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EFFECTIVENESS OF BENZOCAINE AS ANESTHETIC AT DIFFERENT WATER TEMPERATURES FOR EARLY JUVENILE CURIMBA (*Prochilodus lineatus* Valenciennes, 1836), A NEOTROPICAL FISH SPECIES*

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ABSTRACT

Anesthetics have been used frequently in aquaculture to minimize stress during handling. However, several factors can affect the efficiency of anesthetics. For example, temperature is one of the abiotic factors that control animal metabolism and consequently, the effect of anesthetics. This study aimed to evaluate the effectiveness of benzocaine as an anesthetic for early juveniles of curimba *Prochilodus lineatus* at different water temperatures. Juveniles (4.7 ± 1.6 g and total length of 7.4 ± 0.7 cm) were submitted to anesthesia at concentrations of 30, 40, 50, 60, 70, and 80 mg L^{-1} of benzocaine and temperatures of 22, 25, 28, and 31 °C. The effects were evaluated by measuring the induction time to deep and surgical anesthesia, recovery time, time to appetite return, and 96-h mortality rate. The higher temperatures (25, 28 and 31°C) provided shorter induction times to reach deep anesthesia and at 50 mg L^{-1} of benzocaine, the induction time was between 2 and 3 min. Juveniles at temperatures of 28 and 31 °C showed lower surgical anesthesia induction time at concentrations ranging from 60 to 80 mg L^{-1}. Recovery time was longer at 22 °C at all concentrations. The time to appetite return occurred in the first 24 h after anesthesia and the 96-h mortality rate was lower than 10%. Under these conditions, for deep anesthesia, benzocaine concentration of 50 mg L^{-1} for water temperatures of 25, 28, and 31 °C and 60 mg L^{-1} for 22 °C are recommended. Surgical anesthesia can be performed with 50 mg L^{-1} of benzocaine at all four water temperatures. The differences documented in the present study underline the need for adequate concentrations of anesthetics depending on the prevalent water temperature for Neotropical fish species. This should be considered in recommendations for large-scale use.

Key words: anesthesia; management; fish farm; induction; recovery; aquaculture.

EFICÁCIA DA BENZOCAINA COMO ANESTÉSICO EM DIFERENTES TEMPERATURAS DE ÁGUA PARA JUVENIS DE CURIMBA (*Prochilodus lineatus* Valenciennes, 1836), UMA ESPÉCIE DE PEIXE NEOTROPICAL

RESUMO

Os anestésicos têm sido usados com frequência na aquicultura para minimizar o estresse durante o manejo. Entretanto, vários fatores podem afetar a eficiência do anestésico. Por exemplo, a temperatura é um dos fatores abióticos que controlam o metabolismo animal e, consequentemente, os efeitos anestésicos. Este estudo teve como objetivo avaliar a eficácia da benzocaína como anestésico em juvenis de curimba *Prochilodus lineatus* em diferentes temperaturas da água. Juvenis (4,7 ± 1,6 g e comprimento total de 7,4 ± 0,7 cm) foram submetidos à anestesia nas concentrações de 30, 40, 50, 60, 70 e 80 mg de benzocaína L^{-1} e temperaturas de 22, 25, 28 e 31 °C. Os efeitos foram avaliados medindo-se o tempo de indução à anestesia profunda e cirúrgica, tempo de recuperação, tempo de retorno do apetite e taxa de mortalidade em 96 horas. As maiores temperaturas (25, 28 e 31 °C) proporcionaram menores tempos de indução a anestesia profunda e em 50 mg de benzocaína L^{-1} o tempo de indução foi entre 2 e 3 min. Juvenis nas temperaturas de 28 e 31 °C apresentaram menor tempo de indução a anestesia cirúrgica nas concentrações variando de 60 a 80 mg L^{-1}. O tempo de recuperação foi superior a 22 ° C em todas as concentrações. O tempo de retorno do apetite ocorreu nas primeiras 24 horas após a anestesia e a taxa de mortalidade após 96 horas foi inferior a 10%. Nestas condições, para anestesia profunda, recomenda-se a concentração de 50 mg L^{-1} de benzocaína nas temperaturas de 25, 28 e 31 °C e 60 mg L^{-1} para 22 °C. A anestesia cirúrgica pode ser realizada com 50 mg de benzocaína L^{-1} nas quatro temperaturas. As diferenças documentadas no presente estudo reforçam a necessidade de adequar a concentração do anestésico à temperatura da água para as espécies de peixes neotropicais, devendo ser reconsiderada na recomendação para uso em larga escala.

