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ELIMINATION OF METHYLTESTOSTERONE FROM INTENSIVE MASCULINIZATION SYSTEMS: USE OF ULTRAVIOLET IRRADIATION OF WATER

Eleventh Work Plan, Water Quality and Availability Research 1 (11WQAR1)
Final Report

Carl B. Schreck
Oregon Cooperative Fishery Research Unit
Department of Fisheries and Wildlife
Oregon State University
Corvallis, Oregon

Guillermo R. Giannico and Grant W. Feist
Department of Fisheries and Wildlife
Oregon State University
Corvallis, Oregon

Wilfrido M. Contreras-Sánchez, Luis E. Pascual-Valencia, Ulises Hernández-Vidal, and Bernardita Campos-Campos
Laboratorio de Acuacultura
División Académica de Ciencias Biológicas
Universidad Juárez Autónoma de Tabasco
Villahermosa, Tabasco, México

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ABSTRACT

This study tested the hypothesis that 17α-methyltestosterone (MT) could be eliminated from the water used in intensive sex-inversion systems using sunlight and UV sterilizers. Different concentrations of MT diluted in water were exposed to UV or sunlight and measured through time for 48 hours. Water samples were collected from aquaria at the onset of treatments and at 2, 4, 8, 24, and 48 hours. All samples were extracted using ether and the concentration of MT was determined by radioimmunoassay. We also evaluated the elimination of MT in masculinization systems and the masculinizing effects of effluents produced in these systems on tilapia. MT was partially eliminated when water was exposed to direct sunlight; however, MT was completely eliminated from water following exposure to UV. At the end of the treatments, sunlight exposure eliminated between 48 and 62% of the MT detected at the beginning of the trials. When water with MT was exposed to UV light, MT was not detectable after 48 h of treatment. When intensive masculinization systems were used, MT was only detected in 7.1% of the water samples. Five of the detections were from tanks that received MT that had no UV sterilizers and six samples were from tanks with UV sterilizers. Significant masculinization was obtained when MT was administered through the food and the results indicate that UV treatment allowed for higher masculinization rates. Our results also indicate that effluents from masculinization systems can masculinize fish that are not the target of the treatment. This may be due to the sterilizers degrading MT slowly over time or perhaps that the UV degradation products resulted in compounds that increased masculinization. More research is needed regarding treatment methods for masculinization effluents to eliminate the risks of unintended exposure to humans and other non-target organisms.

INTRODUCTION

All-male populations are used in tilapia (*Oreochromis* sp.) aquaculture because the culture of mixed sex populations often results in precocious maturation and early reproduction (Schreck, 1974; Mires, 1995). Furthermore, all-male tilapia populations are desirable because males achieve a larger final size than females (MacIntosh and Little, 1995).

Masculinization of tilapia fry by oral administration of 17α-methyltestosterone (MT) is considered the most successful method employed; however, under certain conditions this technique is sometimes unreliable.

Furthermore, significant "leakage" of MT into the pond environment may occur 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 the steroid or its metabolites.

In recent studies (Contreras-Sánchez, 2001), we found that masculinization of fry through dietary treatment with MT resulted in the accumulation of MT in sediments which produced both intersex fish and females with altered ovarian development. In systems where substrate was not present, there were higher concentrations of MT in the water and lower (sometimes null) masculinization rates than in systems with either soil or gravel. We found that charcoal filtration of water from systems where substrate was not present lowered the amount of MT in water to almost background levels and the treatment resulted in almost complete masculinization of all three broods tested (100, 98 and 100% males, respectively). Apparently, the recommended dose of MT for masculinizing tilapia is higher than needed and a significant portion of it separates from the food and remains either in suspension in the water for the short term or persists in the sediments over the long term (Contreras-Sánchez, 2001).

