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Linalool chemotype essential oil from *Lippia alba* in the anesthesia of fat snook (*Centropomus parallelus*): ventilatory rate, biochemical, antioxidant, and oxidative status parameters

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This study evaluated the anesthetic activity of essential oil from *Lippia alba* (EOLA), linalool chemotype in a euryhaline fish (fat snook *Centropomus parallelus*). In the first experiment, fish were exposed to 30, 80, 130, 180, 200, and 230 μL EOLA L^{-1}. The second experiment evaluated smaller and larger fish with 180 μL EOLA L^{-1}. In the third experiment, ventilatory rates (VR) for up to 120 min in animals during exposure to 5 and 10 μL EOLA L^{-1} were evaluated. In the fourth experiment, fish anesthetized with 30 and 180 μL EOLA L^{-1} were assessed at 0, 30, and 60 min after anesthesia recovery to evaluate biochemical and antioxidant parameters. The best mild and deep anesthesia times were obtained with 30 and 180 μL EOLA L^{-1}, and larger fish had the highest times. The VR increased in fish exposed to EOLA. Blood glucose and whole-body cortisol levels were higher in fish anesthetized with 180 μL EOLA L^{-1}. Fish exposed to EOLA had higher liver glutathione S-transferase and superoxide dismutase activities without affecting catalase and lipid peroxidation levels. The 180 μL EOLA L^{-1} is recommended for fat snook anesthesia because it increases VR, blood glucose, and whole-body cortisol levels and prevents oxidative stress.

Keywords: Anesthesia recovery, Cortisol, Glucose, Lipid peroxidation, Ventilatory rate.

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Este estudo avaliou a atividade anestésica do óleo essencial de *Lippia alba* (OELA), quimiotipo linalool, em um peixe eurialino (robalo-peva *Centropomus parallelus*). No primeiro experimento, peixes foram expostos a 30, 80, 130, 180, 200 e 230 µL OELA L^{-1}. O segundo experimento avaliou peixes pequenos e grandes com 180 µL OELA L^{-1}. No terceiro experimento, avaliou-se taxa ventilatória por até 120 min em animais expostos a 5 e 10 µL OELA L^{-1}. No quarto experimento, peixes anestesiados com 30 e 180 µL OELA L^{-1} foram avaliados nos tempos 0, 30 e 60 min após a recuperação anestésica para verificação de parâmetros bioquímicos e antioxidantes. Os melhores tempos de anestesia leve e profunda foram obtidos com 30 e 180 µL OELA L^{-1}. Peixes maiores apresentaram anestesia e tempos de recuperação mais elevados. A taxa ventilatória aumentou em peixes expostos para OELA comparados ao grupo controle. Os níveis de glicose sanguínea e cortisol corporal foram maiores em peixes anestesiados com 180 µL OELA L^{-1}. Robalos-peva expostos para OELA tiveram maior atividade de glutationa S-transferase e superóxido dismutase no fígado, sem afetar os níveis de catalase e peroxidação lipídica. O uso de 180 µL OELA L^{-1} é recomendado para anestesia de robalo-peva, pois aumentou taxa ventilatória e níveis de glicose sanguínea, cortisol corporal, e preveniu estresse oxidativo.

Palavras-chave: Cortisol, Glicose, Peroxidação lipídica, Recuperação anestésica, Taxa de ventilação.

INTRODUCTION

The use of anesthetics in fish has been encouraged to minimize stress-inducing factors, such as hypermobility and perception of adverse stimuli during fish management (Teixeira *et al*., 2018; Oliveira *et al*., 2019a,b). Anesthetics can cause the inhibition of the respiratory center in the medulla oblongata, resulting in depression of the central nervous system (CNS) and decreasing the ventilatory rate (VR) (Ross, Ross, 2009). However, some anesthetics, mainly those of synthetic origin, depending on concentration and time of exposure, can trigger stress (Parodi *et al*., 2014; Teixeira *et al*., 2017) and induce undesirable collateral effects on metabolism or gill damage in fish (Kiessling *et al*., 2009; Wosnick *et al*., 2018; Oliveira *et al*., 2022), arousing interest in the investigation of anesthetic compounds originating from plants.

Cortisol indicates primary stress in fish and affects secondary stress biomarkers, like blood glucose levels, which increase in response to cortisol levels (Wendelaar Bonga, 1997). Commonly, circulating cortisol levels are typically measured in fish (Sena *et al*., 2016). The whole-body cortisol is an alternative that can detect distinct cortisol levels and measure the physiological stress response (Sink *et al*., 2007) when blood volumes are insufficient to provide measurements of circulating cortisol (Baldisserotto *et al*., 2014; Parodi *et al*., 2014). A more severe consequence of increased primary and secondary stress responses is the occurrence of problems in fish development, reproduction, health, and behavior, which are part of tertiary stress responses (Lemos *et al*., 2018).

Oxidative stress parameters are also considered critical stress indicators in fish (Chowdhury, Saikia, 2020). Oxidative stress can increase the reactive oxygen species (ROS) levels, affecting the maintenance of the cellular redox balance, *i.e.*, cellular homeostasis and its regulation (Lushchak, 2011). In this sense, fish have enzymatic antioxidant defense systems (*e.g.*, glutathione-S-transferase – GST, catalase – CAT, and superoxide dismutase – SOD) that can remove excessive damaging ROS, thus reducing the damage by lipid peroxidation (LPO), playing a critical role in the self-defense system of the body (Sies, 1997; Souza *et al.*, 2018; Copatti *et al.*, 2019).

Lippia alba (Mill.) N. E. Brown (Verbenaceae) is a native bush of South America with several chemotypes, many aromatic and medicinal properties, and low toxicity (Azambuja *et al.*, 2011). The essential oil from *L. alba* (EOLA), linalool chemotype, has shown sedative and anesthetic effects in previous studies with several freshwater fish, such as silver catfish *Rhamdia quelen* (Quoy & Gaimard, 1824) (Cunha *et al.*, 2010; Heldwein *et al.*, 2014), tambacu (*Piaractus mesopotamicus* × *Colossoma macropomum*) (Sena *et al.*, 2016), Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) (Hohlenwerger *et al.*, 2016, 2017), and tambaqui *Colossoma macropomum* (Cuvier, 1816) (Batista *et al.*, 2018), and a few marine species, as sea horse *Hippocampus reidi* Ginsburg, 1933 (Cunha *et al.*, 2011), gilthead sea bream *Sparus aurata* Linnaeus, 1758 (Toni *et al.*, 2015), and meagre *Argyrosomus regius* (Asso y del Rio, 1801) (Cárdenas *et al.*, 2016). However, anesthetics can exhibit different efficiencies in fish that can move from salt to freshwater and vice versa (Sepulchro *et al.*, 2016). However, its effects on these euryhaline fish are still little known.