Palavras-chave: anestesia; manejo; piscicultura; indução; recuperação; aquicultura.

INTRODUCTION

The Neotropical fish "curimba" (*Prochilodus lineatus* Valenciennes, 1836) has been commercially grown in several countries (Avigliano et al., 2017) and has been garnering interest and recognition for its importance to national aquaculture in areas where it is found. Many studies related to *P. lineatus* have been undertaken with the purpose of maximizing the potential of the species in terms of productivity. This species plays an important role in the ecosystem, but its populations have been declining in some regions (Taylor et al., 2006; Piana et al., 2017) such as in the Rio Doce river basin, after the Mariana disaster in Minas Gerais, Brazil. The genus *Prochilodus* includes many endangered species, and *P. lineatus* is classified in the vulnerable category (IBAMA, 2015). Thus, it is essential to intensify research that would enable scaling up the production of the *Prochilodus* genus to meet not only the demands for consumption but also for its repopulation.

Anesthetics are essential for reducing stress in fish for a variety of activities ranging from routine handling to delicate surgical operations. Moreover, anesthetics are widely used in routine aquaculture operations to immobilize animals for transport, spawning, vaccination, and handling (Barton and Iwama, 1991; Jepsen et al., 2002; King et al., 2005; Sneddon 2012). The effectiveness and safety of anesthetics vary among fish species and depends upon water quality, other environmental factors, and the size of the fish (Massee et al., 1995; Gomes et al., 2011; Ribeiro et al., 2015; Priborsky and Velisek, 2018).

Increased temperature has been reported to shorten induction and recovery times for a number of anesthetics in several teleost species (Stehly and Gingerich, 1999; Hoskonen and Pirhonen, 2004; Mylonas et al., 2005). Although some studies have reported on the effects of temperature on warm-water fish species (Ross and Geddes, 1979; Parma de Croux, 1990), the information on the methodologies used are often insufficient and the results conflicting and confusing. Thus, in this study, we evaluated the effects of water temperature and concentration of benzocaine on the induction time to different stages of anesthesia, time to recovery, time to return of appetite, and 96-h mortality rate in early-juveniles of curimba.

MATERIAL AND METHODS

The experiments were approved by the Ethics Committee on Experimental Animals of the Fundação Instituto de Pesca do Estado do Rio de Janeiro, Brazil (protocol: 003/2016). Early juveniles of curimba were purchased from a commercial hatchery and transported by car to the laboratory rearing facilities in Cordeiro, RJ, Brazil. In total, 400 juveniles of curimba were used for anesthetic induction using benzocaine at different temperatures. The curimba juveniles were first acclimated for a minimum of 30 days in a recirculation system in 120-L water tanks that were maintained at a temperature of 28.1 ± 1.2 °C. They were fed three times a day, at 8, 12, and 17 h, with a commercially formulated diet containing 400 g kg^{-1} protein, 350 mg kg^{-1} vitamin C, 80 g kg^{-1} ethereal extract, and 100 g kg^{-1} moisture (levels and guarantees made available by the manufacturer). The experimental units were cleaned daily prior to the first and after the last feed to remove excreta and food residues.

