml) samples were collected with pipettes into 15 ml polypropylene tubes from the sex-inversion tank on days 0, 7, 14, 21, and 28. Five sites were established in the pond (one for each corner and one at the center). Samples were taken in the morning before first feeding, frozen (–20°C), and preserved until processed for MT detection at OSU.

RESULTS

Experiment A: Elimination of MT from the Water of Intensive Sex-Inversion Systems

Table 1. Concentration of 17α-Methyltestosterone in water (ng ml^{-1}) from 5 sampling points measured at different times during four masculinization trials. Treatments were: a) no activated charcoal, b) 2.5 kg of activated charcoal, and c) 5.0 kg of activated charcoal. Non-detectable values are represented with a hyphen.

		Treatments/Sampling Points														
Trial	*Day*	*0.0 Kg of charcoal*					*2.5 Kg of charcoal*					*5.0 Kg of charcoal*				
1		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	7	-	-	0.158	-	-	-	-	-	-	-	-	-	-	-	-
	14	-	-	-	-	-	-	-	-	-	-	-	0.238	-	-	0.230
	21	-	-	-	-	-	-	-	0.310	-	-	-	-	-	-	-
	28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2																
		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9.170
	21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	35	-	-	-	-	-	-	-	0.240	-	-	-	-	-	-	-
3																
		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	28	0.140	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4																
		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	0.250	-	-	-	-	-
	14	-	-	-	-	-	-	0.410	-	-	-	-	-	-	-	-
	21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	28	-	0.160	-	-	-	-	-	-	-	-	-	-	-	-	-
	35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Trial 1 (29 March to 1 May 2002)

The objective of this preliminary trial was to check for possible failures in the filtration system. For this trial vegetal charcoal was used, and the initial density was 2,200 fry m^{-2}. MT was undetectable in almost all of the water samples from each of the experimental units (EU) at all times (Table 1). When MT was detectable, it was seen in one or two sites but never in all simultaneously. MT values in all experimental units ranged from 0.16 to 0.30 ng ml^{-1}. MT was detected in the treatment with no charcoal (EU-1) only on day 7 at site 3 (0.158 ng ml^{-1}) (Figure 2a). In the treatment with 2.5 kg of charcoal (EU-2), MT was detected on day 21 at site 5. MT was detected in the treatment with 5.0 kg of charcoal (EU-3) on day 14 at sites 2 and 3 (0.238 and 0.230 ng ml^{-1}, respectively) (Figure 2c). Adsorption values measured in the granular vegetal charcoal from EU-2 ranged between 92.03 ng g^{-1} (day 14) and 48.40 ng g^{-1} (day 35), while values for EU-3 MT values ranged between 99.58 ng g^{-1} (day 14) and 75.82 ng g^{-1} (day 35) (Figure 3a). In both cases the concentration of MT at day 7 reached 80 ng g^{-1}, increased at day 14, and decreased afterwards as the trial progressed.

Temperature, pH, and DO values ranged between 28.0 to 31.4°C, 7.7 to 8.3, and 3.2 to 8.0 mg l^{-1}, respectively. No specific patterns were observed among the experimental units. Ammonia values ranged between 0.04 and 2.0 mg l^{-1}, showing a steady increase towards the end of the trial.

Trial 2 (6 May to 3 June 2002)

For this trial mineral charcoal was used, and the initial density was 2,800 fry m^{-2}. MT was detected in water only in the treatments with charcoal; the treatment with no charcoal did not show any MT in water at any sampling time (Table 1). The highest MT value measured in water for the entire experiment was seen for the treatment with 5.0 kg of charcoal (EU-3) showing 9.17 ng ml^{-1} on day 14 at site 1 (but not at any other sites); MT was non-detectable afterwards (Figure 4c). MT was detected in the treatment with 2.5 kg of charcoal (EU-2) on day 35 at site 3 (0.24 ng ml^{-1}) (Figure 4b). Adsorption values measured in the granular mineral charcoal from EU-2 and EU-3 ranged between 22.30 and 35.27 ng g^{-1} and remained fairly constant during the trial (Figure 3d and 5). These values indicate a low adsorption capacity for the mineral charcoal. Exposure of charcoal to sunlight at the end of the trial showed that MT concentrations ranged between 17.86 and 37.76 ng g^{-1} for EU-3

