Comparative Biochemistry and Physiology, Part C 155 (2012) 462–468

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

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)

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ARTICLE INFO

Article history:
Received 26 September 2011
Received in revised form 6 December 2011
Accepted 10 December 2011
Available online 16 December 2011

Keywords:
Anesthesia
Essential oils
Shrimp
Eugenol
Oxidative stress

ABSTRACT

The aim of this study was to evaluate the anesthesia induction and recovery times of sub-adult and post-larvae white shrimp (*Litopenaeus vannamei*) that were treated with eugenol and the essential oils (EOs) from *Lippia alba* and *Aloysia triphylla*. Oxidative stress parameters in the hemolymph of this species were also analyzed. The concentrations of eugenol, *A. triphylla* EO and *L. alba* EO recommended for anesthesia were 200, 300 and 750 μL L^{-1} for sub-adults and 175, 300 and 500 μL L^{-1} for post-larvae, respectively. The concentrations studied during the transport of sub-adults were between 20 and 50 μL L^{-1} eugenol, 20–30 μL L^{-1} *A. triphylla* EO and 50 μL L^{-1} *L. alba* EO. For post-larvae, the optimal concentrations for transport were 20 μL L^{-1} eugenol and between 20 and 50 μL L^{-1} *A. triphylla* EO. The white shrimp sub-adults that were exposed to *A. triphylla* EO (20 μL L^{-1}) showed increases in their total antioxidant capacities (150%), catalase (70%) and glutathione-S-transferase (615%) activity after 6 h. *L. alba* EO (50 μL L^{-1}) and eugenol (20 μL L^{-1}) also increased GST activity (1292 and 1315%) after 6 h, and eugenol (20 μL L^{-1}) decreased the total antioxidant capacity (100%). Moreover, concentrations above 30 μL L^{-1} for the EOs of *A. triphylla* and *L. alba* and 20 μL L^{-1} eugenol were effective at inducing anesthesia and improving the antioxidant system against reactive oxygen species (ROS) after 6 h.

1. Introduction

Aquatic animals in culture systems are susceptible to stress caused by capture, handling and transportation, among other stressors. Stress can induce behavioral and physiological changes as well as physical damage and may compromise fish production (Barton, 2002). Anesthetics may be used to reduce the effects of physiological stress in aquatic animals during handling and transport (Di Marco et al., 2011; Pawar et al., 2011).

The signals and responses to pain in shrimp and most crustaceans are not as clearly defined as in vertebrates, but evidence shows that these organisms can experience pain and stress in a manner similar to vertebrates (Elwood et al., 2009). Commonly used drugs do not affect their post-synaptic receptor sites, and they may respond differently to some anesthetics (Ross and Ross, 2008). The topical anesthetic Xylocaine™ reduces the signs of stress in the white shrimp *Litopenaeus vannamei* (Taylor et al., 2004). A few studies have addressed the anesthetic activity of substances of plant origin, such as eugenol (Coyle et al., 2005; Venarsky and Wilhelm, 2006; Akbari et al., 2010) and menthol (Saydmohammed and Pal, 2009), in shrimp. The Pacific white shrimp *L. vannamei* is a tropical species that is naturally distributed along the Pacific coast of Central and South America and is one of the most economically important species cultured worldwide (Zhou et al., 2009).

Like all organisms, crustaceans have an array of defense systems that enable them to meet diverse environmental challenges (Zhou et al., 2009), including constant attack from exogenous and endogenous free radicals, which can lead to serious cellular damage. The antioxidant defense system of this organism includes enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST), which are important components of various detoxification, antioxidant and stress-tolerance pathways. Moreover, the integrated antioxidant

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1532-0456
doi:10.1016/j.cbpc.2011.12.003

system is recognized as an essential component of an organism's self-maintenance ability. The equilibrium between GST and CAT, along with non-enzymatic antioxidants, is important for the effective removal of reactive oxygen species (ROS) (Andrew and Mathew, 1989; Guemouri et al., 1991). Some antioxidant enzymes such as CAT and GST are used as biomarkers of general health status, and the levels of these enzymes can discriminate between shrimp cultivated in intensive and extensive systems (Tu et al., 2008).

Previous studies have reported that eugenol, which is the major constituent of *Eugenia caryophyllata* (Myrtaceae) essential oil (clove oil), has anesthetic properties in some shrimp species, including *Macrobrachium rosenbergii* (Saydmohammed and Pal, 2009) and *Penaeus semisulcatus* (Soltani et al., 2004). The plant *Aloysia triphylla* (L'Herit) Britton (Verbenaceae) grows naturally in South America and was introduced in Europe in the late seventeenth century (Carnat et al., 1999). Traditional ethnobotanical applications of this species include its use in folk medicine as a treatment for insomnia and anxiety and as an analgesic and sedative (Valentão et al., 2002). Recently, the essential oil (EO) of this plant has been patented as an anesthetic for aquatic animals (Patent No PI 016090005905). The EO of *Lippia alba* (Mill.) N.E. Brown (Verbenaceae) has been indicated as an anesthetic for two fish species: the silver catfish *Rhamdia quelen* (Cunha et al., 2010) and the seahorse *Hippocampus reidi* (Cunha et al., 2011). Preparations of *A. triphylla* exhibit antioxidant properties, a powerful superoxide radical scavenging activity and a moderate hydroxyl radical scavenging activity (Valentão et al., 2002). In addition, *L. alba* EO improves the redox state of some silver catfish tissues under both hyperoxia and hypoxia (Azambuja et al., 2011).