Methyltestosterone is a light sensitive hormone, which is subject to photodegradation (Budavari et al., 1989; Sigma Chemical Company, 1994). The type of light most likely responsible for photodegradation is UV-B (wavelengths of 280-315 nm). Methyltestosterone absorbs UV light strongly at a wavelength of 254 nm, which is in the UV-C part of the spectrum (100-280 nm), and absorbs UV weakly in the UV-B area of the spectrum. Unlike UV-B, UV-C is quickly absorbed in the atmosphere and does not reach the earth's surface. Since MT does not absorb UV-B very effectively, treatment with irradiation at 254 nm should be much more effective than exposure to sunlight or UV-B. Virtually nothing is known about the amount of exposure to UV needed to remove MT or of possible metabolites produced during photodegradation. Commercial ultraviolet water sterilizers are currently used by some growers to destroy pathogens. These sterilizers emit UV light at a wavelength of 254 nm.

We propose the use of intensive systems for masculinizing tilapia fry using MT-impregnated food at a large scale where excess MT is eliminated from the water by means of continuous filtration through UV sterilizers. Removal of MT should both increase masculinization rates and reduce the amount entering substrates which could affect other aquatic organisms. This method may allow for the production of large numbers of all-male populations of tilapia fry using a reliable technique compatible with the proposed Best Management Practices for aquaculture systems. Ultraviolet sterilizers are relatively cheap, available in many sizes for different volumes of water in aquaculture systems and can be readily obtained in Southern Mexico.

METHODS AND MATERIALS

Removal of MT from Water by Solar or UV Irradiation (Experiment 1)

This experiment was conducted at the Oregon Cooperative Fish and Wildlife Research Unit laboratory, Department of Fisheries and Wildlife, OSU. The experiment consisted of nine treatments done in duplicate; all exposures lasted for 48 hours.

1) Control water exposed to sunlight;
2) Control water exposed to UV light;
3) 5 mg/l of MT treated water exposed to sunlight;
4) 25 mg/l of MT treated water exposed to sunlight;
5) 50 mg/l of MT treated water exposed to sunlight;
6) 5 mg/l of MT treated water exposed to UV light;
7) 25 mg/l of MT treated water exposed to UV light;
8) 50 mg/l of MT treated water exposed to UV light; and
9) 25 mg/l of MT treated water not exposed to any light.

Untreated control water in aquaria (150 liter) was placed in direct sunlight. Control water in 150 liter aquaria was exposed to UV light at 254 nm in the dark by passing circulating water through a Lifegard® 8 watt UV sterilizer (model UV5) at a comparable rate to that of large masculinization systems. Experimental water was treated with MT at 5, 25 or 50 mg/l and exposed to either sunlight or UV in the same way as for control water. Positive controls were generated by adding MT to water and circulating it through the system in the dark with the UV lamp turned off.

Water samples (2 ml) were collected from the aquaria at 0, 2, 4, 8, 24 and 48 hours. One sampling point was established for each tank at the center of the tank. Samples were obtained, frozen (-80 °C) and preserved until processed for MT.

Radioimmunoassay

For analysis, 0.5 ml of each water sample was 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 Speed-Vac. Each dried extract was reconstituted in 0.5 ml of Phosphate-Buffered Saline containing gelatin (PBSG). Aliquots of the reconstituted extracts were removed to 12x75 mm tubes for determination of MT concentration

by radioimmunoassay (RIA). The RIA methods followed the procedure outlined in Fitzpatrick *et al.* (1986 and 1987). Antiserum specific to MT was 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 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, (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 for water were 87.5%.

Elimination of MT from the Water of Intensive Sex-Inversion Systems (Experiments 2a and 2b)

This experiment was conducted at the Laboratory of Aquaculture at UJAT, Tabasco, Mexico. The experiment consisted of four treatments, done in duplicate:

1) Fry fed control food for 28 days; water not treated with a UV sterilizer;
2) Fry fed control food for 28 days; water treated with a UV sterilizer;
3) Fry fed MT at 60 mg kg^{-1} food for 28 days; water not treated with a UV sterilizer; and
4) Fry fed MT at 60 mg kg^{-1} food for 28 days; water treated with a UV sterilizer.