After laboratory acclimatization, early juveniles of curimba (measuring 7.4 ± 0.7 cm, and weighing 4.7 ± 1.6 g) were subjected to four water temperatures: 22, 25, 28, and 31 °C. Water temperatures varying from 22 to 31°C were evaluated since these are within the range of environmental temperatures typically experienced by Neotropical fish species in Southern Brazil (Garcia et al., 2008). To reach the predetermined temperature of each treatment, the 120-L tanks were put in static system and in thermostatic baths (Hopar®, 300w) and the temperature was gradually adjusted over two days to the corresponding treatments (22, 25, 28 and 31°C). Fish were fed twice a day at apparent satiation and kept at those temperatures for a minimum of 32 days. Each day, before the first and the last feeding, the tanks were siphoned to the waste removal. At that time, about 25% of the total water was renewed at the same temperature of the respective treatments. Physical and chemical monitoring of water was carried out using a HI83203-01 Hanna® Photometer (The total ammonia analysis followed the adaptation of the ASTM Manual of Water and Environmental. Technology, D1426-92, Nessler method), HI9146-04 Hanna® oxygen meter, and HI98130 Hanna® multi-parameter Combo. Water parameters were monitored during acclimatization, induction, recovery tests, and during the 96-h mortality rate evaluation (Table 1).

Table 1. Average (± standard deviation) of water parameters during the acclimatization, induction and recovery tests and 96-h mortality rate evaluation.

Treatments	Temperature (ºC)	Dissolved Oxygen (mg L^{-1})	pH	Eletric condutivity (µS cm^{-1})	Total ammonia (mg L^{-1})
T22ºC	21.8 ± 0.7	9.8 ± 1.5	6.6 ± 0.1	0.3 ± 0.5	0.21± 0.0
T25ºC	25.5 ± 0.4	7.6 ± 1.4	6.3 ± 0.7	0.3 ± 0.4	1.48 ± 0.1
T28ºC	28.8 ± 0.6	6.5 ± 1.4	6.7 ± 0.3	0.3 ± 0.5	1.29 ± 0.1
T31ºC	31.2 ± 1.2	5.7 ± 1.5	6.1 ± 0.9	0.3 ± 0.5	2.57 ± 0.3

Experiment

Early juveniles of curimba were exposed individually to the benzocaine, (Sigma Aldrich®, SLBH1225V), via bath immersions at the concentrations of 30, 40, 50, 60, 70, and 80 mg L^{-1} and temperatures of 22, 25, 28, and 31 °C. The benzocaine was first diluted (1:10) in 98.8% ethanol to reduce its hydrophobic character, and later added to the experimental units at the predetermined concentrations. Fish feed was suspended for a 24-h period before the anesthetic procedure. To ensure that the hyperactivity of fish subjected to immersion in the anesthetic is not because of the solvent used, we exposed the animals to ethanol alone to evaluate any possible hyperactivity due to it.

For the anesthesia induction and recovery tests, one fish was randomly captured at a time and placed in a 2-L beaker containing the desired concentration of the anesthetic to be tested. Criteria for evaluating the stages of induction and recovery after exposure to the anesthetics have been recommended by Hikasa et al. (1986) and Ross and Ross (2008), which characterized deep anesthesia as the complete loss of equilibrium, absence of swimming, reduction in opercular movements and response only to intense tactile stimuli; and surgical anesthesia as the slow irregular opercular movements and loss of reaction to stimuli.

When the fish reached the deep and surgical stages of anesthesia, biometric measurements, such as weight (model BL320H scale, Shimadzu®) and length (total length using a ruler), were obtained to simulate the real handling conditions in fish farms. All recovery procedures were conducted in a 2-L beaker containing anesthetic-free water. After the experiments, the fish from each replicate were pooled and kept in static system with 120-L water tanks and in thermostatic baths, to keep similar water temperature those from the treatments. This management was realized to observe the 96-h mortality rate and time to appetite return. The induction and recovery times were analyzed using a chronometer.

After checking the data for normality by the Cramér-von Mises test and homogeneity of variances by the Levene's test, an analysis of variance (two-way ANOVA) was performed to compare the groups using the Statistical Analysis System-SAS, version 8.0 software. Any significant difference was further tested by the Tukey's multiple comparison test.

