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Research Article

Inhibition of aromatase activity in rainbow trout (*Oncorhynchus mykiss*) larvae to produce neomale fish through immersion with letrozole

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Abstract

Monosex female rainbow trout can be produced by using neomale broodstock. This study aimed to administer Letrozole, an aromatase enzyme inhibitor, in the sexual differentiation process of rainbow trout through the immersion method. Accordingly, three treatments of 250 µg/L (LET250), 500 µg/L (LET500) and 1000 µg/L (LET1000) of letrozole were used. The results of the sex ratio analysis showed a positive effect of the treatments and a significant difference compared to the control group ($p< 0.05$). The best results were obtained for treatment LET1000 with 88 percent of male specimens. The highest concentrations of testosterone and aromatase were measured in treatment LET1000, at 0.281±0.082 ng/g and 0.72±0.31 ng/g, respectively. This treatment also exhibited the lowest concentration of estradiol. The results obtained in this study show the positive effect of letrozole in inhibiting aromatization, which leads to the production of fish with a male phenotype in the drug-treated population. Regarding survival, the use of letrozole, like testosterone, increased mortality in the experimental treatments. In terms of growth rate, the study's results on the treatments' weight showed that the use of letrozole increased growth in the experimental treatments. Regarding morphological malformation, treatment LET1000 with deformed larvae had the highest rate of deformity among the experimental treatments, but was not significantly different from the rate recorded for the control group ($p\geq0.05$). Based on the results obtained, it can be stated that letrozole is an effective drug in changing the sex of rainbow trout and can be used as a substitute for methyltestosterone.

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Introduction

The production of a monosex, all-female rainbow trout populations provides several advantages compared with conventional mixed-sex populations. Female rainbow trout exhibit higher growth rates, higher disease resistance and later maturation than males and such characteristics have encouraged breeders to breed females through the use of juvenile sex reversal techniques (Wang *et al.*, 2018). One practical route to sex reversal is the use of hormones without altering the sex chromosomes after treatment (Hendry *et al.*, 2003). Steroid hormones, steroid-like plant compounds and some drugs can be used to perform the sex reversal process in fish. In this way, steroid hormones are provided to the fish in a specific dose for a specific time, either orally, by immersion, injection, or implantation, before or during the onset of sex differentiation. If hormone therapy is administered during the labile period, the least amount of steroid is needed to achieve the desired sex. The occurrence of the labile period reflects complex events in the gonads that are among the first histological signs of sexual differentiation (Nakamura, 1984). Thus, this period should be synonymous with the stage sometimes referred to as physiological sexual differentiation. One of the drugs that can be used in the field of masculinization is aromatase inhibitors because the aromatase enzyme catalyzes the final step of estrogen biosynthesis (Bhatnagar, 2007). Letrozole is a non-steroidal competitive inhibitor of the aromatase enzyme system that prevents the conversion of androgens to estrogens (Lal et al., 2024). This drug has been used to induce sex change in tilapia, Japanese Flounder, Zebrafish and Guppy (Babiak *et al.*, 2012; Weber *et al.*, 2020). The effect of this drug has also been investigated by immersion on Atlantic salmon (Lee *et al.*, 2003) and orally on Rainbow Trout (Xu *et al.*, 2021), Catfish (Wisdom *et al.*, 2018) and Common Carp (Singh *et al.*, 2015) and has shown positive results. Also, the use of letrozole in combination with the hormone 17-alpha-methyltestosterone to cause sex change in the common Grouper *Epinephelus coioides* showed good performance and the change of gonadal tissue towards the male was completely visible in the treated fish. The effect of letrozole alone and in combination with nano-chitosan at doses similar to methyltestosterone orally on the sex change of rainbow trout was also investigated and the results of this study did not show a significant difference between the percentage of masculinization of the treatments and the control (Alijani *et al.*, 2022). The same results were obtained in the case of using letrozole for *betta splendens* masculinization in the diet method, while the immersion method was significantly more effective (Katare *et al.*, 2015). This study was designed and implemented to investigate the effectiveness of the non-hormonal drug letrozole by the immersion method instead of hormones used for sex reversal of rainbow trout.

