Aquaculture 537 (2021) 736517

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Geraniol and citronellol as alternative and safe phytoconstituents to induce immobilization and facilitate handling of fish

Ednara Ronise L. de Araújo [a], Jhusicleide da Silva e Silva [b], Larissa Matias Lopes [b], Marcelo Ferreira Torres [b], Brenda M.P. Alho da Costa [b], Cristine Bastos do Amarante [c], Moisés Hamoy [d,*], Luis André L. Barbas [b,**], Luís André Sampaio [a,***]

[a] Laboratório de Piscicultura Estuarina e Marinha, Programa de Pós-graduação em Aquicultura, Universidade Federal do Rio Grande, Instituto de Oceanografia, Rio Grande, RS, Brazil
[b] Laboratório de Aquacultura de Espécies Tropicais, Instituto Federal de Educação Ciência e Tecnologia do Pará, Castanhal, PA, Brazil
[c] Coordenação de Ciências da Terra e Ecologia, Museu Paraense Emílio Goeldi, Belém, PA, Brazil
[d] Laboratório de Farmacologia e Toxicologia de Produtos Naturais, Instituto de Ciências Biológicas, Universidade Federal do Pará, Belém, PA, Brazil

ARTICLE INFO

Keywords:
Anaesthetic
Sedation
Electromyography
Fish welfare
Respiratory frequency
Electrocardiography

ABSTRACT

The efficacy of a product as an anaesthetic can be determined through combining behavioural assessment and the evaluation of selected electrophysiological markers, such as the electromyogram (EMG) and electrocardiogram (ECG) for muscle contraction power and cardiac function characterizations, respectively. This study aimed to evaluate the anaesthetic efficacy of geraniol (GRL) and citronellol (CTL) in tambaqui juveniles, *Colossoma macropomum*, through concentration-response trials, and their effects on behaviour and cardiorespiratory responses. Fish (24.78 ± 2.50 g) were assayed into two experimental groups: *I – Behavioural Assessment*: In which six concentrations were tested (10; 30; 50; 70; 90; and 110 μL L^{-1}) for each compound. For both isolates the concentration of 10 μL L^{-1} did not induce any signs of anaesthesia. As for the other concentrations, all fish were anaesthetized and fully recovered according to behaviour evaluation. Concentrations of 70 μL L^{-1} GRL and 90 μL L^{-1} CTL were considered the most effective doses as they promoted anaesthesia and allowed for recovery within appropriate time intervals; *II – Electrophysiological Characterization*: Concentrations of 70 μL L^{-1} GRL and 90 μL L^{-1} CTL were used for the recordings of EMG, ECG, opercular beat intensity and rate (OBI and OBR), and heart rate (HR). For both experiments, nine fish per concentration ($n = 9$) per analysis were used and each animal was considered a replicate. Our results demonstrated that geraniol and citronelol induced full body immobilization, which resulted at least in part, from the myorelaxant properties of these compounds. GRL and CTL at 70 μL L^{-1} and 90 μL L^{-1}, respectively were sufficient to render fish fully and rapidly immobilized. Both products transiently reduced ventilation during anaesthesia, nevertheless allowing for complete recovery after exposure. Although either isolate significantly decreased heart rates, they did not compromise resumption of normal cardiac function. In general, no mortalities were observed and all animals recovered after exposure. Both products could be considered safe alternatives for fish handling and other aquaculture-related activities that might require fish immobilization.

1. Introduction

Intensive aquaculture demands close monitoring and frequent handling of aquatic organisms for biometrics, transport, spawning, and other potentially stressful procedures (Façanha and Gomes, 2005; Ross and Ross, 2008). The use of stress-relieving agents such as anaesthetics

* Correspondence to: M. Hamoy, Laboratório de Farmacologia e Toxicologia de Produtos Naturais, Universidade Federal do Pará, UFPA Campus Belém, Rua Augusto Corrêa n° 01 Guamá, CEP 66075–110 Belém, PA, Brazil.
** Correspondence to: L. A. L. Barbas, Laboratório de Aquacultura de Espécies Tropicais, Instituto Federal de Educação Ciência e Tecnologia do Pará, IFPA Campus Castanhal, BR 316, Km 63 S/N, CEP 68740–970 Castanhal, PA, Brazil.
*** Correspondence to: L. A. Sampaio, Laboratório de Piscicultura Estuarina e Marinha, Universidade Federal do Rio Grande, FURG, Instituto de Oceanografia, CP 474, CEP 96201–900 Rio Grande, RS, Brazil.
E-mail addresses: hamoyufpa@gmail.com (M. Hamoy), andre.barbas@hotmail.com (L.A.L. Barbas), sampaio@mikrus.com.br (L.A. Sampaio).

https://doi.org/10.1016/j.aquaculture.2021.736517
Received 29 October 2020; Received in revised form 11 February 2021; Accepted 12 February 2021
Available online 16 February 2021

has increased in fish farming to mitigate stress, ensure increased yields, better health conditions and survival rates, and also for welfare purposes (Sneddon, 2012; Barbas et al., 2017a, 2017b; Aydın and Barbas, 2020).

A general anaesthetic should act promptly on the central nervous system (CNS) without further complications to the fish. Moreover, anaesthetics are expected to reversibly promote CNS depression with loss of sensation and response to environmental stimuli (Sylvester, 1975; Ross and Ross, 2008). The choice of an anaesthetic is usually associated with economic feasibility and depends on legal constraints.

Several synthetic substances have been used to induce anaesthesia and facilitate stress prevention, including products such as benzocaine and methanesulfonate of tricaine (MS 222), which are commonly used drugs (Gomes et al.,2001; Cotter and Rodnick, 2006; Sneddon, 2012). However, some studies have warned about undesirable effects such as bleeding, hyperglycaemia, loss of mucus, olfactory problems, intense irritability, damage to the corneas, stress and death (Losey and Hugie, 1994; Ross et al., 2007; Sneddon, 2012; Aydın and Barbas, 2020).

On the other side, several studies have shown advantages with the use of natural products, e.g., herbal extractives (essential oils, aqueous extracts, ethanolic and waxy plant extracts, etc.…) which were of similar or superior efficacy, showing less toxicity to fish compared to commonly used synthetic anaesthetics (Inoue et al., 2005; Parodi et al., 2012; Barbas et al., 2016; Barbas et al., 2017a, 2017b). Natural anaesthetics or plant-derived stress relieving products could be less expensive compared to synthetic drugs, without compromising safety and efficacy (Cho and Heath, 2000; Gonçalves et al., 2008).

Citronella grass, *Cymbopogon nardus*, is a plant originated from Sri Lanka and India (Mahalwal and Mahalwal and Ali, 2003) that has shown antibacterial and antioxidant properties in vitro being widely used in teas for its soothing properties (Castro et al., 2010; Park et al., 2015). Its anaesthetic activity has been suggested by Barbas et al. (2017a) when exposing juveniles of tambaqui, *Colossoma macropomum*, to citronella essential oil (EO) in baths. However, there is no information as to which major constituent of this oil would be responsible for the biological and behavioural effects observed in anaesthetized fish, or alternatively, if such effects could be a result of synergistic action among its phytoconstituents.

The major chemical compounds of *C. nardus* are the monoterpenes geraniol and citronellol, which are responsible for the characteristic odour of the EO (Castro et al., 2010). In addition to the use of these compounds in various commercial products, including cosmetics and fragrances, geraniol exerted a broad pharmacological activity as an anti-inflammatory, antioxidant, anti-ulcerative, neuroprotective, acaricidal, and antiseptic agent, inhibiting fungal and bacterial growth (Martins, 2006; Solórzano-Santos and Miranda-Novales, 2012). Moreover it presented cytoprotective and antioxidant effects in animal models subjected to oxidative stress (Tiwari and Kakkar, 2009). Citronellol has shown bacteriostatic and bactericidal activities (Lopez-Romero et al., 2015) as well as antifungal, antispasmodic, analgesic and anticonvulsant properties (De Sousa et al., 2006; Quintans-Júnior et al., 2008). Although these compounds are commercially available at relatively affordable prices, there are no studies on the potential of these isolates as general anaesthetics for fish.

Behavioural assessment is usually reported in literature as a standard methodology for fish anaesthesia evaluation; however, electrophysiological evaluation should be more frequently used to corroborate such observations as they increase the validity, objectivity, and consistency of the data. A number of important biological phenomena are accompanied by cellular electrical manifestations associated with selective ion permeability transport mechanisms (K^+, Na^+ and Cl^-) participating in the generation of membrane potential in cells. These ion fluxes are responsible for the electrical transmission in live animals through different channels, throughout the nervous system and participating in numerous physicochemical and biomechanical processes such as muscle contraction (Mudado et al., 2003; Delattre, 2007) and cardiorespiratory function (De Souza et al., 2019). The evaluation of the electromyogram, electroencephalogram, frequency and cardiorespiratory rhythms allows for a more detailed understanding of the neuropharmacological phenomena that take place in animals exposed to general anaesthetics. For more invasive procedures such as surgeries or euthanasia, recordings of these markers enable the evaluation of the muscle relaxation extent attained and CNS depression. Cardiorespiratory monitoring throughout and after anaesthesia will allow for a life-compatibility assessment and shed light on the impacts that might be implicated with these vital systems (Barbas et al., 2017b; De Souza et al., 2019).