RESULTS

The induction time to both deep and surgical anesthesia was affected by an interaction ($P < 0.05$) between concentration (C) and water temperature (T). In the case of recovery time, temperature was the main factor showing a significant effect ($P < 0.05$) with low temperatures (22 ºC) resulting in increased recovery time ($P < 0.05$) (Table 2).

Table 3 shows induction time to deep anesthesia. In general, using higher concentrations of anesthetic at higher temperatures resulted in a shorter time ($P < 0.05$) to reach this stage of anesthesia. Lower concentrations of the anesthetic (i.e., 30 and 40 mg L^{-1}) ($P < 0.05$) at the higher temperatures of 28 and 31 °C resulted in shorter induction time. On the other hand, at concentrations higher than 50 mg L^{-1}, the induction time was shorter ($P < 0.05$) when temperature was 25 °C and higher.

Table 2. F values and means values (± standard deviation) of induction and recovery times (seconds) of early juveniles of curimba anesthetized with benzocaine at different temperatures.

Statistical	Induction (deep anesthesia)	Induction (surgical anesthesia)	Recovery (from surgical anesthesia)
F values			
Concentration	45.9 **	75.4 **	1.9 ns
Temperature	23.1 *	15.7 *	37.8*
Conc. x Temp.	4.9*	5.8*	1.2 ns
Means for concentration			
30	747 ± 231	1009 ± 263	298 ± 101
40	328 ± 62	406 ± 15	293 ± 97
50	151 ± 27	296 ± 14	285 ± 106
60	95 ± 37	180 ± 13	263 ± 85
70	85 ± 18	136 ± 10	260 ± 91
80	68 ± 10	104 ± 8	254 ± 92
Means for temperature			
22 ºC	326 ± 364	407 ± 434	419 ± 29 a
25 ºC	264 ± 300	394 ± 415	236 ± 22 b
28 ºC	206 ± 212	323 ± 268	226 ± 23 b
31 ºC	189 ± 181	298 ± 245	223 ± 10 b

Statistical analysis used was ANOVA for 6×4 factorial design. Means followed by the same letters on the vertical did not differ by Tukey's test ($p<0.05$). *($p<0.05$); **($p<0.01$); ns (not significative).

Table 3. Interaction (benzocaine concentration x temperature – T.) means values (±standard deviation SD) of induction time (seconds) to deep anesthesia and surgical anesthesia of early juveniles of curimba anesthetized with benzocaine at different temperatures (22, 25, 28 and 31°C).

Benzocaine concentrations (mg L^{-1})						
Deep anesthesia						
T. (°C)	30	40	50	60	70	80
22	1037 ± 254Aa	379 ± 93Ba	193 ± 49Ca	151 ± 81Ca	112 ± 35CDa	81 ± 17Da
25	826 ± 224Aa	384 ± 91Ba	142 ± 47Cab	81 ± 20CDb	80 ± 13CDb	73 ± 20Dab
28	606 ± 156Aab	279 ± 61Bb	137 ± 39Cb	76 ± 17CDb	75 ± 8CDb	61 ± 6Db
31	522 ± 179Ab	270 ± 01Bb	135 ± 41Cb	75 ± 17CDb	73 ± 15CDb	59 ± 17Db
Surgical anesthesia						
T. (°C)						
22	1261 ± 281Aa	423 ± 93Ba	308 ± 75CDa	198 ± 51DEa	141 ± 40Ea	112 ± 31Ea
25	1209 ± 230Aa	414 ± 106Ba	307 ± 79Ca	183 ± 19Da	142 ± 42DEa	108 ± 56Ea
28	822 ± 176Ab	400 ± 80Bab	296 ± 103Ca	175 ± 31CDa	141 ± 46Da	102 ± 28Da
31	746 ± 188Ab	387 ± 83Bb	276 ± 98Ca	167 ± 54Da	120 ± 30Da	94 ± 32Da

Means followed by the same capital letters on the horizontal and lowercase letters on the vertical did not differ by Tukey's test ($p<0.05$).

