

Anesthesia and sedation of map treefrog (*Hypsiboas geographicus*) tadpoles with essential oils

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ABSTRACT: The goal of this study was to investigate the sedative and anesthetic properties of essential oils (EOs) in map treefrog tadpoles (*Hypsiboas geographicus*) and to determine the sedation and deep anesthesia induction times as well as the recovery time. The tadpoles were exposed to one of the EOs from three plant species: *Aniba roseaodora* (EOAR - 25, 50, 100 or 200 μ L L⁻¹), *Lippia origanoides* (EOLO - 13, 25, 50, 100 or 200 μ L L⁻¹), and *Lippia alba* (either chemotype citral [EOL-C - 25, 50, 100 or 200 μ L L⁻¹] or linalool [EOL-L - 50, 75, 100 or 200 μ L L⁻¹]) (n = 8 per replicate). The tadpoles exposed to 25 and 50 μ L L⁻¹ EOL-C and EOL-L, respectively, were not anesthetized within 30min (the maximum time of observation), and those exposed to 200 μ L L⁻¹ EOLO did not recover within 30min. Sedation, deep anesthesia and recovery times showed a concentration-dependent relationship for all EOs tested, with the exception of the recovery with EOLO. The results allowed concluding that all investigated EOs can be used to anesthetize tadpoles of *H. geographicus*, but the use of EOLO must not exceed 100 μ L L⁻¹.
Key words: amphibians, animal welfare, natural anesthetics.

Anestesia e sedação de girinos da perereca geográfica *Hypsiboas geographicus* com óleos essenciais

RESUMO: O objetivo deste estudo foi investigar as propriedades sedativas e anestésicas de óleos essenciais (OEs) em girinos da perereca *Hypsiboas geographicus* e determinar os tempos de indução à sedação e anestesia profunda, bem como o de recuperação. Os girinos foram expostos a um dos OEs de três espécies de plantas: *Aniba roseaodora* (OEAR - 25, 50, 100 ou 200 μ L L⁻¹), *Lippia origanoides* (OELO - 13, 25, 50, 100 ou 200 μ L L⁻¹) ou *Lippia alba* quimiotipos citral (OEL-C - 25, 50, 100 ou 200 μ L L⁻¹) ou linalol (OEL-L - 50, 75, 100 ou 200 μ L L⁻¹) (n = 8 cada repetição). Girinos expostos a 25 e 50 μ L L⁻¹ OEL-C e OEL-L, respectivamente, não foram anestesiados dentro de 30min (tempo máximo de observação) e aqueles expostos a 200 μ L L⁻¹ OELO não recuperaram dentro de 30min. Os tempos de sedação, anestesia profunda e recuperação apresentaram uma relação concentração-resposta para todos os OEs testados, exceto a recuperação com OELO. Os resultados permitem concluir que todos os OEs investigados podem ser usados para anestésiar os girinos de *H. geographicus*, mas o uso de OELO não deve ser superior a 100 μ L L⁻¹.

Palavras-chave: anfíbios, bem estar animal, anestésicos naturais.

INTRODUCTION

Amphibians are commonly exhibited in zoological collections and have a long history of veterinary care in captivity, primarily in research settings, because they are useful as animal models (MITCHELL, 2009; CHINNADURAI & KANE, 2014). The map treefrog (*Hypsiboas geographicus*), family Hylidae, is an amphibian found in the

ecosystems of northern Brazil (PINHEIRO et al., 2012) and has potential use in scientific research.

Many laboratory or field investigations require the use of anesthetics to reduce distress and pain in animals; therefore, sedatives and/or anesthetic products have been used on amphibians for laboratory research purposes. Until recently, synthetic products such as tricaine methanesulfonate (MS 222), a mixture of ketamine/diazepam (HERNÁNDEZ et

al., 2012), isoflurane, medetomidine, benzocaine, propofol (MITCHELL, 2009), a combination of medetomidine/ketamine/meloxicam/butorphanol, meloxicam (CHAI, 2015), sevoflurane, ketamine/diazepam (CHINNADURAI & KANE, 2014) and opioids (STEVENS, 2004) have been studied. Several studies have been performed using adults (MITCHELL, 2009; CHINNADURAI & KANE, 2014), but tadpoles also have been used to identify new anesthetics for amphibians because tadpoles have the same receptors as adults (KRASOWSKI et al., 2001) and because a lower amount of compounds can be used for the tests.

