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Original article

Essential oils from *Citrus* x *aurantium* and *Citrus* x *latifolia* (Rutaceae) have anesthetic activity and are effective in reducing ion loss in silver catfish (*Rhamdia quelen*)

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This study investigated the anesthetic effect of the essential oils (EOs) from the peel of *Citrus* x *aurantium* (EOCA) and *Citrus* x *latifolia* (EOCL) on silver catfish *Rhamdia quelen*. Fish were exposed to different concentrations of EOCA and EOCL to determine time of anesthesia induction and recovery. Induction of anesthesia was observed in all fish exposed to 400, 600 or 800 μL L^{-1} EOCA and 300, 400 or 500 μL L^{-1} EOCL. Another group of fish were exposed for 8 h to 50, 100, or 200 μL L^{-1} of either EOs. Overall, fish exposed to ethanol and both EOs presented higher ventilatory frequencies (VF) than the control group throughout the 8 h of exposure. Net ion (Na^+, K^+ and Cl^-) effluxes and ammonia excretion were significantly lower in fish exposed to 50, 100 or 200 μL L^{-1} of either EOs compared to control fish. Mortality was 37% in fish exposed to 200 μL L^{-1} of either EOs after 8 h. These findings suggest that EOCA and EOCL are useful anesthetics and sedatives for *Rhamdia quelen*, but their usefulness as alternatives to reduce stress in fish transportation at the lower concentrations tested (50-100 μL L^{-1}) deserves further study.

Keywords: Anesthesia, Ion fluxes, Lemon oil, Orange oil, Ventilatory frequency.

O efeito anestésico dos óleos essenciais (OEs) da casca de *Citrus* x *aurantium* (OECA) e *Citrus* x *latifolia* (OECL) em jundiá *Rhamdia quelen* foi investigado. Os peixes foram expostos a diferentes concentrações de OECA e OECL para determinar o tempo de indução e recuperação da anestesia. Todos peixes expostos a 400, 600 ou 800 μL L^{-1} OECA e 300, 400 ou 500 μL L^{-1} OECL foram anestesiados. Outro grupo de peixes foi exposto aos OEs durante 8 h a 50, 100 ou 200 μL L^{-1}. Peixes expostos ao etanol e aos OEs apresentaram VF maior que o grupo controle durante as 8 h de exposição. Os efluxos líquidos de Na^+, K^+, Cl^- e a excreção de amônia foram significativamente menores nos peixes expostos a 50, 100 ou 200 μL L^{-1} dos OEs em comparação com o grupo controle. A mortalidade foi de 37% nos peixes expostos a 200 μL L^{-1} de ambos os OEs após 8 h. Os resultados sugerem que OECA e OECL são anestésicos e sedativos úteis para o jundiá, mas sua utilidade como alternativa para reduzir o estresse no transporte de peixes nas concentrações mais baixas testadas (50-100 μL L^{-1}) necessita de estudos adicionais.

Palavras-chave: Anestesia, Fluxo de íons, Frequência ventilatória, Óleo de laranja, Óleo de limão.

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Introduction

Anesthetics have been used to reduce stress effects in animals caused by capture, handling and transportation (in this situation, at sedative concentrations), avoiding the occurrence of physiological, biochemical and molecular changes (Ross, Ross, 2008; Gressler *et al*., 2012; Parodi *et al*., 2014; Benovit *et al*., 2015; Souza *et al*., 2017). The transportation of live fish, routinely performed in aquaculture, is one of the main factors that cause stress in fish (Barcellos *et al*., 2001; Becker *et al*., 2012). When stress is induced by this factor, fish react by consuming more energy, which impacts considerably on the maintenance of homeostasis (Park *et al*., 2008). Several studies have examined the effect of natural products with anesthetic properties, such as plant extracts and EOs, that coupled with their antioxidant properties are a promising alternative to traditional synthetic drugs (Guénette *et al*., 2007; Becker *et al*., 2012; Zeppenfeld *et al*., 2014) and can help to minimize stress effects (Azambuja *et al*., 2011; Toni *et al*., 2014; Becker *et al*., 2016). In addition, EOs have been recommended for fish anesthesia due to their low costs, easy accessibility, efficacy and environmental safety (Iversen *et al*., 2003; Cunha *et al*., 2011; Pedrazzani *et al.,* 2016).