Similarly, higher anesthetic concentrations and temperatures resulted in shorter times ($P < 0.05$) to reach the surgical stage of anesthesia (Table 3). At concentrations of 30 and 40 mg L^{-1} of benzocaine at temperatures of 28 and 31 °C resulted in shorter induction times ($P < 0.05$). However, at concentrations from 50 to 80 mg L^{-1} there was no significant difference in the induction time between the temperatures.

In all the treatments, the curimba early juveniles showed a return of appetite in 24 h after the anesthesia test. After 96 h following the recovery from anesthesia, no mortality was observed at 22 °C; but 10% mortality occurred for 30, 60, and 80 mg L^{-1} of benzocaine at 25 °C; 10% mortality was observed for all concentrations of the anesthetic at 28 °C and for 40, 60, 70, and 80 mg L^{-1}concentrations at 31 °C. However, our direct observations showed that this mortality resulted from fish interactions (bites) and was not directly related to anesthetic toxicity.

DISCUSSION

The basal metabolism of fish and the physiological processes involved in the uptake and elimination of anesthetics are strongly dependent on temperature. Reduced induction and recovery times with increased water temperature have been reported during anesthesia in a number of fish species (Houston and Woods, 1976; Hoskonen and Pirhonen, 2004; Mylonas et al., 2005). In our study, we identified temperature as an abiotic effect involved in anesthetic metabolism in the early juveniles of curimba.

Adjustments to concentrations in relation to body weight and temperature are necessary in order to obtain induction and recovery times within the range defined ideal for fish anesthetics, i.e., induction within 3 min and recovery within 5 min (Marking and Meyer, 1985). In the present study, early juveniles of curimba were used to evaluate the effectiveness of benzocaine as an anesthetic at different water temperatures. The *Prochilodus* genus is used for aquaculture in Brazil (IBGE, 2016), and the species may be susceptible to a range of temperatures i.e., low temperatures during autumn and winter in the south and southeast regions and high temperatures in the north and northeast during the entire year. In this way, adaptations to the management, mainly to those related to fish metabolism are necessary to avoid stress and mortality.

Benzocaine is an efficient anesthetic agent for inducing deep and surgical anesthesia in early juveniles of curimba. The advantages of benzocaine are low cost, efficacy, and good margin of safety for fish (Gilderhus and Marking 1987; Gilderhus 1989, 1990). For spawning-phase chinook salmon *Oncorhynchus tshawytscha* and Atlantic salmon *Salmo salar*, 30 mg L^{-1} of benzocaine was safe and effective, with narrower safety margins (Gilderhus, 1990). In *Astyanax altiparanae* (3.5 g), deep anesthesia could be successfully induced with 100 mg L^{-1} of benzocaine without affecting water quality parameters and resulting in low mortality (Gimbo et al., 2008). For another Neotropical fish species, tambaqui *Colossoma macropomum* (9.3 g), concentrations higher than 100 mg L^{-1} induced a total loss of equilibrium in less than 3 min. However, at a lower benzocaine concentration (50 mg L^{-1}), a relatively long time (7.37 min) was needed to induce anesthesia, but the recovery was fast (Gomes et al., 2007). For early juveniles of curimba, concentrations ranging from 50 to 60 mg L^{-1} of benzocaine were safe and effective for inducing deep and surgical anesthesia in all tested water temperatures.

Water temperature is one of the main abiotic factors that influence fish metabolism (Sandblom et al., 2014). Shorter induction and recovery times at higher water temperatures were observed in several studies, showing that despite large differences in the response of species to anesthetics, the importance of temperature in determining their effectiveness seems to be consistent. Although anesthetics present differences in the pharmacokinetics

