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Masculinization of dwarf gourami *Trichogaster lalius* through immersion treatment of 17α-methyltestosterone

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ABSTRACT

The present study was conducted to study the effect of 17α-methyltestosterone (17α-MT) on masculinization efficiency through immersion treatment in dwarf gourami, *Trichogaster lalius*. The immersion treatment of 17α-MT at doses of 250, 500, 750 and 1000 µg/l was carried out for 3 h daily on third, fifth and eighth day after hatching. The highest concentrations of 17α-MT produced the highest percentage of males (84.29%). The progeny testing of males from17α-MT treated groups indicated that the female progenies of each of the males tested differ significantly from that of control, indicating that all those males carried XX genotype. The gonado-somatic index of the hormone treated fish revealed significant suppression of the ovarian development.

Key words: 17α-methyltestosterone, Gonadosomatic index, Hormonal profiling, Masculinization, Progeny testing, *Trichogaster lalius*

Ornamental fishes are rapidly gaining immense commercial value in the export trade world over because attractive colouration of the fishes determines their value. In many groups of ornamental fish, the sexes differ according to their colour patterns where the males are more brightly coloured sex (Cogliati *et al*. 2010). Therefore, the culture of all-male stocks of ornamental fish might be economically advantageous as more attractive males fetch higher price than females in ornamental fish trade. Several research studies have been focused on the phenotypic sex reversal in ornamental fish in order to take the advantage of more valuable gender of the fish (Piferrer and Lim 1997).

The main approach to commercial applications of monosex populations is through hormonal and selective breeding that have generated a great amount of interest. Sex reversal, manual sexing, hybridization and super male production are different methods available to obtain desired sex population.The hormonal sex reversal has been used as a valuable key tool in sex manipulation for aquaculture. There are several reports on the use of steroid hormone for successful induction of functional sex in ornamental fish exist (Pandian and Sheela 1995, George and Pandian 1996, Gale *et al*. 1999, Mousavi-Sabet 2011). Sex reversal by immersion treatment with 17α-methyltestosterone (MT) is the most effective and practical method for the production of all male population.

The dwarf gourami (*Trichogaster lalius*), also known as the powder blue dwarf gourami, is the most popular gourami, especially in native region of India, Pakistan and Bangladesh, though in recent years it has become established through releases and escapes in Australia, Columbia and the United States. The male of dwarf gourami is larger, brighter in colour and more attractive than female with male fetching a higher price than female in ornamental fish market.The aim of the present study was to evaluate the effect of 17α-methyltestosterone on masculinization of dwarf gourami (*Trichogaster lalius*).

MATERIALS AND METHODS

The present study was conducted during July 2015-August 2016, to evaluate the effect of 17α-methyl testosterone on masculinization effeciency to *T. lalius.*

Fry production: T. lalius were obtained from local ornamental fish breeders and maintained at the CIFE, Mumbai. The brood fish was developed by feeding daily twice with mosquito larvae and zooplankton. The gravid female and male identified on the basis of their external characteristics and colour, were selected for fry production.

Disinfected, cleaned, filled with fresh filtered water and aerated using an air blower glass aquaria (2×1×1.5 ft) was used for breeding and fry treatment. The female and male brooders (1: 1) were held in aquarium for breeding. In about one or two days of their release in aquarium, the breeding

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took place wherein the eggs were released in batches and fertilized. The resultant fry were used for MT experimentation.

Immersion treatment of 17α-methyl testosterone to fry: Randomly selected fry were subjected to discrete immersion treatment of MT for 3 h daily on third, fifth and eight day after hatching. Stock solution of MT (1 mg/ml) was prepared in absolute alcohol. The four groups of 40 fry each were subjected to discrete immersion of the chemical at 250, 500, 750 and 1,000 µg/l. A control group was maintained without MT and the experiment was conducted in triplicates for each treatment groups including control.

