

Amyris balsamifera essential oil promotes anesthesia in *Colossoma macropomum* (Cuvier, 1818): electrophysiological tools in the indication of a therapeutic window

Axell Timotheo Lima Acioli Lins

axell.ti20@gmail.com

Federal University of Pará (UFPA)

Daniella Bastos de Araújo

Federal University of Pará (UFPA)

Luciana Eiró-Quirino

Federal University of Pará (UFPA)

Clarissa Araújo da Paz

Federal University of Pará (UFPA)

Thaysa de Sousa Reis

Federal University of Pará (UFPA)

Luana Vasconcelos de Souza

Federal University of Pará (UFPA)

Alicia Chaves Manito

Federal University of Pará (UFPA)

Mahara de Souza Libório

Federal University of Pará (UFPA)

Gabriela Paula Goyana

Federal University of Pará (UFPA)

Daniella Rocha Bittencourt

Federal University of Pará (UFPA)

Gabriela Brito Barbosa

Federal University of Pará (UFPA)

Barbarella Matos Macchi

Federal University of Pará (UFPA)

Moisés Hamoy

Federal University of Pará (UFPA)

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Abstract

Currently, anesthesia is widely used in aquaculture, which raises concerns about the choice of anesthetic agent to be administered. *Amyris* sandalwood essential oil has muscle relaxant characteristics due to its chemical composition, which indicates its potential as an anesthetic product. Therefore, this study evaluates the anesthetic potential of *Amyris balsamifera* essential oil in *Colossoma macropomum* as a therapeutic window through behavioral tests and electrocardiographic recordings. The fish were exposed to *Amyris balsamifera* essential oil at concentrations of 20 $\mu\text{L.L}^{-1}$, 25 $\mu\text{L.L}^{-1}$, 30 $\mu\text{L.L}^{-1}$, 35 $\mu\text{L.L}^{-1}$ and 40 $\mu\text{L.L}^{-1}$, in immersion baths. The behavioral test showed that can caused a loss of the postural reflex in fish, with a reversible effect and recovery times dependent on the concentration of oil used. In addition, this study showed that sandalwood essential oil has the potential to decrease heart rate and maintain sinus rhythm with a dose-dependent effect. These findings were made possible by behavioral analysis parameters and electrocardiographic recordings. These results demonstrate the existence of a safe therapeutic window for the use of *Amyris balsamifera* essential oil, given that in the present study, there was a noticeable difficulty in recovering the postural reflex at higher doses, such as 40 $\mu\text{L.L}^{-1}$, and a more immediate recovery at a dose of 20 $\mu\text{L.L}^{-1}$, occurring in an adequate time of up to 5 minutes or less, which guarantees its anesthetic safety.

Introduction

The use of anesthetic agents in fish was initially employed to facilitate handling. However, advancements in knowledge about fish physiology and an increased awareness of the importance of anesthesia for maintaining the welfare of these animals have led to a broader application (Zahl, Samuelsen, Kiessling, 2012). Today, anesthesia is widely used in aquaculture to minimize fish stress and prevent injuries during routine procedures that require handling, such as transportation, weighing, tagging, and vaccination, as well as to ensure welfare in more complex procedures, such as biopsies, surgeries, and euthanasia (Maricchiolo and Genovese 2011; Zahl, Samuelsen, Kiessling 2012; Aydın and Barbas 2020).

The choice of anesthetic agent should consider several parameters. An appropriate anesthetic provides rapid immobilization and an uneventful recovery, while also having high potency, availability, and good cost-effectiveness. Additionally, it should have little or no toxicity and not accumulate in the tissues and organs of the fish, posing no risks for animal and human consumption (Aydın and Barbas 2020).

Anesthetics for fish are divided into two general categories: synthetic and natural (plant-derived) (Aydın and Barbas 2020). Among synthetic anesthetics, the most used is tricaine methanesulfonate, also known as MS-222, as well as benzocaine, quinaldine sulfate, and phenoxyethanol (Maricchiolo and Genovese 2011; Zahl, Samuelsen, Kiessling 2012). However, synthetic compounds have a limited safety margin, making their safety questionable (Taheri Mirghaed, Ghelichpour, Hoseini 2016). Furthermore, these drugs, especially MS-222, have been associated with undesirable side effects on fish behavior and

physiology (Vilhena et al. 2019). Hence, there is a growing need for natural and safer alternatives to synthetic anesthetics (Aydin and Barbas 2020).

Recently, plant-derived products have been increasingly used to replace synthetic anesthetics. Essential oils from various plants, containing different compounds, are used as anesthetics for fish. Among them, clove oil stands out, with eugenol as its main compound (Taheri Mirghaied, Ghelichpour, Hoseini 2016).

Amyris sandalwood essential oil is obtained through steam distillation of the freshly crushed wood of the *Amyris balsamifera* plant, belonging to the Rutaceae family, which primarily grows in the Caribbean region. Due to its scent, which is typical of authentic sandalwood oils, it is sometimes referred to as “West Indian sandalwood oil” (Van Beek et al. 1989; Kucharska et al. 2021). This oil acts as an effective natural muscle relaxant, which is attributed to the presence of large amounts of sesquiterpene alcohols, mainly valerianol (Kucharska et al. 2021). Kačániová et al. (2020) investigated the chemical composition of *Amyris balsamifera* oil; the components identified in the largest quantities were valerianol (23.20%), guaiaol (19.40%), 10-epi- γ -eudesmol (14.80%), and elemol (9.62%).

The electrocardiogram (ECG) is a non-invasive, quick, and low-cost test that records the impulses generated by the heart’s electrical conduction system (Guimarães et al. 2003). This system is essential for regulating the functionality and contraction rhythm of the myocardium (Souza et al. 2018). The electrophysiological response of fish is an excellent biological parameter, as it demonstrates the species’ sensitivity to the tested substance, as well as its cardiotoxicity (Reis et al. 2024; dos Santos. 2024; Cantanhêde et al. 2020). The assessment of cardiac function in fish is done through the analysis of electrocardiogram patterns, heart rate (HR), wave intervals (Q-T and R-R), as well as the duration and amplitude of the QRS complex (Vilhena et al. 2022).

Colossoma macropomum (Cuvier 1818), known as *tambaqui*, is the most farmed native fish species in Brazil and several other countries in South and Central America. In the wild, these fish are widely distributed throughout South America, from the Orinoco River in Venezuela to the Rio de la Plata in Argentina. Because of this, the species has great potential for studies involving anesthetics in tropical species (Merola et al. 1987; Barbas et al. 2016).

Therefore, this study aims to evaluate the anesthetic potential of *Amyris balsamifera* essential oil in *Colossoma macropomum* as a therapeutic window through behavioral testing and electrocardiographic recordings.

Materials and methods

Experimental animals

The fish used for this research were *tambaqui*, *C. macropomum* (n = 108), of both sexes. They were maintained in aquariums at the Experimental Animal Facility of the Laboratory of Pharmacology and Toxicology of Natural Products, at the Federal University of Pará, Institute of Biological Sciences (ICB-

UFPA), in a controlled environment with a temperature range of 25 to 27°C and a 12h light: 12h dark photoperiod. They were fed twice a day with commercial feed (32% protein) until satiety. Simultaneously, siphoning was conducted to clean the aquariums, removing animal excreta, and performing a partial water renewal (about 40% of the tank volume) with water of the same source. During the fish acclimation period (lasting 10 days), water quality variables such as temperature (°C), hydrogen ion potential (pH), dissolved oxygen (DO), ammonia (NH₃⁺), and total hardness were continuously monitored and maintained within the following values: Temp 26,8°C; pH 7,5; DO > 5,0 mg/L; NH₃⁺ 0,1 mg/L; and total hardness 70 NTU.

Drug acquisition, preparation, and preservation

The oil from *Amyris balsamifera* was obtained from the Harmonie laboratory (Registration: 11.938.821/0001-90). The oil was extracted by distillation and steam distillation and was analyzed by the Department of Chemical and Food Engineering (EQA) at the Federal University of Santa Catarina. The analysis method used was High-Performance Gas Chromatography on an AGILENT 7820A Gas Chromatograph under the following conditions: Column: HP-5 30mx0.32mm x 0,25 µm (AGILENT). Temp.: Column: 70°C (0 min), 3°C/min to 250°C. Injector: 250°C Split ratio: 1/50. FID Detector: 260°C. Injection vol.: 1 µl (1% in chloroform). The phytoconstituents present in the oil are described in Table 1, with valerianol being the major component (29.11%) (Figure 1). It has a purity level of 100% and, being an oily substance, was diluted in 70% alcohol at a ratio of 1 ml of oil to 9 ml of alcohol and stored in a refrigerator at 4°C for later aliquoting for the experiment.