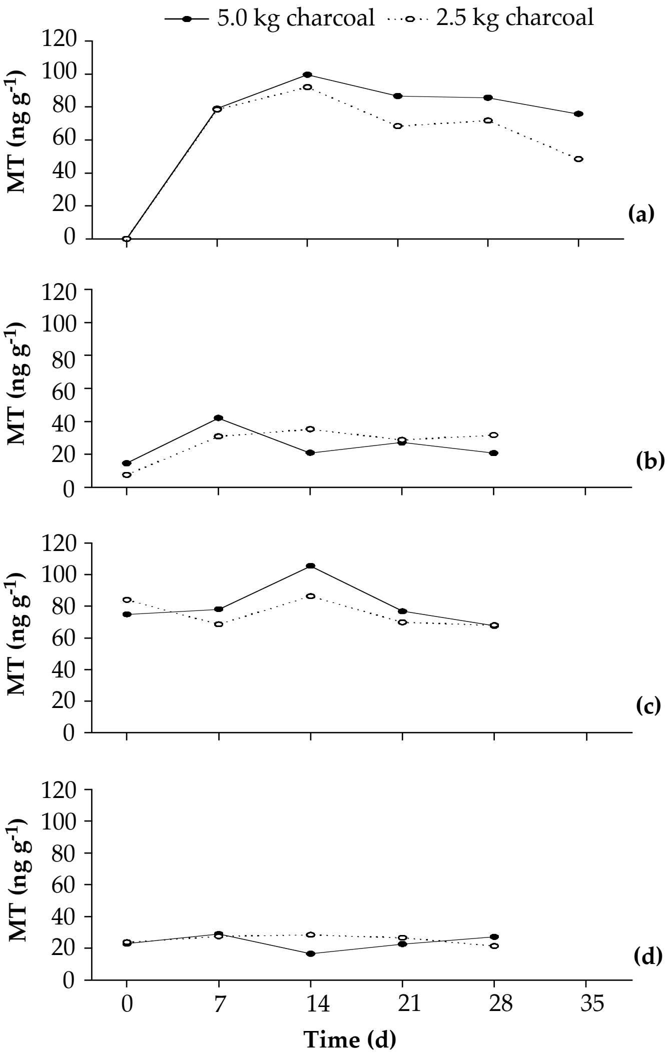

Figure 3. Concentration of 17α-methyltestosterone (MT) (ng g^{-1}) in charcoal during masculinization cycles; (a) Trial 1 using new vegetal charcoal; (b) Trial 2 using new mineral charcoal; (c) Trial 3 reusing vegetal charcoal used in Trial 1; and (d) Trial 4 reusing mineral charcoal used in Trial 2.

