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Scientic Note

EFFICIENCY OF ANESTHETICS ON FEMALE GASTROPOD *Littoraria angulifera*

Gabriela Calvi ZEIDAN[1]
Carlos BARROSO[2]
Guisla BOEHS[1]

[1]Universidade Estadual de Santa Cruz – UESC, Programa de Pós-graduação em Ciência Animal, Laboratório de Moluscos Marinhos – LMM, Rodovia Jorge Amado, km 16, Bairro Salobrinho, CEP 45662-900, Ilhéus, BA, Brasil. E-mail: gboehs@uesc.br (corresponding author).

[2]Universidade de Aveiro – UA, Centro de Estudos do Ambiente e do Mar – CESAM, Departamento de Biologia, Campus Universitário de Santiago, CEP 3810-193, Aveiro, Portugal.

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ABSTRACT

The present study evaluated the effect of three anesthetics at three concentrations, menthol ($C_{10}H_{20}O$) (1%, 1.5%, 2%), eugenol ($C_{10}H_{12}O_2$) (0.5%, 1%, 1.5%) and magnesium chloride ($MgCl_2$) (4%, 7%, 10%) diluted in distilled water, on the gastropod *Littoraria angulifera*. Twenty females were tested for each concentration with shell height varying between 18.3 and 26.1 mm (n = 180), all collected from the same site in the estuary of Barra do Sargi-Uruçuca, Bahia (Brazil). The time of induction and recovery of the sedative effect, as well as the survival rate, were assessed. The animals were kept at a temperature of 25 ± 1 °C and observations were made every 30 minutes since the beginning of the anesthesia for up to 2 hours. After this period, the animals were observed during the following 15 days for survival evaluation. Only $MgCl_2$ caused a sedative effect on *L. angulifera*. At 4% and 7% $MgCl_2$ solutions, 20% and 95% of animals were anesthetized after 2 hours, respectively, and at 10% all animals were anesthetized after 1 hour. In conclusion, *L. angulifera* can be efficiently anesthetized in a 10% $MgCl_2$ solution for 1 h and the recovery occurs after 1 h with no apparent side effects on behavior and survival.

Key words: eugenol; magnesium chloride; mentol; molluscs; sedation.

EFICIÊNCIA DE ANESTÉSICOS EM FÊMEAS DO GASTRÓPODE *Littoraria angulifera*

RESUMO

O presente estudo avaliou o efeito de três anestésicos em três concentrações, o mentol ($C_{10}H_{20}O$) (1%; 1,5%; 2%), o eugenol ($C_{10}H_{12}O_2$) (0,5%; 1%; 1,5%) e o cloreto de magnésio ($MgCl_2$) (4%; 7%; 10%), diluídos em água destilada, sobre fêmeas do gastrópode *Littoraria angulifera*. Para cada concentração foram utilizadas 20 fêmeas (total = 180), com altura da concha entre 18,3 e 26,1 mm, coletadas no mesmo local, no estuário da Barra do Sargi-Uruçuca, Bahia. Foram observados os tempos de indução e de recuperação do efeito sedativo, assim como a sobrevivência. Os animais foram mantidos a uma temperatura laboratorial de 25 ± 1 °C e as observações foram feitas a cada 30 minutos após a anestesia, durante 2 horas. Após o término do experimento, os animais foram observados por 15 dias quanto à sobrevivência. Apenas o $MgCl_2$ causou efeito sedativo sobre *L. angulifera*, sendo que a 4% e a 7% observou-se sedação de 20% e de 95% dos animais, respectivamente, após 2 horas de anestesia, enquanto que a 10%, todos os animais foram anestesiados em 1 hora. Em conclusão, *L. angulifera* pode ser anestesiada de forma eficiente em uma solução de 10% de $MgCl_2$ durante 1 h e a sua recuperação ocorre ao fim de 1 h sem nenhum efeito colateral evidente no comportamento e na sobrevivência.

Palavras-chave: eugenol; cloreto de magnésio; mentol; moluscos; sedação.

INTRODUCTION

Several studies require the anatomical analysis of gastropods (*e.g.* physiology, reproduction, nutrition, ecology, systematics and ecotoxicology), which require relaxation techniques for observation and manipulation of animals with preservation of their original morphological and physiological conditions (MEIER-BROOK, 1976; EMBERTON, 1989). Anesthetics are commonly used in marine gastropods for non-destructive analysis in order to minimize handling stress, reduce mortality, and facilitate in vivo analyzes in aquaculture and ecological studies (BUTT *et al*., 2008; SUQUET *et al*., 2009).

According to KAPLAN (1969), anesthesia consists of temporarily partial or total loss of the body's nervous sensibility. The desirable anesthetic agents characteristics, such as low dosage efficacy, fast induction and recovery from narcotized state, operator safety and low cost, should be combined with the physiology of the species and the induction methods to be employed (MEIER-BROOK, 1976; ROSS and ROSS, 2008).

