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Eugenol as an efficient anesthetic for neotropical fish *Prochilodus nigricans* (Teleostei, Prochilodontidae)

[*Eugenol como um anestésico eficaz para o peixe neotropical* Prochilodus nigricans *(Teleostei, Prochilodontidae)*]

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ABSTRACT

The use of anesthetics in fish farming is essential to reduce stress during management. The present study proposes to evaluate the effect of eugenol as an anesthetic for the management of curimatã (*Prochilodus nigricans)*. Fifty specimens were used (221.34±9.0 g; 25.8 ±1.1 cm), submitted to five treatments with concentrations of 0, 25, 50, 100 and 200 mgL^{-1} eugenol for 10 min. The times required to reach each anesthetic stage and the recovery of each fish were recorded. To check bloodglucose levels individual blood samples were taken from the fish before immersion and after ten minutes of anesthetic exposure. Evaluation of the anesthetic effect ongills was performed by histopathological analysis. The concentration of 50 mgL^{-1} was within the ideal limit of three minutes recommended for surgical anesthesia induction and did not significantly increase glucose levels when compared to the control group (35.7±19.4 mg dL^{-1}), besides not causing gill injuries. At this concentration the fish presented controlled blood glucose levels within the basal levels, besides not taking the risk to have later problems concernig mortality, because this concentration ensures the good health of the gills and good recovery of the animals.

Keywords: aquaculture, pisciculture, curimatã, recovery, anesthesia

RESUMO

O uso de anestésicos em pisciculturas é indispensável para reduzir o estresse durante o manejo. O presente trabalho propôs avaliar o efeito do eugenol como anestésico para manejo do curimatã (Prochilodus nigricans). *Foram utilizados 50 espécimes (221,34±9,0 g; 25,8 ±1,1 cm), submetidos a cinco tratamentos com concentrações de 0, 25, 50, 100 e 200 mg* L^{-1} *de eugenol em exposição de 10 minutos. Foram registrados os tempos necessários para atingir cada estágio anestésico e a recuperação de cada peixe. Para verificação dos níveis de glicose sanguínea, foram realizadas coletas individuais de sangue dos peixes antes da imersão e após os 10 minutos de exposição ao anestésico. A avaliação do efeito do anestésico sobre as brânquias foi realizada por meio de análises histopatológicas. A concentração de 50 mg* L^{-1} *ficou dentro do limite ideal de três minutos preconizado para indução à anestesia cirúrgica e não elevou significativamente os níveis de glicose quando comparada ao grupo controle (35,7±19,4 mg* dL^{-1}*), além de não provocar lesões branquiais. Nessa concentração, os peixes apresentaram níveis de glicose sanguínea controlada, dentro dos teores basais, além de não correrem o risco de apresentarem problemas posteriores em relação à mortalidade, pois essa concentração garante a boa saúde das brânquias e uma boa recuperação dos animais.*

Palavras-chave: aquicultura, piscicultura, curimatã, recuperação, anestesia

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INTRODUCTION

Routine procedures of aquaculture activity such as biometrics, hormonal applications, and transport have strong effects on physiology and can cause high levels of stress in fish, increasing susceptibility to pathogenic and infectious diseases, affecting their reproductive capacity and even death (Hurst, 2007). Thus, the use of appropriate anesthetics at effective concentrations is essential to reduce the action of stressors and minimize mortality in fish farming (Ross and Ross, 2008). Different anesthetics require different concentrations to reach the desired anesthesia stage and their efficacy has intraspecific, and interspecies variations (Teixeira *et al*., 2011). Therefore, the choice of anesthetic should be related to the cost, efficacy, availability and safety of fish, humans and the environment (Marking and Meyer, 1985), as well as pertinent characteristics such as rapid action in the nervous system and the sequelae after the use of these substances (Gonçalves *et al*., 2008).