Therefore, the aims of this study were to determine the optimal concentrations of eugenol and *A. triphylla* and *L. alba* EOs for the induction of anesthesia in *L. vannamei* sub-adults and post-larvae and to evaluate the time required for both anesthesia induction and recovery. In addition, we established the concentrations of these natural products that are suitable for the transport of this species and the effects of the isolated compound and the EOs on oxidative stress parameters in the hemolymph of *L. vannamei* sub-adults.

2. Material and methods

2.1. Animals

L. vannamei sub-adults (15 ± 0.1 g and 10 ± 1 cm) and post-larvae (0.1 ± 0.7 g and 0.5 ± 0.8 cm) were raised at the Marine Station of Aquaculture, Universidade Federal de Rio Grande, Rio Grande do Sul State, Southern Brazil. The animals were collected from rearing ponds that were equipped with biofloc technology and placed in 500 L indoor tanks with continuously aerated clean water at a salinity of 32 ppt, a pH of 8.2 and t 22 °C. The study was conducted in accordance with the ethical committee of animal welfare at the Universidade Federal de Santa Maria, RS, under protocol no. 027176.

2.2. Plant material and essential oil extraction

The plant species *L. alba* and *A. triphylla* were cultivated at São Luiz Gonzaga and Frederico Westphalen, respectively, which are cities in Rio Grande do Sul State, Brazil. The plant materials were identified by the botanist Dr. Gilberto Dolejal Zanetti of the Department of Industrial Pharmacy, UFSM, and voucher specimens (*L. alba* — SMDB no. 10050; *A. triphylla* — SMDB no. 11169) were deposited in the herbarium of the UFSM Department of Biology. The major components of *A. triphylla* EO are Z-citral (29.92%) and E-citral (42.30%), while the predominant compounds in *L. alba* EO are linalool (59.66%) and 1,8-cineole (9.11%). All other constituents occur at concentrations below 5%. Eugenol (99% purity, Odontofarma™, Porto Alegre, Brazil) was purchased commercially.

The *A. triphylla* and *L. alba* EOs were obtained from fresh plants using hydrodistillation, which was performed with a Clevenger apparatus (2 h for *L. alba* and 3 h for *A. triphylla*) according to guidelines from the European Pharmacopoeia (2007). The essential oils were stored at −20 °C in amber glass bottles. The densities were approximately 0.8 for the *L. alba* EO, 0.9 for the *A. triphylla* EO and 0.98 for eugenol.

2.3. Analysis of essential oils

GC–MS TIC analysis was performed using an Agilent-6890 gas chromatograph coupled with an Agilent 5973 mass selective detector under the following conditions: HP-5MS column (5%-phenyl–95%-methylsiloxane, 30 m × 0.25 mm × 0.25 μm); EI–MS: 70 eV; operating conditions: split inlet 1:100; temperature program, 40–260 °C; 40 °C for 4 min; ramp rate, 4 °C/min; carrier gas, He; flow rate, 1 mL min^{-1}; injector and detector temperature, 220 °C; interface temperature 250 °C; Databank NIST 2002.

The constituents of the EOs were identified by comparing their mass spectra with a mass spectral library (NIST, 2002) and by comparison of the Kovats retention index with literature data (Adams, 2001).

2.4. Anesthesia induction and recovery

The experiments involving anesthesia induction and recovery were conducted according to the procedure described by Coyle et al. (2005). Shrimp were classified as stage 1 if they demonstrated a partial loss of equilibrium but were still reactive to touch stimuli and as stage 2 if they demonstrated a complete loss of equilibrium and were not reactive to stimuli. Shrimp were considered recovered from anesthesia when they regained control of their equilibrium and attained an upright position on the bottoms of the aquaria.

The essential oils and eugenol were dissolved in ethanol at a ratio of 1:10 before being added to aquaria containing seawater. To evaluate the time required for anesthesia induction, 16 sub-adults and 30 post-larvae were used for each concentration tested. Each animal was used only once. After induction, the sub-adults and post-larvae were transferred to anesthetic-free aquaria to measure the anesthesia recovery time. The controls were added to aquaria that contained only ethanol at a concentration that was equivalent to the highest concentration used in the experimental conditions (9 mL L^{-1}). The concentrations studied were chosen based on preliminary tests.

2.4.1. Concentrations for short-term anesthesia

Sub-adult animals were transferred to 1 L aquaria (2 animals per aquarium), and the post-larvae were evaluated in 250 mL beakers (10 post-larvae per beaker). The sub-adults were exposed to the following concentrations: 250, 500, 750 or 1000 μL L^{-1} *L. alba* EO; 50, 100, 300 or 500 μL L^{-1} *A. triphylla* EO and 50, 100, 200 or 400 μL L^{-1} eugenol. The post-larvae were exposed to the following concentrations: 400, 500 or 600 μL L^{-1} *L. alba* EO; 100, 300, 400 or 500 μL L^{-1} *A. triphylla* EO and 100, 150 or 175 μL L^{-1} eugenol. The maximum observation time in this experiment was 30 min.