A variety of anesthetics are widely used in marine molluscs for non-destructive manipulation of the organisms, either in ecological studies (PRINCE and FORD, 1985; MCSHANE and SMITH, 1988; VASCONCELOS *et al*., 2006) or in aquaculture (HEASMAN *et al*., 1995; WHITE *et al*., 1996; ACOSTA-SALMON and DAVIS, 2007; BUTT *et al*., 2008). The most commonly used in gastropods are benzocaine, pentoborbital sodium, MS-222, eugenol, menthol and magnesium chloride (MEIER-BROOK, 1976; GIRDLESTONE *et al*., 1989; CULLOTY and MULCAHY, 1992; ARAUJO *et al*., 1995; ACOSTA-SALMON *et al*., 2005; BUTT *et al*., 2008; MAMANGKEY *et al*., 2009).

Menthol ($C_{10}H_{20}O$) is an oil extracted from mint (*Mentha arvensis* L.), also known as mint (LORENZO *et al*., 2002). It is anaestheasic easy to acquire, with low cost (FAÇANHA and GOMES, 2005) and has been encouraged as a narcotic for invertebrates in general and for molluscs in particular (ARAUJO *et al*., 1995).

Eugenol ($C_{10}H_{12}O_2$) is a natural product derived from clove oil, that is rapidly metabolized and excreted by the animals (WAGNER *et al*., 2002).

Magnesium chloride ($MgCl_2$) is a non-toxic, easily administered, efficient anesthetic salt (ACOSTA-SALMON and DAVIS, 2007) widely used in molluscs (BUTT *et al*., 2008; SUQUET *et al*., 2009). Magnesium ion is effective because of its muscle blocking action, competing with the calcium required for synaptic transmission (ROSS and ROSS, 2008).

The aim of the present study is to develop adequate methods to anesthetize the gastropod *Littoraria angulifera* (Lamarck, 1822) for ecotoxicological research. It is an exclusively estuarine species with Neotropical anfiatlantic distribution, is ovoviviparous, and occupies different strata of the mangrove, from regions close to the water level to higher portions in mangrove trunks, where it feeds mainly on fungi and lichens (MERKT and ELLISON, 1998). Although it is observed close to the water and keep gill respiration, it is found above the level throughout the tidal cycle. It is considered a species in transition between the aquatic and terrestrial environments and does not resist submersion for a long time (KOHLMEYER and BEBOUT, 1986; MERKT and ELLISON, 1998; REID, 1999). *Littoraria angulifera* is widely used in taxonomic, physiological and ecological studies, and recently it has been used as a sentinel species for organotin contamination in estuarine environments.

Until now there is no specific anesthesia protocol for *L. angulifera*. In this way, this study analyzed the efficiency of the three anesthetics mentioned above (menthol, eugenol and magnesium chloride) in adult *L. angulifera* females, in terms of concentration, induction and recovery times, as well as their effect on behavior and survival rate after anesthesia.

METHODS

In order to carry out the experiment, 180 adult females of *L. angulifera* were collected in the estuary of Barra do Sargi-Uruçuca, Bahia (14°30'743'S, 39°02'192"W), with shell height between 18.3 and 26.1 mm (Mean: 22.6 ± 2.1 mm) and total weight between 0.53 and 2.53 g (Mean: 1.24 ± 0.43 g). For sexual identification in the field we observed the presence/absence of the penis in males/females. After sampling, the females were taken to the Marine Molluscs Laboratory of the State University of Santa Cruz (LMM-UESC), Ilhéus, Brazil, to perform the experiment. We only selected females because we pretend to use these animals as sentinel organisms to assess organotin contamination in estuarine environments.

After biometry, the females were conditioned (out of water) in three 10L glass containers with a small amount (50 mL) of seawater (30 psu) over the bottom for maintenance of moisture along the adaptation period (15 days). The containers were capped with transparent tulle of 10 mm diameter, at a temperature of 25 ± 1 °C and fed *ad libitum* with lichens and mosses, collected in the same environment where the females were sampled. The experiment was developed in three stages: (a) anesthesia induction, (b) anesthesia recovery and (c) behavior/survival after anesthesia, following instructions from LEGAT *et al*. (2015).