Materials and methods

For drug administration by immersion, three treatments of 250, 500 and 1000 µg/L letrozole were considered, named LET250, LET500, LET1000 and a control group (CTRL) (Katare *et al.*, 2015). By observing

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the implementation of a randomized block design (RBD), three replicates were designed for each treatment and 500 larvae with yolk sacs were used in each replicate. This is the start of the gonadal sensitivity period of rainbow trout and the start of the sexual differentiation period (Piferrer, 2001). The experiment was conducted as follows: for each treatment, the required amount of letrozole was dissolved in alcohol and this solution was added to a pan of water until the concentration of letrozole in the water reached the desired value. The proportion of alcohol was 0.00025 to 0.001 of water. The basket containing the eyed eggs was immersed in the solution for 2 h. This was done 5 times at two-week intervals (Shen *et al.*, 2015). The eggs were aerated during the immersion period using an air stone and an aquarium air pump and the dissolved oxygen level in the water was monitored. After immersion, the eggs were transferred to their original location in the egg storage troughs and normal maintenance and rearing operations were performed on them until sex determination. Biometrics were performed every 20 days during the experimental period. Larvae were maintained in troughs until they weighed 1 g and then transferred to fry ponds. Mortality was recorded daily throughout the study period.

Hormone measurement

Fish were sampled at the end of the exposure period (day 60) to measure sex hormones and aromatase levels. For this purpose,30 larvae from each treatment were completely frozen in a -20°C freezer and dry ice was used to transport them to the laboratory. In the laboratory, frozen larvae in each treatment were pooled separately and hormone and aromatase measurements were performed using an American Microweels device using ELISA method. After sample preparation and dilution, the hormone-specific capture antibody was added to the wells of a 96-well plate and incubated overnight at 4°C. After washing, nonspecific binding sites were blocked with a blocking buffer for 1-2 h at room temperature. For the incubation stage, diluted samples and standards were added to the wells, followed by detection antibody and incubated 1-2h at room temperature. After washing, a substrate solution was added and incubated until color develops and measured absorbance was measured at the specified wavelength using a microplate reader (Diotel *et al.*, 2010).

Sex determination

Sex determination was performed by the histological method in two stages. The first stage was gender determination at a weight of about 10 g and was performed using the Aceto-carmine method, which is a rapid diagnostic method. For sample preparation, tissues were collected and fixed in 10% neutral buffered formalin for 24 h. Fixed tissue was ehydrated through increasing concentrations of ethanol, cleared in xylene and embedded in paraffin. 5-10 µm sections were cut using a microtome and mounted on glass slides. Finally, sections were stained with aceto-carmine solution for 15-30 min, followed by washing in distilled water (Wassermann and Afonso, 2002). The second stage was sex determination at a weight of about 20 g (Fig. 1). After extraction and fixation of the gonads in fixative, staining with hematoxylin-eosin

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and finally preparation of tissue sections and slides from the gonad cross-section. Accordingly, the male-female sex ratio in different treatments was obtained and recorded.

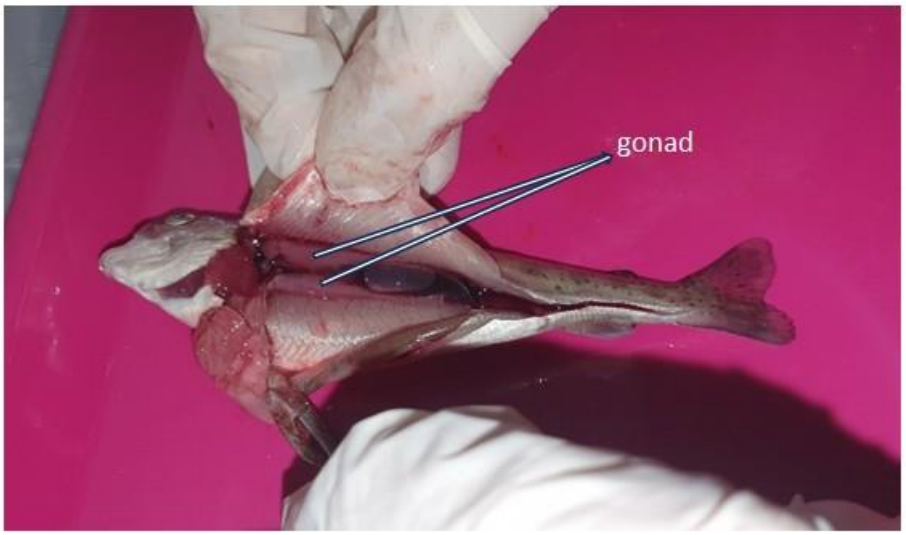

Figure 1: Gonad formation in a 20 g trout.