2.4.2. Concentrations for transport

To evaluate the anesthetic concentration range suitable for shrimp transport, the anesthetic exposure time was fixed at 6 h. Sub-adult animals and post-larvae were maintained in continuously aerated 1 L aquaria (2 animals per aquarium) or 250 mL beakers (5 post-larvae per beaker). Both the sub-adults and post-larvae had five replicates each. The sub-adults were exposed to eugenol (5, 10 or 20 μL L^{-1}), *L. alba* EO (50, 100, 200 or 250 μL L^{-1}) or *A. triphylla* EO (20, 30 or 40 μL L^{-1}). The post-larvae were also exposed to eugenol (10, 20 or 50 μL L^{-1}), *L. alba* EO (100, 200 or 250 μL L^{-1}) or *A. triphylla* EO (10, 20 or 50 μL L^{-1}). The concentrations chosen were below those that

induced deep anesthesia within 30 min in the short-term anesthesia experiment.

2.5. Oxidative stress measurements

The hemolymph of sub-adult white shrimp was collected (n = 6) 6 h after exposure to *A. triphylla* EO (control, control + ethanol or 20, 30 or 40 μL L^{-1} EO), eugenol (control, control + ethanol or 5, 10 or 20 μL L^{-1} eugenol) or *L. alba* EO (control, control + ethanol or 50, 100, 200 or 250 μL L^{-1} EO). The hemolymph was collected directly from the heart of the shrimp using sterile syringes containing an anticoagulant solution (Sotherhall and Smith, 1983), transferred to 1.5 mL polyethylene tubes and stored at −80 °C in an ultra-freezer. For protein quantification and antioxidant enzyme analysis, the hemolymph was centrifuged twice at 3000 and 9000 *g* at 4 °C for 35 and 15 min, respectively, to obtain a pellet. After the centrifugation process, the pellet was resuspended in a 4 °C buffer solution containing Tris base (20×10^{-3} M), EDTA (1×10^{-3} M), dithiothreitol (1×10^{-3} M), KCl (150×10^{-3} M), and PMSF (0.1×10^{-3} M), with the pH adjusted to 7.6. All enzymatic measurements were performed at least in triplicate.

The total antioxidant competence against peroxyl radicals was analyzed by quantifying the ROS in the hemolymph as described by Amado et al. (2009). The total fluorescence production was calculated by integrating the fluorescence units (FU) along the time of the measurement after adjusting the FU data to a second order polynomial function. The results were expressed as the area difference in FU × min in the same sample with and without the addition of 2,2-azobis-2-methylpropionamidine dihydrochloride (ABAP) and were standardized to the ROS area without ABAP (background area). The relative difference between the ROS area with and without ABAP was considered to be a measurement of the antioxidant capacity. A large area of difference corresponded to a low antioxidant capacity because high fluorescence levels were obtained after the addition of ABAP, indicating a low competency for neutralizing peroxyl radicals. Catalase (CAT; EC 1.11.1.6), activity was analyzed as described by Beutler (1975) by determining the initial decomposition rate (1 min) of H_2O_2 (10 mM) at 240 nm. The results were expressed as CAT units mg $protein^{-1}$, where 1 unit (U) is the amount of enzyme that hydrolyzes 1 μmol of H_2O_2 per min and per mg of protein at 30 °C and a pH of 8.0. Glutathione-S-transferase (GST; EC 2.5.1.18), activity was determined by monitoring (3 min) the formation of a conjugate between 1 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (Habig et al., 1974; Habig and Jakoby, 1981). The results were expressed as GST units mg $protein^{-1}$, where 1 unit (U) is defined as the amount of enzyme that conjugates to 1 μmol of CDNB per min and per mg protein at 25 °C and a pH of 7.0. In both cases, the measurement conditions and substrate concentration were selected to guarantee maximum enzymatic activity.

2.6. Statistical analyses

The data were expressed as the mean ± standard error. The relationship between the time needed for anesthesia induction and the concentration of the anesthetic used was determined using Slide Write Plus version 4.0 software (Advanced Graphics Software, Inc., Rancho Santa Fe, CA, USA). To verify the homogeneity of variances, all data were submitted to Levene's test. The data for the anesthesia recovery times (n = 16 sub-adults and 30 post-larvae) and enzymatic activities (n = 6) were analyzed using one-way ANOVA followed by Newman–Keuls post-hoc comparisons. This analysis was performed using Statistica version 7 software (StatSoft, Tulsa, OK, USA), and the significance level was set at $P<0.05$.

3. Results

3.1. Short-term anesthesia induction and recovery

Mortality was not observed in either the sub-adults or post-larvae for any anesthetic at any concentration tested in this experiment. Ethanol alone failed to induce anesthesia.

Increasing concentrations of eugenol and the *L. alba* and *A. triphylla* EOs proportionally decreased the time required for the induction of anesthesia stages 1 and 2 in the sub-adult shrimp. Increasing concentrations of *L. alba* EO proportionally decreased the time that was required for anesthesia recovery, but this relationship was not observed with *A. triphylla* EO or eugenol (Table 1).

Eugenol induced deep anesthesia in sub-adult shrimp at 50 μL L^{-1} (23 min), and rapid (less than 4 min) anesthesia induction and recovery were observed at 400 μL L^{-1} (Fig. 1A). The lowest concentration that was needed to induce stage 2 anesthesia in post-larvae was 150 μL L^{-1}, and rapid (less than 4 min) induction and recovery from deep anesthesia were observed at 175 μL L^{-1} (Fig. 2A).