Fat snook (*Centropomus parallelus* Poey, 1860) is a euryhaline fish species from the family Centropomidae that inhabits a wide range of salinities in marine and estuarine waters of the western coast of the Atlantic Ocean, from southern USA (State of Florida) to southern Brazil (State of Santa Catarina) (Tsuzuki *et al.*, 2007). It is an opportunistic carnivore, appreciated by consumers for its flesh quality and low-fat content. It presents a high-value market (Cerqueira, Tsuzuki, 2009). In addition, since fat snook is euryhaline, it can be inhabited inland, away from the coast (Wosnick *et al.*, 2018), and using anesthetics can facilitate management and transportation procedures in this species by reducing stress.

This study aimed to evaluate the efficacy of EOLA as an anesthetic for fat snook juveniles, analyzing time to induce anesthesia, ventilatory rate (VR), and biochemical, antioxidant, and oxidative stress parameters. To our knowledge, this is the first study describing EOLA's anesthetic activity in a euryhaline fish.

MATERIAL AND METHODS

Locations, and animals. Vouchers were deposited in the ichthyological collection of the Museu de Biologia Professor Mello Leitão of the Universidade Vila Velha UVV), Vila Velha, ES, Brazil (MBML 12877). Two hundred and thirty-two fat snook specimens were purchased from the Laboratório de Piscicultura Marinha (LAPMAR), Florianópolis, SC, Brazil, and transferred to the Laboratório de Ictiologia Apliacada at UVV. During acclimation (ten days), fish were distributed in six 500 L fiberglass tanks containing 400 L of water, with constant aeration and physical and biological

filters. The animals were fed commercial feed containing 54% crude protein (INVE Aquaculture Nutrition, Salt Lake City, USA) three times a day (08:00, 12:00, and 17:00 h) until apparent satiety.

The water quality parameters were measured during the acclimatization (twice a week) and experimental (every day) periods and remained stable. The water quality parameters for dissolved oxygen (5.92 ± 0.13 mg O_2 L^{-1}; 73.98 ± 1.41% saturation) and water temperature (26.83 ± 0.05 °C) were monitored with the aid of an oximeter (YSI oximeter OD 200), pH (7.33 ± 0.04) with the assistance of a pH meter (YSI pH 100), and conductivity (33.88 ± 1.17 µS cm^{-1}) and salinity (30.28 ± 0.05 ppt) using a conduct meter (YSI conductivity EC 300). Total ammonia (0.25 ± 0.05 mg N-NH_3 L^{-1}) was measured by the indophenol method, and alkalinity (117.34 ± 3.10 mg $CaCO_3$ L^{-1}), and nitrite (0.22 ± 0.08 mg N–NO_2 L^{-1}) by titration using colorimetric reactions from a commercial kit (Alcon Ltd. – Camboriú, SC, Brazil). The tanks were cleaned daily at 17:30 h with a siphon to remove excess feces and residues.

Essential oil from *Lippia alba*. The specimens of *L. alba* were cultivated in Frederico Westphalen, RS, Brazil, and its leaves were collected in January 2013 (summer). The EOLA was obtained from fresh plant leaves by hydrodistillation for 2 h using a Clevenger-type apparatus (European Pharmacopeia, 2007). The EOLA was stored at -20 °C until composition analysis and biological assays. The chemical composition of EOLA was determined using gas chromatography-mass spectrometry (GC-MS), as described in detail by Simões *et al*. (2017). The same EOLA investigated in the current study was used in a previous study (Simões *et al*., 2017); its main constituents are linalool (48.69%), eucalyptol (10.51%), β-myrcene (9.70%), and β-caryophyllene (4.19%).

Experimental procedures. Before use, EOLA was diluted 1:10 in absolute ethanol. First, pilot tests (n = 6 fish per concentration) were performed in aquariums containing 1 L of water and constant aeration under conditions similar to those of experiments to choose the most appropriate concentrations to be used in the experiments. The pilot test tested the following concentrations: 10, 20, 30, 40, 50, 60, 80, 100, 120, 130, 140, 150, 160, 180, 200, 220, 230, and 250 µL EOLA L^{-1}. A control group was submitted to the same handling process using water only. The concentrations above 30 µL EOLA L^{-1} caused anesthesia, while 10 and 20 µL EOLA L^{-1} caused only sedation. In this study, four experiments were performed. Only small fish (6.03 ± 0.09 g; 9.30 ± 0.05 cm) were used in experiments 1, 3, and 4. In experiment 2, larger fish (38.49 ± 2.07 g; 16.55 ± 0.26 cm) were also used. The same observers accompanied the experiments. Before each experiment, the fish fasted for 24 h.

Experiment 1: Anesthetic induction. Seventy animals were used to test six different EOLA concentrations: 30, 80, 130, 180, 200, and 230 µL L^{-1}. A control group was transferred to aquariums containing only ethanol (2,070 µL L^{-1}) at a concentration equivalent to the dilution used for 230 µL L^{-1}, totaling 7 treatments. The procedure involved transferring fish (n = 10 per treatment, with one fish used at a time) to aquariums containing 1 L of water and constant aeration. Mild anesthesia (partial loss of balance and erratic swimming) and deep anesthesia (complete loss of balance and cessation of swimming) were evaluated according to Small (2003).

The animals that reached deep anesthesia were rinsed in clean water and transferred to recovery aquariums containing 3 L of water without EOLA and constant aeration to estimate the recovery time (behavior similar to the fish kept in the maintenance tanks, *i.e.*, swimming and equilibrium without alterations). The lowest concentration, capable of inducing partial loss of balance and erratic swimming without causing deep anesthesia, was indicated for mild anesthesia in fish. For deep anesthesia in fish, we chose the lowest concentration capable of causing complete loss of balance and cessation of swimming in less than 3 min and with a recovery of less than 5 min (Small, 2003; Sena *et al.*, 2016).

Experiment 2: Test with larger fish. For this experiment, larger fish (n = 10) were exposed to the concentration indicated in experiment 1 for smaller fish, against which they were compared (*i.e.*, 180 μL EOLA L^{-1}). The evaluations for anesthesia and recovery were performed following the same procedures described in experiment 1, where the anesthesia and recovery times were compared between larger and smaller fish. However, aquariums containing 3 L of water were used to evaluate anesthesia, and aquariums with 6 L of water were used to assess recovery. Both aquariums had constant aeration.

