THE APPLICATION OF ULTRASOUND TO PRODUCE ALL-MALE TILAPIA USING IMMERSION PROTOCOL

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ABSTRACT

Immersion protocols have been unsuccessful in consistently producing all-male tilapia at a high enough ratio for them to be commercially viable. This study explored ultrasound to improve on the results of previous immersion studies. Experiments were carried out to evaluate immersion with ultrasound as a sex-reversal procedure by 1) assessing duration of treatment (one vs. two hours) on the efficacy of hormone to sex-reverse female tilapia and 2) examining the efficacy of various androgens. Due to low survival in experiment 2, experiment 3 was conducted with fewer treatments that included only two hormones (trenbolone acetate, TBA and 17α-methyldihydrotestosterone, MDHT) at two concentrations (100 and 250 μg l^{-1}) and with ultrasound (cavitation level).

Two-hour treatments with ultrasound resulted in a significantly higher percentage of males (92%) than one-hour (73%) treatments. Ultrasound treatment resulted in significantly higher percentages of males (94%) compared to treatments without ultrasound (89%). Two of the three replicates of the TBA-250 μg l^{-1} treatment in the third experiment resulted in 100% males and also in the highest percentage of males (98%). Variability within and between treatments with ultrasound was significantly lower (91 to 98%) than treatments with no ultrasound (83 to 94%). While there was no concentration effect, treatment of fry in TBA-250 μg l^{-1} and ultrasound resulted in significantly higher percentages of males (98.5%) than treatment with MDHT and ultrasound (90.5%). This study thus demonstrated the potential of a short-term immersion protocol using ultrasound to more predictably produce all-male tilapia seed.

INTRODUCTION

Nile tilapia (*Oreochromis niloticus*), one of the most commonly cultured species, is a prolific breeder. Males usually grow faster than females, so propagation of all-male populations is desirable to control reproduction and increase production. Methods to create all-male populations have been developed using various kinds of androgens (Pandian and Sheela, 1995). A number of hormones have been found to be effective, although only 17α-methyltestosterone (MT) is used for commercial production.

Current methods to masculinize populations involve administration of androgen incorporated in feed. Using this technique, newly hatched larvae held in hapas or ponds are fed directly on the pond surface. This method contaminates the environment. An inevitable inefficiency of this method lies in the uneven distribution of hormone as feeding hierarchies develop and some fish get more food than others. Loss of hormone results from uneaten food as well as dissolution from feed in the water column. Although dissolved hormones have been shown to break down in the water column within a week, long-term persistence of hormones has been documented in pond sediments (Fitzpatrick and Schreck, 1999). As an alternative to feeding, immersion of fish in hormone-containing solution has been used to masculinize salmonids and tilapia (Piferrer and Donaldson, 1989; Feist et al., 1995; Contreras-Sánchez et al., 1999).

In a previous study, Fitzpatrick et al. (1998) achieved variable masculinization of ten-day-old tilapia fry (20 to 92%) by using 17α-methyldihydrotestoterone (MDHT), trenbolone acetate (TBA), and MT. Immersing fish in MT solution for 2 and 48 hours resulted in only 20 to 25% males. Gale et al. (1999) achieved better results (73 to 92% male) when immersion treatment was applied for three hours on both day 10 and day 13 after fertilization. This inconsistent inversion of sex may be, in part, due to insufficient and nonuniform uptake of hormone by tilapia larvae.

A novel method that uses cavitation level ultrasound at low frequency has been used to enhance transport of compounds in human as well as fish tissues (Mitragotri et al., 1995; Bart et al., 2001). While Bart et al. (2001) used a cavitation effect to enhance delivery of a target compound (calcein) across fish skin and gills, Mitragotri et al. (1995) used a modified (reduced voltage) ultrasonic bath to achieve the cavitation effect to enhance permeation of insulin-like protein across human skin tissue. Both methods resulted in enhanced uptake of dissolved materials.

