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2-Phenoxyethanol as an anesthetic for *Rhamdia quelen*: a comparison with eugenol

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Abstract: Aquaculture handling requires fast anesthesia, assuming that action-time is within 3 min and recovery in <5 min. This study aimed to verify the ideal concentration (IC) of 2-Phenoxyethanol (2-Phe) to induce anesthesia for *Rhamdia quelen* and to compare its effects on stress parameters in *R. quelen* anesthetized with Eugenol. To accomplish this goal, two assays were carried out. 1°) seven 2-Phe concentrations were tested: 300, 400, 500, 550, 600, 650, and 700 µL L^{-1} to verify IC. When increasing 2-Phe concentrations, induction times decreased while recovery times increased. The IC of 2-Phe to anesthetize *R. quelen* was 700 µL L^{-1} (induction 2.98 ± 0.34 min and recovery time 2.75 ± 0.35 min). 2°) stress parameters evaluation. Fish were divided into three groups: Control (baseline values, without anesthetic), 2-Phe (700 µL µL L^{-1}), and Eugenol (50 mg L^{-1}). Levels of cortisol, glucose, and lactate were measured in the fish as indicators of stress at two different times, time zero (T_0 = anesthetized) and time one (T_1 = one hour after anesthesia). The animals in groups 2-Phe and Eugenol showed a significant increase in glucose values when compared to the control group. The serum lactate at T_0 of the Eugenol and 2-Phe groups did not increase when compared to the control group; however, at T_1, a significant increase was observed on both anesthetics. No significant differences were found in plasma cortisol levels among the treatments and times. Therefore, 700 µL L^{-1} of 2-Phe can be considered a suitable anesthetic for *R. quelen*.

Key words: Anesthesia, Cortisol, Glucose, Lactate, Phenylglycol, Silver catfish.

Resumo: 2-Fenoxietanol como anestésico para *Rhamdia quelen*: comparação com eugenol. Os manejos na aquicultura requerem anestesia rápida, com tempo de indução em até 3 min e recuperação em <5 min. Este estudo teve como objetivo verificar a concentração ideal (CI) de 2-Fenoxietanol (2-Phe) para induzir anestesia em *Rhamdia quelen* e comparar seus efeitos sobre parâmetros de estresse em *R. quelen* anestesiada com Eugenol. Para atingir este objetivo, dois ensaios foram realizados. 1°) foram testadas sete concentrações de 2-Phe: 300, 400, 500, 550, 600, 650 e 700 µL L^{-1} para verificar a CI. Aumentando as concentrações de 2-Phe, os tempos de indução diminuíram, enquanto os tempos de recuperação aumentaram. A CI de 2-Phe para anestesiar *R. quelen* foi de 700 µL L^{-1} (Indução 2,98 ± 0,34 min e tempo de recuperação 2,75 ± 0,35 min). 2°) avaliação dos parâmetros de estresse. Os peixes foram divididos em três grupos: Controle (valores basais, sem anestésico); 2-Phe (700 µL µL L^{-1}) e Eugenol (50 mg L^{-1}). Os níveis de cortisol, glicose e lactato foram medidos nos peixes como indicadores de estresse em dois momentos diferentes, tempo zero (T_0 = imediatamente após a anestesia) e tempo um (T_1 =

uma hora após a anestesia). Os animais dos grupos 2-Phe e Eugenol apresentaram aumento significativo nos valores de glicose quando comparados ao grupo controle. O lactato sérico em T_0 dos grupos Eugenol e 2-Phe não aumentou quando comparado ao grupo controle; entretanto, em T_1, observou-se aumento significativo em ambos os anestésicos. Não foram encontradas diferenças significativas nos níveis de cortisol sérico entre os tratamentos e tempos. Portanto, 700 µL L^{-1} de 2-Phe pode ser considerado um anestésico adequado para *R. quelen*.

Palavras-chave: Anestesia, Cortisol, Glicose, Lactato, Fenilglicol, Jundiá.