Drug-induced mortality and assessment of morphological malformation

To detect possible side effects of drug use, the survival rate of drug treatments, as well as the deformity of larvae up to 1 g in weight, were recorded and compared with the control group. The malformations of the treatments were examined quantitatively and qualitatively from the stage of hatching to reaching a weight of 1 g. Qualitatively, malformations in the treatments were divided into four types: spine, eyes, jaw and other malformations (fins, yolk sac, gill cover).

Data analysis

For statistical analysis of the data, their status was first checked using the Kolmogorov-Smirnov test for normality and the Levene test for homogeneity of variances. To compare the significance of the difference between the means, one-way analysis of variance (One-way ANOVA) and Duncan's test were used. All tests were performed with SPSS software version 23.0 at a confidence level of 95%. Data were presented as Mean±SD (Tan *et al.*, 2020).

Results

Sex determination

The results of sex determination performed by the hematoxylin-eosin method and after the fish reached a weight of 20 g are shown in Figure 3. As shown in this figure, the best results were obtained for treatments LET1000 (1000 µg/lit LET) and LET500 (500 µg/lit LET) with 88 and 83 percent male, respectively. The results obtained in these treatments were not significantly different from treatment LET250 (250 µg/lit LET) with 65 percent male, but were significantly different from the control group (p<0.05). The percentage of fish that did not have a specific sex is also presented in Figure 3. Gonads of male fish and gonads of female fish are shown in Figure 2.

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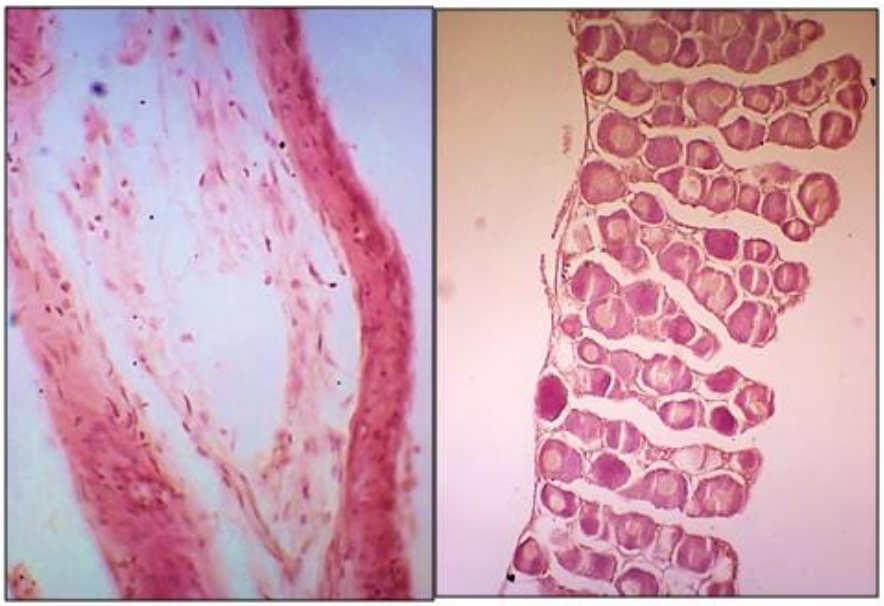

Figure 2: Gonad of male specimen (left side) and gonad of female specimen (right side) in the H&E method.