Table I Chemical composition *Amyris balsamifera* essential oil

TR	Identification	Percentage (%)
24.56	Caryophyllene	0.79
26.21	α -Himachalene	0.65
26.99	β -Chamigrene	0.46
27.13	α -Curcumene	0.54
27.63	α -Zingiberene	1.36
27.78	β -Dihydroagarofuran	1.11
28.16	β -Bisabolene	0.35
28.24	cis- α -Bisabolene	0.25
28.47	7-epi- α -selinene	0.39
28.75	β -Sesquiphellandrene	2.21
29.40	α -Cadinene	0.53
29.56	Selina-3,7(11) -diene	0.71
29.81	Elemol	8.38
30.34	trans-Nerolidol	0.26
31.92	5-epi-7-epi- α -Eudesmol	1.42
32.25	7-epi-cis-sesquisabinene hydrate	0.52
32.48	10-epi- γ -eudesmol	10.10
32.90	γ -Eudesmol	6.76
33.15	8-epi- δ -eudesmol	0.43
33.33	Hinesol	1.39
33.57	β -Eudesmol	7.02
33.88	Valerianol	29.11
34.03	α -Eudesmol	13.34
34.53	Elemol acetate	0.59
35.19	8,9-dehydro-Cycloisolongifolene	0.55
37.80	Drimenol	2.28
Minor compounds	2.32	

High Resolution Gas Chromatography of *Amyris balsamifera*, Harmonie Aromatherapy, Florianópolis, SC, Brazil, 2023

Description of the behavioral experiment

For the behavioral assessment of animals subjected to the anesthetic, the study will be divided into the following groups: behavioral assessment of fish exposed to *Amyris balsamifera* essential oil (BAEO) at concentrations of 20 $\mu\text{L.L}^{-1}$, 25 $\mu\text{L.L}^{-1}$, 30 $\mu\text{L.L}^{-1}$, 35 $\mu\text{L.L}^{-1}$, and 40 $\mu\text{L.L}^{-1}$, in immersion baths.

For each treatment, n=9 per treatment was used, including the vehicle group with n=9 animals, totaling 45 animals. Behavioral assessment was conducted for 5 minutes for induction or until the loss of the postural reflex, while recovery was assessed for 5 minutes or until the postural reflex was restored.

Description of the electrophysiological experiment

Tambaqui specimens ($22,9 \pm 2,5\text{g}$) were randomly distributed into the following experimental treatments: a) control (baseline electrocardiogram recording); b) fish exposed to the vehicle; c) 20 $\mu\text{L.L}^{-1}$; d) 25 $\mu\text{L.L}^{-1}$; e) 30 $\mu\text{L.L}^{-1}$; f) 35 $\mu\text{L.L}^{-1}$; and g) 40 $\mu\text{L.L}^{-1}$, fish subjected to immersion baths with different concentrations of *Amyris balsamifera* essential oil (BAEO).

All recordings had a duration of 5 minutes of cardiac activity. For each recording, n = 9 per treatment was used, with the control and vehicle groups each having n = 9 individuals, resulting in a total of 63 animals. The analysis of electrocardiographic records was conducted during the period of 270-300s of contact during induction and during the anesthetic recovery.

Obtaining the electrocardiogram (ECG)

For the analysis and monitoring of cardiac function, electrodes made of 925 silvers with a diameter of 0,3 mm and a length of 10 mm were crafted, without being coupled. The position for the reference electrode was chosen according to the cardiac vector and was placed on the ventral portion of the specimens, 0,2 mm before the end of the left opercular cavity (reference electrode). The recording electrode was inserted 2,0 mm before the end of the right opercular cavity. After the insertion of the electrodes, they were connected to a high-impedance amplifier (Grass Technologies, Model P511) for electrocardiographic record acquisition. The following parameters were analyzed: heart rate (bpm), amplitude (mv), duration of the QRS complex (ms), R-R (ms), P-Q (ms), and Q-T (ms) intervals.

Records of opercular beat.

For the analysis and monitoring of respiratory function through opercular beats, electrodes made of 925 silvers with a diameter of 0,3 mm and a length of 5,0 mm were crafted and placed on the left operculum

to record activity during the immersion bath. Each recording lasted 5 minutes, and the frequency of opercular beats and the power detected by the electrode were analyzed.

Recording and analysis of data

The electrodes for ECG acquisition were connected to a digital data acquisition system via a high-impedance differential amplifier (Grass Technologies, Model P511), configured for filtering at 0,3 and 300 Hz, and amplification of 2000X. The signals were monitored by using an oscilloscope (ProteK, Model 6510). The recordings were made continuously. They were digitized at a rate of 1 kHz on a computer with a data acquisition card (National Instruments, Austin, TX), and stored on a hard drive for subsequent processing by using a specialized software (LabVIEW express).

The analysis of the obtained records was made possible using a tool developed with Python version 2.7 programming. For mathematical processing, the Numpy and Scipy libraries were used. For graphing, the Matplotlib library was used. The graphical interface was developed by using the PyQt4 library (Souza-Monteiro et al. 2015). The amplitude graphs highlighted the potential differences between the reference and recording electrodes. Additionally, signals from the records were obtained at 1000 samples per second.

Statistical analysis

The data were analyzed to meet the prerequisites of normality and homogeneity of variances by using the Kolmogorov-Smirnov and Levene tests, respectively. Comparisons of the mean values of the analyzed data were made by using one-way ANOVA, followed by Tukey's test. For mean analysis and graph creation, GraphPad Prism® 8 software was used. P-values were considered statistically significant for the following values: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Results

Behavioral analysis showed that BAEO caused loss of the postural reflex in fish, and higher concentrations resulted in shorter latencies for the onset of postural reflex loss. Fish treated with 20 μL . L^{-1} had an average latency for postural reflex loss of 251.3 ± 26.10 s, which was longer than in the other groups. The group treated with 25 μL . L^{-1} had an average latency of 192.4 ± 16.36 s, 30 μL . L^{-1} 133.7 ± 10.05 s), 35 μL . L^{-1} (122.7 ± 8.86 s), and 40 μL . L^{-1} (99.89 ± 7.88 s), all of which were different from each other. The vehicle-treated group showed no behavioral changes (Figure 2 A).

The recovery of the postural reflex in the group treated with 20 μL . L^{-1} of BAEO occurred in 120.0 ± 12.89 s, which was shorter than in the other groups: 25 μL . L^{-1} (154.1 ± 9.62 s), 30 μL . L^{-1} (187.4 ± 9.42 s), 35 μL . L^{-1} (207.6 ± 18.89 s), and 40 μL . L^{-1} (257.7 ± 17.03 s). All groups exhibited recovery times dependent on the concentration used, with higher concentrations resulting in longer recovery times for the postural reflex, showing that the effect was reversible but occurred more slowly in the groups receiving higher concentrations (Figure 2B).

The normal electrocardiogram of the *tambaqui* presented a sinus rhythm and an average heart rate of 82.89 ± 5.30 bpm. The P wave, the QRS complex, and the T wave can be identified (Figure 3 A, B, and C). The interval between R-R discharges (ms), the P-Q intervals (ms), and the Q-T intervals (ms) remained regular, considering the cardiac parameters of normality. In this way, the interference of treatments with increasing concentrations of BAEO was measured. For the control group, the heart rhythm was sinusoidal during the 300-second recording (Figure 3).

The cardiac activity in the control group showed an average heart rate of 82.89 ± 5.30 bpm, similar to the vehicle group ($p = 0.999$), with a sinus rhythm and the presence of all cardiac deflections in the electrocardiogram (Figure 3 and 4A). In a 30-second amplification, all ECG elements can be observed: the P wave, representing atrial contraction; the QRS complex, representing ventricular contraction; and the T wave, representing the ventricular repolarization period. A 5-second amplification allowed for the evaluation of intervals during treatment with BAEO in an immersion bath and its recovery (Figure. 4 A, B, C, D, E and F).

During immersion treatment with $20 \mu\text{L. L}^{-1}$, the fish showed a 14.47% decrease in heart rate compared to the control group (Figure 4 B). The group treated with $25 \mu\text{L. L}^{-1}$ of BAEO showed a 24.67% decrease (Figure 4 C), while the group treated with $30 \mu\text{L. L}^{-1}$ had a 28.95% decrease (Figure 4 D). The group treated with $35 \mu\text{L. L}^{-1}$ exhibited a 28.14% decrease (Figure 4 E), and the group treated with $40 \mu\text{L. L}^{-1}$ showed a 31.63% decrease (Figure 4 F). The treated fish exhibited bradycardia with maintenance of sinus rhythm, which was intensified with increasing concentrations of BAEO. (Figure 4 B, C, D, E and F).

Heart rate decreased during treatment with increasing concentrations of BAEO. The control group had an average of 82.89 ± 5.30 bpm, like the vehicle group ($p = 0.999$). However, these values were higher than those of the other treated groups. The group treated with $20 \mu\text{L. L}^{-1}$ had an average of 70.89 ± 2.26 bpm, which was higher than the other treated groups. The group treated with $25 \mu\text{L. L}^{-1}$ (62.44 ± 2.40 bpm) was similar to the group treated with $30 \mu\text{L. L}^{-1}$ ($p = 0.188$) and $35 \mu\text{L. L}^{-1}$ ($p = 0.419$), but both were higher compared to the group treated with $40 \mu\text{L. L}^{-1}$. The groups treated with $30 \mu\text{L. L}^{-1}$ (58.89 ± 2.26 bpm) were similar to the groups treated with $35 \mu\text{L. L}^{-1}$ and $40 \mu\text{L. L}^{-1}$ ($p = 0.419$) (Figure 5A).

The mean amplitude of the QRS complex in the control group was 1.90 ± 0.50 mV, which was like the other groups ($F [6, 56] = 2.22$; $p = 0.0541$) (Figure 5B).