Eight concrete tanks (5.0 x 1.0 x 1.0 m) were used as experimental units. Each tank had a recirculation system equipped with a 1/2 hp centrifugal pump connected to a PVC pipeline used to recirculate water from the bottom of the tank. At the end of the returning section, the pipeline was perforated with several holes to create a water curtain as water returned to the concrete tank. Those tanks that were UV treated were adapted with a 25 watt Emperor Aquatics™ UV water sterilizer (model 02025). Water flow was 42 l/min. When corrected for water volume and flow rate, the UV sterilizer used in this experiment emitted 5 to 10 fold less UV light (depending on the vendor) when compared to the system used in experiment 1. A ball valve was adapted to the exit of each pump to send water to tanks used to determine biological activity of MT and photodegraded MT metabolites that might be present in the effluent (described below).

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 2,500 fry/m^3 (n = 12,500) for experiment 2a; however, the number of fish used in the second trial (2b) varied because of fry availability at the time of the trial (n = 2,200).

Commercial control and MT-impregnated (60 mg MT/kg) diets were used in the experiment (Silver Cup®, 40% protein). Fry were fed MT or control diet four times a day, for 4 weeks. Feeding rate was 20% of calculated body weight until fish reached 15 mm (approximately 15 days) and then 10% of calculated body weight through the rest of the treatment (Popma and Green 1990). After 28 days of dietary treatment, fry were counted to estimate survival and fed with regular fish food. At 70-90 days 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 and lengths of sampled fish were recorded at that time.

In each of the experimental units, dissolved oxygen, pH and temperature were measured daily. Ammonia, nitrates and nitrites were measured weekly. To maintain water quality, food not eaten and feces were siphoned out of the tank, water exchange (40-50%) was performed three times a week.

Water (2 ml) samples for MT detection were collected from each treatment group once a week. Samples were frozen (–20°C) and preserved until processing. All samples were extracted using diethyl ether and the concentration of MT determined by RIA. At the end of a two-month grow-out period, a sub-sample of the tilapia in each experimental unit (100) were killed with an overdose of anesthetic (MS-222) to determine if the treatment with MT resulted in masculinization.

Determination of Biological Activity of Photodegraded MT Metabolites (Experiments 3a and 3b)

Water used in these experiments came from the tanks used in experiments 2a and 2b and consisted of four treatments, done in duplicate:

1) Fry fed control food for 28 days; tanks received control water not treated with a UV sterilizer;
2) Fry fed control food for 28 days; tanks received control water treated with a UV sterilizer;
3) Fry fed control food for 28 days; tanks received MT water not treated with a UV sterilizer; and
4) Fry fed control food for 28 days; tanks received MT water treated with a UV sterilizer.

Eight plastic tanks (3.0 x 0.5 x 0.3 m) were used as experimental units. Each tank received water from a corresponding concrete tank used in the previous experiment.

Every morning, previous to the first feeding, ninety percent of the water volume was replaced.

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, counted and randomly assigned to each of the experimental units. In experiment 3a (water from experiment 2a), each tank was stocked with 2,000 fry, while in experiment 3b (water from experiment 2b) each tank was stocked with 1,100 fry.

Fry were fed control diet for 4 weeks (Silver Cup™, 40% protein). Feeding rate, sex identification, and sampling procedures were the same as previously described.

Statistical analysis. Data from the first experiment were analyzed graphically using Sigma Plot™. Percent values of MT in water were compared between either control or treated water, exposure to UV, sunlight or darkness, and for exposure time in treated water using ANOVA with $p < 0.05$. Fish sex ratios were compared using contingency tables with a Chi square test. Differences in growth were determined using an analysis of covariance (ANCOVA) using survival as the covariate factor.

RESULTS

Removal of MT from Water by Solar or UV Irradiation (Experiment 1)

MT was partially eliminated when water was exposed to direct sunlight; however, MT was completely eliminated from water exposed to UV (Fig. 1). At the end of the treatments, sunlight exposure eliminated between 48 and 62% of the MT detected at the beginning of the trials. When water with MT was exposed to UV light, MT decreased more than 50% in two cases (5 and 50 ng/ml) after 4 h of treatment. By 24 h, MT reached less than 6 ng/ml in the treatment with 50 ng/ml and was not detectable in the other two treatments. At the end of the trial MT was not detectable in any of the treatments. When water with MT was kept in the dark, the concentration of MT in the tanks remained stable ranging between 14 and 20 ng/ml.

