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ISSN 0792 - 156X

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PUBLISHER:
Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL
Phone: + 972 52 3965809
http://siamb.org.il

The Israeli Journal of Aquaculture - Bamidgeh, IJA_64.2012.703, 6 pages

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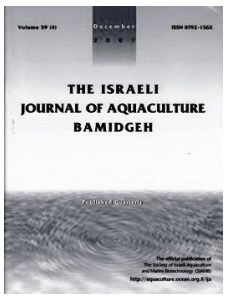

Effect of Nonsteroidal Aromatase Inhibitor on Sex Reversal of *Oreochromis mossambicus* (Peters, 1852)

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(Received 4.3.11, Accepted 17.4.11)

Key words: *Oreochromis mossambicus*, letrozole, 17α-methyltestosterone, aromatase

Abstract

In the present study, the efficacy of letrozole, a potent nonsteroidal aromatase inhibitor, and 17α-methyltestosterone on gondal sex differentiation and sex reversal was examined in Mozambique tilapia (*Oreochromis mossambicus*). Among the doses tried in the experiment, 100 mg letrozole, 200 mg letrozole, and 50 mg 17α-methyltestosterone/kg produced male dominated populations (97-100% males). The combination of 100 mg letrozole+25 mg 17α-methyltestoster/kg yielded 100% males while the combination of 50 mg letrozole+25 mg 17α-methyltestosterone/kg yielded 92% males. The control group consisted of 48.05% males and 51.95% females, close to the normal sex ratio of 1:1. Survival ranged 85.61-94.31% in the treated groups, significantly higher than 73.83% in the control (p<0.05). In general, the letrozole and combination treatments resulted in a slight alteration in the gonadosomatic index of the tilapia. Administration of letrozole alone or in combination with 17α-MT did not adversely affect the proximate composition of the muscle. Results suggest that letrozole and 17α-methyltestosterone have the potential to produce 100% male populations in Mozambique tilapia and that inhibition of aromatase activity influences sex differentiation in Mozambique tilapia.

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Introduction

Successful control of reproduction is a key factor in the commercial production of tilapia. Uncontrolled reproduction leads to overcrowded conditions and results in the production of small, stunted, low-quality fish. The use of steroid hormones to induce sex reversal or enhance growth in fishes is receiving increased importance (Pandian et al., 1999). In several classes of non-mammalian vertebrates, sex differentiation can be influenced by manipulation of environmental factors such as temperature (Kitano et al., 1999). Oestrogens act as female inducers and androgens function as male inducers. The hormonal balance between oestrogens and androgens appears to be crucial in the process of sexual differentiation in developing gonads. This balance relies on the availability and activity of steroid-synthesizing enzymes and, in particular, the cytochrome P450 aromatase complex (P450). The importance of P450 in the gonadal differentiation of fish has been demonstrated in studies using aromatase inhibitors (Afonso et al., 2001). Such studies show that aromatase inhibitors can inhibit aromatase enzyme activity by catalyzing the biosynthesis of oestradiol-17b from its precursor testosterone, and can result in reduced oestrogen production (Steele et al., 1987).

Mozambique tilapia (*Oreochromis mossambicus*) has a fast growth rate, good food conversion efficiency, and good flavored meat. *Oreochromis mossambicus* was introduced into India in 1952 from Sri Lanka to study its suitability for culture. Tilapia are widely distributed in India and consumed in many parts of the world. In tilapia, males grow much faster than females (Pullin, 1984) and, hence, all-male tilapia populations are often preferred for aquaculture. Monosex populations may be obtained by manual sexing of fingerlings, hybridization techniques, chromosome manipulation, or reversing sex by hormone treatment. Sex differentiation is brought about by the male and female sex inducing substance, which is controlled by sex genes. Gonadal hormones, i.e., androgens and estrogens, are sex inducers and can be used to manipulate sex. Direct treatment of androgens or estrogens during sex differentiation in the early stage of gonadogenesis results in the production of monosex or sterile fish.