L. alba EO at a concentration of 500 μL L^{-1} promoted deep anesthesia in sub-adult shrimp after approximately 30 min, and the shortest times for the induction of stage 2 anesthesia (10 min) and recovery (7 min) were observed at 1000 μL L^{-1} *L. alba* EO (Fig. 1B). The post-larvae that were exposed to 500 μL L^{-1} of the same EO exhibited the fastest induction into deep anesthesia (9.5 min); however, the shortest recovery time (6.5 min) was observed at 600 μL L^{-1} EO. A concentration of 250 μL L^{-1} *L. alba* EO was unable to induce any stage of anesthesia in post-larvae (Fig. 2B).

The minimum *A. triphylla* EO concentration capable of inducing stage 2 anesthesia in sub-adult shrimp was 100 μL L^{-1} (approximately 16 min were required for anesthesia induction). *A. triphylla* EO at a concentration of 500 μL L^{-1} produced the shortest stage 2 anesthesia induction time (5 min), but the shortest recovery time was observed at 300 μL L^{-1} EO (approximately 10 min) (Fig. 1C). A concentration of 300 μL L^{-1} *A. triphylla* EO resulted in shorter induction and recovery times (less than 10 min) in post-larvae. The anesthesia recovery time in the shrimp that were exposed to 400 μL L^{-1} EO exceeded 30 min (Fig. 2C).

3.2. Concentrations for transport

None of the eugenol concentrations tested (5, 10 or 20 μL L^{-1}) were able to induce any stage of anesthesia within 6 h in sub-adult shrimp. Exposure to 20 μL L^{-1} *A. triphylla* EO promoted stage 2 of anesthesia in *L. vannamei* sub-adults within 96 min, but 30 and 40 μL L^{-1} of this EO only induced stage 1 of anesthesia, without a significant difference in anesthesia induction time. The time required for recovery was approximately 30 min in shrimp that were exposed to

Table 1
The relationship between the time (min) (y) required for *Litopenaeus vannamei* sub-adults to reach each stage of anesthesia and the concentration (μL L^{-1}) of each anesthetic used (x). The stages were identified following the recommendations in Coyle et al. (2005).

Substance	Stage 1	Stage 2	Recovery
A. triphylla	Ln y = 4.7 + 114/x r^2 = 0.99	1/y = (0 + 6.7) x r^2 = 0.99	–
L. alba	1/y = 0.01 − 1489/x^2 r^2 = 0.99	1/y = 0.02 − 380/x^2 r^2 = 0.99	1/y = 0 − 1.9210/x r^2 = 0.99
Eugenol	Ln y = (11.1 + 1.1) Ln (x) r^2 = 0.98	Ln y = (11 − 0.98) Ln (x) r^2 = 0.96	–

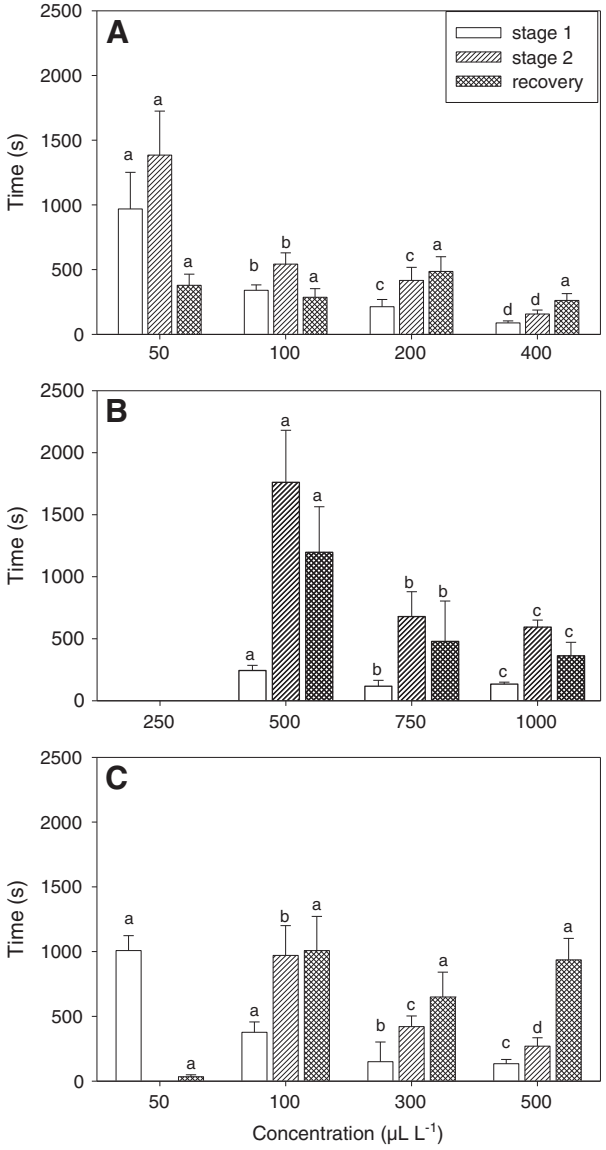

Fig. 1. The time required for induction and recovery from anesthesia in eugenol — (A), *L. alba* EO — (B) and *A. triphylla* EO — (C) treated *Litopenaeus vannamei* sub-adults. The different letters over the bars indicate significant differences in the time required for anesthesia induction and recovery between concentrations.