Introduction

In aquaculture, fish are routinely manipulated, and this can cause stress, pain, and even death of the animals (Barata et al., 2016). To comply with animal use ethics, anesthetics are used (Gomułka et al., 2015; Czerniak et al., 2018; Kizak et al., 2018; Uehara et al., 2019). When submitted to an anesthetic, fish feel less pain and become calmer, thus facilitating and improving handling, transportation, and even surgery (Hoseini et al., 2015; Barata et al., 2016; Mazandarani & Hoseini, 2017). According to Hoseini et al. (2018), anesthetics can be divided between plant derivatives and non-plant derivatives.

Anesthetics can be administered by immersing the fish in an anesthetic solution that is absorbed through the gills, the main route of absorption, followed by diffusion into the blood and migrating to the central nervous system (CNS) (Ross & Ross, 2008). The ideal anesthetic should induce anesthesia in less than three minutes and recovery in less than five minutes without presenting toxicity (Marking & Meyer, 1985).

2-Phenoxyethanol (2-Phe), also known as Ethylene glycol monophenyl ether, PHE-G, PHE-S, PHE, PhG, and Phenylglycol, is a colorless, aromatic and moderately oily liquid (Barata et al., 2016) soluble in water (Barata et al., 2016; Czerniak et al., 2018; da Silva et al., 2019). According to Czerniak et al. (2018), it is an effective and safe anesthetic for fish, in addition to having antimicrobial and antifungal activities (Ross & Ross, 2008).

Eugenol is obtained from the distillation of some parts of plants of the genus *Eugenia* (*E. aromaticum* and *E. caryophyllata*) (Priborsky & Velisek, 2018). Because of its anesthetic properties, it is also commonly used for anesthesia in fish (Bolasina et al., 2017; Rucinque et al., 2017; Almeida et al., 2018; Takatsuka et al., 2019; He et al., 2020). Eugenol is recommended for anesthesia in *R. quelen* because it does not change the levels of blood stress indicators (Cunha et al., 2010; Corso et al., 2019), and its use has been evaluated as an anesthetic for several other fish species (Bolasina et al., 2017; Rucinque et al., 2017; Almeida et al., 2018; Medeiros Júnior et al., 2018; Mirghaed et al., 2018; Romaneli et al., 2018; Yousef et al., 2018; de Oliveira et al., 2019a; de Oliveira et al., 2019b; Takatsuka et al., 2019; He et al., 2020).

Since one of the purposes of anesthesia is to reduce management stress, it is important to observe the physiological responses of fish through the hypothalamic-pituitary-interrenal (HPI) axis, which, after being triggered, results in changes in the levels of cortisol, glucose, lactate and other biochemical and ionoregulatory parameters (Toni et al., 2013). Studies that take these factors into account have been carried out by many researchers to determine whether or not a given anesthetic causes stress in fish (Cunha et al., 2010; da Cunha et al., 2010; Barbas et al., 2016; Barata et al., 2016; Berlinsky et al., 2016; Corso et al., 2019). However, to the best of our knowledge, no research has ever evaluated the implications of 2-Phe and its ideal concentration (IC) in *Rhamdia quelen*. Therefore, the present work aimed to verify the IC of 2-Phe and its effects on stress parameters in *R. quelen* in comparison with Eugenol.

Materials and methods

The study was carried out at the Aquaculture Laboratory, Federal Institute of Education, Science and Technology Catarinense - Araquari, Brazil. All procedures were approved by the Ethics Committee on the Use of Animals (protocol number 252/2018).

Rhamdia quelen juveniles were purchased from a commercial hatchery. The animals were fed *ad libitum* once a day, at noon, with commercial feed (32% crude protein; 7% crude fiber; 6.5% ether extract; 14% mineral matter; 3.5% calcium) and fasted for 24 h before the trial.

Experiment I - Verification of ideal concentration of 2-Phe: Eighty animals (126.47 ± 41.14) grams were used, and they remained in two 100 L polyethylene tanks (density 50.59 g L^{-1}) with a flow-through system and natural photoperiod. During the seven days of acclimatization, the water quality parameters

were monitored daily (Table I). The experiment was conducted in a completely randomized design, in which the fish were subjected to seven concentrations (300, 400, 500, 550, 600, 650, and 700 µL L^{-1}, 10 fish/concentration) of 2-Phe (monophenyl ether of ethylene glycol, SIGMA ALDRICH - Spain). A control group was submitted to a solution containing only the proportion of alcohol used (700 µL L^{-1}) in the highest concentration evaluated. The 2-Phe was previously diluted in 95% ethyl alcohol in the proportion of 1:10 to facilitate its solubilization (da Silva et al., 2019).

