Nauplius
THE JOURNAL OF THE BRAZILIAN CRUSTACEAN SOCIETY

e-ISSN 2358-2936
www.scielo.br/nau
www.crustacea.org.br

ORIGINAL ARTICLE

Anesthetic and sedative effects of plant-derived essential oils on red swamp crayfish (*Procambarus clarkii*) at different concentrations and temperatures

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ABSTRACT

Limited studies have been conducted on the use of anesthetic agents during handling, cooking, and transportation of crayfish species. This study was carried out to evaluate the anesthetic effects of clove (*Syzygium aromaticum*), spearmint (*Mentha spicata*) and chamomile (*Matricaria chamomilla*) essential oils on red swamp crayfish (*Procambarus clarkii* (Girard, 1852)) at temperatures of 17 and 23 °C. The study was performed in 10 L plastic containers filled with 3 L of dechlorinated freshwater to determine induction and recovery times and stages under laboratory conditions. Five concentrations (200, 350, 500, 750, and 1000 µL/L) of essential oils were used. This study found that the induction time at 1000 µL/L was significantly lower than 200 and 350 µL/L at 17 °C ($p < 0.05$) and there was no statistical difference between the five concentrations at 23 °C ($p < 0.05$). Recovery at 1000 µL/L was markedly higher than 200, 350, and 500 µL/L at 23 °C ($p < 0.05$). Red swamp crayfish exposed to clove oil reached Stage 6, which is identified as a total loss of equilibrium, using 1000 µL/L at both temperatures. For spearmint oil, induction time at 1000 µL/L was significantly lower than 200, 350, and 500 µL/L at 17 °C, induction time at 200 µL/L was the highest at 23 °C ($p < 0.05$), and Stage 5 (partial loss of equilibrium) was recorded as the maximum stage reached. Recovery time at 1000 µL/L was the highest at 17 °C, and recovery at 200 and 300 µL/L were lower than 750 and 1000 µL/L at 23 °C ($p < 0.05$). No significant differences were recorded in the induction and recovery times of chamomile oil for all the concentrations at both 17 and 23 °C ($p > 0.05$) and the crayfish reached a maximum of Stage 3 (deep sedation) at 1000 µL/L at 23 °C. Overall, clove and spearmint essential oils were proven to be the most successful at providing effective anesthesia to the red swamp crayfish. However, the length of induction and

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SUBMITTED 17 September 2021
ACCEPTED 14 April 2022
PUBLISHED 28 November 2022

DOI 10.1590/2358-2936e2022035

recovery times may be a disadvantage for some procedures. In addition, it has been revealed that chamomile oil may have only a sedative effect and is therefore unsuitable to be used as an anesthetic.

Keywords

Carcinoculture, chamomile, eugenol, herbal anesthetics, spearmint

Introduction

Decapods (Crustacea: Decapoda), characterized by ten jointed legs, are used for food, bait, ornamental purposes, and neuroscience experiments (Ghanawi et al., 2019). With the increasing importance of animal welfare in recent decades, the possibility of these animals feeling pain should not be ignored (Adams et al., 2019). Hence, the use of anesthetics in crustaceans seems to be more important to facilitate handling and transport procedures.

In aquaculture, sedatives and anesthetics are commonly used to reduce stress and manage upkeep during operations, such as capturing, weighing, marking, handling, transferring, vaccinations, and surgeries (Gunkel and Lewbart, 2007; Harmon, 2009; Zahl et al., 2012; Aydın and Barbas, 2020). Anesthesia methods for decapods are not well established compared to finfish species, as most of the operations do not use anesthetics or sedatives on account of the lack of current legislative regulations (Cowing et al., 2015). In carcinoculture, recent techniques for anesthesia or euthanasia can be listed as ice shocking, heating, magnesium chloride injection, carbon dioxide exposure, and electro-stunning (Ross and Ross, 2008; Cooper, 2011; Fregin and Bickmeyer, 2016). Although these techniques are frequently applied in practice, not all of them are useful for specific intentions such as scientific experiments or transferring, and some procedures might also advance concerns for crustacean welfare (Adams et al., 2019; Ghanawi et al., 2019). Coyle et al. (2005) declared that the use of anesthetic substances on decapods would provide successful findings, however, they recommended further investigation before putting anesthetic usage into practice. The main reason being that various concentrations and durations of anesthetic agents would result in differences in functional and behavioral reactions. Additionally, decapods need different dosages of an anesthetic compared to those required for similar induction stages of fish species (Coyle et al., 2004).

