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# USE OF 17β-ESTRADIOL FOR *Leporinus macrocephalus* FEMINIZATION*

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## ABSTRACT

The aim of this study was to evaluate the effects of the use of a diet supplemented with 50 or 100 mg $kg^{-1}$ 17β-estradiol ($E_2$) for *Leporinus macrocephalus* feminization. Thus, one hundred and fifty fingerlings with 50 days old post-hatch were randomly distributed in fifteen experimental tanks of 90 L and fed for 60 days on a diet supplemented with 50 or 100 mg $kg^{-1}$ of $E_2$. At the end of the experiment, sex ratios were determined through histological and macroscopic observations. Histologically, the differentiated ovaries were evidenced by the presence of numerous nests of oogonia and oocytes in primary growth stage. The female ratio (77%) for the group treated with 100 mg $kg^{-1}$ $E_2$ was significantly higher than those of control (52%) and 50 mg $kg^{-1}$ treatment (48%) groups. These results obtained in this initial study indicated that 100 mg $kg^{-1}$ of $E_2$, administered over 60 days, was the most effective treatment for 50 days old *L. macrocephalus* post larval feminization. However, future studies with variations in the application range can bring even better results.

**Keywords:** sex inversion; monosex fish populations; aquaculture-species; native fish.

## UTILIZAÇÃO DE 17β-ESTRADIOL PARA FEMINIZAÇÃO DE *Leporinus macrocephalus*

## RESUMO

O objetivo do estudo foi avaliar o efeito do 17β-estradiol ($E_2$) na feminização de *Leporinus macrocephalus*. Dessa forma, 150 alevinos com 50 dias de idade foram distribuídos aleatoriamente em 15 tanques experimentais de 90 L e alimentados por 60 dias com dieta suplementada com 50 ou 100 mg $kg^{-1}$ de $E_2$. No final do experimento, as proporções sexuais foram determinadas por meio de observações histológicas e macroscópicas. Histologicamente, os ovários diferenciados foram evidenciados pela presença de numerosos ninhos de oogonia e oócitos em fase crescimento primário. O percentual de fêmeas (77%) do grupo tratado com 100 mg $kg^{-1}$ $E_2$ foi significativamente maior comparado ao grupo controle (52%) e ao grupo 50 mg $kg^{-1}$ (48%). Os resultados obtidos neste estudo inicial indicaram que 100 mg $kg^{-1}$ de $E_2$, administrados durante 60 dias, foi o tratamento mais efetivo na feminização de *L. macrocephalus* com 50 dias de idade. No entanto, estudos futuros com variações no intervalo de aplicações podem trazer ainda melhores resultados

**Palavras-chave:** inversão sexual; populações de peixes monosexo; espécies aquícolas; peixes nativos.

## INTRODUCTION

One of the major challenges of Brazilian aquaculture is the lack of technological packages for the creation of important native species of fish. According to the yearbook of Brazilian fish farming, there was a drop in native fish production, especially due to the lack of technological packages development (Peixe BR, 2019). The production of monosex fish populations is one of the alternatives that can help to boost the cultivation of native fish (Fernandino and Hattori, 2019). Monosex batches provide high economic profitability in production, adding value to the exclusive production of the sex with the best growth rates (Singh, 2013; Budd et al., 2015; Thuong et al., 2017; Alcántar-Vázquez, 2018; Vidal-López et al., 2019). Among the native species of fish considered of great commercial potential, the *Leporinus macrocephalus* deserves to be

highlighted, since it is a large-sized fish, commercially relevant for fisheries and aquaculture (Pereira et al., 2017). This fish is endemic of the Paraguay River basin and it is a valuable species in aquaculture programs (Morelli et al., 2007; Hashimoto et al., 2010; Muñoz et al., 2011). This species was, according to official fish production data, one of the ten most produced native fish in Brazil in 2018 (IBGE, 2018), mainly due to its high-quality meat (Duke Energy International-Geração Paranapanema S/A., 2003) and acceptance for commercial, subsistence, and sport fishing purposes (Petrere Junior et al., 2002; Giamas and Vermulm Junior, 2004). *Leporinus macrocephalus* is considered of great commercial potential because it presents fast growth in the initial stages, rusticity to the handling and resistance to the temperature variations (Soares et al., 2000; Riffel et al., 2012; Capodifoglio et al., 2015).