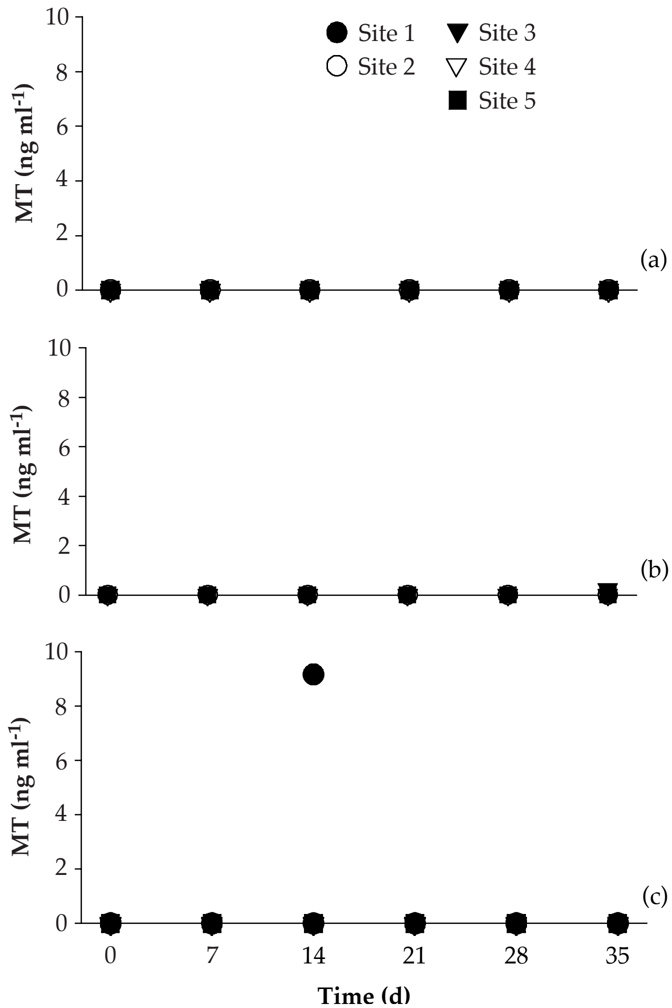

Figure 4. Concentration of 17α-methyltestosterone (MT) in water from five sampling points (sites 1–5) measured at different times during masculinization Trial 2. Treatments were: (a) no activated charcoal (EU-1); (b) 2.5 kg of activated charcoal (EU-2); and (c) 5.0 kg of activated charcoal (EU-3). For this trial new mineral charcoal was used in the filtration systems.

and 26.59 and 31.27 ng g^{-1} for EU-2, showing a tendency to increase after 48 h of exposure (Figure 3a and 5). However, some charcoal samples were not completely dry (the amount of water remaining was inversely proportional to the number of hours of sunlight exposure); therefore, the amount of charcoal weighed out prior to extraction was less for earlier sampling dates. This probably accounts for the higher levels seen at 48 h.

Temperature, pH, and DO values ranged between 28.3 to 30.9°C, 6.8 to 8.7, and 3.0 to 7.2 mg l^{-1}, respectively. No specific patterns were observed among the experimental units. Ammonia, nitrite, and nitrate values ranged between 0.06 to 2.45, 0.91 to 3.04, and 3.10 to 18.12 mg l^{-1}, respectively. These parameters showed a steady increase towards the end of the trial.

Trial 3 (15 June to 8 July 2002)

Vegetal charcoal used in the first trial was reused after being exposed to sunlight and stored for 75 d in black plastic bags. The initial density was 2,450 fry m^{-2}. MT was detected in water from the treatment with no charcoal only at day 28 at site 2 (0.14 ng ml^{-1}) (Table 1 and Figure 6a). Adsorption values measured in the granular vegetal charcoal from EU-2 and EU-3 ranged between 67.50 and 105.50 ng g^{-1} (Figure 3c). The highest value was observed in the treatment with 5.0 kg of charcoal at day 14; however, MT values remained fairly constant during the trial. Exposure of charcoal to sunlight at the end of this trial showed that MT concentrations ranged between 38.94 and 75.85 ng g^{-1} for EU-2 and 31.84 and 88.47 ng g^{-1} for EU-3, showing a tendency to increase after 48 h of exposure (Figure 3b and 5).

Temperature, pH, and DO values ranged between 28.0 to 31.1°C, 6.5 to 7.7 and 3.5 to 8.0 mg l^{-1}, respectively. Once again no specific patterns were observed among the experimental units. Ammonia, nitrite, and nitrate values ranged between 0.13 to 3.48, 0.24 to 1.82, and 0.44 to 14.39 mg l^{-1}, respectively.

Trial 4 (16 July to 17 August 2002)

Mineral charcoal used in the second trial was re-used after being exposed to sunlight for 48 h and stored for 45 d in black plastic bags. The initial density was 2,945 fry m^{-2}. MT was