Citrus (Rutaceae) includes several species of plants that produce some of the most cultivated fruits in the world, including oranges, bergamots and lemons, which have an appreciable content of essential oil in their peel (Kummer *et al.,* 2013). Essential oils (EO) are extracted from the pericarp or peel of the fruits and consist mainly of monoterpenes and sesquiterpenes and phenylpropanoids, metabolites that confer their organoleptic characteristics (Astani *et al*., 2010; Acar *et al*., 2015). Brazil is one of the main suppliers of the EOs of orange, lemon and other citrus fruits, with *Citrus x aurantium* L., *Citrus x sinensis* and *Citrus x latifolia* being the species of major commercial importance (Bizzo *et al*., 2009; Mattos Jr *et al*., 2010). EOs of plants of this genus have been used in the treatment of anxiety or insomnia in humans (Lehrner *et al*., 2005), and oils of different species have shown promising results in preclinical trials using rodents. The most noteworthy among these are *C. x sinensis* (Faturi *et al*., 2010) and C. *x limon* (Ceccarelli *et al*., 2004) in rats, and *C. x latifolia* (Gargano *et al*., 2008) and *C. x aurantium* (Carvalho-Freitas, Costa, 2002; Pultrini *et al*., 2006) in mice. Sedative and anxiolytic-like effects have been described for the EO obtained from the peel of *Citrus x aurantium* in rats and mice (Leite *et al*., 2008; Costa *et al*., 2013). However, the literature does not present reports of their anesthetic effects on fish.

Mindful of this, the aim of this study was to evaluate the effectiveness of the EOs of *C. x aurantium* (EOCA) and *C. x latifolia* (EOCL) as anesthetics and sedatives for the silver catfish, *Rhamdia quelen* (Quoy & Gaimard). In addition, we investigated ion regulatory balance, survival rates, ventilatory frequency (VF) and water parameters during long-term exposure to EOs, to analyze their possible use as sedative in transport.

Materials and Methods

Animals. The experiments were conducted in the Fish Physiology Laboratory at the Universidade Federal de Santa Maria (UFSM), Rio Grande do Sul (RS), Brazil. Silver catfish (mean ± SEM, 17.88 ± 3.42 g; 12.86 ± 1.35 cm) (voucher UFRGS 22661) were obtained from a fish culture near Santa Maria, southern Brazil and acclimated to the laboratory conditions for seven days. Dissolved oxygen and temperature were measured with a Y5512 oxygen meter (YSI Inc., Yellow Springs, OH, USA). The pH was verified with a DMPH-2 pH meter (Digimed, São Paulo, SP, Brazil). Total ammonia nitrogen (TAN) and un-ionized ammonia (NH_3) levels were determined as described by Zeppenfeld *et al*. (2014).

The experimental protocol was approved by the Ethical and Animal Welfare Committee of the UFSM under registration number 074/2014.

Essential oil extraction, analysis and identification of constituents. The fruits were purchased from a local market in Santa Maria. Immediately after purchase, fruits were washed in tap water and peeled. The peels were processed for extracting EOCA and EOCL by hydrodistillation for 3 h in a Clevenger apparatus (Council of Europe, 2007) and stored at −4 °C until utilization. The qualitative analysis of the EOs was performed by gas chromatography-mass spectrometry-total ion chromatogram using an Agilent 7890A gas chromatograph coupled with an Agilent 5975C mass selective detector and employing a HP-5MS column (5% phenyl, 95% methylsiloxane, 30 m × 0.25 mm i.d. × 0.25 mm) as described by Pinheiro *et al*. (2016). The constituents were identified by comparison of the Kovats retention index and their mass spectra with data from the mass spectral library (NIST, 2010) and the literature (Adams, 2009). The quantitative analysis was performed with an Agilent 7890A gas chromatograph with flame ionization detection using the same parameters described by Pinheiro *et al*. (2016). The percentage of the chemical components was based on peak area normalization. The yields of EOCA and EOCL were 0.92% (v/w) and 0.45% (v/w), respectively.

Experiment 1: Anesthetic induction and recovery. Juvenile fish were individually transferred to aquaria containing 1 L of continuously aerated water and the EOs previously diluted in ethanol (1:10). Concentrations of 100, 200, 400, 600 or 800 μL L^{-1} of EOCA and 100, 200, 300, 400 or 500 μL L^{-1} of EOCL were used. These concentrations were determined after a pre-treatment trial. Ethanol controls were also tested at the same concentrations used for the dilutions corresponding to the highest EO concentrations. To evaluate the time required for anesthesia induction, 10 juveniles were used for each concentration and EO tested, and each juvenile was used only once, according to Silva *et al*. (2013a). All fish were fasted the day prior to sampling. The stages of anesthesia observed in the

present study are an adaptation of those detailed by Small (2003). The induction times were recorded during sedation, which were characterized by the decreased reactivity to external stimuli, and anesthesia, in which the total loss of equilibrium, cessation of locomotion and a lack of response to tactile stimuli occurred. The maximum observation time was 30 min. After induction, juveniles were transferred to anesthetic free aquaria to measure recovery time. Animals were considered to be recovered when they showed normal swimming behavior and response to external stimuli. After recovery, fish were grouped according to the anesthetic protocol and transferred into continuously aerated 250 L aquaria, where they were observed for 48 h for any signs of abnormal behavior, disease or mortality.