Tambaqui, *C. macropomum*, is a native species to the Amazon River, Orinoco and its tributaries, being a common fish species in floodplain lakes and an economically important fish to many Northern Latin American countries. It is characterized by its resistance to farming conditions and diseases, showing good growth rates. Such features were essential for the improvement of its rearing conditions (Saint-Paul, 1986; Valladão et al., 2016).

Different studies have used juveniles of tambaqui in anaesthesia trials (Façanha and Gomes, 2005; Roubach et al., 2005; Barbas et al., 2016). This species has been reinforced as a promising in vivo model for tests with novel anaesthetics, in which not only the anaesthetic potential of herbal products has been evaluated, but also their stress relieving and antioxidant properties (Barbas et al., 2016; Barbas et al., 2017a, 2017b; De Souza et al., 2019; Aydın and Barbas, 2020).

Thus, this study aimed to evaluate the anaesthetic-like properties of geraniol and citronellol in tambaqui juveniles through concentration-response trials and their effects on behaviour, muscle contraction power and cardiorespiratory modulation.

2. Material and methods

2.1. Acquisition of the plant constituents

The oils were purchased from a commercial establishment (AROMACH ingredients™) and stored in amber glass bottles at 4 °C until use. According to the manufacture's information the products were certified for purity as follows: geraniol (GRL) (68.2%) and citronellol (CTL) (96.0%).

2.2. Animals and acclimation period

All procedures in this study were approved by the Animal Ethics Committee of the Federal Institute of Pará/IFPA Castanhal – Protocol # 6686081118 (ID 000021).

For this study, juveniles of tambaqui, *Colossoma macropomum* were purchased from a commercial farm with initial average weight of 3.0 ± 0.5 g. Prior to the experiments, animals were acclimated for 30 days to a recirculation system (six 300–L fibre tanks connected to a biofilter), continuously aerated, at a density of 33 fish per tank (~ 0.35 kg/m^3) and photoperiod set at 12 h Light: 12 h Dark. Water quality parameters were controlled and maintained as follows: pH 6.3 ± 0.6, temperature 28.5 ± 0.6 °C and dissolved oxygen 5.3 ± 0.4 mg L^{-1} were measured using a multiparameter equipment (HANNA™ HI9828). Total ammonia nitrogen (TAN) 1.97 ± 0.29 mg L^{-1} N-NH_3 was determined following methodology of Unesco (1983), nitrite 0.16 ± 0.02 mg L^{-1} N-NO_2 was evaluated according to Bendschneider and Robinson (1952), and alkalinity (57.7 ± 8.8 mg $CaCO_3$ L^{-1}) was evaluated by titration according to Eaton et al. (2005) guidelines.

The feed (commercial feed - 32% protein) was supplied three times daily until satiety.

For the trials, fish were assayed in groups (treatments) for the evaluation of times to anaesthesia and recovery (latencies) (Experiment I) and the effects of the compounds on selected electrophysiological markers (Experiment II) as described ahead.

2.3. Experiment I: behavioural assessment

Stock solutions of the compounds were prepared by pre-dilution in ethanol (96%) at a 1:9 ratio (oil: ethanol). Thereafter, anaesthetic efficacy of the oils was tested in six concentrations (10; 30; 50; 70; 90; and 110 μL L^{-1}) for each compound: GRL and CTL. After acclimation, nine fish (24.78 ± 2.50 g) per concentration were used and each animal was considered a replicate ($n = 9$). Latencies (in seconds) to anaesthesia and recovery stages were visually assessed and recorded with a digital stopwatch. For the anaesthesia tests, animals were transferred to aquaria (30*L*) containing 15 L water added by the respective anaesthetic concentration. For recovery evaluation an identical aquarium was used with the same volume of clean water. Fish were observed individually, always by the same observer and used only once. Two control groups (9 animals per group) were monitored in an aquarium similar to the one used in the tests. Sham fish were handled in anaesthetic/ethanol-free water and the other control group was exposed to ethanol-only added water (vehicle control) at a concentration which corresponded to the highest volume used to dilute the highest concentration of oil. The same water from the maintenance tanks was used throughout the behavioural evaluation.

The maximum observation time was 30 min, including the controls. Behavioural cues associated with induction and recovery were characterized according to Park et al. (2008) with modifications as suggested by Barbas et al. (2016): agitation (A1), loss of equilibrium and erratic swimming (A2) and absence of or minimum opercular beating with loss of reaction to tail pinch stimulus (A3) were used as behavioural indicators of anaesthesia induction; erratic swimming and recovery of equilibrium (R1), normal opercular beating and normal swimming (R2) were used as markers to evaluate recovery.

Upon reaching A3 stage, the fish were measured, weighed and immediately transferred to the anaesthetic-free aquarium, for the observation and registration of the different stages of recovery. The water was completely changed and the aquariums were washed after each test. After full recovery (R2), animals were handled back to their maintenance tanks, according to their respective treatments, and monitored for 72 h to check for mortalities.

2.4. Experiment II: electrophysiological characterization

The concentrations used for the recordings were 70 μL L^{-1} GRL and 90 μL L^{-1} CTL, as they promoted deep and fast immobilization, i.e., anaesthesia (< 3 min) (see results in Table 1). For the recordings of the electrical signals and analyses of the acquired data, the methodologies of Barbas et al. (2017a) and De Souza et al. (2019) were followed.

The juveniles of tambaqui (24.78 ± 2.50 g) were assayed into four groups: a) control (basal), b) fish submitted to GRL induction and recovery, c) fish submitted to CTL induction and recovery, and d) fish submitted to ethanol (vehicle control) and recovery (if applicable). Recordings of electromyography (EMG), opercular beat intensity (OBI) and opercular beat rate (OBR), electrocardiography (ECG) and heart rate (HR) were then carried out. Nine fish per electrophysiological recording were used ($n = 9$), including controls and ethanol-exposed fish for each indicator. Mean values for EMG and ECG (including calculations of HR) were obtained from the same animals, and the same procedure was used for the OBI and OBR mean values which were measured in the same group of fish. The same water from the maintenance tanks was used during the recordings.

After recordings, the experimental animals were killed with a blow to the head followed by mechanical destruction of the brain.

Table 1
Latencies to induction and recovery stages (seconds ± SD) in tambaqui, *Colossoma macropomum* juveniles exposed to anaesthetic concentrations of geraniol (GRL) and citronellol (CTL).

[μL L^{-1}]		Latencies to stages of induction (s)			Latencies to stages of recovery (s)	
		A1	A2	A3	R1	R2
GRL	10	–	–	–	–	309 ± 69
	30	–	192 ± 43^{a}	528 ± 163^{a}	137 ± 53	313 ± 78
	50	–	104 ± 32^{b}	240 ± 42^{b}	169 ± 59	331 ± 51
	70	–	71 ± 17^{bc}	191 ± 40^{b}	146 ± 49	263 ± 45
	90	–	73 ± 13^{bc}	170 ± 30^{b}	189 ± 41	369 ± 100
	110	–	52 ± 9^{c}	130 ± 33^{b}	179 ± 96	294 ± 120
CTL	10	–	–	–	–	449 ± 125
	30	–	153 ± 55^{a}	422 ± 140^{a}	160 ± 23^{a}	329 ± 40^{a}
	50	–	104 ± 31^{b}	293 ± 84^{b}	215 ± 55^{ab}	353 ± 49^{a}
	70	–	79 ± 23^{b}	261 ± 68^{b}	261 ± 77^{bc*}	609 ± 265^{b*}
	90	–	87 ± 18^{b}	183 ± 22^{bc}	323 ± 77^{c*}	657 ± 72^{b*}
	110	35 ± 7	67 ± 19^{b}	103 ± 36^{c}	314 ± 66^{c*}	699 ± 132^{b*}

Dashes denote absence of any stage after a 30 min observation. Times to reach the different stages of induction or recovery are cumulative. Lower case letters in the same column indicate significant differences between concentrations within the same anaesthetic and values with an asterisk indicate significant differences within the same concentrations between anaesthetics (two-way ANOVA, Tukey's test, $P < 0.05$), $n = 9$.

2.5. Statistical analyses

To verify the homogeneity of variances and normality, data were submitted to Levene and Kolmogorov-Smirnov tests, respectively. After assumptions were met, comparisons of mean latencies for induction and recovery stages were made using two-way ANOVA (with concentrations and anaesthetic being used as factors) followed by Tukey's test. Comparisons among mean amplitudes (power) of EMG and OBI, mean heart rate (HR) and mean opercular beat rate (OBR) were performed using one-way ANOVA followed by Tukey's test. Moreover, Pearson correlation was carried out between times to A3 or R2 stages and increasing concentrations of GRL or CTL. The GraphPad Prism™ 5 software was used for the analyses and the minimum significance level was set at $p < 0.05$ in all cases (Zar, 1996).

3. Results

3.1. Behavioural assessment

Control fish and fish exposed to ethanol (vehicle) did not show any signs of anaesthesia after 30 min observation. No mortality was observed during exposure to the anaesthetics or throughout 72 h in observation. All results for latencies to anaesthesia and recovery are shown in Table 1.