In the present study, an immersion protocol combined with ultrasound exposure was expected to increase transport of hormones from water into fish. Increased transport was expected to result in a more consistent and higher rate of masculinization of tilapia fry. The objective was to assess the effect of cavitation level ultrasound on sex inversion using four types of hormones—MT, androstenedione (AN), MDHT, and TBA—at various concentrations and immersion durations.

METHODS AND MATERIALS

Three experiments were conducted to evaluate variation in hormone type, duration of exposure, and influence of cavitation level ultrasound. Three-day-old fertilized eggs were collected from the Chitralada strain of Nile tilapia from broodfish held at the Asian Institute of Technology (AIT) aquaculture research ponds in Thailand. Fertilized eggs (three days post-fertilization, dpf) from 11 females were pooled and incubated in hatchery trays as described by Little et al. (1993). The hatched larvae were collected on the second day of incubation. Three days after hatch, a high-protein (40% crude protein) diet consisting primarily of fishmeal was fed five times per day. Temperature throughout the experimental period was maintained at 27 to 29°C.

Experiment 1 was a factorial (2 × 2 × 2) randomized block design. Variables were two hormones (AN and MT), two concentrations (100 and 500 µg l^{-1}), and two treatment durations (1 and 2 h) with three replicates for each treatment. Experiment 2 used a similar design consisting of three hormones (MT, MDHT, and TBA) and three concentrations (50 µg l^{-1} for MT and 250 or 100 µg l^{-1} for MDHT and TBA). In experiment 3, variables included two hormones (MDHT and TBA) at two concentrations (100 and 250 µg l^{-1}) with or without ultrasound. A total of 150 larvae per treatment, consisting of 50 larvae per replicate, were subjected to ultrasound.

The immersion solutions were prepared by mixing hormones MDHT, TBA, AN, and MT with water containing 0.5 ml l^{-1} of ethanol vehicle, resulting in final concentrations of 50 (MT), 100 (AN and MT), 250 (MDHT and TBA), and 500 µg l^{-1} (AN and MT). Steroids were obtained from Sigma Chemical Company (St. Louis, Missouri). Fifty 10-dpf fry were randomly picked for each replicate and immersed in a modified ultrasound bath (Bransonic 1210, Danbury, Connecticut) containing 500 ml of solution at the treatment concentration of hormone. With the exception of the first experiment, all treatments used two-hour exposure to hormones. Three levels of controls were used, in which the first did not receive ultrasound, the second did not receive hormones, and the third did not receive either ultrasound or hormones.

The ultrasound bath was modified by reducing the voltage. Voltage was divided by placing a series of 10 to 60 W/5W resistors connected to the transducer. An analog meter (Class 2.5, Mu 45) was used to read the voltage output that drove the transducer. Voltage ranging from 190 to 520 V was fixed in six arbitrary incremental steps (190, 230, 420, 440, 480, and 520 V). At step three, when the voltage was 420, cavitation effects (microbubble formation, growth, and collapse) were clearly visible with the aid of a hand-held magnifying glass. Larvae were subjected to each step during the preliminary trials, and 420 V was found to be the highest level that could be delivered without causing mortality. Mortality associated with 420 V of ultrasound exposure was less than 3%, similar to that of the control, and was therefore selected for the two following experiments.

Larvae (10 dpf) receiving ultrasound were held in a bath solution for two hours with continuous pulse at 47 (± 6%) kHz. Fry were removed after treatment and placed in an aquarium with continuous aeration until 13 dpf. On day 13 they were subjected to the same treatment as described above and allowed to recover in the aquarium three or seven days prior to stocking in hapas in a pond.

Mortality was recorded at each step of the process. Survival rates were also assessed between stocking and harvest. Sex ratio was determined after 144 days post-hatch by simple dissection and observation of maturing gonads. Observation *in situ* (40X) revealed the presence of eggs or sperm. Difference in sex ratios between six treatments was analyzed using analysis of variance and chi-square test (α ± 0.05; Zar, 1984).

