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Research Article

Effect of 17α-methyl testosterone, tamoxifen, and letrozole on growth performance and sex reversal of rainbow trout (*Oncorhynchus mykiss*)

Irani A.[1*]; Noori F.[1]

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Abstract

Sex reversal and producing a monosex population is one of the most preferred growth promotion techniques of rainbow trout culture. Thus, the effects of 17α-methyltestosterone (2 mg/kg), tamoxifen (2, 20, and 100 mg/kg), letrozole (2, 20, and 100 mg/kg), and a combination of tamoxifen (100 mg/kg) and letrozole (100 mg/kg) on growth, masculinization and serum steroid content of rainbow trout were investigated in this research. Ethanol-dissolved chemicals were sprayed on commercial trout diet, and ethanol was evaporated overnight. The fish were fed the treated diet for two months and afterward, they were fed a commercial diet for six months. Results showed that 17α-methyltestosterone reversed the sex of rainbow trout effectively. The proportion of males, intersex, and females in this group were 76.67%, 10%, and 13.33%, respectively. In contrast with 17α-methyltestosterone, using tamoxifen and letrozole showed no effect on sex reversal of rainbow trout. Growth performance was adversely affected by all chemical-treated diets. However, compensatory growth occurred during first month after ending treatment period.

Keywords: Masculinization, Rainbow trout, Sex reversal

1-Artemia and Aquaculture Research Institute, Urmia University, Urmia, Iran.
*Corresponding author's E-mail: a.irani@urmia.ac.ir

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Introduction

Sex determination is strongly related to genes in higher vertebrates; consequently, it is challenging to reverse the sex in these vertebrates. Conversely, in fish sex differentiation process can be easily affected by internal and external conditions (Piferrer *et al*., 1994). Thus, for sex reversal in fish, oral administration of steroid hormones is used by aquaculturists.

Fish sex is separated into genotype, determined by the genes responsible for gonads formation, and phenotype, the appearance of ovary or testis. The genotype of sex, related to genes, is determined at the time of fertilization, whereas, phenotype differentiation, the appearance of male or female sex organs, occurs in embryonic and larval development stages. This process is changed with administration of exogenous androgens and estrogens. Because monosex population of some cultured species, having a higher growth rate in comparison with mixed sexes, it is preferred by aquaculturists. For example, male tilapia, female common carp, and female rainbow trout are preferred for culture.

Monosex populations can be achieved either by directly treating fish with hormones to produce preferred sex or by indirect method with two steps; in the first step, embryos/ larvae/ juveniles are treated with androgens or estrogens to produce neo male (XX♂), neo female (XY♀, ZZ♀), or super male (YY♂) populations. In the second step, these sex-reversed fish are used as breeders for producing all female or all-male populations (Hoga *et al*., 2018). Similar to humans, sex determination system in rainbow trout is XX/XY type; thus, to obtain all-female population, XX males are produced by administration of androgens in the first step, and these sex-reversed males are used for mating with normal females in the second step. Among androgens, 17α-methyltestosterone (MT) is numerously used for producing neo male population of teleost fishes (Asadi Eidivand et al., 2022).

In addition, effects of chemicals such as tamoxifen (TM) and letrozole (LZ), with anti-estrogenic activities, on sex differentiation process and sex reversal have been studied in many species of vertebrates (Singh and Srivastava, 2015; Alijani *et al*., 2022**)**. It is well demonstrated that TM and LZ suppress estradiol production in mammals (Bhatnagar *et al*., 2001; Simpson *et al*., 2002) and fish (Kwon *et al*., 2000; Sun *et al*., 2007; Singh *et al*., 2012). Based on acting mechanisms and pathways, there are two groups of anti-estrogenic chemicals. Chemicals, including TM, directly affect actions of estrogens because they inhibit the binding of estrogens to estrogen receptors. Additionally, some studies indicated that masculinization effect of TM is related to suppression of ovary-type P450arom mRNA expression (Kitano *et al*., 2007; Hulak *et al*., 2010). In contrast to the first group, chemicals like LZ act indirectly by inhibiting the excretion of estrogens. In this process, LZ inhibits aromatase activities, the enzyme that plays a crucial role in turnover of estrogens (Sun *et al*.,

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2011). Consequently, ovarian development is suppressed, and sex differentiation leads to development of testis (Singh and Srivastava, 2015).