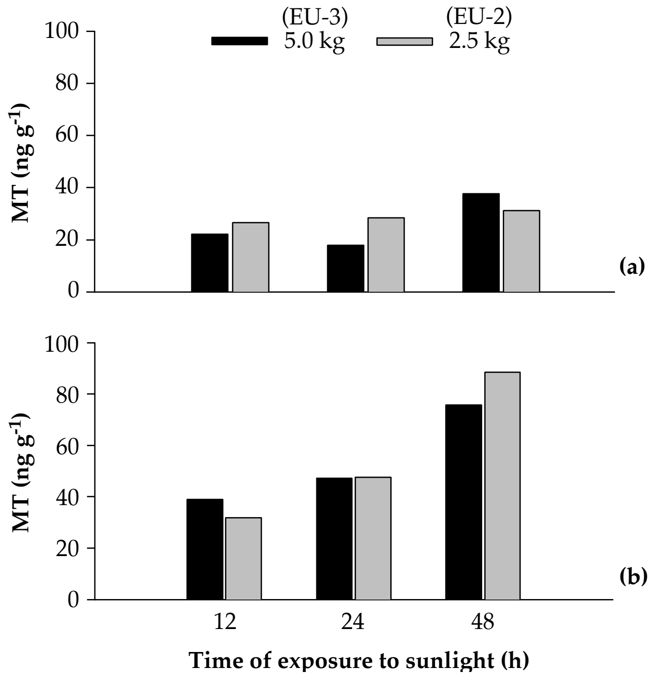

Figure 5. 17α-methyltestosterone (MT) concentrations in charcoal after being exposed to sunlight; (a) mineral charcoal used in Trial 2 and (b) vegetal charcoal used in Trial 3. Legend identifies the amount of charcoal used in each treatment.

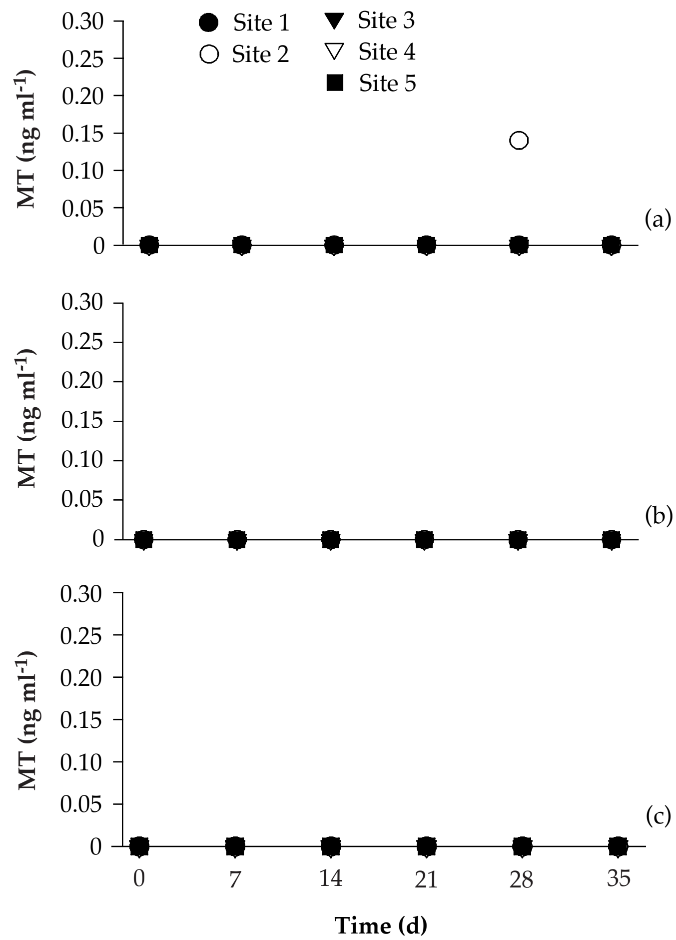

Figure 6. Concentration of 17α-methyltestosterone (MT) in water from five sampling points (sites 1–5) measured at different times during masculinization Trial 3. Treatments were: (a) no activated charcoal, (b) 2.5 kg of activated charcoal, and (c) 5.0 kg of activated charcoal. For this trial vegetal charcoal used in the first trial was reused in the filtration systems.

detected in water only in the treatments with no charcoal and with 2.5 kg of charcoal (Table 1). The treatment with 5.0 kg of charcoal showed no MT in the water at any sampling time. The MT values for the treatment with 2.5 kg of charcoal (EU-2) were 0.25 ng ml^{-1} on day 7 at site 5 and 0.41 ng ml^{-1} on day 14 at site 2 (Figure 7b). MT returned to non-detectable levels afterwards. A lower MT value (0.16 ng ml^{-1}) was measured in the treatment with no charcoal (EU-1) on day 28 at site 2. Adsorption values measured in the granular mineral charcoal from EU-2 and EU-3 ranged between 16.52 and 28.83 ng g^{-1} and remained fairly constant during the trial (Figure 3d). These values indicate, once again, a low adsorption capacity for the mineral charcoal.