The appropriate anesthetic at its optimal concentration should minimize negative impacts of management, reduce stress on fish, produce anesthesia less than or equal to three minutes, and recovery should occur within 5 to 10min (Marking and Meyer, 1985; Ross and Ross, 2008). These characteristics include eugenol (4-allyl-2-methoxyphenol), a compound derived from clove oil, viscous liquid obtained by distillation of the leaves, buds, flowers and stem of *Eugenia aromatica* or *Eugenia caryophyllata* (Mylonas *et al*., 2005). Eugenol is rapidly eliminated from the bloodstream, relatively low cost, has good efficacy and is readily available in nature, and is considered safe for animals, the environment and those who handle it (Woody *et al*., 2002). Eugenol has been found to be an acceptable anesthetic with potential for use in aquaculture and has been widely used in several studies with different fish species (Moreira, 2009; Rotili *et al*., 2012), but studies to verify likely immediate effects on fish structures such as gills are incipient.

The gills are organized in a system of progressive subdivisions, giving rise to the gill slits, separated by septa with the gill arch, where the rows of gill filaments are located, whose surfaces give rise to the folds that constitute the lamellae, primary sites of gas exchange (Nogueira *et al*., 2008). The anesthetic solution is captured by the gills, through which the anesthetic is absorbed and eliminated (Ross and Ross, 2008), and side effects caused by anesthetics may affect gill health and endanger the survival of fish after anesthesia (Oliveira *et al*., 2009).

Curimatã (*Prochilodus nigricans*), is a freshwater fish distributed in the Amazon and Tocantins River basins (Fricke *et al.,* 2020), due to its high consumption in Brazil it is widely cultured in fish farms in the northern and northeastern Brazil. In this way, the objective of this study was to evaluate the effect of different concentrations of eugenol as an anesthetic and to determine its ideal dose for *P. nigricans*.

MATERIALS AND METHODS

The current study was approved by the Ethics Committee on Animal Experimentation of the UEMA Veterinary Medicine course - (33/2016). This study was conducted at the Laboratory of Reproduction of Aquatic Organisms and the Laboratory of Fisheries and Aquatic Ecology (LABPEA), both of the State University of Maranhão (UEMA). Fifty specimens of *P. nigricans* with a total average length of 25.8±1.1 cm were used, weighing an average of 221.34±9.0 g. The fish were acclimated in a masonry tank and fed ad libitum twice daily with commercial feed (28% CP). During this period, the water temperature was 29.47±0.30 °C, oxygen dissolved at 6.15±1.12 mg L^{-1} and pH 7.35±0.22. Feeding was stopped 24 hours before the experimental procedure.

Eugenol (Sigma®), due to its oily nature, was diluted in ethanol PA, resulting in a stock solution at a concentration of 100 mg mL^{-1} (1:10) in accordance with Rotili *et al*. (2012). For exposure and recovery 80 liter capacity aquariums were used, containing 40 liters of water and provided with constant aeration. To verify the influence of anesthetic concentration on fish exposure, five treatments with eugenol concentrations of 0, 25, 50, 100 and 200 mg L^{-1} were performed. For each concentration 10 fish were individually immersed, randomly collected and exposed to anesthetic concentration for 10 min. The different stages of anesthesia were recorded according to the criteria proposed by Ross and Ross (2008) described in Table 1.

Table 1. Stages of anesthesia in fish*

Stage	Description	Physiological and behavioral signs
0	Normal	Reactive to external stimuli; normal opercular beats; normal muscle reaction.
I	Light sedation	Reactive to external stimuli, reduced movement, slower opercular beats, normal balance.
II	Deep sedation	Total loss of reactivity to external stimuli except for strong pressure; slight fall in opercular movement; normal balance.
III	Narcosis	Partial loss of muscle tone; erratic swimming, increased opercular movements; reactive only to strong tactile stimulation or vibration.
IV	Deeper anesthesia	Total loss of muscle tone; total loss of balance; slow but regular opercular beat.
V	Surgical anesthesia	Total loss of reaction to even massive stimulation; slow and irregular opercular movements; slow heartbeat; total loss of all reflexes.

*Modified from Ross and Ross (2008).

