Anesthesia using eugenol for two weight classes of *Piaractus mesopotamicus*: Evaluation of induction time, recovery time, behavioral response and serum glucose

.

ABSTRACT

Aims: The use of anesthetics in fish ensures that they can undergo common handling in fish farming without causing damage to production. The aim of the present study was to determine the induction and recovery times of *Piaractus mesopotamicus* submitted to eugenol anesthesia.
Study design: 100 *P. mesopotamicus* were used, divided into two weight classes (Class I weighing between 275 and 460 grams; and Class II weighing between 461 and 680 grams).
Methodology: The fish (n=10) were submitted to eugenol anesthesia in five dosages (50.0; 75.0; 100.0; 125.0 and 150.0 mg L^{-1}) and the induction times to anesthesia and recovery to normal activity, as well as fish behavior during this stage.
Results: There were statistical differences (p = .05) between anesthesia and fish recovery. For fish belonging to class I, the best dose to apply is 50 mg L^{-1}, while for class II, the recommended dose is 75 mg L^{-1}. Glucose was higher (p = .05) in animals submitted to a dose of 75 mg L^{-1} compared to those stunned at 50 mgL^{-1}, but there was no effect between the other doses as well as between weight classes.
Conclusion: Larger fish are induced to deep anesthesia and return to normal behavior before smaller fish. Considering the induction times to anesthesia and total recovery, it is recommended to use a dose of 50 mg L^{-1} for class I and 75 mg L^{-1} for class II.

Keywords: Handling; Management; Sedation; Welfare

1. INTRODUCTION

The farming of freshwater fish is constantly growing in Brazil, and a large number of species with potential for aquaculture can be found in the territory [1]. One of them is the pacu (*P. mesopotamicus*), which presents rusticity, well accepted to the most different breeding systems, low protein requirement, high fillet yield [2], pleasant flavor [3] and high commercial value [4].

Intensified fish farming requires adequate management methods to prevent injuries during routine activities such as sampling, transportation, induced reproduction, egg collection, grading, and other related processes [5, 6, 7, 8, 9, 10, 11], all of which can result in economic losses in production. Consequently, the use of anesthetics has emerged as an effective alternative [12, 13], primarily shortening the metabolism of the animals [14] and allowing desensitization [15]. This results in reduced motility, minimizing abrasions. Without such precautions, implications could range from loss of appetite to death [16], particularly in severe cases when stressors become chronic. Glucose serves as a valuable tool in analyzing fish anesthesia, aiding in the assessment of animal stress [17].

There is a large variety of anesthetic products available on the market, ranging from plant to industrial origin. When used in fish farms, all these products must ensure the well-being of the fish and the environment [18, 19]. However, the effects of such drugs depend on the species, developmental stage, body condition, health [19], and water quality such as temperature and pH [20]. Therefore, studies must be carried out in order to reach the most diverse species and their particularities as well as the places where they are inserted.

The selection of the ideal active ingredient depends on its effectiveness, availability, cost, and safety in handling, for both fish, humans and the environment [19, 8]. Additionally, according to Roubach& Gomes [21] the ideal time for fish anesthesia during biometrics and manipulations is typically one to three minutes. For healthy assessments and surgical interventions, the recommended duration is three to five minutes. Quick recovery, typically not exceeding five minutes, is also essential.

The objective of this work was to assess the behavioral characteristics exhibited during the anesthetic activity and the times at which these characteristics were observed in two weight classes of *P. mesopotamicus* when subjected to five different anesthetic doses.

2. MATERIAL AND METHODS

The study was conducted at the Aquaculture Laboratory of UniversidadeEstadual do Oeste do Paraná - Unioeste, Campus of Toledo, PR, Brazil. A total of 100 specimens of pacu (*P. mesopotamicus*) were categorized into two weight classes. Class I consisted of individuals weighing 380.63 ±57.88 grams (ranging from 275 to 460 grams), with an average length of 25.77 ±1.34 cm, while class II included specimens weighed 545.87 ±65.70 grams (ranging from 461 to 680 grams), with an average length of 29.27 ±1.14 cm.