and pharmacodynamics in the body of fish (Zahl et al., 2012), there is a pattern when temperature is changed. The alterations on induction and recovery times provoked by temperature observed in this study for early juveniles of curimba with benzocaine are similar to those reported using others anesthetics (Houston and Woods, 1976; Dawson and Gilderhus, 1979; Sylvester and Holland, 1982; Hikasa et al., 1986; Stehly and Gingerich, 1999; Woolsey et al., 2004; Mylonas et al., 2005) and using benzocaine for juveniles and adults of the brown trout Salmo trutta, northern pike *Esox lucius*, common carp *Cyprinus carpio*, largemouth bass *Micropterus salmoides* (Dawson and Gilderhus, 1979) and for the striped bass *Morone saxatilis* juveniles (Gilderhus et al., 1991). This feature is the result of an increased oxygen demand due to rises in the basal metabolism leading to superior respiration and blood flow and thus accelerated physiological processes involving absorption and elimination of the anesthetic. Furthermore, the solubility of oxygen diminishes as the water temperature rises and may lead to an additional need for enhanced respiration and blood flow (Zahl et al., 2009).

The induction and recovery times are dependent on the pharmacokinetic properties, where a short induction in early juveniles of curimba indicates a fast uptake and absorption and a long recovery time indicates a slow clearance and elimination. In fact, we observed shorter induction and recovery times with increasing water temperature, more precisely with fish acclimated and exposed to water temperature ranging from 25 ºC to 31 °C. Those water temperatures are similar to what is recommended for tropical species, 25 to 28 °C (Parker and Davis, 1981; Frascá-Scorvo et al., 2001). The juveniles kept at 31 °C were an exception that showed kinetics of absorption and elimination of benzocaine, indicated by the observed induction and recovery times, similar to fish exposed to 25 and 28 °C.

The temperature-related effects of an anesthetic could be explained by the dependence of metabolic rate on temperature, and at 22 °C temperature, which is lower than temperatures recommended for Neotropical fish species (Parker and Davis, 1981; Frascá-Scorvo et al. 2001), the curimba juveniles required a higher benzocaine concentration to reach deep anesthesia and a longer time to eliminate the anesthetic agent. The temperature of 22 °C decreased the metabolic rate of early juveniles of curimba, directly resulting in alterations in the required concentration to achieve the desired induction and recovery times. Also, slow recovery from anesthesia at low temperatures was related to the thermal requirements for the species whitefish Coregonus lavaretus, perch *Perca fluviatilis*, and roach *Rutilus rutilus* (Hoskonen and Pirhonen, 2004).

High survival rates were observed in curimba juveniles 96 h after anesthesia, regardless of temperature; moreover, fish showed return of appetite 24 h after the procedure in all treatments. The mortality observed for early juveniles of curimba after the procedure was a result of fish interaction (bites) and had no direct relation to anesthetic toxicity. Bittencourt et al. (2012) evaluated the induction and recovery time of goldfish (*Carassius auratus*) exposed to two anesthetics and did not observe mortality in 96 h after anesthesia even using benzocaine concentrations of up to 112.5 mg L^{-1}. The benzocaine concentration used with curimba in the present study was in the usual range for inducing anesthesia in fish and only superior concentrations may lead to fish intoxication (Ross and Ross, 2008).

The differences in induction and recovery times documented in the present study underline the need to examine the response of each Neotropical fish species of interest for aquaculture to anesthetic exposure with changing environment factors, such as temperature, prior to its recommendation for large-scale use. Moreover, recommendations should take into consideration the optimal temperature indicated for fish farming, since inappropriate anesthetic concentration may induce adverse physiological consequences, resulting in depressed growth and survival.

Benzocaine is an effective anesthetic for juveniles of curimba, as observed for Parma De Croux (1990) at temperatures of 20 and 25 ºC; however, the information on the methodologies used are often insufficient and the results conflicting and confusing. Our results bring up about times of induction, recovery, survival and time to appetite return in a wider range of temperature variation i.e. 22 to 31 °C. In such case, water temperature is an important factor that affects the efficacy of this anesthetic, visualized in the current investigation by the induction and recovery times observed as well as the responsiveness to external stimuli. Higher water temperature results in shorter induction and recovery times. Also, for deep anesthesia, we recommend a benzocaine concentration of 50 mg L^{-1} for temperatures of 25, 28, and 31 °C and 60 mg L^{-1} for 22 °C. The surgical anesthesia can be performed with 50 mg L^{-1} of benzocaine at these four temperatures.

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