

Clove oil as an anaesthetic for Australian redclaw crayfish *Cherax quadricarinatus*

Joly Ghanawi¹ | Ghazi Saoud² | Caline Zakher³ | Samer Monzer³ | Imad Patrick Saoud³

¹University of Edinburgh, Edinburgh, UK

²American Community School, Beirut, Lebanon

³American University of Beirut, Beirut, Lebanon

Correspondence

Imad Patrick Saoud, American University of Beirut, Beirut, Lebanon.
Email: is08@aub.edu.lb

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Abstract

Crustaceans are aquacultured both for food and as ornamental organisms. Pain and distress are not entirely understood in crustaceans, but the industry is moving towards requiring protection for the welfare of these animals during handling. In the present study, we evaluated the efficacy of clove oil as an anaesthetic for redclaw crayfish (*Cherax quadricarinatus*) as a model for freshwater crustaceans. We also studied how factors such as body weight and sex of redclaw could affect this efficacy. The whole experiment was replicated in two consecutive years. Redclaw juveniles were sorted into three size classes: small (<5 g), medium (5–12 g) and large (12–37 g). At least 10 males and 10 females from each size class were placed individually in water containing clove oil concentrations of 375 and 500 µl/L. Both concentrations induced rapid induction and recovery times, with 500 µl/L being the more effective concentration of the two. Induction and recovery times increased with the increase in crayfish size. No significant differences were found in induction and recovery times between male and female crayfish. Results suggest that clove oil is an effective anaesthetic for redclaw.

KEYWORDS

anaesthetic, *Cherax quadricarinatus*, clove oil, redclaw

1 | INTRODUCTION

Crustaceans including crayfish are used for human consumption, ornamentals, public aquariums and scientific trials (e.g. neurobiology). The need for humane treatment of crustaceans during handling has been discussed for decades (e.g. Baker, 1955; Gunter, 1961), especially that animal welfare is becoming more important when considering its final use. For example, if an animal product is perceived as having been treated inhumanely, sales might be impacted (Broom, 2007). Recently, concerns have been raised as to how crustaceans are treated in fisheries and aquaculture in terms of capture, sorting, transportation, killing and cooking (Elwood, Barr, & Patterson, 2009). Although pain in crustaceans is not understood, there are suggestive data that crustaceans respond to signs of pain and distress. If proven true, there would be a need to protect the welfare of

these animals during their various uses (Broom, 2007; Elwood, 2012; Elwood et al., 2009).

A number of anaesthetic agents are used to reduce physiological stress in aquatic animals during activities such as netting, handling, transportation, tagging, weighing, surgeries and vaccinations (Ross & Ross, 2008; Zahl, Samuelsen, & Kiessling, 2012). Some of the common anaesthetics used on poikilothermic organisms include tricaine methanesulfonate (MS-222), 2-phenoxyethanol, benzocaine and metomidate (Popovic et al., 2012; Readman, Owen, Knowles, & Murrell, 2017; Santos, Ghanawi, & Saoud, 2015; Weber, Peleteiro, Logarcía, & Aldegunde, 2009). The most widely used fish anaesthetic and the only approved anaesthetic by the U.S. Food and Drug Administration is MS-222 (Popovic et al., 2012). Although MS-222 can anaesthetize fish, it was found ineffective for most crustaceans (Coyle, Durborow, & Tidwell, 2004). Moreover, crustaceans appear

to need higher concentrations of an anaesthetic as compared to those required to anaesthetize fish (Coyle et al., 2004).

Some traditional methods used to anaesthetize edible crustaceans include cooling, heating, bubbling holding water with carbon dioxide, immersion in magnesium salts, ethanol and electro-stunning (Fregin & Bickmeyer, 2016). Although these methods have been commonly used, not all are useful for purposes such as experimental trials or transportation, and some methods might also raise issues of animal welfare.

Clove oil, isolated from *Eugenia caryophyllata* L. Merr. & Perry (Myrtaceae), used to be widely utilized in dentistry for its antiseptic and analgesic properties (Chaieb et al., 2007). The active ingredient in clove oil is eugenol, but the oil also contains lesser amounts of other components such as β -caryophyllene and benzyl alcohol (Chaieb et al., 2007). Clove oil is restricted in its use on fish that are intended for human consumption despite the fact that it is safe for human use (Priborsky & Velisek, 2018). Nevertheless, some commercial products such as AQUI-STM (AQUI-S New Zealand) contain eugenol as a main constituent and are for fish use in some countries such as Australia, Chile, Finland, New Zealand and Faroe Islands but not in the EU and the USA (reviewed by Priborsky & Velisek, 2018).