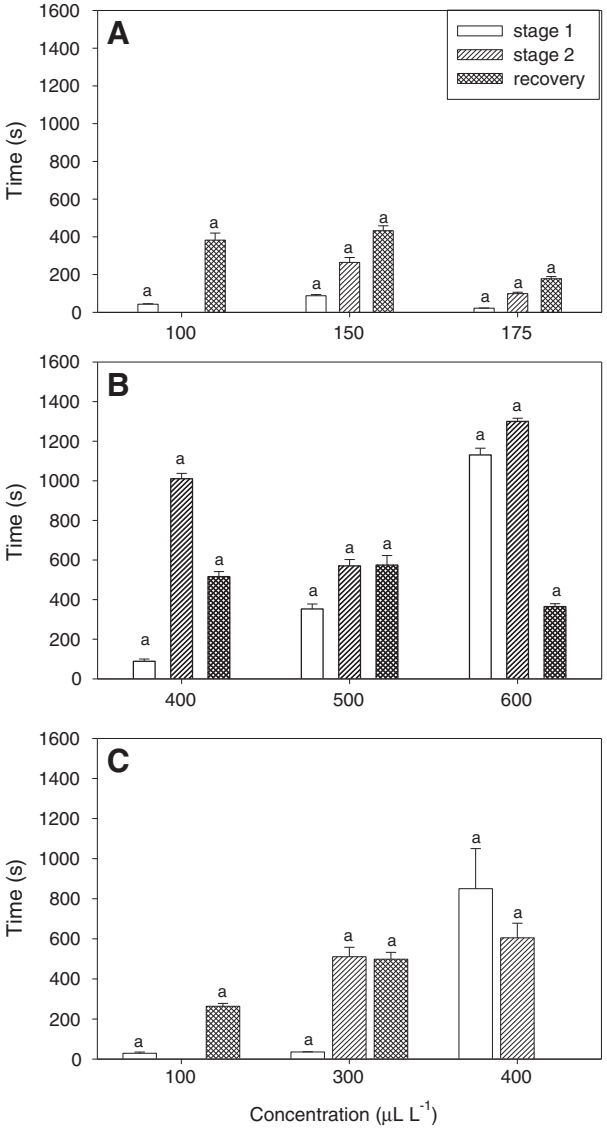

Fig. 2. The time required for induction and recovery from anesthesia in eugenol — (A), *L. alba* EO — (B) and *A. triphylla* EO — (C) treated *Litopenaeus vannamei* post-larvae. The recovery bar for 400 μL L^{-1} *A. triphylla* EO is not shown because the time exceeded 30 min. The different letters over the bars indicate significant differences in the time required for induction and recovery from anesthesia between concentrations.

all three concentrations. *L. alba* EO caused stage 2 of anesthesia at the highest concentrations studied (100, 200 and 250 μL L^{-1}), and the time required for anesthesia induction increased with increasing concentrations. At these concentrations, the recovery time was longer than 70 min. After 6 h, 37 and 25% mortality were observed in the sub-adults treated with 200 and 250 μL L^{-1} *L. alba* EO, respectively (Table 2).

In the post-larvae, both eugenol and *A. triphylla* EO (10 μL L^{-1} each) were unable to induce any stage of anesthesia. The exposure of post-larvae to 20 μL L^{-1} *A. triphylla* EO did not induce any stage of anesthesia, but eugenol induced anesthesia stage 1 in 170 min. A concentration of 50 μL L^{-1} eugenol or *A. triphylla* EO induced anesthesia up to stage 2, and recovery was faster in those anesthetized with eugenol (20 min) than with *A. triphylla* EO (over 120 min). All of the *L. alba* EO concentrations tested led to stage 2 of anesthesia (Table 2), but the post-larvae that were exposed to 250 μL L^{-1} progressed rapidly to death. The mortality at this concentration was 100%.

3.3. Oxidative stress

The white shrimp exposed to 20 μL L^{-1} *A. triphylla* EO exhibited a significantly higher total antioxidant capacity (lower relative area) than the shrimp receiving other treatments ($P<0.05$). The white shrimp exposed to 20 μL L^{-1} eugenol exhibited a significantly lower total antioxidant capacity (higher relative area) compared with the other treatments ($P<0.05$), but those shrimp exposed to *L. alba* EO did not display any significant difference from the controls ($P<0.05$) (Fig. 3A).

Exposing white shrimp to 20 μL L^{-1} *A. triphylla* EO significantly increased CAT activity compared to other treatments. The CAT activity in the white shrimp that were treated with eugenol did not differ from that resulting from the other treatments ($P>0.05$). The white shrimp exposed to 50 μL L^{-1} *L. alba* EO exhibited the lowest CAT activity, followed by those treated with 100 μL L^{-1} *L. alba* EO (Fig. 3B).

The GST activity in the white shrimp treated with 20 and 30 μL L^{-1} *A. triphylla* EO was increased compared to the GST activity in the controls ($P<0.05$), and GST activity reached a maximum at

Table 2
The time for anesthesia induction and recovery in *L. vannamei* sub-adults and post-larvae following a total exposure time of 6 h. N = 16 for sub-adults and 30 for post-larvae.

