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BIOLOGY

Hesperozygis ringens essential oil as an anesthetic for *Colossoma macropomum* during biometric handling

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ABSTRACT: This study evaluated the effectiveness of the essential oil of *Hesperozygis ringens* (EOHR) for anesthesia of *Colossoma macropomum* by documenting hematological and blood biochemical responses after biometric handling. In Experiment 1, juveniles (14.12 ± 3.53 g) were exposed to different concentrations of EOHR: 0 (control), 75, 150, 300 and 450 µL L^{-1} (n=10 fish for each concentration), to determine times for induction and recovery from anesthesia, as well as its effects on ventilatory frequency (VF). Based on these results, Experiment 2 evaluated the effects of 0 (control), 75 (with induction and recovery times outside that recommended for fish anesthesia) and 150 µL L^{-1} EOHR (within recommended times) on hematological and biochemical variables of juveniles (20.52 ± 3.47 g) after anesthesia and after 24 h of recovery (n = 6 fish for each concentration and collection time). Survival was 100%. Induction time showed a quadratic effect of EOHR concentration. Recovery time did not differ among EOHR concentrations. Concentrations between 150 and 450 µL L^{-1} EOHR caused rapid induction (< 3 min) and recovery (< 5 min). EOHR concentration affected VF. The concentration of 150 µL L^{-1} EOHR had little influence on hematological and biochemical parameters of *C. macropomum* of 20 g.

Key words: deep anesthesia, fish handling, plant essential oil, tambaqui, triglycerides.

Óleo essencial de *Hesperozygis ringens* como anestésico para *Colossoma macropomum* durante manipulação biométrica

RESUMO: Este estudo avaliou a eficiência do óleo essencial de *Hesperozygis ringens* (EOHR) para anestesia de *Colossoma macropomum*, documentando as respostas hematológicas e bioquímicas do sangue após o manuseio biométrico. No experimento 1, juvenis (14,12 ± 3,53 g) foram expostos a diferentes concentrações de EOHR: 0 (controle), 75, 150, 300 e 450 µL L^{-1} (n = 10 peixes para cada concentração), para determinar os tempos de indução e recuperação da anestesia, bem como seus efeitos na frequência ventilatória (VF). Com base nesses resultados, o experimento 2 avaliou os efeitos de 0 (controle), 75 (com tempos de indução e recuperação fora do recomendado para anestesia de peixes) e 150 µL L^{-1} EOHR (dentro dos tempos recomendados) sobre variáveis hematológicas e bioquímicas de juvenis (20,52 ± 3,47 g) após a anestesia e após 24h de recuperação (n = 6 peixes para cada concentração e tempo de coleta). A sobrevivência foi de 100%. O tempo de indução mostrou efeito quadrático da concentração de EOHR. O tempo de recuperação não diferiu entre as concentrações de EOHR. Concentrações entre 150 e 450 µL L^{-1} EOHR causaram rápida indução (< 3 min) e recuperação (< 5 min). As concentrações de EOHR afetaram a VF. A concentração de 150 µL L^{-1} de EOHR teve pouca influência nos parâmetros hematológicos e bioquímicos de *C. macropomum* de 20 g.

Palavras-chave: anestesia profunda, manejo de peixe, óleo essencial de planta, tambaqui, triglicerídeos.

INTRODUCTION

Tambaqui, *Colossoma macropomum* is a fish species of the Amazon and Orinoco Rivers basins (REIS, 2003; BRIAN et al., 2004) that is important for aquaculture in northern South America (SEVILLA & GÜNTHER, 2000; VALLADÃO et al., 2018). In Brazil, the production of *C. macropomum* stands out in relation to that of other native freshwater fish species (PEIXE BR, 2022). The successful production of this species is due to its rapid growth, omnivorous feeding behavior, high commercial value and good acceptance by consumers (MORAIS & O'SULLIVAN, 2017; ARAÚJO-DAIRIKI et al., 2018; WOYNÁROVICH & VAN ANROOY, 2019). Furthermore, *C. macropomum* demonstrates resistance to hypoxic conditions

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(NEVES et al., 2020; NEVES et al., 2022), thus being considered a rustic fish. Although, *C. macropomum* is highly resistant to rearing conditions, over-handling can be harmful (as for example, biometric and transport) (MORAIS & O'SULLIVAN, 2017).