Experiment 3: Ventilatory rate (VR). Tests were conducted in aquariums with 5 L of water and constant aeration. In each aquarium, squares (4.8 cm^2) were marked in the background and behind to determine the VR of the fat snook exposed to 5 and 10 μL EOLA L^{-1}. In a pilot study, these concentrations caused only sedation (decreased reactivity to external stimuli) (Small, 2003). Two control groups were also evaluated; one control group was kept only in water, and another was kept in water plus ethanol (90 μL L^{-1}). The VR (count of opercular movements in beats min^{-1}) was individually analyzed in 32 fat snooks (n = 8 per treatment) at 0, 20, 40, 60, and 120 min during exposure to the anesthetic solution. At one time, fish was filmed concurrently from the front and above the same test aquarium for 10 min each time.

Experiment 4: Stress responses. One hundred and twenty animals were exposed individually to 30 and 180 μL EOLA L^{-1} (concentrations indicated for mild and deep anesthesia, respectively; see results of the first experiment) in aquariums containing 1 L of water and constant aeration. A control group was transferred to aquariums (1 L) containing only ethanol (1,620 μL L^{-1}). A second control group with fish kept in aquariums (1 L) containing only water was also performed. After the fish reached deep anesthesia or after 4 min (control groups), the animals were transferred to recovery aquariums (3 L) without EOLA. The fish were evaluated at 0, 30, and 60 min after anesthesia recovery for blood glucose, whole-body cortisol, antioxidant enzymes, and oxidative stress parameters.

Experimental analysis in experiment 4. As the fish were small, collecting only one drop of blood (from the caudal vein) was possible. Blood was collected in fish after transferring to recovery aquariums at 0 (no recovery), 30, and 60 min. Blood glucose levels were analyzed of 5 fish per treatment at each time using microfilm strips and a digital glucometer (Accu-Chek Active, Roche ™) immediately after blood collection

and are expressed as mg dL^{-1}. Then, fish were euthanized with lethal benzocaine hydrochloride (250 mg L^{-1}) and frozen at -80 °C for future analysis of whole-body cortisol.

The whole-body cortisol was extracted using the validated method described by Sink *et al*. (2007). This method was chosen due to inadequate blood volume to measure plasma cortisol accurately. This method was selected because the fish were too small to collect enough blood for plasma analysis. The mean detection accuracy of spiked samples was 94.3%, which was tested by calculating the recoveries from samples spiked with known amounts of cortisol (50, 25, and 12.5 ng mL^{-1}). All values were adjusted for recovery following the "Cortisol value = measured value × 1.0604" equation. Whole-body cortisol levels were measured (in duplicate) using a commercially available enzyme-linked immunosorbent assay kit (EIAgen™ Cortisol test, BioChem Immuno Systems). The whole-body cortisol levels are expressed as ng g of $tissue^{-1}$. This kit was fully validated for fish tissue extracts using the method proposed by Sink *et al*. (2007) and described in detail by Parodi *et al*. (2014). There was a strong positive correlation between the curves (R^2 = 0.89), and the samples had low inter- (CV of 7–10%) and intra-assay (CV of 5–9%) variations.

Another 5 fish per treatment each time were euthanized with the lethal concentration of benzocaine hydrochloride for liver collection. The liver was collected at the exact times of the blood collection and then preserved at -80 °C until analysis of oxidative stress parameters.

The liver was weighed and homogenized (1:4, w/v) in Tris buffer 20 mM (pH 7.4), sucrose 0.5 mM, KCl 0.15 mM, and 1 mM protease inhibitor (PMSF). The samples were centrifuged at 10,000 x g for 20 min (4 °C). The resulting supernatant fraction was used for glutathione S-transferase (GST), catalase (CAT), superoxide dismutase (SOD), and lipid peroxidation (LPO) assays. All assays (in triplicate) were carried out using a spectrophotometer (Spectramax Plus 384, Molecular Devices) at 25 °C.

Protein content was determined by the Bradford method (Bradford, 1976) adapted to the microplate. Enzymatic activities were determined at 25 °C and expressed as activity per mg of protein. The samples showed no differences in protein content.

The LPO was assessed by Fe^{2+} oxidation in the presence of xylenol orange (FOX, ferrous oxidation-xylenol orange assay) as described by Jiang *et al*. (1991). The homogenized samples were treated with 10% trichloroacetic acid and centrifuged. The supernatants were applied to a solution containing 900 mL of FOX reagent in 90% (v/v) methanol and incubated at 37 °C for color development prior to colorimetric measurement at 560 nm. The LPO concentrations were expressed as nmol mg $protein^{-1}$.

The GST activity was determined by measuring the increase in absorbance at 340 nm, incubating reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates (Keen *et al*., 1976). The enzyme activity was calculated as µmol GS-DNB min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}.

The CAT activity was determined following the method described by Beutler (1975), based on the consumption of H_2O_2 recorded at 240 nm. The CAT activity was defined as the difference in the absorbance per unit of time (extinction coefficient 40 mM^{-1} cm^{-1}) and expressed as µmol min^{-1} mg $protein^{-1}$.

The SOD activity was determined, according to McCord, Fridovich (1969), by measuring the absorption of the reduction of cytochrome C by the xanthine oxidase/

hypoxanthine system at 550 nm. One unit of SOD is the amount of the enzyme that inhibits by 50% the reduction of cytochrome C. The SOD activity was expressed as IU mg protein^{-1}.

Statistical analyses. All data are presented as mean ± standard error of the mean (SEM). Levene's test tested the homogeneity of variances between treatments. Experiments 1 and 2 were analyzed using one-way ANOVA, while experiments 3 and 4 were analyzed using a two-way ANOVA (time × treatment). After ANOVA, Tukey post hoc tests were performed. In addition, experiment 1 (mild and deep anesthesia) was also evaluated by power regression analysis (concentration × time). Significance was set at a critical level of 95% ($P < 0.05$).

RESULTS

Fish showed no mortality during or after 72 h of exposure to EOLA in the experiments.

Experiment 1: Anesthetic induction. Applying 2,070 μL L^{-1} of ethanol alone did not induce sedation or anesthesia. The regression results showed that higher concentrations of EOLA resulted in a shorter time for fish anesthesia. However, no significant relationship was found between the EOLA concentrations and the anesthetic recovery time. At a 30 μL EOLA L^{-1} concentration, the fish reached mild anesthesia at 127.4 s. Fat snooks were deeply anesthetized only at concentrations above 80 μL EOLA L^{-1}. The concentrations of 180 μL EOLA L^{-1} induced the shortest deep anesthesia and recovery times, with times of 184.6 and 163.4 s, respectively ($P < 0.05$) (Fig. 1).

Experiment 2: Tests with larger fish. Fat snooks of larger size showed mild and deep anesthesia and recovery times significantly higher than those of smaller size when exposed to 180 μL EOLA L^{-1} ($P < 0.05$) (Fig. 2).