Essential oils (EOs) extracted from plants have been gaining interest as potential sedatives and/or anesthetics for aquatic animals (SILVA et al., 2013; TONI et al., 2015). However, there are no studies regarding the effects of EOs on amphibians, and the anesthetic effects produced by EOs can vary depending on their chemical composition and animal species exposed (CUNHA et al., 2010; PARODI et al., 2012; TONI et al., 2015). *Aniba rosaeodora* Ducke is a large tree reaching up to 30m in height that is native to the Amazon region. Its EO is known as rosewood oil, and its anti-inflammatory, sedative and hypothermic effects are attributed to its main compound, linalool. Experimentation with rodents has demonstrated the sedative effect of linalool-rich rosewood oil (ALMEIDA et al., 2009), and isolated linalool had a similar sedation profile as that of the EO at a proportional concentration in silver catfish (*Rhamdia quelen*) (HELDWEIN et al., 2014).

Lippia alba is a shrub found in the United States of America (Florida and Texas), Central and South America and India (HENNEBELLE et al., 2008). It has a large range of EO composition, which varies depending on the geographic origin of the plant. Consequently, the EO is classified into different chemotypes according to its major constituents: citral, linalool, β -caryophyllene, tagetenone, limonene, carvone, myrcene, γ -terpinene, camphor-1,8-cineole and estragole (STASHENKO et al., 2004; HENNEBELLE et al., 2008; TELES et al., 2012). The EO of *L. alba* chemotype linalool (EOL-L) can induce fish and shrimp sedation and anesthesia (CUNHA et al., 2010; PARODI et al., 2012). *Lippia origanoides* Kunth is an aromatic shrub found from southern North America to northern South America (SARRAZIN et al., 2015). Its EO has an analgesic effect on mice (OLIVEIRA et al., 2014), and in the Amazonian region, thymol and carvacrol are its main compounds (SANTOS et al., 2004; OLIVEIRA et al., 2007).

The goal of this study was to investigate the sedative and anesthetic effects of the EOs from *A. rosaeodora* (EOAR), *L. origanoides* (EOLO) and *L. alba* (chemotype citral, EOL-C, and chemotype linalool, EOL-L) on tadpoles of *H. geographicus*.

MATERIALS AND METHODS

Essential oils

Aniba rosaeodora, *L. origanoides* and *L. alba* (chemotype citral) leaves were collected in Santarém, Pará State, northern Brazil, and *L. alba* (chemotype linalool) leaves were collected in Santa Maria, Rio Grande do Sul state, southern Brazil. The EOs were extracted from fresh leaves of the plants by hydrodistillation for 2h using a Clevenger-type apparatus (EUROPEAN PHARMACOPOEIA, 2007) and stored at -4°C in amber glass bottles until composition analysis. The analysis was performed using a gas chromatograph (Agilent 6890) coupled to a mass-selective detector (Agilent 5973) using an HP5-MS column (5% phenyl, 95% methylsiloxane, 30m \times 0.25mm inner diameter \times 0.25mm) as described by SILVA et al. (2013). The constituents of the EOs were identified by comparison of the Kovats retention index and mass spectra with a mass spectral library (NIST, 2014) and literature data (ADAMS, 2001).

Biological tests

Tadpoles of *H. geographicus* ($1.52 \pm 0.26\text{g}$; $5.44 \pm 0.51\text{cm}$) were collected from the Fish Production Station UAGRO/SAGRI/SEDAP – Santarém/PA and immediately transferred to aquaria. The EOs and concentrations tested were as follows: EOAR - 25, 50, 100 or $200\mu\text{L L}^{-1}$; EOL-C - 25, 50, 100 or $200\mu\text{L L}^{-1}$; EOLO - 13, 25, 50, 100 or $200\mu\text{L L}^{-1}$; and EOL-L - 50, 75, 100 or $200\mu\text{L L}^{-1}$. For each concentration and EO, eight animals were placed in aquaria with 500mL of water and the EO (the EO was previously diluted in ethanol, 1:10), and the times for inducing sedation, deep anesthesia and full recovery (Table 1) were recorded. The sedation and deep anesthesia stages were in accordance with stages 2 and 4 described by SCHOETTGER & JULIN (1967). The maximum observation time was 30min, and each animal was used only once. Preliminary tests indicated that lower EO concentrations had no sedative or anesthetic effect up to 30min of observation. The same procedure was conducted with one group of eight animals exposed to the highest concentration of ethanol used for dilution of the EOs. Experimental methodologies were approved by the Ethical and

Table 1 - Stages of anesthesia and recovery of *Hypsiboas geographicus* (adapted from SCHOETTGER & JULIN, 1967).