Three anesthetic aqueous solutions were tested: 1%, 1.5%, 2% menthol ($C_{10}H_{20}O$; Vetec Química Fina); 0.5%, 1%, 1.5% eugenol ($C_{10}H_{12}O_2$; Vetec Química Fina); 4%, 7%, 10% magnesium chloride ($MgCl_2$; Farmax). The anesthetics were measured considereing their weight (magnesium chloride) and volume (menthol and eugenol) for solutions preparation, according to their respective concentrations, where they were diluted in distilled water. All solutions were prepared three days before the bioassay and were kept in sterilized glasses, in an aphotic environment. The concentrations were defined according to studies already performed with other gastropod species (RUNHAM *et al*., 1965; GIRDLESTONE *et al*., 1989; ACOSTA-SALMON and DAVIS, 2007) and bivalves (ARAUJO *et al*., 1995; ACOSTA-SALMON *et al*., 2005; ALIPIA *et al*., 2014).

For the experiment, the temperature (25 °C) and solution aeration conditions were kept constant throughout the period. The solutions were placed in containers with 300 ml of each anesthetic solution. Females were immersed individually in these solutions, totaling 20 replicates per concentration/anesthetic. The sedative effect was observed at intervals of 30, 60, 90 and 120 minutes and was classified as: (I) non-anesthetized, (II) partially anaesthetized (opening of the operculum but sensitive to mechanical stimulus), and (III) fully anesthetized (opening of the operculum, total relaxation of the soft part and absence of sensitivity to mechanical stimulus). For the observation of sedation evolution degree under different concentrations throughout the experiment, it was calculated the cumulative percentage of fully anesthetized animals (III) (accumulated percentage of anesthetized females from time zero to final time, 120 minutes) for each interval of observation.

After the anesthesia induction of 2 h, the specimens were transferred to containers with sea water at 30 psu for the recovery step. The duration and observation times were the same as those used in the first step (induction). For this step, the following classification was used: (a) animals not recovered – animals still anesthetized; (b) partially recovered - decreased mobility and deficient closure of the operculum after mechanical stimulation; (c) fully recovered - animals that resumed motion and the capacity to close the operculum spontaneously. At the end of the induction and recovery stages, the females were then reconditioned emmersed in the glass containers under the same adaptation conditions prior to experiment, for behavior and survival observation for 15 days after the bioassay.

RESULTS

Only magnesium chloride proved to be an effective anesthetic for *Littoraria angulifera* (Figure 1A). Menthol and eugenol showed no anesthetic efficacy at any concentration tested, even after the 2 hours stipulated for the experiment. Regarding the recovery time (Figure 1B), at 4% concentration, all animals (100%) recovered completly in 30 minutes. At 7% concentration, the animal's recovery started faster than those submitted to 10% concentration, however, the total sensorineural recovery was only reached at the end of 90 minutes. At 10% concentration, all animals were partially recovered after 30 minutes and fully recovered after 1 hour. There was no mortality in the three experiments within the following 15 days, demonstrating the non-lethal effects of the anesthetics for the concentrations tested.

As summarized in Table 1, in the 4% $MgCl_2$ solution, the anesthesia effect was observed only after 90 minutes in 10% of females the and just a small percentage of animals (20%) were fully anesthetized (stage III) at the end of 2 hours. At the same time, 60% of animals were partially anesthetized (stage II) and 20% showed normal behavior (stage I). At concentrations of 7% and 10% $MgCl_2$, 50% and 60% of females were partially anesthetized (stage II), respectively, the remainder to about 30% to 10% in stage I and 20 to 30% in stage III. The concentration of 10% was the most efficient because all animals were fully anesthetized after 1 hour. At 7% concentration, 95% of animals were fully anesthetized only after 2 hours.

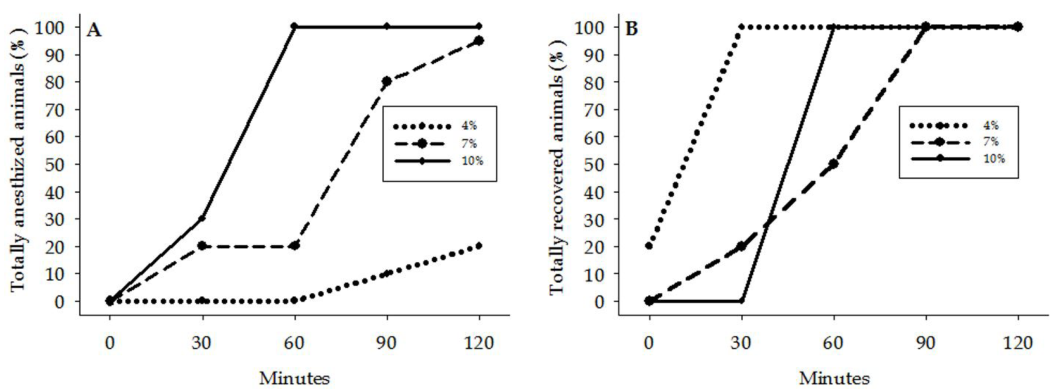

Figure 1. Cumulative percentage of *Littoraria angulifera* females exposed to different concentrations of magnesium chloride solution: A = Females fully anesthetized during exposure; B = females fully recovered after narcotization.