The animals were quickly captured with the help of a dip net, one at a time, and transferred to an observation glass aquarium with a capacity of 10 L containing 3 L of water and the concentration of the anesthetic to be evaluated while under constant aeration. The stages of anesthesia were observed (Table II), and the induction times were recorded until the fish reached stage II of anesthesia at which time they presented with total loss of balance and muscle tone, low frequency of opercular movements, and strongly attenuated reflexive responses, according to the methodology employed by Weber et al. (2009).

Then the animals were quickly removed, weighed, and placed in an aquarium supplied with aeration, containing only water, where recovery times were observed (Weber et al., 2009). The maximum observation time for induction and recovery was 30 min. Then, they were transferred to polyethylene tanks containing approximately 50 L of water and grouped according to the anesthetic concentration to which they were submitted. The tanks were in a closed system with recirculation and constant aeration. They remained in these conditions for 24 hours under observation.

Experiment II - Evaluation of physiological parameters: After determining the IC of 2-Phe, it was evaluated and compared to that of Eugenol, which is known to mitigate stress responses (Cunha et al., 2010). An experiment was carried out in a completely randomized design, arranged in a 2 × 2 + 1 factorial scheme, and it consisted of two anesthetics (Eugenol and 2-Phenoxyethanol) and two times after the anesthetic exposition, Time 0 and 1, plus a control treatment. Forty animals (118.93 ± 40.01 g) were used to measure plasma levels of cortisol, lactate, and glucose. The fish were divided into three groups: 10 fish for the control group (baseline values, without anesthetic or alcohol exposition), 15 fish for the 2-Phe treatment group (700 µL L^{-1}), and 15 fish for the Eugenol treatment group (50 mg L^{-1}, Biodinâmica, Chemicals and Pharmaceuticals - Brazil).

The groups were placed in 50 L polyethylene tanks with a flow-through system and natural photoperiod. During seven days of acclimatization, the water quality parameters were monitored daily (Table I). The fish were divided into five groups: 10 fish for the control group (baseline values, without anesthetic); 7 fish for 2-Phe (700 µL L^{-1}) treatment group $T_{0;}$ 8 fish for 2-Phe (700 µL L^{-1}) treatment group $T_{1;}$ 7 fish for the Eugenol (50 mg L^{-1}) treatment group $T_{0;}$ and 8 fish for eugenol (50 mg L^{-1}) treatment group $T_{1.}$ Treatment T_0 blood was collected immediately after anesthesia, Treatment T_1 blood was collected one hour after anesthesia.

After anesthesia and weighing, blood extraction was started, using 1 mL syringes (23 G needles), rinsed internally with Ethylenediaminetetraacetic acid (EDTA).

Table I. Water quality parameters (mean ± standard deviation) monitored during the experiments.

	Dissolved oxygen (mg L^{-1})	**pH**	**Temperature (ºC)**	**Total ammonia (mg L^{-1})**	**Nitrite (mg L^{-1})**
Experiment I: Ideal concentration	7.10 ± 0.40	7.50 ± 0.50	24.50 ± 0.50	0.25 ± 0.23	0.07 ± 0.11
Experiment II: Stress avaliation	6.80 ± 0.70	7.10 ± 0.20	25.10 ± 0.80	0.00 ± 0.00	0.00 ± 0.00

Approximately 0.50 mL were collected by puncture of the caudal vein. From the moment of capture to the withdrawal of blood, the maximum time for this procedure was 1 min for all groups. After collection, the blood was stored in a 1.50 mL Eppendorf and placed in a box with thermal capacity containing ice, and it remained there until the end of the collection. After the collection, the blood was centrifuged at 11600 × g for 5 min at 4 ºC. The plasma obtained was transferred to another Eppendorf and stored in liquid nitrogen at –196 °C until the analyses were performed.

Commercial kits were used to measure glucose concentrations (GLUCOSE PAP - LABTEST DIAGNÓSTICO - Minas Gerais, Brazil), lactate (LACTATO - LABTEST DIAGNÓSTICO - Minas Gerais, Brazil) and cortisol (Immulite 2000 - Siemens Healthcare Diagnostics - São Paulo, Brazil). Glucose and lactate were determined by enzymatic-colorimetric tests and cortisol by chemiluminescence, according to the methodology employed by da Silva et al. (2021).