In general, anaesthetic sedation techniques for crustaceans are not as developed as for fish and there is no legal requirement to administer anaesthetics for most procedures. However, there are suggestions that crustaceans respond to pain and distress, and thus, suitable anaesthetics will soon be required by granting agencies. Clove oil is an attractive anaesthetic to use because of its cost, safety and effectiveness in a number of crustaceans including the American lobster (*Homarus americanus*) (Waterstrat & Pinkham, 2005), the giant freshwater prawn (*Macrobrachium rosenbergii*) (Manush & Asimkumar, 2009) and *Macrobrachium tenellum* (Palomera, Zaragoza, Galván, & Vega-Villasante, 2016). There is limited knowledge on the effectiveness of clove oil on the Australian redclaw crayfish (*Cherax quadricarinatus*) and how factors such as sex and weight affect this effectiveness.

The Australian redclaw crayfish is a decapod endemic to Australia and Papua New Guinea. The popularity of the species in the aquarium and aquaculture trades has led to wide translocations (e.g. South Africa, South-East Asia, the United States, Mexico). It is attractive for commercial farming because of its physiological, biological and commercial attributes (Ghanawi & Saoud, 2012). Redclaw is a sexually dimorphic, eurythermal, mesohaline species that can grow rapidly, tolerate high stocking densities and can tolerate wide ranges of water quality conditions (e.g. pH, low oxygen levels). The aim of the present study was to evaluate the efficacy of clove oil as an anaesthetic for redclaw crayfish and assess how factors such as body weight and sex could affect this efficacy.

2 | MATERIALS AND METHODS

2.1 | Redclaw crayfish

The crayfish used for the experimental trials were obtained from stocks maintained at the aquatic animal facility at the American University of Beirut. The crayfish were manually size-sorted into

three weight range categories: small (<5 g), medium (5–12 g) and large (12–37 g). They were held in glass aquaria (52 L, 58 × 30 × 30 cm; L × W × H) for the duration of the trials. Water temperature in holding tanks was measured using a thermometer and maintained at approximately $21.8 \pm 0.5^\circ\text{C}$ using submerged heating elements. pH was maintained at 8.2 ± 0.2 and hardness at 974.7 ± 59.2 mg/L. Dissolved oxygen concentration and salinity were measured using a YSI Model 85 oxygen meter (Yellow Springs Inc.). Salinity was 2.4 ± 0.12 ppt, and dissolved oxygen concentration was 6.48 ± 1.84 mg/L. The crayfish were offered feed once daily to maintain good health and fasted 24 hr prior to the experiments. Sex was identified by observing the gonopore of each individual after anaesthesia but prior to placing in the recovery tank. Crayfish that were moulting or had only one cheliped or any other poor physical condition were excluded from the trials.

2.2 | Clove oil preparation

Clove oil (82%–87% eugenol) was purchased from Carolina Biological Supply Co., Burlington, NC, USA. The clove oil was dissolved in 96% ethanol at 1:10 v:v ratio. Nine concentrations were prepared from the stock solution of clove oil: 50, 100, 250, 375, 500, 600, 700, 750 and 1,000 $\mu\text{L/L}$. A pilot study was performed using the nine concentrations, and the minimum clove oil concentrations that were found to be effective were 375 and 500 $\mu\text{L/L}$. In the present manuscript, effective denotes causing anaesthesia in <10 min of exposure but not resulting in mortality after treatment. Low cost, ease of administration and human safety are not part of the definition. At concentrations <375 $\mu\text{L/L}$, most crayfish required more than 10 min to reach stage I anaesthesia, and at concentrations greater than 500 $\mu\text{L/L}$, too many animals died within 24 hr of treatment. Accordingly, 375 and 500 $\mu\text{L/L}$ were the two concentrations used for the actual experiment.

2.3 | Experimental design

The experimental set-up consisted of an induction glass tank (4 L of the anaesthetic solution), a recovery tank (5 L of fresh aerated water) and a balance to weigh the crayfish. Both the induction and recovery tanks were aerated using a submerged air diffuser to maintain an adequate DO level. A stopwatch was used to record the induction and recovery times as soon as the animal was placed in a solution. A plastic probe was used to prod the animal to assess its condition during anaesthesia and recovery stages.