Temperature, pH, and DO values ranged between 22.0 to 32.0°C, 6.5 to 7.9, and 1.4 to 8.0 mg l^{-1}, respectively. Ammonia, nitrite, and nitrate values ranged between 0.25 to 5.16, 0.30 to 3.65, and 0.44 to 45.16 mg l^{-1}, respectively. These compounds showed a steady increase towards the end of the trial.

Masculinizing Efficiency of MT and Survival

The efficacy of MT for masculinizing Nile tilapia fry was high during the entire experiment. No significant differences were found between trials or treatments (Chi square test, $P > 0.05$). Treatment with 0.0, 2.5, and 5.0 kg of charcoal yielded an average of 93.8, 93.5, and 94.0% males, respectively, while the average percent of males for the control groups was 51.7 (Figure 8a). Control groups had significantly higher survival than treatment groups (Figure 8b), while no significant differences were observed between MT-treated groups (43.5, 41.5, and 47.0%, respectively).

Study B: Implementation of Safe Intensive Sex-Inversion System in Local Tilapia Farms

Vegetal charcoal was used at both farms, and one masculinization cycle was performed at each farm. MT was detected only once at each place. In the trial performed at the Jose Narciso Rovirosa farm (JNF), 0.57 ng ml^{-1} of MT was detected at site 1 on day 21, while 0.46 ng ml^{-1} was found in the pond used at the Rio Playa farm (RPF) at site 3 on day 28 of experimentation (Table 2 and Figure 9). MT was not detected at any other sampling times or sites.

Masculinization efficiency was high at both farms, reaching 97% at JNF and 99% at RPF, while the proportion of males for the control treatments were 48 and 38%, respectively (Figure 10a). Chi square tests indicated a significantly higher proportion of males for the MT-treated groups ($P < 0.001$). Survival for the MT-treated groups was 80% for JNF and 72% at RPF, while 80 and 78% of the fish survived in the control groups at each farm, respectively (Figure 10b). Average temperature, DO, pH, and ammonia were 28.6 and 28.0°C, 3.0 and 4.4 mg l^{-1}, 7.6 and 8.4, and 3.3 and 0.26 mg l^{-1}, respectively, for RPF and JNF.

DISCUSSION

Recent studies in our laboratory have shown that MT can be detected in the water during feeding of MT-impregnated diets,