Different techniques are being used to mitigate the effects of stress caused by routine practices in fish farms, including the use of anesthetics (SINK & NEAL, 2009; SOUZA et al., 2019; FERREIRA et al., 2021a; ANANIAS et al., 2022). Anesthetic compounds have been a tool used to promote complete immobilization of fish and/or prevent the physiological effects of stress on animals, providing greater safety for both the animal and the handler (VELISEK & SVOBODOVA, 2004; ROSS & ROSS, 2008).

Eugenol is the most widely used natural anesthetic in aquaculture (AYDIN & BARBAS, 2020); however, high concentrations of eugenol (> 250 mg L^{-1}) can cause partial lamellar fusion and necrosis in the gills of fish (ABDEL-FATTAH et al., 2005). Thus, alternative studies based on concentration-response assays have evaluated the sedative and/or anesthetic properties of several essential oils (EOs) from plants for use in biometric handling of several fish species. For example, EO of *Ocimum gratissimum* L. for *Lophiosilurus alexandri* (BOAVENTURA et al., 2020) and *Oreochromis niloticus* (FERREIRA et al., 2021b), EO of *Ocimum basilicum* for *C. macropomum* (VENTURA et al., 2021), EOs of *Ocimum americanum* and *Lippia alba* for *O. niloticus* (RUCINQUE et al., 2021), and EO *Lippia sidoides* for *C. macropomum* (BRANDÃO et al., 2021). These anesthetic efficacy studies in fish are based on a fast induction time of anesthesia (< 3 min) and a short recovery time (< 5 min) as described by KEENE et al. (1998) and ROSS & ROSS (2008); and their ideal concentrations depend on the fish species and size (ROSS & ROSS, 2008; READMAN et al., 2017; FERREIRA et al., 2020).

In this sense, the EO of *Hesperozygis ringes* (EOHR), a plant of the family Lamiaceae and native to southern Brazil (DAWOOD et al., 2021), has presented sedative and anesthetic properties for *Rhamdia quelen* (SILVA et al., 2013; TONI et al., 2014; TONI et al., 2015). Thus, considering the discussed facts, and the lack of information on the use of EOHR with Amazonian round fish, the present study aimed to evaluate different concentrations of EOHR for anesthesia of juveniles of *C. macropomum* and its effects on induction and recovery times, ventilatory frequency, and hematology and blood biochemistry after biometric handling.

MATERIALS AND METHODS

Fish acclimation

Juveniles of *C. macropomum* used in Experiment 1 were acclimatized in a recirculating aquaculture system (RAS) with five 42-L (useful volume) rectangular tanks for two weeks, at a density of 10 fish per tank. For Experiment 2, the animals were acclimated and distributed in six tanks at a density of six fish per tank. The water of the RAS was maintained at a temperature of 28.40 ± 0.75 ºC, with pH of 6.68 ± 0.13 (multiparameter probe Hanna HI98130), dissolved oxygen levels of 4.61 ± 0.36 mg L^{-1} (determined by the oximeter EcoSense® DO200A) and total ammonia of 0.14 ± 0.05 mg L^{-1} (measured with the colorimetric AlfakitLabcon kit). Two water changes were performed during the week, with replacement of 40% of the useful volume of the RAS. The fish were fed an extruded commercial feed (2–3 mm in diameter), containing 360 g kg^{-1} crude protein, 65 g kg^{-1} ether extract, 30 g kg^{-1} calcium and 6 g kg^{-1} phosphorus as described by the manufacturer, and offered up to satiety twice a day (8:00 and 15:00 h). All fish were fasted for 24 h prior to their respective experiment.

Essential oils are hydrophobic and need a dilution vehicle to mix with water. Therefore, in the present study, 5 mL of ethanol was added to all studied concentrations, including the control group (0 μL L^{-1}) (RIBEIRO et al., 2015).

Experiment 1 – Anesthetic effect of EOHR for C. macropomum

To induce anesthesia, fish were exposed to different concentrations of EOHR, based on TONI et al. (2014), as follow: 0, 75, 150, 300 and 450 μL L^{-1}. Fifty juveniles of *C. macropomum* (9.17 ± 0.84 cm and 14.12 ± 3.53 g) were distributed in a completely randomized design. Ten animals from the same tank were used for each concentration, with each fish being considered a replicate. Control fish (0 μL L^{-1}) were observed for 10 min to simulate anesthesia induction and another 5 min to simulate recovery.