Experiment 2: Ventilatory frequency (VF) and ion fluxes. This experiment was conducted to evaluate the ventilatory frequency (per minute) of the fish exposed to EOCA or EOCL at concentrations of 50, 100 or 200 μL L^{-1} for 0, 0.5, 1, 2, 4 or 8 h, to evaluate the efficacy of these EOs to be used for fish transport at sedative concentrations. This experiment lasted 8 h because it is the usual time of transport of juveniles in South Brazil (Tondolo *et al.*, 2013; Cunha *et al.*, 2017) and the concentrations chosen were those which only induced sedation in the anesthetic induction experiment. Groups of fish were also exposed to water (control) and ethanol (1800 μL L^{-1}). One fish per aquarium (11 cm x 16 cm x 11 cm, n = 8 aquaria each concentration and EO; fish 16.59 ± 3.18 g; 12.70 ± 0.80 cm) was maintained with 1 L of water and the respective anesthetic concentrations. The VF was quantified by visually counting 20 successive opercular movements and measuring the elapsed time with a chronometer (adapted from Alvarenga, Volpato, 1995).

Water samples (5 mL) were collected at the beginning and at the end of exposure. Chloride levels were determined according to Zall *et al*. (1956) and Na^+ and K^+ levels with a B262 flame spectrophotometer (Micronal, São Paulo, Brazil). Net ion fluxes (*j*net) and ammonia excretion were calculated according to the equation:

$$j\text{net} = V([\text{ion}_1] - [\text{ion}_2])/Mt$$

Where $[\text{ion}_1]$ and $[\text{ion}_2]$ are the ion or ammonia concentrations in the water at the beginning and end of the exposure, respectively, V is the water volume (in L), M is the mass of the fish (in kg) and *t* is the duration of the experiment (h).

Statistical analysis. All data are represented as mean ± SEM, and were subjected to Levene's test to check homogeneity of variances. Evaluation of the anesthetic activity was performed by regression analysis (concentration × times of sedation, anesthesia induction and recovery). The comparisons of anesthetic activity between EOs (concentration 400 μL L^{-1}), ventilatory frequencies and net ion fluxes were analyzed using Kruskal-Wallis ANOVA test, followed by the multiple comparison of mean ranks for all groups. Differences between means were tested at the 95% probability level. Analysis was performed using the software STATISTICA ver. 7.0 (SPSS, Chicago, IL, USA).

Results

Essential oil analysis and identification of constituents. The major chemical components of EOCA were limonene (93.89%), linalool (2.60%) and β-pinene (1.71%). A total of 20 substances were identified in EOCL, the major constituents were limonene (49.73%), γ-terpinene (11.12%), α and β-citral (9.17%) and β-pinene (9.14%).

Experiment 1: Anesthesia induction and recovery in *R. quelen* exposed to EOCA and EOCL.

No mortality was registered throughout exposure to the EOs nor up to 48 h after the exposure. The concentrations of EOCA at 200–300 μL L^{-1} or EOCL at 100–200 μL L^{-1} caused only light sedation in 30% of the fish throughout the 30 min evaluation period. Increasing the concentration of the EOs proportionally decreased the time required for sedation and anesthesia induction, which occurred in all animals at 400–800 μL L^{-1} EOCA or 300–500 μL L^{-1} EOCL. An inverse relationship between the concentration of EO and the time to recover from anesthesia was observed with EOCA, but not with EOCL. All EOCL concentrations tested induced anesthesia within 7 min or less and recovery in 10 min or less. The lowest time of anesthesia induction (less than 3 min) and recovery (less than 5 min) was observed at 500 μL L^{-1} EOCL (Tab. 1).