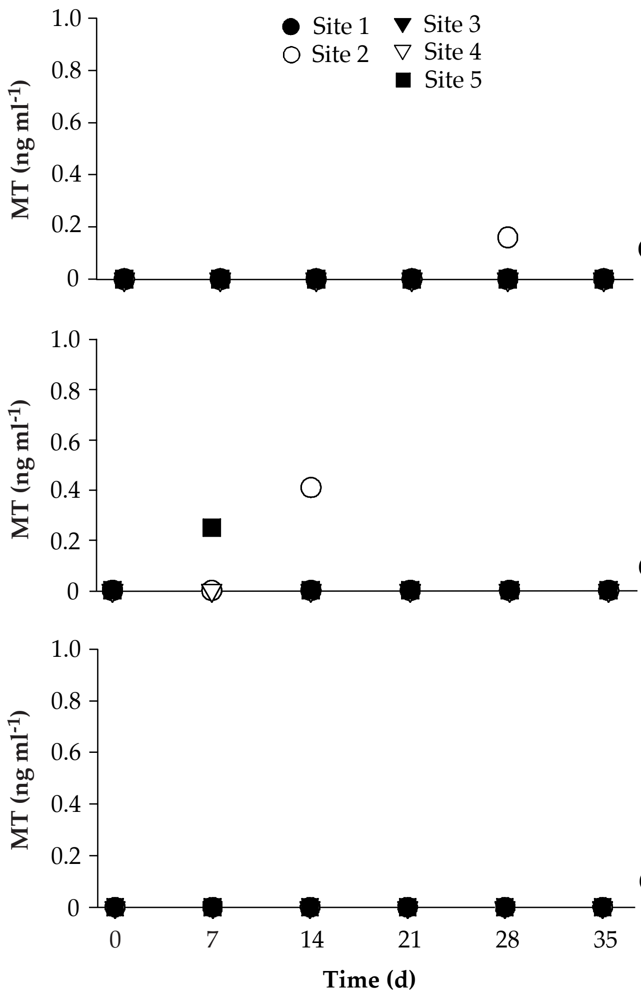

Figure 7. Concentration of 17α-methyltestosterone (MT) in water from five sampling points (sites 1–5) measured at different times during masculinization Trial 4. Treatments were: (a) no activated charcoal (EU-1), (b) 2.5 kg of activated charcoal (EU-2), and (c) 5.0 kg of activated charcoal (EU-3). For this trial mineral charcoal used in the second trial was reused in the filtration systems.

and the steroid eventually accumulated and remained in the sediments of aquaria and ponds for several weeks (Fitzpatrick et al., 2000; Contreras-Sánchez 2001; Contreras-Sánchez et al., 2001). In the current study, we determined that during the masculinization of tilapia fry in intensive systems, MT could be efficiently removed from the water using chemical or biological filtration. In general MT was not detectable in the water at the end of the trials in any of the systems that had a filtration system. MT levels in water were detected at low levels at different times, but the compound was never seen consistently at all sites within a tank. The MT RIA was relatively insensitive (10 pg per tube compared with typically 1 to 3 pg per tube) and we suspect that these few detections of low MT were spurious. From all of the samples examined, MT was detected only 4.17% of the time. MT concentrations varied between 0.14 and 9.17 ng ml^{-1} and the appearance of the largest value occurred as an isolated case. All the other values were below 1.0 ng ml^{-1}. We speculate that the single high value may have been caused by a particle of fecal material captured during sampling that may have contained high levels of MT.

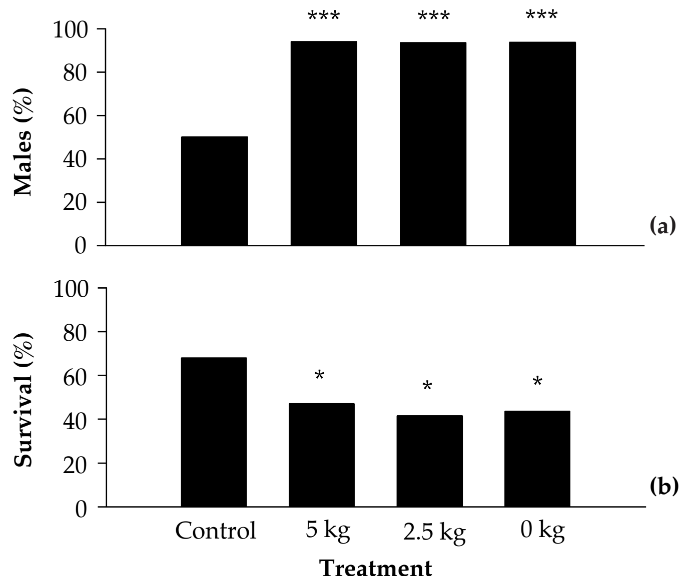

Figure 8. Mean percent of males (a) and survival (b) of Nile tilapia fry obtained after masculinization trials. Percents were obtained from four masculinization cycles. Asterisks represent significant differences between the treatments and the control groups (Chi square test, $P < 0.05$).

Surprisingly, MT levels showed the same pattern of variability and low values in the systems with and without charcoal. This may be caused by two factors:

1) biodegradation of MT in the biological portion of the filter with no charcoal and
2) sunlight degradation of the MT molecules.

Biotransformation of steroids by bacteria and fungi has been reported by several authors (Datcheva et al., 1989; Ahmed et al., 1996; Oppermann et al., 1996).

This well-known process has been employed as a method for

Table 2. Concentration of 17α-Methyltestosterone in water (ng ml^{-1}) from 5 sampling points measured at different times during four masculinization trials at the farms José Narciso Rovirosa (JNF) and Rio Playa (RPF). Filters contained 3.0 kg of activated charcoal. Non-detectable values are represented with a hyphen.

