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**Aquaculture**

# Menthol and eugenol as natural anesthetics for early juveniles of curimba

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**ABSTRACT** - The objective of this study was to evaluate menthol and eugenol as anesthetics for early juveniles of curimba, *Prochilodus lineatus*. Juveniles of 4.0±0.5 g and a total length of 8.8±0.1 cm were exposed to the anesthetics menthol (20, 30, 40, 50, 60, 70, and 80 mg $L^{-1}$) and eugenol (20, 30, 40, 50, 60, and 70 mg $L^{-1}$) up to deep anesthesia. The anesthetic effects were evaluated measuring the induction time to deep anesthesia (characterized by loss of equilibrium, absence of swimming, reduction of opercular movements, and responses only to intense tactile stimuli), recovery time, time to appetite return, and mortality rate after 96 h of procedure. The concentrations between 60 to 80 mg of menthol $L^{-1}$ provided the lowest time of induction. Increased concentrations led to a decrease in recuperation time. The concentrations between 50 to 70 mg of eugenol $L^{-1}$ provided the lowest induction times; however, recovery time was not affected by eugenol concentrations. The return to appetite was observed 24 h after anesthesia, while the survival after 96 h was >90%. Concentrations of 60 and 50 mg $L^{-1}$ of menthol and eugenol, respectively, are recommended for effective anesthesia with limited side effects.

Key Words: aquaculture, physiology, *Prochilodus lineatus,* stress

## Introduction

In Brazil, anesthesia has been studied aiming to minimize stress and, thereby, decrease suffering of animals during handling procedures (Bertozi Junior et al., 2014; Ribeiro et al., 2015a,b). The concentration and efficacy required for induction may vary in relation to species, age, size, and water quality parameters (Walsh and Pease, 2002; Woody et al., 2002; Gomes et al., 2011).

Eugenol and menthol are among the main natural anesthetics used in aquaculture. Eugenol is a natural product obtained from the distillation of the extract of clove (*Eugenia caryophyllata*) leaves, stems, and roots (Inoue and Moraes, 2007). The efficiency of eugenol has been observed for several species, such as rainbow trout (*Oncorhynchus mykiss*; Keene et al., 1998), pacamã (*Lophiosilurus alexandri*; Ribeiro et al., 2013), Nile tilapia (*Oreochromis niloticus*; Vidal et al., 2008; Ribeiro et al., 2015b), matrinxã (*Brycon cephalus*; Barbosa et al., 2007; Vidal et al., 2007), pacu (*Piaractus mesopotamicus*; Gonçalves et al., 2008), lambari (*Astyanax altiparanae*; Pereira-da-Silva et al., 2009), silver catfish (*Rhamdia quelen*; Cunha et al., 2010; Gomes et al., 2011), and fat snook (*Centropomus parallelus*; Souza et al., 2012).

Menthol is an essential oil extracted from plants of the genus *Mentha* (Patel et al., 2007). This drug may be used for fish anesthesia, as observed for pacu (Gonçalves et al., 2008), tambaqui (*Colossoma macropomum*; Façanha and Gomes, 2005), fat snook (Souza et al., 2012), dourado (*Salminus brasiliensis*; Pádua et al., 2010), and Nile tilapia (Simões and Gomes, 2009).

Curimba (*Prochilodus lineatus*) has social, ecological, and economic importance in South America (Jorge et al., 2011). The species has excellent growth performance and potential for aquaculture (Graeff and Tomaselli, 2011). However, studies on the effects of eugenol and menthol in early juveniles of curimba were not tested yet in curimba. Therefore, the present study aimed to evaluate the efficiency of eugenol and menthol as anesthetics for early juveniles of the species.

## Material and Methods

Early juveniles of curimba were purchased from a commercial hatchery and transported by car to the laboratory

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rearing facilities in Cordeiro, RJ, Brazil (22º01'03.88" S and 42º21'24.14" W). All procedures were carried out according to the international practices for animal use and care under the control of a local ethical committee on animal use (case no. 003/2016).

