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Anesthetic effect and acute toxicity of *Citrus sinensis* essential oil in betta

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ABSTRACT

This study investigated the use of *Citrus sinensis* essential oil (EOCS) in *Betta splendens*, evaluating toxicity, induction and recovery times to anesthesia, its action on agonist behavior, and on male collective transport. To toxicity, fish were exposed to EOCS at different concentrations during 48 h. The induction test was performed to evaluate sedation, anesthesia, and recovery under different concentrations. To assess agonist behavior, male subjects were maintained with different dosages of EOCS, assessing opercular beat and fin expansion, while exposed to untreated animals. In the mass transport study, males were divided into two treatments with two levels of EOCS, for 6 h, also analyzing gill histometry at the end of transport. The results obtained show that the mean lethal concentration in 48 h of exposure to EOCS was calculated at 49.17 $\mu L \cdot L^{-1}$. The shortest anesthesia induction time was at 300 $\mu L \cdot L^{-1}$ EOCS. EOCS was able to reduce agonist behavior in males individual. Histometric analyzes revealed a reduction in the height of gill filaments in fish transported with 20 $\mu L \cdot L^{-1}$ EOCS. EOCS can be used as a sedative and anesthetic agent for *B. splendens*.

Keywords: Behavior; Limonene; Orange; Ornamental fish; Stress.

Efeito anestésico e toxicidade aguda do óleo essencial de *Citrus sinensis* em betta

RESUMO

Este estudo investigou o uso do óleo essencial de *Citrus sinensis* (OECS) em *Betta splendens*, avaliando a toxicidade, tempos de indução e recuperação, sua ação sobre o comportamento agonista e no transporte coletivo de machos. Para a toxicidade, peixes foram expostos ao OECS em diferentes concentrações durante 48 h. O teste de indução foi realizado para avaliar a sedação, anestesia e recuperação sob diferentes concentrações. Para avaliar o comportamento agonista, indivíduos machos foram mantidos com diferentes dosagens de OECS, avaliando batimento opercular e expansão das nadadeiras, enquanto expostos a animais não tratados. No estudo de transporte coletivo de machos, foram divididos em dois tratamentos com dois níveis de OECS, durante 6 h, analisando também a histometria branquial ao final do transporte. Os resultados obtidos mostram que a concentração letal média em 48 h de exposição ao OECS foi calculada em 49,17 $\mu L \cdot L^{-1}$. O menor tempo de indução à anestesia foi com 300 $\mu L \cdot L^{-1}$ OECS. O OECS foi capaz de reduzir o comportamento agonista em indivíduos machos. As análises histométricas revelaram uma redução na altura dos filamentos branquiais dos peixes transportados com 20 $\mu L \cdot L^{-1}$ OECS. O OECS pode ser usado como agente sedativo e anestésico para o *B. splendens*.

Palavras-chave: Comportamento; Limoneno; Laranja; Peixe ornamental; Estresse.

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INTRODUCTION

Approximately 2 billion of ornamental fish are transported every year, and they experience high levels of stress during packaging, transport, and retrieval (Vanderzwalmen et al., 2020). Exposure to such stressful conditions triggers physiological and behavioral changes that can negatively affect fish health and increase mortality (Can et al., 2018; Ferreira et al., 2020). In freshwater fish, mortality can be higher than 5%, while in marine fish caught in the wild, the mortality percentage is much higher, reaching 80% (Vanderzwalmen et al., 2020). In this way, the use of anesthetics during aquaculture practices becomes essential to ease working during farming process and mitigate the effects of stress (Aydin and Barbas, 2020; Silva et al., 2020).

To date, several chemical anesthetics have been effective in sedating and anesthetizing fish, such as tricaine methanesulfonate (MS-222), 2-phenoxyethanol, quinaldine, and benzocaine (Can et al., 2018). However, the use of these products is often limited by price and unavailability, especially in Latin American countries (Ventura et al., 2021). Additionally, many of them have been reported as stressors or have caused undesirable side effects, such as increased muscle tone, behavioral changes, mucus hypersecretion, corneal damage, and skin and gill irritation (Aydın and Barbas, 2020). Due to the importance of these problems, recent research has explored the anesthetic effects of essential oils (EOs) in fish, as well as their effects on biochemical, anti-stress, electrophysiology, histological parameters and sensory evaluation of fillet (Souza et al., 2019; Santos et al., 2020; Silva et al., 2020; Teixeira et al., 2017).