Non-steroidal aromatase inhibitors such as fadrozole have been used to manipulate the sex ratio and gonadal development in Nile tilapia (Kwon et al., 2002), carp (Tzchori et al., 2004), Japanese flounder (Kitano et al. 2000), Japanese medaka (Adam and Brouwer, 2006), gilthead seabream (Wong et al., 2006), and blue gill sunfish (Gao et al. 2010). 17α-Methyltestosterone is an effective and widely-used androgen in inducing sex inversion in teleosts. Functional males with spermiation have been obtained by oral administration of exogenous methyltestosterone in Japanese flounder (Kitano et al., 2000) and grouper (Tanaka et al., 2000. A non-paradoxical dose of 17α-methyltestosterone effects the sex ratio, growth, and gonadal development in Nile tilapia, *Oreochromis niloticus* (Phelps and Okoko, 2010).

The non-steroidal aromatase inhibitor, letrozole, induced sex inversion in the protogynous red spotted grouper, *Epinephelus akaara* (Li et al., 2005) and inhibited oocyte growth in the Japanese medaka, *Oryzias latipes* (Sun et al., 2007). The present study evaluates the effect of letrozole, alone or in combination with 17α-methyltestosterone, on the growth, survival, sex ratio, gonadosomatic index, and proximate composition of Mozambique tilapia.

Materials and Methods

Preparation of diets. Diets were prepared by mixing the ingredients, namely rice bran, fishmeal, wheat flour, ground nut oil cake, tapioca flour, and mineral/vitamin mix (Agrimin-mineral mix with amino acid methionine and lysine), and hand kneading the mixture with a sufficient quantity of water to obtain the required soft consistency dough. Letrozole (trade name Letronat, NATCO, Hyderabad) and 17α-methyltestosterone (Sigma Chemicals, St. Louis, USA) were incorporated into the diet as follows: the desired quantities of letrozole and 17α-MT were dissolved in 50 ml 90% ethanol/kg feed and thoroughly mixed with the feed using an electric mixer to achieve final concentrations of 50, 100, and 200 mg/kg.

Experimental design. Mature male and female tilapia were selected based on the shape and size of the genital papilla, which is accentuated during the breeding season and can be used to identify the sex of individuals. The tilapia were bred in a cement cistern (1 × 1 × 1 m) at a sex ratio of 1:4 (male:female). After spawning, developing eggs were robbed from the mouths of females and incubated in round fiberglass tanks (55 x 44.5 cm) with a continuous flow of water. After hatching, 5-6 day fry (first feeding fry) were randomly distributed into six treatment groups in triplicate tanks at 30 fry per tank. The first group was fed the control diet. The other five groups were fed diets containing 100 mg/kg letrozole, 200 mg/kg letrozole, 50 mg/kg 17α-MT, 50 mg/kg letrozole+25 mg/kg 17α-MT, or 100 mg/kg letrozole+25 mg/kg 17α-MT. The fry were fed to apparent satiation for 90 days.

Evaluation of growth, survival, sex ratio. Upon termination of the experiment, surviving fish were harvested, counted, individually weighed, and measured for length. All fish were sacrificed by anesthetizing with quinaldine (10 mg/kg). The phenotypic sex of the treated and control fish was determined based on secondary sexual characters such as the shape of the belly, size of the genital papilla, and body coloration. The sex was also determined by examining the gonads. Fish with recognizable ovarian or testicular portions were classified female or male, respectively. The dissected gonads were examined to confirm the sex and stage of maturity.

Gonadosomatic index and proximate composition. The gonadosomatic index (GSI) was calculated according to GSI (%) = (gonad wt x 100)/somatic wt. Proximate composition of the tilapia flesh was analyzed using methods of the AOAC (1975). Muscle was collected from the trunk region of the fish, avoiding bony parts. To estimate the moisture content, the muscle was dried at 105°C for 15-16 h until a constant weight was obtained. The resulting dry matter was powdered and stored in airtight polythene covers for further analysis. The dry samples were analyzed for crude protein, crude fat, and total ash by following standard methods. Crude protein was estimated using a Kjeltec 1002 Auto Distilling Unit (Foss Tecator, Hoganas, Sweden), crude fat was estimated using a Soxtec 1043 Extraction Unit (Foss Tecator, Sweden), and crude fiber was estimated using a Fibretec Extraction Unit. (Pelican Equipments, Chennai India).

Statistical analysis. The chi-square test was used to test the equality of the numbers of male and female fish, excluding sterile fish, in each group. Significant differences between treated and control samples were tested by ANOVA and *t* test. Duncan's multiple range test was used to determine which pairs of means significantly differ.