Statistical analysis:

Experiment I – Verification of ideal concentration of 2-Phe: The data were assessed for normality by the Shapiro-Wilk test and homogeneity of variance by the Bartlett test and subsequently subjected to analysis of variance. The means were compared using the Scott-Knott test, and regression models were adjusted, both with 5% significance using R software, version 3.2.3 (R Core Team, 2019). The adjustments of the models were based on the significance of the parameters and the coefficient of determination.

Experiment II - Evaluation of physiological parameters: The data were assessed for normality by the Shapiro-Wilk test and homogeneity of variance by the Bartlett test and subsequently subjected to analysis of variance. The means were compared using the Tukey test, and the control treatment was compared using Dunnett's test, both with a 5% significance level, using R version software, v. 3.2.3 (R Core Team, 2019).

Results

Experiment I – Verification of ideal concentration: When submitted to the highest ethyl alcohol (700 µL L^{-1}) concentration used, anesthetic induction did not occur. Mortality rates were not observed throughout the experiment.

The concentrations of 300, 400, 500, 550, 600, 650, and 700 µL L^{-1} of 2-Phe induced *R. quelen* to stage II of anesthesia (Fig. 1A). Among these, the concentrations 500, 550, 600, 650, and 700 µL L^{-1} of 2-Phe did not differ statistically ($P > 0.05$). The concentrations of 300 and 400 µL L^{-1} were statistically different ($P < 0.05$), between themselves and from the other concentrations. A strong correlation ($R^2 = 0.82$) developed between the increase in concentration and the reduction in induction time (Fig. 1C).

Regarding the recovery times, the concentrations of 550, 600, 650 and 700 µL L^{-1} were similar ($P > 0.05$), while they differed statistically ($P < 0.05$) from the concentrations of 300, 400 and 500 µL L^{-1}, which did not present statistical differences ($P > 0.05$) among themselves (Fig. 1B). A strong correlation ($R^2 = 0.97$) also developed between the concentration used and the time required for complete recovery (Fig. 1D).

Experiment II - Evaluation of physiological parameters: For the physiological parameters evaluated within the same anesthetic between treatments, T_0 and T_1 of the Eugenol group showed no statistical differences ($P > 0.05$) in glucose values, lactate, and cortisol. A similar result was observed for 2-Phe with values of glucose, lactate, and cortisol (Fig. 2).

When comparing Eugenol and 2-Phe at the same time (T_0 Eugenol with T_0 2-Phe or T_1 Eugenol with T_1 2-Phe), no statistical differences ($P > 0.05$) were observed among the means in any of the stress indicators evaluated. Plasma cortisol levels did not differ statistically ($P > 0.05$) among the control, T_0 Eugenol, T_1 Eugenol, T_0 2-Phe, and T_1 2-Phe (Fig. 2). A significant increase ($P < 0.05$) in plasma glucose levels was observed in treatments when compared with the control group (Fig. 2). Plasma lactate levels did not increase significantly ($P > 0.05$) when treatments at T_0 were compared to the Control group (Fig. 2). However, the increase in levels was significant ($P < 0.05$) when comparing the T_1 treatments with the Control group (Fig. 2).

Discussion

Experiment I – Verification of ideal concentration: The absence of anesthetic properties in alcohol is commonly reported (da Silva et al., 2019; Weber et al. 2009). In addition, the excellent ability to improve the solubilization of molecules (Benovit et al., 2012) makes alcohol a diluent suitable for oily substances.