The time required to reach each anesthetic stage was measured by a digital stopwatch and after ten minutes of exposure the fish were removed for biometrics and then transferred to the recovery aquarium, where the time to reach their normal balance was recorded. Active swimming was measured. To check bloodglucose levels, individual blood samples were taken from fish before immersion and after ten minutes of exposure. With the aid of a heparinized 1ml syringe, blood was collected by caudal puncture and immediately after collection the blood glucose content was checked with the aid of a digital glucometer (OneTouch Ultra®).

The anesthetic effect on the gills of *P. nigricans* was evaluated by histological analysis. After anesthetic recovery, the fish were euthanized by the medullary section and the first gill arch of each specimen was removed and fixed in 10% formaldehyde and kept in 70% alcohol until the usual histological technique was applied. The first gill arch was dehydrated in increasing series of alcohols, diaphanized in xylol, impregnated and embedded in paraffin (Velloso *et al*., 2012). Cross sections of approximately 5 μm thickness were stained with Hematoxylin and Eosin (HE). The slides were read by a light microscope using 10x, 40x objectives and the lesions found were photomicrographed in AXIOSKOP - ZEIS photomicroscope. The classification of gill alterations according to the lesion importance factor was made according to a scale of Bernet *et al*. (1999) in: I (minimal pathological importance), II (moderate pathological importance), and III (marked pathological importance).

The data were analyzed by one-way analysis of variance. When the F value indicated significant differences ($P<0.05$), the means were compared by the Tukey test. The results are presented as mean±standard deviation, all analyses were performed by Bioestat software, version 5.0.

RESULTS AND DISCUSSION

The specimens of *P. nigricans* isolated from different anesthetics of eugenol have indicative sizes of adult fish, including average amplitude data of the first sexual maturation of species recorded by Mota and Rufino (1997). The individuals showed no significant difference in the total weight and total length ($P>0.05$) (Table 2), and the fish demonstrated healthy external exposure during anesthesia, manipulation procedures, and 24h after exposure, with no mortality.

In culture systems, the use of anesthetic is essential during management procedures, however, for the ideal use of this anesthetic, it is necessary to understand the dose required for a given size of fish, since different size fish respond differently to the anesthesia time when submitted to the same anesthetic concentrations (Roubach *et al*., 2005), in addition, the ideal use of the anesthetic avoids mortality and minimizes management costs. Juvenile fish can be anesthetized at lower concentrations than adults of the same species, as they have comparatively

smaller branchial surfaces, capable of better absorbing the anesthetic (Roubach *et al*., 2005). This statement corroborates the study by Souza *et al*. (2015) who, when evaluating the effect of eugenol on *Lutjanus synagris*, observed that the larger the individual, the more resistant to sedation with this anesthetic.

In our study, *P. nigricans* did not undergo the stage of mild sedation in any of the treatments. On the other hand, higher concentrations of eugenol (100 mg L^{-1} and 200 mg L^{-1}) triggered a series of behaviors in *P. nigricans*, such as hyperactivity, cough, and regurgitation. These behaviors probably contributed to accelerating the opercular beat of this species, with a consequent increase in the flow of water over the gills, contributing to faster anesthesia. When exposed to a lower concentration of eugenol (50 mg L^{-1}), *P. nigricans* immediately reduced their opercular beats, causing longer anesthesia compared to other concentrations (Table 3).

Table 2. Biometric data of *Prochilodus nigricans* anesthetized with different concentrations of eugenol

Concentration (mg L^{-1})	WT (g)	LT (cm)
0	220.8±36.3[a]	27.1±3.2[a]
25	207.5±51.9[a]	26.3±3.1[a]
50	232.5±48.0[a]	24.0±1.2[a]
100	221.7±39.6[a]	25.7±1.5[a]
200	224.2±65.6[a]	25.8±2.8[a]

Where: WT = total weight of the individual; LT = total length of the individual. Data presented by means and standard deviation.