RESULTS

Experiment 1: Duration of Treatment

The use of ultrasound during immersion was expected to reduce the time required to achieve masculinization. Of the two duration treatments (1 and 2 h) assessed using AN and MT at concentrations of 100 or 500 µg l^{-1}, the two-hour treatment was found to yield a significantly larger percentage of males (92%) than the one-hour treatment (73%) ($P < 0.05$; Figure 1).

The immersion treatments using MT resulted in a higher average percentage of males (90%) compared with AN treatment (75%) when ultrasound was applied. Controls were subjected to immersion but not treated with ultrasound. While controls had one replicate, treatments had two replicates due to limited number of fry and time to conduct the trials. Percentage of males from MT treatment was higher than AN treatment (75 and 65%, respectively) regardless of the duration of treatment. This suggested higher efficacy of MT for sex inversion compared to AN. Efficacy of MT was especially amplified when the ultrasound was applied. Greater variation between replicates treated with AN also indicated that AN is a less useful hormone for sex inversion in tilapia using this protocol.

Survival immediately after the first treatment (10 dpf) was lower (85%) compared to the control (99%). After the second treatment (13 dpf), survival was further reduced (79%) compared to the control (97%). Prior to stocking (7 d post-treatment), overall survival was 53% in the ultrasound-only treatment group and high in both hormone immersion-only and control groups (74 and 76%, respectively; Figure 2). Treatment durations of one or two hours did not affect survival. Survival after seven weeks of rearing was again slightly lower in the treatments than in the control (43 and 55%, respectively).

The difference in survival between treatment and control during the early part of the experiment could be due to acoustic stress caused by ultrasound. This study was conducted during the early part of July, when dissolved oxygen in the seed collection ponds was chronically low (2.2 to 3.1 mg l^{-1}

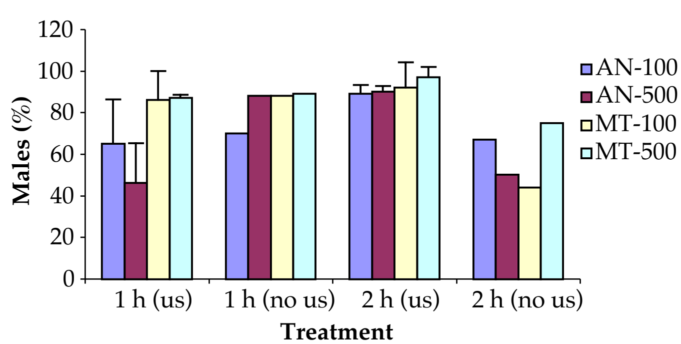

Figure 1. Percent males at harvest of Nile tilapia, immersed in AN and MT at 100 and 500 µg l^{-1} for one or two hour duration with or without ultrasound (us). The control was treated without ultrasound and without hormone but immersed in 5% ethanol solution.

due to overcast days), which may have affected the health of broodfish and subsequent health conditions of larvae. Another weakness of this study was the low number of replicates (two) per treatment, which made it difficult to carry out some statistical comparisons.

Experiment 2. Comparison of MT, MDHT, and TBA

The second experiment repeated the procedures of the first experiment, except it used three replicates per treatment and a lower MT dose (50 μg l^{-1}). It also tested MDHT and TBA as hormones at 100 and 250 μg l^{-1}.

Percent males resulting from this procedure was high in all treatments and significantly higher than in the control. The largest number of males was obtained from TBA-100 and TBA-250 treatments (97 and 96%, respectively). The lowest percentage of males resulted from immersion only in MDHT-250