Table 1. Results, in absolute numbers (individuals), about test of magnesium chloride ($MgCl_2$) anesthetic efficiency in *Littoraria angulifera*. Induction: I = not anesthetized; II = partially anesthetized; III - totally anesthetized. Recovery: (a) not anesthetized; (b) partially recovered; (c) fully recovered. n = 60.

	Induction (min)														
	0			**30**			**60**			**90**			**120**		
	I	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**
4%	20			20			20			8	10	2	4	12	4
7%	20			6	10	4	2	14	4		4	16		1	19
10%	20			2	12	6			20			20			20
	Recovery (min)														
	a	**b**	**c**	**A**	**b**	**c**	**a**	**b**	**c**	**a**	**B**	**c**	**a**	**b**	**c**
4%	4	12	4			20			20			20			20
7%	19			4	12	4		10	10			20			20
10%	20				20				20			20			20

DISCUSSION

There are several products used as anesthetics that can be successfully applied in gastropods for different non-destructive studies. Three anesthetics were tested in the present work, in order to verify which one was more efficient for *L. angulifera* females.

The results obtained for menthol and eugenol show that the solutions prepared were not good anesthetics for *L. angulifera*, in contrast to what has been observed in other aquatic species (WAGNER *et al*., 2002; FAÇANHA and GOMES, 2005). BILBAO *et al*. (2010), testing several anesthetics, founded that eugenol at a concentration of 0.5% diluted in alcohol 50% was efficient to anesthetize the gastropod *Haliotis tuberculata*, which may indicate that this anesthetic efficiency could be related with the solvent used: only distilled water was used in the present study because alchool may interfere with metabolic functions of interest for our ecotoxicological studies (to be reported elsewhere). Regarding menthol, this has been used as a narcotic for some aquatic invertebrate species. ARAUJO *et al*. (1995) evaluated the relaxing effect of this product on different freshwater molluscs and concluded that despite the efficiency variability between species, an efficient relaxation was achieved only for long exposures (above 24 hours). We did not test the efficiency of the anesthetics for a long-term relaxation in *L. angulifera* because this compromises the survival of the animals as they do not resist submerged for much time (REID, 1999). Some authors suggest that litorinids such as *L. angulifera* that inhabit the mangrove supralittoral in the adult phase and remain above the water level throughout the tidal cycle represent an intermediate stage in evolution of terrestrial gastropods to the marine environment, being adapted to the aerial respiration through the reduction of their gill lamellae size and the formation of air sacs in the mantle cavity (LENDERKING, 1954; BERRY, 1963; REID *et al*., 2010). In this way, although these organisms present gill respiration and are frequently observed close to the water, they do not resist the submersion for long periods (KOHLMEYER and BEBOUT, 1986). On the other hand, according to OSTRENSKY *et al*. (2008) and GOMES *et al*. (2001), shorter exposure periods of aquatic organisms to anesthetics will favour faster recoveries, which may benefit the animal's health.

The results obtained for magnesium chloride show that this is an effective anesthetic for *L. angulifera* females, similarly to what has been described for other gastropod (ACOSTA-SALMÓN *et al*., 2005) and bivalve species (BUTT *et al*., 2008; SUQUET *et al*., 2009; ALIPIA *et al*., 2014). Although this anesthetic could cause the relaxation of a small proportion of specimens at the lowest concentration tested (4%), the most notorious response leading to a complete sedaction of all animals occurred at 10%. Several authors which also used this anesthetic to narcotise marine gastropods, apply concentrations between 7% and 7.5% (BRYAN and GIBBS, 1991; BARROSO *et al*., 2000; SOUSA *et al*., 2005; CASTRO *et al*., 2007; SOUSA *et al*., 2009; SANTOS *et al*., 2011), but we proved that the concentration of 7% is less efficient for *L. angulifera*. Hence, we recommend for this species the use of 10% $MgCl_2$. This concentration is the most efficient because it allows a rapid anesthesia induction and a quick recovery. Both factors may contribute to the reduction of the anesthesia stress caused to the animals, which is benefic when one pretends to release them back to nature or to use them in further experiments, assuring a higher survival and better condition.

CONCLUSIONS

The magnesium chloride ($MgCl_2$) at a 10% of concentration in distilled water was efficient to anesthetize females of *Littoraria angulifera* after one hour, with no apparent side effects on animal's health. Menthol ($C_{10}H_{20}O$) and eugenol ($C_{10}H_{12}O_2$) were not efficient to anesthetize this gastropod with the solvent used and further studies should attempt new methods to dissolve these compounds in order to obtain proper anesthetic solutions for this species.

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