The anesthetic effect of different levels of clove essential oil on freshwater crayfish (*Astacus leptodactylus*)

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Abstract

Freshwater crayfish from the Aras Dam are considered one of the country's high-value fishery export resources. Freshwater crayfish are exported live at low temperatures. Limited studies have been conducted on the welfare and use of anesthetic agents in stressful situations such as handling, cooking, manipulation, sampling, and transportation. In the present study, the efficacy of 0, 50, 100, 200, 400, 800, 1200, and 1600 mg/L of clove essential oil (Pars Iman Daru, PI-222) as an anesthetic for freshwater crayfish (*Astacus leptodactylus*) was evaluated. Also, the duration of anesthesia induction and recovery time of freshwater crayfish were investigated with the aim of determining the appropriate anesthetic concentration for young crayfish weighing approximately 20 ± 3 grams. The results of the present study showed that the appropriate concentration of clove essential oil for anesthesia of freshwater crayfish in Aras Dam, considering the reduction in the time to reach stage 4 of anesthesia and also a significant reduction in the time to recover from anesthesia, was 1200 mg/L. According to the results of the present study, clove essential oil at a concentration of 1200 mg/L can be used as a safe and inexpensive anesthetic in freshwater crayfish for anesthesia and stress control in manipulations such as sampling and surgery.

Keywords: Anesthesia, Clove essential oil, Freshwater crayfish, *Astacus leptodactylus*

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Introduction

Freshwater crayfish is a species with economic export value in Iran whose exploitation has been limited to the extraction of water resources behind dams, especially the Aras Dam. In recent years, researchers have succeeded in artificially propagating and breeding it in in closed circuit systems, and according to the results, this species can be considered a cultivated species with high economic potential (Jalili and Nekouiefard, 2024). Therefore, it is necessary to conduct studies on the possibility of anesthesia with different substances in order to provide optimal welfare conditions during the process of harvesting, transporting, rearing, and sampling for laboratory studies, which can cause physical damage and physiological stress, and ultimately increase susceptibility to infections caused by pathogens or death (Roth and Skra, 2021; Zhu *et al.,* 2023). Anesthetics are classified into synthetic and natural types. Ethanol, MS222, and magnesium chloride are used as synthetic anesthetics in aquaculture. Clove oil, a natural anesthetic in aquaculture, is one of the most widely used aquatic anesthetics due to its efficacy, low toxicity risk, antibacterial properties, and antioxidant effects (Spandana *et al.,* 2018). Some of the common anesthetics used in aquatic animals include clove oil, tricaine methane sulfonate (MS222), 2-phenoxyethanol, benzocaine, and metomidate (Mirzargar and Seidger, 2023). The most commonly used fish anesthetic and the only one approved by the US Food and Drug Administration is MS222 (Popovic *et al.,* 2012). Although MS222 is highly effective in anesthetizing fish, it has been shown to be ineffective for most crustaceans. It also requires higher concentrations of anesthetic to anesthetize crustaceans compared to fish (Coyle *et al.,* 2004).

Clove oil, isolated from the clove plant (*Eugenia caryophyllata*), is widely used in dentistry due to its antiseptic and analgesic properties. The active ingredient in clove oil is eugenol, but it also contains smaller amounts of other compounds such as β-caryophyllene and benzyl alcohol (Chaieb *et al.,* 2007). Clove oil is an attractive anesthetic for use due to its cost, safety, and effectiveness in a number of crustaceans, including American lobster (*Homarus americanus*) (Waterstrat and Pinkham, 2005), giant freshwater shrimp (*Macrobrachium rosenbergii*) (Manush and Asimkumar, 2009), and *Macrobrachium tenellum* (Aréchiga-Palomera *et al.,* 2016).

To date, there has been no study on the effectiveness of clove oil on *Astacus leptodactylus*. Assessment of the effectiveness of an anesthetic involves observing behaviors such as active locomotion and aggression (Cowing *et al.,* 2015). For example, clove oil induces struggle in cuttlefish. However, it limits their control over arm movements, while magnesium chloride induces erratic behavior (Yang *et al.,* 2020). The stages of anesthesia in crustaceans are different from those in fish and include several stages (Table 1).

Table 1: Stages of crustacean anesthesia (based on De Souza Valente, 2022).