The average RR interval for the control group was 726.1 ± 43.05 ms, like the vehicle group ($p = 0.999$), and lower compared to the other groups. The group treated with $20 \mu\text{L. L}^{-1}$ had an average RR interval of 838.9 ± 25.44 ms, which was lower than the other treated groups. The group treated with $25 \mu\text{L. L}^{-1}$ (961.8 ± 36.49 ms) was similar to the group treated with $35 \mu\text{L. L}^{-1}$ ($p = 0.1844$). The groups treated with $30 \mu\text{L. L}^{-1}$, $35 \mu\text{L. L}^{-1}$, and $40 \mu\text{L. L}^{-1}$ were similar ($p = 0.2298$) (Figure 5 C).

The average PQ interval for the control group was 95.78 ± 3.15 ms, showing no difference compared to the vehicle group ($p = 0.999$), $20 \mu\text{L. L}^{-1}$ ($p = 0.966$), $25 \mu\text{L. L}^{-1}$ ($p = 0.997$), $30 \mu\text{L. L}^{-1}$ ($p = 0.990$), and $35 \mu\text{L. L}^{-1}$.

L^{-1} ($p= 0.103$). The group treated with $35 \mu\text{L. } L^{-1}$ ($106.3 \pm 8.83 \text{ ms}$) was like the group treated with $40 \mu\text{L. } L^{-1}$ ($p= 0.629$). The group treated with $40 \mu\text{L. } L^{-1}$ was higher than the other groups (Figure 5 D).

The mean duration of the QRS complex for the control group during induction was $16.22 \pm 1.7 \text{ ms}$, similar to the vehicle group ($p= 0.999$), but smaller than the other groups. The groups treated with BAE0 were like each other ($p= 0.6164$) (Figure 5 E).

For the control group, the mean QT interval during induction was $273.7 \pm 20.87 \text{ ms}$, like the vehicle group ($p= 0.9927$), but smaller than the other groups. The vehicle group was similar to the group treated with $25 \mu\text{L. } L^{-1}$ ($p = 0.0734$). The group treated with $20 \mu\text{L. } L^{-1}$ ($310.6 \pm 20.36 \text{ ms}$) was similar to the groups treated with $25 \mu\text{L. } L^{-1}$ ($p= 0.999$) and $30 \mu\text{L. } L^{-1}$ ($p= 0.977$). The group treated with $40 \mu\text{L. } L^{-1}$ ($345.4 \pm 13.68 \text{ ms}$) was like the groups treated with $30 \mu\text{L. } L^{-1}$ ($p= 0.0672$) and $35 \mu\text{L. } L^{-1}$ ($p=0.999$) (Figure 5 F).

During the recovery from treatment with BAE0 at concentrations of $20 \mu\text{L. } L^{-1}$, $25 \mu\text{L. } L^{-1}$, $30 \mu\text{L. } L^{-1}$, $35 \mu\text{L. } L^{-1}$ and $40 \mu\text{L. } L^{-1}$, reversibility of the electrocardiographic changes was observed (Figures 6 A, B, C, D, and E). This reversibility was observed to be slower in the groups treated with higher concentrations of BAE0.

During recovery from treatment, the group treated with $20 \mu\text{L. } L^{-1}$ was like the control group ($p= 0.999$) (Figure 6 A). The fish treated with $25 \mu\text{L. } L^{-1}$ showed 94.36% recovery of cardiac function compared to the control group (Figure 6 B). The group treated with $30 \mu\text{L. } L^{-1}$ of BAE0 showed 93.56% recovery (Figure 6 C), the group treated with $35 \mu\text{L. } L^{-1}$ showed 93.29% recovery (Figure 6 D), and the group treated with $40 \mu\text{L. } L^{-1}$ showed 88.46% recovery (Figure 6 E). The fish treated exhibited slow reversibility but maintained sinus rhythm. (Figure 6).

During recovery, the control group had an average heart rate of $82.89 \pm 5.30 \text{ bpm}$, which was like the vehicle group, and treated with $20 \mu\text{L. } L^{-1}$ ($p = 0.999$). The groups treated with $20 \mu\text{L. } L^{-1}$ ($82.67 \pm 2.64 \text{ ms}$) were similar to the group treated with $25 \mu\text{L. } L^{-1}$ ($p= 0.0557$). The groups treated with $25 \mu\text{L. } L^{-1}$ ($78.22 \pm 1.85 \text{ bpm}$) were similar to the groups treated with $30 \mu\text{L. } L^{-1}$ and $35 \mu\text{L. } L^{-1}$ ($p= 0.996$). The group treated with $40 \mu\text{L. } L^{-1}$ ($73.33 \pm 3.16 \text{ ms}$) was like the groups treated with $30 \mu\text{L. } L^{-1}$ ($p= 0.0804$) and $35 \mu\text{L. } L^{-1}$ ($p= 0.1138$) (Figure 7 A).

The amplitude of the QRS complex during recovery for the control group was $1.90 \pm 0.502 \text{ mV}$, which was like the other groups ($F [6, 56] = 0.1912$, $p=0.9781$) (Figure 7 B).

During recovery, the mean RR interval for the control group was $726.1 \pm 43.05 \text{ ms}$, which was like the vehicle ($p=0.999$), $20 \mu\text{L. } L^{-1}$ ($p=0.999$), and $25 \mu\text{L. } L^{-1}$ ($p=0.053$) groups. The groups treated with $25 \mu\text{L. } L^{-1}$ ($767.2 \pm 18.08 \text{ ms}$) were similar to $30 \mu\text{L. } L^{-1}$ ($p=0.998$) and $35 \mu\text{L. } L^{-1}$ ($p=0.995$) groups. The group treated with $40 \mu\text{L. } L^{-1}$ ($817.0 \pm 37.31 \text{ ms}$) was like the group treated with $35 \mu\text{L. } L^{-1}$ ($p=0.053$). (Figure 7 C).

The PQ interval during recovery for the control group was 95.78 ± 3.15 ms, which was like the other groups ($F [6, 56] = 0.8004$, $p=0.573$) (Figure 7 D).

During recovery, the duration of the QRS complex in the control group had a mean of 16.22 ± 1.78 ms, which was like the other groups ($F [6, 56] = 2.674$, $p=0.0236$) (Figure 7 E).

During recovery, the QT interval for the control group had a mean of 273.7 ± 20.87 ms, which was like the vehicle group ($p= 0.990$), $20 \mu\text{L. L}^{-1}$ ($p= 0.982$) and $25 \mu\text{L. L}^{-1}$ ($p= 0.2256$), $30 \mu\text{L. L}^{-1}$ ($p= 0.1214$) and $40 \mu\text{L. L}^{-1}$ ($p= 0.1085$) groups. However, it was lower than the group treated with $35 \mu\text{L. L}^{-1}$ (Figure 7 F).

The opercular activity in the control group showed a mean frequency of 83.78 ± 3.80 movements per minute, maintaining a breathing rhythm with a mean amplitude of 3.9 mV. The energy distribution in the spectrogram was more intense up to a frequency of 10 Hz. During the recording period, the respiratory rhythm was maintained, as detected by the potential difference between the electrodes implanted in the opercular cavity, allowing for the evaluation of the mean power of movements during the induction and recovery phases of BAE0 treatment (Figure 8 A, B, and C).

The opercular beat recordings demonstrated that the mean for the control group was 83.78 ± 2.40 (opercular movements per minute) (opm), which was like the vehicle group ($p= 0.995$). During treatment with BAE0, a concentration-dependent decrease was observed (Figure 9 A, B, C, D, E, and F). The control and vehicle groups had higher values compared to the other groups. The group treated with $20 \mu\text{L. L}^{-1}$ showed a reduction in opercular activity of 55.96%; $25 \mu\text{L. L}^{-1}$ showed a reduction of 55.96%, $30 \mu\text{L. L}^{-1}$ had a decrease of 60.74%, $35 \mu\text{L. L}^{-1}$ showed a reduction of 61.80% and $40 \mu\text{L. L}^{-1}$ (66.84 %).

For opercular movements, the control and vehicle groups had higher values compared to the other groups. The group treated with $20 \mu\text{L. L}^{-1}$, with a mean opercular beat of 36.89 ± 2.261 opm, was like the group treated with $25 \mu\text{L. L}^{-1}$ ($p= 0.999$) and with $30 \mu\text{L. L}^{-1}$ ($p= 0.0756$). The group treated with $30 \mu\text{L. L}^{-1}$ was similar to the group treated with $35 \mu\text{L. L}^{-1}$ ($p= 0.995$). The group treated with $40 \mu\text{L. L}^{-1}$ was like the group treated with $35 \mu\text{L. L}^{-1}$ ($p=0.0509$) (Figure 9 G).

A decrease in the power of opercular movement records (opm) was observed during the induction of treatment with different doses of BAE0 in *Colossoma macropomum*. The control group showed a mean power of 0.90 ± 0.16 mV² /Hz, which was like the vehicle group ($p= 0.846$), but higher than the other groups. The groups treated with $20 \mu\text{L. L}^{-1}$ (0.075 ± 0.021 mV² /Hz) were similar to the other treated groups ($p= 0.977$) (Figure 9 H).