Fish were individually placed in 1-L beaker with water from the cultivation system itself and constant aeration for the evaluation of anesthesia induction and recovery times. Anesthesia induction time (seconds) was recorded using a digital timer (Taksun Ts1809), which was started at the moment fish first made contact with the anesthetic solution and stopped by the absence of swimming and loss of balance and consciousness (deep anesthesia) (SMALL, 2003; ROSS & ROSS, 2008). In addition, opercular beats per minute (ventilatory frequency, VF) were counted during induction (from the first contact of fish with the anesthetic solution until the deep anesthesia) through visualization and the use of a manual counter, following Alvarenga & Volpato (1995) with modifications. After deep anesthesia, weight and total length biometrics were performed, a procedure that lasted about 40 s. Fish total length was measured with a ruler and weight using a digital scale (Marte AD5002). The animals were then placed in 1-L beaker with clean water (without anesthetic) to assess recovery time and VF. The fish were considered recovered when they showed movements and normal swimming equilibrium (SMALL, 2003; ROSS & ROSS, 2008).

At the end of the procedures, the fish of each EOHR concentration evaluated were grouped and replaced in their respective original tanks in RAS (fish acclimation); to assess survival and return to food search for up to 48 h post-anesthesia and handling procedure. During this period the fish were fed twice a day until apparent satiety and evaluated possible mortality.

Experiment 2. Hematological and biochemical responses of C. macropomum anesthetized with different EOHR concentrations

Based on the results of Experiment 1, a new assay with concentrations of 0, 75 and 150 µL L^{-1} of EOHR was performed. These concentrations were chosen because one had times outside (75 µL L^{-1}) and the other within (150 µL L^{-1}) those recommended for rapid fish anesthetic induction (< 180 s) and recovery (< 300 s) (KEENE et al., 1998; ROSS & ROSS, 2008), in addition to a control group. Thirty-six juveniles of *C. macropomum* (10.79 ± 0.66 cm and 20.52 ± 3.47 g) were distributed in a completely randomized design in a factorial scheme (3 x 2), being three EOHR concentrations and two blood collection periods (1 h post-anesthesia and 24 h post-recovery), with six fish for each concentration and collection time. Each animal was used only once and was considered a replicate. The same methodologies described in Experiment 1 were performed.

Blood collection was performed by tail puncture using heparinized syringes and an additional 10% sodium heparin was added to the total volume of blood collected. Individual blood samples were used to measure hemoglobin values using a commercial colorimetric kit (Bioclin®) followed by reading in a UV/VIS spectrophotometer (Biochrom Libra S21-S22). Blood was then centrifuged at 1792 G-force for 10 min. Aliquots of separated plasma were used to determine glucose, triglycerides and cholesterol values through respective commercial kits (Bioclin®). Protein samples were measured using a Goldberg manual refractometer.

Statistical analysis

Homoscedasticity of variances and normality of the data were tested by Levene's test and the Shapiro-Wilk test, respectively. Regression analysis was performed for anesthesia induction and recovery times ($P < 0.05$). Two-way ANOVA was performed for blood variables, followed by Tukey's post-hoc test ($P < 0.05$). Nonparametric results (VF) were analyzed using the Kruskal-Wallis test ($P < 0.05$). Data were presented as mean ± standard deviation. Data analysis was performed using R and Infostat software.

RESULTS

Experiment 1

Survival was 100% and all fish resumed feeding within 30 h after anesthesia and handling. Anesthesia induction time showed a quadratic effect of EOHR concentration ($P < 0.05$) with a minimum value at 353.33 µL L^{-1} (Figure 1A). Anesthesia recovery time was not influenced by EOHR concentration ($P > 0.05$) (Figure 1B).

During anesthesia induction, the lowest VFs were observed for animals exposed at low concentrations of EOHR (75 and 150 µL L^{-1}) ($P < 0.05$) (Figure 2A), and the VF for fish anesthetized with 150 µL L^{-1} EOHR was similar to from the control group (0 µL L^{-1}, non-anesthetized animals). The highest VFs were recorded for fish anesthetized with 300 and 450 µL L^{-1} EOHR. During recovery from anesthesia, VFs at 75 and 150 µL L^{-1} were lower than those for fish of the control group ($P < 0.05$) (Figure 2B).