A total of 150 juveniles of curimba, weighing 4.0±0.5 g, with a total average length of 8.8±0.1 cm, were used for anesthetic induction by menthol and eugenol. The juveniles of curimba were acclimated for a period of 30 days in a recirculation system in 120-L water tanks. They were fed three times a day (at 8:00, 12:00, and 17:00 h) a commercially formulated diet containing 400 g protein $kg^{-1}$, 350 mg $kg^{-1}$ vitamin C, 80 g ether extract $kg^{-1}$, and 100 g moisture $kg^{-1}$ (levels and guarantees made available by the manufacturer). The experimental units were cleaned prior to the first and after the last feed for the withdrawal of excreta and food residues. The water quality indicators remained in the range considered adequate for the maintenance of tropical fish species (Arana, 2004). Water temperature was kept at 25.2±0.8 °C; dissolved and saturated oxygen at 7.0±0.4 mg $L^{-1}$ and 86.0±3.9 %, respectively; pH at 6.7±0.3; electric conductivity at 0.6±0.1 µS $cm^{-1}$; total dissolved solids at 0.3±0.1 ppt; and unionized ammonia nitrogen ($NH_3$-N) at 0.15±0.03 mg $L^{-1}$. Physical and chemical monitoring of water was carried out using a HI83203-01 Hanna Photometer, HI9146-04 Hanna oxygen meter, and HI98130 Hanna multi-parameter Combo.

The experiments were performed in a completely randomized design, in which the treatments were menthol (20, 30, 40, 50, 60, 70, and 80 mg $L^{-1}$) and eugenol (20, 30, 40, 50, 60, and 70 mg $L^{-1}$) concentrations. Ten fish randomly chosen were used in each treatment. Eugenol (Escamaforte®) and menthol (CRQ−1006310100) were first diluted (1:10) in ethanol 98.8% to reduce their hydrophobic character, and later added to the experimental units in accordance with the predetermined concentrations. Fish feed was suspended for a 24-h period before the anesthetic procedure. The hyperactivity of fish subjected to immersion in an anesthetic should not be attributed to ethanol used as a solvent. Therefore, early juveniles of curimba were subjected to an ethanol (98.8%) solution at similar quantity of that used in the highest concentrations of anesthetics to evaluate the possible hyperactivity.

For the induction analysis and recovery from anesthesia, fish were randomly captured one at a time and placed in a 2-L beaker containing the concentration of anesthetic to be tested. The behavioral characteristics of the deep anesthesia (stage 4 of anesthesia) used in this study followed the recommendations suggested by Ross and Ross (2008), basically characterized by loss of equilibrium, absence of swimming, reduction of opercular movements, and responses only to intense tactile stimuli. The non-reaction to stimuli was verified by touching the fish on the side using a glass rod. When the individuals reached the stage 4 of anesthesia, their biometry and weight (model BL320H Shimatzu scale) measurements (total length using a ruler) were carried out to simulate the actual handling conditions in fish farming. For recovery, all individuals were placed in anesthetic-free water in 2-L beakers. To assess the anesthetic recovery stages, the criteria proposed by Hikasa et al. (1986) were adopted. After the experiments, fish from each replicate were pooled and kept in 50-L tanks in a recirculating water system to observe the return to appetite and the survival rate after 96 h.

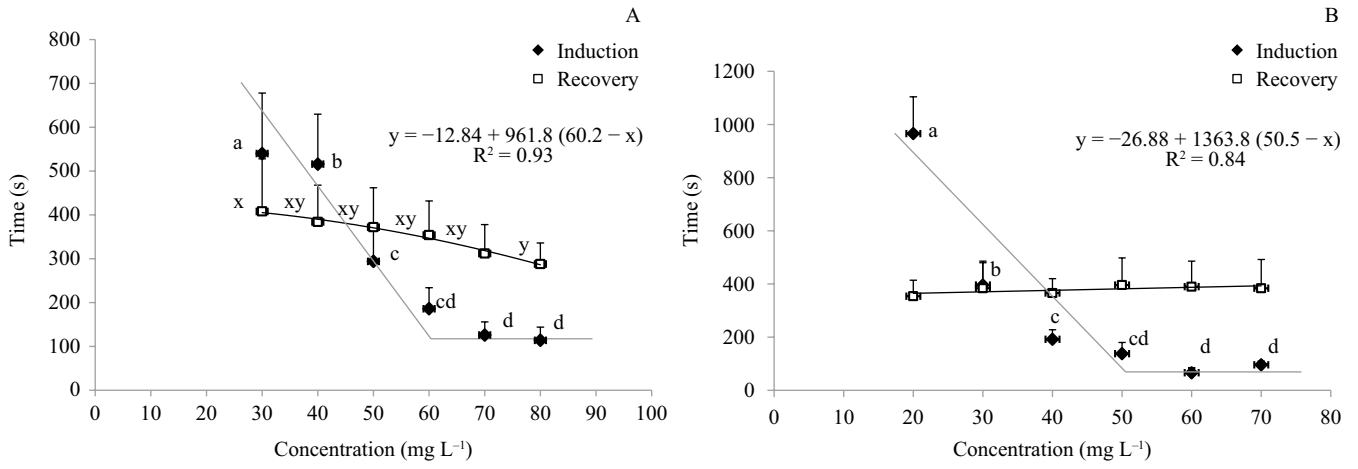
An ANOVA and the Tukey's test for the comparison of means were performed using the SAS software (Statistical Analysis System, version 8.0). The results of the induction and recovery time were analyzed using a non-linear regression for the function with best adjustment of data.