During the recovery period following treatment with BAE0, the frequency of opercular movement per minute (opm) showed reversibility, approaching that of the control group (Figures 10 A, B, C, D, E and F). The spectrograms indicated an increase in power during recovery from the treatment. During recovery, the opercular activity of the control group, with an average of 83.78 ± 3.80 opm, was like the vehicle group ($p= 0.999$), the group treated with $20 \mu\text{L. L}^{-1}$ ($p= 0.876$), $25 \mu\text{L. L}^{-1}$ ($p= 0.998$), $30 \mu\text{L. L}^{-1}$ ($p= 0.999$),

and 35 $\mu\text{L. L}^{-1}$ ($p = 0.059$). The group treated with 40 $\mu\text{L. L}^{-1}$ (60.67 ± 7.550 opm) was lower than the other groups (Figure 10 F).

The recovery of power in opercular movement recordings (opm) was observed during the recovery period after treatment with BAEO in *Colossoma macropomum*. The control group had an average power of 0.90 ± 0.16 mV^2/Hz , which was like the vehicle group ($p = 0.846$), the group treated with 20 $\mu\text{L. L}^{-1}$ ($p = 0.9826$), 25 $\mu\text{L. L}^{-1}$ ($p = 0.8668$), and 30 $\mu\text{L. L}^{-1}$ ($p = 0.998$). However, it was higher than the groups treated with 35 $\mu\text{L. L}^{-1}$ and 40 $\mu\text{L. L}^{-1}$ (Figure 10 G).

Discussion

In this study, we demonstrate for the first time that sandalwood essential oil (*Amyris balsamifera*) caused a reduction in the latency for the onset of loss of the postural reflex, increased the recovery latency of the postural reflex, and decreased heart rate in a concentration-dependent manner in *tambaqui* (*Colossoma macropomum*). These findings were achieved through behavioral analysis and ECG recordings.

In this regard, BAEO shows that at higher concentrations (40 $\mu\text{L. L}^{-1}$), the induction time (7,88s) for the loss of postural reflex is shorter. In contrast, lower concentrations (20 $\mu\text{L. L}^{-1}$) indicate a longer induction time (26,10s). In other studies, on anesthetics in fish, this relation was also found using different types of drugs such as spilanthol, cunaniol, piper divaricatum, and nepeta cataria (Vilhena et al. 2022; Hamoy et al. 2023; dos Santos et al. 2024; Vilhena et al. 2022; Garcia et al. 2024).

Based on the behavioral evaluation, it was possible to confirm the effectiveness of the essential oil of *Amyris balsamifera* through the assessment of the recovery from the loss of postural reflex to normality within an appropriate time, that is, within 5 minutes or less, a period considered ideal to ensure the anesthetic safety of a drug (Ross & Ross 2008; Ghanawi et al. 2013; Hoseini et al. 2019). Additionally, it is possible to identify a dose-dependent effect of the drug, in which the effect becomes more pronounced as the concentration of the drug administered to the *tambaqui* increases. That is, in the present research results, there is a noticeable difficulty in recovering the postural reflex at higher doses, such as 40 $\mu\text{L. L}^{-1}$, and a more immediate recovery at the dose of 20 $\mu\text{L. L}^{-1}$. This dose-dependent effect has also been observed in other studies evaluating behavioral responses to anesthesia in fish, such as with sodium bicarbonate (Oliver et al. 2020); eugenol (da Paz et al. 2024); and spilanthol (Garcia et al. 2024).

During anesthetic recovery, *Amyris balsamifera* at different concentrations demonstrated maintenance of sinus rhythm and almost complete reversibility of cardiac activity within 5 minutes, the maximum recommended time for the resumption of normal behavior after anesthesia (Ross & Ross 2008). At a concentration of 25 $\mu\text{L. L}^{-1}$, electrical activity corresponded to 94,36% of the control activity, and at a concentration of 40 $\mu\text{L. L}^{-1}$, it corresponded to 88,46%. As for the electrocardiographic traces, the R-R interval increased due to the prolonged duration of the QRS complex and the QT interval in proportional

relation to the concentrations, demonstrating a dose-dependent effect, yet like the control traces. That said, complete reversibility was not achieved, but there was a tendency toward recovery to the initial condition over time.

From this perspective, this oil has a major component, with approximately 29,11% valerian oil (valerianol). This information is highly relevant because various *in vivo* scientific studies have shown that, at the cellular level, this essential oil acts on serotonin 5-hydroxytryptamine 1A (5-HT_{1A}) and γ -aminobutyric acid (GABA) receptors, resulting in its ability to promote sedation in rats (Wang 2022). Although this finding is related to an animal species far removed from the one addressed in this article, it also supports the findings in our manuscript, as our results showed a loss of postural reflex and a decrease in opercular beating, both evident signs of sedation in fish (Vergneau-Grosset 2022). This occurs because fish are animals with a Central Nervous System and have serotonergic receptors, such as 5-HT₁.

These receptors are subtypes of serotonin receptors (5-HT) that, when activated, can regulate neuronal excitability, directly affecting the response to anesthetic stimuli. Recent studies indicate that activation of 5-HT₁ receptors, especially 5-HT_{1A}, can modulate calcium currents (CaV_{VV2.2}) in sensory neurons, which impacts the response to anesthesia and pain control (Khan 1997; Bhattarai 2014; Prasad 2015; Anselmi 2022; Li 2022). Moreover, GABAergic receptors, such as GABA_A ion channels, mediate the main form of fast inhibitory neurotransmission in the central nervous system by allowing chloride ions (Cl⁻) to enter neuronal cells, resulting in membrane hyperpolarization and decreased neuronal excitability. This leads to the regulation of neuronal activity, contributing to effects such as sedation and anesthesia (Möhler 2010; Crestani 2015; Snigirov 2018). These factors are sufficient to trigger molecular events like those observed in other species exposed to the same substance.

In addition to the pharmacological effects on the central nervous system (CNS) presented so far, cardiovascular events can be presumed, as there is interaction and interconnection between them. Furthermore, other compounds highlighted in the essential oil chromatography are alpha-eudesmol, estimated at 13,34%, and beta-eudesmol, at 7,02%, both sesquiterpenes. Terpene compounds are important subcomponents of aromatic substances with sedative potential. Studies indicate that GABA_A receptors are particularly sensitive to the action of these compounds (Milanos et al. 2017).

Beta-eudesmol is the most explored sesquiterpene compound due to its greater bioactive potential and low toxicity (Acharya et al. 2020). Its physiological effect on the nervous system is notable, as it acts as a non-competitive blocker of nicotinic acetylcholine receptors (nAChR) at the neuromuscular junction, which impairs nerve impulse transmission and causes muscle relaxation and pain relief (Kimura et al. 2012), relating to the sedative and muscle-relaxing effects observed with the application of *A. balsamifera*.

Monitoring of opercular beat frequency revealed a reduction ranging from 55.96% to 66.84%, depending on the administered dose. These results demonstrate a decrease in frequency like that obtained with the exposure of *tambaqui* to propofol (Souza et al. 2019) and menthol at 100 mg. L⁻¹ (Alho da Costa et al.

2022), and greater than that observed with citronella essential oil at 600 µL. L⁻¹ (Barbas et al. 2017), a concentration significantly higher than the Amyris sandalwood essential oil used in this study. The strength of opercular movements was also analyzed. It showed a relatively uniform decrease among the groups treated with different doses compared to the control group, and comparable to that observed with etomidate at 4 mg. L⁻¹ (Reis et al. 2024). These results indicate that Amyris sandalwood oil exhibits a suppression effect on respiratory activity comparable to, or even greater than, other essential oils and drugs generally used as anesthetics in *tambaqui*.

The depression of opercular activity showed reversibility during recovery. Fish exposed to Amyris sandalwood oil regained both the frequency and intensity of opercular beats. During the recovery period following immersion, the opercular beat frequency in the treated groups demonstrated reversibility like the control group, except for the group subjected to the 40 µL. L⁻¹ dosage, which showed less reversibility compared to the other groups in the study. Similar to the recovery behavior of frequency in relation to SAEO, groups exposed to citronella oil (600 µL. L⁻¹) also showed opercular beats close to those of the control group, supporting our manuscript (Barbas et al. 2017). Showing a divergent behavior, in the recovery period after immersion in menthol oil (100 mg. L⁻¹), opercular beats were increased compared to the control groups (Alho da Costa et al. 2022).