The fish were captured at the Development Center for Fish Farming in Net Tanks, located in the Biological Refuge in the municipality of Santa Helena, PR, Brazil. They were transported in tanks equipped with constant oxygenation for a duration of 90 minutes to reach the Laboratory. Upon arrival, the fish were placed in 20 tanks of 500 liters where they were acclimated for ten days before the start of the experiment. During this acclimation period, the fish were fed four times a day with a commercial diet containing 32% crude protein, provided until apparent satiation. The experiment was conducted in glass aquariums with a useful volume of 80 L, although they were filled with only 30 liters of water, sourced from the acclimation tanks where the fish were held prior to the experiment.

Water quality parameters were assessed at various stages of the experiment: before the addition of the anesthetic solution and the fish in the induction tank, before transferring the fish in the recovery tank, and after removing the animal from each recovery tank. Parameters such as dissolved oxygen, pH, electrical conductivity and water temperature were monitored. The average values of water quality in the aquariums are presented in Table 1. It's important to note that all measured parameters remained within the recommended range for tropical climate fish, as defined by Proença (1994) [22].

Eugenol solutions were prepared by diluting eugenol in (98.8%) ethyl alcohol, resulting in a stock solution with a concentration of 10 g L^{-1} for ease of handling. Concentrations of 50.0, 75.0, 100.0, 125.0, and 150.0 mg L^{-1} of eugenol were tested on both weight classes of *P. mesopotamicus*. Each test was conducted individually, starting from the lowest concentration to the highest. Ten fish (n = 10) were exposed individually to each treatment. A control group underwent the same handling procedures but without the anesthetic solution in the aquarium. The time required for the observed behavioral patterns to appear was recorded using a digital stopwatch. The aquarium water was completely changed after each test, and the anesthetic stages were determined based on the fish's behavior during exposure to the molecule.

Table 1. Water quality parameters of induction and recovery tanks, before transferring and after removal of fish.

Aquariums introducing fish into the aquarium				
	Temperature (°C)	**Oxygen (mg L^{-1})**	**Conductivity (µS cm^{-1})**	**pH**
Induction	25.42	5.17	98	6.41
Recovery	25.5	4.95	97	6.39
Aquariums After removing the fish from the aquarium				
	Temperature (°C)	**Oxygen (mg L^{-1})**	**Conductivity (µS cm^{-1})**	**pH**
Induction	25.29	5.22	98	6.41
Recovery	25.75	4.55	97	6.35

To determine the absence of reaction to any stimulus, the side of each fish was gently touched with a glass rod. Anesthetic recovery was performed individually, in a 30 L aquarium without anesthetic, equipped with continuous aeration. Following recovery, the fish were transferred to 500 L tanks with constant aeration and daily feeding for 96 hours to monitor mortality. Behavioral characteristics of the fish at different anesthetic stages were evaluated from the beginning of anesthetic induction (Table 2) adapted from [23, 24, 25].

Table 2. Behavioral characteristics of *P. mesopotamicus* according to different anesthetic stages.

Stage	Behavior characteristic
I	Acceleration in the opercular beat, euphoria and restlessness
II	Decreased opercular beat, reduced swimming,
III	Imbalance, erratic swimming, lateral decubitus
IV	Absence of reaction to stimuli
Recovery	Normal swimming condition, touch sensitivity, escape ability

For the analysis of blood glucose (mg dL^{-1}), three fish submitted to each eugenol dosage were captured, as well as three animals that were not submitted to sedation, and 1.0 mL of blood was collected. Samples were centrifuged at 2,500 rpm for five minutes. To carry out the analyses, specific kits from Gold Analisa Diagnóstica® 82 were used, the analyses were processed in a spectrophotometer and carried out according to the kit manufacturer's instructions.

2.1 Statistical analysis

The data obtained were grouped by the treatments and submitted to the normality of ShapiroWilk test [26] and were evaluated through simple regression analysis. To determine the possible effects of eugenol doses on the stages of anesthesia and recovery and to verify the possible effects of glucose and classes of weight and anesthetic dose, analysis of variance (two-way ANOVA), with subsequent multiple comparisons of means by Tukey's test [27] at a 5% significance level. Statistical analyses were carried out using the R software [28].