Stage	Behavioral response
Sedation	Partial loss of equilibrium and decreased reactivity to external stimuli
Deep anesthesia	Total loss of equilibrium, cessation of locomotion (swimming activity) and no response to strong external stimuli
Full recovery	Full recovery of equilibrium, swimming activity and response to external stimuli

Animal Welfare Committee of the Universidade Federal do Pará (process n° 42/2012) and Instituto Chico Mendes de Conservação da Biodiversidade – ICMBio and Sistema de Autorização e Informação em Biodiversidade - SISBIO (n° 24072-1/2010).

Statistical analyses

The data were expressed as the means \pm SD. Evaluation of anesthetic activity was performed by regression analysis (concentration \times time of anesthesia induction; concentration \times time of recovery from anesthesia) using Sigma Plot 11.0 software.

RESULTS

There were no mortalities throughout the experiment. Ethanol at the highest concentration used for dilution of the EOs did not lead to sedation or anesthesia. Time to induce sedation or anesthesia and recovery showed a concentration-dependent relationship for all EOs tested. There was a reduction in the induction time to sedation and anesthesia with an increase in EO concentration, and the recovery time was longer with an increase in EO concentration. The exception was the recovery time with EOLO, since animals exposed to 200 μ L L⁻¹ did not recover within 30min (Table 2).

The major component identified in EOAR and EOL-L was linalool (88.6 and 50.6%, respectively). Carvacrol (40.7%) and citral (54.2%: E-citral 29.8%, Z-citral 24.4%) were the major components of EOLO and EOL-C, respectively.

DISCUSSION

The growing presence of amphibians as pets and in zoological institutes has increased the interest in research with these animals. Since many procedures can cause discomfort or pain, some sedative and/or anesthetic substances have been tested in adults (MITCHELL, 2009; HERNÁNDEZ et al., 2012; CHINNADURAI & KANE, 2014) or tadpoles (KRASOWSKI et al., 2001). However,

clove oil is lethal to *Rhinella marina* (HERNÁNDEZ et al., 2012) adults and causes respiratory depression in adult leopard frogs (*Rana pipiens*). Another side effect noted with clove oil in the leopard frog was that 50% of the frogs had a prolapsed stomach after being removed from the clove oil solution (MITCHELL, 2009).

The present study demonstrated that the EOs in the range of concentrations tested were efficient in the induction of sedation and anesthesia in *H. geographicus* at a maximum observation time of 30min and did not exceed this same time for recovery (in general). No side effects or mortalities were observed.

Several studies conducted by our research group demonstrated that EOs are a useful tool in aquaculture procedures because they can induce fish and shrimp sedation and anesthesia (CUNHA et al., 2010; PARODI et al., 2012; TONI et al., 2015). The lowest EOL-L concentration to induce anesthesia of *H. geographicus* tadpoles was 75 μ L L⁻¹ and the lowest concentration to induce anesthesia within 5min was 100 μ L L⁻¹. These results demonstrated that this frog is relatively susceptible to anesthesia with EOL-L, since the lowest EOL-L concentration to induce anesthesia in fish is 50-200 μ L L⁻¹ and to induce anesthesia within 5min is 50-450 μ L L⁻¹ (CUNHA et al., 2010; TONI et al., 2015; HOHLENWERGER et al., 2016). The lowest EOAR concentration to induce anesthesia of *H. geographicus* tadpoles within 5min (200 μ L L⁻¹) was somewhat higher than that of EO-L, but the lowest concentration to induce anesthesia was lower (25 μ L L⁻¹). This result is likely related to the higher linalool percentage in EOAR than EO-L, since the concentration of linalool to induce sedation in silver catfish is comparatively lower than that of EO-L (HELDWEIN et al., 2014).

The lowest EOLO concentration to induce anesthesia of *H. geographicus* was lower than the concentration of the other EOs tested, but the lowest EOLO concentration to induce anesthesia within 5min was the same as that of EOL-L and EOL-C. There are no studies of anesthesia with EOLO, but the EO of the carvacrol chemotype of *Lippia*

Table 2 - Time (in seconds) for inducing sedation and deep anesthesia and for recovery of *Hypsiboas geographicus* tadpoles exposed to the essential oils of *Aniba rosaedora* (EOAR), *Lippia origanoides* (EOLO) and *Lippia alba* (chemotypes citral, EOL-C, and linalool, EOL-L). *no recovery within 30min.