Elimination of MT from the Water of Intensive Sex-Inversion Systems (Experiment 2a; n=12,500 fry/tank)

The total amount of MT added to each tank via feeding during the entire experiment was 463.87 mg, starting with 6.2 mg at day 1 and finishing with 47.7 on day 28. During this experiment MT was only detected in 7.1% of the total amount of samples processed, five samples were from tanks that received MT and had no UV sterilizers and six samples were from tanks with UV sterilizers. Amounts of MT detected ranged between 0.5 and 1.5 mg of MT/tank in systems without UV sterilizers and 0.4 and 1.6 mg of MT/tank in systems with UV sterilizers. The percent of MT remaining in the water varied between 1.4 to 13.5% of the total amount fed in a particular day for tanks with no UV sterilizers and 0.7 to 3.4% for tanks with UV sterilizers. This indicates that the tanks with no UV sterilizers had an average of four times more MT than those thanks that had UV sterilizers installed.

Masculinization

Fish that received MT and had UV sterilizers installed had a significantly higher percentage of males than those that did not receive UV light treatment (98.41 ± 1.0% and 91.75 ± 1.9%, respectively; $P < 0.001$, Fig. 2). Both groups of MT-treated fish had significantly higher percentages of males than controls ($P < 0.001$) which averaged 50.0 ± 12.38% males (controls with no MT and water exposed to UV light) and 69.55 ± 1.07% males (controls with no MT and water not exposed to UV light). No significant differences were found between the two control groups ($P = 0.62$).

Growth

The analysis of covariance (using mortality as the covariable) indicated that there was no significant differences in growth between treatments ($P = 0.23$ for weight and $P = 0.34$ for length). Mortality was different between treatments having a significant effect on fish growth ($P = 0.024$). Data for growth and survival are shown in Table 1.

Determination of Biological Activity of Photodegraded MT Metabolites (Experiment 3a; n=2000 fry/tank)

During this experiment MT was detected in 5.3% of the total amount of samples of effluents analyzed. Two samples were from tanks that received effluents with MT and had no UV treatment and six samples were from tanks that received effluents with MT and were treated with UV sterilizers. Amounts of MT detected ranged between 0.4 and 0.8 mg of MT/tank in systems without UV treatment and 0.4 and 1.4 mg of MT/tank in systems with UV treatment.

Fish that were exposed to effluents from experiment 2a had similar sex proportions to that observed in the treatments where fish were fed with or without MT (Fig. 2). A higher number of males was observed in the tanks that received MT-treated effluents exposed to UV (91.83 ± 3.9% males), while tanks that received MT-treated effluents not exposed to UV had 78.36 ± 0.7% males ($P = 0.005$). Tanks that received effluents from control tanks with or without UV had a significantly lower ($P = 0.001$) number of males (66.59 ± 3.04 and 64.51 ± 5.59% males, respectively). No significant differences were found between control groups ($P = 0.77$). No significant differ-

ences were found in growth or mortality (P = 0.80 and 0.76, respectively; Table 2).

Elimination of MT from the Water of Intensive Sex-Inversion Systems (Experiment 2b; n=2,200 fry/tank).

The total amount of MT added to each tank via feeding during the entire experiment was 81.61 mg. The initial amount used (day 1) was 1.1 mg and the final amount on day 28 was 7.9 mg. Since MT was rarely detected in experiment 2a, MT was not measured by RIA in this experiment.

Masculinization

Results were similar to those obtained in experiment 2a (Fig. 3). Fish that received MT and had UV sterilizers installed had a significantly higher percentage of males than those that did not receive UV light treatment (96.5 ± 0.5% and 88.0 ± 5.0%, respectively; P = 0.002). These two treatments of MT-treated fish had a significantly higher percentage of males than the controls (P < 0.001) that averaged 45.13 ± 2.87% males (controls with no MT and water exposed to UV light) and 45.5 ± 9.5% males (controls with no MT and water not exposed to UV light). No significant differences were found between these two control groups (P = 0.99). No significant differences in growth or survival were found between treatments (P > 0.05; Table 3).