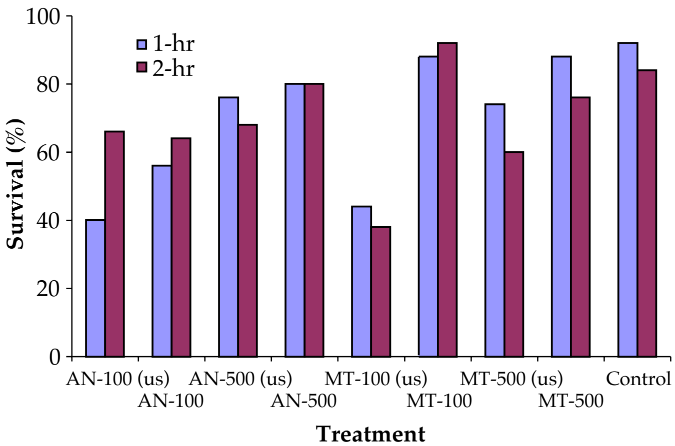

Figure 2. Percent survival at harvest of Nile tilapia immersed in AN and MT at 100 and 500 μg l^{-1} for one or two hour duration with or without ultrasound (us). The control was treated without ultrasound and without hormone but immersed in 5% ethanol solution.

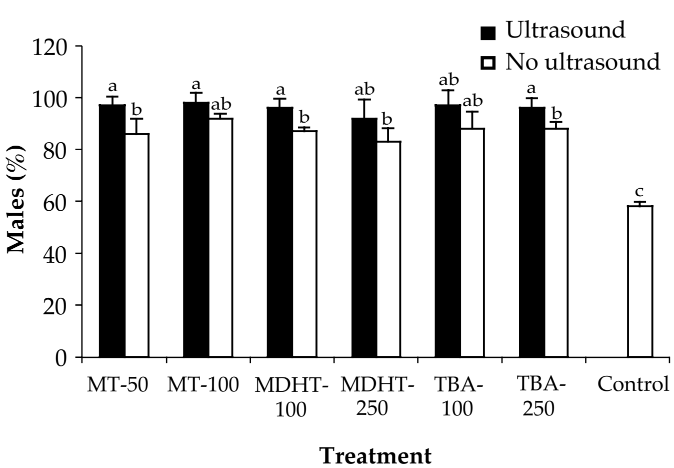

Figure 3. Percent males at harvest of Nile tilapia fry immersed in MT at 50 or 100 μg l^{-1} and MDHT or TBA at 100 or 250 μg l^{-1} with or without ultrasound (us). The control was treated without ultrasound and without hormone but immersed in 5% ethanol solution. Treatments with different letter superscripts indicate mean values that are significantly different ($P < 0.05$).

(83%). No significant difference was found between fish treated with MT at 50 and 100 μg l^{-1}. While the effect of ultrasound was clearly evident for some hormone exposures, no difference in the effectiveness of ultrasound was observed among all three hormones and concentrations examined (Figure 3). The controls in this experiment were slightly male-biased (58%).

Although mortality measured immediately after both sets of ultrasound treatments was less than 4%, survival was severely affected by bacterial infection prior to stocking in the pond. All treatments were similarly affected and mean survival at harvest ranged from only 18 to 56% (Figure 4).

Experiment 3. Comparison of MDHT and TBA with and without Ultrasound

In order to verify the results from the second experiment, which had low survival at harvest in all treatments due to infection, a third experiment was carried out with only two hormones (MDHT and TBA), two concentrations (100 and 250 μg l^{-1}), and with or without ultrasound treatments.

Ultrasound resulted in significantly higher ($P < 0.05$) percentages of males (94%) compared to treatments without ultrasound (89%). The exception was MDHT-250, which had similar values between ultrasound treatments (Figure 5). Two of the three replicates of the TBA-250 treatment resulted in 100% males. Moreover, this entire treatment also resulted in the highest percentage of males (98%). Variation in percentage of males among fish not treated with ultrasound was significantly higher (83 to 94%) than variation among fish treated with ultrasound (91 to 98%). Within a treatment, fish not subjected to ultrasound were also slightly more variable in percentage of males (83 to 95%) than fish treated with ultrasound (89 to 100%). Although sex ratio was skewed toward males in the controls, those treated with ultrasound but no hormone also had a higher number of males ($P < 0.05$) than controls without hormone and ultrasound.