Tab. 1. Time (s) required for induction and recovery from anesthesia using the essential oils of *Citrus x aurantium* (EOCA) and *Citrus x latifolia* (EOCL) in *Rhamdia quelen* (n = 10 each concentration). $^1Y = 10^{3.043-0.00097X}$ ($r^2 = 0.83$); $^2Y = 10^{3.361-0.00082X}$ ($r^2 = 0.92$); $^3Y = 10^{3.243-0.00087X}$ ($r^2 = 0.89$); $^4Y = 10^{2.672-0.0013X}$ ($r^2 = 0.97$); $^5Y = 10^{3.070-0.0017X}$ ($r^2 = 0.92$); ^{6}No significant relationship or difference between concentrations ($P > 0.05$). Equations represent relationships between the times of sedation, anesthesia or recovery and concentrations of EOs, where y = time to reach the stages (seconds) and x = EO concentrations (μL L^{-1}). *significantly different from the same concentration of EOCA ($P < 0.05$). Values are means ± SEM.

	Concentration (μL L^{-1})		
	EOCA		
	400	600	800
[1]Sedation	590 ± 83.7	208.1 ± 85.4	212 ± 74.2
[2]Anesthesia	1076.3 ± 102.8	876 ± 108.5	493.2 ± 115.7
[3]Recovery	703.2 ± 74.5	845.1 ± 141.1	349 ± 129.0
	EOCL		
	300	400	500
[4]Sedation	219.2 ± 19.5	144 ± 23.3*	117.4 ± 22.5
[5]Anesthesia	403 ± 12.4	216.2 ± 22.5*	175 ± 20.6
[6]Recovery	371.4 ± 56.7	517.1 ± 68.6	298.3 ± 64.5

Sedation and anesthesia with 400 μL L^{-1} EOCL was faster than with the same concentration of EOCA. Ethanol at the highest concentration used (7200 μL L^{-1}) to dilute the EOs did not produce any anesthetic effect when applied alone.

Experiment 2: Water parameters, net ion fluxes and ammonia excretion.

In the second set of experiments, the dissolved oxygen levels, temperature and NH_3 levels in the water did not exhibit any significant difference between the treatments before and after 8 h exposure to EOCA and EOCL. However, pH levels were significantly lower in groups exposed to all concentrations of either EOs (Tab. 2).

Fish of the control group presented net Na^+ influx and net K^+ and Cl^- effluxes. Exposure to ethanol, 50 μL L^{-1} EOCA or 200 μL L^{-1} EOCL provoked significantly higher net Na^+ efflux compared to control fish, while 50 or 100 μL L^{-1} EOCL significantly increased net Na^+ influx compared to control and ethanol-exposed fish. Fish exposed to 100 or 200 μL L^{-1} EOCA and 50 or 100 μL L^{-1} EOCL significantly increased net K^+ influx compared to control and ethanol-exposed fish. Fish exposed to 50 or 200 μL L^{-1} EOCA and 100 or 200 μL L^{-1} EOCL reduced net Cl^- effluxes and 50 μL L^{-1} EOCL significantly increased net Cl^- effluxes compared to control and ethanol-exposed fish. Ethanol did not significantly change net K^+ or Cl^- fluxes (Figs. 1a–b). Ammonia excretion was significantly lower in fish exposed to all concentrations of either EOs than in the control and ethanol groups throughout the 8 h exposure (Figs. 1c–d).

No mortality was recorded in fish exposed to 50μL L^{-1} or 100 μL L^{-1} EOCA or EOCL throughout or after exposure. However, mortality was 37% after 8 h exposure to 200 μL L^{-1} of either EOs.

Ventilatory frequency (VF). Ventilatory frequency decreased with time in all treatments. Fish exposed to ethanol, 50, 100 or 200 μL L^{-1} EOCA presented higher VF compared to the control group throughout the first 4 h of exposure, however at 200 μL L^{-1} EOCA after 8 h of exposure reduced VF was observed. Fishes exposed to 50 or 100μL L^{-1} EOCL had increased VF between 0.5–8 h compared to control fish, while those exposed to 200 μL L^{-1} EOCL had increased VF at the moment of exposure and after 8 h, but this was reduced between 0.5–2 h compared to control group (Tab. 3).

Discussion

Yields of both EOs obtained during this study can be considered good (EOCA was 0.92% v/w and EOCL 0.45% v/w) when compared to literature reports on the yield of *Citrus* oils, of 0.4% (Bizzo *et al*., 2009). *Citrus* oils represent a viable economic alternative considering their potential, yield and low acquisition cost. It is important to consider that the use of waste originating from the manufacture of juices of tropical and subtropical fruits reduces environmental contamination (Kobori, Jorge, 2005).