3. RESULTS AND DISCUSSION

Honorato & Nascimento (2016) [29] describe that the gills of fish have multiple purposes, including the promotion of gas exchange and the use of anesthetics can cause adverse reactions to the fish, such as stopping respiratory process and insufficient gas and ionic exchange between the blood and the Water. This effect mixed with the intense movement of the animals may have resulted in a slight increase in oxygen concentrations in the induction aquariums. On the other hand, during the recovery there is a great consumption of oxygen, which can be observed by the value presented in the recovery aquarium before the introduction of the fish, and in the recovery aquarium after the removal of the fish. There was a reduction of approximately 0.40 mg L^{-1} (please see Table 1), which allows us to infer that when the fish is placed in clean water again, it tries to get rid of the anesthetic drug by breathing quickly and consuming greater amounts of oxygen.

Regarding to the behavior of the fish when anesthetized (Table 2), in anesthesia stage I, which according to Wood (2002) [30] indicate visibly slow or erratic opercular movement, *P. mesopotamicus* in both weight classes demonstrate the opposite effect, appearing agitated, accelerated opercular movement and euphoria. This indicates that in a short time the animals were under the effect of the product placed in the water. For this stage of anesthesia, there were differences for the tested dose, that is, the more eugenol contained in the solution, the shorter the time for the fish to behave in this way (Figure 1A).