Experiment 3: Ventilatory rate (VR). Comparing the differences between treatments, the VR was significantly higher in fish at 10 μL EOLA L^{-1} at times between 20 and 120 min of recovery and at 5 μL EOLA L^{-1} at times of 60 and 120 min of recovery compared to the control and ethanol groups ($P < 0.05$).

The two tested EOLA concentrations also showed differences over time. The VR was significantly higher in fish exposed to 5 μL EOLA L^{-1} at times between 40 and 120 min, compared to 0 min ($P < 0.05$). Similarly, the VR in fish at 10 μL EOLA L^{-1} was significantly lower at 0 min than at other times ($P < 0.05$). In addition, at the time 20 min to 5 μL EOLA L^{-1} and at times 20 and 40 min to 10 μL EOLA L^{-1}, the VR was significantly lower than at the time 120 min of recovery ($P < 0.05$) (Fig. 3).

Experiment 4: Blood glucose and whole-body cortisol. Blood glucose levels were significantly higher in fish anesthetized with 180 than with 30 μL EOLA L^{-1} at all the times evaluated ($P < 0.05$). The treatment 180 μL EOLA L^{-1} also showed blood glucose levels significantly higher than the ethanol group (1,620 μL L^{-1}) at 0 min and the control group at 60 min after recovery ($P < 0.05$). Fish exposed to 30 μL EOLA L^{-1}

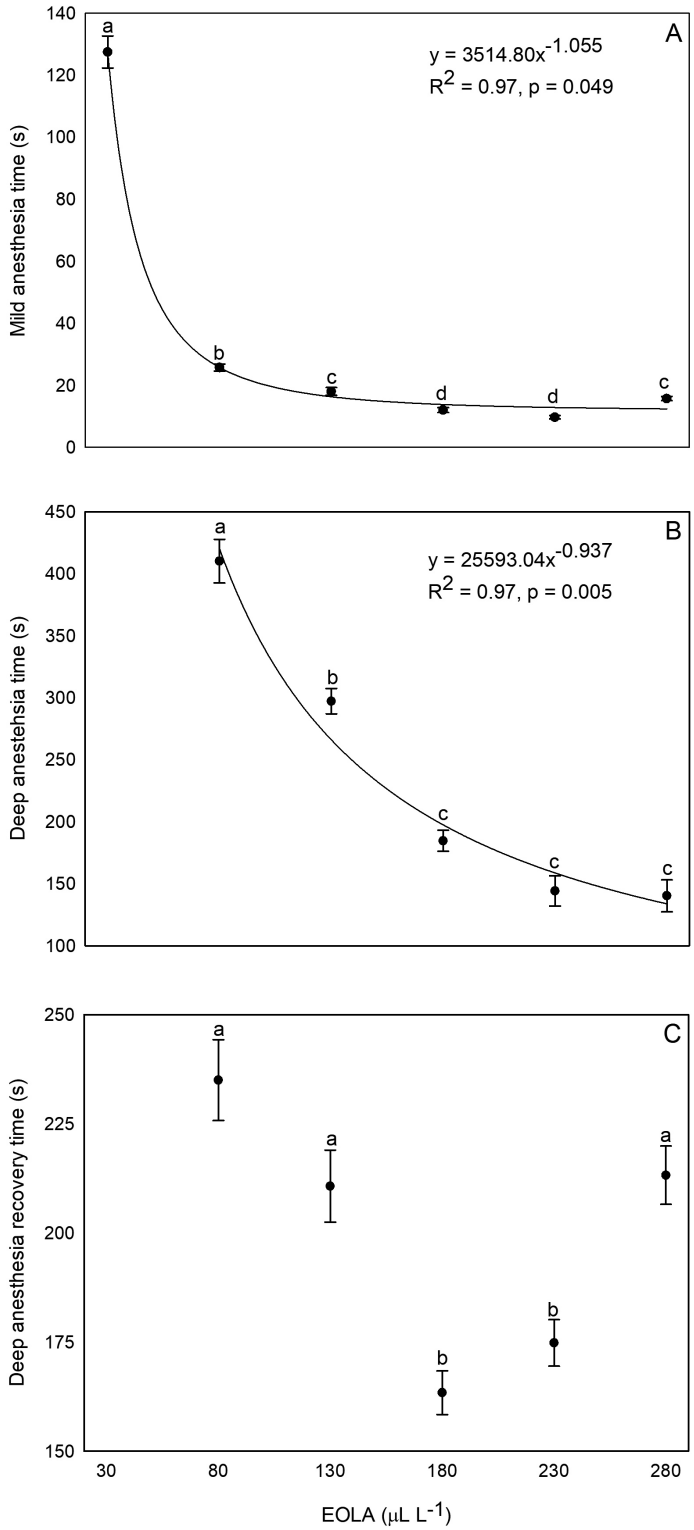

FIGURE 1 | Time (s) required for mild and deep anesthesia and recovery in fat snook angelfish (*Centropomus parallelus*) with increasingly essential oil from *Lippia alba* (EOLA) concentrations. Data are presented as the mean ± SEM (n = 10 fish per treatment). Different letters indicate significant differences between treatments. One-way ANOVA and Tukey's tests were used to determine statistical significance ($P < 0.05$). Mild and deep anesthesia times showed regression.

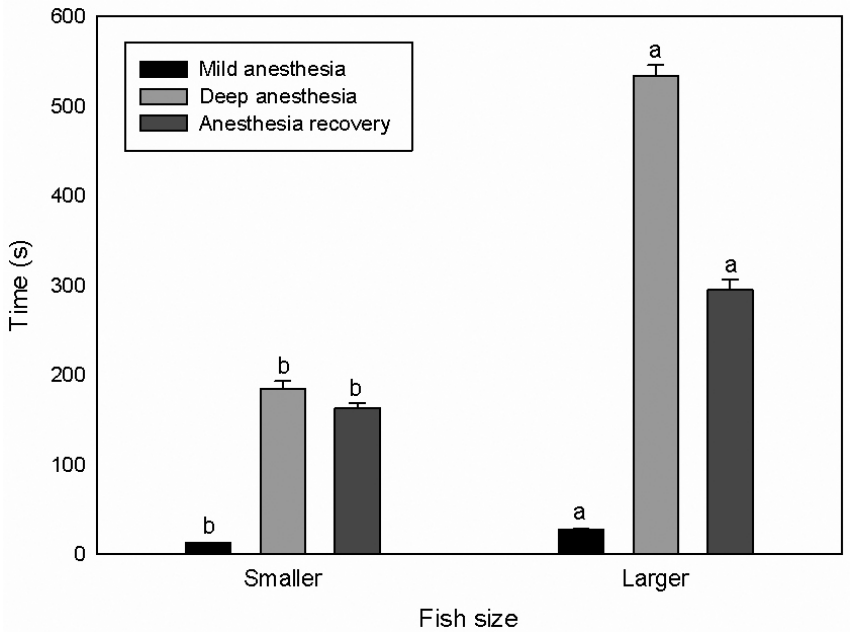

FIGURE 2 | Time (s) required for mild and deep anesthesia and recovery in fat snook (*Centropomus parallelus*) exposed to essential oil from *Lippia alba* (180 µL L^{-1}). Smaller fish = 6.03 ± 0.09 g; 9.30 ± 0.05 cm. Larger fish = 38.49 ± 2.07 g; 16.55 ± 0.26 cm. Data are presented as the mean ± SEM (n = 10 fish per treatment). Different letters indicate significant differences between fish body size classes. One-way ANOVA and Tukey's tests were used to determine statistical significance ($P < 0.05$).