Table 3. Stages of behavior (in seconds) of *Prochilodus nigricans* adults exposed to different concentrations of eugenol

Dose (mg L^{-1})	Stage II	Stage III	Stage IV	Stage V	Recovery
25	16.3±3.9[a]	26.7±4.9[a]	38.6±5.0[a]	77.2±13.5[a]	148.67±15.0[a]
50	29.3±8.2[b]	44.2±9.5[b]	61.8±9.3[b]	162.8±30.2[b]	398.3±41.4[b]
100	12.5±2.3[a]	17.7±3.5[a]	23.8±4.0[c]	30.2±3.4[c]	687.0±134.9[c]
200	13.3±3.6[a]	23.7±7.4[a]	34.5±9.4[ac]	51.4±20.0[a]	1032.1±115.4[d]

Where: Stage II - deep sedation; stage III- narcosis; stage IV - deep anesthesia; stage V- surgical anesthesia. Lower case letters indicate difference between treatments (P<0.05). There was no gradual increase in the behavior of *P. nigricans* exposed to different concentrations of eugenol, where the animals went from the normal stage to deep sedation (stage II). We can deduce that even in low concentrations, this anesthetic has a faster effect on physiology, affecting behaviors and accelerating the stages of anesthesia in this species. Our results corroborate with Vidal *et al*. (2008) when observing that *Oreochromis niloticus* exposed to concentrations of 200 and 250 mg L^{-1} of eugenol, did not show an evident progressive transition from the behaviors characteristic of the initial stages of anesthesia to the deeper stages, due to the speed of action of eugenol in the body.

The lowest concentration (25 mg L^{-1}) evaluated in this experiment presented induction times similar to the highest concentrations, not differing from the concentration of 200 mg L^{-1} in the different stages of anesthesia (P>0.05), however, it was significantly less than the time to induce the concentration of 50mg L^{-1} in the different stages (P<0.05). When submitted to the lowest concentrations, *P. nigricans* maintained its normal behavior regarding the opercular beat, not decreasing as in the concentration of 50 mg L^{-1}. This behavior probably contributed to greater absorption of the anesthetic over time, reaching levels of deep anesthesia similar to the higher concentrations.

The exposure of *P. nigricans* to 50 mg L^{-1} of eugenol revealed an average time of induction at all stages of anesthesia, being higher than the other treatments (P<0.05). For deep anesthesia induction, the meantime was 61.8±9.3 s. Results found by Park *et al*. (2008) when evaluating a similar eugenol concentration in *Epinephelus bruneus*, recorded an average induction time of 66s, staying within the ideal limit of three minutes

for surgical anesthesia induction, recommended by Marking and Meyer (1985).

The concentrations of 100 mg L^{-1} and 200 mg L^{-1} of eugenol accelerated the sedative effect on the animals, evidenced by hyperactivity and irritation behaviors, confirmed by the rapid movement and coughing behavior, with regurgitation reflex. Hyperactivity and irritation behavior were also observed by Vidal *et al*. (2007) for juveniles of *Brycon cephalus* and *Colossoma macropomum* and for other fish species. According to Mylonas *et al*. (2005) this reaction is caused by the anesthetic and not by the alcohol used during the preparation of the solution, as *Sparus aurata* and *Dicentrarchus labrax*, exposed separately to alcohol did not show euphoria or irritation behaviors, when compared with the presence of eugenol (Collins, 1985).

In culture systems, the deep anesthesia stage is recommended for biometrics and quick handling, as it causes the total loss of balance of the fish, allowing activities to be carried out safely, reducing stress, and minimizing mortality risks (Simões and Gomes, 2009). Although the total time for fish recovery is less important in a field situation, a quick and complete resumption of normal physiological activities is recommended as soon as possible (Walsh and Pease, 2002).