Previous studies have presented synthetic anesthetics for use in carcinoculture, including halothane, MS-222 (tricaine methanesulfonate), lidocaine-HCl, ketamine-HCl, 2-phenoxyethanol and quinaldine sulfate (Obradović, 1986; Brown et al., 1996; Coyle et al., 2005). These chemicals are commonly used, but come with several disadvantages such as high cost, supply difficulties and lack of thorough testing in decapods (Akbari et al., 2010; Cowing et al., 2015). For instance, MS-222 can be toxic and may evoke undesirable responses (Souza et al., 2018). Potentially, applying plant-based essential oils with anesthetic properties may offer more safety for crustaceans (Morgan et al., 2001; Souza et al., 2018). Plant-derived essential oils, especially clove (*Syzygium aromaticum*) oil, can be considered as an advantageous alternative to chemical-based anesthetics because they are considerably cheaper, non-toxic and easy to implement. Clove oil applications have been used in various marine and freshwater fish species and several decapods including the American lobster, *Homarus americanus* H. Milne Edwards, 1837 (Waterstrat and Pinkham, 2005), Norway lobster, *Nephrops norvegicus* (Linnaeus, 1758) (Cowing et al., 2015), black tiger shrimp, *Penaeus monodon* Fabricius, 1798 (Jiang et al., 2020), *Macrobrachium* Spence Bate, 1868 species, including *Macrobrachium rosenbergii* (De Man, 1879) (Coyle et al., 2005; Vartak and Singh, 2006), *Macrobrachium nipponense* (De Haan, 1849) (Xinlong et al., 2007), *Macrobrachium tenellum* (Smith, 1871) (Aréchiga-Palomera et al., 2016), grass shrimp, *Palaemonetes sinensis* (currently *Palaemon sinensis* (Sollaud, 1911)) (Li et al., 2018a; 2018b), and the three-spot swimming crab, *Portunus sanguinolentus* (Herbst, 1783) (Premarantha et al., 2016). However, spearmint (*Mentha spicata*) essential oil has only

been used on several freshwater fish, including the common carp, *Cyprinus carpio* Linnaeus, 1758 (Roohi and Imapoor, 2014; 2015; Chaharborji et al., 2019) and Atlantic salmon, *Salmo salar* Linnaeus, 1758 (Danner et al., 2011), and the authors stated that this was effective in inducing an anesthetized state. Also, the potential anesthetic effect of chamomile (*Matricaria chamomilla*) is a new topic of study in aquaculture. Positive results have been reported on common carp, *C. carpio* (see Al-Niaeem et al., 2019), electric blue hap, *Sciaenochromis fryeri* Konings, 1993, and yellow lab, *Labidochromis caeruleus* Fryer, 1956 (Can et al., 2017).

The red swamp crayfish, *Procambarus clarkii* (Girard, 1852), is a species that inhabit burrows in muddy substrates, such as wetlands and marshes and originates from North Eastern Mexico and Louisiana, United States. This species is the most cultivated crayfish in the world with a production ratio of 99.9% (FAO, 2018) and it is important for both the aquaculture and aquarium trade. The crayfish can tolerate extreme water quality conditions, i.e., low temperature or oxygen levels, pollution, as well as drought (Barbaresi and Gherardi, 2000; Cruz and Rebelo, 2007).

To build on previous studies, this research was designed to test the potential use of plant-derived essential oils as anesthesia on the red swamp crayfish, in order to decrease or prevent stress during transportation or manipulation. Accordingly, the aim of this study was to investigate the anesthetic efficacy of clove, spearmint, and chamomile essential oils on the red swamp crayfish. However, another important objective of this study was to determine the optimum and efficient concentrations of the essential oils tested, because Teixeira et al. (2017) warned that an anesthetic concentration above what is required represents a waste of essential oil and unnecessary expense. Specifically, induction and recovery levels at different concentrations and temperatures were evaluated.

Material and Methods

Ethical approval

The present study was carried out in accordance with animal welfare and the ethics requirements and complied with the guidelines of the EU Directive 2010/63/EU for animal experiments.

Experimental conditions and crayfish

The study was carried out in the Tropical Aquaculture Laboratory, Faculty of Fisheries, İzmir Katip Çelebi University, İzmir, Turkey. Red swamp crayfish (*P. clarkii*), obtained from a commercial facility (Çelik Aquaculture, İzmir, Turkey), were stocked in 240 L glass aquariums (with 5 cm diameter PVC pipes on the floor for each individual) and kept for 14 days to adapt to these new external conditions. The crayfish were fed with commercial bottom feed (Art Akua Bottom Food; 50% crude protein and 10% crude lipid) once daily to satiation. The uneaten food and feces were removed from the aquariums daily. The experiment was carried out in 10 L plastic aquariums and each concentration for both temperatures was tested in triplicate. Thirty specimens with an average weight of 5.32 ± 0.35 g, an average total length of 58.03 ± 1.05 mm, and an average carapace length of 28.57 ± 0.58 mm, were used for testing of each essential oil. The crayfish were starved for 24 h before exposing the anesthetic agents.

Water parameters

Carreira et al. (2017) stated the optimum growth temperature for red swamp crayfish (*P. clarkii*) ranges from 20 to 27 °C. Based on this information, we aimed to measure the response of the anesthetic effects of essential oils used in the study, both under the optimum and closest to the optimum conditions, by determining the temperatures as ± 3 °C (17 and 23 °C), which are above and below the minimum growth temperature of this species.

Concentration experiments at different temperatures were carried out in the autumn months in two different laboratories in the same unit. The Aquarium Laboratory is constantly heated by air conditioners and all 23 °C experiments were performed in this laboratory. In another laboratory without air conditioning, an aquarium was placed with the same equipment as in the Aquarium Laboratory and the average water temperature was measured at around 17 °C. In the evening before the low temperature experiments, the crayfish were transferred to another laboratory with 23 °C water. The water was allowed to gradually cool overnight and after about 12 h, experiments at 17 °C were started in this laboratory. These procedures continued daily until the trials were over.

