

USE OF EUGENOL AS AN ANAESTHETIC FOR *Geophagus brasiliensis* JUVENILES

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ABSTRACT

The eugenol is the active ingredient of clove oil and it has shown to be effective and safe as fish anesthetic. In this study were evaluated the effects of different concentrations of eugenol for anesthesia, recovery time, blood glucose and hematocrit percentage of pearl cichlid (*Geophagus brasiliensis*) juveniles. Concentrations of 50, 80, 100, 120, 150, 170 and 200 mg L⁻¹ eugenol were evaluated in terms of time to achieve the different stages of anesthesia and recovery in a completely randomized design (CRD) with 12 repetitions. These results were tested using the Kruskal-Wallis test with subsequent Dunn test. The evaluated concentrations had anesthetic action of the deep induction 172.57 ± 25 s (50 mg L⁻¹) and the period of full recovery of 516.25 ± 102 s (200 mg L⁻¹), with statistical difference between the treatments at all the stages. The eugenol effect on blood glucose and on hematocrit percentage at 0 h after deep anesthesia was evaluated for concentrations of 0; 50; 80; 100; 150; 200 mg L⁻¹, in a completely randomized design with five replications, and the results were assessed by One-Way ANOVA. Glucose and percentage of hematocrit: no statistically significant differences ($P>0.05$) were observed for these variables. All concentrations of eugenol showed to be effective as an anesthetic for *G. brasiliensis* and did not affect the survival, blood glucose and the percentage of hematocrit. To minimize adverse effects and achieve faster recovery is indicate the use of 50-80 mg L⁻¹ of eugenol for pearl cichlid juveniles.

Keywords: anesthesia; clove oil; fish; pearl cichlid

UTILIZAÇÃO DE EUGENOL COMO ANESTÉSICO EM JUVENIS DE *Geophagus brasiliensis*

RESUMO

O eugenol, substância ativa do óleo de cravo, tem demonstrado ser eficiente e seguro como anestésico para peixes. Neste estudo foram avaliados os efeitos de diferentes concentrações de eugenol na anestesia, tempo de recuperação, glicemia e percentual de hematócrito de cará (*Geophagus brasiliensis*). Concentrações de 50, 80, 100, 120, 150, 170 e 200 mg L⁻¹ de eugenol foram avaliadas em relação aos tempos para atingir os diferentes estágios de anestesia e de recuperação, em desenho inteiramente casualizado (DIC), com 12 repetições. Os resultados foram analisados pelos testes de Kruskal-Wallis e teste Dunn. As concentrações avaliadas produziram ação anestésica em período de indução profunda de $172,57 \pm 25$ s (50 mg L⁻¹) e no período de total recuperação de $516,25 \pm 102$ s (200 mg L⁻¹), com diferença estatística entre os tratamentos para todas as fases avaliadas. Foi avaliado o efeito do eugenol sobre a glicose no sangue e sobre o percentual de hematócrito, em 0 h após anestesia profunda, nas concentrações 0, 50, 80, 100, 150 e 200 mg L⁻¹, em DIC com cinco repetições, e os resultados avaliados através de ANOVA uma via. Não foram observadas diferenças estatísticas significativas ($P>0,05$) para glicose e hematócrito. Todas as concentrações de eugenol avaliadas apresentaram eficiência como anestésico para *G. brasiliensis* e não afetaram a sobrevivência, a glicose sanguínea e o percentual de hematócrito. Para minimizar efeitos adversos não avaliados e obter recuperação mais rápida, indica-se o uso de 50 a 80 mg L⁻¹ de eugenol para juvenis de carás.

Palavras chave: anestesia; cará; óleo de cravo; peixe

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INTRODUCTION

There are evidences of the similarity of the fish nociceptive system with other vertebrates, and the use of anesthetics has been indicated to reduce the stressful impact of handling, especially during routine procedures, such as weighing, immunization, blood collection, identification, experimental surgery and veterinary procedures (SNEDDON, 2012; BITTENCOURT *et al.*, 2013). Studies have evaluated rates of anesthesia and recovery by monitoring the rate of gill ventilation, maintenance of equilibrium and the response capacity to external stimuli and handling, as well as the pharmacokinetics of the anesthetic (SNEDDON, 2012).