Citrus sinensis essential oil (EOCS) is an EO extracted from the orange peel, is rich in bioactive substances, such as limonene, myrcene, and linalool. Limonene is found in greater proportion (> 90%) in this EO, and its presence may be directly related to biological activities, for example, antibacterial effects against *Lactobacillus plantarum* and *Escherichia coli* bacteria (Raspo et al., 2020). In the literature there are few works on species of the genus Citrus as an anesthetic or sedative in fish. However, Lopes et al. (2018) showed that *Citrus latifolia* and *Citrus aurantium* EOs have an anesthetic effect on *Rhamdia quelen*, in addition, this work points out that these EOs can be used during routine procedures, since they promoted sedation and reduced the ventilatory frequency in fish.

Originally from central Thailand and the lower Mekong, betta *Betta splendens* was initially bred for its use in gambling matches similar to cock fights (Zhang et al., 2022), but it was due to their exuberance, presenting a variety of fin shapes and colors that *B. splendens* has become one of the most popular species of freshwater aquarium fish (França et al., 2021; Pattanasiri et al., 2017a). Thus, the objective of this work was to study the acute toxicity of the essential oil of *C. sinensis*, the induction and recovery times of anesthesia, its action on the behavior and collective transport of adult males of *B. splendens*.

MATERIALS AND METHODS

Study site and animals

A total of 196 male and female *B. splendens* at 6 months of age (1.1 ± 0.08 g and 3.02 ± 0.21 cm total length) were obtained from animal reproduction at the Marine Biology Station (EBM), from the Federal Rural University of Rio de Janeiro (UFRRJ), Mangaratiba, Rio de Janeiro, Brazil, where all experiments were carried out.

The fish were initially sexed and stored for 15 days for acclimatization before the experiments. The males were stored individually in 2 L aquariums, and the females were stored collectively in a 100 L box. During this period, the animals received commercial feed for ornamental fish (40% crude protein), provided twice a day until apparent satiation. The feed supply was suspended 24 h before the experiments. During acclimatization, the following water quality parameters were monitored: temperature (28.00 ± 0.11°C), pH (7.40 ± 0.20), dissolved oxygen (7.00 ± 0.06 $mg \cdot L^{-1}$; Akso® multi-parameter meter model AK88, São Leopoldo, Rio Grande do Sul, Brazil), and un-ionized ammonia (0.001 ± 0.02 $mg \cdot L^{-1}$; Alfakit, Florianópolis, Santa Catarina, Brazil).

Citrus sinensis essential oil

The essential oil used in the present study was commercialized by FERQUIMA®, São Paulo, Brazil and the main constituents of EOCS were limonene (93.20%), myrcene (1.60%), α-pinene (0.40%), sabinene (0.25%) and 4.55% of the constituents were unidentified as minor compounds.

Acute toxicity of EOCS in B. splendens

The lethal concentration of EOCS for *B. splendens* was determined using an acute toxicity test over an exposure period of 48 h. The concentrations used were predetermined using a preliminary tolerance test in which the values chosen for the acute toxicity test corresponded to the lowest concentration (60 $\mu L \cdot L^{-1}$) capable of causing 100% mortality and the highest concentration (20 $\mu L \cdot L^{-1}$) that did not cause mortality among fish, as described by Pattanasiri et al. (2017a). The fish (n = 36) were transferred individually to aquariums containing 1 liter of water, where concentrations of 20, 30, 40, 50, and 60 μL·L^{-1} EOCS were evaluated, with six fish per concentrations (three males and three females). As a control, the highest concentration of ethanol (540 μL·L^{-1}) used in the EO dilution was used.

Fish tolerance to EOCS was monitored through quantitative descriptive analysis during exposure to the product. Regarding mortality, the following criteria were considered: absence of opercular movement and reaction to any external stimuli. With the confirmation of death, the fish were weighed, measured, and identified.

Anesthesia induction and recovery

Of both sexes, 48 *B. splendens* were used to assess the anesthetic activity of EOCS. The fish were individually transferred to continuously aerated aquariums with a capacity of 1 L (n = 6), where they were exposed to concentrations of 25, 50, 100, 150, 200, 250, and 300 μL·L^{-1} EOCS previously diluted in ethanol (1:10). The control group comprised ethanol at a concentration equivalent to the dilution used for the highest EO concentration. Each animal was used only once, and the maximum exposure time to EO was 30 min.

The sedation, anesthesia, and recovery times were adapted from Small (2003) as follows: Stage 1-mild sedation, with less reactivity to external stimuli; Stage 2-deep sedation, with partial loss of balance and irregular swimming; and Stage 3-deep anesthesia, with total loss of balance, cessation of locomotion, and absence of response to tactile stimuli. After induction, the fish were transferred to aquaria with 1 L of aerated water and free of EO for recovery. All times were measured with a digital chronometer and expressed in seconds. After the experimental procedures, the fish were grouped according to the concentration tested. The males were kept individually and then monitored for 48h to observe the incidence of mortality.