The water parameters, including temperature, pH and dissolved oxygen of the stock aquariums were checked daily with AZ 84051 Combo Water Quality Meter during the study. Mean values were recorded as 25.61 ± 0.24 °C for temperature, 7.05 ± 0.11 for pH, and 8.85 ± 0.57 ppm for dissolved oxygen.

Essential oils

Clove (*Syzygium aromaticum*), spearmint (*Mentha spicata*), and chamomile (*Matricaria chamomilla*) essential oils were purchased from Med World Distribution and Marketing, İstanbul, Turkey. The main compounds of essential oils were listed as eugenol for clove oil, carvone for spearmint oil and apigenin for chamomile oil. Ethanol (96%) was used as a solvent and the anesthetic solutions were prepared at a 1:10 ratio (v:v). A total of 12 concentrations (20, 30, 40, 50, 75, 100, 150, 200, 350, 500, 750, and 1000 µL/L) of each essential oil were prepared. A related test, conducted by Ghanawi et al. (2019) using Australian red claw crayfish (*Cherax quadricarinatus* (von Martens, 1868)), found that only concentrations of 200 µL/L and above are effective as anesthesia. Consequently, low concentrations were not included in their study, since it has been noted that concentrations of 200 µL/L and above are effective. However, in our study, we have included low concentrations in order to test the effectiveness on red swamp crayfish.

Experimental setup

Two plastic aquariums (10 L) to test induction and recovery were filled with 24 h rested freshwater at temperatures of 17 and 23 °C. The mean pH was measured at 6.99 ± 0.33 and the mean dissolved oxygen at 9.12 ± 0.54 ppm. An air pump with two outlets (Atman Champion CX-0088) connected with air stones was released into the freshwater in the induction (3 L) and recovery (5 L) aquariums to maintain dissolved oxygen levels. The solutions placed in the water were mixed in order to ensure they were homogeneous and it was checked whether there was any dissolution. The experiment was conducted in a dimly lit condition to minimize stress. Crayfish were left individually in plastic aquariums and the time of induction and recovery was determined with a digital chronometer. To assess the induction and recovery stages, crayfish were prodded periodically with a glass stick.

Determination of induction stages

A separate experiment was carried out using an identical experimental setup. A specimen was placed in a 10 L plastic aquarium and anesthesia levels were determined for each essential oil's concentration at two temperatures with three replicates for one hour total. The identified stages (Tab. 1) at 15, 30, 45, and 60 min for clove (CLO), spearmint (SPO), and chamomile (CHO) oils are presented, respectively.

Table 1. Identification of induction and recovery stages in crayfish (the stages were rearranged from Vartak and Singh (2006) and Xie et al. (2010) with considered experimental observations).

	Stage	Description	Behavior
Induction	1	Normal	There is a slight loss of activity in crayfish.
	2	Light sedation	Crayfish try to rise to the surface of the water. Increased movement begins in swimmerets.
	3	Deep sedation	Crayfish move in an uneasy manner. Irregular movements seen in the maxillipeds and very rapid movement begin in swimmerets.
	4	Stagnation	Crayfish were stationary, remaining in one place. Irregular movements begin in the chelipeds, walking legs, and antennae.
	5	Partial loss of equilibrium	Crayfish gradually lose control over their chelipeds and walking legs. There is some movement in the antennae and antennules.
	6	Total loss of equilibrium	Complete loss of control is seen in the chelipeds and walking legs. Crayfish are immobilized.
	7	Death	Stiffening and loss of color are seen in the body and extremities.
Recovery	1	Partial revival of equilibrium	Irregular movements are seen in the chelipeds, walking legs, and antennae.
	2	Complete revival of equilibrium	Regular movements are seen in the chelipeds, walking legs, and antennae. Looseness and relaxation are observed in the crayfish.
	3	Normal	Crayfish swim freely.

Statistical analysis

The Shapiro-Wilk W and Levene tests were implemented respectively, to verify normality and homogeneity of variance before further analysis was undertaken. One-way analysis of variance (ANOVA) was used for assessing the significance in stages 3, 4, 5, and 6 induction times at different concentrations of clove and spearmint essential oils, and two-way ANOVA was performed for the analysis of the data of stage 2 induction and for recovery times at different concentrations of three essential oils at two temperatures. Differences between the experimental groups were ranked using Tukey's multiple range test. All means were presented with standard errors (± SE). For statistical assessment of the study data, statistical software (Statgraphics Centurion XVI, Statpoint Technologies Inc., The Plains, VA) was used (Zar, 1999). Differences were considered significant at the 95% confidence interval.