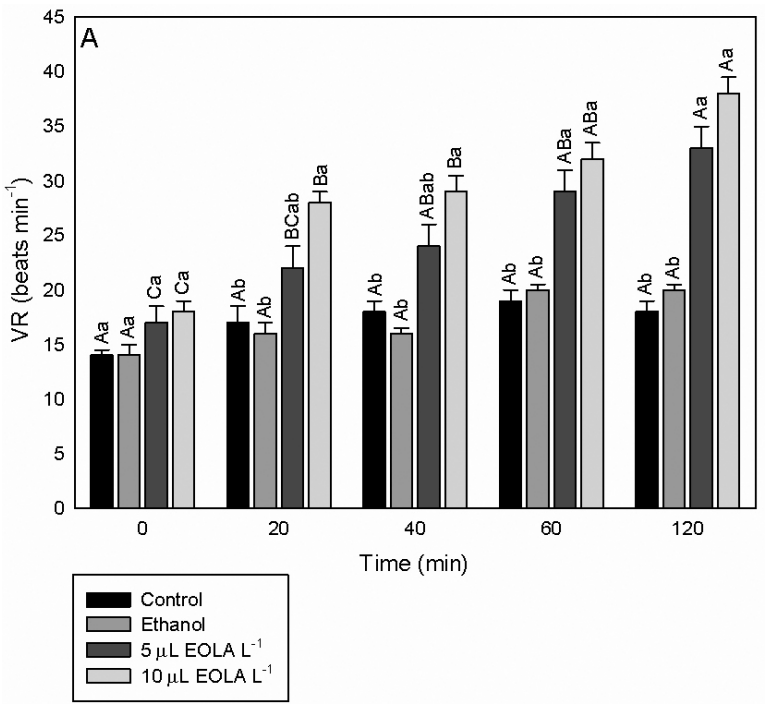

FIGURE 3 | Ventilatory rate (VR) of fat snook (*Centropomus parallelus*) during exposure to the essential oil from *Lippia alba* (EOLA). Data are presented as the mean ± SEM (n = 8 fish per treatment). Capital letters indicate significant differences between time points within the same treatment. Lowercase letters indicate significant differences between treatments at the same time point. Two-way ANOVA and Tukey's tests were used to determine statistical significance ($P < 0.05$).

had blood glucose levels significantly lower than other groups at times 30 and 60 min after recovery (except for the ethanol group at the time 30 min) ($P < 0.05$). At the time 0 min, the values of blood glucose were significantly lower in all groups than at the time 30 min, and this difference was maintained at the time 60 min for fish exposed to ethanol and 180 µL EOLA L^{-1} ($P < 0.05$) (Fig. 4A).

The whole-body cortisol values at 0 min were significantly lower in the ethanol group than in fish exposed to 30 µL EOLA L^{-1} ($P < 0.05$). Fish exposed to 180 µL EOLA L^{-1} had whole-body cortisol levels significantly higher than those exposed to 30 µL EOLA L^{-1} at 30 min and the control group at 60 min after recovery ($P < 0.05$). In the control and ethanol groups, whole-body cortisol levels were significantly higher at 30 min than at 0 min ($P < 0.05$). In the fish anesthetized with 180 µL EOLA L^{-1}, whole-body cortisol levels were significantly higher at times 30 and 60 min than at the time 0 min ($P < 0.05$) (Fig. 4B).

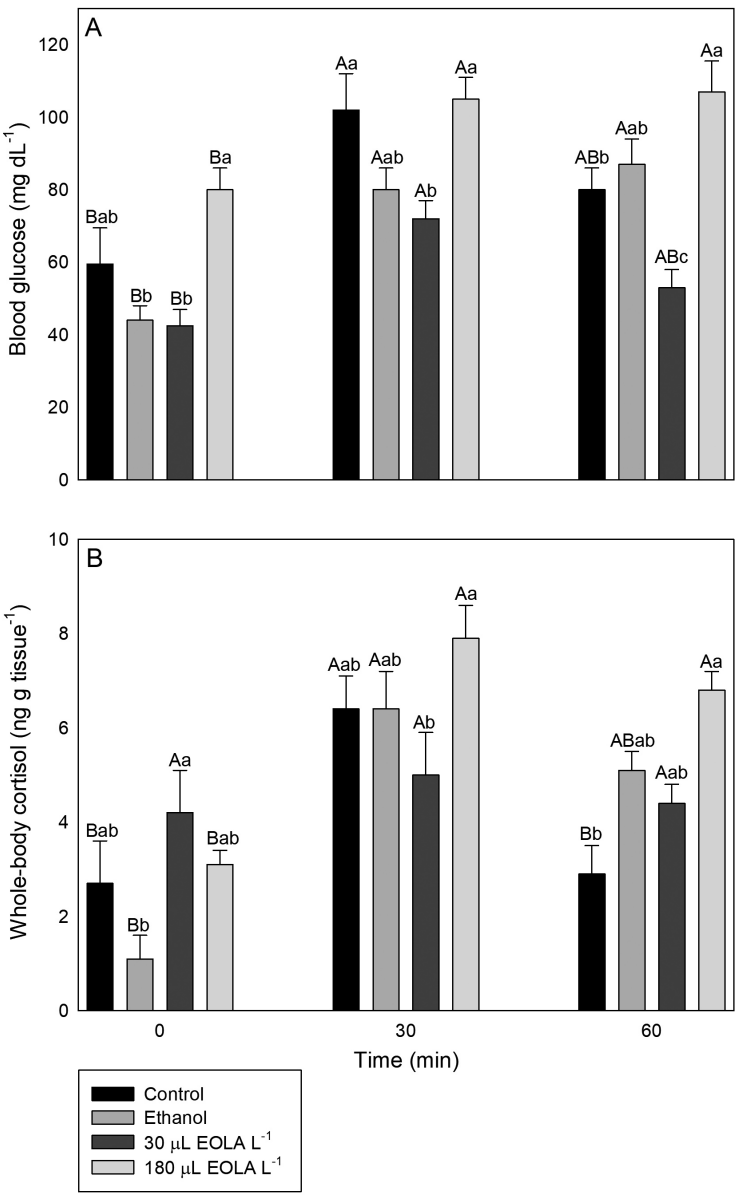

FIGURE 4 | Blood glucose (A) and whole-body cortisol (B) levels after transferring to recovery aquariums of anesthetized fat snook (*Centropomus parallelus*) with essential oil from *Lippia alba* (EOLA). Data are presented as the mean ± SEM (n = 5 fish per treatment each time). Capital letters indicate significant differences between time points within the same treatment. Lowercase letters indicate significant differences between treatments at the same time point. Two-way ANOVA and Tukey's tests were used to determine statistical significance ($P < 0.05$).