Agitation (A1) was not observed upon exposure to either anaesthetic, except at 110 μL L^{-1} CTL after 35 ± 7 s. For both GRL and CTL exposure, the concentration of 10 μL L^{-1} did not induce A1, A2 and A3 stages, however, mild sedation was attained with resumption of normal swimming (R2) after 309 ± 69 s and 449 ± 125 s for GRL and CTL, respectively. Time to reach A3 stage in group exposed to anaesthesia with GRL was longer at 30 μL L^{-1} (528 ± 163 s) ($p < 0.05$) relative to the other concentrations within the same anaesthetic. Likewise, the same pattern is observed for CTL exposure, with 90 and 110 μL L^{-1} concentrations showing even lower latencies ($p < 0.05$) compared to other concentrations. Yet, times to reach A3 stage were similar within the same concentrations and between anaesthetics, not showing significant differences.

Regardless of concentration, GRL did not elicit a dose-dependent response after induction, showing no significant differences among mean times in recovery. On the other hand, R2 in CTL-exposed fish was positively correlated with increasing concentrations, being shorter ($p < 0.05$) at 30 and 50 μL L^{-1} than at the concentrations of 70, 90 and 110 μL L^{-1}. As for the recovery response within the same concentration and between anaesthetics, return times were longer ($p < 0.05$) for CTL-exposed fish at 70, 90 and 110 μL L^{-1}.

Irrespective of concentration or time to R2, all fish were considered fully recovered in the behavioural evaluation.

For GRL (Fig. 1A), the higher the concentration of the product, the shorter the induction time (A3) was. The same pattern of induction with CTL was observed (Fig. 1B). On the other hand, during recovery (R2) no clear pattern could be observed, as time for recovery from GRL exposure did not vary significantly over increasing concentrations. On the other hand, the CTL trial showed a clear opposite correlation in time vs. concentration. In the equations, "y" corresponds to the time required for deep anaesthesia (A3) while "x" corresponds to the GRL and CTL concentrations used.

3.2. Electrophysiological responses

Since no differences ($p > 0.05$) were observed between mean amplitude and frequency values of the control (basal) and ethanol (vehicle control) groups for any of the measured electrophysiological markers, only mean values of the former (basal) were used for comparison purposes with GRL and CTL-exposed groups.

3.2.1. Electromyography (EMG)

Electromyographic tracings showed continuous activity with intense muscle contraction under normal conditions (Fig. 2A). Signal intensity increased in frequencies up to 20 Hz, as observed in the correspondent frequency spectrogram (Fig. 2B). Mean power amplitude observed in the control (basal values) corresponded to 7.62 ± 0.72 mV^2 / Hz x 10^{-3} (Fig. 3).

Dorsal muscle contraction activity is observed in Fig. 2C for GRL-exposed fish, following with myorelaxation after 100 s, as also evidenced by the frequency spectrogram (Fig. 2D). In earlier moments of the induction, i.e., during the first half of the record (Ind I_{GRL}) the average amplitude (10.67 ± 0.86 mV^2 / Hz x 10^{-3}) was significantly higher than that of the basal group. Mean amplitude significantly reduced to 0.14 ± 0.06 mV^2 / Hz x 10^{-3} in the second half of the induction (Ind II_{GRL}) as observed in Fig. 3.

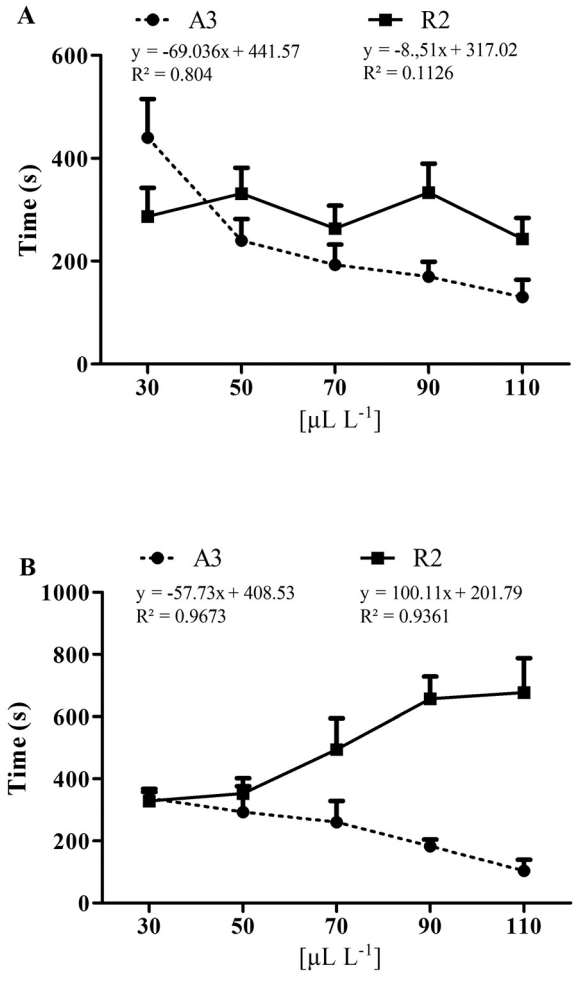

Fig. 1. Trends of induction (A3) and recovery (R2) in juvenile tambaqui, *Colossoma macropomum* exposed to short-term baths with increasing concentrations of geraniol (A) and citronellol (B).

Differently from GRL, CTL induced a more intense excitability, which is reflected by high amplitude spikes in the first 100 s (Fig. 2E) also corroborated by the intensity of the signal in the colorimetric scale of the frequency spectrogram (Fig. 2F) within the same interval, following with muscle relaxation afterwards. This high mean amplitude is quantified in Fig. 3 with higher averages (15.69 ± 3.23 mV^2 / Hz x 10^{-3}, $p < 0.001$) relative to the control in the first half of the induction (Ind I_{CTL}), whereas during the second half of the induction with CTL (Ind II_{CTL}), mean amplitude of 0.26 ± 0.10 mV^2 / Hz x 10^{-3} was significantly lower ($p < 0.001$) compared to the control and Ind I_{CTL}.

During recovery post-anaesthesia low amplitude tracings were initially observed for both products. The resumption of muscle activity progressed over time, and muscle contraction power augmented during the second half of the tracings (Fig. 2G and I), as also evidenced by the frequency spectrograms in both cases (Fig. 2H and J). Mean amplitude of tracings for the whole recovery interval were 5.99 ± 1.15 and 2.63 ± 0.92 mV^2 / Hz x 10^{-3} for GRL and CTL-exposed fish, respectively, thus returning ($p > 0.05$) to basal values. Further, it was clear that mean amplitudes increased significantly during recovery as it can be seen when mean values were compared against values during Ind II in both cases (Fig. 3).

3.2.2. Opercular beat intensity (OBI) and opercular beat rate (OBR)

The tracings in the control group (Fig. 4A) showed constant and regular waves, also corroborated by the pattern attained in the frequency spectrogram (Fig. 4B) during normal beats. The mean power in the control was 3.29 ± 0.71 mV^2 / Hz x 10^{-3} (Fig. 5A) while OBR was calculated to be at 114.2 ± 4.74 bpm (Fig. 5A and B).

Upon contact with GRL and within the 150 s of the recordings, a transient apnea was observed, followed later on by an adaptation to a regular beating (Fig. 4C). This reduction in amplitude can be confirmed by the respective mean OBI value 0.52 ± 0.16 mV^2 / Hz x 10^{-3} (Fig. 5A). Nevertheless, the OBR in Ind I_{GRL} is higher ($p < 0.05$) (122.8 ± 4.06 bpm) than that of the basal group, and the frequency spectrogram (Fig. 4D) showed higher intensity of the signal mainly in frequencies below 10 Hz. However, during Ind II_{GRL}, OBR decreased to 62.44 ± 7.27 bpm ($p < 0.001$) compared to the control and compared to the first half of the induction period (Fig. 5B).

CTL decreased the opercular beat power throughout the induction period (Fig. 4E) which was reduced ($p < 0.001$) relative to basal recordings (Fig. 5A). A more irregular distribution and less intense signal can be seen in the CTL frequency spectrogram (Fig. 4F). Although a reduced mean amplitude value for OBI was recorded (0.27 ± 0.10 mV^2 / Hz x 10^{-3}) compared to the control, the mean OBR was 121.8 ± 6.67 during Ind I_{CTL}, being unchanged compared to the control. Later, during Ind II_{CTL}, the rate reduced to 62.44 ± 7.27 bpm ($p < 0.001$) relative to the control (Fig. 5B).

During recovery, both products proved to be reversible. However, GRL seemed to allow for a faster recovery of the movements (Fig. 4G) as tracings showed a relative greater amplitude and better energy distribution in the frequency spectrogram (Fig. 4H) compared to the CTL's spectrogram as per the colour pattern attained (Fig. 4J). The OBI mean values were higher ($p < 0.001$) compared to induction values for both products, 1.79 ± 0.41 and 1.39 ± 0.18 mV^2 / Hz x 10^{-3} for GRL and CTL, respectively (Fig. 5A). Moreover, increased OBRs ($p < 0.001$) for GRL (88.44 ± 2.40 bpm) and CTL (78.44 ± 6.62 bpm) during recovery were also observed compared to mean OBRs in the second half of the induction in either case (Fig. 5B).