By choosing an anesthetic, it is important to consider its efficacy for the species of fish, their availability, cost and to be ease of use, as well as toxicity to fish, humans and the environment (AKBULUT *et al.*, 2010; 2011; HEO and SHIN, 2010). Eugenol, the active substance of clove oil, has shown to be effective and safe as an anesthetic for fish, with good speed of action and faster inducing deep anesthesia (JAVAHERY *et al.*, 2012), besides being considered a natural anesthetic (SANTOS SANCHEZ *et al.*, 2014a, b). This anesthetic has been evaluated for different handling, especially during immunizations, breeding, transportation and conducting research (BECKER *et al.*, 2012; PÁDUA *et al.*, 2012). Due to the fact that each one requires different anesthetic concentration to induce the desired anesthesia stage, and this concentration may vary between species and sizes classes (HOSEINI *et al.*, 2013). It is necessary to know the optimal concentration for each species of fish, because the use of wrong concentrations may cause unwanted effects, such as an increased stress and mortality of animals exposed to the drug (HOSEINI *et al.*, 2010; HOSEINI and GHELICHPOUR, 2011; HOSEINI and NODEH, 2011).

In Brazil there are recent reports of using eugenol (2-methoxy-4-(2-propenyl)phenol), the main component of clove oil (70-90% by weight) or just clove oil as an anesthetic for several species like *Centropomus undecimalis* (BERNARDES JÚNIOR, *et al.*, 2013), *Pimelodus britskii* (BERTOZI JÚNIOR *et al.*, 2014), *Rhamdia quelen* (CUNHA

et al., 2010; GOMES *et al.*, 2011; BECKER *et al.*, 2012; 2013; SUTILI *et al.*, 2014), *Oreochromis niloticus* (MOREIRA *et al.*, 2010; 2011), *Cyprinus carpio* (BITTENCOURT *et al.*, 2013), *Colossoma macropomum* (INOUE *et al.*, 2011), *Pseudoplatystoma reticulatum* (SANTOS SANCHEZ *et al.*, 2014b), *Gymnotus aff. inaequilabiatus* (PÁDUA *et al.*, 2012), *Brycon hilarii* (FABIANI *et al.*, 2013) among others. However, there are few studies investigating the physiological responses of fish when exposed to anesthetics and to date no study of anesthesia was carried out with pearl cichlid (*Geophagus brasiliensis*).

The pearl cichlid is distributed in coastal basins of eastern and southern Brazil and Uruguay. It is an important fish for artisanal fishing and show great potential for aquaculture due to their characteristics of robustness, easy handling and feeding, and ability of reproduce easily in the tanks and ponds. However there are few published data concerning the production of the pearl cichlid (AMARAL *et al.*, 2011; MALABARBA *et al.*, 2013) and many of the studies of this species are still in the development process, especially in southern Brazil. As an example, this study evaluated the effects of different concentrations of commercial cloves oil (eugenol) on anesthesia and recovery time, blood glucose and hematocrit percentage of pearl cichlid (*G. brasiliensis*).

MATERIAL AND METHODS

The study was conducted at the Research Center Herman Kleerekoper of the State Foundation Agricultural Research - FEPAGRO Aquaculture and Fisheries - Terra de Areia (RS), in 2013. The procedures adopted were approved by the Ethics Committee on the Use of Animals (protocolled under the number 22/2013 - CEUA - IPVDF). Juveniles of pearl cichlid (114) were used with an average weight (\pm SD) of 17.47 ± 7.61 g (wet weight after reaching the stage of deep anesthesia in semi-analytical digital scale, 0.001 g, Shimadzu®, BL 320 H) and average total length (\pm SD) of 8.76 ± 1.76 cm (vernier caliper), belonging to the stock of animals in this research center. The animals were acclimated for three days in a 1000 L tank with aeration and daily replacement of 50% of the volume of water. Fish

were fed once daily with commercial extruded feed (32% crude protein) until apparent satiation. Animals did not show morphological characteristics to differ the sex.