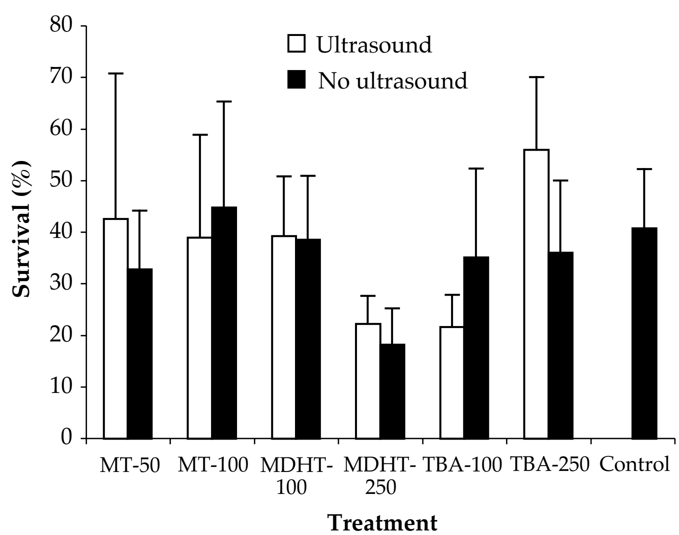

Figure 4. Percent survival at harvest of Nile tilapia fry immersed in MT at 50 or 100 μg l^{-1} and MDHT or TBA at 100 or 250 μg l^{-1} with or without ultrasound (us). The control was treated without ultrasound and without hormone but immersed in 5% ethanol solution.

Larvae treated in TBA resulted in a significantly higher percentage of males (94%) compared to MDHT (89%; $P < 0.05$). Furthermore, TBA treatment, when combined with ultrasound, resulted in a significantly greater number of males (98.5%) than MDHT with ultrasound (90.5%). There was no significant difference in the percentage of males resulting from fry in no-ultrasound treatments using the two hormones ($P > 0.05$). Higher concentrations of hormones (250 mg l^{-1}) did not result in a greater number of males for any treatment combination.

Survival to harvest for treated fish (90%) was not significantly different from controls (95% for fish with ultrasound and no hormones, 94% for fish with no ultrasound and no hormones; Figure 6). The survival of ultrasound-treated fry was 98.5% after the first and second treatments. Low mortality (< 2%) was observed during the treatment period, the post-treatment period, and at stocking (98.34%). This low mortality was not significantly different from control fish.

DISCUSSION

In a similar study Gale et al. (1999) used MT and MDHT immersion for 3 h at a rate of 500 and/or 100 µg l^{-1} and achieved an average of 92% males in the most effective treatment. Unfortunately, variability in the percentage of males between treatments ranged from 52 to 92%, with a mortality rate as high as 80% in one control. This enormous variation in the percentage of males between treatments and between similar experiments, although difficult to explain, could be partly because of nonuniform and differential diffusion of hormones. In comparison, our study using TBA and MDHT immersion for two hours at 100 and 250 µg l^{-1}, with continuous pulse ultrasound, had low mortality rates. The most effective treatment (TBA at 250 µg l^{-1}) resulted in 98% males, and in this treatment two replicates had 100% males. These results are comparable to those seen in larvae fed MT (Macintosh and Little, 1995). The number of males in any treatment never fell below 90% (90.5 to 98.4%) when ultrasound was used. Reduced variability in percentage of males between and within treatments may be due to a more uniform transport of hormones into fish larvae when ultrasound is applied. More focused studies are needed to identify the portal of entry and the kinetics of hormone movement across tissue that is facilitated by ultrasound.

TBA was clearly the most potent hormone, even when not exposed to ultrasound. At a higher concentration of TBA, immersed fry without ultrasound exposure resulted in more males (94%) than the MDHT treatment with ultrasound (91%). Despite lower concentrations (250 µg l^{-1}), more fish per batch (n = 50), and a higher density (1 fish $(5\ ml)^{-1}$) used in this study, the percentage of males was higher in all TBA treatments than in previous studies using other hormones (Varadaraj and Pandian, 1987; Leone and Ridha, 1993; Gale et al., 1999).