Results

Red swamp crayfish exposed to chamomile oil reached Stage 2 at maximum, except 1000 µL/L at 23 °C. In order to compare the induction times of all essential oils tested in this stage, times of induction to Stage 2 depending on different temperatures of three plant-based essential oils on red swamp crayfish are shown in Fig. 1. As seen in Fig. 1A, significant differences were found in the crayfish exposed to clove oil between the 17 and 23 °C at concentrations of 200, 350, 500, and 750 µL/L ($p < 0.05$). At 17 °C, induction time at 1000 µL/L was significantly lower than 200 and 350 µL/L ($p < 0.05$). There was no statistical difference between the five concentrations at 23 °C ($p > 0.05$). Significant differences were recorded between the two temperatures at all concentrations of spearmint oil, which was used for red swamp crayfish anesthesia ($p < 0.05$) (Fig. 1B). Induction time at 1000 µL/L was significantly lower than 200, 350, and 500 µL/L at 17 °C, and induction time at 200 µL/L was the highest at 23 °C ($p < 0.05$). There were no differences in chamomile oil's induction times between all concentrations at both 17 and 23 °C ($p > 0.05$) (Fig. 1C). In two-way ANOVA, crayfish exposed to clove and spearmint oils were influenced by both concentration ($p = 0.0000$ for both essential oil) and temperature ($p = 0.0000$ for both essential oil). Interactions were recorded regarding the independent factors in clove ($p = 0.0012$) and spearmint ($p = 0.0063$) oils. The crayfish exposed to chamomile oil were only influenced by the temperature ($p = 0.0004$).

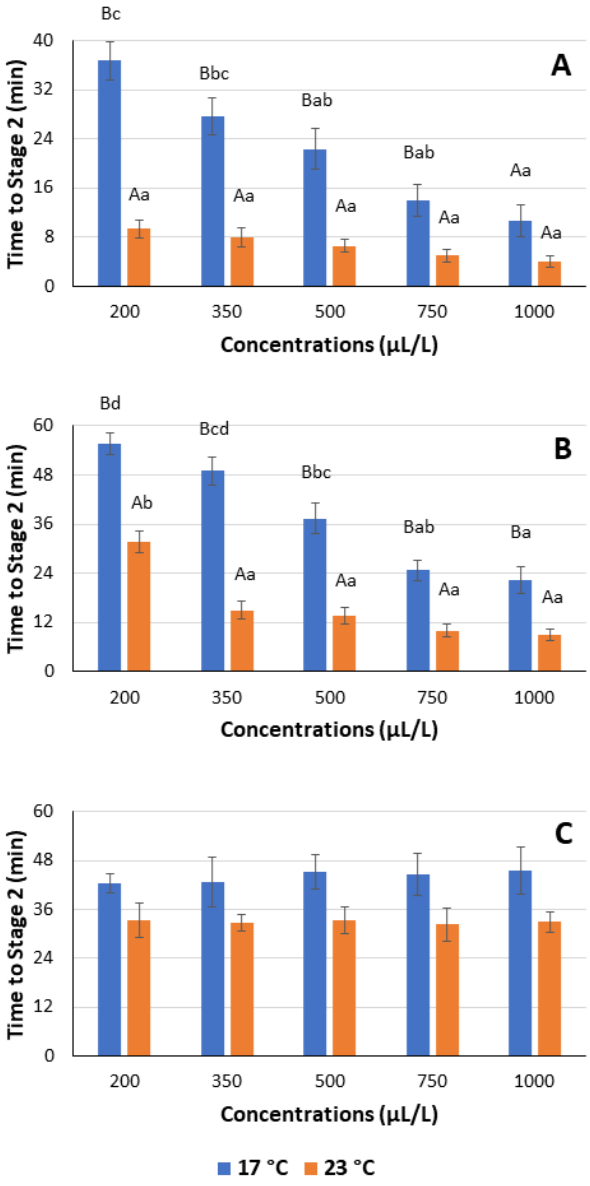

Figure 1. Times to Stage 2 induction (min) for three different plant-based essential oils used as a natural anesthetic for red swamp crayfish. (A) Clove oil (in two-way ANOVA, $p = 0.0000$ for concentration, $p = 0.0000$ for temperature, and $p = 0.0012$ in interaction); (B) spearmint oil (in two-way ANOVA, $p = 0.0000$ for concentration, $p = 0.0000$ for temperature, and $p = 0.0063$ in interaction); (C) chamomile oil (in two-way ANOVA, $p = 0.9901$ for concentration, $p = 0.0004$ for temperature, and $p = 0.9896$ in interaction). Different uppercase letters indicate significant differences between temperatures at the same concentration. Different lowercase letters indicate significant differences between concentrations at the same temperature ($p < 0.05$).

Times to Stages 3 and 4 inductions of red swamp crayfish exposed to 1000 µL/L clove oil at 23 °C were lower than all concentrations at both temperatures, except 750 µL/L at 23 °C ($p < 0.05$) (Fig. 2A, B). No crayfish exposed to spearmint oil reached Stage 3 at 200 and 350 µL/L at 17 °C and 350 µL/L at 23 °C, and also only 4 groups (1000 µL/L at 17 °C and 500, 750, and 1000 µL/L at 23 °C) reached Stage 4. Induction times to Stage 3 of crayfish exposed to 500, 750, and 1000 µL/L spearmint oil at 23 °C, and times to Stage 4 at 1000 µL/L at 23 °C were the lowest ($p < 0.05$) (Fig. 2C, D).