Evaluation of the aggressive behavior of *B. splendens* exposed to EOCS

Based on experiment II, three concentrations of EOCS were chosen because they induced only light sedation in fish. In this phase, 24 male *B. splendens* were used. The fish were individually transferred to 1 L aquariums, where 10, 20, and 30 μL·L^{-1} EOCS (n = 6 each concentration), previously diluted in 1:10 ethanol, were evaluated. As a control, the highest concentration of ethanol (270 μL·L^{-1}) used in the EO dilution was used. To evaluate the agonist behavior and breathing frequency of atmospheric air during male-male interactions, an aquarium containing a male animal exempt from EOCS treatment was used next to the aquarium with the treated fish, thus allowing the fish to see each other and interact through the glass (methodology adapted from Alton et al., 2013). The exposure time of the animals to the EOCS was 30 min, and during the visual contact between the fish, aggressive behavior was characterized by counting the number of times the operculum opened and the fins expanded (caudal, dorsal, and anal). This behavior was evaluated according to the definitions of Greene and Szalda-Petree (2022). In addition, the aerial respiratory rate of the fish was quantified by counting the number of times the fish emerged to capture oxygen (Alton et al., 2013).

Transport procedure

In this phase, another 80 adult male *B. splendens* were used in two different treatments (n = 40 fish per treatment) in quadruplicate were evaluated: fish transported without EO (control) or with 20 μL·L^{-1} of EOCS, both for 6 h. It was decided to use concentration of 20 μL·L^{-1} in this experiment because it induced sedation, but not anesthesia, in the six hours of transport. After the fasting period (24 h before transport), 4 fish not subjected to transport (basal group) were collected.

The fish (n = 10 per bag) were distributed in 8 plastic bags, with 5 L of water and air. The plastic bags were stored in styrofoam boxes and transported in a closed car, simulating routine practices of transporting ornamental fish. The water quality parameters were monitored before (0 h) and after transport (6 h) using an Akso® multi-parameter meter (model AK88) to check the temperature, pH, and dissolved oxygen, while total ammonia and non-ionized ammonia (NH3) were determined using a commercial colorimetric kit.

Morphometric Analysis

After the transport period, the fish were euthanized by section of the spinal cord to remove the gills that were kept at −4°C until histological analyses. Samples were collected from 4 fish per treatment before and after transport. The gills were conditioned in 10% buffered formalin for 48 h. After the fixation period, the gill arches were dehydrated in an increasing series of ethyl alcohol (70, 80, 90, and 99.99% (absolute) and xylene for 1 h, embedded in paraffin for 3 h. Subsequently, with the aid of a microtome (Leica RM2245®), blocks resulting from histological processing were cut at a thickness of 5 μm. The prepared slides were stained with hematoxylin and eosin according to Luna (1968).

The slides were taken to an optical microscope for observation and photographed using the Leica Application Suite (LAS) software (40× magnification), then the height in microns (μm) of 10 gill filaments was measured. The height was obtained from the base to the apex of each filament, and the parameters evaluated according to the protocol previously described by Heluy et al. (2020) and Adamek-Urbańska et al. (2021).

Statistical analyses

The quantitative results of the experiments on agonist behavior and respiratory rate, anesthesia induction and recovery were analyzed using SAS software (SAS Institute, 2008). The data were previously converted by logarithmic transformation and then submitted to the Shapiro–Wilks and Bartlett tests to verify the normality and homoscedasticity of the data. Then, linear regression was performed. Histometric data were analyzed using the Infostat statistical software (2017 version) and submitted to preliminary exploratory analysis, with the aim of eliminating discrepant data. After the preliminary analyses, the data were submitted to analysis of variance and, in case of a significant effect, the means were compared by Duncan's test at 5% probability. For toxicity analysis and determination of the LC50 of the EOCS during 48 h of exposure, the TSK test (Trimmed Spearman–Karber) was used without eliminating the lower and upper limits of the mortality data, as previously described by Hamilton et al. (1977).

Figure 1. Female *Betta splendens*, after exposure to 60 μL·L^{-1} of the EOCS during the acute toxicity test. Note the presence of bulging of the abdomen (a, b, c), in addition to color change (a and c) compared to the animal in the control group (d).

RESULTS

Acute toxicity of EOCS in *B. splendens*

The results showed that the lowest concentration of EO (20 μL·L^{-1}) provided a higher percentage of fish survival (100%), while the lowest percentage of survival (0%) was obtained at 60 μL·L^{-1} EOCS. Moreover, 33% of the fish survived when using 50 μL·L^{-1} EOCS. The LC50 of EOCS capable of causing 50% mortality at 48 h of exposure was estimated at 49.17 μL·L^{-1}. In addition, animals exposed to concentrations of 50 and 60 μL·L^{-1} EOCS showed severe damage to the fins, and the highest dose (60 μL·L^{-1}) caused bulging of the fish abdomen, mainly in females (Fig. 1).