The experiment was performed in June 2018 and then replicated with new animals in June 2019. Data from the two experiments were pooled and analysed together. Crayfish of each body weight category and sex were individually placed in one of the two concentrations (375 and 500 $\mu\text{L/L}$) of clove oil. Each crayfish was first placed in the anaesthetic bath (induction tank); then, the time to reach stages I and II was recorded. There were at least 10 replicate animals per treatment each year, and the treatments were anaesthetic concentration, size and sex. The stages of anaesthesia

were adapted from Vartak and Singh (2006) and defined as follows: stage I was when the animal exhibited partial equilibrium loss, some body movements and reduced reaction to external stimuli (when touched or prodded with a plastic probe), and stage II was the time from the end of stage I until the animal exhibited limited or no movements, complete loss of equilibrium (when turned over the animal could not upright itself) and little to no reaction to external stimuli. Recovery was then recorded in another tank (recovery tank) (5 L clean fresh aerated water) with no anaesthetic. Recovery I was when the animal started exhibiting some reactions to external stimuli (when touched with a plastic probe) but not started walking yet. Recovery II was from the end of recovery I until the animal started walking slowly and exhibited more movements and reactions to external stimuli. Each stage was timed separately so that statistical comparisons among individual stages were possible. The maximum exposure time to the anaesthetic was 10 min. If no change was observed after 10 min, the concentration was considered inefficient. Individual crayfish weight (g) was measured and sex was identified as soon as stage II was reached and before placing in recovery tank. At the end of the experiment, the crayfish were moved to a holding tank and monitored for 24 hr.

2.4 | Statistical analysis

One-way ANOVA was used to test for differences in induction and recovery times between the two tested clove oil concentrations. Data from both sexes and size classes were pooled for the statistical analysis. Interactions between treatments were not assessed. Because 500 µl/L was more effective than 375 µl/L, ANOVA was used to evaluate differences in induction and recovery times between male and female crayfish at 500 µl/L clove oil irrespective of size class. Finally, ANOVA was used to study differences in induction and recovery times of crayfish of various size classes at 500 µl/L irrespective of sex. The Student–Newman–Keuls means separation

test was used to identify differences among size classes. Significance level for all analyses was set at $p < .05$. All analyses were performed using SAS (V.9.2; SAS Institute Inc.).

3 | RESULTS

The induction and recovery times for stage 1 and 2 anaesthesia for two concentrations of clove oil are shown in Table 1. Induction times to stage I were significantly greater for clove oil concentration of 375 µl/L than for 500 µl/L (Table 1). There were no differences in recovery times of the crayfish between the two treatment concentrations. Induction times to stages I and II and recovery times to stages I and II for the large crayfish group were significantly greater than the induction times to stages I and II and recovery times for the smaller crayfish (Table 2). Although there appears to be an increasing trend in time of anaesthesia and recovery from small to large crayfish at 500 µl/L, the difference between small- and medium-sized crayfish was not significant. No significant differences were found in induction and recovery times between male and female crayfish anaesthetized at 500 µl/L clove oil (Table 3). Additionally, none of the experimental animals died within 24 hr of being exposed to the anaesthetic.

4 | DISCUSSION

Clove oil has the criteria of an ideal anaesthetic, which means rapid induction and recovery times, easy administration to the animal, low dose effectiveness, low cost and non-toxic to humans (Priborsky & Velisek, 2018). Results of the present study indicate that clove oil is an effective anaesthetic for redclaw crayfish as it was for other crustaceans including *Nephrops norvegicus* (Cowing, Powell, & Johnson, 2015) and grass shrimp (*Palaemonetes sinensis*) (Li, She, Han, Sun, Liu, & Li, 2018).

TABLE 1 Time (sec) required by *C. quadricarinatus* to reach stage I, stage II, recovery I and recovery II using two concentrations of clove oil anaesthetic. Results from crayfish in the various size classes and genders were pooled

Anaesthetic concentration (µl/L)	Time to stage I (sec) (mean ± SE)	Time to stage II (sec) (mean ± SE)	Time to recovery I (sec) (mean ± SE)	Time to recovery II (sec) (mean ± SE)
375 (n = 43)	493.02 ^a ± 22.28	321.97 ^a ± 26.35	289.55 ^a ± 18.80	285.58 ^a ± 22.30
500 (n = 41)	432.93 ^b ± 16.21	313.18 ^a ± 21.52	290.64 ^a ± 24.15	250.93 ^a ± 21.15

Note: Values (mean) with different superscript are not significantly different from each other.

TABLE 2 Time (sec) required by *C. quadricarinatus* from three size categories (small, medium, large) to reach stage I, stage II, recovery I and recovery II using clove oil concentration of 500 µl/L. Data from both sexes were pooled by size and analysed

Size	Time to stage I (sec) (mean ± SE)	Time to stage II (sec) (mean ± SE)	Time to recovery I (sec) (mean ± SE)	Time to recovery II (sec) (mean ± SE)
Small (n = 16)	345.06 ^b ± 27.75	238.07 ^b ± 34.47	273.63 ^b ± 28.87	202.50 ^b ± 31.29
Medium (n = 42)	475.07 ^a ± 17.15	316.05 ^{a,b} ± 31.67	250.92 ^b ± 19.28	280.37 ^{a,b} ± 21.17
Large (n = 26)	516.92 ^a ± 22.74	352.21 ^a ± 23.00	383.33 ^a ± 28.87	313.35 ^a ± 33.32

Note: Values (mean) in the same column with different superscript are significantly different from each other.