Similar results confirming the strong correlation between concentration and induction time were found in *Solea senegalensis* (Weber et al., 2009), *Argyrosomus regius* (Barata et al., 2016),

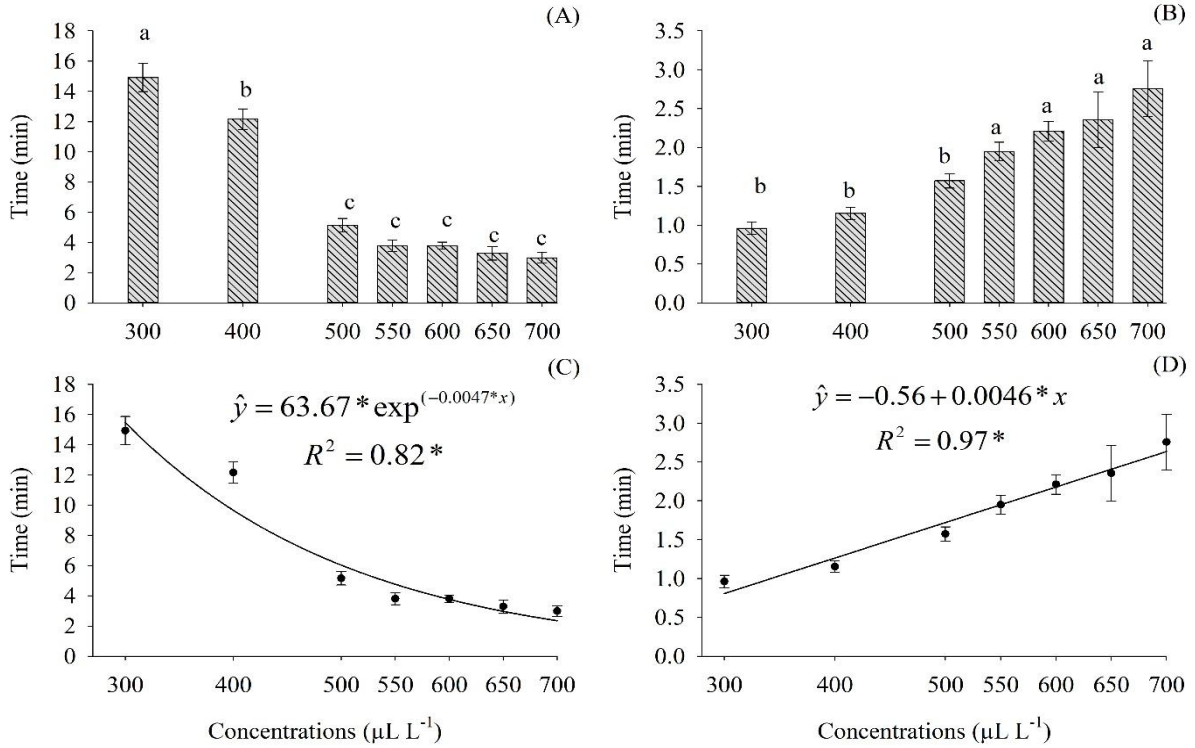

Figure 1. *Rhamdia quelen* induction (A) and anesthetic recovery times (B) submitted to different concentrations of 2-Phenoxyethanol. Regression curves adjusted for the induction time (C) and recovery time (D). Vertical bars indicate the standard error of the mean, and different letters represent a statistically significant difference ($P < 0.05$).

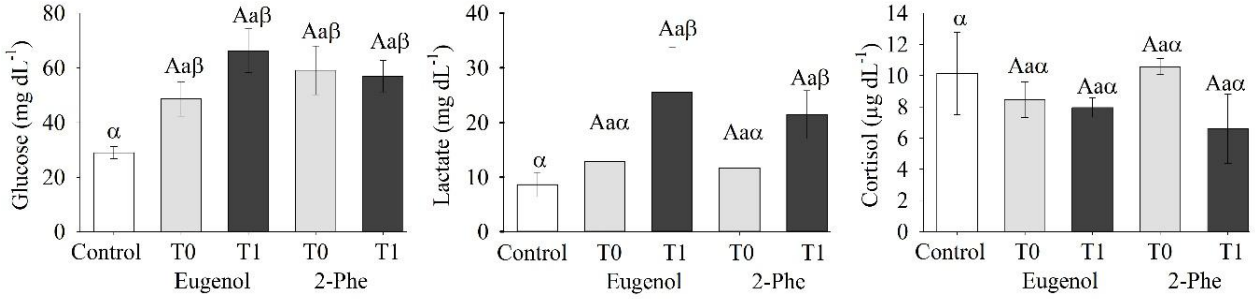

Figure 2. Average values of glucose and lactate in mg dL^{-1} and cortisol in µg dL^{-1} after being submitted to different anesthetics (Eugenol 50 mg L^{-1}, 2-Phe 700 µL L^{-1}) and treatments T0 (zero time, anesthetized) and T_1 (one hour after anesthesia), plus the control treatment (baselines values, without anesthetic). Vertical bars indicate the standard error of the mean and means followed by the same letter do not differ ($P > 0.05$). Uppercase letters compare the treatment (T_0 and T_1) within the same anesthetic. Lowercase letters compare the T_0 or T_1 treatment, depending on the different anesthetics. Greek letters compare the factorial with the control by Dunnett's test at the 5% level.