*Leporinus macrocephalus* females show higher growth rates than males (Reidel et al., 2004), which is a factor that can be used to increase productivity without the need to enlarge the area of cultivation, and, in addition, to reduce the time of slaughter. The use of endocrine techniques for sexual inversion is widely used in fish production, and it directs the formation of monosex populations for the genus that presents zootechnical advantages (Devlin and Nagahama, 2002; Cnaani and Levavi-Sivan, 2009; Singh, 2013; Örn et al., 2016; Thuong et al., 2017; Alcántar-Vázquez, 2018; Vidal-López et al., 2019). In aquaculture this may represent significant economic gain, adding value to the product if only individuals from the sex with the best growth rates are produced. Thus, in order to foster fish farming industry, it is necessary to develop technological packages for the creation of native species considered of great commercial potential. However, there are few studies in the literature that carry out the production of monosex population of freshwater tropical native species. Thus, this study aimed to evaluate the effects of the use of a diet supplemented with 50 or 100 mg $kg^{-1}$ of 17β-estradiol ($E_2$) for *Leporinus macrocephalus* feminization.

## MATERIAL AND METHODS

### Ethical note

This study was conducted in agreement with the precepts of the National Council for the Control of Animal Experimentation (CONCEA) and was approved by the Animal Ethics and Welfare Committee from UNESP, Jaboticabal, SP, Brazil, under permission number 015279/10.

### Fish culture

Fifty days old fingerlings obtained from a fishing farm were transferred to the Fish Reproduction Laboratory at the Aquaculture Center of Unesp – CAUNESP, and kept in 500 L indoor stock-tanks (ca. 1 fish 5 $L^{-1}$) during 10 days for acclimation before the experiment. During acclimatization, the animals were manually fed a pelleted balanced commercial diet (moisture content (max) 10.0%; crude protein (min) 32.0%; ethereal extract (min) 10.0%; fibrous matter (max) 7.0%; ash (max) 10.0%; calcium (max) 1.2%; phosphorus (min) 1.2%) corresponding to 3.0% of total body weight four a day. External biological filters and constant aeration ensured water quality.

### Experimental Protocols

After the acclimation period, 150 fingerlings (4.35 ± 0.92 cm, 2.12 ± 1.36 g) were randomly distributed in 15 experimental tanks of 90 L in a density of about 1 fish/9 L. A completely randomized design was applied with an equal number of replicates per treatment (N = 5). Experimental diets were enriched with two different concentrations (50 and 100 mg $kg^{-1}$) of 17β-estradiol ($E_2$) (Sigma Co., USA). To incorporate $E_2$ into manufactured feed, we used a well-established method of spraying ethanol-dissolved steroids onto the food (Lin et al., 2012; Singh, 2013). This procedure involved making stock solutions (1 ppt) of $E_2$ dissolved in 100% ethanol which was added by spraying to ensure uniform distribution of the hormone. Then the feed was distributed in trays under the light, for 48h for the complete evaporation of the ethanol. Control diets were prepared in the same way, but without the addition of steroid. The fish were fed to satiation, by hand, four times daily throughout the experiment, with feeding times spread over a 12 h period. External biological filters and constant aeration ensured water quality. Water temperature was kept at 27 ± 1°C and photoperiod was 12L: 12D. The leftover food and feces were removed weekly, by the drain in the tanks.

### Sex determination and histological examination

Sixty days after the onset of the treatments, two fish per replicate were randomly selected and euthanized with a lethal dose of benzocaine (2 g $L^{-1}$). Biometric analyses were performed by determination of the individual total weight (g) and standard length (cm). Food intake was calculated by recording the consumption in a period of 24 hours. Its calculation was obtained by the daily subtraction between the food quota offered and the leftovers from the subsequent day (Pereira et al., 2015). To determine the sexual differentiation, the gonads were photographed and analysed by stereomicroscope. However, for the definitive diagnosis of the sex, we collected gonad samples from all animals for histological evaluation, aiming to verify the presence of germ and somatic cells in presumptive testicles and ovaries. The cranial, medial, and tail regions of the gonad's tissues were fixed in Bouin solution for routine histological procedures, embedded in Historesin™ for histological preparation, and stained with hematoxylin-floxin. The changes in sex ratios were determined by light microscopy. The gonadal tissues were classified as undifferentiated and differentiated. For the sex characterization, the following criteria were adopted: for males, the presence of spermatogonia cysts and spermatocytes were sought, and for females, the presence of oocytes in primary growth. Undifferentiated gonadal tissues were characterized by the presence of gonocytes only (detectable by light microscopy). The effects of $E_2$ on gonadal differentiation were estimated considering the sexual proportions between the treatments.