Experiment 2

There was no interaction between EOHR concentration and blood collection period for hemoglobin, plasma protein, glucose, triglycerides and cholesterol ($P > 0.05$) (Figure 3). Hemoglobin (6.92 ± 1.12 g dL^{-1}) was not affected by EOHR concentration ($f = 0.21$; $P = 0.8103$) nor by blood collection period ($f = 0.40$; $P = 0.5312$) (Figure 3A). The highest plasma glucose values (103.86 ± 12.18 mg dL^{-1}) were observed at 1 h post-anesthesia and biometric handling ($f = 52.60$; $P < 0.0001$) (Figure 3B). Similar behavior was observed for the effect of blood collection time, where plasma protein (4.91 ± 0.23 g dL^{-1}) was increased 1 h post-anesthesia ($f = 14.92$; $P = 0.0006$) (Figure 3C). However, EOHR concentration had no effect for plasma glucose and

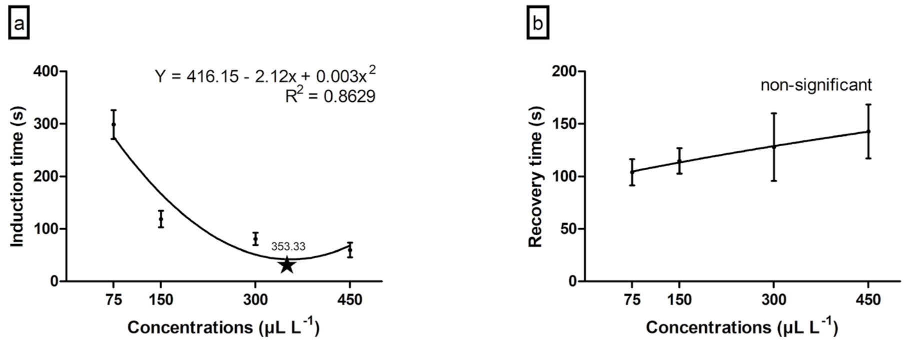

Figure 1 - Anesthesia induction (a) and recovery (b) times (mean ± standard deviation) for *Colossoma macropomum* exposed to different concentrations of the essential oil of *Hesperozygis ringens* (star represents the minimum value of the equation).

protein (P> 0.05). Triglycerides, conversely, were affected by both EOHR concentration and blood collection time. Fish anesthetized with 75 and 150 µL L^{-1} EOHR had lower plasma triglycerides values (123.65 ± 28.27 and 138.96 ± 36.43 mg dL^{-1}, respectively) than the control group (182.68 ± 59.39 mg dL^{-1}) (f = 11.02; P = 0.0003) (Figure 3D). Regarding blood collection period, the highest values of triglycerides were observed 1 h after anesthesia and handling (f = 27.05; P< 0.0001). Plasma cholesterol (132.05 ± 20.20 mg dL^{-1}) was also not affected by ETOH concentration (f = 0.96; P = 0.3942) nor blood collection period (f = 2.61; P = 0.1165) (Figure 3E).

DISCUSSION

EOHR was able to cause anesthesia in juveniles of *C. macropomum*. Furthermore, it caused only small changes in hematological and biochemical parameters after handling biometrics. SOUZA et al. (2019) reported that the composition of plant EOs and; consequently, their anesthetic effects may vary according to plant part used for oil extraction, collection site, plant variety and climate. In this study, the essential oil was extracted from fresh leaves of *H. ringens,* through the hydrodeslitation process (duration of 3 h). The literature cites that the main components of EOHR responsible for causing anesthesia in fish are pulegone (95.18%) and limonene (1.28%) (TONI et al., 2014). However, in general, plant EOs have shown benefits in mitigating stress effects caused by fish biometric handling (HOSEINI et al., 2019; SOUZA et al., 2019). Although, EOs required dilution in ethanol, this compound at low concentrations does not cause mortality or anesthetic induction in fish (RIBEIRO et al., 2015; BOAVENTURA et al., 2020; ANANIAS