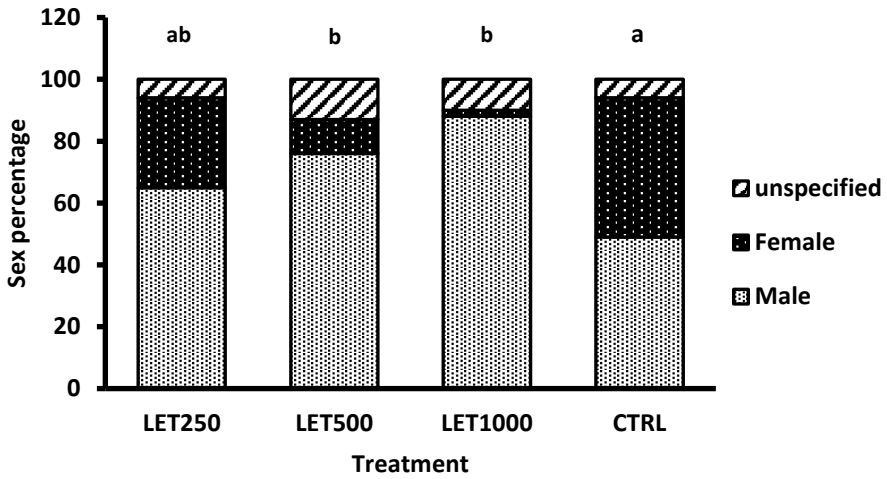

Figure 3: Sex percentage of experimental treatments and the control group

Hormonal and endocrine measurement

The results of measuring the hormones testosterone, estradiol and aromatase enzyme levels in all treatments are presented in Figure 4. As shown in Figure 4, the amount of estradiol hormone was measured at the highest level in treatment CTRL on day 60, at 0.93±0.05 ng/g and the level of estradiol was lower in other treatments. The lowest level of estradiol was observed in treatment LET1000, which was significantly different from the other treatments (p<0.05). Examination of the amount of testosterone hormone in the experimental treatments showed that the concentration of this hormone in treatment LET1000 was higher than in other treatments (0.281±0.082). The aromatase enzyme in all experimental treatments was higher than in the control groups and the highest amount was observed in treatment LET1000.

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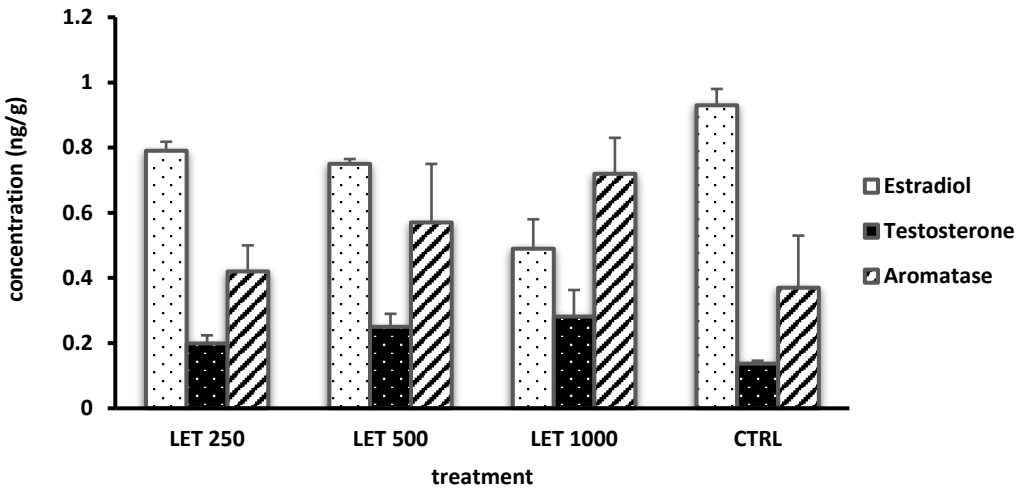

Figure 4: Concentration of testosterone, estradiol, and aromatase enzyme in treatments.

Growth rate

The initial weight of the larva at the beginning of the project was 0.128±0.027g. The average weight of the treatments was measured at 10day intervals and the growth rate was calculated based on that. Figure 5 shows the final weight of the treatments on day 60 of the research and day 130. On day 60, there were significant differences between the drug treatments and control. As can be seen from the weight of the treatments at the end of the experiment (day 130), growth was higher in treatment LET1000 than in the other treatments. In terms of weight at the end of the experiment, no significant difference was observed between the letrozole treatments, but a significant difference was observed in weight between the drug treatments and the control group (Fig. 5).