The different tested concentrations of eugenol were prepared from a stock solution of 100 mg mL⁻¹ commercial eugenol (Eugenol U.S.P. 99-100.5%, Biodinâmica®), diluted in ethylic alcohol in a ratio of 1:10.

For the assessment of the different stages of anesthesia and return the protocol of ROSS and ROSS (1999) adapted by VIDAL *et al.* (2007) was used (Chart 1). The concentrations tested were: 50, 80, 100, 120, 150, 170 and 200 mg L⁻¹. The

experiment was carried out in a completely randomized design (CRD), consisting of seven treatments and twelve repetitions (n = 84). The animals were fasted between 12 and 16 hours before the study. The individuals were collected from the tanks with dip nets and individually placed into glass jars, filled with 4 L of water filtered in the Cuno® filter (filter element with a 25 µm pore), and constant aeration. Dissolved oxygen content, temperature and pH in the water, were kept between 8.0 ± 0.6 mg L⁻¹, 22.5 ± 1.5 °C and 6.0, respectively during the experiment, measured by digital equipments like oximeter (YSI® 55 model) and pH meter (Hanna®, HI 98183 model).

Chart 1. Stages of anesthetic induction and recovery, according ROSS and ROSS (1999), adapted by VIDAL *et al.* (2007).

STAGES OF ANESTHETIC INDUCTION	
Stage I	Reacts to external stimuli, reduced movements, slow opercular movements, normal equilibrium.
Stage II	Total loss of reactivity to external stimuli except heavy pressure, mild reduction of opercular movement, normal balance.
Stage III	Partial loss of muscle tone, erratic swimming, increased opercular movements, reactive only strong vibration or tactile stimulation.
Stage IV	Complete loss of muscle tone, complete loss of equilibrium, opercular rate slow but regular.
Stage V	Total absence of reaction, even a strong stimulus, opercular movements slow and irregular, slow heartbeat, complete loss of all reflexes.
STAGES OF ANESTHETIC RETURN	
Stage I	Reappearance of opercular movements.
Stage II	Partial return of balance and ability to swim.
Stage III	Complete recovery of the balance.
Stage IV	Swimming and reaction to external stimuli still vacillating.
Stage V	Total restoration of balance and ability to swim.

The anesthetic for each concentration was renovated after each individual observation, obtained from the standard solution. Then, the fish were placed into glass jars with natural water without anesthetic and with aeration, for monitoring the stages of recovery. The survival was observed until 24 hours after anesthetic induction.

The effects of different concentrations of eugenol (50, 80, 100, 150, 200 mg L⁻¹) on blood glucose and hematocrit percentage, 0 h after reaching the stage V of anesthesia, were evaluated

and compared to individuals without anesthesia (control), with six treatments and five replications (n = 30). Blood was collected by section of caudal fin. Glucose was determined using digital portable glucometer, after the use of one drop of fresh blood directly to the reagent strip (Accu-Chek Active, Roche®). The hematocrit percentage (GOLDENFARB *et al.*, 1971) was determined in duplicate using microhematocrit centrifuge (Fanem®).

All results were assessed for normality by the Shapiro-Wilk test, to homoscedasticity by SNHT

and the results were expressed as mean value \pm standard deviation. Possible effects of the concentration of eugenol on the different stages of anesthesia and recovery were analyzed using the Kruskal-Wallis test with Dunn's post hoc test (CALLEGARI-JACQUES, 2007). Correlation analyzes (Pearson) between different concentrations and time of anesthesia and recovery were performed for each evaluated stage. To evaluate the results obtained for glucose content and hematocrit percentage was performed by One-Way analysis of variance (ANOVA) followed by the Tukey's test. For all analyzes, the level of significance adopted was 95% ($\alpha = 0.05$), using the XLSTAT® software Version 2014.5.01 (XLSTAT, 2014).