Both EOCA and EOCL were shown to contain limonene as the major component, in agreement with previous analysis of Sharma, Tripathi (2008) and Kummer *et al*. (2013). Besides EOCA and EOCL, other EOs that showed anxiolytic-like effects in animal models, such as *C. x aurantium*, *C. x reticulata*, *Foeniculum vulgare* and *Lippia alba* (limonene-carvone chemotype), have limonene as a major component (Gargano *et al*., 2008; Faturi *et al*., 2010; Saiyudthong, Marsden, 2011; Hatano *et al*., 2012; Mesfin *et al*., 2014). Limonene directly binds to the adenosine A_{2A} receptor, which may induce sedative effects (Park *et al*., 2011) and also demonstrated direct vasorelaxant effects (Kang *et al*., 2013). Vale *et al*. (2002) also reported the sedative and motor relaxant effects of limonene as a result of direct activity on the central nervous system (CNS). Additionally, the intraperitoneal injection of EOCA promoted a depressant activity in the CNS of mice, and this activity was attributed to limonene (Carvalho-Freitas, Costa, 2002).

Tab. 2. Water parameters after 8 h exposure of *Rhamdia quelen* to the essential oils of *Citrus x aurantium* (EOCA) and *Citrus x latifolia* (EOCL) added to the water. *significantly different from control ($P < 0.05$). Values are means ± SEM. Dissolved oxygen and un-ionized ammonia were expressed as mg/L^{-1} and temperature as °C.

Parameter	Concentration (μL L^{-1})				
	EOCA				
	Control	Ethanol	50	100	200
Dissolved oxygen	5.3 ± 0.09	5.4 ± 0.13	5.8 ± 0.12	5.8 ± 0.11	5.3 ± 0.13
pH	6.93 ± 0.05	6.73 ± .09	6.14 ± .06*	6.09 ± .06*	6.18 ± 0.06*
Temperature	23.5 ± 0.06	23.6 ±0.13	23.6 ± 0.04	23.8 ± 0.02	23.7 ± 0.05
Un-ionized ammonia	0.010.014 ± 0.09	0. 007±0.06	0.011 ±0.04	0.009 ± 0.03	0.011 ± 0.02
	EOCL				
	Control	Ethanol	50	100	200
Dissolved oxygen	5.4 ± 0.19	5.2 ± 0.09	5.4 ± 0.07	5.5 ± 0.08	5.7 ± 0.11
pH	7.21 ± 0.02	7.13 ± 0.02	6.23 ± 0.03*	6.19 ± 0.05*	6.12 ± 0.05*
Temperature	23.17 ± 0.02	23.30 ± 0.02	23.18 ± 0.04	23.19 ± 0.02	23.21 ± 0.03
Un-ionized ammonia	00. 0.034 ± 0.01	0.037± 0.01	0.054 ± 0.01	0.025 ± 0.001	0.037 ± 0.012