## Results

Exposure to ethanol solvent did not provoke any behavioral change. Menthol and eugenol concentrations proved efficient in promoting induction at the deep anesthetic phase in early juveniles of curimba. As concentrations increased, there was a decrease ($P<0.05$) in the induction and recovery time. Juveniles of curimba did not reach deep anesthesia after 30 min (1800 s) of exposure to the 20 mg menthol $L^{-1}$. Thus, this concentration was withdrawn from the statistical analysis. The longest induction time ($P<0.05$) was verified at the lowest concentration of 30 mg menthol $L^{-1}$, whereas the shortest times ($P<0.05$) were observed in the 60 to 80 mg menthol $L^{-1}$ concentrations ($P<0.05$). Juveniles subjected to anesthesia showed a recovery time inversely proportional to menthol concentrations, i.e., it was verified that, as the anesthetic concentrations increased, the recovery time decreased ($P<0.05$) (Figure 1).

Eugenol proved to be efficient in anesthetizing early juveniles of curimba, with concentrations presenting statistical differences for the time required to induce the deep anesthesia phase (Figure 1). The longest induction time ($P<0.05$) occurred at lower concentrations of 20 mg eugenol $L^{-1}$, whereas the shortest induction time ($P<0.05$) occurred at a concentration of 70 mg eugenol $L^{-1}$. Regarding the recovery period, no difference was detected ($P>0.05$) relative to the tested concentration (Figure 1).

In all treatments, the early juveniles of curimba showed a return to appetite in 24 h after the anesthesia test. After 96 h following the recovery from anesthesia, a 10%




Means followed by the same letters (a-d for induction; x-y for recovery) did not differ by Tukey's test (P<0.05).

Figure 1 - Induction and recovery time to reach anesthesia (seconds) of early juveniles of curimba (*Prochilodus lineatus*) subjected to menthol (A) and eugenol (B) concentrations.

mortality occurred for 30, 50, 60, and 80 mg menthol $L^{-1}$ concentrations. For eugenol, a 10% mortality was observed in 20 to 70 mg $L^{-1}$ concentrations. In our observations, this mortality resulted from fish interactions (bites) and was not directly related to anesthetic toxicity.

## Discussion

Hyperactivity was observed in fish subjected to immersion in an anesthetic and should not be attributed to ethanol, generally used as a solvent, but to the anesthetic itself (Vidal, et al., 2008; Readman, et al., 2013). Therefore, early juveniles of curimba were subjected to ethanol solution; these animals showed no reactions or hyperactivity. The present study provides insights into adequate techniques for the use of the anesthetics menthol and eugenol for sedation of early juveniles of curimba.

The concentrations tested in this study fall within the suggestions given by Marking and Meyer (1985), who considered a good anesthetic as that which induces deep anesthesia from 1 to 3 min (60 to 180 s), and recovery at 5 min (300 s). In general, there were differences in methods and fish size during the anesthesia process. Smaller fish have a larger gill surface area ratio in relation to body weight than larger fish and, thus, have a superior area for anesthetic absorption through the gills (Woody et al., 2002; Ross and Ross, 2008). The present study provides information about the use of menthol and eugenol as anesthetics for early juveniles of curimba (4 g).