	Substance	Concentration (μL L^{-1})	Time (min)		
			Stage 1	Stage 2	Recovery
Sub-adults	*A. triphylla*	20	93 ± 18	96 ± 13	30 ± 18
		30	117 ± 12	Not achieved	30 ± 9
		40	146 ± 47	Not achieved	30 ± 12
	L. alba	50	45 ± 0.1	Not achieved	45 ± 1
		100	40 ± 4	131 ± 22	70 ± 0.5
		200	103 ± 18	153 ± 7[a]	120 ± 1
		250	95 ± 16	172 ± 3[b]	120 ± 11
Post-larvae	*A. triphylla*	10	Not achieved	Not achieved	0
		20	Not achieved	Not achieved	0
		50	75 ± 5	165 ± 3	Above 120
	L. alba	100	100 ± 2	240 ± 1	70 ± 2
		200	65 ± 18	40 ± 15	65 ± 17
		250	65 ± 3	46 ± 6	No recovery[c]
	Eugenol	10	Not achieved	Not achieved	0
		20	170 ± 6	Not achieved	5 ± 10
		50	95 ± 5	175 ± 13	20 ± 2

The values represent the means ± SEM.
[a] 37% mortality.
[b] 25% mortality.
[c] When post-larvae reached stage 2, they progressed to death.

40 μL L^{-1} EO. The white shrimp exposed to all of the eugenol concentrations exhibited significantly higher GST levels than the control group, and those exposed to 5 μL L^{-1} eugenol displayed the highest GST activity ($P<0.05$). The GST activity in the shrimp exposed to all tested concentrations of *L. alba* EO was significantly higher than that in the controls ($P<0.05$) (Fig. 3C).

The addition of alcohol to the water did not significantly alter the TOSC, CAT and GST activities compared to the control group.

4. Discussion

4.1. Short-term anesthesia induction and recovery

An effective anesthetic must provide rapid sedation, anesthesia and recovery times (Soltani et al., 2004). In the current study, eugenol induced rapid and deep anesthesia in *L. vannamei*. At 175 μL L^{-1} eugenol, post-larvae were anesthetized in 4.1 min, and sub-adults were anesthetized in 2.6 min at 400 μL L^{-1}. The shortest deep anesthesia induction time (approximately 21 min) that was elicited by eugenol in the shrimp *M. rosenbergii* was obtained at 800 μL L^{-1} (Saydmohammed and Pal, 2009), while anesthesia stage 2 was reached by all of the animals only after 60 min of treatment with 300 μL L^{-1} (Coyle et al., 2005). The same authors verified that treating this species with 25–100 μL L^{-1} eugenol provoked only light sedation (stage 1) within 60 min. In juvenile (1.8–2.1 g) *P. semisulcatus*, 100, 150 and 200 μL L^{-1} clove oil (80% eugenol) induced deep anesthesia in 5, 3 and 2.2 min, respectively (Soltani et al., 2004). Therefore, eugenol is more effective at anesthetizing *L. vannamei* than *M. rosenbergii*, and lower concentrations are also effective at anesthetizing *P. semisulcatus*.

There was no relationship between eugenol concentration and recovery time, but both *L. vannamei* sub-adults and post-larvae exhibited the shortest recovery times (4.3 and 3.36 min) at the highest eugenol concentrations tested (400 and 175 μL L^{-1}, respectively). In *M. rosenbergii* (Coyle et al., 2005; Saydmohammed and Pal, 2009) and *P. semisulcatus* (Soltani et al., 2004), increasing clove oil or eugenol concentrations correlated with longer anesthesia recovery times. The recovery of *M. rosenbergii* that were exposed to 800 μL L^{-1} eugenol was longer than 55 min (Coyle et al., 2005; Saydmohammed and Pal, 2009), and the fastest recovery time in *P. semisulcatus* was observed at 50 μL L^{-1} (Soltani et al., 2004).

The EOs of *L. alba* and *A. triphylla* showed concentration-dependent anesthetic activity in silver catfish (Cunha et al., 2010; Patent No PI016090005905), and this pattern was also be detected in sub-adult *L. vannamei*. The concentration range of *A. triphylla* EO that produced the shortest deep anesthesia induction time (3.5–1.2 min) in silver catfish was 300–800 μL L^{-1}, with recovery times between 9.4 and 18.3 min (Patent No PI016090005905). The *L. vannamei* sub-adults that were exposed to 500 μL L^{-1} *A. triphylla* EO were anesthetized in 4.48 min, with recovery within 15 min. Therefore, this EO seems to have effects on *L. vannamei* at a concentration that is equivalent to the concentration that induced anesthesia in the silver catfish. However, this EO is less effective in post-larvae because it takes approximately 10 min to induce deep anesthesia. Sub-adult shrimp quickly reach stage 2 of anesthesia when exposed to 750 to 1000 μL L^{-1} *L. alba* EO, recovering after 6.6 to 8.3 min. In post-larvae, the shortest stage 2 anesthesia induction time was obtained using 500 μL L^{-1} *L. alba* EO. The concentrations for sub-adults (but not for post-larvae) are above the range recommended for silver catfish (300–500 μL L^{-1}) (Cunha et al., 2010).