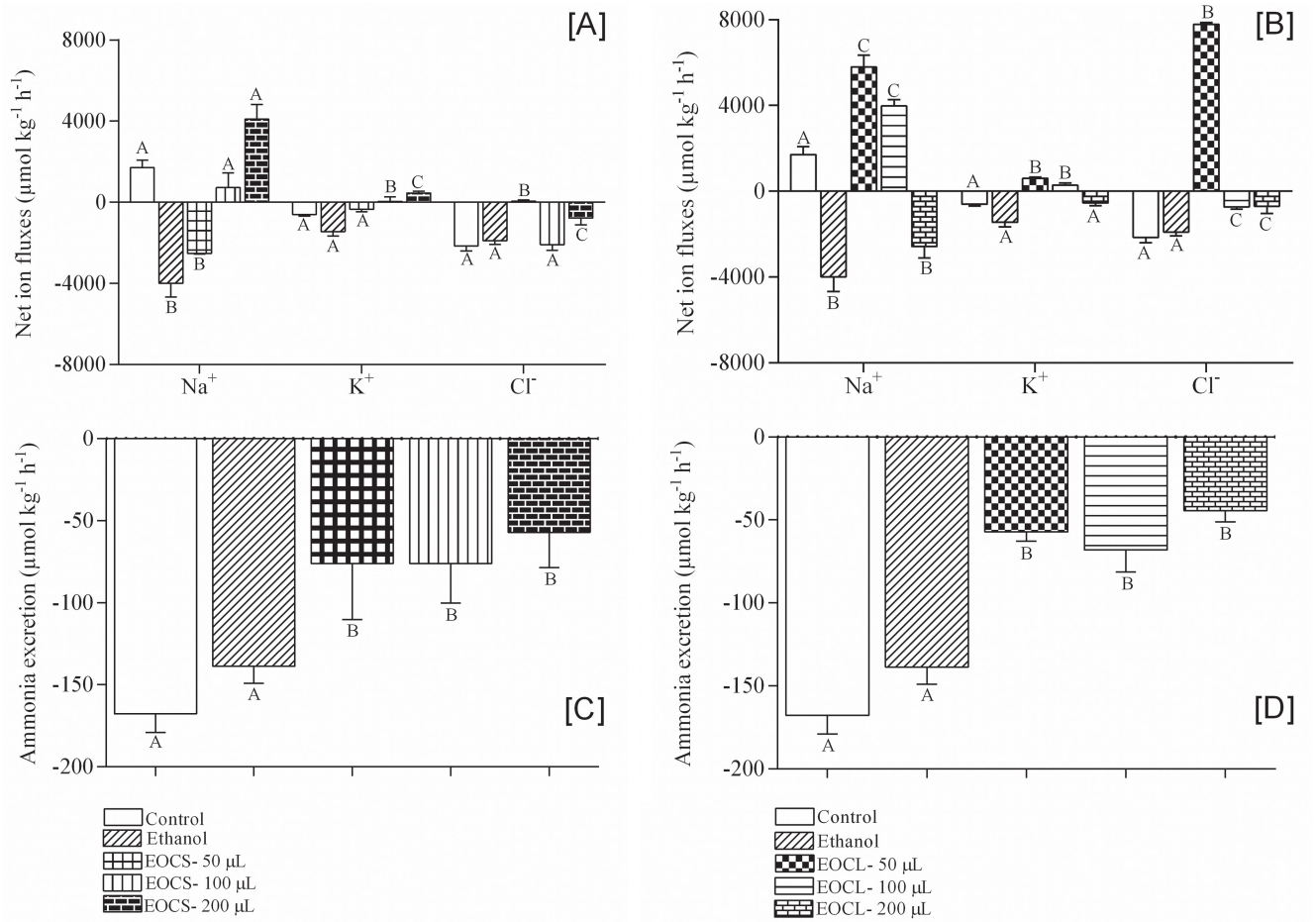

Fig 1. Net ion (Na^+, K^+, Cl^-) fluxes (**a-b**) and ammonia excretion (**c-d**) in silver catfish through 8 h of exposure to essential oils of *Citrus x aurantium* (EOCA) and *Citrus x latifolia* (EOCL). Values are means ± SEM. Different letters indicate significant differences between treatments ($P < 0.05$). Positive values indicate net influxes and negative values net effluxes.

Tab. 3. Ventilatory frequency (opercular or buccal movements min^{-1}) of *Rhamdia quelen* maintained in water with the essential oils of *Citrus x aurantium* (EOCA) or *Citrus x latifolia* (EOCL). Values are means ± SEM. Different capital letters in the rows indicate significant difference between treatments at the same time ($P < 0.05$). Different lowercase letters in the columns indicate significant difference between times in the same treatment ($P < 0.05$).

Time (h)	Concentration (µL L^{-1})				
	EOCA				
	Control	Ethanol	50	100	200
0	27.9 ± 0.4^{Ba}	29.0 ± 1.6^{Aa}	27.6 ± 0.5^{Ba}	28.5 ± 0.6^{Aa}	29.1 ± 1.1^{Aa}
0.5	24.0 ± 1.7^{Bb}	27.8 ± 1.6^{Ab}	25.6 ± 1.1^{Bc}	27.3 ± 1.3^{Ab}	27.4 ± 0.8^{Ab}
1	23.7 ± 2.2^{Bc}	27.6 ± 1.5^{Ab}	26.1 ± 0.9^{Ab}	26.8 ± 1.2^{Ac}	25.7 ± 2.5^{Ac}
2	24.0 ± 2.3^{Bb}	28.0 ± 1.7^{Ab}	26.2 ± 0.8^{Ab}	26.4 ± 1.1^{Ac}	25.4 ± 1.2^{Ac}
4	22.6 ± 0.9^{Bd}	27.1 ± 2.3^{Ab}	24.7 ± 0.9^{Ad}	24.3 ± 1.6^{Ad}	24.2 ± 1.6^{Ad}
8	23.4 ± 0.6^{Ac}	23.5 ± 1.3^{Ac}	23.4 ± 1.0^{Ae}	23.3 ± 1.4^{Ae}	20.9 ± 1.1^{Be}
	EOCL				
	Control	Ethanol	50	100	200
0	27.9 ± 0.5^{Ba}	29.0 ± 1.6^{Ab}	28.6 ± 0.9^{Aa}	29.0 ± 0.8^{Ab}	28.1 ± 0.8^{Aa}
0.5	24.0 ± 1.7^{Bb}	30.8 ± 1.6^{Aa}	28.8 ± 1.0^{Aa}	30.7 ± 1.2^{Aa}	22.8 ± 0.9^{Cc}
1	23.7 ± 2.2^{Bc}	30.6 ± 1.5^{Aa}	26.7 ± 1.0^{Ac}	30.1 ± 0.6^{Aa}	21.3 ± 0.9^{Cd}
2	24.0 ± 2.3^{Bb}	29.0 ± 1.7^{Ab}	25.6 ± 0.3^{Ad}	29.0 ± 0.6^{Ab}	20.5 ± 1.5^{Ce}
4	22.6 ± 0.9^{Bd}	27.1 ± 2.3^{Ac}	25.7 ± 0.5^{Ad}	28.5 ± 0.8^{Ac}	23.2 ± 1.4^{Bb}
8	22.3 ± 0.6^{Cd}	23.5 ± 1.3^{Bd}	27.8 ± 1.4^{Ab}	26.3 ± 0.9^{Ad}	23.1 ± 1.9^{Bb}