Induction times to Stage 5 and Stage 6 of red swamp crayfish exposed to clove and spearmint oils are presented in Tab. 2. The lowest induction times to Stage 5 and Stage 6 were found in 1000 µL/L clove oil at 23 °C ($p < 0.05$). Induction times to Stage 5 of 500 and 750 µL/L clove oil at 23 °C were lower than 750 µL/L clove oil at 17 °C and 500 and 750 µL/L spearmint oil at 23 °C ($p < 0.05$).

The 15 min induction stages of red swamp crayfish are detailed in Tab. 3. At 17 °C, the crayfish exposed to clove oil entered the Stage 6 (total loss of equilibrium) after 1 h (1000 µL/L concentration). It was noted that crayfish exposed to clove oil at 200, 350, and 500 µL/L concentrations were able to pass to Stage 4 (stagnation) and the crayfish exposed to the anesthetic medium at 750 µL/L were able to pass to Stage 5 (partial loss of equilibrium). At 23 °C, the crayfish entered Stage 6 (after 1 h) at concentrations of 750 and 1000 µL/L. It was noted that at 200 µL/L, they most often entered Stage 4 and at 350 and 500 µL/L, they entered Stage 5. At the end of the 1 h period at 17 °C, the crayfish exposed to spearmint oil were able to enter Stage 2 at 200 and 350 µL/L, Stage 3 at 500 and 750 µL/L, and Stage 4 at 1000 µL/L. It has been noted that at 200 and 350 µL/L, they could pass to Stage 2 (light sedation), and at 500 and 750 µL/L, they entered Stage 3 (deep sedation). At the end of 1 h at 23 °C, it was observed that the crayfish moved to Stage 2 at 200 µL/L, to Stage 3 at 350 µL/L, and to Stage 5 at 500, 750, and 1000 µL/L concentrations. Similar findings were obtained in red swamp crayfish exposed to chamomile oil for both temperatures (17 and 23 °C). It was recorded that the crayfish, in which 1000 µL/L chamomile oil was used, were able to reach to the third stage only at 23 °C and after one h.

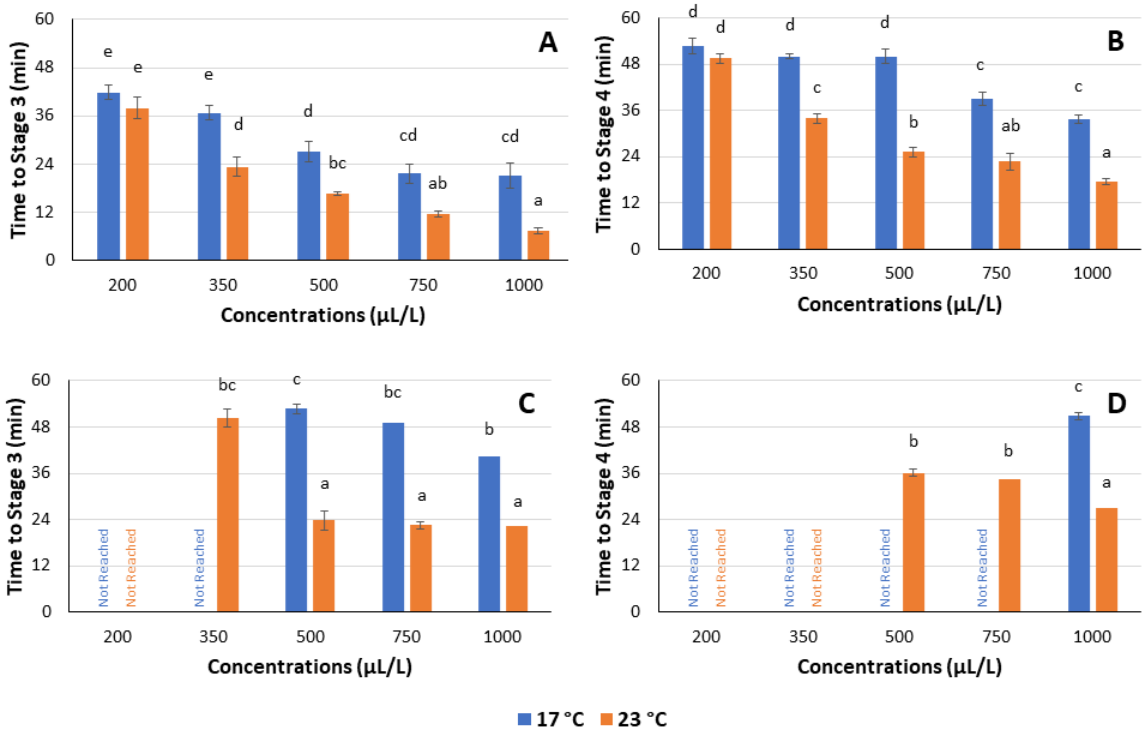

Figure 2. Times to Stages 3 and 4 inductions (min) of clove and spearmint essential oils used as a natural anesthetic for red swamp crayfish. (A) and (B) Clove oil; (C) and (D) spearmint oil. The significances between the concentrations were analyzed using one-way ANOVA. Different letters in each graphic indicate the statistical differences between the concentrations for both temperatures ($p < 0.05$).