TABLE 3 Time (sec) required by male and female *C. quadricarinatus* to reach stage I, stage II, recovery I and recovery II at clove oil concentration of 500 µl/L. Data from all size classes were pooled and analysed

Sex	Time to stage I (sec)	Time to stage II (sec)	Time to recovery I (sec)	Time to recovery II (sec)
Female (n = 37)	487.39 ^a ± 22.83	318.67 ^a ± 27.91	292.79 ^a ± 23.75	275.39 ^a ± 24.20
Male (n = 34)	467.69 ^a ± 19.61	348.32 ^a ± 27.03	285.46 ^a ± 29.54	290.30 ^a ± 27.19

Note: Values (means) with the same superscript are not significantly different from each other.

The effectiveness of an anaesthetic depends on the dose and other factors such as body weight and gender (Ross & Ross, 2008; Santos et al., 2015; Li et al., 2018). Results of the present study indicated that clove oil concentrations of 375 and 500 µl/L induce rapid induction and recovery times, with 500 µl/L being the more effective. However, scientists or aquaculturists wanting to transport or handle redclaw for a long period might prefer low sedation and use lower concentrations even if those do not completely knock out the animal. Clove oil concentrations that are between 375 and 500 µl/L could be used for procedures that require full anaesthesia for short periods of time such as excessive handling or tagging (Cowing et al., 2015).

The effective concentration of an anaesthetic depends on the species being tested. Li et al. (2018) reported that concentrations of eugenol ranging from 100 to 500 µl/L were effective to anaesthetize the shrimp *P. sinensis* and also noted that the most effective and safe dose of eugenol that induced anaesthesia in *P. sinensis* was 200 µl/L. Vartak and Singh (2006) tested various clove oil concentrations in postlarval (15–125 mg/L) and juvenile (75–1,000 mg/L) freshwater prawn *Macrobrachium rosenbergii*. The authors concluded that clove oil was suitable only for postlarval prawn and a concentration of 15 mg/L was suitable for transportation of up to 3 hr. Clove oil has a density nearly equal to that of water, so 15 mg/L is practically equal to 15 µl/L, which is much less than the results of the present work suggest is necessary for redclaw. Parodi et al. (2012) reported that concentrations of 175 and 400 µl/L induced rapid and deep anaesthesia in postlarval and subadult white shrimp (*L. vannamei*), respectively, similar to the present findings.

Results of the present study indicated that induction and recovery times increased with the increase in crayfish size. The reason for the increased induction and recovery times might be related to the need of larger animals to consume less oxygen relative to their body size than smaller animals (Clarke & Johnston, 1999; Oikawa, Takeda, & Itazawa, 1994). As the uptake and elimination of clove oil or other anaesthetic agents is affected by rate of oxygen consumption, ratio of body volume to gill surface area and rate of gill perfusion, it is expected that body size would have an inverse relationship to anaesthetic efficiency (Oikawa & Itazawa, 1985; Zahl et al., 2012). Thus, in smaller crayfish the relative gill surface area is larger and anaesthetic absorption and elimination is faster than that for medium and larger crayfish. Similar results were reported for the grass shrimp *P. sinensis* (Li et al., 2018).

Factors such as lipid solubility, water solubility, degree of ionization, chemical stability and molecular weight have an effect of

how fast an anaesthetic agent is spread across the body of an animal (Hunn & Allen, 1974). Clove oil is highly lipophilic and thus absorbed easily by lipid-rich body tissues such as fat and brain (Summerfelt & Smith, 1990; Zahl et al., 2012). In fish, we expect that females accumulating fat for egg production might need less time to reach anaesthesia and have slower elimination of the anaesthetic or longer recovery times (Zahl et al., 2012). Mature redclaw females in the process of becoming gravid might be more easily anaesthetized by clove oil, but that is not something we observed in the present study. We did not see any differences in induction and recovery times between male and female crayfish, but also no females were obviously gravid or berried. Cowing et al. (2015) reported differences in induction and recovery times between berried and non-berried female *Nephrops*, and the authors attributed the differences to the absorption of eugenol by females that had lipid rich eggs. In conclusion, clove oil is an effective anaesthetic for redclaw (*C. quadricarinatus*) of various sizes and both genders. If complete anaesthesia is required, then we recommend using 500 µl/L. If light anaesthesia is needed for long transportation, then we believe a concentration of 100 µl/L is acceptable although we have not tested this concentration for a period longer than 15 min.

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