Experiment 4: Oxidative stress parameters. At 30 min after recovery, the fat snooks exposed to 30 μL EOLA L^{-1} had significantly higher liver GST activity than the fish submitted to the other treatments at the same time (30 min) or than this same treatment (30 μL EOLA L^{-1}) at 0 min ($P < 0.05$). At 60 min, the group 180 μL EOLA L^{-1} had significantly higher liver GST activity than the control and ethanol groups at the same time (60 min) or than this same treatment (180 μL EOLA L^{-1}) at other times ($P < 0.05$) (Fig. 5A). At 60 min of recovery, the fish from the EOLA groups had significantly higher liver SOD activity than the control group (only water). Still, they did not differ from the ethanol group ($P < 0.05$). In addition, fish exposed to 30 μL EOLA L^{-1} showed significantly higher liver SOD activity at 60 min than at 0 min ($P < 0.05$) (Fig. 5B). The different treatments did not change liver CAT and LPO levels ($P > 0.05$) (Figs. 5C, D).

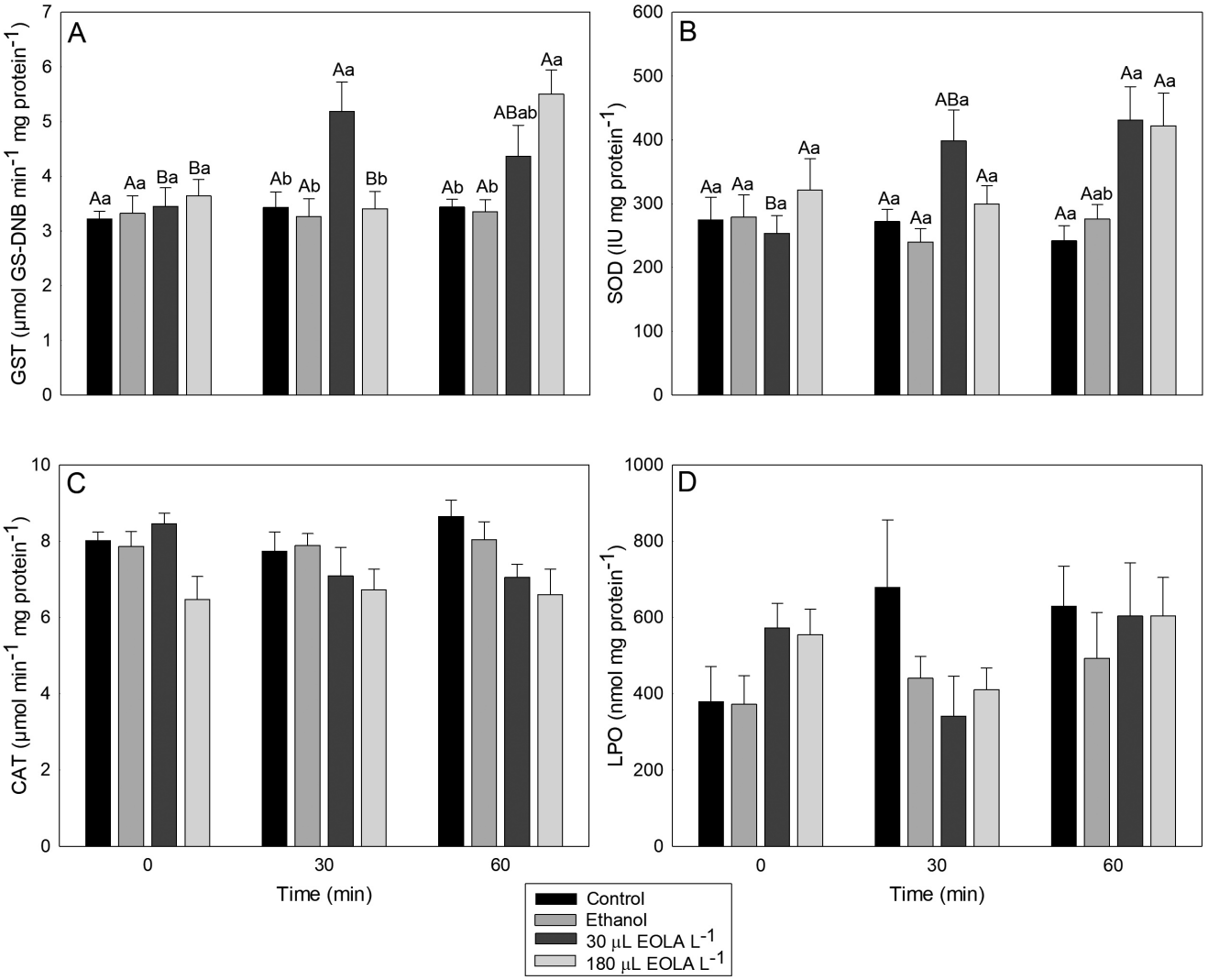

FIGURE 5 | Antioxidant and oxidative stress parameters in the liver after transferring to recovery aquariums of fat snook (*Centropomus parallelus*) anesthetized with the essential oil from *Lippia alba* (EOLA). A = GST (glutathione S-transferase). B = SOD (superoxide dismutase). C = CAT (catalase). D = LPO (lipid peroxidation). Data are presented as the mean ± SEM (n = 5 fish per treatment each time). Capital letters indicate significant differences between time points within the same treatment. Lowercase letters indicate significant differences between treatments at the same time point. Two-way ANOVA and Tukey's tests were used to determine statistical significance ($P < 0.05$).