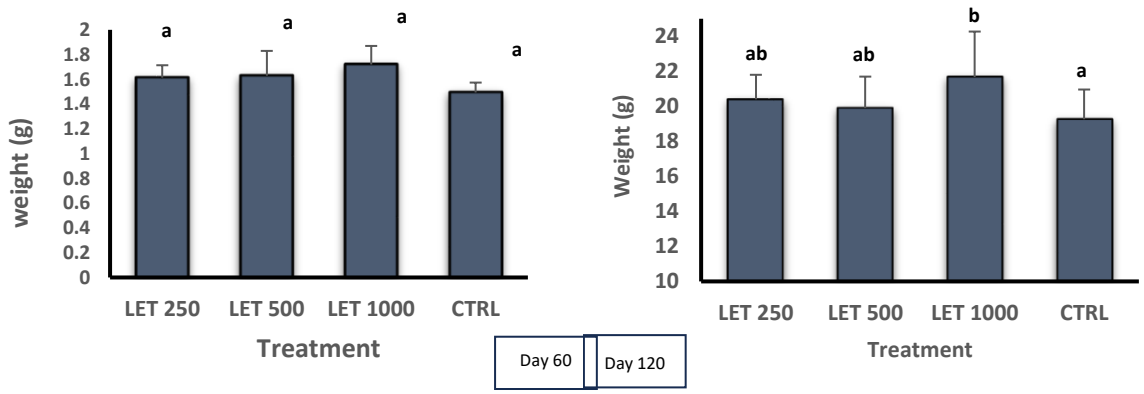

Figure 5: Growth rate of the treatments on days 60 and 130.

Survival

As shown in Table 1, the treatment LET1000 had the highest mortality and the lowest survival, which was significantly different from the other treatments. Among the immersion treatments, treatment LET250 had the highest survival and its difference with the control group was not significant.

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Table 1: Survival percentage of different research treatments at the end of day 60. CTRL: control group.

Treatment	LET250	LET500	LET1000	CTRL
Survival (%)	75±3	61±4	57±6	78±3

Assessment of morphological malformation

Fig. 8 shows that quantitatively, treatment LET1000 with deformed larvae had the highest rate of deformity among the experimental treatments, but this rate was not significantly different from the rate recorded for the control group ($p \geq 0.05$). The rate of deformity in treatment LET250 was 5.2% (78 malformed larvae), in treatment LET500 was 4% (60 malformed larvae), in treatment LET1000 was 6.46% (97 malformed larvae) and in CTRL was 4.8% (72 malformed larvae). Also, treatment LET500 had the highest and treatment LET250 had the lowest rate of deformity in the spine (Fig. 8). The rate of jaw deformity of the larva for each treatment is shown in Figure 8. Treatment LET1000 recorded the highest and treatment CTRL recorded the lowest number of deformed eye larva. Treatment LET1000 had the highest rate of deformed larvae in fins, yolk sac and gill cover (Fig. 8). Some of the deformities in larvae of treatments are shown in Figure 7.

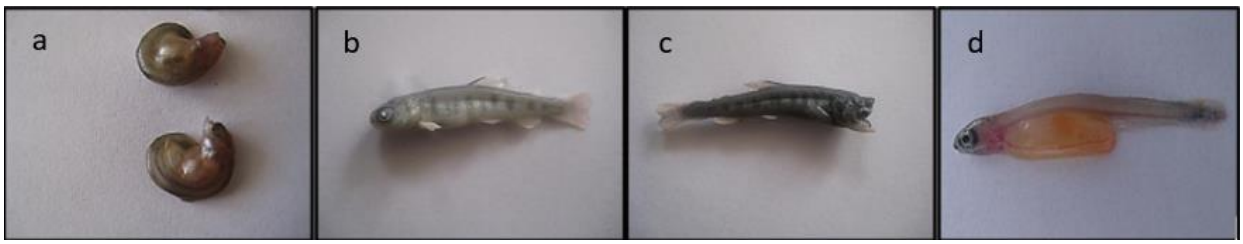

Figure 7: Types of deformities in experimental treatments. Spine (a), eyes (b), jaw(c), and healthy larva (d).