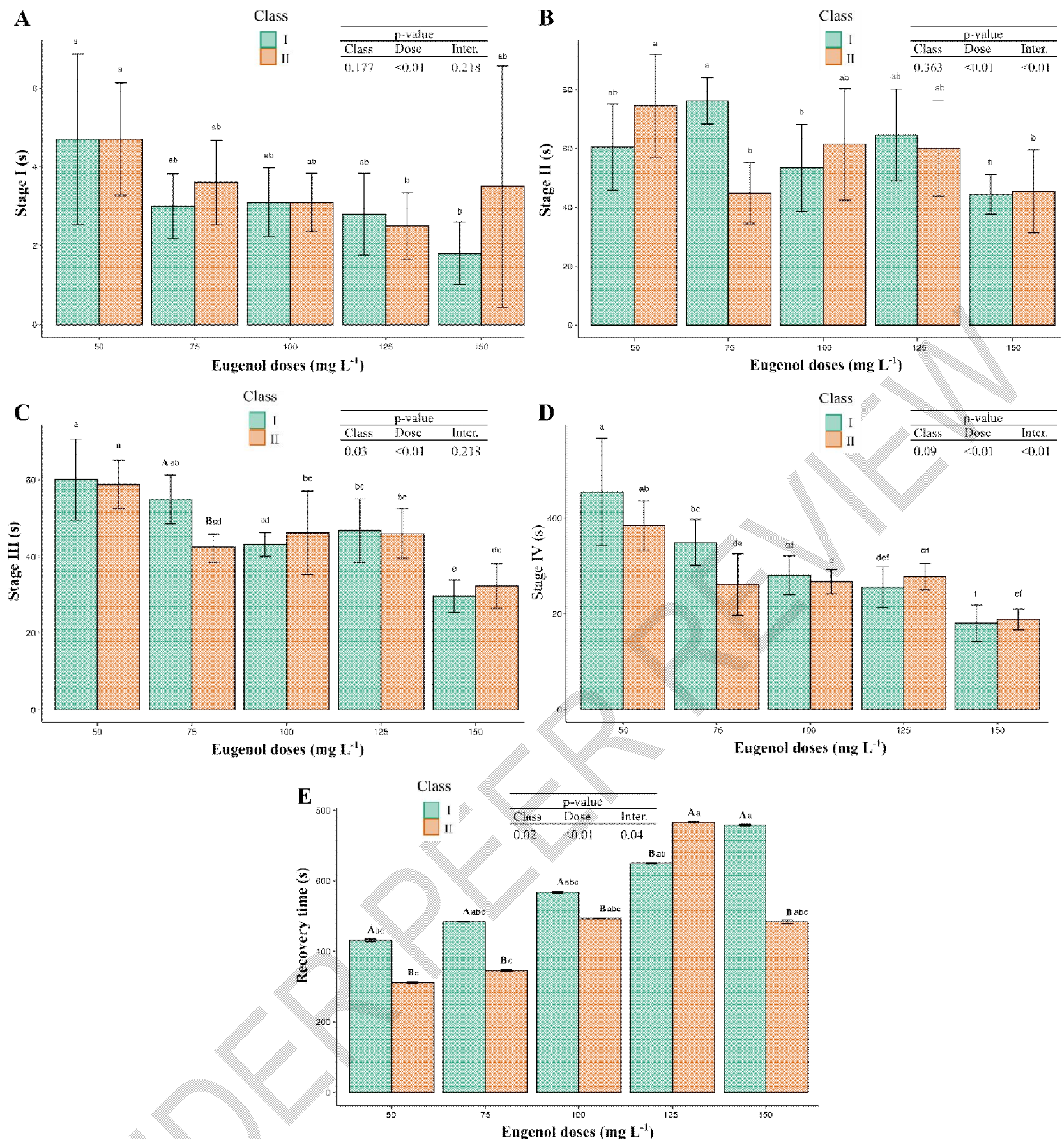


Fig. 1. Time (s) of anesthesia and recovery of *P. mesopotamicus* submitted to eugenol anesthesia in two weight classes. Green class I; Light orange class II. Uppercase letters interaction between weigh class and anesthetic doses and distinct lowercase letters indicate statistical differences for anesthetic dose according to Tukey's (p = .05).

During stage II of anesthesia there were significant differences (p = .05) for the tested dose (Figure 1B), same behavior in the first stage, in which with the increase of eugenol content in the water, the sedative effect occurs more quickly. The behavioral characteristic observed for this stage is a reduction in opercular beating and swimming, as well as contact with the surface of the aquarium, possibly in an attempt to stay in a position of escape from any type of threat. Roubach& Gomes [21] and Roubach, Gomes, Fonseca & Val (2005) [12] using the protocol proposed by [31] observed that first the fish lose reactions and then the balance and decrease their opercular beats, similar to what was observed in the current study.

During stage III of anesthesia, the *P. mesopotamicus* specimens showed differences in the sedation time for the eugenol doses applied in the water (p = .05) and there was also an interactive effect between the classes and doses used. Among the behavioral characteristics observed during this stage are: difficulties in maintaining the swimming position, erratic swimming and lateral decubitus position (Figure 1C).

In stage IV, there was a reduction in the induction time as the applied dosage increased and it can be seen that the animals completely lost the ability to react to the applied stimuli (Figure 1D). Stage IV anesthesia corresponds to the loss of sensitivity by the fish, this is influenced both by the weight class of the fish and by the level of eugenol used for sedation. And, from the moment they regain their senses, the first reaction is the return of the opercular beats, partial return of balance, ability to swim and later ability to escape the submitted impulses. Patterns that fit the configuration proposed by [32].

The results found for the effect of anesthesia in fish demonstrate that the weight class influences only the stage IV, of deep anesthesia, and the recovery to the normal condition of movement. However, the anesthetic dose interferes in all stages of anesthesia induction and also in the recovery of the fish (Figure 1E), and larger fish have a faster effect in both processes, deep induction and recovery, in addition, there is an interaction between the class of fish weight and the anesthetic dose used during the development of stages II, III and IV.

Fish belonging to class I (275 to 460 grams) reached deep anesthesia with a maximum of seconds when the applied dose was the lowest among all those tested (50 mg L^{-1}), but when the dose was 150 mg L^{-1}, this process took approximately 44 seconds, linear 144 reduction in induction time as the dose was increased. This allows us to infer that *P. mesopotamicus*responds quickly to eugenol, similar to that reported by Roubach, Gomes, Fonseca & Val (2005) [12] for tambaquis (*Colossomamacropomum*).

For the recovery of the animals belonging to class I, it was verified that there is an increase in time up to a maximum limit (approximately 111.0 mg L^{-1} of eugenol) and subsequently a decrease (Figure 2A). However, as the same dose is used to verify both behaviors, sedation and recovery, it is preferable to put a concentration of 50 mg L^{-1} of eugenol in the aquarium water, as this will make the fish completely sedated in just 113 seconds and recover their normal behavior in 215 seconds (less than 5 minutes), without mortality, and ensuring a shorter recovery time for the animals. Roubach, Gomes, Fonseca & Val (2005) [12] found *C. macropomum* recovery times ranging from three to seven minutes, depending on the dose used. For the *P. mesopotamicus* in the current experiment, at none of the doses did the fish take more than 382 seconds (6.5 minutes), although this is longer than the recommended time for fish recovery, which is five minutes [21], it 8 is still outside the critical range of over ten minutes [33].