In this study, we observed that the recovery time of *P. nigricans* had a progressive increase with the increase in eugenol concentrations ($P<0.05$). Considering the importance of the recovery time for safe use in management, we suggest concentrations of 25 mg L^{-1} and 50 mg L^{-1} of eugenol for *P. nigricans.* The recovery times in these treatments were within the maximum limit considered safe by Ross and Ross (2008). Concentrations of 100 mg L^{-1} and 200 mg L^{-1}, on the other hand, showed an average recovery time above 10 minutes, therefore, above the recommended time for fish safety. According to Park *et al*. (2008), the optimal concentration of anesthetic should minimize the negative impacts of management, reduce stress on fish, produce anesthesia in a period of less than or equal to 3min and allow recovery within a maximum period of 10 min.

The *P. nigricans* specimens had an average blood glucose value of 21.8±8.7 mg L^{-1}, before exposure to the anesthetic (control). Exposure to concentrations of 25 mg L^{-1} and 50 mg L^{-1} did not significantly increase glucose levels when compared to the control group, with values of 39.7±21.4 mg L^{-1} and 35.7±19, 4 mg L^{-1}, respectively (Figure 1), indicating that adult *P. nigricans* can be exposed to these concentrations without additional stress. On the other hand, 100 mg L^{-1} and 200 mg L^{-1} of eugenol significantly increased the blood glucose levels of these animals compared to the control group (Figure 1) ($P<0.05$), indicating a higher level of stress in these concentrations .

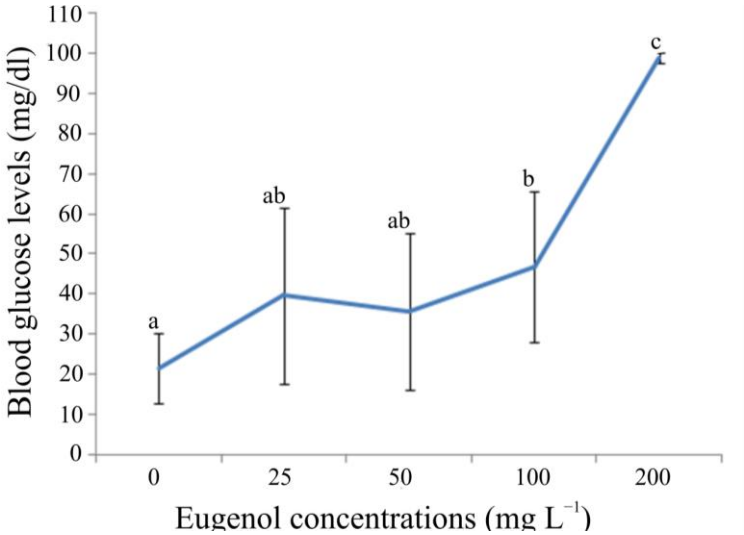

Figure 1. Bloodglucose levels of *Prochilodus nigricans* exposed to different concentrations of eugenol. Lower case letters indicate difference between treatments ($P<0.05$).

Studies that aim to evaluate the physiological stress of fish submitted to anesthetics through analysis of cortisol and glucose are important since these physiological effects vary between species, stage of development, and metabolic state of the animal (Moreira 2009). A study reveals that fish exposed to anesthetics generally increase levels of cortisol and blood glucose in response to the stress of this solution (Mommesen *et al*., 1999).

In addition to the possible effects on the physiology of fish, eugenol can also cause undesirable reactions in the morphology of some tissues. The gills are a multifunctional organ, which is directly exposed to the actions of the anesthetic in the environment through absorption and transport. According to Santana *et al*. (2016), concentrations of anesthetics outside the ideal limit for a given species can cause mild, moderate, and severe histological changes. In our experiment, we observed that fish submitted to concentrations of 0 mg L^{-1} (control group), 50 mg L^{-1} and 200 mg L^{-1} of eugenol did not show branchial lesions. Fish exposed to concentrations of 25 mg L^{-1} and 100 mg L^{-1} showed the following changes: disorganization of the lamellae, partial fusion of the lamellae, congestion and aneurysms (Figure2).