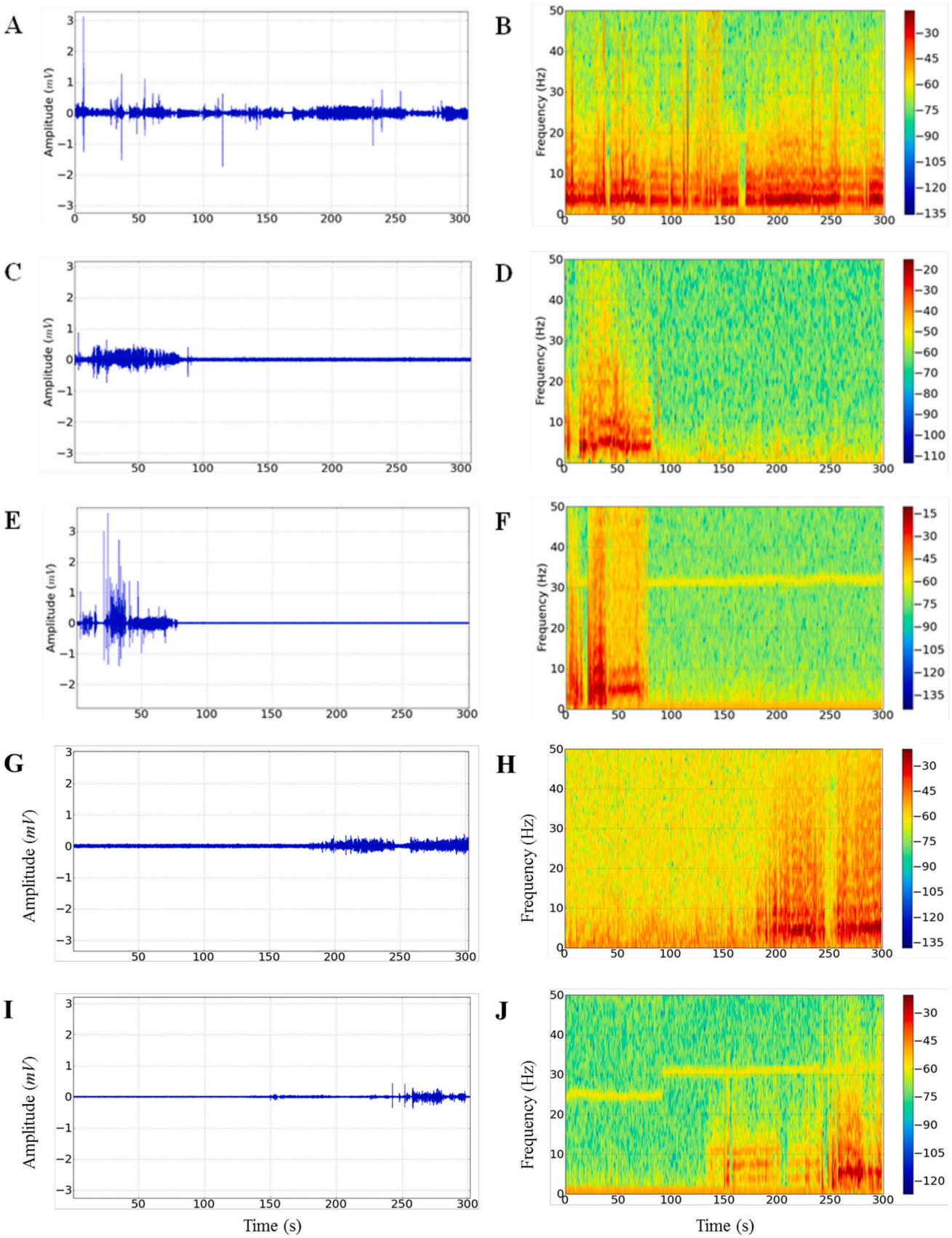

Fig. 2. Electromyographic records (EMG) of tambaqui, *Colossoma macropomum* in the basal state (A and B); submitted to short-term baths with geraniol (GRL) at 70 μL L^{-1} (C and D), citronellol (CTL) at 90 μL L^{-1} (E and F) and during recovery after anaesthesia with GRL (G and H) and CTL (I and J). Recordings were performed for 300 s. A colorimetric scale is used in the frequency spectrograms whereby the reddish colours indicate a more intense electrical signal over time and across different frequencies.

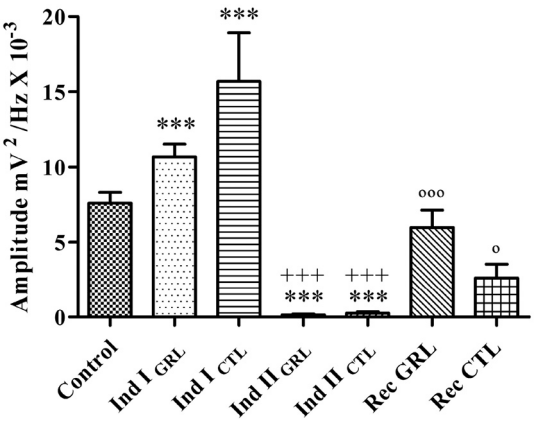

Fig. 3. Mean amplitudes recorded in the electromyogram (EMG) of *Colossoma macropomum* juveniles submitted to anaesthesia with geraniol (GRL) and citronellol (CTL). Recordings performed for 300 s in the controls, fish undergoing anaesthetic baths with GRL at 70 μL L^{-1}, CTL at 90 μL L^{-1} and in recovery post-anaesthesia (Rec GRL and Rec CTL). The Ind I and Ind II periods correspond to the mean amplitude within standardized intervals from 1 to 150 s and 150 to 300 s, respectively. *** Indicates significant differences ($p < 0.001$) from Ind I to control; +++ Indicates significant differences ($p < 0.001$) from Ind II to Ind I within the same anaesthetic; ° Indicates significant differences (°, $p < 0.05$; °°°, $p < 0.001$) in Recovery compared to Ind II relative to the same anaesthetic, [ANOVA and Tukey's test ($p < 0.05$, $n = 9$)].

3.2.3. Electrocardiographic recordings (ECG)

The basal electrocardiogram of tambaqui is presented in Fig. 6A. Fig. 6B presents a 2-s snapshot, showing the P wave, QRS complex and T wave. The average beat rate was 104.0 ± 4.00 bpm in sham fish.

During the induction with GRL, heart rate decreased, but the amplitude of the ECG tracings was maintained, as can be seen by the regularity of the P and T waves and the amplitude of QRS complex (Fig. 6C and D). The mean frequency of heart beats in GRL-exposed fish was 59.56 ± 3.58 bpm, which was lower ($p < 0.001$) compared to the control group. Upon recovery, there was a gradual reversibility of the effect with return of amplitude without major changes in the tracings and maintenance of sinus rhythm (Fig. 6E and F), with an increase of heart rate to 70.22 ± 2.73 bpm ($p < 0.001$) in relation to the induction response (Fig . 7).

On the other hand, during induction with CTL some arrhythmia was observed, showing irregularities in the tracings, reduced amplitude, elevation of the T wave and distortion of the QRS complex at some points (Fig. 6G and H). Upon contact with CTL, a decreased heart rate 48.44 ± 2.19 bpm was observed relative to the control ($p < 0.001$) (Fig. 7). During recovery, a reduced amplitude (Fig. 6I and J) and a significantly depressed heart rate (56.00 ± 2.45 bpm) was still observed ($p < 0.001$) compared to responses during induction (Fig. 7).

4. Discussion

Throughout exposure, both products induced a reversible full body immobilization and significantly modulated electrophysiological responses with distinctive characteristics. GRL and CTL did not elicit agitated behaviour during exposure except for the concentration of 110 μL L^{-1} in the latter. Although anaesthetic induction should preferably occur with low or no hyperactivity, such a reaction is commonly observed in animals exposed to anaesthetic baths with different substances, as reported in other studies (Gomes et al., 2001; Ross and Ross, 2008; Barbas et al., 2016; Barbas et al., 2017a; De Souza et al., 2019; Vilhena et al., 2019).

Anaesthetics act on the fish CNS, which are usually anaesthetized in baths added with products that can be rapidly absorbed through the gills and thus enter the bloodstream (Ross and Ross, 2008). The time to render fish anaesthetized is important as it can influence the stress response and a long induction time can negatively impact recovery (Sladky et al., 2001; Woody et al., 2002; Zahl et al., 2009; Barbas et al., 2016). Ideally, an anaesthetic should induce rapid immobilization and allow for a fast recovery in an as much as possible stress-free condition. Our findings showed that induction time was inversely correlated with concentration, which corroborates other similar studies on fish (Barbas et al., 2016; Barbas et al., 2017a; Fujimoto et al., 2017; De Souza et al., 2019; Vilhena et al., 2019).