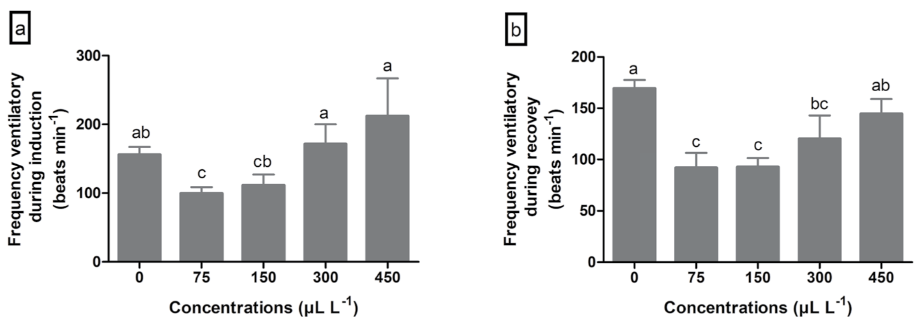

Figure 2 - Ventilatory frequency during anesthesia induction (a) and recovery (b) (mean ± standard deviation) of *Colossoma macropomum* exposed to different concentrations of essential oil of *Hesperozygis ringens* (Experiment 1). Means followed by different letters differ significantly according to the Kruskal-Wallis test (P < 0.05).

et al., 2022), as observed for the control animals of the present study. In Experiment 1, the survival of juveniles of *C. macropomum* was 100% for all evaluated EOHR concentrations and all fish resumed their search for food within 30 h post-anesthesia associated with biometric handling. TONI et al. (2014) also reported no mortality for *R. quelen* 48 h poist-anesthesia with EOHR. Furthermore, EOHR was not to be a stressor for *R. quelen* (SILVA et al., 2013). Given the above, EOHR can be considered safe and can be tested for different species.

It is recommended that an anesthetic for fish cause rapid anesthesia induction (within 3 min) and recovery (within 5 min) (KEENE et al., 1998; ROSS & ROSS, 2008). According to such recommendations, the present study indicated the use of EOHR at concentrations between 150 and 450 µL L^{-1} for anesthesia of juveniles of *C. macropomum* of 14 g. For *R. quelen*, concentrations of 300 and 450 µL L^{-1} EOHR were effective for complete loss of consciousness (deep anesthesia). This variation in the appropriate concentration of a particular anesthetic may be related to fish species and size (ROSS & ROSS, 2008; RIBEIRO et al., 2015; TARKHANI et al., 2017). Thus, there is a need for prior assessments of EOHR for each species and size.

The measurement of ventilatory frequency (VF) is a non-invasive method that indicates possible physiological changes in the respiratory system of fish caused by acute stressors, such as the manipulation and use of anesthetics (ALVARENGA & VOLPATO, 1995; BARRETO & VOLPATO, 2004; TONI et al., 2014; SILVA et al., 2019; ANANIAS et al., 2022). In the present study, VF during induction and recovery from anesthesia were reduced for fish anesthetized with 150 µL L^{-1} EOHR, which corresponds to the lowest concentration to be recommended for juveniles of *C. macropomum*. In this way, it was possible to achieve complete immobilization of animals for biometric analysis. ANANIAS et al. (2022) described a similar behavior when anesthetizing *L. alexandri* with 50 mg L^{-1} menthol.

In Experiment 2, the use of EOHR did not change hemoglobin values. This finding was also observed for juveniles of *C. macropomum* anesthetized with EO of *O. gratissimum* (BOIJINK et al., 2016). Hyperglycemia was observed 1 h post-anesthesia with EOHR and biometric handling. Fish in stressful situations release circulating catecholamines that activate the interrenal pituitary hypothalamus axis, affecting the synthesis of the hormone cortisol (BARTON, 2002). As a result of this increase in cortisol,