Although treatments were significantly different from the controls, controls were observed to have male-biased sex ratios (57%). The reason for such bias was not clear, and others (Gale et al., 1999) have reported a similar male-biased phenomenon. Similarly, controls that were exposed to ultrasound without hormones had a higher percentage of males (69%) and were significantly different ($P < 0.05$) from controls that did not receive hormones or ultrasound (57%). Ultrasound itself may have an effect on sex reversal, which needs further examination.

Immersion and ultrasound exposure did not significantly affect mortality in this experiment. Post-treatment evaluation immediately and three days after ultrasound exposure indicated no survival difference between treatments and controls. High survival was observed in all treatments (82 to 96%). The lowest survival (82%) was observed in treatment (TBA-250) without ultrasound, while the highest survival (96%) was observed in the treatment MDHT-100 with ultrasound. Controls had similar survival to the treatments.

ANTICIPATED BENEFITS

Three experiments were successfully conducted to assess the effect of ultrasound on production of all-male tilapia over six months. Each subsequent study benefited from lessons learned in the previous experiment. In all experiments the effect of ultrasound was clearly evident. The first experiment indicated that two-hour immersion combined with ultrasound produced consistently larger percentages of males than one-hour treatments. The second experiment illustrated that MT, MDHT, and

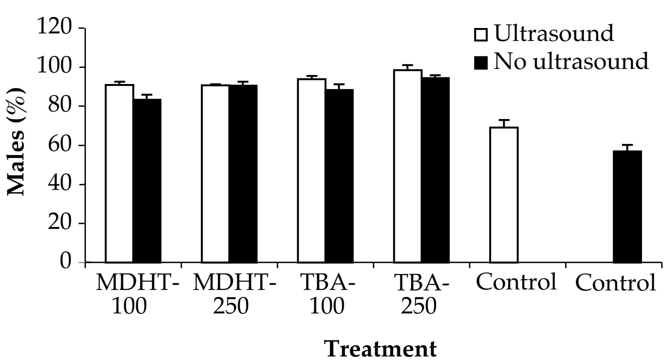

Figure 5. Percent males at harvest of Nile tilapia fry immersed in MDHT or TBA at 100 or 250 µg l^{-1} with or without ultrasound (us). The first control was treated with ultrasound and without hormone, and the second control was immersed in 5% ethanol solution without either ultrasound or hormone treatment. Treatments with different letter superscripts indicate mean values that are significantly different ($P < 0.05$).

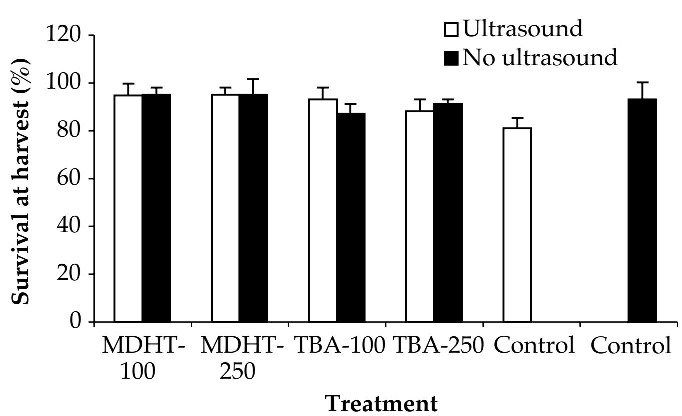

Figure 6. Percent survival at harvest of Nile tilapia fry immersed in MDHT or TBA at 100 or 250 µg l^{-1} with or without ultrasound (us). The first control was treated with ultrasound only, and the second control was immersed in 5% ethanol solution without either ultrasound or hormone treatment.

TBA are all potent inducers of masculinization in tilapia. However, the results were clouded by high mortality across all treatments, including the control. The third experiment was conducted to verify the observation of the second experiment with fewer variables. TBA at 250 µg l^{-1} was found to be the most effective hormone in consistently producing a large percentage (98 to 100%) of male tilapia. The results clearly indicate that this technique has the potential to replace the currently practiced technique of feeding testosterone for sex reversal of tilapia.

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