DISCUSSION

Various studies have indicated that an anesthetic is more effective when it has fast action (< 3 min) and a short recovery time (< 5 or 10 min) (Small, 2003; Ross, Ross, 2009; Sena *et al*., 2016; Teixeira *et al*., 2017; Oliveira *et al*., 2019b). In addition, lower anesthetic concentrations may provide a higher safety margin for fish welfare and avoid essential oil wastage (Teixeira *et al*., 2017). In this sense, the present study recommends 180 μL EOLA L^{-1} as the minimum effective concentration for deep anesthesia of fat snook. In addition, considering that the concentration used for long-term anesthesia should be the minimum possible to avoid deep anesthesia (Oliveira *et al*., 2019a), 30 μL EOLA L^{-1} is viable for mild anesthesia in this species. In line with our results, previous studies also found EOLA as a potential anesthetic for silver catfish (300 μL EOLA L^{-1} (Cunha *et al*., 2010; Heldwein *et al*., 2014; Souza *et al*., 2018), tambacu (200 μL EOLA L^{-1}) (Sena *et al*., 2016), seahorse (150 μL EOLA L^{-1}) (Cunha *et al*., 2011), gilthead sea bream (100–200 μL EOLA L^{-1}) (Toni *et al*., 2015), and Nile tilapia (500 μL EOLA L^{-1}) (Hohlenwerger *et al*., 2016).

In the current study, the main compound of EOLA was linalool (48.69%). Linalool is a constituent of several essential oils whose depressor activities on the CNS are well-described in rodents and humans (Dobetsberger, Buchbauer, 2011). Souza *et al*. (2018) verified that EOLA chemotype linalool is a safe and effective anesthetic since it did not significantly change the expression of several hypothalamus-pituitary-interrenal (HPI) axis genes in silver catfish. The anesthetic effect of EOLA is related to the GABAergic system in silver catfish (Heldwein *et al*., 2012). Still, Heldwein *et al*. (2014) did not detect the direct interaction of linalool with the benzodiazepine site of GABA receptors in the same species. Eucalyptol (the second main compound in EOLA in this study; 10.51%) has anticonvulsive effects in mice (Galindo *et al*., 2010). Myrcene (the third main compound in EOLA in this study; 9.70%) acts at both central and peripheral sites, mediating endogenous opioids and α2-adrenoreceptors in mice (Rao *et al*., 1990). So, linalool could have interacted with other compounds (such as eucalyptol and β-myrcene) to cause the anesthetic effects in fat snook.

Several factors can affect the time for fish to reach anesthesia, such as water quality (Sneddon, 2012), essential oil composition (Limma Netto *et al*., 2016; Souza *et al*., 2018), and size (Sneddon, 2012; Oliveira *et al*., 2022). Body weight is a determining factor in defining the best time for anesthetic induction, where smaller fish would be more easily anesthetized (Tarkhani *et al*., 2016). This information agrees with the current study because larger fish (with higher body weight) took more time to be anesthetized than smaller fish. Therefore, 180 μL EOLA L^{-1} is the ideal concentration to anesthetize larger fish. The gill surface of smaller fish is proportionally more prominent to the body than in larger fish (Hoseini *et al*., 2013), maximizing the contact and diffusion capacity of the essential oil. This action was corroborated by our results, where larger fat snooks took about twice as long as smaller ones to be mildly and deeply anesthetized and recover from anesthesia. Similarly, larger freshwater angelfish *Pterophyllum scalare* (Schultze, 1823) showed longer anesthesia and recovery times than smaller ones (Oliveira *et al*., 2022).

Anesthetics (regardless of causing mild or deep anesthesia) at low concentrations commonly have a sedative effect (Oliveira *et al*., 2019b). Concentrations between 10 and 20 μL EOLA L^{-1} were indicated for transporting silver catfish, seahorse, Nile tilapia,

and tambacu (Cunha *et al*., 2010, 2011; Becker *et al*., 2012; Hohlenwerger *et al*., 2016; Sena *et al*., 2016). These same concentrations caused sedative effects for fat snook in a pilot test, and, therefore, we investigated their impact on the VR. In fish, sedative substances can reduce VR and metabolic stress (Becker *et al*., 2018). Essential oils at low concentrations (causing only sedation) reduced VR: EOLA (10–20 μL EOLA L^{-1}) in Nile tilapia (Hohlenwerger *et al*., 2017) and silver catfish (5–10 μL EOLA L^{-1}) (Becker *et al*., 2018), essential oil from *Aloysia triphylla* (*Aloysia citrodora* Paláu) in Nile tilapia (20–30 μL EOLA L^{-1}) (Teixeira *et al*., 2018), and essential oil from *Lippia sidoides* (syn. *Lippia origanoides* Kunth) in angelfish freshwater (10 and 15 mg EOLA L^{-1}) (Oliveira *et al*., 2022). On the other hand, in the current study, 10–20 μL EOLA L^{-1} increased VR.

A possible explanation for this finding would be that the presence of the anesthetic in the water could cause transitory stress, increasing the VR (Sneddon, 2012; Becker *et al*., 2018). A consequence of increased VR is increased oxygen absorption from the water. Higher oxygenation should increase the tissue oxygen concentration, a precursor to ROS (Nitz *et al*., 2020a). In this situation, animals could increase their antioxidant defenses to avoid damage to cellular homeostasis. This was verified in the present study when the fish were anesthetized with 30 and 180 μL EOLA L^{-1}, where exposure time and anesthetic concentration strongly influenced liver GST and SOD activity. On the other hand, the anesthetic did not change liver CAT and LPO values. GST is an important enzyme catalyzing LPO products and other metabolites and transforming xenobiotics into more easily excreted substances (Lushchak *et al*., 2009). SOD and CAT protect against oxidative damage (Pandey *et al*., 2003). The LPO acts as a cell lesion mechanism provoked by free oxygen radicals (Copatti *et al*., 2019). If the antioxidant system does not work well, LPO, which is highly toxic for fish, may occur (Mirzargar *et al*., 2022). In the current study, the cellular function must not have been impaired in fat snook exposed to EOLA, as liver LPO levels did not differ from non-anesthetized groups. The results also suggest that EOLA did not cause oxidative stress in this species. Although EOLA did not influence liver CAT activity, the increase of liver GST linked to SOD activity could minimize oxidative damage (LPO) during temporary changes resulting from physiological and biochemical adjustments of recovery from anesthesia (Souza *et al*., 2018), contributing to the primary antioxidant defense system.

Although few studies evaluated antioxidant responses and oxidative stress in anesthetized fish with linalool chemotype EOLA, the results found by these authors were similar to those recorded in our study. Anesthesia with EOLA showed an increase in the antioxidant capacity of silver catfish, increasing liver GST, SOD, and CAT activity, besides reducing liver LPO levels (Azambuja *et al*., 2011; Salbego *et al*., 2017; Souza *et al*., 2018) and cururu stingray *Potamotrygon wallacei* Carvalho, Rosa & Araújo, 2016, increasing brain SOD, and CAT activity, besides reducing brain LPO levels (Finamor *et al*., 2023). Therefore, in an integrative analysis of our study with the studies mentioned, it is demonstrated that EOLA can potentially improve antioxidant responses in fish anesthesia.