Results

At the end of treatment, there were far more male fish in the treated groups than in the control, indicating up to 100% sex reversal (Table 1). Moisture, ash, and fiber tended to be lower, and fat and protein higher, in the treated groups than the control (Table 2).

Table 1. Effect of feeding diets containing letrozole and/or 17α-methyltestosterone on growth, survival, sex ratio, and gonadosomatic index (GSI) of Mozambique tilapia.

Dose (ppm)		*Growth*			*Sex ratio*				*GSI (%)*	
Letrozole	*17α-MT*	*Body wt (g)*	*Total length (cm)*	*Survival (%)*	*N*	*Males (%)*	*Females (%)*	*P-value*	*Males*	*Females*
0 (Control)	0	6.99^{a}	7.77	73.83^{a}	42	48.05^{a}	51.95	0.312	0.48±0.07	1.11±0.31
100	0	11.80^{ab}	9.45	91.26^{b}	52	97.83^{b}	2.17	0.002	0.43±0.04	1.97
200	0	15.64^{bc}	10.30	94.23^{b}	51	100^{b}	0	0.001	0.41±0.04	0
0	50	19.09^{c}	10.35	85.61^{ab}	47	100^{b}	0	0.001	0.54±0.03	0
50	25	25.17^{d}	11.80	93.05^{b}	53	92.31^{b}	7.69	0.003	0.54±0.02	0.79
100	25	36.20^{e}	13.25	94.31^{b}	50	100^{b}	0	0.001	0.39±0.03*	0.8

Different superscripts indicate significant differences (chi-square test; $p<0.05$) between treatments.
P-values indicate differences from the theoretical 50:50 sex ratio (chi-square test).
* significantly different from the control group when *t* test applied ($p<0.05$)

Table 2. Proximate composition (%±SE) of fish fed letrozole alone or in combination with 17α-methyltestosterone (n = 3).

Letrozole	*17α-MT*	*Moisture*	*Crude protein*	*Crude fat*	*Total ash*	*Crude fiber*
0 (Control)	0	78.84±0.43	15.50±0.15	3.05±0.15	1.63±0.04	1.87±0.01
100	0	76.77±0.68	16.49±0.47	3.14±0.04	1.56±0.08	1.78±0.01
200	0	77.68±0.24	15.52±0.24	3.28±0.10	1.60±0.06	1.83±0.15
0	50	78.07±0.41	15.38±0.58	3.56±0.04	1.50±0.15	1.58±0.06
50	25	76.43±0.24	15.87±0.47	4.12±0.18	1.52±0.18	1.86±0.20
100	25	76.53±0.05	15.80±0.28	3.60±0.05	1.58±0.03	1.70±0.01

Discussion

Results of the present investigation demonstrate that it is possible to manipulate the sex ratio of *O. mossambicus* by oral administration of letrozole, a non-steroidal aromatase inhibitor. Dietary administration of letrozole alone (100 and 200 mg/kg) produced 97-100% males. Similarly, fadrozole, a potent non-steroidal aromatase inhibitor (Steele et al., 1987), induced sex reversal of genetic females to phenotypic males in Nile tilapia (Kwon et al., 2000, 2002) and in chinook salmon that were immersed for 2 h during the bipotent period (three days after hatching) in 1 or 10 mg/kg fadrozole, in 10 mg/kg 17α-MDHT, in 10 mg/kg 17α-MT+1 mg/kg fadrozole, or in 10 mg/kg 17α-MT+10 mg/kg fadrozole (Piferrer et al., 1994). The combination of 17α-MT and fadrozole resulted in 98-100% males (Piferrer et al., 1994). Aromatase may play a role in the process of sex differentiation in chinook salmon (Piferrer et al., 1994). In the present study, a combination of 17α-MT and letrozole resulted in the production of 92-100% males, indicating that the aromatase inhibitor might have prevented the conversion of 17α-MT or natural androgens into estrogens as suggested by Piferrer et al. (1994). The combination of 100 letrozole+25 mg 17α-MT consistently yielded a high percentage of males, suggesting that letrozole prevents the aromatization of aromatizable MT which can then reestablish its full androgenic potency, a finding that is strongly supported by Piferrer et al. (1994). While 17α-MT at 50 mg/kg without letrozole consistently led to 100% sex reversal in the present study, higher doses of 17α-MT could lead to induction of females due to the aromatization of MT (Piferrer et al., 1994).