Menthol, which is the main component of the EOs of plants from the genus *Mentha*, was able to induce stage 2 of anesthesia in *M. rosenbergii* at 800 μL L^{-1}, but only after 207 min (Saydmohammed and Pal, 2009). This finding suggests that the EOs from *A. triphylla* and *L. alba* have superior efficacy for inducing deep anesthesia in shrimp. Anesthesia stage 2 was reached in *L. vannamei* with 300 μL L^{-1} Aqui-S™, which is an isoeugenol-based anesthetic, and recovery took over 30 min. After 30 min, 100 μL L^{-1} Aqui-S™ induced deep anesthesia in only 13% of the shrimp, and the percentage of anesthetized animals remained the same even after 60 min. The commercial anesthetic quinaldine (300 mg L^{-1}) was effective at inducing stage 2 of anesthesia in only 20% of the shrimp by 45 min, and recovery took approximately 18 min. Both 100 mg L^{-1} quinaldine and 100 mg L^{-1} MS-222 were unable to induce any stage of anesthesia in *M. rosenbergii* (Coyle et al., 2005). In *L. vannamei*, 800 μL L^{-1} lidocaine induced deep anesthesia in 2.5 min, with recovery in 24 min. Halothane also promoted rapid anesthesia (2.5 min) at 2.5 mL L^{-1}, but recovery took 17 min (Guzmán-Sáenz et al., 2010).

4.2. Long-term anesthesia induction and recovery

Anesthetics have been used for fish transportation because they reduce the stress and agitation of the fish (Singh et al., 2004; Park et al., 2009). However, if the fish are too heavily sedated, they lose equilibrium, cease swimming and may experience mechanical injury from hitting the tank walls; thus, anesthetic concentrations that only

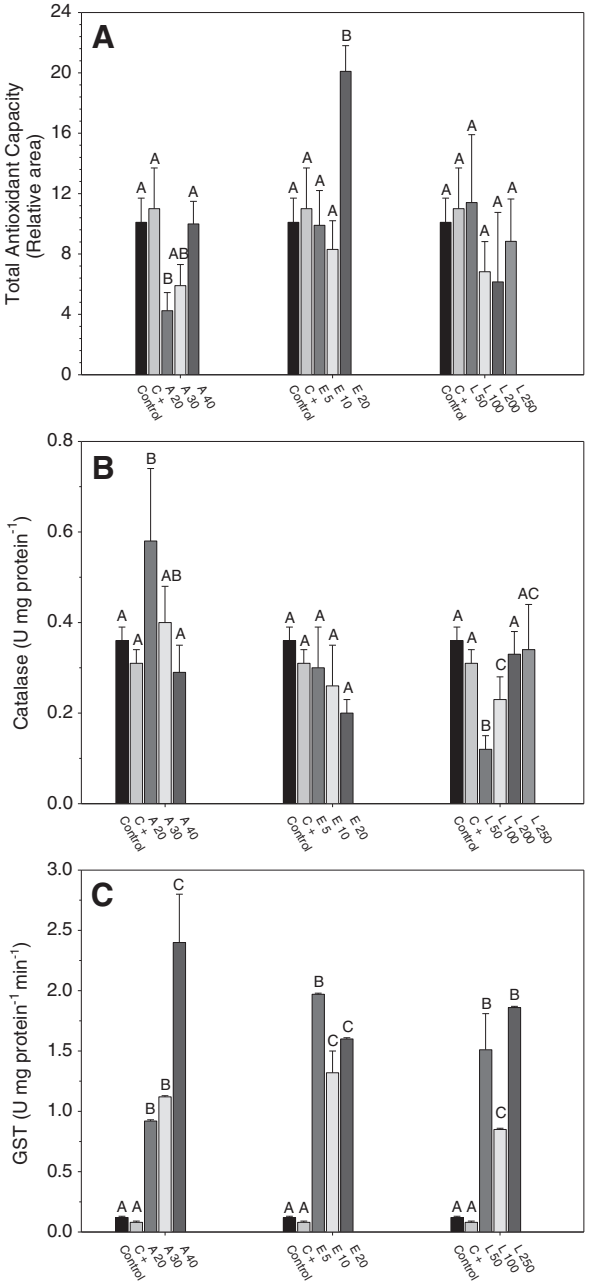

Fig. 3. The total oxyradical scavenging capacity against peroxyl radicals (A), catalase activity (B) and glutathione-S-transferase activity (C) in the hemolymph of *L. vannamei* sub-adults after 6 h of exposure to *A. triphylla* EO (A), eugenol (E) and *L. alba* EO (L). The data are expressed as the means ± SEM (n = 6). The different letters over the bars indicate significant differences between the treatments that used the same anesthetic ($P<0.05$). C+: Control plus ethanol.

induce sedation are recommended (Wagner et al., 2003). Although most operations in crustacean culture can be conducted without anesthesia, the rapid movement of shrimp may cause handling problems. In addition, their cannibalistic nature and sharp rostrum can present problems during transportation. Consequently, there has been some interest in investigating shrimp anesthetics, particularly for use in transport (Akbari et al., 2010).

Low concentrations of eugenol (5, 10 or 20 μL L^{-1}) were not sufficient to induce sedation (stage 1) in sub-adult *L. vannamei*. Because sub-adults went into deep anesthesia when exposed to 50 μL L^{-1} eugenol, the best range of eugenol for transport seems to be 20–50 μL L^{-1}. However, a concentration of 20 μL L^{-1} eugenol can be considered adequate for the post-larvae of this species because it only induced sedation over 6 h, with a 5–10 min recovery after this extended exposure time. The post-larvae of *Fenneropenaeus indicus* are more sensitive to eugenol, as the recommended concentration range for 12 h of transport is 1.3–3.7 μL L^{-1}, and 4 μL L^{-1} eugenol provoked mortality after 4 h (Akbari et al., 2010).