Determination of Biological Activity of Photodegraded MT Metabolites (Experiment 3b; n=1100 fry/tank).

Contrary to the findings obtained in experiment 3a, no significant differences in male percentage

(P > 0.12) were found between treatments in this experiment (Fig. 3). Tanks that received MT-treated effluents exposed to UV had 52.78 ± 3.7% males while tanks that received MT-treated effluents not exposed to UV had 48.20 ± 0.24% males. Tanks that received effluents from the control groups with or without UV had 47.24 ± 1.24 and 44.5 ± 1.15% males, respectively. No significant differences in growth or survival were found between treatments (P > 0.05; table 4).

DISCUSSION

Results from this research indicate that MT in water can be partially removed by solar exposure and completely removed when water is exposed to UV light for 48 hours. It is known that MT is a light sensitive steroid, which is subject to photodegradation (Budavari et al., 1989; Sigma Chemical Company, 1994); however we do not know of any studies which have examined either the intensity of light needed or the exposure time required to eliminate MT after exposure to sunlight or UV. Under a laboratory setting, UV light could alter the structure of MT so that it was no longer detectable or recognized by the antibody used in our RIA after 48 hours of exposure with almost all of the steroid being degraded by 24 hours. Our field trials at UJAT indicated that exposure of water to UV light during masculinization resulted in approximately 8% more males being produced compared to water that was not exposed to UV. This may be due to the ability of UV to degrade enough of the MT to reduce paradoxical feminization or perhaps the decomposition products of the steroid resulted in compounds which were more effective than MT itself for masculinization. Several researchers have shown that steroid metabolites and conjugates are equally, or more, potent than their parent compound (Somogyi et al., 1976; Pelissero, 1993; Sundaram, 1995).

Even though MT was not detectable in a majority of the masculinization and effluent water samples, masculinization of fish from experiment 3a indicated that MT (or a decomposition product not detectable by our RIA) was present in sufficient quantities to induce sex reversal. The intensity of the lamp used during the field trial at UJAT was 5 to 10 fold less (depending on the type used) than that employed at OSU. Since MT was delivered daily in a pulsatile manner, the strength of the UV sterilizer may have been insufficient to eliminate enough MT to prevent masculinization of fish. This possibility is strengthened by results from the second trial (experiment 3b) where the amount of MT that fish were exposed to in effluents was 3 fold less than in the first experiment (3a). Either the amount of MT present had not reached the threshold level needed for masculinization, or the UV sterilizer was capable of eliminating enough MT so that the amount of the steroid remaining was insufficient to induce sex reversal.

We have previously reported that considerable amounts of MT leak into the environment during dietary treatments, remaining in the water for several minutes and potentially accumulating in sediments (Contreras, 2001). We have also shown that the amount of MT remaining in the water and sediments was not sufficient to masculinize fish, but some females had altered ovarian development. Budworth and Senger (1993) reported that testosterone injected into rainbow trout (*Oncorhynchus mykiss*) leaked out of the fish and eventually reached other fish present in the system. Other studies have reported that exposure of non-targeted organisms to MT can result in skewed sex ratios. Gomelsky *et al*. (1994) found significant masculinization of common carp (*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. Incidental sex reversal of tilapia has also been reported (Abucay and Mair, 1997 and Abucay *et al*.,

1997). These authors indicated that in aquaria and concrete tanks, sex ratios are significantly skewed when non-target fish are housed in the same tank where groups of fish are fed with MT.

The possibility also exists that MT is being photodegraded into intermediary decomposition products which may be more potent than MT itself for inducing sex reversal. More research is needed regarding treatment methods for masculinization effluents to eliminate the risks of unintended exposure to humans and other non-target organisms.

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Table 1. Growth and survival of fish used in experiment 2a fed control or MT-treated food.