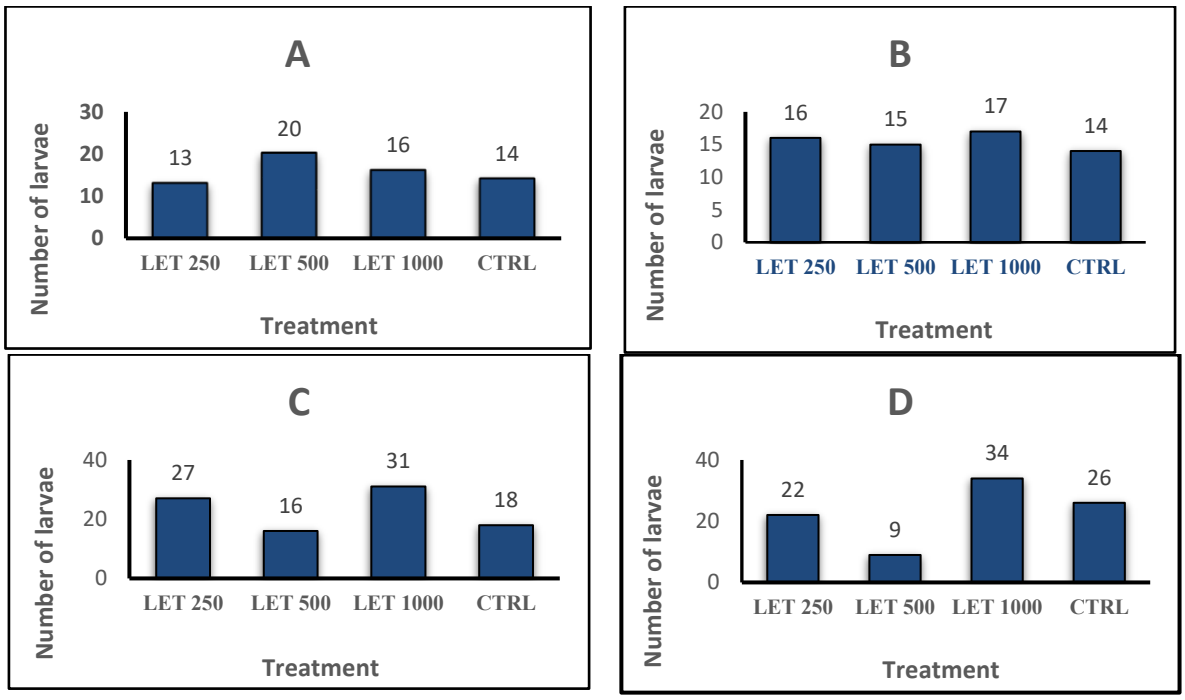

Figure 8: Number of spine malformations (A), eye malformations (B), jaw malformations (C), and other malformations (D) in treatments.