Alosa pseudoharengus (Berlinsky et al., 2016), *Cyprinus carpio* (Czerniak et al., 2018), and *Astyanax bimaculatus* (da Silva et al., 2019). This situation can be attributed to higher concentrations that induced anesthesia more quickly, which allowed fish to be removed from the anesthetic tank and placed in clear water earlier than fish exposed to lower concentrations (Mylonas et al., 2005).

The animals submitted to a concentration of 700 µL L^{-1} (2-Phe) were induced to the second stage of anesthesia within 2.98 min, recalling that an ideal anesthetic should induce fish to that stage within 3 minutes (Marking & Meyer, 1985). This was the lowest effective concentration observed for *R. quelen*. This concentration was similar to that found by Weber et al. (2009) to anesthetize *S. senegalensis* (74 ± 4 and 213 ± 15 g, 600 µL L^{-1}) and by Czerniak

Table II. Stages of anesthesia and recovery from anesthesia employed as endpoints in the present study. Adapted from Weber et al. (2009).

Stage		Description
Anesthesia		
	I	Partial loss of equilibrium Fish retain some body movements
	II	Total loss of equilibrium and muscular tone Low frequency of opercular movements Strongly attenuated reflexive responses
	III	Imperceptible opercular movements Total loss of spinal reflexes
Recovery		
	I	Lack of equilibrium Fish do not shows any body movements
	II	Fish begin to recover opercular movements Recovery of equilibrium and some body movement Recovery of the frequency of opercular movements
	III	Opercular frequency slightly higher than in pre-anaesthesia Similar to pre-anaesthesia Normal swimming

et al. (2018) in *C. carpio* (107 ± 20.90 g, 600 µL L^{-1}).

Similar results confirming the strong correlation between concentration and recovery time were found in *D. sargus* L. and *D. puntazzo* C. (Tsantilas et al., 2006), *A. regius* (Barata et al., 2016), *C. carpio* (Czerniak et al., 2018) and *A. bimaculatus* (da Silva et al., 2019). According to Benovit et al. (2012), the accumulation of lipophilic substances in adipose tissue can lead to increases in anesthetic recovery times.

Even with different recovery times, none of the concentrations used took more than 5 min for full recovery of the fish, which, according to Marking & Meyer (1985), is suitable for an ideal anesthetic. When testing the concentrations of 400 to 800 µL L^{-1} in *C. carpio* and 200 to 600 µL L^{-1} in *A. bimaculatus*, Czerniak et al. (2018) and da Silva et al. (2019), respectively, also observed a recovery time of less than 5 min at all concentrations used.

Experiment II - Evaluation of physiological parameters: A rapid anesthetic induction causes only a minor stress response. Therefore, it can reduce disturbances in the plasma levels of stress indicators (Hoseini & Nodeh, 2013; Mirghaed et al., 2018), which may justify the absence of some increases.

When testing *R. quelen* with 100 and 300 µL L^{-1} of linalool and citral extracted from *Lippia alba*, de Freitas Souza et al. (2018) observed that the two concentrations of both compounds maintained plasma cortisol levels at times 0, 10 min, 4 h, and 8 h after anesthesia, equal to the baseline group, while the groups containing only alcohol and control remained the same at baseline at time 0 and 10 min, increasing significantly after 4h and reducing baseline levels only after 8h. When submitting *R. quelen* to a stressful situation, Barcellos et al. (2001) observed that serum cortisol increased from time 0, remaining higher than the baseline group during the 24 h of the experiment.