Declarations

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by the Animal Ethics Committee (CEUA—UFPA) number. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

Conceived and designed the experiments: A.T.L.A.L. and M.H. Performed the experiments: A.T.L.A.L., L.V.d.S., L.L.d.R., G.B.B and M.H. Writing-original draft and editing: all authors. Financial support and administrative support: M.H. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

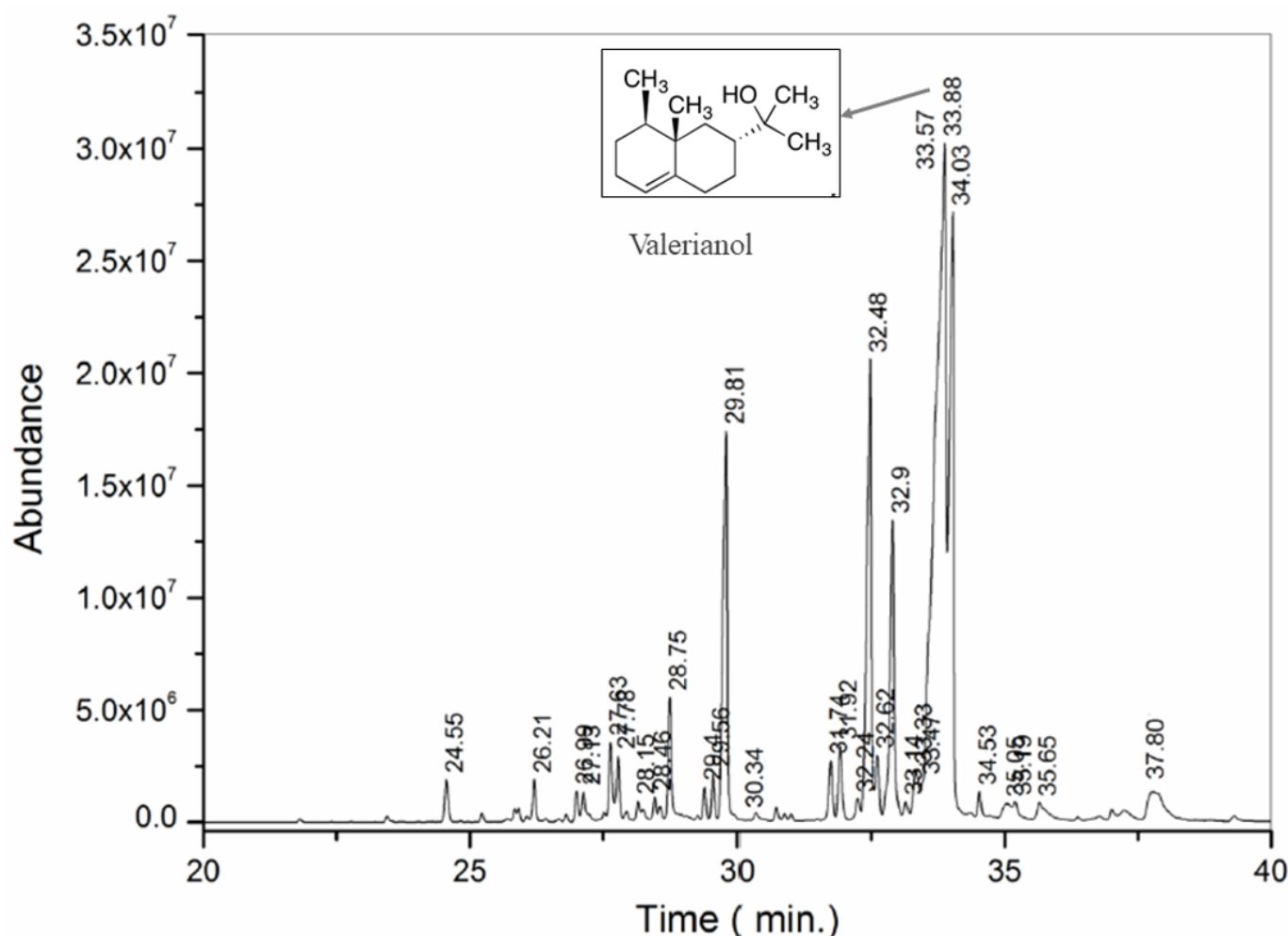


Figure 1

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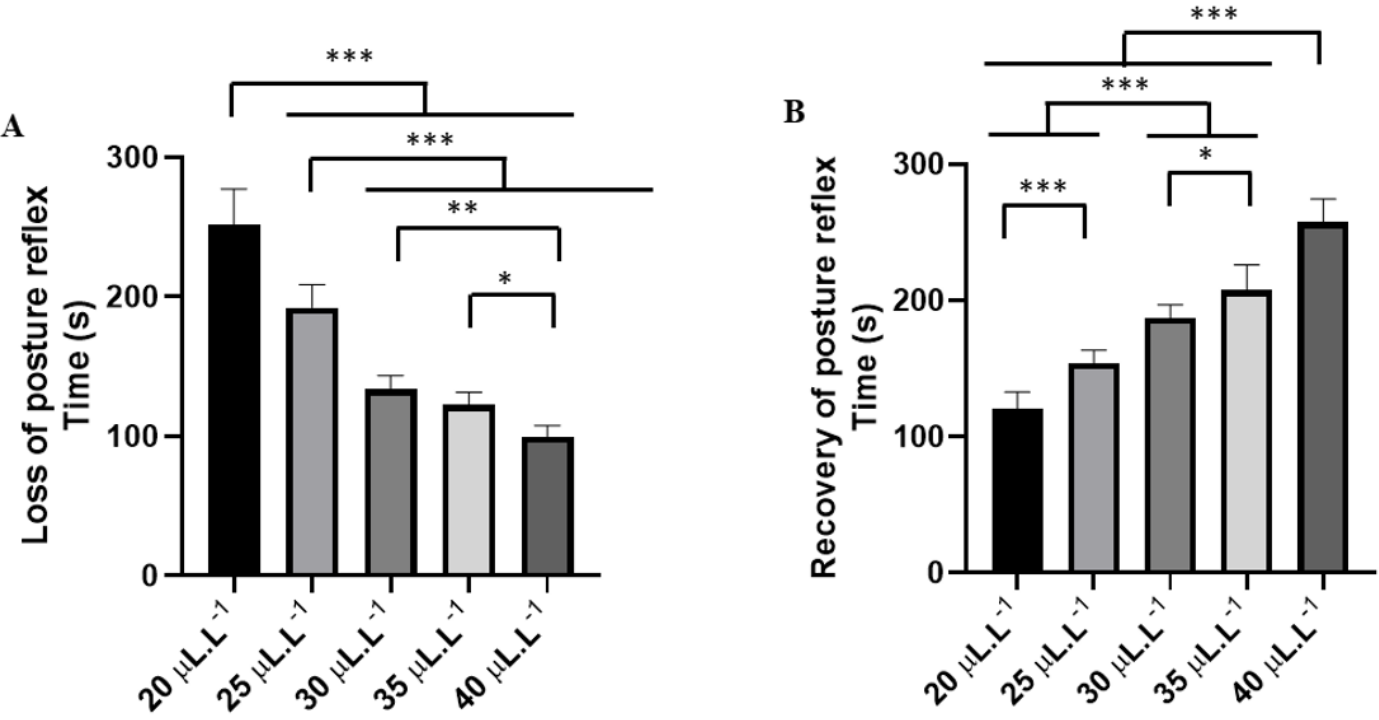


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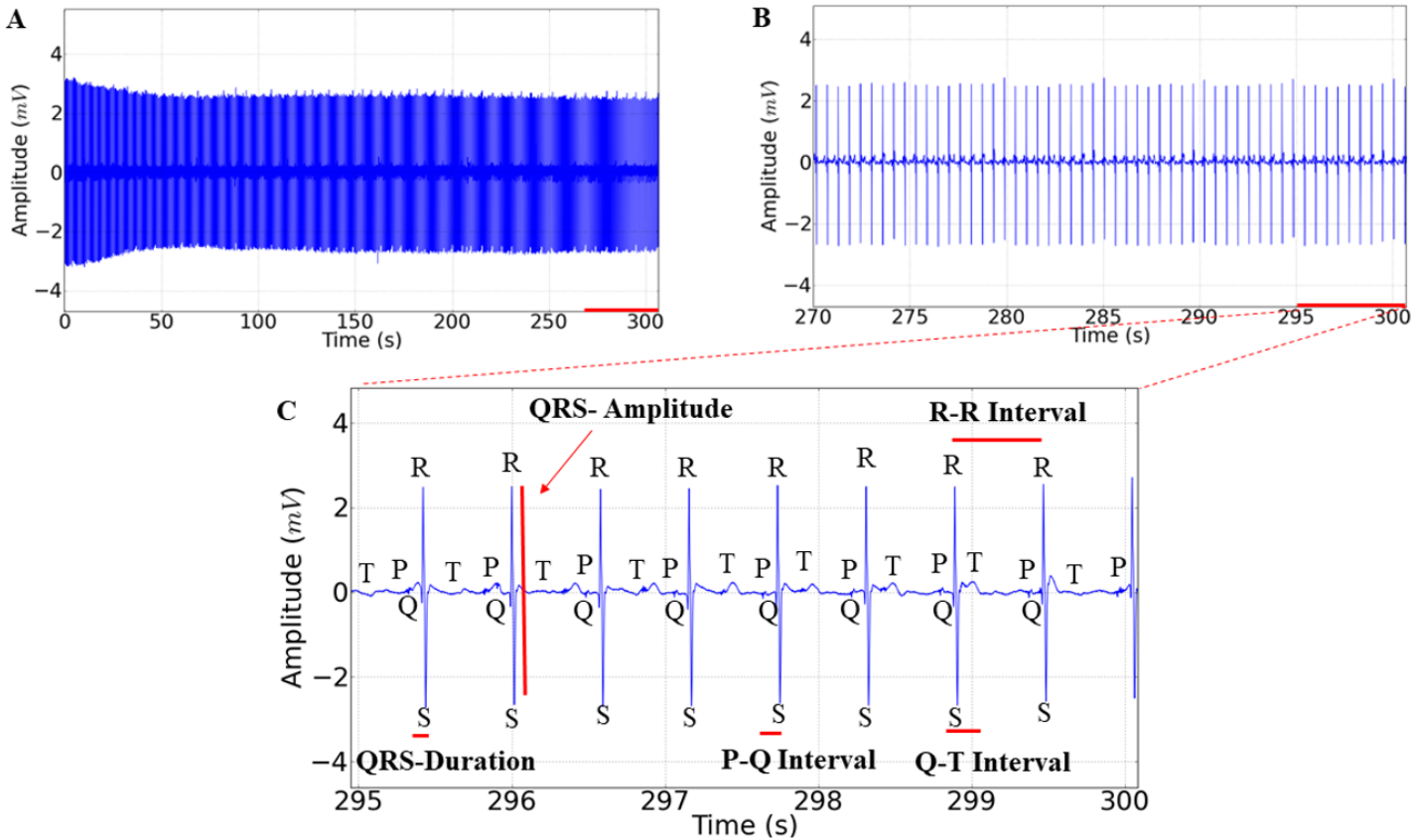