Anesthesia induction and recovery

Regarding anesthesia induction and recovery times, the increase in EOCS concentration significantly decreased the time required for sedation and induction of deep anesthesia and increased the recovery time (Fig. 2). The shortest induction time of deep anesthesia was at a concentration of 300 μL·L^{-1}, which differed significantly from concentrations between 150 and 250 μL·L^{-1} EO (Fig. 2c). There was a positive significant relationship between recovery time and EOCS concentration: the higher the concentration of EOCS used, higher the recovery time (Fig. 2d). Ethanol at the highest concentration (2700 μL·L^{-1}) did not produce any anesthetic effects in the animals. The induction of sedation and anesthesia was not accompanied by mortality within the tested range or during recovery, nor was residual mortality observed in the first 48 h of observation after the experiments.

Evaluation of the aggressive behavior of *B. splendens* exposed to EOCS

The results indicate that the EOCS reduced the incidence of aggressive behaviors in the *B. splendens*. The increase in EOCS concentrations used reduced the agonistic behavior of fish ($p < 0.05$) (Fig. 3a). Regarding the air respiratory rate, a decreasing linear effect was observed; that is, the higher the concentration of EO used, the lower the capture of atmospheric air (Fig. 3b). Ethanol, when applied alone, did not influence the agonist behavior of the fish, nor did it change the air respiratory rate.

Transport procedure

Results show that the EOCS did not change in the water parameters at the end of the transport procedure; in the two groups evaluated, dissolved oxygen, temperature, pH,

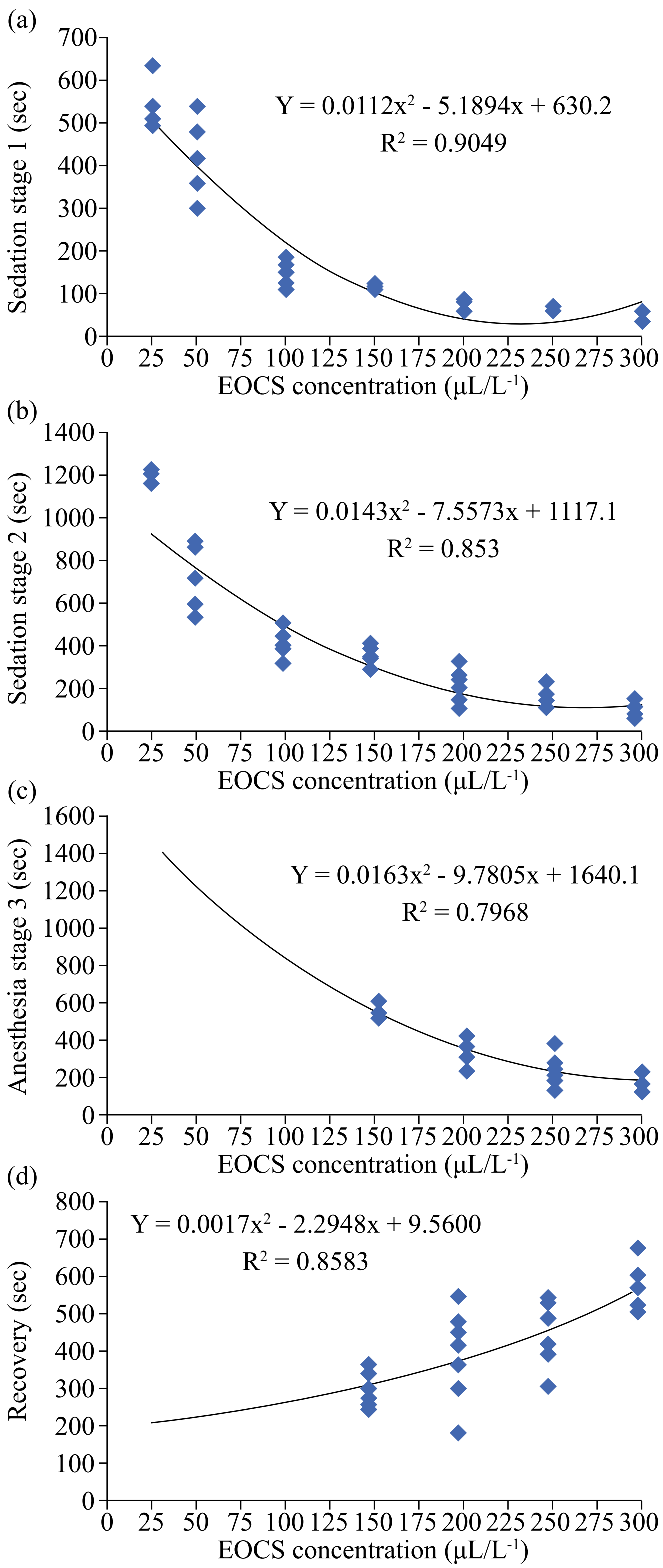

Figure 2. Sedation, anesthesia and recovery of *Betta splendens* exposed to different concentrations of *Citrus sinensis* essential oil. Stage 1 and 2 sedation (a and b, respectively), induction of deep anesthesia (c), and recovery times (d).