Concentrations of 70 μL L^{-1} GRL and 90 μL L^{-1} CTL were sufficient to render fish fully immobilized in approximately three minutes, and thus complying with the guidelines as proposed for fish anaesthesia (Ross and Ross, 2008; Barbas et al., 2017a, 2017b). Compounds used in this study are the major components of the *C. nardus* essential oil, and concentrations used herein were much lower than those reported by Barbas et al. (2017a) when investigating the effects of citronella essential oil as an anaesthetic for *C. macropomum* juveniles. While the citronella essential oil at 600 μL L^{-1} was recommended as a suitable concentration to promote fast and deep anaesthesia in *C. macropomum*, our findings suggest that a 6.5 to 8.5-fold reduction in concentration for an effective anaesthesia is possible when using these isolated citronella essential oil derivatives. Thus, it could represent reduction of costs depending on the technology used for the oil extractions.

Regardless of anaesthetic concentration, induction time may vary among specimens, which could be related to variations in the rate of water flow across the gills (Treves-Brown, 2000) and the level of anaesthetic depression depending on the purpose of the procedure (biometrics, surgery, transportation and others). Herein, concentrations of 30 μL L^{-1} and above induced all stages of anaesthesia for either product, however, the animals showed distinct responses during recovery.

While GRL promoted timely recovery irrespective of the concentration used, the group exposed to CTL oil had longer recovery periods, i.e., more than 5 min to resumption of normal swimming in anaesthetic-free water, and thus not complying with the time threshold criteria for recovery. Recovery time response was inversely proportional to induction time and directly correlated to increments in concentration. However, prediction of fish recovery after anaesthesia is always an issue, as several studies have reported irregular responses during that stage (Mylonas et al., 2005; Barbas et al., 2016; Barbas et al., 2017a; De Souza et al., 2019; Vilhena et al., 2019).

Electrophysiological responses seemed to be consistent with a general anaesthetic effect. According to the EMG data, there were clear differences in muscle contraction power in the first half of the induction, showing excitability during exposure in both cases in relation to the basal group. When under anaesthesia, fish are expected to show CNS depression and increased muscle relaxation, which in turn will lead to reduced motor coordination, and impairing the righting reflex and normal swimming behaviour. During the second half of the record the oils promoted loss of muscle tonus with general immobilization, as corroborated by the low amplitude in tracings, which were overlapped by A3 stage. These results are in line with those reported by De Souza et al. (2019) working with *Nepeta cataria* essential oil in *C. macropomum* juveniles and by Fujimoto et al. (2017) who reported a depression in muscle contraction power in fish anaesthetized with clove oil.

Reversibility of the effects occurred for both products as per the EMG tracing patterns observed during recovery. Overall, there was a gradual and progressive recovery in muscle contraction power post-exposure, showing partial reversibility of the muscular tonus without indication of excitability or spasms throughout the recordings. Mean amplitude values for REC_{CTL} are below basal and REC_{GRL} values, indicating the need for a longer time for resumption of the muscle contraction power as also reported in other studies using the same species as a model (Barbas et al., 2017a; De Souza et al., 2019; Vilhena et al., 2019). Indisputably, these results show that the myorelaxant effects of GRL and CTL are reversible.

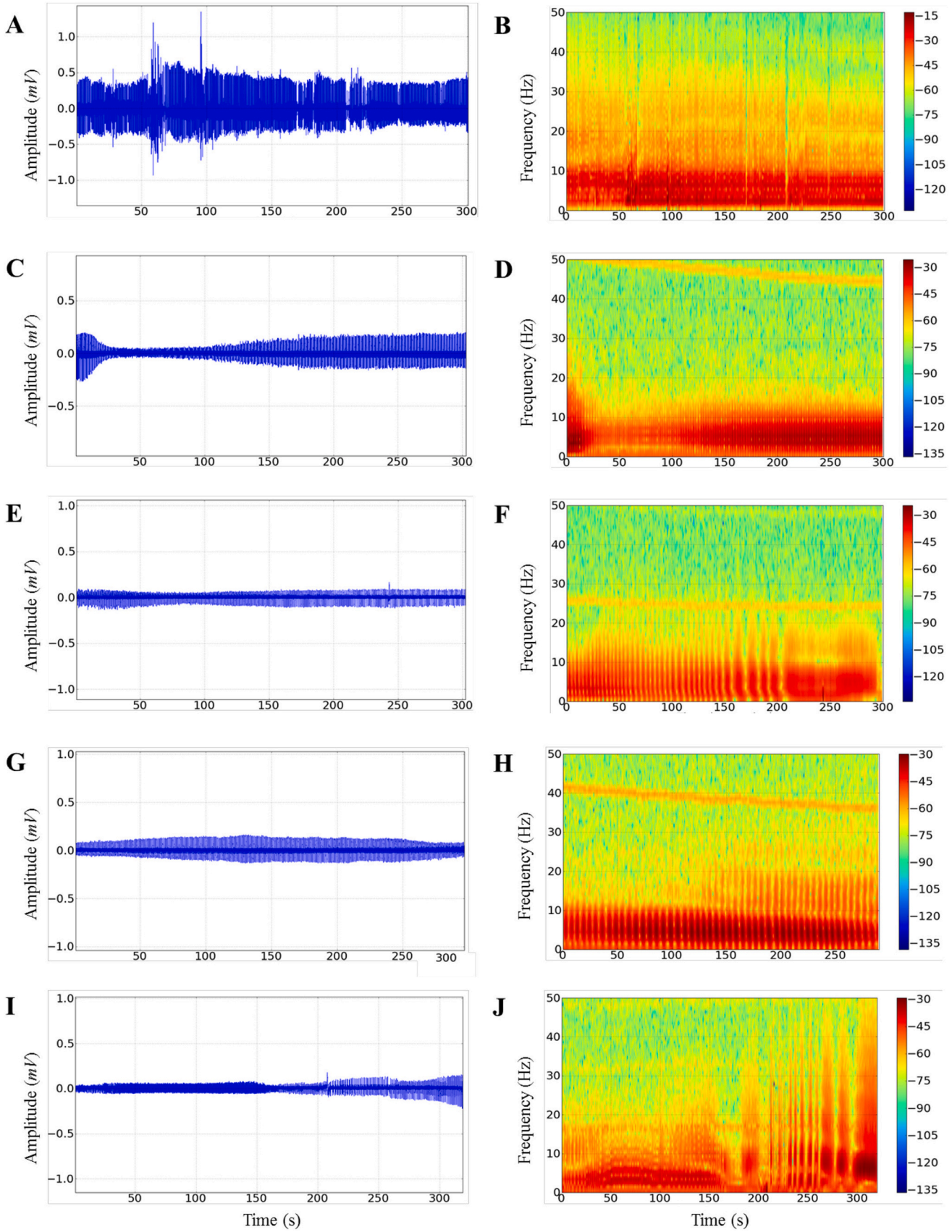

Fig. 4. Opercular beat intensity (OBI) records of tambaqui, *Colossoma macropomum* in the basal state (A and B), submitted to baths with geraniol (GRL) at 70 μL L^{-1} (C and D) and citronellol (CTL) at 90 μL L^{-1} (E and F) and during recovery after anaesthesia with GRL (G and H) and CTL (I and J). Recordings were performed for 300 s. A colorimetric scale is used in the frequency spectrograms whereby the reddish colours indicate a more intense electrical signal over time and across different frequencies.

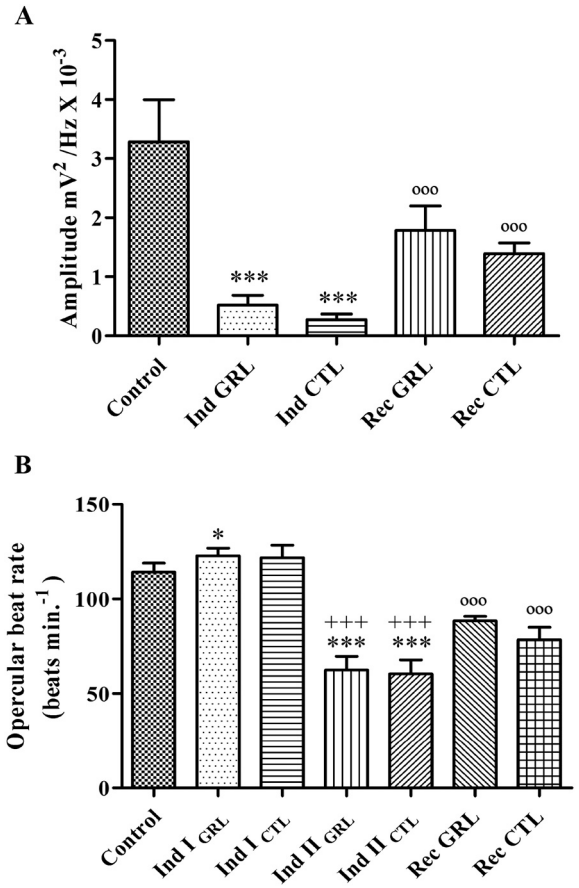

Fig. 5. Record of mean amplitude of opercular beat intensity (OBI) (A) and opercular beat rate (OBR), in beats per minute (bpm) (B) of *Colossoma macropomum* juveniles submitted to anaesthesia with geraniol (GRL) and citronellol (CTL). Recordings made in 300 s in the controls, fish submitted to anaesthetic baths with GRL at 70 μL L^{-1}, CTL at 90 μL L^{-1} and during recovery after anaesthesia (Rec GRL and Rec CTL). The Ind I and Ind II periods correspond to the average amplitude of standardized intervals from 1 to 150 s and 150 to 300 s, respectively. * Indicates significant differences (*, $p < 0.05$; ***, $p < 0.001$) from Ind I to control; +++ Indicates significant differences ($p < 0.001$) from Ind II to Ind I within the same anaesthetic; °°° Indicates significant differences ($p < 0.001$) of Recovery in relation to Ind II within the same anaesthetic, [ANOVA and Tukey's test ($p < 0.05$, $n = 9$)].