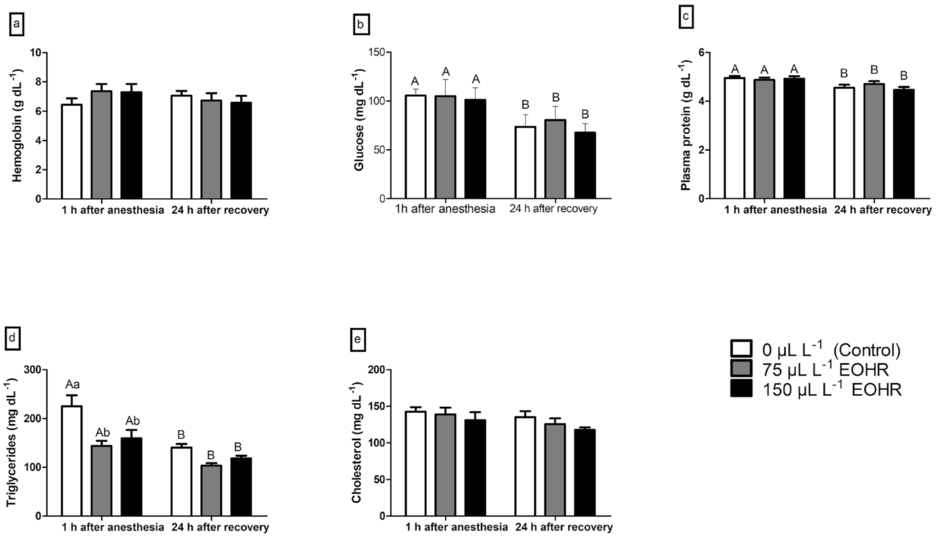

Figure 3 - Hematological and biochemical parameters for *Colossoma macropomum* measured 1 h post-anesthesia with essential oil of *Hesperozygis ringens* (EOHR) and 24 h post-recovery. Control group: 5 mL ethanol. a) Hemoglobin, b) Glucose, c) Plasma protein, d) Triglycerides and e) Cholesterol. Values (mean ± standard deviation) (N = 6 animals per treatment). The data were analyzed by two-way ANOVA, followed by Tukey's post-hoc test ($P < 0.05$). Different lowercase letters indicate a significant difference between concentrations at the same time (1 h post-anesthesia or 24 h post-recovery). Different capital letters indicate a significant difference between times for a given concentration.

the animal organism can trigger gluconeogenesis and glycolysis (increase in glucose), processes for the body to escape or overcome the new conditions imposed by the environment (PANKHURST, 2011; JEREZ-CEPA & RUIZ-JARABO, 2021). According to TONI et al. (2014) the concentration of 300 µL L^{-1} EOHR was not able to mitigate the effects of stress caused by biometric handling in *R. quelen*, which confirmedthe results of the present study. However, plasma glucose values returned to their normal values 24 h post-recovery. This glucose behavior was also recorded for different species anesthetized with EO from plants (TONI et al., 2014; TEIXEIRA et al., 2017; SANTOS et al., 2020; FERREIRA et al., 2021a), indicating the rapid recovery of fish when anesthetized. Thus, this finding can be understood as the result of triggering glycogenolysis and increasing glucose during the anesthesia and manipulation; or it can also be explained as the result of the fasting time to which the fish were submitted, which may have decrease glycogen stock and caused glycemia, as described by RIBEIRO et al. (2019) and FERREIRA et al. (2021a).

The highest values of plasma protein (globulin and albumin fractions) were observed 1 h post-anesthesia and handling. This finding can also be explained by the increase in cortisol caused by a stressor, which can consequently affect albumin synthesis (CUNHA et al., 2010), and also by the catabolic activity of proteins (MOMMSEN et al., 1999) in this case of stress. However, fish in situations of hypoxia (similar to deep anesthesia) can use proteins as an energy source (VIJAYAN et al., 1991; RIBEIRO et al., 2019; NEVES et al., 2020; PORTO et al., 2021).Conversely, the use of EOHR for *R. quelen* did not change plasma protein values after anesthesia (TONI et al., 2014).

Low triglyceride levels were observed 24 h post-recovery and during biometry in fish anesthetized with EOHR. This demonstrates possible lipid modulation between the liver and the production of phospholipids and cholesterol (JUN et al., 2015), suggesting that the catabolism of this metabolite (triglycerides) may have been preserved (VELISEK et al., 2005). This finding helps explain the non-effect of anesthesia and handling on cholesterol values for any of the factors (EOHR concentration and collection time) evaluated in this study. Thus, it can be inferred that the concentration of 150 µL L^{-1} EOHR was able to prevent the use of lipids as an energy source after biometric handling of *C. macropomum* of 20 g.

CONCLUSION

Concentrations between 150 and 450 µL L^{-1} EOHR are recommended for anesthesia of juveniles of *C. macropomum* of 14 g, as they demonstrate induction times of less than three minutes and recovery times of less than five minutes. However, the concentration of 150 µL L^{-1} EOHR (most appropriate) was able to reduce VF during anesthesia with minimal influence on hematological and biochemical parameters after biometric handling of *C. macropomum* of 20 g.

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BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

All protocols were approved by the Ethics Committee on the Use of Animals (CEUA - nº 64/2021) of the Universidade Federal de Minas Gerais (UFMG). Thus, the authors assume full responsibility for the presented data and are available for possible questions, should they be required by the competent authorities.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. Allauthors critically revised the manuscript and approved of the final version.

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