Farm	Day	3.0 Kg of charcoal				
JNF		1	2	3	4	5
	0	-	-	-	-	-
	7	-	-	-	-	-
	14	-	-	-	-	-
	21	0.570	-	-	-	-
	28	-	-	-	-	-
RPF		1	2	3	4	5
	0	-	-	-	-	-
	7	-	-	-	-	-
	14	-	-	-	-	-
	21	-	-	-	-	-
	28	-	-	0.460	-	-

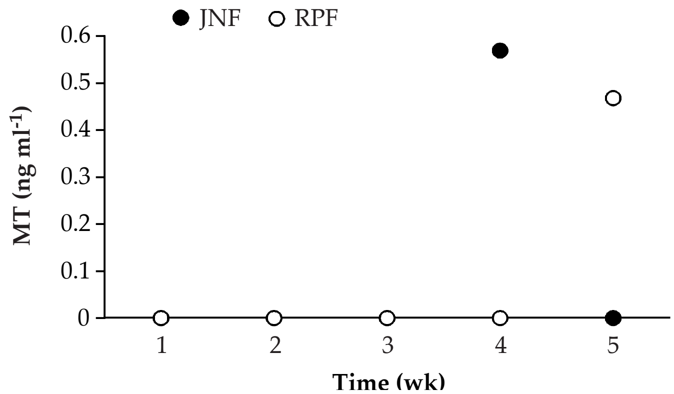

Figure 9. Concentration of 17α-methyltestosterone (MT) in water measured at different times during masculinization trials conducted at the Jose Narciso Rovorisa Farm (JNF) and the Rio Playa Farm (RPF).

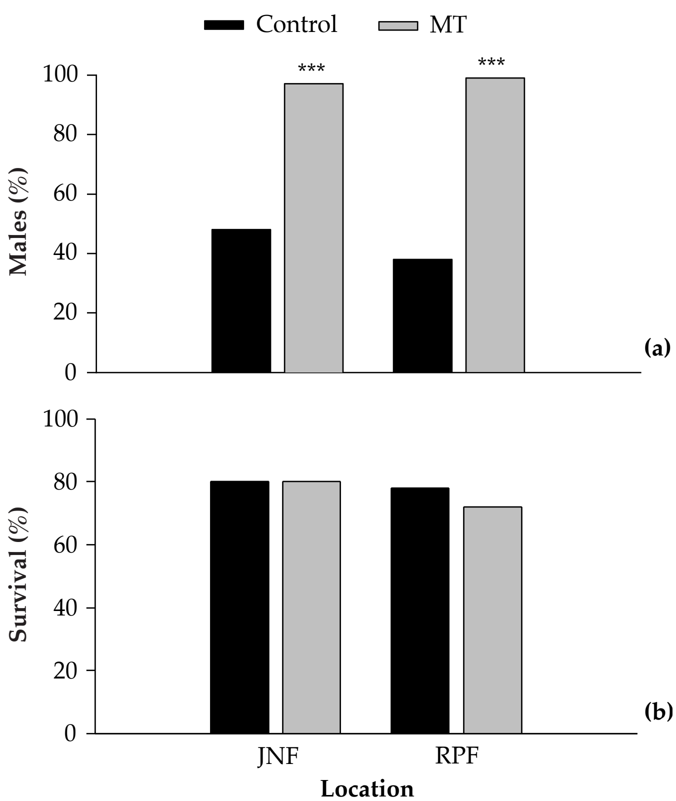

Figure 10. Mean percent of males (a) and survival (b) of Nile tilapia fry obtained after masculinization trials at the Jose Narciso Rovirosa Farm (JNF) and the Rio Playa Farm (RPF). Percentages represent a single value from a masculinization cycle. Asterisks represent significant differences between MT-treated and control groups (Chi square test, $P < 0.05$).

preparation of hydroxysteroids used in research as well as for product development (Holland, 1999). Despite this, little research has been reported regarding bacterial transformation of steroids in the water environment.
Degradation of steroids by exposure to sunlight is a well-known process, and some companies that sell synthetic steroids recommend keeping them in the dark. Budavari et al. (1989) and Sigma Chemical Company (1994) have indicated that methyltestosterone is a light-sensitive steroid subject to photodegradation. The RAS used in our experiments allowed water to be exposed to both bacteria and sunlight. This system may, therefore, have created conditions that are favorable for these two processes to metabolize MT. The flow rate created by the pump (60 l per minute) allowed for at least 9 complete passes each day of the entire volume of water through the biofilter, which had a large surface area that was in constant contact with the water. The water curtain created by the perforated return pipe also allowed for constant exposure of water to sunlight for at least 8 h a day.