### Statistical analysis

Data normality was verified using the Cramer-von Mises test. Homoscedasticity was checked through the Fmax test. The food intake and body weight were analyzed by comparing different treatments with a one-way analysis of variance (ANOVA). The Chi - square test ($\chi^2$) was used to analyze gonadal differentiation between treatments. A threshold of $P \leq 0.05$ was set to infer statistical significance.

## RESULTS

### Growth and survival

We did not observe significant differences in the body weight of the animals among groups. Mortality was not observed in any of the experimental groups. There was no difference for food intake among the treatments and controls ($P = 0.45$; Table 1).

### Sex ratio and macroscopic evaluation of gonads

Animals treated with 100 mg kg$^{-1}$ $E_2$ showed significantly higher female ratio (77.78%) than control (57.14%) and 50 mg kg$^{-1}$ treatment (62.50%) ($\chi^2$, $P = 0.01$, Table 1). No male animals were observed in any group up to the end of the experimental period. Undifferentiated gonads, found mainly in the control treatment, were constituted by a pair of translucent, homogeneous and elongated structures, located in the dorsal region of the celomatic cavity, parallel to the swim bladder (Figure 1A). On the other hand, ovaries, which were frequent in the treatment 100 mg kg$^{-1}$ $E_2$, presented a thicker aspect in the cranial region in relation to the caudal one. In addition, the its coloration changed from translucent to slightly brownish-gray, with the presence of blood vessels (Figure 1B).

**Table 1.** Average values (± standard error) of food intake, body weight and sex ratio (female) of fingerlings fed with different concentrations (50 and 100 mg kg$^{-1}$) of $E_2$.

| Parameters | Treatments | | |
|---|---|---|---|
| | Control | $E_2$ (50 mg kg$^{-1}$) | $E_2$ (100 mg kg$^{-1}$) |
| Food intake (g) | 15.04 ± 0.80 | 14.27 ± 0.43 | 14.01 ± 0.91 |
| Body weight (g) | 21.53 ± 1.08 | 21.26 ± 0.38 | 21.33 ± 0.74 |
| Female ratio (%) | 57.14 ± 4.7 $^{b}$ | 62.50 ± 2.6$^{b}$ | 77.78 ± 1.8$^{a}$ |