Concentrations of up to 5 μL L^{-1} eugenol were sufficient for 30 min of *Micropterus salmoides* transport (Cooke et al., 2004), but the recommended concentrations of eugenol for the transport (12–24 h) of the fish *Haplochromis obliquidens* are 18–20 μL L^{-1} (Kaiser et al., 2006). Therefore, the range of eugenol that is optimal for the transport of *L. vannamei* and *F. indicus* is similar to the range that is observed in fish.

A. triphylla EO at a concentration of 30–40 μL L^{-1} was sufficient to sedate *L. vannamei* sub-adults for 6 h, with complete recovery of the animals observed in 30 min. The *A. triphylla* EO concentration range that is recommended for the transport of *L. vannamei* post-larvae is 20–50 μL L^{-1}. The 10 and 20 μL L^{-1} concentrations of *A. triphylla* EO were unable to promote sedation. At 50 μL L^{-1} EO, the post-larvae progressed to stage 2 of anesthesia and exhibited long recovery times (greater than 120 min). *L. alba* EO can be used at a concentration of 50 μL L^{-1} for the transport of *L. vannamei* sub-adults, but higher concentrations cause death and therefore cannot be used. For post-larvae, the apparent concentration of this EO that is suitable for transport is below 100 μL L^{-1}.

4.3. Oxidative stress

In our study, white shrimp exhibited a rapid modulation of the antioxidant defense system after 6 h of exposure to several anesthetics, and *A. triphylla* EO (20 μL L^{-1}) augmented the total antioxidant capacity (150%) against peroxyl radicals. This natural product exhibits similar antioxidant characteristics to compounds that increase the total antioxidant capacity of human plasma by 49.4% by inhibiting lipid peroxidation during the oxidation process (Dadé et al., 2009).

In the present study, *L. vannamei* that were exposed to 20 μL L^{-1} eugenol decreased their total antioxidant capacity (100%), suggesting that this anesthetic exhibits pro-oxidant behavior. Thus, concentrations of eugenol that are lower than 20 μL L^{-1} are indicated for application in this species.

Exposure to *A. triphylla* EO (20 μL L^{-1}) increased CAT (70%) activity in white shrimp after 6 h of exposure. The increase in the activity of this antioxidant enzyme could be at least partly responsible for the higher total antioxidant capacity observed under this experimental condition. The same result occurs in *Farfantepenaeus paulensis* and *Corbicula fluminea* that are submitted to eyestalk ablation and hypoxia, respectively. The increased CAT activity observed after these treatments indicates enhanced antioxidant potential (Almeida et al., 2004; Lushchak, 2011). The catalase activity in the hemolymph of white shrimp exposed to 50 and 100 μL L^{-1} *L. alba* EO for 6 h decreased by 165 and 55%, respectively, compared to the control group. A similar effect was observed when *Litopenaeus stylirostris* was fed probiotics (*Pediococcus acidilactici*) because of the low stimulation of GST and CAT (Castex et al., 2009). The rapid modulation (i.e., reduced activity) of one of the enzymes involved in H_2O_2 processing should affect oxidative damage because this ROS is the precursor of the hydroxyl radical, which is a chemical species that triggers lipid peroxidation (Hermes-Lima, 2004). After exposure to *A. triphylla* or *L. alba* EOs or eugenol for 6 h, GST activity increased (615; 1292, and 1315%, respectively), which may be a response intended to mitigate the toxic effects of these substances or neutralize the harmful free radicals generated directly or indirectly by these anesthetics. A similar response was observed in *L. vannamei* exposed to pH stress, during which the toxic effects of acidosis increased the GST levels (Zhou et al., 2009). In crabs, exposure to the cyanotoxin microcystin for seven days also increases GST activity, indicating that the detoxification system is activated (Pinho et al., 2003).

A. triphylla EO has antioxidant properties, a powerful superoxide radical scavenging activity and moderate hydroxyl radical scavenging activity (Valentão et al., 2002). These properties can increase GST activity, which is extremely important for the detoxification of peroxyl radicals. Therefore, the EO of this plant not only produced an anesthetic effect but also increased GST activity, thus demonstrating the beneficial effects of this natural product on the transport of *L. vannamei*.

5. Conclusions

These results have demonstrated the effectiveness of eugenol and the *L. alba* and *A. triphylla* EOs in inducing anesthesia in *L. vannamei* at both the sub-adult and post-larvae stages. Compound efficacy and animal survival are dependent on the concentration, exposure time and developmental cycle of this species. These anesthetics could also be utilized in the transport of *L. vannamei* sub-adults and post-larvae. *A. triphylla* EO increased the total antioxidant capacity and CAT and GST activities in response to antioxidants. In addition, GST activity was enhanced upon treatment with all of the tested anesthetics.

Acknowledgments

This study was supported by research funds from the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS/PRONEX, process 10/0016-8) and Conselho Nacional de Pesquisa e Desenvolvimento Científico (CNPq, process 470964/2009-0). B. Baldisserotto, W. Wasielesky Junior, L.O. Garcia and J.M. Monserrat received productivity research fellowships from CNPq. T.V. Parodi, M.A. Cunha and C.G. Heldwein received PhD and MSc fellowships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). The authors are grateful to G. D. Zanetti for the identification of the plant material.

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