We were able to demonstrate that activated charcoal efficiently captures MT, but that exposure to sunlight does not efficiently degrade the steroid. The apparent increase of MT while charcoal is exposed to sunlight may be due to a sampling error, probably related to the different moisture content of the samples. The permanence of MT in the charcoal may be due to the porous structure of the charcoal material, which may hide the compound from direct exposure to sunlight. Data from our experiment also shows that vegetal charcoal has a higher adsorption capacity than mineral charcoal. In the two cases that vegetal charcoal was used (new and reused), levels of MT levels neared 100 ng g^{-1}, while mineral charcoal never reached 50 ng g^{-1}. The higher level may reflect the saturation point for both types of charcoal; however, more research is needed to determine if this is the case.

The relatively low values of MT detected during the experiment do not minimize the potential risks posed by using MT-impregnated feed during aquacultural operations. It is well known that many chemicals can exert biological functions in very low concentrations (parts per million or parts per billion). Concentrations of picograms per milliliter or gram may have a significant biological effect in the context of androgen and estrogen levels in whole fish embryos and larvae. For example, Feist et al. (1990) found that the concentration of sex steroids from whole-body extracts of fish during the stage of sexual differentiation ranged between < 100 to 900 pg g^{-1} for testosterone, 11-ketotestosterone, androstenedione, 17α-hydroxy-20β-dihydroprogesterone, and 17β-estradiol. Given the biological potency of these steroids at low concentrations, protective gloves and clothes should be used by workers who will come in contact with water used in masculinization systems.

The results obtained from intensive masculinization systems implemented at two tilapia farms in Tabasco, Mexico, demonstrate that MT can be removed from the water whilst allowing high masculinization rates and good fry survival. These large systems showed better water quality than the systems used at UJAT, resulting in higher survival rates.

Workshops on Masculinzation and Safe Handling of Steroids

Three workshops were conducted during the development of this study. Twenty-five people attended a workshop implemented in Villahermosa. This workshop was directed towards farmers, extension workers, and students. Two other workshops were implemented at the *ejido* Rio Playa (Comalcalco, Tabasco) and the state farm, Jose Narciso Rovirosa (Teapa, Tabasco). A total of 23 farmers attended these workshops where they received training for the construction of a filtration system and the safe handling of steroids for masculinization purposes.

CONCLUSIONS

The problems associated with contamination of water and sediments are further compounded by the many effects related to bioaccumulation and the transfer of the contaminants and their metabolites through the food web (Kime, 1998). Therefore, it is important to promote the use of preventive measurements such as filtration or biodegradation to remove the steroid and its metabolites. The use of filtration systems in tanks used for intensive masculinization of tilapia fry using MT-impregnated food results in a substantial reduction of the steroid. Despite large variability in the data, it can be concluded that MT is eliminated from the water by the end of the treatments that also allow high masculinization rates. More research is needed to determine if the concentration of MT or its metabolites in effluents may pose a potential environmental risk. We suggest that caution should be exercised because of the risk of unintended MT exposure to pond workers, fish, and other organisms.

The aquaculture industry is already involved in the use of Best Management Practices (BMP) in attempts to respond to increased environmental and regulatory pressures (Boyd and Schmittou, 1999). If usage of MT is not approached with preventive measurements, the use of hormones for sex inversion in aquaculture may become another environmental concern for the industry. We recommend the use of activated charcoal filtration systems to eliminate excess MT and potentially increase masculinization.

ANTICIPATED BENEFITS

The elimination of the anabolic steroid MT in the water from sex-inversion tanks provides key information related to the elimination of residual MT from masculinization systems. The successful implementation of filtration systems represents a step forward in the use of environmentally friendly aquaculture techniques. The workshops conducted at Villahermosa, Teapa, and Rio Playa in Tabasco were well received by farmers, students, and technicians that work in aquaculture in the region. We will continue implementing such workshops to make information available to all interested users.

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