However, the efficacy of TM and LZ is yet to be evaluated in rainbow trout. Thus, effects of tamoxifen (a receptor blocker), letrozole (an aromatase inhibitor), and 17α-methyltestosterone on growth, masculinization, and serum steroid content of rainbow trout were investigated in this study.

Material and methods

Fish and rearing system

All-female larvae of rainbow trout before yolk sac uptake were transferred to Urmia University by nylon bags containing water and oxygen (1:3 ratios) from Rashekan rainbow trout hatchery. After two days of adaptation, the larvae were randomly distributed among 27 tanks, 125 individuals per 25-liter tank (three replicates per treatment). Water flow rate of 1.3-2.9 l/m and an aeration system were established for each tank in a flow-through system. Water temperature, pH, and dissolved oxygen values were 13.1-15.3°C, 7.85-8.04, and 7.96-8.22 mg/L, respectively. The fish were kept in these conditions for 2.5 months. Afterward, all fish were weighed, counted, and transferred to 200-liter tanks. Water flow rate of 5.7-6.4 l/m and an aeration system were established for each tank in a flow-through system. Water temperature, pH, and dissolved oxygen values were 14.8-15.6°C, 7.94-8.08, and 7.11-7.84 mg/L, respectively (Table 1). Under these conditions, the fish were raised for 5.5 months.

Table 1: Physico-chemical factors of the rearing water during experimental period.

Treatment	Temperature (°C)		pH		DO (mg/L)		water flow rate (l/m)	
	1-75 daf	76-240 daf	1-75 daf	76-240 daf	1-75 daf	76-240 daf	1-75 daf	76-240 daf
C	14.3±0.75	15.1±0.26	7.96±0.08	7.99±0.05	8.07±0.11	7.53±0.25	2.13±0.63	6.07±0.23
M	14.1±0.77	15.3±0.29	7.94±0.08	7.98±0.05	8.11±0.13	7.69±0.31	2.06±0.65	5.95±0.25
T2	14.2±0.72	15.1±0.24	7.93±0.09	8.04±0.04	8.05±0.10	7.59±0.24	2.10±0.62	6.01±0.31
T20	14.3±0.70	15.2±0.22	7.96±0.08	8.00±0.04	8.08±0.11	7.42±0.20	2.03±0.59	6.21±0.20
T100	14.3±0.73	15.0±0.28	7.97±0.07	7.98±0.05	8.01±0.14	7.61±0.27	2.25±0.63	6.17±0.26
L2	13.9±0.69	15.3±0.30	7.91±0.09	7.97±0.06	8.05±0.10	7.54±0.21	2.08±0.66	6.13±0.28
L20	14.1±0.74	15.1±0.25	7.96±0.09	8.04±0.04	8.07±0.11	7.52±0.19	2.33±0.60	6.05±0.23
L100	14.3±0.74	15.2±0.27	7.92±0.08	8.01±0.05	8.13±0.09	7.46±0.25	2.16±0.62	6.01±0.22
T/L100	13.9±0.71	15.1±0.20	7.90±0.07	7.98±0.04	7.97±0.15	7.71±0.32	2.19±0.65	5.99±0.24

Data represents mean ± standard deviation; treatments include C: control, M: 17α-methyltestosterone, T2: 2 mg tamoxifen/kg feed, T20: 20 mg tamoxifen/kg feed, T100: 100 mg tamoxifen/kg feed, L2: 2 mg letrozole/kg feed, L20: 20 mg letrozole/kg feed, L100: 100 mg letrozole/kg feed, and T/L100: 100 mg tamoxifen and 100 mg letrozole/kg feed; daf: days after initiation of feeding.