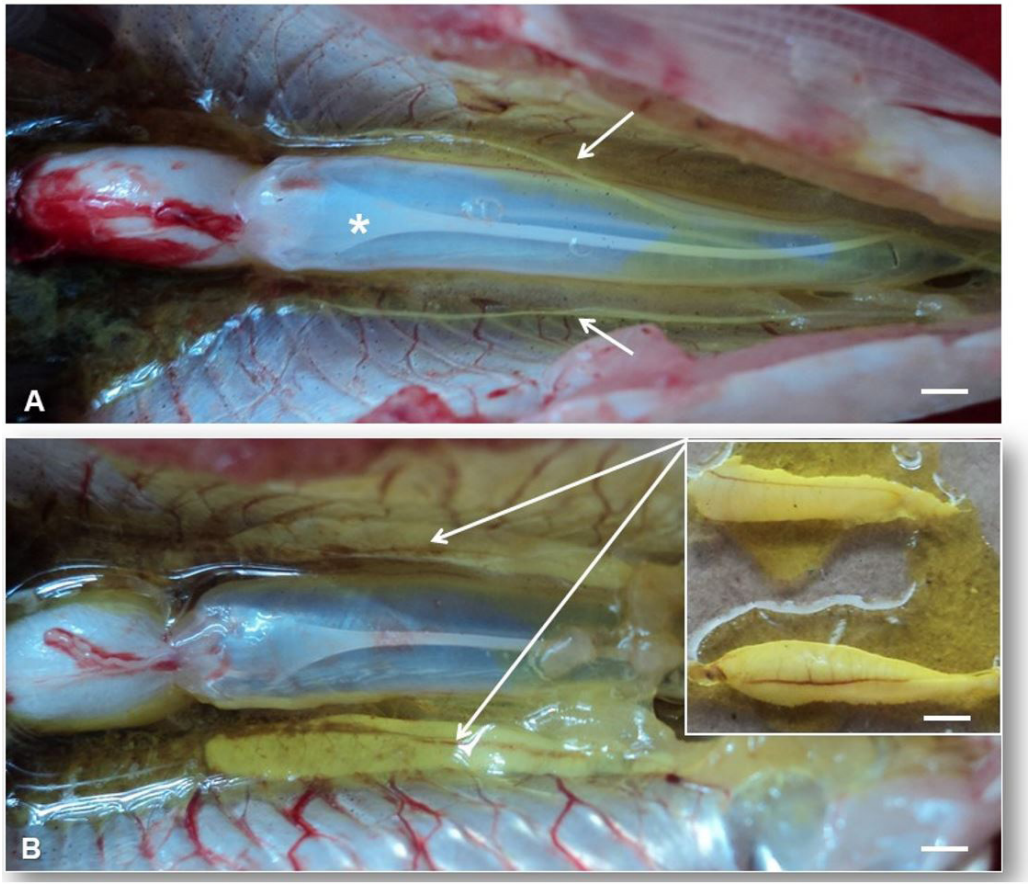


**Figure 1.** (A) Photograph of undifferentiated gonads observed in the control treatment. The gonads (arrows) were located in the dorsal region of the celomatic cavity, parallel to the swim bladder (asterisk). (B) Photograph of the ovary of *Leporinus macrocephalus* fed with 100 mg kg$^{-1}$ of E2. The ovaries showed small blood capillaries throughout the gonadal tissue (arrows). The inset shows details of ovaries extracted from the fish for histological processing. Scale bar = 400 µM.

### Histological evaluation of gonads

Undifferentiated gonads were formed by gonocytes and somatic cells. Somatic cells located close or around gonocytes were had elongated to cubic shape. Their basophilic nuclei shape varied according to cell shape. Gonocytes showed relatively large nuclei, which contained a prominent nucleolus and loose chromatin (Figure 2A). Ovaries were evidenced by the presence of numerous nests of oogonia distributed throughout the germinal epithelium. In addition, ovaries with numerous oocytes in primary growth stage were found (Figure 2B).

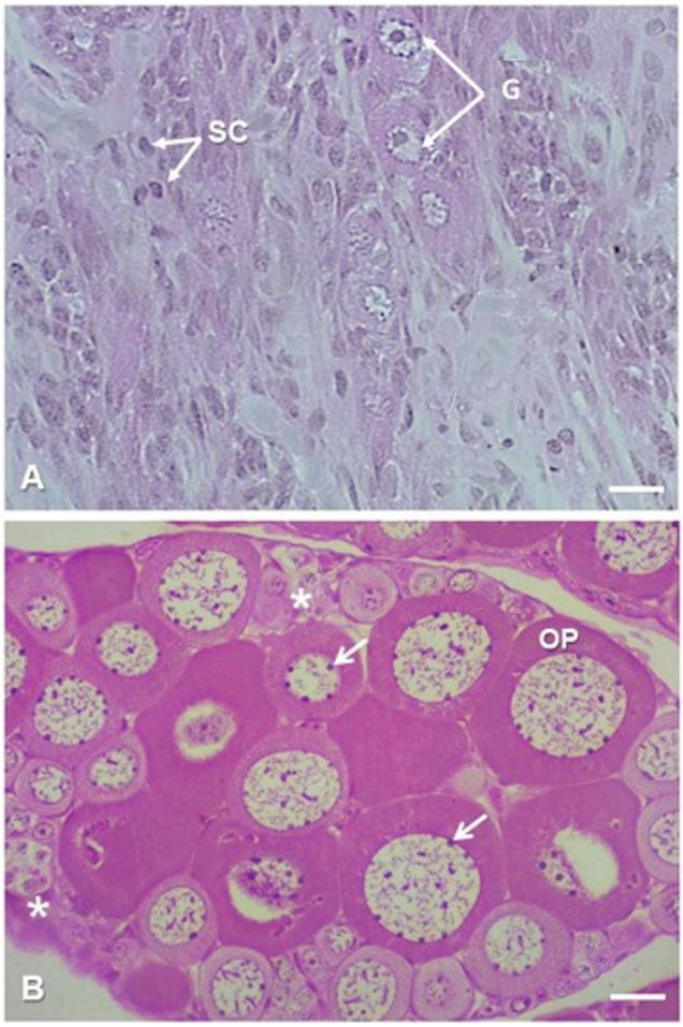


**Figure 2.** (A) Photomicrographs of cross sections of undifferentiated gonads showing the presence of gonocytes (G) and somatic cells (SC). (B) Photomicrographs of cross sections of the ovary of *Leporinus macrocephalus* fed with 100 mg $kg^{-1}$ of $E_2$ showing the presence nests of oogonia (asterisk) and numerous previtellogenic oocytes (OP) in primary growth stage. In this stage multiple nucleoli are displaced to the periphery of the germinal vesicle (arrows). Hematoxylin–floxin. Scale bar: Fig 2A = 20 µM; Fig 2B = 40 µM.