Fry rearing and evaluation of MT treatment: Plastic crates (250 l) were cleaned, dried, filled with fresh water and used for the rearing of fry immediately after treatment for 50 days. Subsequently, after 50 days, the fry were stocked in outdoor FRP tanks (manured with chicken manure) of 500 l capacity for 130 days till maturity. During post-treatment rearing, the fry were fed with sieved zooplankton till maturity. On termination of the experiments, all the surviving fish were harvested and counted. The phenotypic sex of the treated and control fish was determined based on secondary sexual characters, such as the shape of the belly, body colouration and finnage.

Progeny testing: The progeny testing was conducted to confirm sex reversed males.The male from treated and control group were mated with normal females in glass tanks at a ratio of 1:1 (M:F) and the obtained resultant progeny was reared in FRP tank for 160 days and during this period the fry were fed with zooplankton. Upon maturity, sexing was done based on secondary sexual characters like colour, shape of the belly and finnage.

Testosterone analysis: After post rearing period of 160 days, the fishes were sacrificed aseptically and whole body samples as well as different tissues like gonads and liver were dissected out, weighed and kept at –20°C for testosterone assays. Dissected tissues were homogenized with chilled sucrose solution (0.25 M) in a glass tube using tissue homogenizer. During homogenization, the glass tube was continuously kept in ice bath. Then homogenate samples were centrifuged at 5,000 rpm for 20 min at 4ºC in a cooling centrifuge. The supernatant was kept frozen at –20 ºC till further analysis. A 5% homogenate was prepared for all the tissues. The quantification of testosterone was performed according to the protocol provided along with the respected EIA kits, procured from Cayman Chemicals, USA.

Cortisol extraction protocol: Whole body samples were collected, partially thawed, weighed and then homogenized in 500 µl of ice cold 1× phosphate buffered saline (PBS) for biological study to assay cortisol levels. After recording weight (g), the whole body samples were dissected on ice into smaller parts for efficient homogenization. The quantification of cortisol was accomplished using EIA kit procured from Cayman Chemicals, USA.

Gonadosomatic index: The gonadosomatic index (GSI) for treated and control fish was determined using the formula:

$$\text{Gonadosomatic index (\%)} = \frac{\text{Gonad weight (g)}}{\text{Body weight (g)}} \times 100$$

Statistical analysis: Differences in survival, male percentage, gonadosomatic index and hormonal profiling among treatment were subjected to one way ANOVA with Duncan's multiple range test (SPSS 18).

RESULTS AND DISCUSSION

The present study is the first report on the production of a male-dominated population of *T. lalius* with 17α-MT through discrete immersion treatment for 3 h daily on third, fifth and eight day after hatching. The results of immersion treatment of MT are presented in Table 1. The immersion treatment produced 84.29% males with 89.90% survival at a dose of 1000 µg/l. In other groups of immersion treatment, it resulted in 53.85 (250 µg/l), 56.64 (500 µg/l) and 80.01% (750 µg/l) male population with the survival rates of 89.98, 86.96 and 90.90%, respectively, whereas the survival during the treatment period ranged between 81.66 (control) to 65.00% (1000 µg/l), indicated that in all the treated groups survival was significantly lower ($P \leq 0.05$) than that of the control group showing that an increase in doses of the 17α-MT decreased the survival rate (Table 1).

Kirankumar and Pandian (2002) reported 98% masculinization with 71% survival in *Betta splendens* through the immersion of 17α-MT at 900 µg/l for 3 h on

Table 1. Number of fry stocked and recovered, sex composition and survival of *T. lalius* given immersion treatment of 17α-methyltestosterone

Treatment	Treatment period				Post-treatment period				
	No. of fry (Initial)	No. of fry* (Final)	Survival* (%)	No. of fish recovered*	Males*	Females*	Male* (%)	Female* (%)	Survival* (%)
Control	40	32.66±0.88^{c}	81.66±2.20^{c}	29.33±0.66^{c}	14.33±0.66^{a}	15.00±0.00^{d}	48.80±1.1^{a}	51.19±1.19^{c}	89.82±0.81
250 µg/l	40	30.33±0.88^{bc}	75.83±2.20^{bc}	27.33±1.45^{bc}	14.66±0.33^{a}	12.66±1.20^{c}	53.85±1.9^{b}	46.14±1.93^{b}	89.98±2.17
500 µg/l	40	28.33±0.88^{ab}	70.83±2.20^{ab}	24.66±1.20^{ab}	14.00±1.00^{a}	10.66±0.33^{b}	56.64±1.47^{b}	43.35±1.47^{b}	86.96±1.52
750 µg/l	40	29.33±0.88^{b}	73.33±2.20^{b}	26.66±0.88^{bc}	21.33±0.66^{b}	5.33±0.33^{a}	80.01±0.84^{c}	19.98±0.84^{a}	90.90±1.13
1000 µg/l	40	26.00±1.00^{a}	65.00±2.50^{a}	23.33±0.33^{a}	19.66±0.33^{b}	3.66±0.33^{a}	84.29±1.34^{c}	15.70±1.34^{a}	89.90±2.09