β-pinene, another major component of EOCA and EOCL, may contribute to the anesthetic effects of these EOs because D- and L-enantiomers of α- and β-pinene induced sedation and anesthesia in mice (Kasanen *et al*., 1998; Mercier *et al*., 2009). In addition, the EO of the leaves of *Hyptis mutabilis*, containing 7.9% β-pinene, induced anesthesia in silver catfish (Silva *et al*., 2013a). α-pinene is reported as a positive modulator of $GABA_A$ receptors (Komiya *et al*., 2006). These receptors are recognized as important targets for the modulation of sedative, anxiolytic and general anesthetic agents. Additionally, linalool, found in EOCA, has anesthetic effects in silver catfish (both S-(+) and R-(−) linalool isomers) (Silva *et al*., 2017) and common carp, *Cyprinus carpio* Linnaeus (Mirghaed *et al*., 2016), though its mechanism of action seems not to involve the benzodiazepine site of the GABAergic system (Heldwein *et al*., 2014). Citral, another major component of EOCL, increased barbituric sleeping time of mice (Vale *et al*., 2002) and blocks the excitability of rat sciatic nerves (Sousa *et al*., 2015). Citral is also the main component of other EOs that have anesthetic effects in fish: *Aloysia triphylla* (Gressler *et al*., 2012; Parodi *et al*., 2014; Santos *et al*., 2017 *Cymbopogon flexuosus* (Limma-Neto *et al*., 2016) and *L. alba* citral chemotype (Souza *et al*., 2017). The anesthetic effects of citral in fish does not involve the benzodiazepine site of the GABAergic system γ-terpinene, also a major component of EOCL, increases dopamine release from rat brain striatum slices (Fukumoto *et al*., 2006), but to our knowledge, no study so far has analyzed the sedative and/or anesthetic activities of this compound.

Silver catfish exposed to EOCA at 200–300 μL L^{-1} or to EOCL at 100–200 μL L^{-1} were not fully anesthetized, and presented only light sedation. The animals maintained the capacity of reaction to external stimuli, with reduced movements, but normal equilibrium, which would be recommended by Becker *et al.* (2012) for fish transportation. However, when exposed to 400-600 or 800 μL L^{-1} EOCA or 300-400 or 500 μL L^{-1} EOCL, the increasing concentration proportionally decreased the time required for sedation and anesthesia induction. The inverse relationship between the concentration of EO and induction time verified in this study had previously been observed: in *R. quelen* anesthetized with the EOs of *L. alba* (Cunha *et al*., 2010) and *A. triphylla* (Parodi *et al*., 2014); in *Centropomus parallelus* Poey, 1860 with the EO of *Nectandra megapotamica* (Tondolo *et al*., 2013); and in *C. carpio* with the EOs of *Melaleuca alternifolia* and *Mentha spicata* (Hajek, 2011; Roohi, Imanpoor, 2015).

Only the highest concentration of EOCL (500 μL L^{-1}) presented ideal times for induction (less than 3 min) and recovery (less than 5 min) according to Marking, Meyer (1985). However, 300–400 μL L^{-1} EOCL or 800 μL L^{-1} EOCA may also be suitable for anesthesia (up to 8 min). In addition, anesthetic concentrations that showed a longer recovery time may be appropriate for surgical procedures that require a delayed recovery after anesthetic exposure (Prince, Powell, 2000), and the lowest concentrations of EOCA (400–600 μL L^{-1}) would suit this purpose. The EOCL, which contains a lower percentage of limonene (49.7%) than EOCA (93.89%), showed better anesthetic effects, indicating that the other components (γ-terpinene, citral and β-pinene) may be more effective in inducing anesthesia in *R. quelen*. Therefore, it is possible that the activity of the major component is modulated by other minor molecules (Hoet *et al*., 2006).