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Experimental feed

The alcohol evaporation method was used to prepare the chemical-treated diets (Navarro-Martín *et al*., 2009). 17α-methyltestosterone (Aburaihan Pharmaceutical Co., Tehran, Iran), tamoxifen (Iran Hormone Pharmaceutical Co., Tehran, Iran), and letrozole (Soha Pharmaceutical Co., Tehran, Iran) after dissolving in 95% ethanol, were sprayed on the commercial trout diet (Faradaneh Co., Tehran, Iran). 95% ethanol was added to the diet of the control group without the chemicals. Alcohol of the diets was evaporated at room temperature overnight then stored at 4°C in refrigerator. There were nine treatments (with triplicates) containing 0 (control), 2 mg MT/kg feed (M), 2, 20, and 100 mg TM/kg feed (T2, T20, and T100, respectively), 2, 20, and 100 mg LZ/kg feed (L2, L20, and L100, respectively), and combination of 100 mg TM/kg feed and 100 mg LZ/kg feed (T/L100). The fish were fed with treated diets for two months after initiation of the exogenous feeding.

Sampling and measurements

A portable multimeter (WTW, Multi 3630 IDS, Weilheim, Germany) was used to record rearing water temperature, pH, and dissolved oxygen. At the end of the experiment, body weight of all fish from each tank were measured. Weight gain was calculated using the formula "Weight gain=Final weight–Initial weight". The formula "SGR (%day^{-1})=100×(lnWt-lnW0)/t" was applied for calculating specific growth rate (SGR), where Wt and W0 are final and initial weight, respectively, and t is growth time in days (Gisbert and Williot, 1997). Growth retarding rate was calculated using the formula "GRR=(Wc–Wt)/Wc", where Wc and Wt represent mean body weights of control and experimental groups, respectively. For calculating GRR values, means of replicates were used (Shen *et al*., 2015). GSI values were measured using the formula "GSI=(gonad weight/body weight)×100".

For hormonal and histological analyses, 30 fish from each treatment were sampled randomly at the end of the experimental period. The fish were anesthetized with 500 mg/L carnation, and the blood of six specimens of each group was pooled. The samples allowed clotting in serum separator tubes for two hours at room temperature. The samples were centrifuged at 1000 rpm for 15 min at 4°C to obtain serum and stored at -80°C in freezer. Commercial kits (Monobind Inc., Lake Forest, USA) and microplate reader (BioTek, Synergy HT, USA) were used to estimate the serum 17β- estradiol and testosterone.

The fish were dissected after collecting blood. Gonads were weighed and fixed in Bouin solution. Standard histological techniques and microscopic analyses were used for sex determination of experimental fishes (Shen *et al*., 2015).

Statistical analysis

SPSS 22 was used for all statistical analyses of data. Levine's Test was used for Homogeneity of variances testing,

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and Shapiro-Wilk test was used for normality of distribution testing. One-way ANOVA was used to analyze the mean values, then Duncan's *post-hoc* Test was used to separate significantly different groups. All analyses were performed at α=0.05 (Irani and Noori, 2020).

Results

Growth and survival

Values of body weight in the control group were significantly more than T/L100 group 15 days after initiation of the experiment (Fig. 1). In contrast, there were no significant difference among the control, M, T100, L100, and T/L100 groups 60 days after initiation of the experiment (at the end of the hormonal treatment period). Similar results were observed 15 days after ending the hormonal treatment period. There was no significant difference among all groups in the rest of the experimental period (Fig. 2).