Both anaesthetics reduced the power and frequency of the opercular beat throughout the first half of the recovery. In animals exposed to GRL, the OBI decreased by 84.17% during induction and OBR by 45.33% during the second half of the record. For the group in contact with CTL, the OBI was much lower (91.66%) compared to the control, and the reduction in OBR was in the order of 47.08% during second half of the induction. These results are expected in view of the synchronization that occurs between beat power and frequency. Water flow depends on the opercular beat, which in turn is essential for an effective gas exchange across blood and water. As total suppression of ventilation did not occur during baths, severe hypoxia does not seem to represent a threat to life of fish submitted to short-term exposure using these compounds.

The HR in GRL-exposed fish during induction was 42.74% lower than that of the control. The tracings were continuous and regular, allowing for a progressive return of the HR during recovery, with no signs of arrhythmias. Reductions in HR of tambaqui were observed in other studies using different anaesthetics (Barbas et al., 2017a; De Souza et al., 2019).

Although either isolate significantly decreased heart rates, it did not compromise resumption of normal cardiac function. However, CTL caused a more severe depression of the cardiac function with mild arrhythmia. Barbas et al. (2017a) and De Souza et al. (2019) observed transient arrhythmia during recovery of juvenile tambaqui exposed to essential oil of citronella and propofol, respectively. Similarly to our findings, both studies also showed reduction in the OBI. As the flow occurs to the benefit of the arteries, a combination of central and peripheral control of cardiorespiratory interactions is capable of generating synchronization between respiration and the heart function, with a component related to breathing through the vagal nerve innervating to the heart (Taylor, 1992; Taylor et al., 1999).

Geraniol has the ability to inhibit Ca^{2+} currents and prevent arrhythmic effects on the atrial tissue (De Menezes-filho et al., 2014). It could explain our findings, since the control of the heart rate in fish is exerted by the action potentials of the sinoatrial node, i.e., activities generated by the pacemaker tissue in the heart, which involves the activation of K^+, Na^+ and Ca^{2+} channels, where K^+-induced contractions are inhibited by blocking Ca^{2+} channels or by removing Ca^{2+} to the external environment and therefore are dependent on the Ca^{2+} fluxes.

Rhythmic oscillations occurred in animals exposed to CTL. In this group, bradycardia was observed during the induction period (48.44 ± 2.19 bpm) with a reduction of 53.42% in relation to the control, which remained depressed throughout the recovery period (56.00 ± 2.45 bpm). This is likely related to the partial blockade of voltage-dependent Na^+ channels induced by CTL, resulting in the stabilization of excitable membranes, since voltage-dependent Na^+ channels are a major contributor to rapid membrane depolarization (De Sousa et al., 2006). It has been previously reported that this isolated compound determined significant cardiovascular effects, whereas the bradycardic condition can improve cardiac contractility as an adaptive response during anaesthesia (Schwerte et al., 2006; Bastos et al., 2009; Menezes et al., 2010; Santos et al., 2011).

The effects of monoterpenes can occur by various mechanisms due to their structural diversity, as they can be cyclic or acyclic molecules. In addition, changes in vagal tone affect heart rate, and this increase affects bradycardia (Taylor et al., 2010) which is associated with hypoventilation. As suggested by De Souza et al. (2019), these events may occur due to an indirect consequence of central neuronal depression or a result of the direct depressant effect of anaesthetics in the cardiac and respiratory tissues. Presumably, their vasodilatory action appears to be caused by an inhibition of Ca^{2+} influx across the plasma membrane, as studies have concluded that the use of these monoterpenes promotes hypotensive and bradycardic activity (De Sousa et al., 2006; Bastos et al., 2009).

In summary, the present study discussed some effects caused by CTL and GRL at the behavioural and physiological levels in an attempt to make sense of any deleterious implications that could be involved during exposure to these substances. Moreover, the importance of pursuing more specific indicators, such as EEG recordings, could shed more light on the potential of these substances to cause CNS depression in fish. Future studies should focus on a more prolonged monitoring of fish undergoing anaesthesia and recovering from exposure to these compounds to further characterize responses in muscle or any late impacts on cardiac function, especially in the case of exposure to CTL including the characterization of the ECG complex and waves.

5. Conclusion

In conclusion, our results showed that geraniol and citronelol were able to promote full body immobilization, which resulted at least in part, from the myorelaxant properties of these citronella-derived compounds. Concentrations of 70 μL L^{-1} GRL and 90 μL L^{-1} CTL were sufficient to render fish fully and rapidly immobilized. Both products transiently reduced ventilation during anaesthesia, nevertheless allowing for complete recovery after exposure. Although either isolate significantly decreased heart rates, they did not compromise resumption of normal cardiac function. In general, as no mortalities were observed and all animals recovered after exposure, both products could be considered

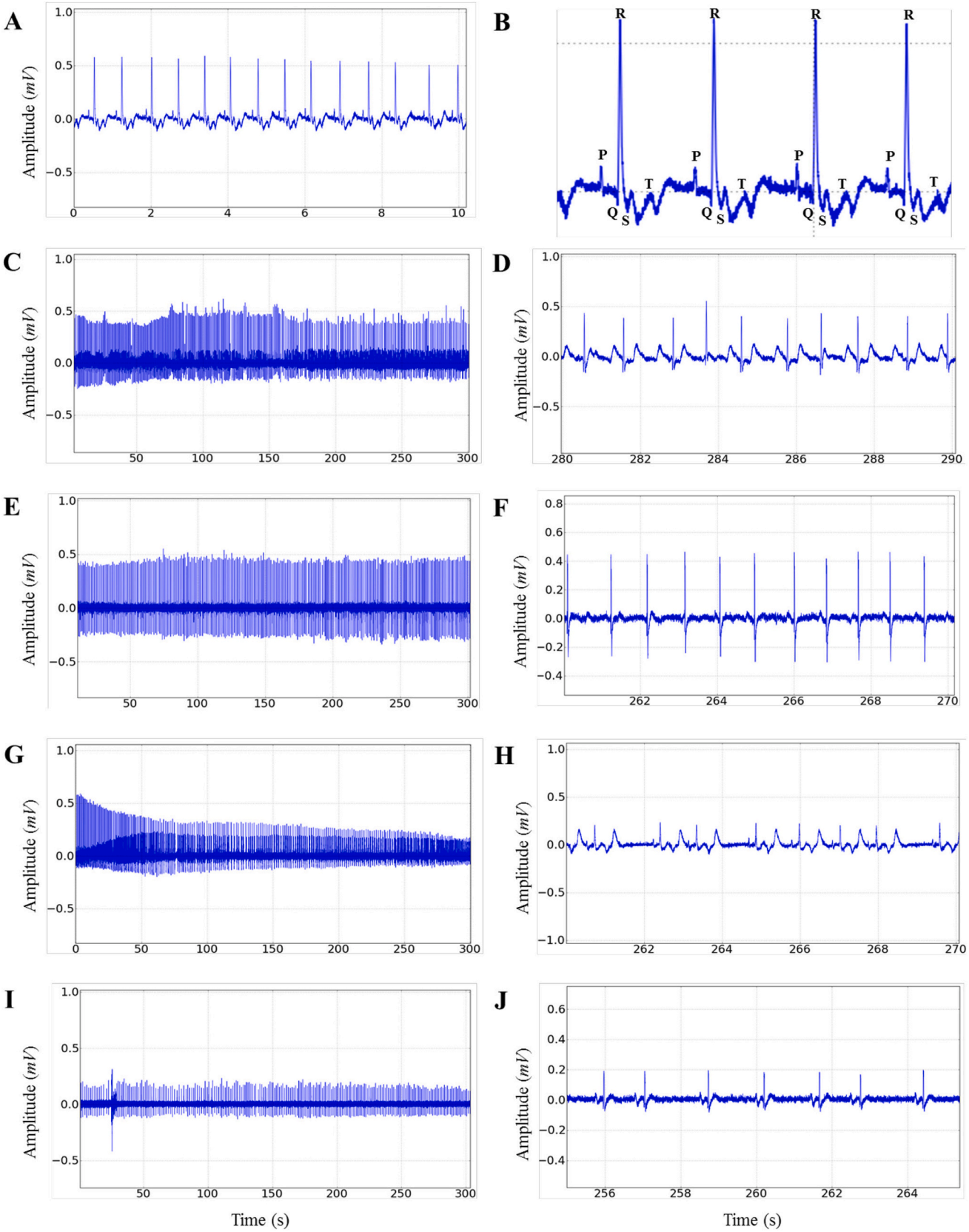

Fig. 6. Normal electrocardiographic (ECG) tracings of tambaqui *Colossoma macropomum* (10 s) (A) and 2-s snapshots showing the P and T waves, and the QRS complex (B); and fish undergoing short-term baths with geraniol (GRL) at 70 μL L^{-1} (C and D) and during recovery after anaesthesia (E and F), citronellol (CTL) at 90 μL L^{-1} (G and H) and during recovery after anaesthesia (I and J). Recordings were all performed for 300 s (left panels) showing 10-s fragment amplifications (right panels except for B panel).