RESULTS

The average time for different stages of anesthesia in the juveniles of pearl cichlid is reported in the Table 1. The time for deep anesthesia (stage V) varied between one and three minutes respectively for concentrations of 200 mg L⁻¹ and 50 mg L⁻¹. A significant difference between treatments ($P < 0.05$) was observed to all stages of anesthesia.

A state of euphoria with intense agitation was observed at doses between 100 and 170 mg L⁻¹,

which can be viewed with the increase in time for the fish to reduce the movements of both swimming and operculum (Stage I). Apparently this behavior did not affect the other stages of anesthesia. The analyzed results showed a negative correlation between the time for the stages of anesthesia and eugenol concentrations evaluated, however were not significant ($P > 0.05$) (Table 1).

Table 2 displays the times required for juveniles of pearl cichlid to achieve the different stages of recovery from anesthesia after biometrics. Statistical differences between the concentrations of eugenol in all stages of recovery were observed ($P < 0.05$). Positive linear correlations between concentrations and recovery times were observed, however, were not statistically significant ($P > 0.05$). The total recovery time ranged from eight to three minutes, respectively for concentrations of 200 mg L⁻¹ and 50 mg L⁻¹. No fish mortality was observed over the 24 h after the anesthetic induction tests.

No statistically significant differences were observed in juvenile pearl cichlid exposed to different concentrations of commercial eugenol for the variables glucose and hematocrit percentage at 0 h after deep anesthesia and treatment without anesthesia ($P > 0.05$) (Table 3).

Table 1. Anesthetic induction time in seconds (mean \pm standard deviation) of *Geophagus brasiliensis* subjected to different concentrations of commercial eugenol, K values and probability (Kruskal-Wallis test) and correlation coefficient (r) between concentration and time to each stage evaluated.

Eugenol (mg L ⁻¹)	Time to Induction (s)				
	Stage I ¹	Stage II ¹	Stage III ¹	Stage IV ¹	Stage V ¹
50	29.4 \pm 11 ^{ab}	74.8 \pm 10 ^b	110.0 \pm 28 ^c	137.1 \pm 20 ^c	172.6 \pm 25 ^c
80	28.8 \pm 7 ^{ab}	63.2 \pm 10 ^{ab}	86.6 \pm 14 ^{bc}	99.1 \pm 10 ^{bc}	119.4 \pm 19 ^{bc}
100	40.2 \pm 12 ^b	61.1 \pm 16 ^{ab}	91.5 \pm 21 ^{bc}	114.1 \pm 18 ^{bc}	137.5 \pm 23 ^{bc}
120	31.8 \pm 9 ^{ab}	57.2 \pm 12 ^a	74.7 \pm 14 ^{ab}	90.6 \pm 13 ^{ab}	111.4 \pm 16 ^{ab}
150	34.2 \pm 5 ^{ab}	56.4 \pm 10 ^a	74.1 \pm 11 ^{ab}	92.1 \pm 10 ^{ab}	110.5 \pm 19 ^{ab}
170	32.0 \pm 6 ^{ab}	50.2 \pm 7 ^a	60.7 \pm 9 ^a	78.6 \pm 10 ^a	92.0 \pm 10 ^a
200	24.0 \pm 8 ^a	49.7 \pm 12 ^a	61.5 \pm 6 ^a	88.9 \pm 12 ^{ab}	90.7 \pm 15 ^a
K (observed)	17.4	32.0	49.0	47.0	56.4
P-value	0.008	<0.0001	<0.0001	<0.0001	<0.0001
r	-0.13	-0.56	-0.66	-0.66	-0.74

¹According to ROSS and ROSS (1999), adapted by VIDAL *et al.* (2007); different letters in the column indicate significant differences by Dunn's test ($P < 0.05$).

Table 2. Recovery time in seconds (mean \pm standard deviation) of *Geophagus brasiliensis* subjected to different concentrations of commercial eugenol, K values and probability (Kruskal-Wallis test) and correlation coefficient (r) between concentration and time to each stage evaluated.