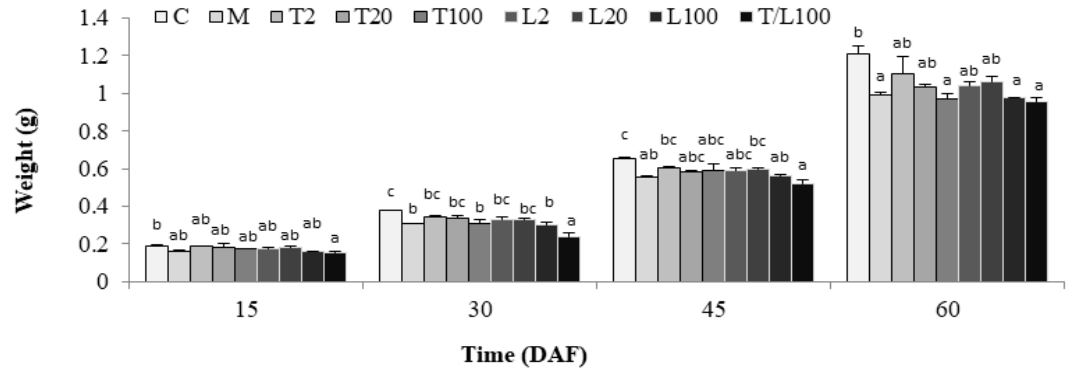


Figure 1: Mean values of rainbow trout body weight during hormonal treatment period. Error bars show standard deviation. Different superscripts represent significant differences among treatments, one-way ANOVA, α<0.05, C: control, M: 17α-methyltestosterone, T2: 2 mg tamoxifen/kg feed, T20: 20 mg tamoxifen/kg feed, T100: 100 mg tamoxifen/kg feed, L2: 2 mg letrozole/kg feed, L20: 20 mg letrozole/kg feed, L100: 100 mg letrozole/kg feed, and T/L100: 100 mg tamoxifen and 100 mg letrozole/kg feed; DAF: days after initiation of feeding.

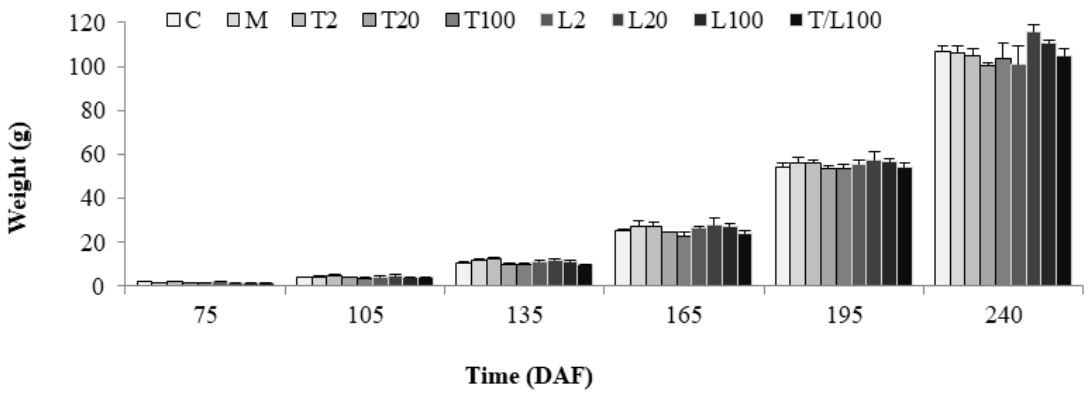


Figure 2: Mean values of rainbow trout body weight after hormonal treatment period. Error bars show standard deviation. C: control, M: 17α-methyltestosterone, T2: 2 mg tamoxifen/kg feed, T20: 20 mg tamoxifen/kg feed, T100: 100 mg tamoxifen/kg feed, L2: 2 mg letrozole/kg feed, L20: 20 mg letrozole/kg feed, L100: 100 mg letrozole/kg feed, and T/L100: 100 mg tamoxifen and 100 mg letrozole/kg feed; DAF: days after initiation of feeding.

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Growth suppression, especially in M, T100, L100, and T/L100 groups, started 15 days after initiation of the hormonal treatment. Growth retarding rate increased during the first month, while it was almost constant during the second month (Fig. 3). Compensatory growth occurred 45 days after ending the hormonal treatments, as there were no significant difference in the growth performances among the groups from 105 DAF onwards.