$\mu\text{L L}^{-1}$	Sedation	Anesthesia	Recovery
-----EOAR-----			
25	1475.87 \pm 53.41	1741.12 \pm 83.33	64.62 \pm 38.23
50	414.25 \pm 87.85	787.82 \pm 241.63	114.87 \pm 43.67
100	174.50 \pm 16.16	468.25 \pm 74.94	245.75 \pm 4.85
200	207.00 \pm 78.63	282.25 \pm 45.61	382.87 \pm 111.62
Equations	$y = 188.17 + 7397.72e^{(-0.07x)}$; $r^2 = 0.999$	$y = 337.31 + 4105.51e^{(-0.04x)}$; $r^2 = 0.992$	$y = 682.24 [1 - e^{(0.004x)}]$; $r^2 = 0.994$
-----EOLO-----			
13	279.87 \pm 10.89	483.63 \pm 73.27	302.50 \pm 44.75
25	223.25 \pm 64.62	598.25 \pm 195.71	381.50 \pm 52.99
50	179.25 \pm 13.26	361.62 \pm 141.07	450.12 \pm 51.51
100	147.37 \pm 25.27	260.62 \pm 57.96	332.87 \pm 226.91
200	74.87 \pm 19.20	103.75 \pm 33.70	*
Equations	$y = 61.58 + 240.9^{(-0.013x)}$; $r^2 = 0.965$	$y = -45.62 + 651.90e^{(-0.007x)}$; $r^2 = 0.890$	-
-----EOL-C-----			
25	195.37 \pm 101.71	-	24.25 \pm 6.43
50	112.00 \pm 32.37	416.12 \pm 151.79	87.42 \pm 33.76
100	70.25 \pm 11.18	182.12 \pm 21.09	476.50 \pm 171.27
200	64.50 \pm 14.78	140.00 \pm 17.24	422.75 \pm 63.45
Equations	$y = 64.21 + 360.88e^{(-0.04x)}$; $r^2 = 1.000$	$y = 138.95 + 1779.4^{(-0.037x)}$; $r^2 = 1.000$	$y = -342.06 + 821.65 [1 - e^{(-0.02x)}]$; $r^2 = 0.853$
-----EOL-L-----			
50	267.37 \pm 98.53	-	130.85 \pm 56.35
75	143.12 \pm 26.27	559.25 \pm 125.11	165.12 \pm 100.13
100	200.37 \pm 74.19	294.85 \pm 50.34	174.12 \pm 71.03
200	79.12 \pm 20.44	207.75 \pm 44.65	186.00 \pm 128.26
Equations	$y = 57.31 + 366.29e^{(-0.013x)}$; $r^2 = 0.742$	$y = 207.42 - 22919.64e^{(-0.056x)}$; $r^2 = 1.000$	$y = 189.03 [1 - e^{(-0.025x)}]$; $r^2 = 0.973$

Equations represent relationships between the times of sedation, anesthesia or recovery and concentrations of EOs, where y = time to reach the stages (seconds) and x = EO concentrations ($\mu\text{L L}^{-1}$).

sidoides, which contains 68% carvacrol, the main compound of EOLO, can anesthetize silver catfish (SILVA et al., 2013). Recovery of *H. geographicus* within 30min was not obtained after anesthesia with 200 $\mu\text{L L}^{-1}$ EOLO. Silver catfish anesthetized with EO of *L. sidoides* also did not recover normal behavior within 30min at most concentrations tested, and this EO causes mucous loss and mortality (SILVA et al., 2013). These side effects of EOLO were not observed in *H. geographicus*, probably because the percentage of carvacrol was lower (41.7%) in EOLO.

Hypsiboas geographicus is also very susceptible to anesthesia with EOL-C because the lowest EO-C concentration to induce anesthesia was 50 $\mu\text{L L}^{-1}$, and the lowest EOL-C concentration to induce anesthesia within 5min was in the 100-200 $\mu\text{L L}^{-1}$ concentration range. No anesthesia studies with EOL-C have been performed thus far, but the lowest concentration of *Aloysia triphylla* EO, whose main

component is citral (72.2%: E-citral 42.3%, Z-citral 29.9%) (PARODI et al., 2012), to induce anesthesia in silver catfish is 100 $\mu\text{L L}^{-1}$, and an anesthesia induction within 5min requires 300-600 $\mu\text{L L}^{-1}$ (depending on the strain) (PARODI et al., 2014).

Anesthesia recovery times of *H. geographicus* were within appropriate times (less than 7min) with all EOs tested, with the exception of 200 $\mu\text{L L}^{-1}$ EOLO. This recovery time was similar to that observed in some fish species anesthetized with EOL-L (CUNHA et al., 2010; TONI et al., 2015; HOHLENWERGER et al., 2016) and *A. triphylla* EO (PARODI et al., 2014). Therefore, while all studied EOs are effective sedatives and anesthetics for tadpoles of *H. geographicus*, EOLO has a narrow safety range, and its use must not exceed 100 $\mu\text{L L}^{-1}$. In conclusion, the present study showed promising perspectives for the utilization of these EOs for clinical procedures in amphibians.

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