## DISCUSSION

*Leporinus macrocephalus* fed with diets supplemented with 100 mg $kg^{-1}$ of $E_2$ had significantly higher female ratios, being an effective alternative to promote the process of feminization in this species.

Although sexual steroids have a direct influence on the growth and survival of teleosts (Piferrer, 2001; Lin et al., 2012; Thuong et al., 2017; Alcántar-Vázquez, 2018; Vidal-López et al., 2019), in this study, we did not observe mortality and differences in food intake or growth among treatments and controls. According to Vidal-López et al. (2019), estrogens show no anabolic effect in most teleost. This agrees with that we observed in our study, since exposure to estrogen did not cause a significant reduction or increase in growth rate. Thus, 100 mg $kg^{-1}$ of $E_2$ may be used for this purpose in *L. macrocephalus*, since it did not cause productive losses.

It has been previously shown that the sexual differentiation in fish is regulated by sex steroid hormones (Singh, 2013; Pradhan and Olsson, 2015; Hoga et al., 2018) and indirectly by steroidogenic enzymes (Baroiller and D'Cotta, 2001; Tokarz et al., 2015; Di Rosa et al., 2016; Koyama et al., 2019). However, environmental factors (Hunter and Donaldson, 1983; Devlin and Nagahama, 2002; Piferrer et al., 2012; Díaz and Piferrer, 2015) and hormonal manipulations may intensify this process (Piferrer, 2001; Lin et al., 2012; Alcántar-Vázquez et al., 2015; Marín-Ramírez et al., 2016; Juárez-Juárez et al., 2017). In this concern, the most conventional route of ovarian development involves the transcription of the *Cyp 19a1a* gene and the production of the enzyme aromatase complex (P450arom). This enzyme is the main key in estrogen synthesis (Vernetti et al., 2013; Göppert et al., 2016), which is responsible for inducing and maintaining ovary development (Devlin and Nagahama, 2002; Nishimura and Tanaka, 2014; Lau et al., 2016). Thus, it is possible that diets supplemented with $E_2$ increase the circulating levels of this hormone in the treated fish, promoting the gonadal differentiation during the initial development of this species. In fact, this has been seen in several studies (Marín-Ramírez et al., 2016; Juárez-Juárez et al., 2017; Alcántar-Vázquez, 2018; Vidal-López et al., 2019) in which diets supplemented with $E_2$ promote fish gonadal differentiation.

The action of $E_2$ on gonadal differentiation in fish is well documented in the literature (Nagahama and Yamashita, 2008; Thuong et al., 2017; Alcántar-Vázquez, 2018; Vidal-López et al., 2019), and this steroid binds to specific nuclear receptors, which are three main subtypes in fish (α, β1 and β2) (Thomas et al., 2006). Such receptors are expressed at different sites and they also regulate ovarian development and growth (Pankhurst, 2016). In addition, during the early stages of growth, $E_2$ stimulates the first mitotic divisions of oogonia (Miura et al., 2002; Lubzens et al., 2017) and the synthesis of cortical alveoli in oocytes (Kwok et al., 2005; Luckenbach et al., 2013; Lubzens et al., 2017).

## CONCLUSION

In this study, the use of diets supplemented with 100 mg $kg^{-1}$ of $E_2$ demonstrated to be an alternative to intensify the process of feminization in *L. macrocephalus*. Monosex fish production is a breakthrough for aquaculture, and several studies showed the efficiency of this technique to increase productivity. In this concern, here we have brought promising results for application of this methodology to *L. macrocephalus*, in which females present higher growth rates than males. However, future studies are needed to investigate the mechanism of action of $E_2$ in feminization in this species and to clearly determine the best dose and exact time for administration of diets enriched with $E_2$ to increase the rate of sexual inversion.

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