SGR values were low on 15 DAF and increased afterward (Fig. 4). The values decreased gradually from 60 DAF onwards. There were significant differences between the control and T/L100 groups on 15 and 30 DAF.

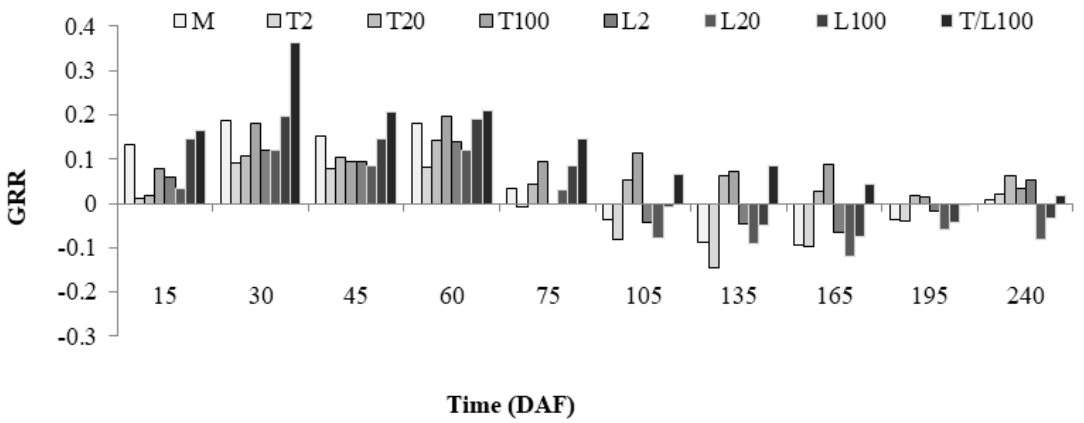


Figure 3: Growth retarding rate of rainbow trout body weight during experimental period, M: 17α-methyltestosterone, T2: 2 mg tamoxifen/kg feed, T20: 20 mg tamoxifen/kg feed, T100: 100 mg tamoxifen/kg feed, L2: 2 mg letrozole/kg feed, L20: 20 mg letrozole/kg feed, L100: 100 mg letrozole/kg feed, and T/L100: 100 mg tamoxifen and 100 mg letrozole/kg feed; DAF: days after initiation of feeding.

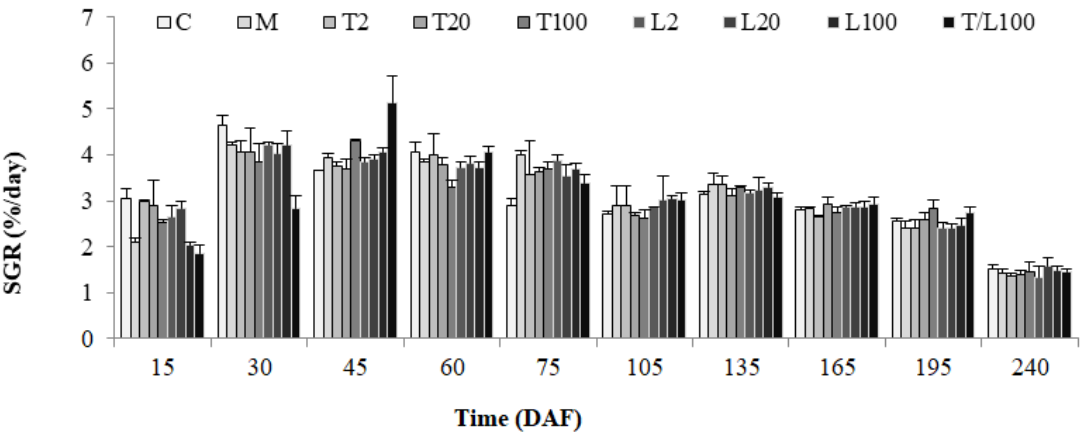


Figure 4: Mean SGR values of rainbow trout during the experimental period; error bars show standard deviation. C: control, M: 17α-methyltestosterone, T2: 2 mg tamoxifen/kg feed, T20: 20 mg tamoxifen/kg feed, T100: 100 mg tamoxifen/kg feed, L2: 2 mg letrozole/kg feed, L20: 20 mg letrozole/kg feed, L100: 100 mg letrozole/kg feed, and T/L100: 100 mg tamoxifen and 100 mg letrozole/kg feed; DAF: days after initiation of feeding.