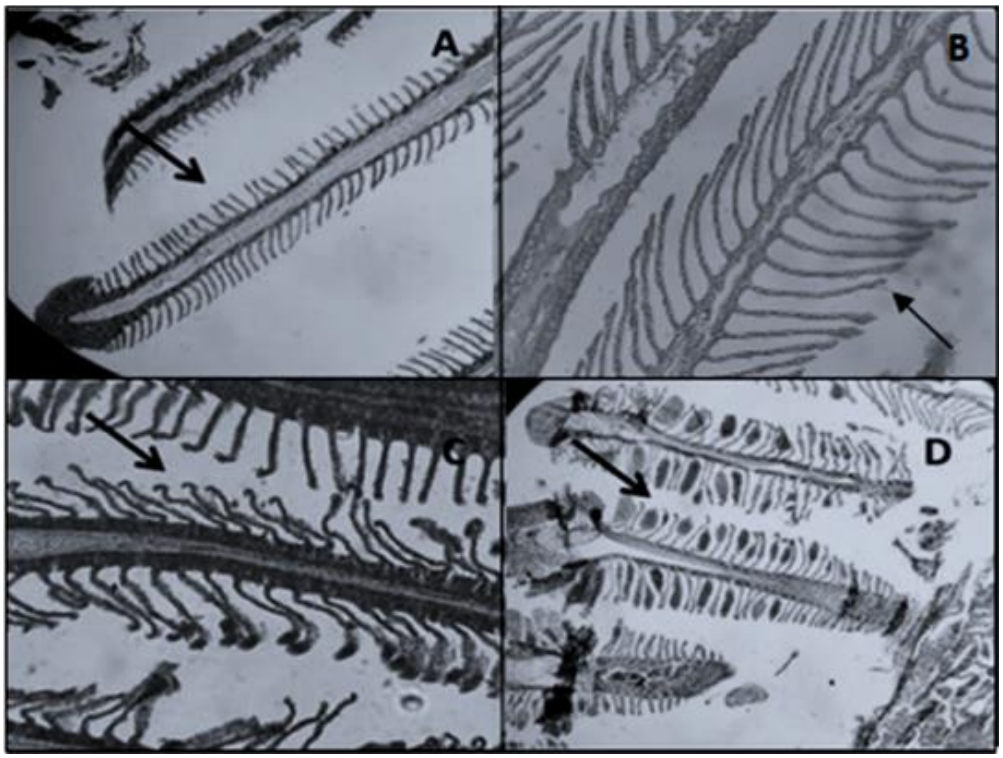

Figure 2. Histology of the gills of *Prochilodus nigricans* exposed to different eugenol concentrations. (A and B) arrow indicating lamellae with normal characteristics (50 mg L^{-1} and 200 mg L^{-1}); (C) arrow indicating disorganization in the lamellae (100 mg L^{-1}); (D) arrow indicating congestion (25 mg L^{-1}).

In the present study, the damage caused to the gills of *P. nigricans* at concentrations of 25 mg L^{-1} and 100 mg L^{-1} may be related to the individual's behavior in relation to treatment, since fish exposed to 25 mg L^{-1} maintained the normal opercular rhythm contributing to a greater exchange of water by the gills for a long time, increasing the substance's absorption in this tissue. These lesions are evaluated as circulatory disorders, being considered as mild damage, and can be reversible when the animal is directed to a controlled environment (Bernet *et al*., 1999).

The branchial epithelium represents a surface in continuous contact with the environment, thus being an important target of pollutants or substances that are present in the water (Wong and Wong, 2000). In general, lifting of the epithelium

and circulatory disorders (such as aneurysms) in fish gills can indicate acute toxicity, where contact with the toxic agent, in an approximate period of 24 hours, can accumulate in the tissue and its effects appear immediately or over a few days (Vanderoost *et al.*, 2003).

CONCLUSIONS

The appropriate concentration of eugenol anesthetic for rapid management of adult *Prochilodus nigricans* is 50 mg L^{-1}. At this concentration the fish will have their blood glucose levels controlled within the basal levels and will not run the risk of further mortality problems, as the anesthetic at this concentration ensures good gill health and good animal recovery.

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