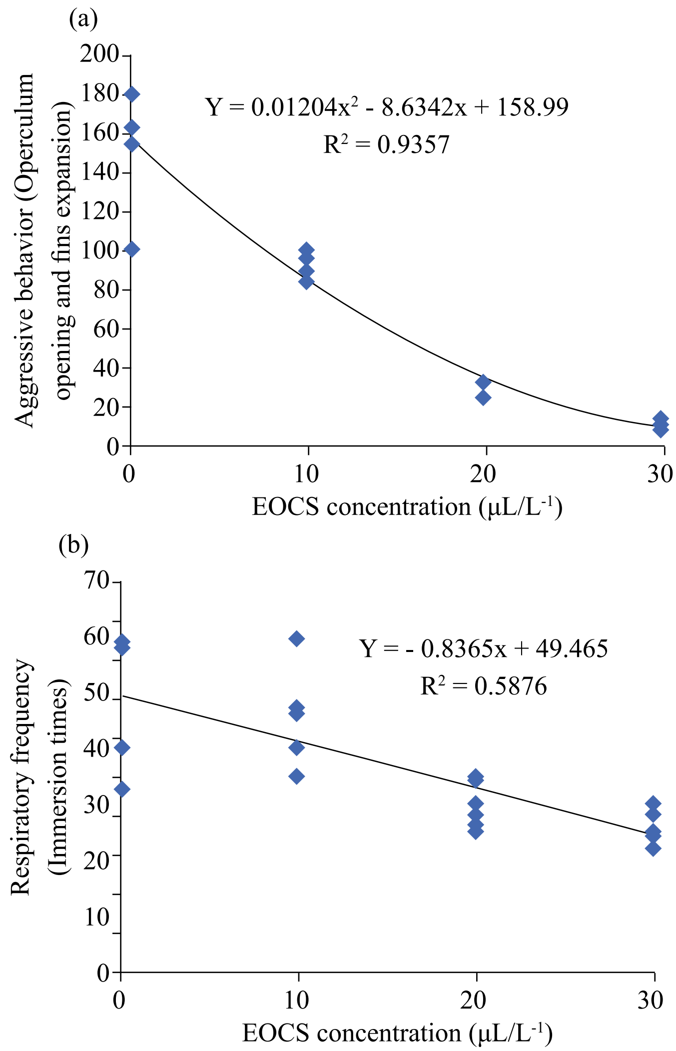

Figure 3. Agonist behavior (a) and air respiratory rate (b) of adult males of *Betta splendens* during 30 min of exposure to different concentrations of *Citrus sinensis* essential oil.

total ammonia, and un-ionized ammonia were 7.73 mg·L⁻¹, 26.7°C, 6.6, 0.5 mg·L⁻¹ N-NH3 and 0.001 mg·L⁻¹ N-NH3, respectively. As expected, the concentration of EOCS used did not induce deep anesthesia throughout the 6 h of transportation. In addition, it maintained the integrity of the fish without causing the incidence of mortality during the experiment or in the first 48 hours after the procedure. Fish that were not transported or transported with 0 and 20 µL·L⁻¹ EOCS did not show gill lesions or cellular changes. However, when comparing histometric data with Duncan's test, a significant reduction of gill filament height was observed in the group exposed to 20 µL L⁻¹ EOCS ($p < 0.0001$) compared to the basal group and control group (Table 1).

Table 1. Height of *Betta splendens* gill filaments exposed to EOCS during transport. Means followed by the same letters do not differ by Duncan's test at 5% probability ($p < 0.05$). CV (%) = coefficient of variation.

	Treatments			CV (%)	p-value
	Basal group	0.0 μL·L⁻¹ EOCS	20 μL·L⁻¹ EOCS		
Height (μm)	60.12 a	55.62 a	43.57 b	14.55%	0.0001

DISCUSSION

The use of substances that can reduce stress responses during transport can be beneficial for fish, especially when these animals are transported for long distances. It is recommended that fish be sedated (decrease in metabolic rate and loss of reactivity to external stimuli) through transport, but that equilibrium must be maintained (Pirhonen and Schreck, 2003; Summerfelt and Smith, 1990). In this sense, EO can constitute a valuable alternative to traditional anesthetics because they present many desirable qualities as such as short induction and recovery times, least noxious and economically viable (Bolasina et al., 2017; Can et al., 2018; Mitjana et al., 2014). However, even EOs at certain concentrations can be highly toxic to fish, leading to death (Pattanasiri et al., 2017a; Waristha et al., 2011) or severe histological changes in fish gill tissues (Brandão et al., 2021).