With exception of 45 DAF, lowest FCR values were observed in control group during the hormonal treatment period (Fig. 5). Values of M, T100, L100, and

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T/L100 groups were higher than those of other groups during this period, whereas there were no significant difference among the experimental groups from 75 DAF onwards.

Sex reversal and GSI

Histological examination of gonads showed that sex reversal occurred only in the group treated with 17α-methyltestosterone. Proportions of males, intersex, and females in this group were 76.67%, 10%, and 13.33%, respectively.

Mean GSI values in males of group M were significantly more than those in other groups. In contrast, the values in females of this group were significantly lower than those in other groups (Fig. 6). There was no significant difference among the rest of the groups.

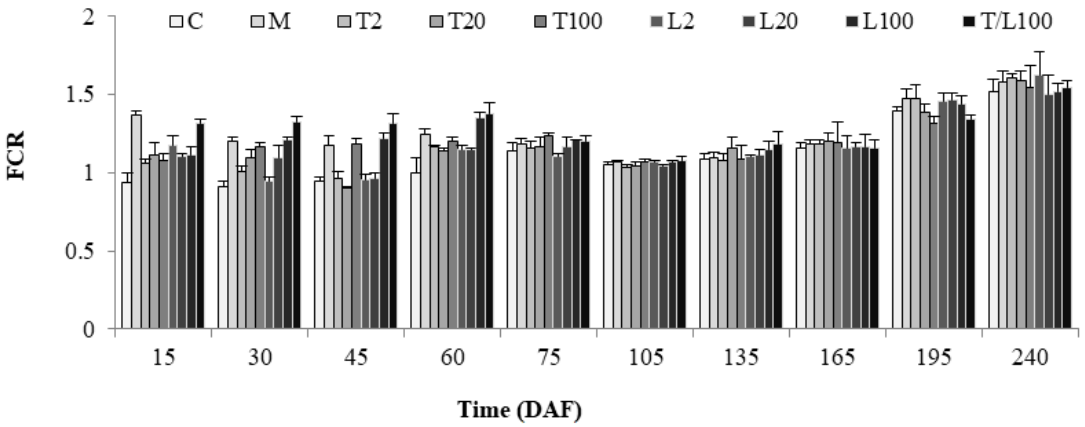


Figure 5: Mean FCR values of rainbow trout during the experimental period. Error bars show standard deviation. C: control, M: 17α-methyltestosterone, T2: 2 mg tamoxifen/kg feed, T20: 20 mg tamoxifen/kg feed, T100: 100 mg tamoxifen/kg feed, L2: 2 mg letrozole/kg feed, L20: 20 mg letrozole/kg feed, L100: 100 mg letrozole/kg feed, and T/L100: 100 mg tamoxifen and 100 mg letrozole/kg feed; DAF: days after initiation of feeding.