*Data expressed as mean±SE; mean values in the same column with different superscript differ significantly ($P<0.05$).

second, fifth and eight dph. In the present study, the discrete immersion treatment was conducted for 3 h daily on third, fifth and eighth day after hatching which may be the reason for the production of only 84.29% male population in the present study. Lee *et al*. (2004) investigated single or double immersion treatments in MDHT at 400 µg/l for 120 min during the period 0–28 d after median hatch resulted in significant masculinization in *Salmosalar*. Immersion treatments were most effective when conducted more than 14 d after median hatch. The two immersion treatments 7 or 14 d apart resulted in 100% masculinization,whereas single immersions yielded only 77% masculinization. In the present study, only single immersion treatments were conducted. Silarudee and Kongchum (2008) suggested that hormonal immersion using MT for 24 h at 250 µg/l produced only 65.48% males in flower horn fish. The present study produced only 53.85% males at 250 µg/l MT which may be because of 3 h immersion of MT daily on third, fifth and eighth day after hatching. The present study produced 84.29% male population at 1000 µg/l MT compared to the study conducted by Kumar and Haniffa (2011) who found immersion with the 17α-MT for 6 h at 400 mg/l produced 85% males in platy and 75% in koi carp. Haniffa *et al.* (2004) investigated the single immersion for 3 h in MT (100 µg/l) and produced 80–82% males in *H. fossilis* eggs treated 20 h after fertilization. In *T. lalius* it was not possible to use the fertilized eggs for immersion treatment due to the smaller size of eggs, which are easily susceptible to the handling stress that hampered fertilization and led to higher mortality.

The results of progeny testing of males from treated and normal males of immersion treatment are shown in Table 2. The males from 17α-MT treated groups when mated with normal females produced 50.87 (control), 74.26, 85.47, 90.43 and 100% female population. The percentage of male and female significantly (P<0.05) changed in each progeny group when compared with control group (Table 2).

Similar results were reported in black crappie (Gomelsky *et al*. 2002), *B. splendens* (Kirankumar and Pandian 2002) where dominant female progenies were reported when 17α-MT treated fish were crossed with normal female. Haugen *et al*. (2011) observed 100% females in the offspring of *Gadusmorhua* hermaphrodites treated with 17α-MT. Galbreath *et al*. (2003) obtained 100% female progeny of *Salvelinus fontinalis* by the cross between MDHT treated male parents and genotypically female.

The results of the effect of immersion treatment of 17α-MT on gonadosomatic index of male and female are shown in Table 3. The gonadosomatic index of male fish varied significantly between different groups. In the females, suppression of ovarian development was observed in the treated groups compared to control (Table 3).

MT treatment indicated certain amount of suppression of ovarian development in *B. splendens* (Jessy and Varghese 1988, Kirankumar and Pandian 2002, James and Sampath 2006), *X. helleri* (James and Sampath 2006). Bhandari *et al*. (2006) also reported that the GSI of 11KT-treated fish was significantly lower (P<0.05) than that of the control fish in *Epinephelus merra*. Bharadwaj and Sharma (2000) reported that GSI of MT treated sterile groups were significantly lower (P<0.01) than those of males and females in *Cyprinus carpiocommunis*.