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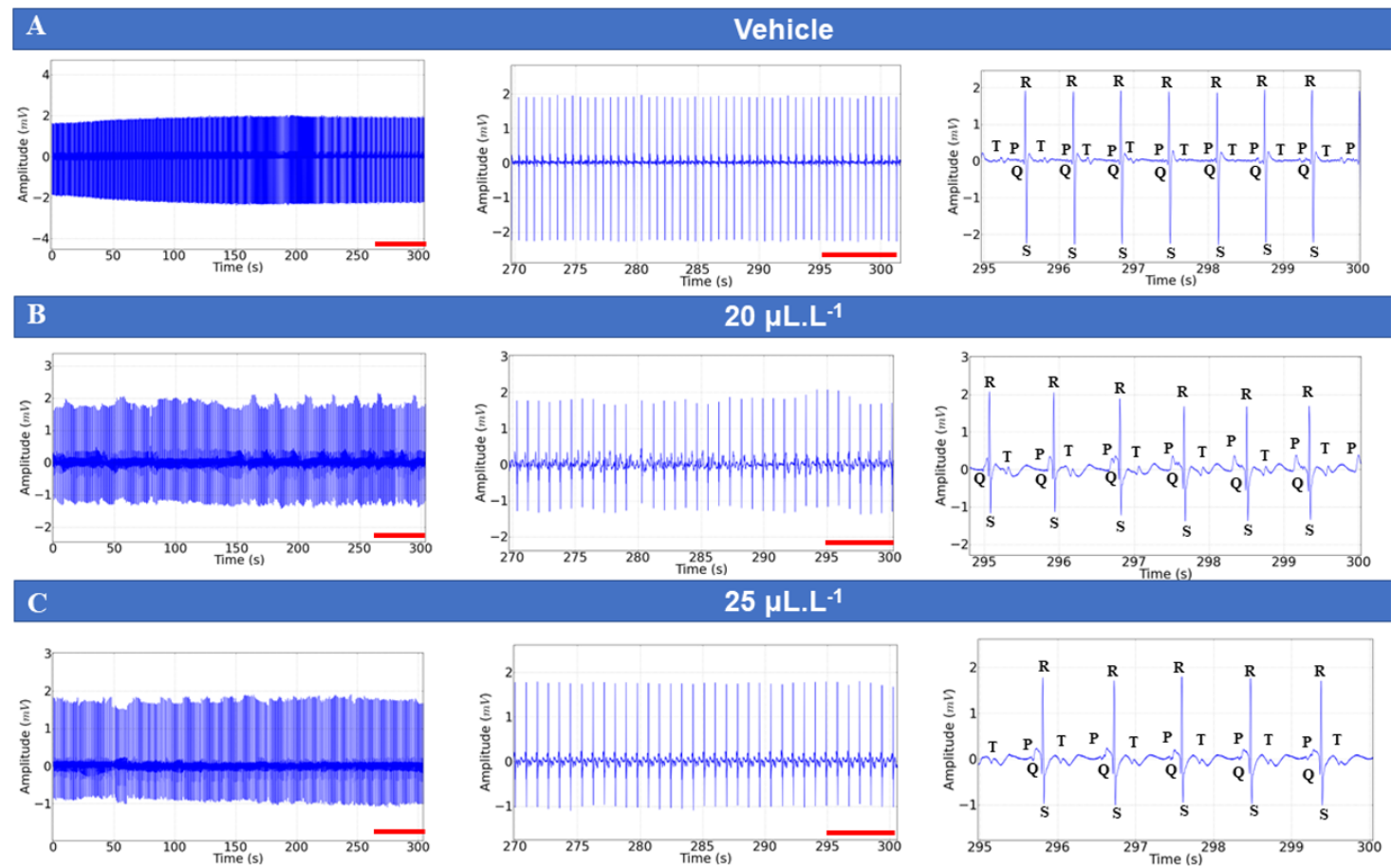


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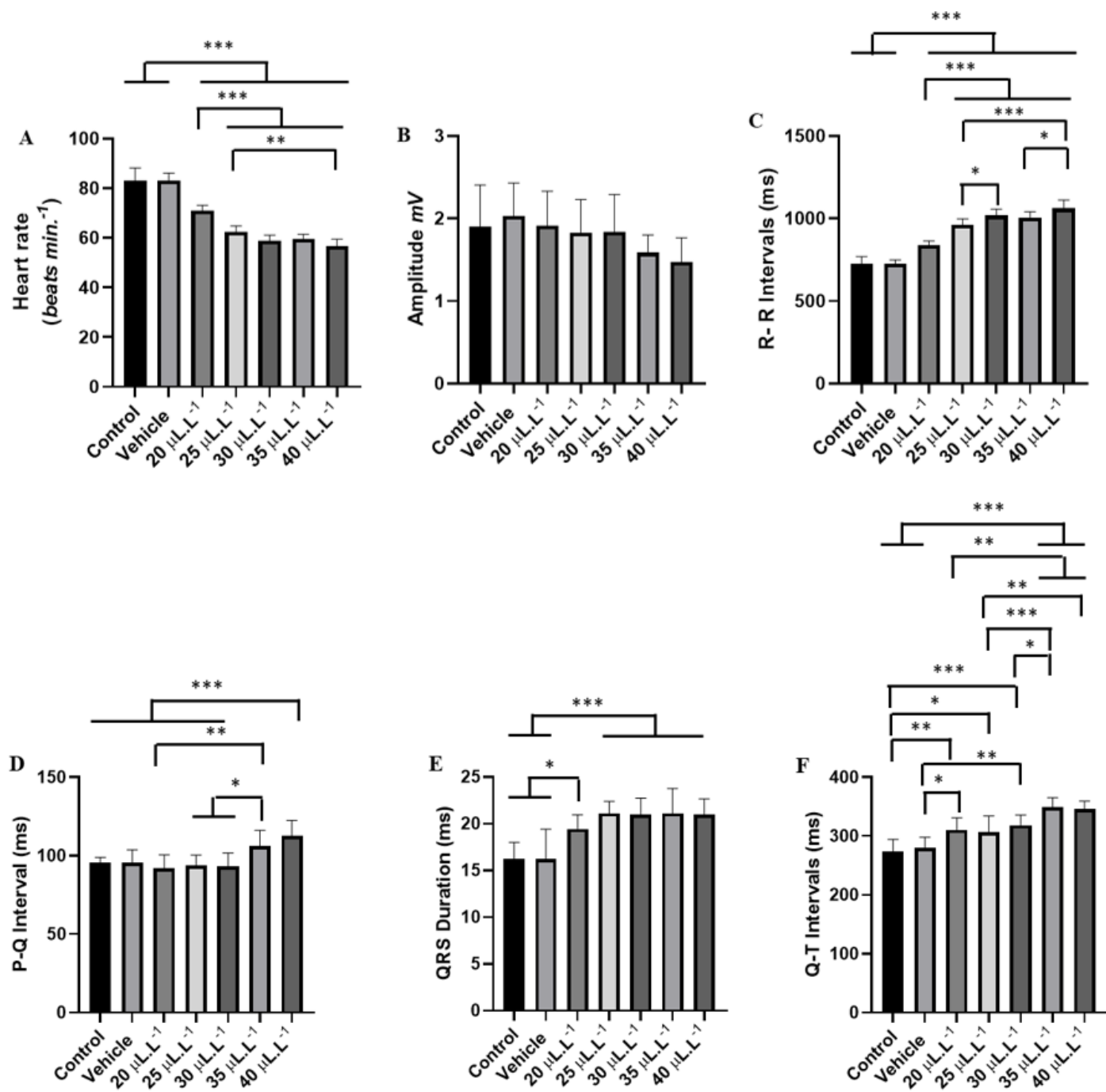