Eugenol (mg L ⁻¹)	Time to Recovery (s)				
	Stage I ¹	Stage II ¹	Stage III ¹	Stage IV ¹	Stage V ¹
50	13.3 \pm 13 ^{ab}	60.3 \pm 34 ^a	126.7 \pm 58 ^a	151.3 \pm 68 ^a	175.1 \pm 68 ^a
80	10.2 \pm 9 ^{ab}	125.2 \pm 40 ^{ab}	173.8 \pm 44 ^{ab}	207.4 \pm 43 ^{ab}	233.2 \pm 50 ^{ab}
100	13.3 \pm 9 ^{ab}	187.1 \pm 58 ^b	233.4 \pm 52 ^{bc}	326.3 \pm 58 ^{bcd}	363.0 \pm 92 ^{bcd}
120	12.4 \pm 11 ^{ab}	119.9 \pm 26 ^{ab}	182.3 \pm 33 ^{ab}	210.4 \pm 42 ^{ab}	266.4 \pm 38 ^{abc}
150	19.8 \pm 12 ^b	129.8 \pm 48 ^{ab}	269.0 \pm 49 ^{bc}	372.6 \pm 83 ^{cd}	378.0 \pm 77 ^{cd}
170	7.4 \pm 9 ^a	137.6 \pm 34 ^b	208.4 \pm 38 ^{abc}	249.0 \pm 29 ^{abc}	278.8 \pm 37 ^{abc}
200	14.7 \pm 8 ^{ab}	200.5 \pm 121 ^b	354.5 \pm 85 ^c	438.9 \pm 98 ^d	516.2 \pm 102 ^d
K (observed)	13.3	27.9	45.9	57.1	51.0
P-value	<0.05	<0.0001	<0.0001	<0.0001	<0.0001
r	0.03	0.39	0.68	0.66	0.68

¹According to ROSS and ROSS (1999), adapted for VIDAL *et al.* (2007); different letters in the column indicate significant differences by Dunn's test ($P < 0.05$).

Table 3. Values of glucose and hematocrit percentage (mean \pm standard deviation) for juveniles of *Geophagus brasiliensis* exposed to different concentrations of commercial eugenol.

Eugenol (mg L ⁻¹)	Glucose (mg dL ⁻¹)	Hematocrit (%)
0	172.0 \pm 78	33.1 \pm 18
50	144.0 \pm 12	44.2 \pm 4
80	118.2 \pm 53	42.6 \pm 11
100	116.2 \pm 22	39.9 \pm 5
150	172.8 \pm 63	44.3 \pm 5
200	224.6 \pm 12	35.6 \pm 9
F value	0.9463 ns	1.1037 ns

ns: no significant effect by One-Way ANOVA ($P \geq 0.05$).

DISCUSSION

In addition to animal species, the weight and size of the fish, the gill surface area in relation to body weight, sex, sexual maturity, content of lipids, the physical condition and health, the density storage and water quality parameters (pH, temperature, salinity, oxygen and minerals) may also affect the efficiency of the anesthetic in fish (HOSEINI *et al.*, 2013). The efficiency of eugenol as an anesthetic for *G. brasiliensis* was demonstrated in the experimental conditions for all concentrations evaluated, although the fish were not separated by sex, demonstrating the fact that the organisms were possibly in appropriate

conditions for the species and are a homogeneous group may have aided in the efficiency of this anesthetic.

According to PARK *et al.* (2009), the concentration of drug is considered optimal when the period is no larger than 180 s to induction and 600 s to recovery. All concentrations of eugenol evaluated in this study did not exceed the times indicated by these authors. These findings seem to indicate that the concentrations studied were effective and safe to anesthetic induction and recovery for pearl cichlid. Similar dosages of eugenol for anesthesia of the tilapia, anesthesia and recovery times were between the safe values (MOREIRA *et al.*, 2011).