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Discussion

Letrozole, an aromatase inhibitor, has been studied to control sex and sexual maturation processes in a variety of aquatic species with positive results. Some species that have responded positively to letrozole include Mozambique tilapia, siamese fighting fish, zebrafish, common grouper and yellow catfish. The effects of this drug on sexual physiological processes in rainbow trout have also been reported (Akbary *et al.*, 2015). This study showed that letrozole can significantly affect the synthesis of endogenous estrogens from endogens and delay sexual development. The results of the analysis of testosterone and estradiol hormones in the present study also showed that letrozole had a positive effect on blocking the aromatase enzyme and preventing the production of estradiol, so that in the samples taken in the drug treatments containing letrozole, the level of estradiol was significantly lower than in the control group. On the other hand, the amount of testosterone hormone produced in letrozole treatments was significantly higher than the control and since the fish were not given an exogenous hormone, this hormone was endogenous and resulted from preventing the conversion of testosterone to estradiol by blocking the function of the aromatase enzyme. These results were obtained despite the higher aromatase levels in the experimental treatments, indicating that although the aromatase levels were high, the conversion of testosterone to estradiol was inhibited. The high aromatase levels in these treatments could be due to the positive interaction between the presence of testosterone and its converting enzyme in the body. In this study, the sex ratio in the letrozole treatments was different from the control and the LET1000 treatment showed the highest rate of masculinization among the letrozole treatments, indicating the positive effect of increasing the dose of letrozole on the sex reversal of this fish. Also, the highest male sex ratio in the experimental treatments belonged to the LET1000 immersion treatment. One of the advantages of the immersion method over the oral methods of sex change in rainbow trout is the possibility of starting the treatment at the same time as the start of the sexual differentiation period of this fish, which is not possible in the oral method. As stated by Wang *et al.*, 2021, the sensitive period of sexual differentiation or labile period is different in different aquatic species and in rainbow trout, the beginning of this period is before the fish begins to actively feed (Wang *et al.*, 2021). In oral administration, due to the inability of fish larvae to feed on the yolk sac period, part of this period is practically lost and it seems that drug administration by immersion can cover a longer period of this sensitive period of sexual differentiation in rainbow trout. Low efficiency of sex change in oral administration was reported by Alijani *et al.* (2022), who reported that the highest sex ratio in the experimental treatments was 70%. He suggested using the immersion method to investigate the effect of letrozole on rainbow trout, which was implemented in this study and showed positive results. Of course, the low dose of the drug (maximum 3 mg/kg feed) was also stated as another reason for the not very high efficiency of sex change in that experiment. In another study, letrozole was tested orally at a higher

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dose of Letrozole 50 mg/kg, which showed better results (Xu *et al.*, 2021). The results showed that all letrozole-treated groups exhibited higher mortality and consequently lower survival rates than the control group.

The lower survival in the immersion treatments can be attributed to the experimental method. Although aeration was performed regularly during the experiment, water withdrawal during treatment may cause stress in the larvae. Repeating this procedure several times can exacerbate its negative effect, especially in weaker fish. However, it seems that the use of letrozole, like the use of methyltestosterone, increases the mortality rate in fish, which has been observed and reported in other similar studies (Alijani *et al.*, 2022). The growth results of the experimental treatments showed that letrozole had a positive effect on growth rate at the end of the experiment (day 130) in the tested treatments. These data are different from the results presented in the study by Alijani *et al.* (2022), which may be related to the higher dose of the drug in the present study. According to Yamazaki (1976), the increase in fish growth as a result of hormonal treatment depends on the amount of hormone consumed. The amount of letrozole used in this study was up to 50 times higher than that in the study mentioned. In the study by Qin *et al.* (2020), it was also observed that the growth of drug treatments containing letrozole was greater than that of fish in the control group, although this difference was not significant. A previous experiment reported an increase in the growth of rainbow trout larvae in response to the administration of 17-alpha-methyltestosterone in the salmon diet (Bashti *et al.*, 2018). Other reports have also been presented on the potential value of the hormone 17-alpha-methyltestosterone in the cultivation of coho, chinook and rainbow trout (Kobayashi *et al.*, 2006). Also stated that the proximity to external estrogens and androgens can interfere with the growth and development processes of the natural reproduction of fish (Atar *et al.*, 2009) did not observe any significant difference in growth rate in rainbow trout during the first 60 days of the period. However, a significant difference in growth rate was observed during the period from 60 to 180 days of age. Therefore, continued use of this drug may lead to better results regarding the growth of drug treatments. Based on the results obtained, it can be stated that letrozole is an effective drug in changing the sex of rainbow trout and can be used as a substitute for methyltestosterone, with a positive effect in inhibiting aromatization, controlling estrogen production and increasing endogenous testosterone, which leads to the production of fish with a male phenotype in the drug-treated population.

Conclusion

Based on the results obtained, LET1000 treatment showed the highest percentage of sex differentiation. Moreover, the highest levels of testosterone and aromatase enzyme were measured in this treatment, in contrast to esteradiol hormone. These findings, together with the better growth results of this treatment, could lead to LET1000 treatment being recommended as the superior letrozole treatment for use in

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masculinization of rainbow trout larvae. However, lower survival and and higher incidence of morphological deformities observed in this treatment should be carefully considered when evaluating its overall efficacy and practical applicability. Overall, the present findings suggest that letrozole has the potential to induce sex reversal in rainbow trout and may provide an alternative to conventional methyltestosterone-based approaches.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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