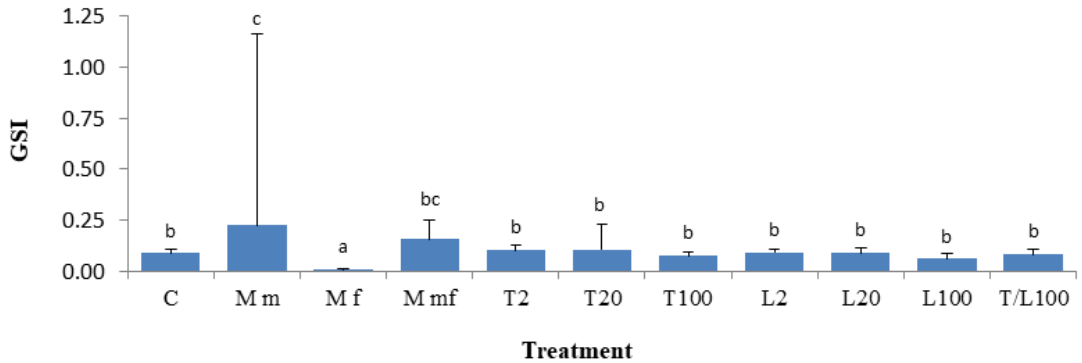


Figure 6: Mean GSI values of rainbow trout during the experimental period. Error bars show standard deviation. Different superscripts represent significant differences, one-way ANOVA, α<0.05; C: control, M: 17α-methyltestosterone, T2: 2 mg tamoxifen/kg feed, T20: 20 mg tamoxifen/kg feed, T100: 100 mg tamoxifen/kg feed, L2: 2 mg letrozole/kg feed, L20: 20 mg letrozole/kg feed, L100: 100 mg letrozole/kg feed, and T/L100: 100 mg tamoxifen and 100 mg letrozole/kg feed; DAF: days after initiation of feeding.

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Steroids

Tamoxifen and letrozole caused no significant change in both testosterone and estradiol of experimental groups in comparison with the control group, whereas sex-reversed males in the 17α-methyltestosterone treated group showed significantly higher and lower concentrations of testosterone and estradiol, respectively, compared to other groups. Testosterone concentration in intersex fish was also significantly higher than that in females of all groups (Fig. 7).

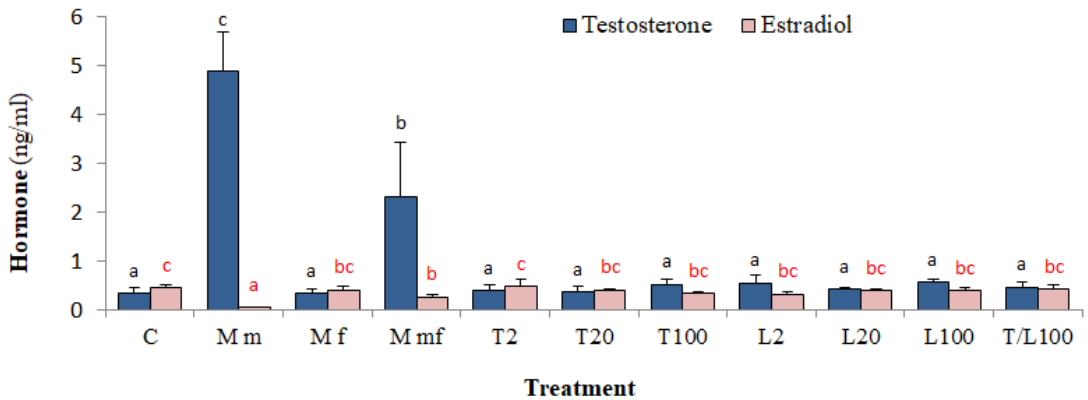


Figure 7: Testosterone and estradiol values of rainbow trout during the experimental period. Error bars show standard deviation. Different superscripts represent significant differences, one-way ANOVA, α<0.05; C: control, M: 17α-methyltestosterone, T2: 2 mg tamoxifen/kg feed, T20: 20 mg tamoxifen/kg feed, T100: 100 mg tamoxifen/kg feed, L2: 2 mg letrozole/kg feed, L20: 20 mg letrozole/kg feed, L100: 100 mg letrozole/kg feed, and T/L100: 100 mg tamoxifen and 100 mg letrozole/kg feed; DAF: days after initiation of feeding.