Number	Stage/ Definition	Physiological and behavioral symptoms
1	Un anesthetized	Responsive to stimuli and awake, Normal behavior, Normal body orientation
2	stage I-Sedation	Partial loss of equilibrium, Reduced mobility, Responsive to stimuli,
3	Stage II- Initial anesthesia	Loss of equilibrium, Lack of limb withdraw, Slow antennae withdraw, weak response to stimuli, Loss of defense behavior
4	StageIII- Surgical anesthesia	Unconsciousness, Unresponsive to stimuli, Immobility (including limb and antennae), Relaxation of abdominal flaps and chelae, Preserved scaphognathite pumping
5	StageIV- Nervous System depression	Respiratory arrest, Cardiac arrest, Potential death

Despite extensive research on the effects of various anesthetics, including clove essential oil, on various species of farmed fish, there is little information on freshwater crayfish anesthesia. This study aimed to evaluate the effect of different concentrations of clove essential oil based on determining factors such as anesthesia induction duration, recovery time from anesthesia, and freshwater crayfish behavior in terms of anesthesia and ensuring the welfare of freshwater crayfish during manipulation and induction of stressful conditions.

Materials and methods

300 freshwater crayfish with an average weight of 20±3 grams were collected from the Aras Reservoir Lake. The freshwater crayfish were transferred to the Aquaculture and Breeding Laboratory of the National Artemia Research Center in a unolith and near ice (Nekouiefard, 2016). Freshwater crayfish transferred to the laboratory were stored in 1000-liter polyethylene tanks and fed with commercial shrimp feed (Faradaneh Company) at a rate of 1% of body weight daily during storage (Mazlum *et al.,* 2011). In this study, clove essential oil with the trade name PI222, a product of the Pars Iman Darou Herbal Medicine Development Knowledge-Based Group, was used. This study was conducted in 8 experimental groups with 3 replications. 144 freshwater freshwater crayfish weighing (20±3 g) were selected after 24 hours of starvation and transferred to plastic containers containing different concentrations of clove essential oil: 0, 50, 100, 200, 400, 800, 1200, and 1600 mg/L at a density of 6 individuals per tank. The time it took for the freshwater crayfish to reach different stages of anesthesia, according to their appearance and behavioral characteristics, as well as the time they recovered from anesthesia, was recorded separately with a stopwatch based on Table 1, and the average of each repetition was calculated (De Souza Valente, 2022). The weight and total length of crayfish were determined using a digital scale with an accuracy of 0.1 g and a ruler with an accuracy of 1 mm (Seidgar *et al.,* 2022). During the experiment, the water temperature range (25±2°C) and dissolved oxygen (7.0±0.2

mg/L) were maintained constant by an aquarium heater and aeration.

Statistical analysis

Before performing ANOVA, the normality of the data was checked using the Kolmogorov-Smirnov test. If the data were not normal, they were transformed. One-way analysis of variance was used to analyze normal data, and after the significance of ANOVA, Duncan's test was used to compare the means of different experimental groups. The minimum significance level of the tests was $p<0.05$ and the data was reported as mean±standard deviation. SPSS version 20 software was used to perform statistical analyses and Excel version 20 software was used to draw graphs.

Results

The results showed that low concentrations of 50 and 100 mg/L of clove essential oil had no significant effect on *A. leptodactylus* anesthesia (Unanesthetized). The duration of different stages of anesthesia and recovery from anesthesia for different concentrations of clove essential oil (PI222) on freshwater crayfish is shown in Table 2. The results showed that the duration of reaching stages I and II of anesthesia increased significantly with increasing clove essential oil concentration from 200 to 800 mg/L ($p<0.05$). However, there was no significant difference between the experimental groups of 800, 1200 and 1600 mg/L ($p>0.05$). The time to reach stage III and IV of anesthesia increased significantly among the experimental groups with increasing clove essential oil concentration from 200 to 1200 mg/L ($p<0.05$). However, there was no significant difference between the experimental groups of 1200 and 1600 mg/L ($p>0.05$).

Table 2: Time of different stages of anesthesia and recovery from anesthesia (minutes) for different amounts of clove essential oil (PI222) on freshwater crayfish of Aras Dam.

Anesthesia stage (minutes)	Different levels of clove essential oil (mg/liter)				
	200	400	800	1200	1600
I	45.00±4.45^{a}	11.67±2.08^{b}	6.17±0.47^{c}	4.13±0.15^{c}	3.43±0.40^{c}
II	53.67±5.51^{a}	21.67±3.06^{b}	10.0±2.00^{c}	5.84±0.37^{c}	4.78±0.15^{c}
III	132.3±11.68^{a}	32.33±2.52^{b}	21.33±1.53^{c}	7.65±1.07^{d}	6.83±0.76^{d}
IV	315.0±22.91^{a}	106.6±10.41^{b}	54.67±4.16^{c}	21.67±3.21^{d}	17.23±1.66^{d}

The values presented in the table represent the mean ± SD. Numbers in a column with different letters are significantly different at the $p<0.05$ level.