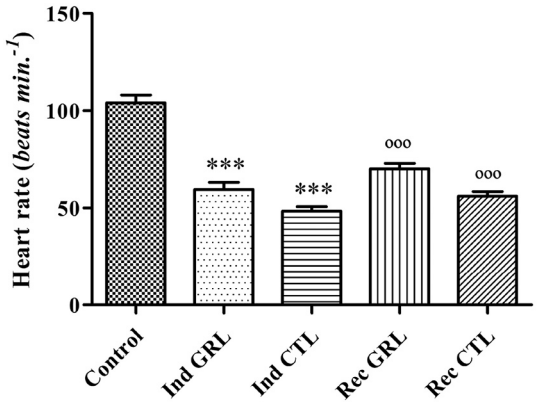

Fig. 7. Heart rate (HR), in beats per minute (bpm) of *Colossoma macropomum* juveniles submitted to anaesthesia with geraniol (GRL) and citronellol (CTL). Recordings made in 300 s in the controls, fish submitted to anaesthetic baths with GRL at 70 μL L^{-1} (Ind GRL), CTL at 90 μL L^{-1} (Ind CTL) and during recovery post anaesthesia (Rec GRL and Rec CTL). *** Indicates significant differences ($p < 0.001$) of Induction relative to the control; °°° Indicates significant differences (°°°, $p < 0.001$) of Recovery in relation to Induction within the same anaesthetic, [ANOVA and Tukey's test ($p < 0.05$, $n = 9$)].

safe alternatives for fish handling and other aquaculture-related activities that might require short-term fish immobilization.

Author statement

Conceptualization and formal analyses: E.R.L.A., M.F.T., C.B.A., L.A.L.B., M.H. and L.A.S.; Data curation: E.R.L.A., J.S.S., L.M.L., B.M.P.A.C., L.A.L.B. and M.H.; Writing – original draft: E.R.L.A., L.A.L.B., M.F.T. and M.H. All authors contributed equally to the review & editing of this manuscript's final version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

E.R.L.A is grateful to the Coordination for the Improvement of Higher Education Personnel (Brazilian CAPES) for the scholarship provided. Authors are also thankful to students and staffs of the Tropical Species Aquaculture Laboratory (LAET/IFPA – Castanhal) and Natural Products Toxicology Laboratory (UFPA – Belém) for their technical support during the experiments. L.A.S is a research fellow of the National Council for Scientific and Technological Development – CNPq, Brazil.

References

Aydın, B., Barbas, L.A.L., 2020. Sedative and anesthetic properties of essential oils and their active compounds in fish: a review. Aquaculture 520, 1–9. https://doi.org/10.1016/j.aquaculture.2019.734681.

Barbas, L.A.L., Stringhetta, G.R., Garcia, L.O., Figueiredo, M.R.C., Sampaio, L.A., 2016. Jambu, *Spilanthes acmella* as a novel anaesthetic for juvenile tambaqui, *Colossoma macropomum*: secondary stress responses during recovery. Aquaculture 456, 70–75. https://doi.org/10.1016/j.aquaculture.2016.01.026.

Barbas, L.A.L., Hamoy, M., De Mello, V.J., Barbosa, R.P.M., De Lima, H.D.S.T., Torres, M. F., Do Nascimento, L.A.S., Da Silva, J.K.R., Andrade, E.H.A., Gomes, M.R.F., 2017a. Essential oil of citronella modulates electrophysiological responses in tambaqui *Colossoma macropomum*: a new anaesthetic for use in fish. Aquaculture 479, 60–68. https://doi.org/10.1016/j.aquaculture.2017.05.027.

Barbas, L.A.L., Maltez, L.C., Stringhetta, G.R., de Garcia, L.O., Monserrat, J.M., da Silva, D.T., Heinzmann, B.M., Sampaio, L.A., 2017b. Properties of two plant extractives as anaesthetics and antioxidants for juvenile tambaqui *Colossoma macropomum*. Aquaculture 469, 79–87. https://doi.org/10.1016/j.aquaculture.2016.12.012.

Bastos, J.F., Moreira, I.J., Ribeiro, T.P., Medeiros, I.A., Antoniolli, A.R., De Sousa, D.P., Santos, M.R., 2009. Hypotensive and vasorelaxant effects of citronellol, a monoterpene alcohol, in rats. Basic Clin. Pharmacol. Toxicol. 106, 331–337. https://doi.org/10.1111/j.1742-7843.2009.00492.x.

Bendschneider, K., Robinson, R.J., 1952. A new spectrophotometric method for the determination of nitrite in sea water. J. Mar. Res. 11, 87–96.

Castro, H.G., Perini, V.B.M., Santos, G.R., Leal, T.C.A.B., 2010. Evaluation of content and composition of the essential oil of *Cymbopogon nardus* (L.) in different harvest times. Rev. Ciênc. Agron. 41, 308–314. https://doi.org/10.1590/S1806-66902010000200020.

Cho, G.K., Heath, D.D., 2000. Comparison of tricaine methanesulphonate (MS222) and clove oil anestesia effects on the physiology of juvenile chinook salmon *Oncorhynchus tshawytscha* (Walbaum). Aquac. Res. 31, 537–546. https://doi.org/10.1046/j.1365-2109.2000.00478.x.

Cotter, P.A., Rodnick, K.J., 2006. Differential effects of anaesthetics on electrical properties of the rainbow trout (*Oncorhynchus mykiss*) heart. Comp. Biochem. Physiol. A 145, 158–165. https://doi.org/10.1016/j.cbpa.2006.06.001.

De Menezes-Filho, J.E.R., Gondim, A.N.S., Cruz, J.S., De Souza, A.A., Santos, J.N.A., Conde-Garcia, E.A., Sousa, D.P., Santos, M.S., Oliveira, E.D., Vasconcelos, C.M.L., 2014. Geraniol blocks calcium and potassium channels in the mammalian myocardium: useful effects to treat arrhythmias. Basic Clin. Pharmacol. Toxicol. 115, 534–544. https://doi.org/10.1111/bcpt.12274.

De Sousa, D.P., Gonçalves, J.C.R., Quintans-Júnior, L., Cruz, J.S., Araújo, D.A.M., Almeida, R.N., 2006. Study of anticonvulsant effect of citronellol, a monoterpene alcohol, in rodents. Neurosci. Lett. 401, 231–235. https://doi.org/10.1016/j.neulet.2006.03.030.

De Souza, A.S.L., Peret, A.C., Hamoy, M., De Souza, R.A.L., Torres, M.F., Barbas, L.A.L., 2019. Propofol and essential oil of *Nepeta cataria* induce anaesthesia and marked myorelaxation in tambaqui *Colossoma macropomum*: implications on cardiorespiratory responses. Aquaculture 500, 160–169. https://doi.org/10.1016/j.aquaculture.2018.10.017.

Delattre, E., 2007. Fundamentos de eletrofisiologia: potenciais de membrana. Medicina 40, 378–393.

Eaton, A.D., Clesceri, L.S., Rice, E.W., Greenberg, A.E., 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed. American Public Health association, Springfield. (8 p.).

Façanha, M.F., Gomes, L.C., 2005. A eficácia do mentol como anestésico para tambaqui (*Colossoma macropomum*, Characiformes: Characidae). Acta Amaz 35, 71–75.

Fujimoto, R.Y., Pereira, D.M., Silva, J.C., Oliveira, L.C.A.O., Inoue, L.A.K.A., Hamoy, M., Mello, V.J., Torres, M.F., Barbas, L.A.L., 2017. Clove oil induces anaesthesia and blunts muscle contraction power in three Amazon fish species. Fish Physiol. Biochem. 44, 245–256. https://doi.org/10.1007/s10695-017-0430-8.

Gomes, L.C., Chippari-Gomnes, A.R., Lopes, N.P., Roubach, R., Araujo-Lima, C.A.R.M., 2001. Efficacy of benzocaine as an anesthetic in juvenile tambaqui *Colossoma macropomum*. J. World Aquacult. Soc. 32, 426–431. https://doi.org/10.1111/j.1749-7345.2001.tb00470.x.

Gonçalves, A.F.N., Santos, E.C.C., Fernandes, J.B.K., Takahashi, L.S., 2008. Menthol and eugenol as benzocaine substitutes in anesthetic induction of pacu juveniles. Acta Sci. Anim. Sci. 30, 339–344. https://doi.org/10.4025/actascianimsci.v30i3.1081.

Inoue, L.A.K.A., Afonso, L.O.B., Iwama, G.K., Moraes, G., 2005. Effects of clove oil on the stress response of matrinxã (*Brycon cephalus*) subjected to transport. Acta Amaz 35, 289–295. https://doi.org/10.1590/S0044-59672005000200018.