According to the results obtained for *B. splendens,* the LC50 at 48 h of exposure to EOCS was 49.17 $\mu L \cdot L^{-1}$, and the highest percentages of survival were obtained with 20 and 30 $\mu L \cdot L^{-1}$ EOCS (100 and 83%, respectively). Waristha et al. (2011) investigated the toxicity of clove EO in male *B. splendens* at six months of age and observed that the (LC50-96h) of clove oil was 26.3 $\mu L \cdot L^{-1}$. Thus, EOCS can be considered less toxic than clove essential oil. However, it is necessary to consider the longer exposure time of fish to clove EO. In another study, Pattanasiri et al. (2017a) evaluated different concentrations of clove oil and eugenol for the toxicity of these products to *B. splendens* and found that, at 48 h, the LC50 of clove oil and eugenol was in the range of 30.63 $\mu L \cdot L^{-1}$ and 29.95 $\mu L \cdot L^{-1}$, respectively.

As for anesthesia induction and recovery in *B. splendens* the lowest concentration of EOCS used in the present study (25 $\mu L \cdot L^{-1}$) caused only mild sedation at approximately 8.3 min, and a concentration of 150 $\mu L \cdot L^{-1}$ was necessary to induce deep anesthesia in this species within 10 min. Waristha et al. (2011) found a similar sedation induction time for *B. splendens* with 20 $\mu L \cdot L^{-1}$ clove EO (approximately 12 min). Pattanasiri et al. (2017a) studied the sedative and anesthetic effects of clove EO and eugenol on *B. splendens* and observed that 25 $\mu L \cdot L^{-1}$ of both products could sedate fish in 18.7 min and 13.7 min, respectively; similarly, 50 $\mu L \cdot L^{-1}$ of the two anesthetics caused deep anesthesia within 20 min.

The results show that EOCS can provide optimal sedation induction times for *B. splendens*. However, only concentrations of 200, 250 and 300 $\mu L \cdot L^{-1}$ of EOCS caused anesthesia more rapidly. In general, anesthesia or sedation should be induced rapidly, preferably in less than 3 minutes, and with minimum accompanying hyperactivity or other stress (Ross, L. and Ross, B., 2008). Lopes et al. (2018) found high induction times for *R. quelen* anesthetized with 400, 600, and 800 $\mu L \cdot L^{-1}$ of *Citrus* x *aurantium* EO. According to the authors, the highest concentration of *C.* x *aurantium* EO (800 $\mu L \cdot L^{-1}$) provided the best induction time for this species (8.2 min).

According to Lopes et al. (2018), the sedative and anesthetic effects of citrus EOs in fish are caused by additive and/or synergistic interactions between several compounds, such as limonene, β-pinene, α-pinene, and γ-terpinene. Limonene has been identified as one of the main components of EOs involved in fish anesthesia (Becker et al., 2018) and this compound can bind directly to the adenosine A_{2A} receptor, producing anesthetic effects in fish (Park et al., 2011). In this since, *Aloysia triphylla* EO containing 21.6% limonene was effective in anesthetizing *Serrasalmus rhombeus* and *Cinnamomum camphora* EO, which presents 30% limonene, produced different anesthetic stages in *Amphiprion ocellaris* (Almeida et al., 2018; Pedrazzani and Ostrensky Neto, 2016). In the present study, the EOCS used presented 93.2% limonene; thus, its anesthetic effect on *B. splendens* was expected.

In addition, β-pinene, another major component of EOCA, may contribute to the anesthetic effect (Kasanen et al., 1998; Mercier et al., 2009). The EO of the leaves of *Hyptis mutabilis*, containing 7.9% β-pinene, induced anesthesia in *R. quelen* (Silva et al., 2013). α-pinene is reported as a positive modulator of GABAA receptors (Komiya et al., 2006). These receptors are recognized as important targets for the modulation of sedative, anxiolytic and general anesthetic agents.

Our results also showed the existence of an inverse relationship between the concentration of EO used and the induction anesthetic time; that is, as the concentration of EOCS increased, the induction time decreased. Regarding recovery, an effect opposite to that of induction was observed since the higher the concentration, the longer the time spent for the fish to reach full recovery. In this sense, similar results of anesthetic induction and recovery have already been reported for other ornamental fish species, such as *Carassius auratus* with EO of *Aniba rosaeodora* (Kizak et al., 2018), *Sciaenochromis fryeri* and *Labidochromis caeruleus* with EO of *Pelargonium graveolens* (Can et al., 2018), and *Aulonocara nyassae* with benzocaine and menthol (Ferreira et al., 2020).