Discussion

Oral administration effects of 17α-methyltestosterone (2 mg/kg), tamoxifen, (2, 20, and 100 mg/kg), letrozole (2, 20, and 100 mg/kg), and a combination of tamoxifen (100 mg/kg) and letrozole (100 mg/kg) on growth, GSI, steroids, and sex reversal of rainbow trout were investigated in this study. The hormones have been used for sex reversal of several farmed species to produce a monosex population. There are several economic advantages to rear the most profitable gender, which possesses more growth characteristics (Taranger *et al*., 2010; Singh, 2013; Hoga *et al*., 2018). 17α-methyltestosterone (MT) is numerously used for producing neomale population, and all-female population can be produced with these neomale breeders (Piferrer, 2001). In addition, chemicals like tamoxifen and letrozole, with anti-estrogenic activities, have been used for sex reversal in mammals, birds, amphibians, reptiles, and fishes (Singh and Srivastava, 2015**)**.

In the current study, growth performance was significantly affected by all administrated chemicals during two-month treatment period, and suppression of growth occurred, especially at 17α-methyltestosterone and high dosages of both tamoxifen and letrozole

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administrations. Compensatory growth occurred one month after the chemical treatment period. Similar findings were reported by Shen *et al*. (2015) for use of 17α-methyl testosterone in yellow catfish *Tachysurus fulvidraco*. In contrast, they achieved different results in use of letrozole, as low dose (20 mg/kg) of letrozole enhanced growth performance, while in higher doses (50 and 100 mg/kg) growth was not affected (Shen *et al*., 2015). Betancur *et al*. (2014) reported promotion of growth performance in low-dose of LZ (25 mg/kg) in treated red tilapia. This effect could be observed until one month after the treatment period. In their study there was no significant difference among growth values of the control, 17α-methyltestosterone (60 mg/kg), and high dosage (100 mg/kg) of letrozole-treated groups.

These findings indicate significant differences in effects of hormones and other chemicals on growth performance of farmed fish, which could be due to species-specific characteristics. However, these adverse effects were not permanent, and usually compensatory growth occurred during the first month after ending chemical treatment period.

In this study, GSI values in tamoxifen and letrozole-treated groups were not affected, as there were no significant differences between the above-mentioned groups and the control group. Similarly, sex differentiation of rainbow trout was not affected by oral administration of tamoxifen and letrozole, whereas GSI values and sex differentiation of the 17α-methyl testosterone-treated group were significantly influenced as GSI values of males were significantly more than those of the rest. While, in females of the group M, GSI values were significantly lower than those in other groups, which means that the ovary development was suppressed by 17α-methyl testosterone administration.

In contrast to our results, tamoxifen and letrozole showed significant effects on sex reversal of warm-water fishes, like common carp, catfish, and tilapia. A study of tamoxifen and letrozole influences on common carp (*Cyprinus carpio*) and Nile tilapia (*Oreochromis niloticus*) by Singh and Sirvastava (2015) indicated that 100 mg letrozole/kg feed made about 79% masculinization in common carp, and 88% masculinization in Nile tilapia, while the same dose of tamoxifen brought about 63% and 78% masculinization, respectively (Singh and Sirvastava 2015). Oral administration of letrozole (20, 50, and 100 mg/kg feed) produced about 75-83% males in yellow catfish (*Tachysurus fulvidraco*) that were significantly higher than male ratio in the control group, while 17α-methyl testosterone did not affect sex reversal of yellow catfish (Shen *et al*., 2015).

Thus, non-effectiveness of tamoxifen and letrozole on sex reversal in this study might be a result of rainbow trout physiology because this fish is considered a cold-water fish with different physiological requirements in comparison with warm-water fishes.

Monosex population of some cultured species like rainbow trout, having higher

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growth rate in comparison with mixed sexes, is preferred by aquaculturists. Thus, 17α-methyltestosterone is commonly used for masculinization of this species. In the current study, this hormone effectively changed sex proportion of rainbow trout. In addition to 17α-methyltestosterone, use of chemicals with anti-estrogenic activities (tamoxifen and letrozole) for sex reversal, has been attempted in some ornamental and warm-water fishes. In contrast with 17α-methyltestosterone, tamoxifen and letrozole showed no effect on sex reversal of rainbow trout in this study. Growth performance was adversely affected by all chemical-treated diets. However, compensatory growth retardation occurred during first month after ending the treatment period.

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