Lopez-Romero, J.C., González-Ríos, H., Borges, A., Simões, M., 2015. Antibacterial effects and mode of action of selected essential oils components against *Escherichia coli* and *Staphylococcus aureus*. Evid. Based Comp. Alt. 2015, 1–9. https://doi.org/10.1155/2015/795435.

Losey, G.S., Hugie, D.M., 1994. Prior anesthesia impairs a chemically mediated fright response in a gobiid fish. J. Chem. Ecol. 20, 1877–1883. https://doi.org/10.1007/BF02066229.

Mahalwal, V.S., Ali, M., 2003. Volatile constituents of *Cymbopogon nardus* (Linn.) Rendle. Flavour Fragr. J. 18, 73–76. https://doi.org/10.1002/ffj.1144.

Martins, R.M., 2006. Estudio "in vitro" de la acción acaricida del aceite esencial de la gramínea citronela de java (*Cymbopogon winterianus* Jowitt) no carrapato *Boophilus microplus*. Rev. Bras. Plant Med. 08, 71–78.

Menezes, I.A.C., Barreto, C.M.N., Antoniolli, A.R., Santos, M.R.V., De Sousa, D.P., 2010. Hypotensive activity of terpenes found in essential oils. Z Naturforsch. C. J. Biosci. 65, 562–566. https://doi.org/10.1515/znc-2010-9-1005.

Mudado, M.A., Moreira, T.H., Cruz, J.S., 2003. O início da era dos canais iônicos. Clínica Hoje 33, 56–58.

Mylonas, C.C., Cardinaletti, G., Sigelaki, I., Polzonetti-Magni, A., 2005. Comparative efficacy of clove oil and 2-phenoxyethanol as anesthetics in the aquaculture of European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) at different temperatures. Aquaculture 246, 467–481. https://doi.org/10.1016/j.aquaculture.2005.02.046.

Park, J.-Y., Kim, J.-Y., Jang, S.-H., Kim, H.-J., Lee, S.-J., Park, S.-C., 2015. Biological activities and acute oral toxicity of citronella and lemongrass oil. Korean J. Vet. Res. 55, 13–20. https://doi.org/10.14405/kjvr.2015.55.1.13.

Park, M.O., Hur, W.J., Im, S.Y., Seol, D.W., Lee, J., Park, I.S., 2008. Anaesthetic efficacy and physiological responses to clove oil-anaesthetized kelp grouper *Epinephelus bruneus*. Aquac. Res. 39, 877–884. https://doi.org/10.1111/j.1365-2109.2008.01941.x.

Parodi, T.V., Cunha, M.A., Heldwein, C.G., De Souza, D.M., Martins, Á.C., Garcia, L.O., Wasielesky Jr., W., Monserrat, J.M., Schmidt, D., Caron, B.O., Heinzmann, B., Baldisserotto, B., 2012. The anesthetic efficacy of eugenol and the essential oils of

Lippia alba and *Aloysia triphylla* in post-larvae and sub-adults of *Litopenaeus vannamei* (Crustacea, Penaeidae). Comp. Biochem. Physiol. C 155, 462–468. https://doi.org/10.1016/j.cbpc.2011.12.003.

Quintans-Júnior, L.J., Souza, T.T., Leite, B.S., Lessa, N.M.N., Bonjardim, L.R., Santos, M. R.V., Blank, A.F., Antoniolli, A.R., 2008. Phythochemical screening and anticonvulsant activity of *Cymbopogon winterianus* Jowitt (Poaceae) leaf essential oil in rodents. Phytomedicine 15, 619–624. https://doi.org/10.1016/j.phymed.2007.09.018.

Ross, L.G., Ross, B., 2008. Anaesthetic and Sedative Techniques for Aquatic Animals. Blackwell Publishing, Oxford, UK (228 p).

Ross, L.G., Blanco, J.S., Martínez-Palacios, C., Racotta, I.S., Cuevas, M.T., 2007. Anesthesia, sedation and transportation of juvenile *Menidia estor* (Jordan) using benzocaine and hypothermia. Aquac. Res. 38, 909–917. https://doi.org/10.1111/j.1365-2109.2006.01642.x.

Roubach, R., Gomes, L.C., Fonseca, F.A.L., Val, A.L., 2005. Eugenol as an efficacious anaesthetic for tambaqui, *Colossoma macropomum* (Cuvier). Aquac. Res. 36, 1056–1061. https://doi.org/10.1111/j.1365-2109.2005.01319.x.

Saint-Paul, U., 1986. Potential for aquaculture of south American freshwater fishes: a review. Aquaculture 54, 205–240. https://doi.org/10.1016/0044-8486(86)90329-7.

Santos, M.R.V., Moreira, F.V., Fraga, B.P., De Sousa, D.P., Bonjardim, L.R., Quintans-Júnior, L.J., 2011. Cardiovascular effects of monoterpenes: a review. Rev. Bras 21 (4), 764–771. https://doi.org/10.1590/S0102-695X2011005000119.

Schwerte, T., Prem, C., Mairösl, A., Pelster, B., 2006. Development of the sympatho-vagal balance in the cardiovascular system in zebrafish (*Danio rerio*) characterized by power spectrum and classical signal analysis. J. Exp. Biol. 209, 1093–1100. https://doi.org/10.1242/jeb.02117.

Sladky, K.K., Swanson, C.R., Stoskopf, M.K., Loomis, M.R., Lewbart, G.A., 2001. Comparative efficacy of tricaine methanesulfonate and clove oil for use as anesthetics in red pacu (*Piaractus brachypomus*). Am. J. Vet. Res. 62, 337–342. https://doi.org/10.2460/ajvr.2001.62.337.

Sneddon, L.U., 2012. Clinical anesthesia and analgesia in fish. J. Exot. Pet Med. 21, 32–43.

Solórzano-Santos, F., Miranda-Novales, M.G., 2012. Essential oils from aromatic herbs as antimicrobial agents. Curr. Opin. Biotechnol. 23, 136–141. https://doi.org/10.1016/j.copbio.2011.08.005.

Sylvester, J.R., 1975. Factors influencing the efficacy of MS-222 to striped mullet (*Mugil cephalus*). Aquaculture 6, 163–169. https://doi.org/10.1016/0044-8486(75)90067-8.

Taylor, E.W., 1992. Nervous control of the heart and cardiorespiratory interactions. In: Hoar, W.S., Randall, D.J., Farrell, A.P. (Eds.), Fish Physiology. Vol. XIIB. Academic Press, New York, pp. 343–387.

Taylor, E.W., Jordan, D., Coote, J.H., 1999. Central control of the cardiovascular and respiratory systems and their interactions in vertebrates. Physiol. Rev. 79, 855–916. https://doi.org/10.1152/physrev.1999.79.3.855.

Taylor, E.W., Leite, C.A.C., Skovgaard, N., 2010. Autonomic control of cardiorespiratory interactions in fish, amphibians and reptiles. Braz. J. Med. Biol. Res. 43, 600–610. https://doi.org/10.1590/S0100-879X2010007500044.

Tiwari, M., Kakkar, P., 2009. Plant derived antioxidants-geraniol and camphene protect rat alveolar macrophages against t-BHP induced oxidative stress. Toxicol. in Vitro 23, 295–301. https://doi.org/10.1016/j.tiv.2008.12.014.

Treves-Brown, K.M., 2000. Anaesthesia. Em. In: Treves-Brown, K.M. (Ed.), Anaesthetics in Applied Fish Pharmacology. Kluwer Academic Publishers, Dordrecht, Netherlands, pp. 206–217.

Unesco, 1983. Chemical methods for use in marine environmental monitoring. In: Manual and Guides 12. Intergovernamental Oceanographic Commissiony, France, Paris.

Valladão, G.M.R., Gallani, S.U., Pilarski, F., 2016. South American fish for continental aquaculture. Rev. Aquac. 10, 351–369. https://doi.org/10.1111/raq.12164.

Vilhena, C.S., Nascimento, L.A.S., Andrade, E.H., Silva, J.K.R., Hamoy, M., Torres, M.F., Barbas, L.A.L., 2019. Essential oil of *Piper divaricatum* induces a general anaesthesia-like state and loss of skeletal muscle tonus in juvenile tambaqui, *Colossoma macropomum*. Aquaculture 510, 169–175. https://doi.org/10.1016/j.aquaculture.2019.05.057.

Woody, C.A., Nelson, J., Ramstad, K., 2002. Clove oil as an anaesthetic for adult sockeye salmon-field trials. J. Fish Biol. 60, 340–347. https://doi.org/10.1006/jfbi.2001.1842.

Zahl, I.H., Kiessling, A., Samuelsen, O.B., Hansen, M.K., 2009. Anaesthesia of Atlantic cod (*Gadus morhua*) – effect of pre-anaesthetic sedation, and importance of body weight, temperature and stress. Aquaculture 295, 52–59. https://doi.org/10.1016/j.aquaculture.2009.06.019.

Zar, J.H., 1996. Biostatistical Analysis, 3rd ed. Prentice-Hall, New Jersey, USA. (662 p).