Male *B. splendens* are naturally aggressive fish, with well-studied and standardized behavioral responses. According to the literature, intimidation behaviors in this species include lateral displays, gill flaring, and fin spreading, while the main attack behavior is the bite (Castro et al., 2006; Greene and Szalda-Petree, 2022). In our study, fish treated with EOCS showed less intimidation behaviors, such as gill flaring, and fin spreading. Indicating that possibly the EO used has properties on the mechanisms that trigger aggressive behavior in this species.

In *B. splendens*, aggressive behavior is the result of a polygenic structure (Zhang et al., 2022) and is also associated with several neuro-physio-endocrine factors. In the same work, the authors hypothesized that, during a fight between two animals, there is an increase in air breathing, which is related to greater efficiency in maintaining a metabolism associated with lactate, due to the greater need for muscle contraction responsible for the attacks.

This was the first work to describe the use of EO in the reduction of aggressiveness in fish. The use of EOCS minimized the incidence of aggressive behavior by *B. splendens*; thus, the oxygen demand may not have been altered, and consequently, fish emerged less from the water surface to capture oxygen (Castro et al., 2006), as seen in the present study. EOCS may also have influenced fish through its sedative effects, as sedated fish tend to decrease respiratory activity at the expense of decelerating their metabolism (Teixeira et al., 2018).

In addition to EOs, other plant-based products showed positive effects when administered to live fish transport water. For example, the use of a 260 $\mu L \cdot L^{-1}$ water conditioner based on *Aloe vera* during simulated transport lasting 1 h significantly reduced the number of bites in *Poecilia reticulata*. In *Xiphophorus variatus*, this same product at a concentration of 125 $\mu L \cdot L^{-1}$ caused reduced levels of erratic swimming and aggressive behavior after international transport of approximately 30 h (Vanderzwalmen et al., 2020).

The use of fluoxetine, a serotonin inhibitor, also helps to reduce aggressive behavior in *B. splendens* (Eisenreich and Szalda-Petree, 2015). Future studies should be carried out to elucidate the genetic and metabolic mechanisms that act to reduce this behavior in fish.

According to Becker et al. (2012), the anesthetic concentrations used during the transport of live fish should induce, at most, stage 2 anesthesia (deep sedation stage). Light sedation allows fish to maintain balance, swimming activity, and breathing (Santos et al., 2020). In addition, the sedative concentrations of EOs are sufficient to improve some physiological indicators during fish transport, thereby contributing to the maintenance of homeostasis (Salbego et al., 2017). In the present study, the concentration of EOCS used to transport *B. splendens* for 6 h was 20 $\mu L \cdot L^{-1}$, since exposure to this concentration in this period only caused sedation in the fish, without anesthesia.

In sedation, the fish showed less mobility in the plastic transport bag but maintained balance and normal swimming activity. Deeply sedated fish tend to lose balance and the ability to swim and may suffocate when remaining at the bottom of the transport container (Parodi et al., 2014). Similar sedation was observed in *B. splendens* transported with clove oil (10 $\mu L \cdot L^{-1}$) by Pattanasiri et al. (2017b) and in *R. quelen* with eugenol (1.5 or 3.0 $\mu L \cdot L^{-1}$) or *Lippia alba* EO (10 or 20 $\mu L \cdot L^{-1}$) by Salbego et al. (2017).

Betta splendens is known for its aggressiveness; extremely territorial males of this species can initiate aggressive fights in defense of their territory (Castro et al., 2006; Craft et al., 2003). Agnostic interactions in males of this species can usually last for more than 1 h and such disputes involve stereotyped social displays with biting/blows and mouth blocking (Vu et al., 2021). In the case of direct fights, serious damage can occur to one of the fish, causing death (Castro et al., 2006). Therefore, it is recommended to pack the males of this species individually to avoid injuries and stress during transport (Monticini, 2010).

As this study also sought to assess the to evaluate the viability of EOCS in the collective transport of males of this species, it was decided to pack the fish collectively. Thus, it was possible to observe that the fish transported with 20 $\mu L \cdot L^{-1}$ EOCS had intact fins, while the animals in the control group showed damaged fins. Therefore, the reduction of aggressive behavior, as observed with the addition of EOCS, is desirable, as it can contribute to the maintenance of fish well-being and allow the storage of more than one male *B. splendens* per plastic transport bag.