The results of the present study demonstrated that the increase in menthol concentrations led to a decrease in induction and recovery times for early juveniles of curimba. A similar response was observed in *S. brasiliensis* (Pádua et al., 2010), *O. niloticus* (Mello et al., 2012), and fat snook (Souza et al., 2012). For fat snook, increased menthol concentration did not affect recovery time. Pádua et al. (2010) anesthetized juveniles of *S. brasiliensis* and found that the longest anesthetic induction time was observed at the concentration of 60 mg menthol $L^{-1}$, and the shortest time was verified at the concentration of 150 mg menthol $L^{-1}$. For lambari, induction time decreased, and recovery time increased linearly with the increase in the menthol concentration (Pereira-da-Silva et al., 2014). In addition, menthol has an anesthetic effect and attenuates the stress response in lambari; 50 mg $L^{-1}$ was the most effective concentration for inducing deep anesthesia within 60 s, and was safe up to 360 s exposure. The 60 mg menthol $L^{-1}$ concentration suggested for *P. lineatus* showed that animals can reach deep anesthesia within 180 s and recover 332 s after being induced.

The concentration suggested in this study for the anesthetic induction of 4 g curimba was 50 mg eugenol $L^{-1}$. Concentrations of 20 mg eugenol $L^{-1}$ provided the longest time for curimba juvenile sedation, more than 30 min. For silver catfish, eugenol concentrations between 20 to 30 mg $L^{-1}$ induced stage 4 anesthesia within 15 min (900 s) (Cunha et al., 2010). In addition, rapid induction to stage 4 of anesthesia was observed with limited effects on the fish at a concentration of 50 mg eugenol $L^{-1}$. This value was similar to that suggested by Diemer et al. (2012) for juveniles of jundiá (*Rhamdia voulezi*). In early life stages of Nile tilapia, with an average weight of 2 and 11 g, 50 mg eugenol $L^{-1}$ induced anesthesia within 3 min (Ribeiro et al., 2015b). Pereira-da-Silva et al. (2009) studied the effectiveness

of eugenol on early juveniles of lambari and found that concentrations between 50 and 150 mg $L^{-1}$ promoted deep anesthesia in <3 min; however, mortalities following higher dosages were documented (150 mg $L^{-1}$).

Previous studies have shown that proper immersion anesthesia may decrease the incidence of adverse effects and lead to a milder recovery (Acerete et al., 2004; Zahl et al., 2009). In the present study, there were no differences in recovery time in early juveniles of curimba anesthetized with eugenol in concentrations from 20 to 70 mg $L^{-1}$. Eugenol was also effective in inducing deep anesthesia in juveniles (mean weight = 3.31 g) of matrinxã (*Brycon cephalus*). Concentrations of 50-100 mg eugenol $L^{-1}$ were effective in inducing anesthesia with a relatively fast recovery time (Vidal et al., 2007). For groups of Nile tilapia ranging from 0.02 to 11 g, the recovery time was between 53.07 to 184.31 s. Eugenol is an efficient anesthetic for tambaqui; juveniles exposed to 35-135 mg eugenol $L^{-1}$ recovered within 350 to 1235 s, respectively (Roubach et al., 2005). In fact, concentrations of 50-100 mg eugenol $L^{-1}$ provided a recovery time higher than the ideal (300 s), however, out of the critical range (>600 s) (Marking and Meyer, 1985). Similarly, early juveniles of curimba showed a recovery time of 354 to 390 s.

High survival was observed in juveniles of curimba 96 h after anesthesia with menthol and eugenol (>90%); moreover, fish showed return to appetite 24 h after the procedure. For Nile tilapia between 0.02-11 g, after 24 h of testing with the eugenol concentrations from 50 to 200 mg $L^{-1}$, a 100% survival was observed, and all fish resumed eating (Ribeiro et al., 2015b). Elevated mortality of lambari was observed when fish were exposed to surgical anesthesia using 75 and 100 mg eugenol $L^{-1}$. The unique safe concentrations for surgical anesthesia in lambari was 50 mg eugenol $L^{-1}$. However, for deep anesthesia, concentrations of 50 to 125 mg eugenol $L^{-1}$ resulted in no mortality for the species (Pereira-da-Silva et al., 2009). Generally, 20-70 mg eugenol $L^{-1}$ resulted in elevated survival and demonstrated the possibility of effective handling of animals and avoiding increased mortality. The mortality observed for early juveniles of curimba after the procedure was a result of fish interaction (bites) and not a direct relation to anesthetic toxicity.

## Conclusions

Menthol and eugenol proved to be efficient in inducing deep anesthetic stages in early juveniles of curimba, and the recommended concentrations are 60 and 50 mg $L^{-1}$, respectively.

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