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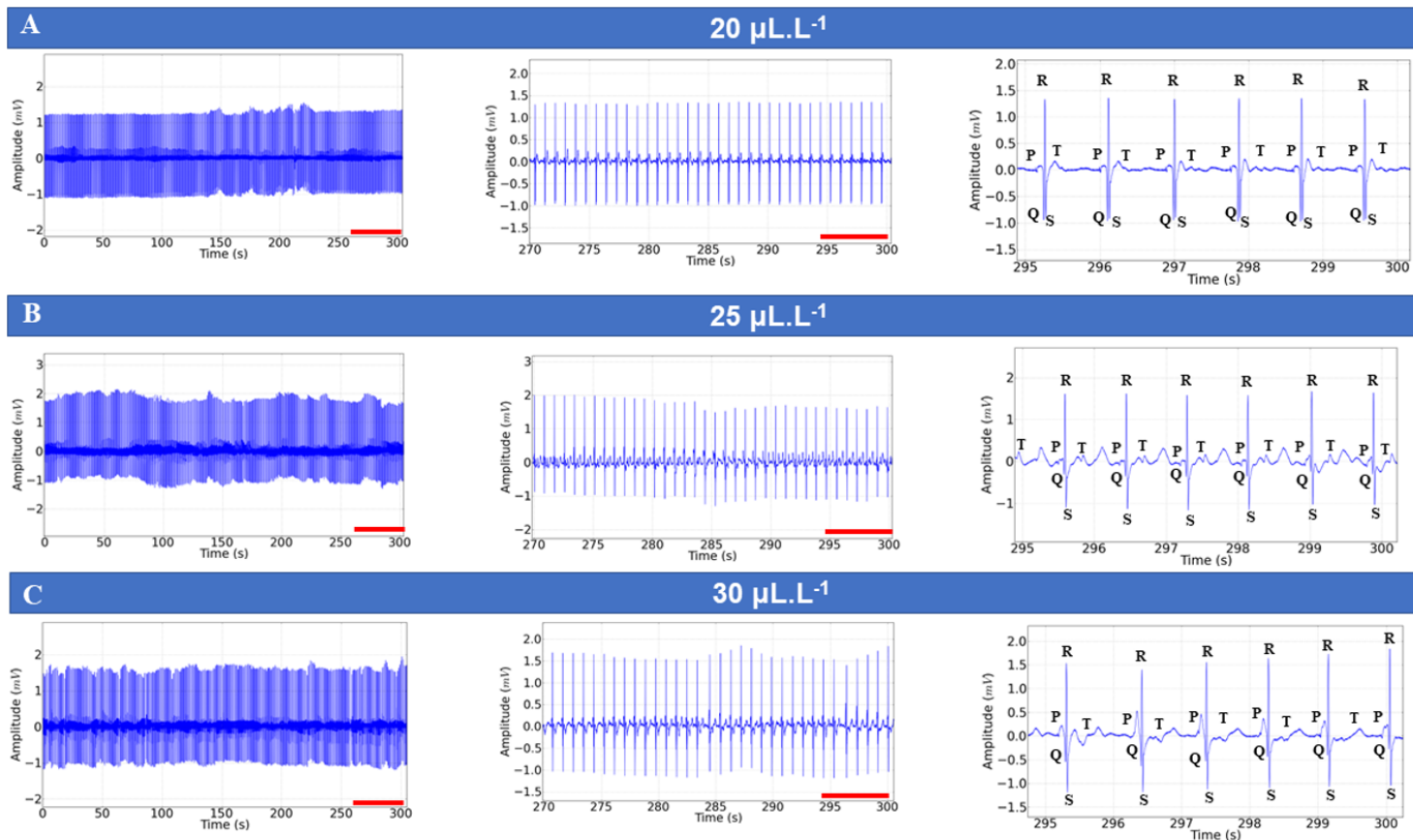


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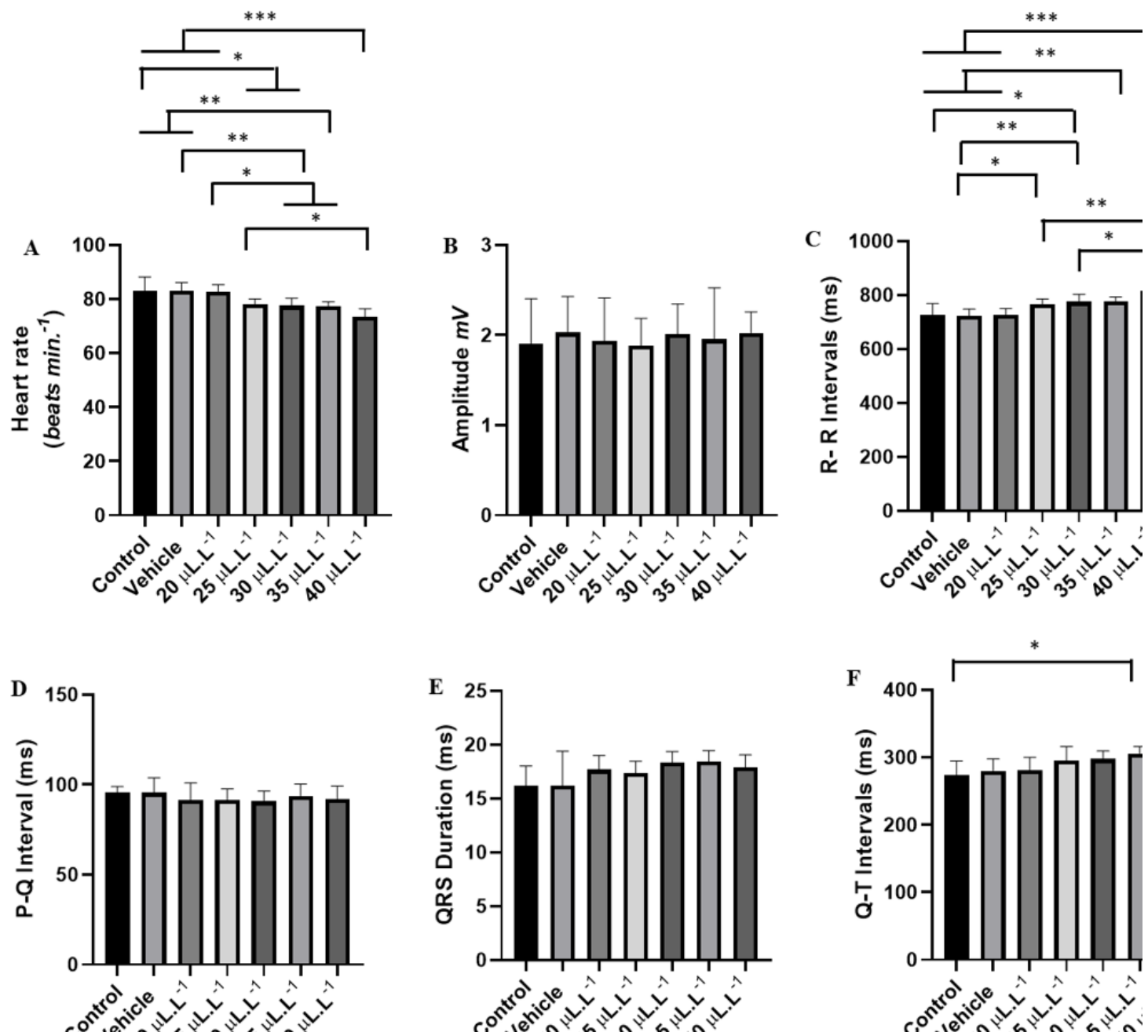


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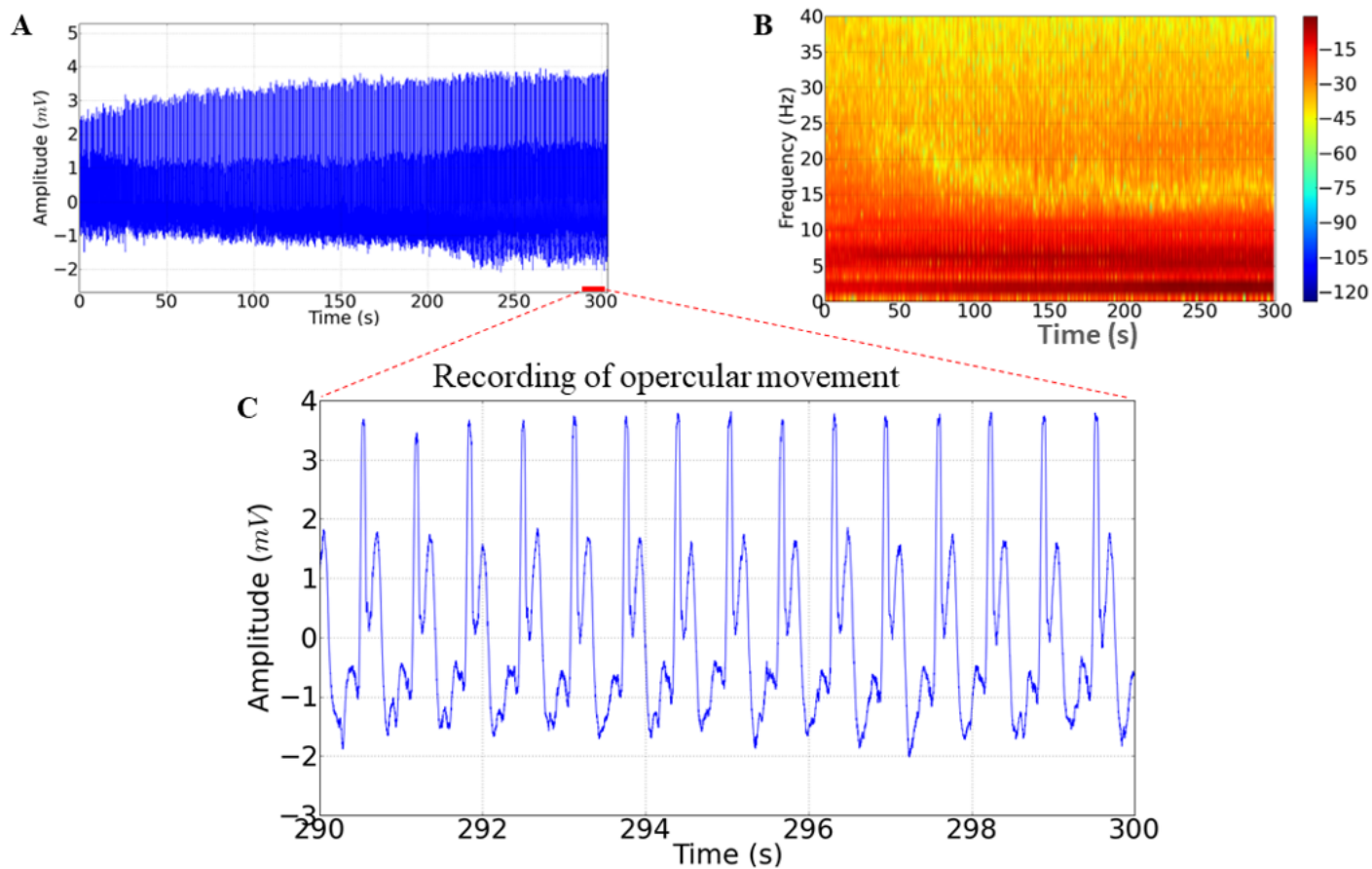