Previous studies show that the use of EOs in fish transport can bring several benefits, such as maintenance water quality, reduction stress and improves the visual appearance of fish after transport (Aydın and Barbas, 2020; Oliveira et al., 2022; Teixeira et al., 2018). In this regard, Pattanasiri et al. (2017b), showed that the addition of clove oil (10 $\mu L \cdot L^{-1}$) to the transport water of males *B. splendens* can improve the visual quality of the fish. The authors did not carry out collective transport of the animals, but identified that fish from the control group had lower visual quality compared to fish transported in the presence of essential clove oil. Similarly, Wang et al. (2021) evaluated the addition of eugenol (0, 10, and 20 $\mu L \cdot L^{-1}$) and thymol (0, 10, and 20 $\mu L \cdot L^{-1}$) in the transport water of juveniles of *Ictalurus punctatus* and recommended the use of eugenol in the concentration range of 10 to 20 $\mu L \cdot L^{-1}$ and thymol at 10 $\mu L \cdot L^{-1}$ to transport this species over long distances since they promoted lower levels of serum cortisol in fish.

Fish gills are responsible for many important physiological processes, such as water exchange and salt and play an important role in removing ammonia (Koca and Kara, 2022). Thus, changes in gill tissues are of concern as they can impair normal physiological functions (Oliveira et al., 2022). Here we show that the administration of 20 $\mu L \cdot L^{-1}$ of EOCS in the transport water of *B. splendens* does not cause any histological or cellular changes. However, histometric analyzes showed a reduction in the height of the gill filaments of fish exposed to EOCS. This change in the height of the filaments can be considered an adjustment to the imposed conditions, once the gill epithelium is in direct contact with external environment, which makes it highly susceptible to environmental variations (Santos et al., 2020).

According to Brandão et al., (2021), the changes suffered by the gill tissue indicate the development of adaptive strategies by fish to ensure the maintenance of important physiological functions. In addition, aquatic organisms when exposed to external elements in lethal or sublethal concentrations may present different biochemical, physiological, histological and morphometric changes in the gill tissues (Koca and Kara, 2022). In *Pterophyllum scalare*, the histopathological analysis of the gills showed that exposure to 10 $\mu L \cdot L^{-1}$ of *Lippia sidoides* oil or *Cymbopogon citratus*, under transport conditions for 8 h, caused adjustment alterations, such as epithelium displacement and fusion of secondary lamellae (Oliveira et al., 2022). Similarly, Santos et al. (2020) found different types of histopathological changes associated with a combination of tea tree and clove oils (10.4 $\mu L \cdot L^{-1}$) during the transport of juvenile tambaqui *Colossoma macropomum*.

The use of essential oils as sedatives in the transport of fish must be carried out carefully, as they can affect some parameters of the water and compromise the survival of the animals (Lopes et al., 2018). In the present study, the concentrations of EOCS used in the experimental transport did not cause changes in the water quality or mortality rate of male *B. splendens* at the end 6 h of transport. In contrast to these results, Pattanasiri et al. (2017b) obtained higher levels of dissolved oxygen, lower levels of un-ionized ammonia, a reduction in water pH, and a lower mortality rate for *B. splendens* transported in water containing clove essential oil. The maintenance of the pH observed in the present study may have occurred due to the aerial facultative respiration of the beta compared to other fish species previously studied. In this case, as there is no direct incorporation of CO_2 into the water, there is no acidification of the water associated with the dissolution of this compound in an aqueous medium.

CONCLUSION

Based on the results of the present study, the LC50 in 48 h of the essential oil of *C. sinensis* was 49.17 $\mu L \cdot L^{-1}$. *Citrus sinensis* essential oil has the potential to be used as a sedative and anesthetic agent for *B. splendens*, providing acceptable induction times for this species. In addition, a concentration of 20 $\mu L \cdot L^{-1}$ of *C. sinensis* essential oil is recommended for the collective transport of *B. splendens*, as such a concentration did not cause mortality or reduced visual quality of animals transported together. The water quality parameters were not changed during transport, despite the recovery time being above that recommended for fish.

ETHICAL APPROVAL

The experiment was approved by the Ethics Committee on the Use of Animals (CEUA/UFRRJ/IZ), process 0108-08-2020.

CONFLICT OF INTEREST

Nothing to declare.

DATA AVAILABILITY STATEMENT

All dataset were generated or analyzed in the current study.

AUTHOR CONTRIBUTIONS

Conceptualization: Silva RC, Ramos LRV; **Data curation:** Silva LR, França IF, Pantoja BTS; **Formal analysis:** Pereira MM, Ramos LRV; **Research:** Silva RC, França IF, Silva LR, Pantoja BTS; **Methodology:** Silva RC, Ramos LRV, Lopes JM; **Supervision:** Ramos LRV, Lopes JM; **Writing - Preparation of original draft:** Silva RC, Ramos LRV; **Writing - Proofreading and editing:** Lopes JM, Pereira MM.

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