Table 2. Induction times to Stage 5 and Stage 6 from Stage 1 of red swamp crayfish exposed to different concentrations of clove and spearmint oils at two temperatures

Essential oil	Temperature	Concentration (µL/L)	Time to Stage 5 Induction (min)	Time to Stage 6 Induction (min)
Clove oil	17 °C	750	51.23±1.50^{cd}	N/A
		1000	42.84±0.81^{bc}	54.96±1.73^{b}
	23 °C	350	43.96±1.05^{bc}	N/A
		500	39.97±1.94^{b}	N/A
		750	37.24±2.61^{b}	51.10±2.70^{b}
		1000	26.13±0.79^{a}	39.73±1.66^{a}
Spearmint oil	23 °C	500	54.11±2.63^{d}	N/A
		750	49.88±1.32^{cd}	N/A
		1000	42.48±2.49^{bc}	N/A

Note: The significances between the concentrations were analyzed using one-way ANOVA. Different letters in the same column indicate the statistical differences between the concentrations for induction times at 17 and 23 °C ($p < 0.05$).

Table 3. The 15 min induction stages of red swamp crayfish exposed to clove (CLO) spearmint (SPO), and chamomile (CHO) oils for 1 h.

Temperature	Concentration (µL/L)	15 min	30 min	45 min	60 min
17 °C	200	1 | 1 | 1	2 | 1 | 1	3 | 2 | 2	4 | 2 | 2
	350	1 | 1 | 1	2 | 1 | 1	3 | 2 | 2	4 | 2 | 2
	500	2 | 1 | 1	3 | 1 | 1	3 | 2 | 2	4 | 3 | 2
	750	2 | 1 | 1	3 | 2 | 1	4 | 3 | 2	5 | 3 | 2
	1000	2 | 1 | 1	3 | 2 | 1	5 | 3 | 2	6 | 4 | 2
23 °C	200	2 | 1 | 1	2 | 2 | 1	3 | 2 | 2	4 | 2 | 2
	350	2 | 1 | 1	3 | 2 | 1	5 | 2 | 2	5 | 3 | 2
	500	2 | 2 | 1	4 | 3 | 1	5 | 4 | 2	5 | 5 | 2
	750	3 | 2 | 1	4 | 3 | 1	5 | 4 | 2	6 | 5 | 2
	1000	3 | 2 | 1	5 | 4 | 1	6 | 5 | 2	6 | 5 | 3

Note: Induction stages in each cell were presented as CLO | SPO | CHO.

Recovery times of red swamp crayfish exposed to three essential oils are given in Fig. 3. A significant difference was found in the crayfish exposed to clove oil between the 17 and 23 °C at the 750 µL/L ($p < 0.05$). Recovery times at 200 and 350 µL/L were significantly lower than 750 and 1000 µL/L at 17 °C ($p < 0.05$). Recovery at 1000 µL/L was markedly higher than 200, 350, and 500 µL/L at 23 °C ($p < 0.05$) (Fig. 3A). There were significant differences between 17 and 23 °C at all concentrations of spearmint oil ($p < 0.05$). Recovery time at 1000 µL/L was the highest at 17 °C, and recovery at 200 and 300 µL/L were lower than 750 and 1000 µL/L at 23 °C ($p < 0.05$) (Fig. 3B). There were no differences in chamomile oil recovery times between all concentrations at both 17 and 23 °C ($p > 0.05$) (Fig. 3C). In two-way ANOVA, recovery of crayfish exposed to clove and spearmint oils were influenced by both concentrations ($p = 0.0000$ for both essential oil) and temperatures ($p = 0.0000$ for clove oil and $p = 0.0003$ for spearmint oil). An interaction was noted regarding the independent factors in spearmint oil ($p = 0.0000$). The crayfish exposed to chamomile oil was not influenced by any factor.