The concentration range of EOCL required to induce anesthesia is similar to the EO of *Cinnamomum camphora* (30% limonene) in *Amphiprion ocellaris* Cuvier, 1830 (Pedrazzani, Ostrensky Neto, 2016) but overall, both EOCL and EOCA are less effective than other EOs tested in *R. quelen*, such as those from *L. alba* (Cunha *et al*., 2010), *Ocimum gratissimum* (Silva *et al*., 2012), *H. mutabilis* (Silva *et al*., 2013a), *Hesperozygis ringens* (Silva *et al*., 2013b), *A. triphylla* (Parodi *et al*., 2014) and *Ocimum americanum* (Silva *et al*., 2015).

Most fish anesthetics have an inhibitory effect on the respiratory system, which results in a lower ventilatory frequency (Keene *et al*., 1998), but this effect can vary depending on the concentration of the anesthetic (Roohi, Imanpoor, 2015). Ethanol increased VF in silver catfish, and EOCA and EOCL avoided this increase only for some periods in fish exposed to 200 μL L^{-1}, but these concentrations also provoked mortality. In *C. carpio* anesthetized with spearmint oil, VF first increased and then gradually decreased with increasing concentration of anesthetic (Roohi, Imanpoor, 2015) compared to control fish. Eugenol and the EO of *L. alba* (Becker *et al*., 2012; Hohlenwerger *et al*., 2016) also reduced VF in fish, but the EO of *H. ringens* did not change this parameter compared to control group (Toni *et al*., 2015).

Overall, the sedative concentrations of EOCA and EOCL that did not provoke mortality in silver catfish increased VF compared to control and ethanol groups. The higher VF could indicate higher stress and/or metabolism and the lower water pH observed at the end of 8 h exposure to all EOs concentrations could be related to carbon dioxide excretion by the fish, corroborating this hypothesis. Although water carbon dioxide concentration was not measured in the present study, aquaria were aerated all the time, and in this situation carbon dioxide usually remains low (Colt, Orwicz, 1991; Colt, Kroeger, 2013). However, CO_2 removal is not immediate and therefore can still react with carbonates from the water, reducing water pH (Moran, 2010; Colt *et al.*, 2012), as observed in the present study. In addition, the decrease of water pH could be due, at least partially, to higher net acidic equivalent loss, which could be provoked by stimulation of gill H^+-ATPase, as observed by Toni *et al.* (2014) in silver catfish exposed to the EO of *H. ringens*, or of the Na^+/H^+ antiporter, which would explain the higher net Na^+ influx induced by some concentrations of EOCA and EOCL.

Stress increases ion branchial efflux (McDonald *et al.*, 1991), but both EOs reduced ion loss at some concentrations

and also reduced ammonia excretion, which could be also related to a reduction of the metabolism. Net ion fluxes provided a good correlation with the stress of transport for silver catfish (Becker *et al.*, 2012, 2013; Parodi *et al.*, 2014; Zeppenfeld *et al.*, 2014; Garcia *et al.*, 2015). Sedation with eugenol and the EOs of *L. alba* and *A. triphylla* also reduced ammonia excretion and ion loss in silver catfish (Becker *et al*., 2012, 2016; Parodi *et al*., 2014; Zeppenfeld *et al*., 2014), methanolic extract of *Condalia buxifolia* and the EOs of *Cunila galioides* and *Origanum majorana* decreased ion loss in silver catfish (Becker *et al*., 2013; Cunha *et al.*, 2017). Eugenol also reduced Cl^- efflux during and after transport of *Salmo salar* (Iversen *et al*., 2003). The addition of linalool, a compound found in EOCA composition (2.60%), to the water of transport, also reduced ammonia levels in the water post-transport of *C. carpio*, but as there was also an increase on serum urea levels in the linalool-treated fish, Mazandarani *et al.* (2017) supposed that this compound may provoke improper ammonia excretion in the fish and conversion of ammonia to urea. It is not possible to rule out that the lower ammonia excretion induced in silver catfish by the EOs tested in the present study may cause the same effect because plasma ammonia or urea levels were not determined. Additional studies analyzing ammonia and urea excretion and plasma levels in fish exposed to the tested EOs must be performed to clarify this doubt.

The results suggest that EOCA and EOCL are effective anesthetics for silver catfish, but their usefulness as alternatives to reduce stress in fish transportation at the lower concentrations tested (50-100 $\mu L\ L^{-1}$) deserves further study because some results indicate that these EOs may increase metabolism.

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