In addition, Oliveira *et al*. (2022) verified that the essential oil from *L. sidoides* (a plant of the same genus used in this study) can cause irreversible changes in gills. A commitment of branchial O_2-sensitive chemoreceptors can lead to a greater VR in fish because these structures exert dominant control over ventilatory reflexes (Burleson, Smatresk, 2000). However, there is still no proof that the branchial changes provoked

by anesthetics cause detrimental effects on the O_2-sensitive chemoreceptors. Another possibility for the increase of VR verified in the current study would be the increases in arterial and venous O_2 tension of fat snook, which can cause hyperventilation (Burleson, Smatresk, 2000). A situation that causes hyperoxygenation commonly increases the fish metabolism (Nitz *et al*., 2020b) because, during recovery, fish can increase VR immediately to blow off CO_2 to maintain acid-base balance (Burleson, Smatresk, 2000). The fish of the present study were kept in aquariums with continuous aeration during the sedation recovery period. The aeration could have contributed to increasing gill oxygenation, with a consequent increase in VR to reinforce metabolic demand.

Similarly, Kiessling *et al*. (2009) verified higher VR in Atlantic salmon *Salmo salar* Linnaeus, 1758 during recovery from anesthesia with benzocaine, MS-222, and isoeugenol. Becker *et al*. (2012) showed an increase in VR in silver catfish after 30 min of sedation with EOLA or eugenol; however, the VR was reduced at 60 min of exposure. Thus, our data indicate that fish can utilize VR to compensate for gill alterations or acid-base disturbances, such as during anesthesia recovery. In addition, we evaluated only smaller fish, which should have a higher VR than larger fish.

It is recognized that using anesthetics in fish is much more complex than previously described in the literature (Readman *et al*., 2017). In fish, using anesthetics is usually pointed as a stress reducer (Sena *et al*., 2016; Hohlenwerger *et al*., 2017). However, their direct application can increase stress responses since unventilated anesthesia causes depression of the CNS, impairing net ion fluxes, VR, and metabolism (Ross, Ross, 2009; Teixeira *et al*., 2018; Oliveira *et al*., 2022). Cortisol is the leading indicator of primary stress responses in fish and involves a series of neuroendocrine responses. It can trigger glycogenolysis and gluconeogenesis and increase blood glucose levels (Sena *et al*., 2016; Teixeira *et al*., 2017; Oliveira *et al*., 2019a). Additionally, this hormone is crucial for euryhaline fish since it interacts with other hormones (*e.g*., growth hormone, prolactin) and stimulates an increase in the functional area of ionocytes and a decrease in the gill permeability to maintain ionic balance (McCormick *et al*., 2008; Copatti, Baldisserotto, 2021).

Euryhaline fish can maintain blood glucose levels constant within their optimum salinity range (Herrera *et al*., 2009). However, if the anesthetic does not suppress the activation of the HPI axis during stress, a rapid release of catecholamines and cortisol might occur, increasing glucose metabolism (Barton, 2002). In our study, 180 μL EOLA L^{-1} could not avoid stress because it increased whole-body cortisol and blood glucose levels during recovery. Whole-body cortisol techniques have been previously used to evaluate the stress of small fishes, being useful as a general indicator of stress (Ramsay *et al*., 2006; Baldisserotto *et al*., 2014). A stress event, such as handling or fish perception of anesthetic presence (Souza *et al*., 2018), can stimulate catecholamine and cortisol release, which induces liver glycogenolysis and an increase of blood glucose (Wendeelar Bonga, 1997; Barton, 2002) to ensure the energy supply with a possible higher metabolic demand. Another possibility for the rise in whole-body cortisol is its relationship with VR, which, when increased, can cause a more rapid recovery from anesthesia and thereby trigger an ability to react to external signals and pay the oxygen debt acquired during the anesthesia (Kiessling *et al*., 2009). In the present study, the anesthesia caused stress in fat snooks, increasing oxygen demand, as demonstrated by our results for VR and blood glucose levels.

Previous studies performed with fat snook found similar results. Wosnick *et al.* (2018) reported a relationship between the increase in blood cortisol and glucose levels after anesthesia with benzocaine (50 mg L^{-1}), regardless of the salinity of exposure of the fish (5–30 ppt). Elevated blood glucose levels were also observed in transporting fish with essential oil from *Nectandra megapotamica* (Spreng.) Mez (300 μL L^{-1}), whose values were higher in individuals adapted to seawater than freshwater (Tondolo *et al.*, 2013). Parodi *et al.* (2016) verified that adding essential oil from *A. triphylla* (20 μL L^{-1}), although it had reduced blood cortisol levels, increased blood glucose levels in fat snook after transport. Interestingly, both EOLA and the essential oil from *A. triphylla* are not aversive to silver catfish and zebrafish, *Danio rerio* (Hamilton, 1822) (Bandeira Junior *et al.*, 2018) and avoided plasma cortisol rise in silver catfish (Cunha *et al.*, 2010; Parodi *et al.*, 2014, respectively), but EOLA was not as efficient for reduce cortisol levels in fat snooks (current study), tambacu (Sena *et al.*, 2016), and meagre (EOLA) (Cárdenas *et al.*, 2016), demonstrating that the effect of anesthetics can be different according to the management conditions (*e.g.*, anesthetic chemical compounds, anesthetic concentration, salinity, ambient temperature) and species.

In conclusion, the best mild and deep anesthesia times in fat snook juveniles for EOLA were obtained with 30 and 180 μL EOLA L^{-1}, respectively, and these times were lower in smaller than larger fish. The EOLA (mainly at the highest concentration) increased VR, whole-body cortisol, blood glucose, and liver GST and SOD values. The transfer of EOLA across the gills is presumably regulated by branchial ventilation; therefore, the increase of VR is possibly related to the rise of whole-body cortisol and blood glucose levels in fish anesthetized with 180 μL EOLA L^{-1}. Finally, the rise of liver GST and SOD activities found in fish exposed to 30 and 180 μL EOLA L^{-1} demonstrated that EOLA effectively prevented oxidative stress and can be used for anesthesia in fat snook.

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AUTHORS' CONTRIBUTION

Larissa N. Simões-Bueno: Investigation, Methodology, Validation, Visualization, Writing-original draft.

Carlos E. Copatti: Formal analysis, Investigation, Validation, Visualization, Writing-original draft, Writing-review and editing.

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ETHICAL STATEMENT

The methodology of this experiment was approved by the Ethical Committee on the Use of Animals of the Universidade Vila Velha, Vila Velha, ES, Brazil (Process number 218/2012).

COMPETING INTERESTS

The author declares no competing interests.

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