Treatment	Length (mm) (mean ± SD)	Weight (g) (mean ± SD)	% Survival (mean ± SD)
CONTROL	31.00 ± 0.62	0.445 ± 0.02	43.61 ± 3.75
CONTROL-UV	28.07 ± 0.38	0.412 ± 0.02	58.73 ± 26.75
MT	25.38 ± 0.37	0.263 ± 0.01	55.6 ± 13.33
MT-UV	29.80 ± 0.49	0.509 ± 0.02	48.21 ± 9.47

Table 2. Growth and survival of fish used in experiment 3a exposed to effluents from 2a.

Treatment	Length (mm) (mean ± SD)	Weight (g) (mean ± SD)	% Survival (mean ± SD)
CONROL	34.64 ± 0.46	0.754 ± 0.03	48.86 ± 8.66
CONTROL-UV	28.07 ± 0.38	0.698 ± 0.03	49.07 ± 5.56
MT	33.49 ± 0.35	0.704 ± 0.02	54.6 ± 4.11
MT-UV	34.49 ± 0.47	0.764 ± 0.03	40.48 ± 12.23

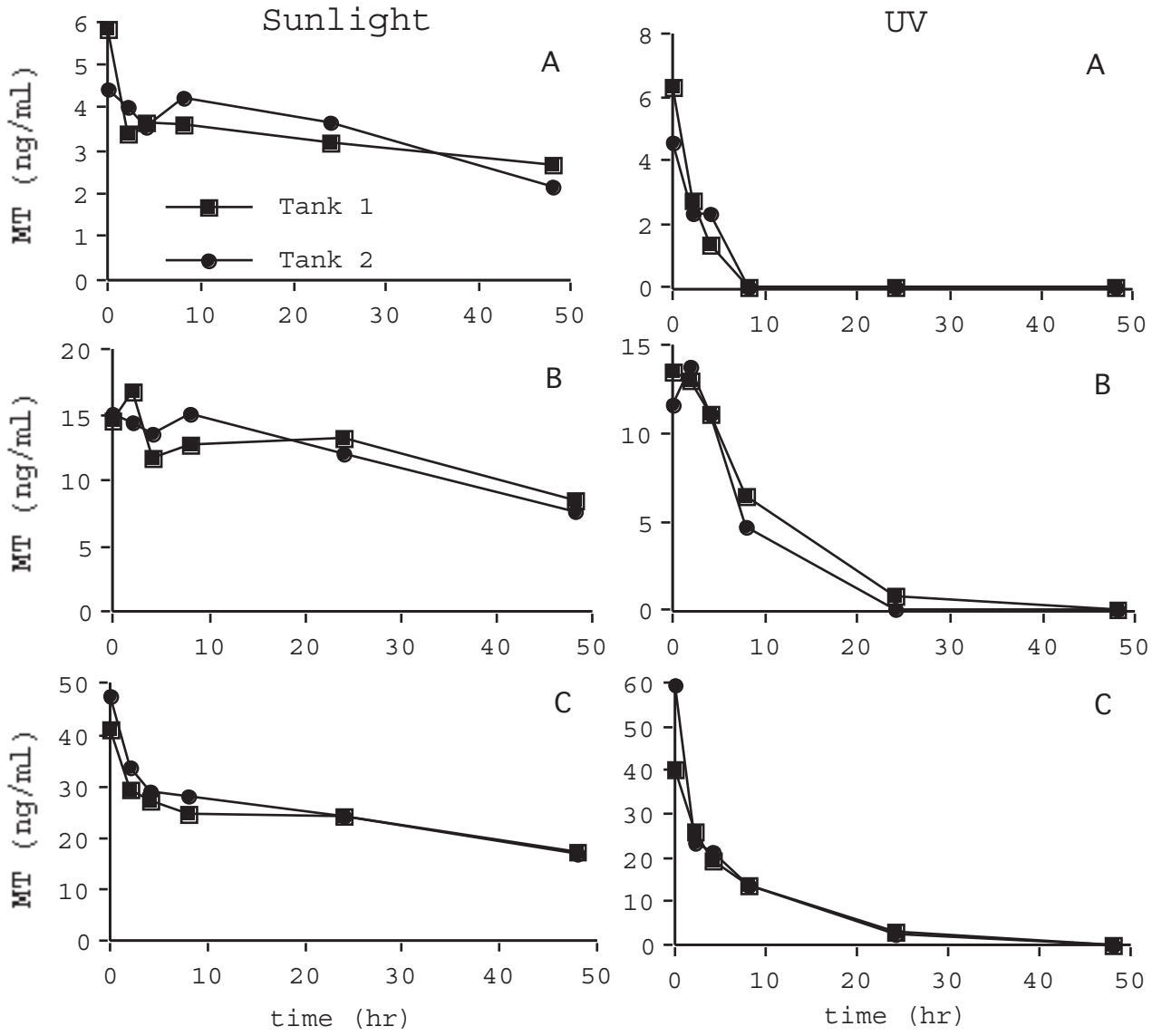
Table 3. Growth and survival of fish used in experiment 2b fed control or MT-treated food.