When evaluating the possible stressful effects caused by exposure to 500 µL L^{-1} of 2-Phe and 80 µL L^{-1} of clove oil (composed mainly of Eugenol) in *S. senegalensis*, Weber et al. (2011) observed that clove oil increased the plasma cortisol levels after 20 and 30 min of exposure, while in the group anesthetized with 2-Phe, plasma cortisol levels increased at 20 min, but decreased at 30 min of exposure. However, in our experiment, where the animals remained exposed to Eugenol and 2-Phe for less than 3 min, the cortisol values remained the same ($P > 0.05$) as those of the control group.

2-Phe is believed to inhibit the activity of N-methyl-D-aspartate (NMDA)-type glutamate receptors, reducing pain (Musshoff et al., 1999). Activation of NMDA receptors increases the excitability of neurons, resulting in the opening of a nonselective ion channel for cations and allowing the flow of Na^+ and Ca^{2+} into and K^+ out of the cell (Priborsky & Velisek, 2018). The inhibition of the NMDA receptor is responsible for the analgesic effect (Grasshoff et al., 2006).

Cunha et al. (2010) considered the 50 mg L^{-1} Eugenol concentration to be suitable for anesthesia in *R. quelen* (194.89 ± 12.50 g) after observing a reduction in serum cortisol levels when comparing the control group with the groups collected 0 h, 1 h, and 4 h after anesthesia. Cunha et al. (2010) also

observed no increase in cortisol levels when comparing time 0 h with 4 h after anesthesia, while in the control group, the animals collected after 4 h showed an increase in cortisol when compared to time 0 h. The results found by Corso et al. (2019) corroborate this statement since they also found that the concentration of 50 µL L^{-1} of Eugenol reduced the plasma level of cortisol in *R. quelen* (500 ± 10 g) when compared to the control group. For Iversen et al. (2003), the maintenance of plasma cortisol levels in fish anesthetized with Eugenol results from the effect of blocking the transmission of sensory information to the hypothalamus.

Barcellos et al. (2001) observed that the plasma glucose of *R. quelen* has its maximum peak 1 h after the stressful event. This increase is induced by catecholamines and corticosteroids, and an increase is usually observed after anesthesia and some stressful event (Kristan et al., 2012). In the experiment by Weber et al. (2011), after 30 min of exposure to 2-Phe, glucose at the baseline level could not be maintained. Similarly, clove oil increased plasma glucose after 20 and 30 min of exposure in *S. senegalensis*. Meanwhile, de Freitas Souza et al. (2018) observed an increase in glucose levels 10 min after the exposure of *R. quelen* to linalool and citral, and the levels returned to values similar to the baseline level only after 8 h of anesthesia with linalool.

De Freitas Souza et al. (2018) observed an increase in lactate levels in *R. quelen* anesthetized with linalool and citral 10 min after exposure, only returning to baseline levels after 4 h. The increase in plasma lactate levels is the result of anaerobic metabolism and is usually seen after anesthesia (Trushenski et al., 2012). Accumulation is believed to be associated with the effects of hypoxia caused by bradypnea during anesthesia or by arousal just before anesthesia (Matsche, 2017).

Conclusion

2-Phe can be used for anesthesia in *R. quelen* in concentrations from 700 µL L^{-1} when a fast anesthetic induction (up to 3 min) is required. It induced the animals to hyperglycemia at all time periods evaluated and increased plasma lactate one hour after exposure (T_1). Nevertheless, it managed to cancel the primary stress response, preventing an increase in cortisol levels. Therefore, 2-Phe can be considered a safe anesthetic for use in *R. quelen*.

Ethics statement

All procedures were approved by the Ethics Committee on the Use of Animals (protocol number 252/2018).

Authors' contributions

Eduardo da Silva - Execution of research and writing of the manuscript; Gabriel Tobias Deschamps - Execution of research; Fabiano de Lima Matter - Blood analysis; Manuel Aldegunde - Revised the manuscript and provided guidance on the technical part of aquaculture; Deivisson Ferreira da Silva - Statistical analysis; Carlize Lopes - Revised the manuscript and provided guidance on the technical part of anesthesiology; Adolfo Jatobá - Revised the manuscript, provided guidance on the technical part of aquaculture and collaborated in sampling blood; Robilson Antônio Weber - Guided the entire execution and writing of the research project and article.

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Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest.

Availability of data and material

The data and materials that support the conclusions of this study are available with the author for correspondence, upon reasonable request.

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