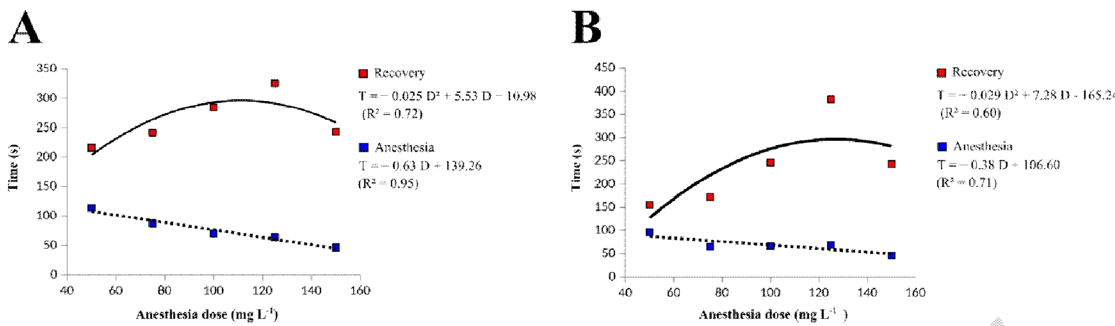


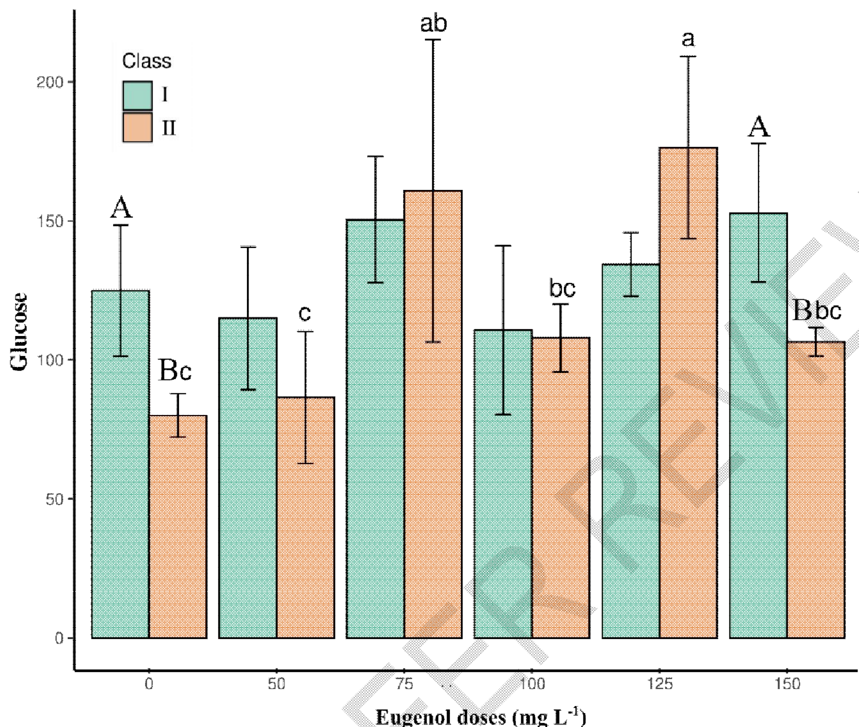
Fig. 2. Induction and recovery time of *P. mesopotamicus* class I (275 to 460 g) and class II (461 a 680 g) submitted to eugenol anesthesia.