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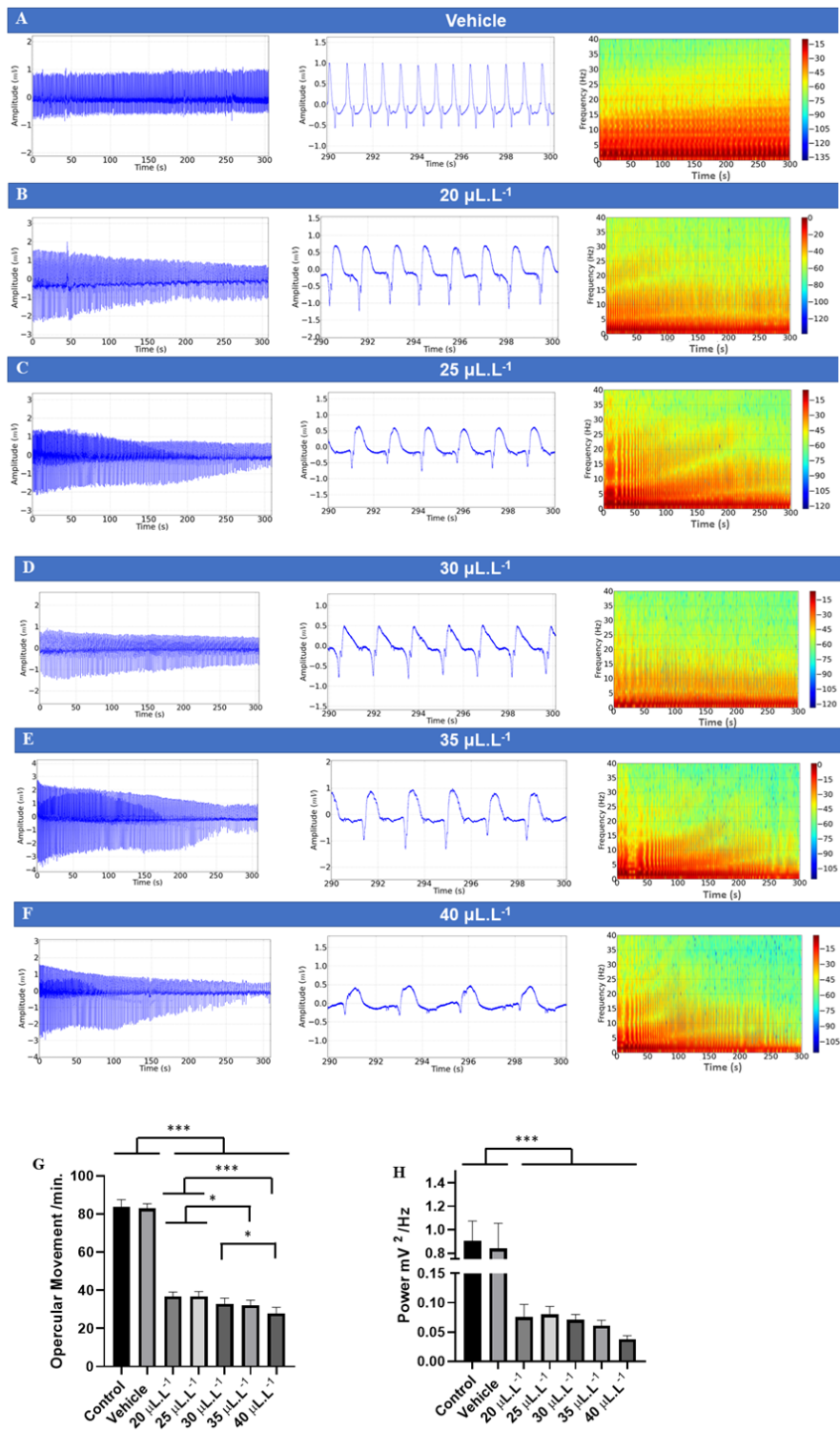


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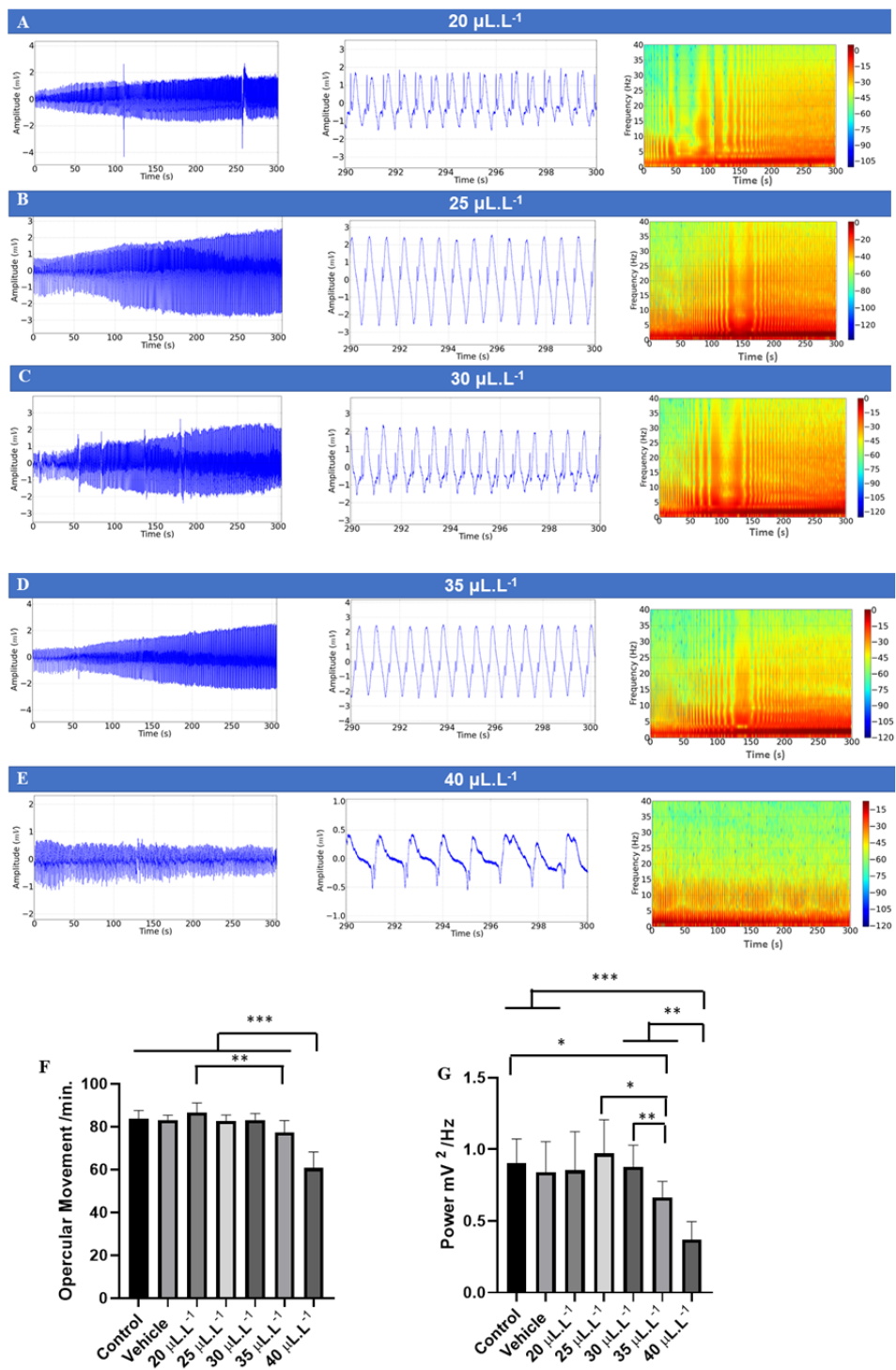


Figure 10

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