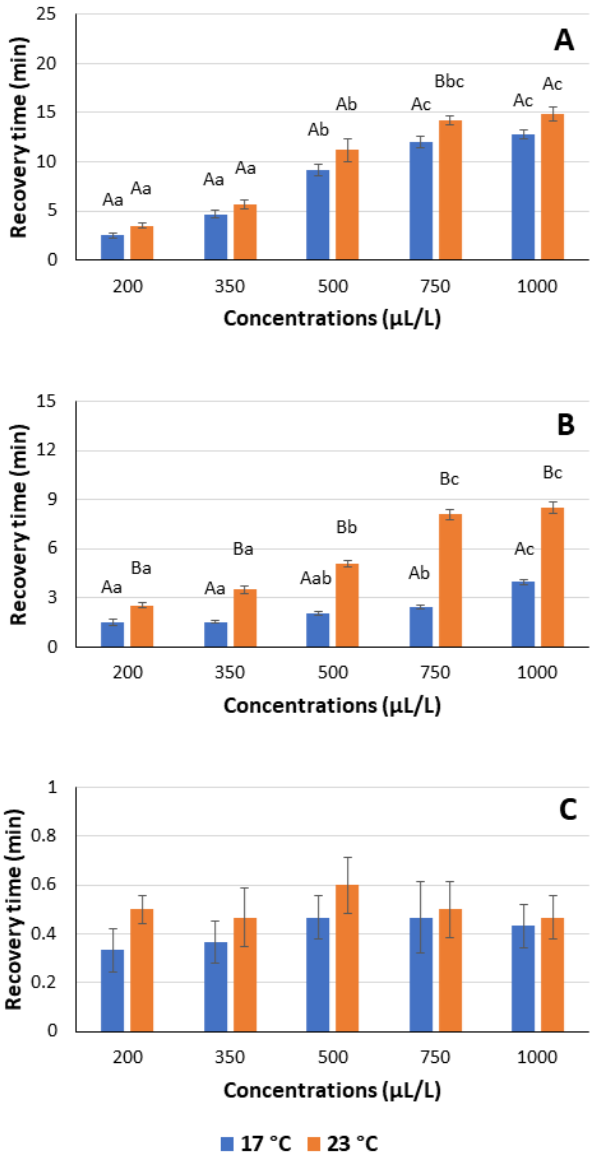

Figure 3. Recovery times (min) for three different plant-based essential oils used as a natural anesthetic for red swamp crayfish. (A) Clove oil (in two-way ANOVA, $p = 0.0000$ for concentration, $p = 0.0003$ for temperature and $p = 0.7499$ in interaction); (B) spearmint oil (in two-way ANOVA, $p = 0.0000$ for concentration, $p = 0.0000$ for temperature, and $p = 0.0000$ in interaction); (C) chamomile oil (in two-way ANOVA, $p = 0.7585$ for concentration, $p = 0.1642$ for temperature, and $p = 0.9511$ in interaction). Different uppercase letters indicate significant differences between temperatures at the same concentration. Different lowercase letters indicate significant differences between concentrations at the same temperature ($p < 0.05$).

Discussion

In this study, although red swamp crayfish exposed to 1000 µL/L clove oil at 17 and 23 °C reached Stage 6 induction (total loss of equilibrium), the recovery times were longer at this concentration. No crayfish exposed to spearmint oil reached Stage 6, but lower induction and recovery times to Stage 5 (partial loss of equilibrium) were found at 1000 µL/L at 23 °C, which is significantly similar to 350 µL/L clove oil at 23 °C for both induction and recovery times. Crayfish exposed to chamomile oil did not reach beyond Stage 2 (light sedation), except at 1000 µL/L at 23 °C. Similar induction stages were recorded for each essential oil at 1000 µL/L at 17 °C and 750 µL/L at 23 °C (Stage 6 for clove oil, Stages 4 and 5 for spearmint oil and Stage 2 for chamomile oil).

The present research investigated five different concentrations (200, 350, 500, 750, and 1000 µL/L) of clove, spearmint, and chamomile essential oils, the highest concentration of 1000 µL/L reached the maximum stage depending on essential oil characteristics on red swamp crayfish (*P. clarkii*). Previous studies demonstrated that different kinds of anesthetic substances could be used for crayfish species, but concentration was also important as well as anesthetic type (Ross and Ross, 2008; Ghanawi et al., 2019). Cowing et al. (2015) stated that successful anesthesia stages could be related to higher concentrations. Former studies revealed that the induction time decreased with the further concentrations of eugenol (300, 600, and 900 µL/L) on Norway lobster (*N. norvegicus*) and clove oil (150, 200, and 250 µL/L) on three-spotted crab (*Po. sanguinolentus*) (Cowing et al., 2015; Premarathna et al., 2016). Higher concentrations of clove and spearmint oils might be useful in full anesthesia operations, including surgery, tagging and handling, etc. (Gunkel and Lewbart, 2007; Saydmohammed and Pal, 2009), while lower concentrations that result in the crayfish being lightly sedated could be used for operations such as transferring (Cowing et al., 2015).

Clove and spearmint oils caused successful anesthesia on red swamp crayfish (*P. clarkii*) by immersion, but both essential oils extended induction and recovery durations. We found that the induction times decreased and the recovery times increased with the further concentrations as in the previous studies conducted with Australian red claw crayfish, *C. quadricarinatus* (see Ghanawi et al., 2019), giant river prawn, *M. rosenbergii* (see Vartak and Singh, 2006), longarm river prawn, *M. tenellum* (see Aréchiga Palomera et al., 2016), Chinese grass shrimp, *Pa.*

sinensis (see Li et al., 2018a), and black tiger shrimp, *Pe. monodon* (see Jiang et al., 2020). However, a successful recovery depends on rapid absorption and elimination from tissues (Cupp et al., 2016) and this becomes more difficult as the amount of substance entering the body increases (at higher concentrations). Ghanawi et al. (2019) studied clove oil's induction and recovery times on *C. quadricarinatus* at Stage I and II, separately. They stated that the induction times (at 21.8 °C) were 8.2 min for Stage I and 5.35 min for Stage II at 375 µL/L and 7.2 min for Stage I and 5.2 min for Stage II at 500 µL/L. Stage I and II of Ghanawi et al. (2019) correspond to Stage 5 and 6 in our study, respectively. It is evident that in this study conducted with red swamp crayfish, induction times were longer than in the same stages. There are limited studies on anesthesia in crayfish species in the literature and these were generally conducted on the effects of MS-222 and some synthetic anesthetics. Therefore, further studies are needed to fully describe the responses of crayfish species to different anesthetic agents. For example, immersion application of MS-222 has been reported to be ineffective in European crayfish (*Astacus astacus* (Linnaeus, 1758)), virile crayfish (*Orconectes virilis*, currently *Faxonius virilis* (Hagen, 1870)), and red swamp crayfish (*P. clarkii*) (Obradović, 1986; Brown et al., 1996; Ewing and Medler, 2020). Therefore, these studies should be increased in order to more comprehensively compare the duration of anesthesia and the response of crayfish to anesthetic agents in crayfish species and to explain the effects of species and size differentiation on induction and recovery times.