Fish belonging to class II, with weights ranging between 461 and 680 grams, there was a linear reduction in anesthesia times as the eugenol content increased, with the longest time verified being 96 seconds, when the dose of 50 mg L^{-1}, and the shortest time of seconds when the highest dosage (150 mg L^{-1}) was used. For the animals' recovery, there was a quadratic effect, and deriving the regression equation, the level of eugenol that provides longer time under sedation effect is 126.8 mg L^{-1} (Figure 2B). These fish are larger, have less ability to resist the drug and anesthesia occurs more quickly. For this phase, the use of 75 mg L^{-1} is recommended, as anesthesia occurs completely in 65 seconds and the return to normal activity in 172 seconds, ensuring good recovery and no mortality.

Vidal et. al, (2008) [9] determining the values of the lethal concentration of eugenol for piavuçu (*Leporinus macrocephalus*), found that 50% of the fish did not return to activity after 30 minutes, when they remained for ten minutes at a concentration of 45.13 mg L^{-1}, and 99% did not. They resume normal activities in the same time interval when the eugenol concentration is 65.05 mg L^{-1}. In the present study, none of the animals remained in water with anesthetic for more than two minutes, possibly this was decisive for the non-occurrence of mortality. Even if the intention was to verify the time of deep anesthesia, which is the state to perform surgical processes, to avoid greater wear on the fish and greater exposure to the product, they can be removed from the container with the anesthetic when they reach stage II and III, as success for biometrics is guaranteed due to the fish reducing their swimming capacity and losing balance.

Regarding blood glucose, the animals showed differences only in relation to the anesthetic dose, and the animals belonging to class II submitted to the concentration of 125 mgL^{-1} of eugenol had higher glucose values. Animals without submission to the drug showed the lowest value.

Glucose did not show a linear behavior according to the anesthetic doses used, this can be attributed to the hardiness of the *P. mesopotamicus* and to the effect of the product itself, which was safe for the animals. Honorato & Nascimento (2016) [29] report that the increase in glucose levels is a secondary indicator of fish stress and this is motivated by the presence of some stressor to meet the greater energy demand, characteristic of unfavorable situations. In the present experiment, this elevation behavior was observed in 125 mg L^{-1} dose and agitation was observed at the beginning of the animal's contact with the product (Figure 3). [34] evaluating the effect of eugenol in tambaquis, describe a linear behavior in the increase of glucose according to the doses of the anesthetic, and in the highest applied dose (60 mg L^{-1}), the authors found values close to 180 mg dL^{-1} of glucose, mean levels that were similar only in the dose of 125 mg L^{-1} of eugenol in the present study.



p-value		
Class	Dose	Interaction
0.012	0.0014	0.0489

Fig. 3. Glucose values observed in fish weight classes and anesthetic doses used. Green class I; Light orange class II. Uppercase letters interaction between weight class and anesthetic doses and distinct lowercase letters indicate statistical differences for anesthetic dose according to Tukey's (p = .05)

Deriggi, Inoue & Moraes (2006)[35] Suggest that eugenol does not minimize the stressful effects of handling tilapia juveniles, although the authors do not claim that eugenol causes additional stress to the animals, and conclude that the short manipulation of individuals is sufficient to induce the release of catecholamines and subsequent rise in glucose. In any case, with the glucose values observed in the blood of the animals, it is possible to state 201 that the product is safe for use.

Eugenol is a safe substance to be applied during *P. mesopotamicus* sedation, it has a good safety margin both for the applicator and for the fish that are immersed in this solution, and shortly after the animal is removed from the water containing this anesthetizing agent, the fish have the ability to return to their normal activity. Although no mortalities were presented, more studies are needed to determine the lethal dose for this species, because according to Murgas et al., (2010)[36], the exacerbated time of action of the product can cause damage to the central nervous system of these animals.

4. CONCLUSION

The best anesthetic dose for class I fish is 50 mg L^{-1} of eugenol and for class II the best dos e is 75 mg L^{-1}. The behavioral pattern while anesthetized in *P. mesopotamicus* is: during sta ge I of anesthesia, acceleration in the opercular beat, euphoria and agitation; in stage II, dec reased opercular beat, reduced swimming and contact with the side of the aquarium; in stag e III, imbalance, erratic swimming and lateral decubitus; in stage IV, absence of reaction to s timuli; and in stage V, normal swimming condition, sensitivity to touch and ability to escape.

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ETHICAL APPROVAL

The trial was conducted under UNIOESTE Ethical Committee Guide – Protocol 024/2019

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