Treatment	Length (mm) (mean ± SD)	Weight (g) (mean ± SD)	% Survival (mean ± SD)
CONTROL	48.06 ± 0.47	2.01 ± 0.07	46.41 ± 4.78
CONTROL-UV	45.86 ± 0.46	1.79 ± 0.05	47.63 ± 1.52
MT	53.15 ± 0.39	2.61 ± 0.05	56.88 ± 16.51
MT-UV	51.94 ± 0.39	2.47 ± 0.05	75.88 ± 10.9

Table 4. Growth and survival of fish used in experiment 3b exposed to effluents from 2b.

Treatment	Length (mm) (mean ± SD)	Weight (g) (mean ± SD)	% Survival (mean ± SD)
CONTROL	43.14 ± 0.53	1.61 ± 0.06	76.68 ± 1.32
CONTROL-UV	43.13 ± 0.45	1.45 ± 0.05	74.71 ± 2.18
MT	41.08 ± 0.57	1.33 ± 0.05	72.22 ± 0.77
MT-UV	42.81 ± 0.42	1.43 ± 0.04	81.45 ± 0.54

Figure 1. Concentrations of 17a-methyltestosterone (MT) in water over time exposed to either sunlight, ultraviolet irradiation (UV) or darkness. MT was added to water at concentrations of 5, 25, 50 mg/l (A,B and C respectively). The experiment was duplicated (tank 1 and 2).



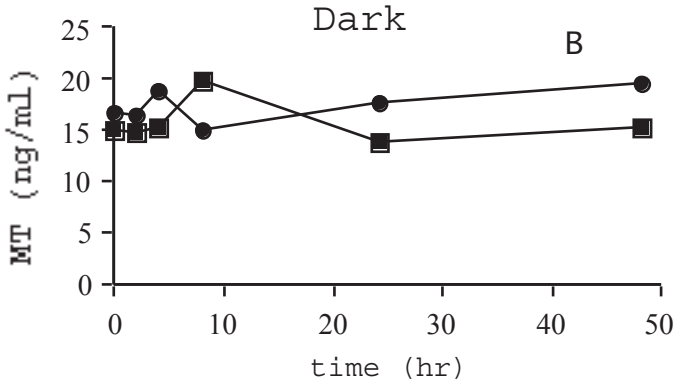


Figure 2. Mean percent (±SE) of male tilapia obtained from experiment 2a. Fry received control (C) or 17a-methyltestosterone (MT) treated food and water was treated with or without UV light (panel a). Percent male fish (experiment 3a) obtained after exposure to the effluents from experiment 2a are shown in panel b. Common letters indicate treatments that were not significantly different (P > 0.05).