Water parameters, predominantly temperature, are critical factors for growth performance, survival rate, and metabolic activities of cultivated species in aquaculture (Rahman, 1982). Red swamp crayfish is a tropical species that live in water temperatures ranging from 20-30 °C (Aiken and Waddy, 1992). This study tested the effects of different temperatures (17 and 23 °C) and their interactions with different concentrations of three plant-based essential oils (clove, spearmint, and chamomile). The induction times of all three essential oils at 23 °C were lower than at 17 °C while a contrary state was noted in recovery times. This can be explained that increasing opercular ventilation and cardiac rates at higher temperatures caused an increase in the permeability of anesthetic into gills and this situation also resulted in increasing the impact of the anesthetic (Javahery et al., 2012). Similar results were reported in previous studies carried out regarding the anesthetic effects of eugenol (clove) and menthol (peppermint) at different temperatures on grass shrimp, *Pa. sinensis* (see Li et al., 2018a; 2018b) and black tiger shrimp, *Penaeus japonicus* Spence Bate, 1888 (see Jiang et al., 2020). However, studies on decapod species pointed out that the respiratory rate typically increases with water temperature (Allan et al., 2006; Huang et al., 2008; Cai et al., 2012) and induction time is reduced with the increasing water transfer in gills (Jiang et al., 2020).

The ratio of the major compounds in essential oils is also an important factor for successful anesthesia (Aydın and Barbas, 2020). For example, Hussen and Sharma (2015) anesthetized the common carp at 5 µL/L clove oil (with 75% eugenol), while Roohi and Imanpoor (2015) inducted the same species at 5 mL/L spearmint oil (with 28.4% carvone). Since spearmint oil generally contains less carvone (60%–70%) compared to the eugenol content of clove oil (70%–90%) and carvone has a limited anesthetic effect than eugenol (de Carvalho and da Fonseca, 2006; Purbosari et al., 2019), corresponding differences in induction times are noted, as in the present study. On the other hand, chamomile oil was identified as a mild sedative (Das et al., 1999) and Avallone et al. (1996) stated that its flavonoid influences the brain's benzodiazepine receptors, which caused sedative effects. The impact of chamomile oil as an anesthetic substance is recently studied in fish species, but no research has been previously conducted on crustaceans. Al-Niaeem et al. (2019) found a total anesthesia time of 63 min at 400 mg/L and 55 min at 450 mg/L on common carp (*C. carpio*). When compared to clove oil, which is the most widely used herbal anesthetic in aquaculture, these durations are very high for this species. Though, it can be said that the anesthetic effect of chamomile oil is weak on fish and crustaceans because of its sedative features.

In conclusion, considering the effects of three plant-derived essential oils tested in this study, 350 µL/L of clove oil can be used for anesthesia on red swamp crayfish. In addition, spearmint oil can be effective when lower stages of anesthesia are needed

such as immobilizing (for example, 350 µL/L for deep sedation and 500 µL/L for stagnation). However, although it is thought that chamomile oil can be used in transportation or post-arrival processes due to its sedative effects, it is unsuitable for red swamp crayfish, as it may have harmful effects at higher concentrations. The results of this study indicate that the water temperature was significant in red swamp crayfish anesthesia. We also suggest that anesthesia applications be performed over 23 °C for this species.

Acknowledgements

The authors are grateful to Asst. Prof. Tuğçe Şensurat Genç for the material support and Mustafa Deniz and Bayram Yaşar Güler for their technical assistance. The authors are also very grateful to Mrs. Holly Aymelek who contributed to the proofreading. Special thanks to Prof. Derya Güroy for the final check of the manuscript. In memory of my father, Ziver Karadal. I dedicate the biggest thanks in my life to him.

Additional Information

Author Contributions

Conceptualization and Design: OK. Performed research: EE, OK. Acquisition of data: EE, OK. Analysis and intepretation of data: EE, OK. Preparation of figures/tables/maps: EE, OK. Writing - original draft: OK. Writing - critical review & editing: OK.

Consent for publication

All authors declare that they have reviewed the content of the manuscript and gave their consent to submit the document.

Competing interests

The authors have no conflicts of interest to declare.

Data availability

All data generated and analyzed during this study are presented in this article.

Funding and grant disclosures

This work was not financially supported.

Study association

The present study is the master's thesis of Ercan Elmas prepared at the Graduate School of Natural and Applied Sciences, İzmir Katip Çelebi University, İzmir, Turkey.

Study permits

Not applicable.

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