The cortisol level of the immersion treatment of MT is presented in Table 3. The level of cortisol in each treatment significantly differed (P<0.05) in male population but not in female, and the level of cortisol increased with 250 to

Table 2. Results of progeny testing of 17α-methyltestosterone treated and control groups

Treatment	No. of fry* (Initial)	No. of fry (Final)*	Survival* (%)	Males*	Females*	Male* (%)	Female* (%)
Control	91.33±11.2[a]	61.66±3.28[b]	69.25±7.37	30.33±2.02[c]	31.33±1.33[a]	49.12±0.87[d]	50.87±0.87[a]
250 µg/ml	68.33±1.76	42.66±2.90[a]	62.43±0.77	11.00±1.00[b]	31.66±2.02[a]	25.73±0.98[c]	74.26±0.98[b]
500 µg/ml	74.00±4.93	50.66±3.66[a]	68.49±2.08	7.33±0.66[ab]	43.33±3.38[b]	14.52±1.28[b]	85.47±1.28[c]
750 µg/ml	77.33±6.35	49.33±2.33[a]	64.14±2.13	4.66±0.33[a]	44.66±2.66[b]	9.56±1.07[a]	90.43±1.07[d]
1000 µg/ml	76.00±7.81	48.66±3.48[a]	64.45±2.07	-	48.66±0.48[b]	-	100[e]

*Data expressed as mean±SE; mean values in the same column with different superscript differ significantly (P<0.05).

Table 3. Gonadosomatic index and hormonal profiling (cortisol and testosterone)

Treatment	Gonadosomatic index (%)		Cortisol level (pg/ml)		Testosterone level (pg/ml)	
	Male*	Female*	Male*	Female*	Male*	Female*
Control	0.24±0.0284[a]	1.43±0.020[d]	146.67±10.13[a]	131.67±7.26	152.67±14.62[a]	97.00±6.65[a]
250 µg/ml	0.36±0.0296[b]	1.15±0.020[c]	173.33±7.31[ab]	136.00±7.02	236.67±18.55[b]	120.00±7.63[ab]
500 µg/ml	0.42±0.0371[bc]	1.09±0.012[b]	185.33±9.13[bc]	139.67±7.85	255.00±18.92[b]	125.67±9.83[b]
750 µg/ml	0.45±0.0120[c]	0.98±0.017[a]	210.67±6.98[cd]	143.00±8.73	283.00±17.5[b]	139.67±6.38[b]
1000 µg/ml	0.46±0.020[c]	0.97±0.020[a]	218.33±10.13[d]	139.33±8.08	280.00±27.83[b]	140.67±6.35[b]

Data expressed as mean±SE; n=3; mean values in the same column with different superscript differ significantly (P<0.05).

1000 µg/l in immersion treatment, indicating that the drug may impose slight stress in male and it increased with increasing dose of 17α-MT.

Joshi (2013) also reported similar observation in *Poecili areticulata* when fadrozole nanoparticles were used for the induction of sex reversal at different doses (negative control, positive control, 50 ppm pure fadrozole without nanoparticles, PLGA nanoparticle (20 ppm) and 5, 25, 37.5 and 50 ppm of FDZ-loaded PLGA NPs) with different period treatment (10, 15 and 30 days).

The results of testosterone levels of the immersion treatment of MT are presented in Table 3. In our study, 17α-MT was found to stimulate testosterone production. Male testosterone level indicated that as the dose of 17α-MT increased, testosterone level also increased; similar observation were found in case of female testosterone level (Table 3).

Sarter *et al.* (2006) found that the plasma MT levels were significantly elevated, in dusky grouper juveniles in response to the MT treatment, both 6 and 12 weeks after beginning the treatment. Bhandari *et al.* (2006) observed significantly low plasma E2 levels in the exogenous 11KT treated sex-changed fish, while both testosterone (T) and 11KT were significantly increased in *Epinephelus merra*.

Interestingly, the highest concentration of 17α-MT (1000 µg/l) produced the highest percentage of males. No intersex was found in this study. Thus, further studies are required to establish an ideal treatment regime for production of all-male *T. lalius* population using 17α-MT and to provide conclusive evidence regarding their efficacy to be used as a sex-reversal agent in *T. lalius*.

The results emanating from this study indicate that the immersion treatment of 17α-MT might be used to produce higher proportion of male *T. lalius*.

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