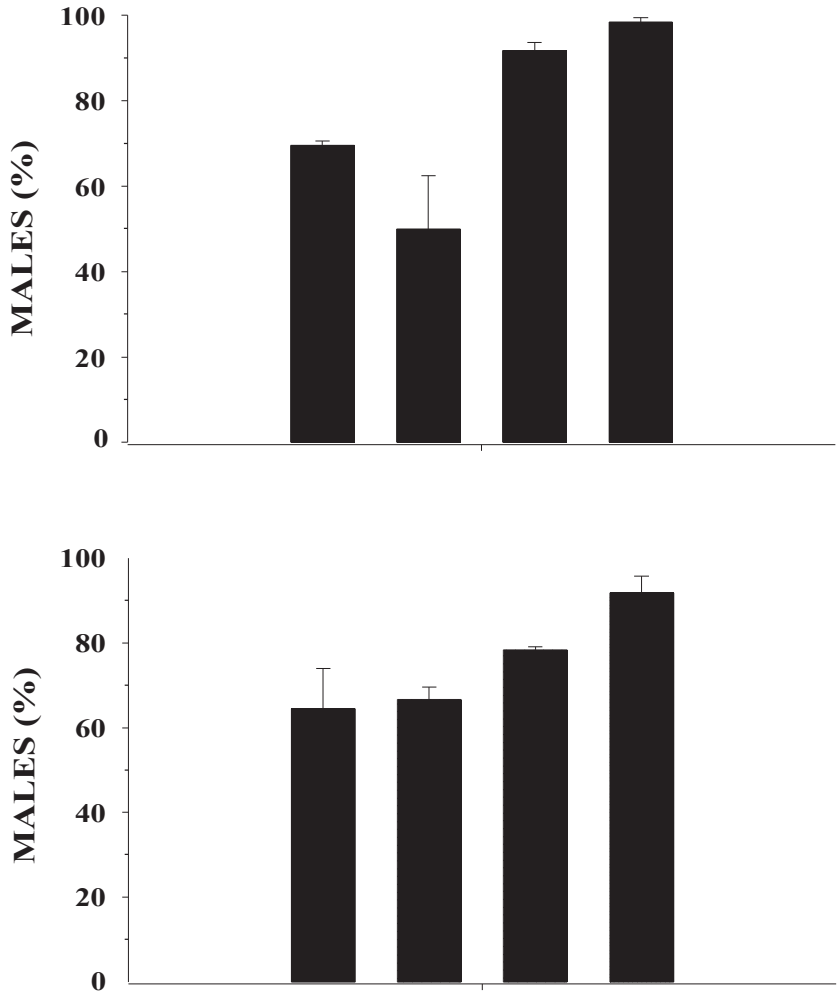
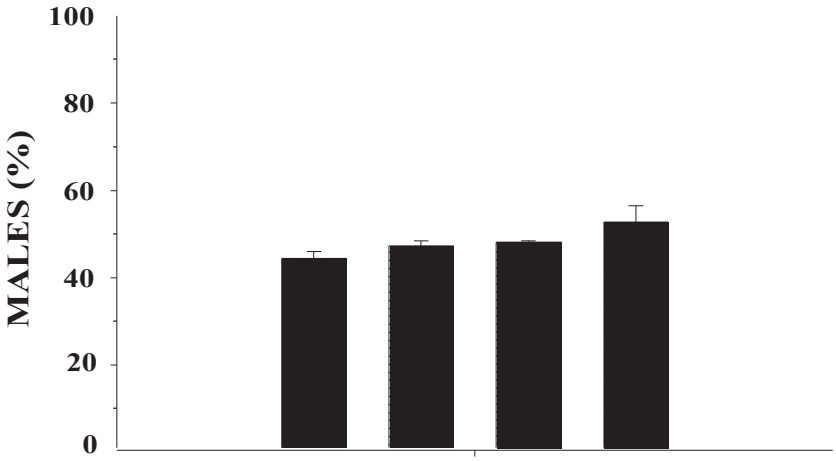


Figure 3. Mean percent (±SE) of male tilapia obtained from experiment 2b. Fry received control (C) or 17a-methyltestosterone (MT) treated food and water was treated with or without UV light (panel a). Percent male fish (experiment 3b) obtained after exposure to the effluents from experiment 2b are shown in panel b. Common letters indicate treatments that were not significantly different (P > 0.05).



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