However, euphoric behavior and swimming movements accelerated in concentrations between 100 and 170 mg L⁻¹ were observed. Signs of hyperactivity were also described for the use of eugenol as an anesthetic for spotted surubim *Pseudoplatystoma corruscans* (VIDAL *et al.*, 2006), "matrinxã" *Brycon cephalus*, cachama *C. macropomum* (VIDAL *et al.*, 2007), Nile tilapia (MOREIRA *et al.*, 2011) and "cachara" *P. reticulatum* (SANTOS SANCHEZ *et al.*, 2014b), what could be related adverse reactions to the drug itself. In this way, it may infer that the dosages of 50 and 80 mg L⁻¹ for *G. brasiliensis* are safer than others evaluated in this study.

Studies have demonstrated that eugenol is an efficient inducer of anesthesia for carps (*C. carpio*) when used at doses lower than 75 mg L⁻¹ and recommended dosage of 37.5 mg L⁻¹ for anesthesia this specie, to avoid drug toxicity (BITTENCOURT *et al.*, 2013). For South American catfish (*R. quelen*) GOMES *et al.* (2011) indicated a dosage of 40 ml L⁻¹ is sufficient to induce anesthesia and compensate for the effects of size of fish and water quality. In the studies of HONCZARYK and INOUE (2009) was reported that doses of 30 and 60 mg L⁻¹ applied by spraying in gills of arapaima (*Arapaima gigas*) were effective for the management of this species. For *Pangasius hypophthalmus* weighing 2 to 20 g, the minimum concentration of eugenol to induce anesthesia in less than three minutes was 53.8 to 81.5 mg L⁻¹ and the maximum concentration that the fish recover at least five minutes was 65.9 to 105.8 mg L⁻¹ (HOSEINI *et al.*, 2013). The time for a fast anesthesia, loss of equilibrium and deep anesthesia is important for blood collection, handling and surgery (HOSEINI *et al.*, 2010; HOSEINI and GHELICHPOUR 2011; HOSEINI and NODEH 2011; FABIANI *et al.*, 2013). In this study, the concentration of 80 mg L⁻¹ is the most appropriate, though having had no significant difference between doses of 50 and 100 mg L⁻¹, because the times for achieve the different stages were more homogeneous. Beyond the verification of the times of anesthesia and recovery of different drugs and their possible concentrations, studies have been conducted to minimize the stress caused by practices that require the handling of fish in farms (MOREIRA *et al.*, 2010).

The blood glucose parameter, and cortisol, it is useful to measure known stress responses (HEO and SHIN, 2010) and increasing hematocrit has been described as an indicator of stress (secondary effect) (SNEDDON, 2012). Has been observed that the use of eugenol may cause a decrease in serum glucose of *Carassius auratus* (MI *et al.*, 2013), *Salmo salar* (IVERSEN *et al.*, 2003), *Oncorhynchus tshawytscha* (CHO and HEATH, 2000), and these authors relate this decline by stress decrease. As well as observed in the present study, SANTOS SANCHEZ *et al.* (2014b), observed no difference in plasma glucose between "cachara" anesthetized with eugenol and control (without anesthetic). An increase in glucose

(20 and 60 mg L⁻¹) and hematocrit (60 mg L⁻¹) after simulated bath 15 minutes with eugenol in *C. macropomum* were observed by INOUE *et al.* (2011), indicating that exposure to the eugenol, for longer periods than those used in this study, can affect these parameters, and demonstrate the effect of this drug on stress. It can be considered that if bath time is no greater than necessary to achieve the state of anesthesia V, as the concentrations of this study, there is no additional stress due exclusively to anesthesia in juvenile pearl cichlid. A small influence of eugenol on stress-related enzymes for *C. auratus* was observed by MI *et al.* (2013) suggesting that the effect of this drug is not toxic and not results in hyperglycemia for that species.

In this study there was no fish mortality after 24 hour exposure those concentrations evaluated. In similar period of observation, MOREIRA *et al.* (2011) have tested clove oil as an anesthetic in tilapia juveniles and did not report mortality.

CONCLUSIONS

The concentrations evaluated of eugenol showed efficiency as an anesthetic for *G. brasiliensis* and did not affect the survival, blood glucose and hematocrit percentage. To minimize adverse effects and promote faster recovery may be indicate the use of 50 to 80 mg L⁻¹ for this species.

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