A series of experiments were carried out by Kwon et al. (2000) in which genetically female Nile tilapia fry were treated with dietary fadrozole during the period of sexual differentiation. Batches of tilapia fry treated with the aromatase inhibitor during the first 30 days following yolk-sac resorption (7-37 days post hatch) showed a dose-dependent increase in the percentage of males from 0 to 200 mg/kg. The percentage of males remained approximately constant (92.5-96.0%) from 200 to 500 mg/kg.

Treatment with androgens such as 17α-MT probably increases the androgen level in the blood. But since 17α-MT can be converted to estrogen by P450 aromatase, it may also increase the estrogen level (Kwon et al., 2000). Treatment with an aromatase inhibitor does not introduce exogenic steroids to the system. Rather, by blocking the transformation of androgens into estrogens, it increases the androgen/estrogen ratio. Therefore, the efficacy of aromatase inhibitor treatment to induce male differentiation is higher than the efficacy of treatment with aromatizable androgens such as 17α-MT (Kwon et al., 2000). In the present study, oral administration of 17α-MT (50 mg/kg) consistently induced 100% masculinization, while the combination treatment (100 letrozole+50 mg 17α-MT) was less effective. The recommended dose of 17α-MT for masculinization of tilapias ranges 5-1000 mg/kg for 11-69 days for *O. mossambicus* (Pandian and Sheela, 1995) and 5-60 mg/kg for 25-59 days for *O. niloticus* (Gale et al., 1999). Sexually undifferentiated fry of *O. mossambicus* (8-10 mm) were masculinized by dietary administration of 17α-MT at 100 mg/kg for 30 days (Basavaraja et al., 1991; Patil and Varghese, 1994). All males were produced in *O. mossambicus* using diets containing 17α-MT at 5, 10, 20, 30, and 40 mg/kg for only 10 days (Pandian and Varadaraj (1987).

In the present study, the growth rate tended to increase with higher dosages. Anabolic steroids in general and androgens in particular accelerate the growth rate in fishes possibly because 17α-MT increases food conversion, activation or secretion of endogenous anabolic hormones, and gene expression in muscle cells (Lone and Matty,

1980; Pandian et al., 1999). A decreased growth rate at higher dosages has been attributed to catabolic action and decreased appetite (Lone and Matty, 1980). In the present investigation, greater growth was recorded in the treated fish throughout the experiment because the treated groups were dominated by males and males grow faster than females in tilapia (Pullin, 1984). Similarly, a higher biomass was reported in several species of fish treated with steroid hormones (Basavaraja et al., 1989).

There was no major effect of letrozole and 17α-MT on the gonadosomatic index, similar to results of Piferrer et al. (1994) who reported that fadrozole-treated genetic females developed into phenotypic males that were indistinguishable from regular males in terms of gonadosomatic index and rate of maturity. Administration of letrozole affected the proximate composition of the *O. mossambicus*; there was a slight increase in crude fat and crude protein in all treated groups. Moisture, total ash, and crude fiber slightly varied between treated and control fish. While we were unable to find literature on the effect of letrozole on the proximate composition of fish muscle, we can compare with studies using 17α-MT. In common carp treated with 17α-MT (2.5, 5.0, 10.0 mg/kg) for 90 days, total lipids and proteins likewise increased while moisture and ash decreased (Lone and Matty, 1980). Protein, fat, and ash increased while moisture decreased in common carp fed 17α-MT (300 mg/kg) or 17β-estradiol (300 mg/kg) for 131 days (Sathyanarayana Rao et al., 1984). Similar observations were reported in common carp treated with 1.0, 2.5, or 10 mg/kg 11-ketotestosterone (Lone and Matty, 1980). Moisture and ash decreased while total protein and lipid increased when common carp were treated with 300 or 400 mg/kg testosterone acetate or 800 mg/kg 17β-estradiol benzoate (Nagaraj and Rao, 1988). Moisture and ash decreased in 17α-MT treated common carp (Basavaraja and Sathyanarayana, 1988).

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