The results of recovery time from anesthesia are shown in Figure 1. The recovery time from anesthesia at 200 and 400 mg/L of anesthetic was significantly longer ($p<0.05$) compared to 1200 mg/L. This could be due to the longer duration of exposure to anesthetic and reaching stage III of anesthesia. Also, the recovery time from anesthesia was significantly longer at a concentration of 1600 mg/L compared to a concentration of 1200 mg/L, which could be due to the high concentration of anesthetic.

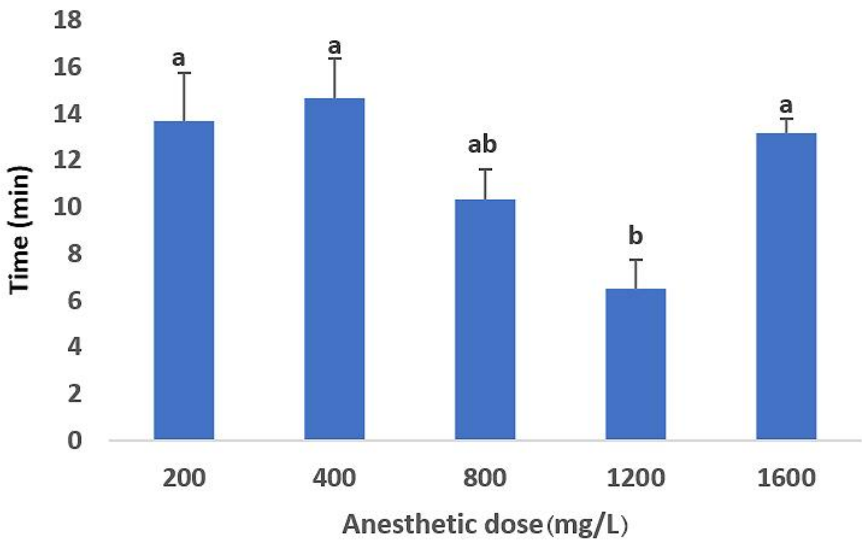

Figure 1: Recovery time from anesthesia in freshwater crayfish using different amounts of clove essential oil (PI222). Different letters indicate statistically significant differences between experimental groups at *p*<0.05.

No significant differences were observed among the other experimental groups (concentrations of 200, 200, 800, and 1200 mg/L).

Discussion and conclusion

Anesthetics are important in inducing relaxation of aquatic animals and reducing stress during transport and handling. To date, few studies have been conducted on the effects of anesthetics in freshwater crayfish, especially, *Astacus leptodactylus*. Previous studies have mostly used clove oil, MS222, ethanol, and magnesium chloride to anesthetize crustaceans such as blue crabs, crayfish, gammarids, Pacific white shrimp, and European lobsters (Stanley *et al.,* 2020; Perrot-Minnot *et al.,* 2021; De Souza Valente, 2022; Rotllant *et al.,* 2023). Also, the results of previous studies showed that the survival rate in crabs exposed to MS222 and magnesium chloride was lower compared to clove oil and ethanol. Also, MS222 and magnesium chloride are not considered effective anesthetics in crabs (Zhu *et al.,* 2023). Clove essential oil meets the criteria of an ideal anesthetic, which means rapid induction and recovery times, easy administration to the animal, low dose efficacy, low cost, and non-toxicity to humans (Velisek and Priborsky, 2018). The results indicate that clove essential oil is an effective anesthetic for freshwater crayfish, as it has been effective for other crustaceans including red claw crabs (Ghanawi *et al.,* 2019), *Nephrops norvegicus* (Cowing *et al.,* 2015), and the grass shrimp *Palaemonetes sinensis* (Li *et al.,* 2018).

Clove essential oil (*E. caryophyllus*) contains a mixture of different compounds, the main active compounds of which are the phenolic eugenol (85-95%) and isoeugenol and methyl eugenol (5-15%) (West *et al.,* 2007). The anesthetic effects of eugenol have been reported in Daphnia (Bownik, 2015, 2016), amphipods (Venarsky and Wilhelm, 2006; Perrot-Minnot *et al.,* 2021), pinniped shrimp (Soltani *et al.,* 2004; Huang *et al.,* 2008; Parodi *et al.,* 2012; Matulovic and Oshiro, 2016; Wycoff *et al.,* 2018), caridean shrimp (Coyle *et al.,* 2005; Aréchiga-Palomera *et al.,* 2016; Li *et al.,* 2018), spiny lobsters (Waterstrat and Pinkham, 2005; Huntsberger, 2012; Cowing *et al.,* 2015), freshwater crayfish (Ozeki, 1975; McRae *et al.,* 1999; Ghanawi *et al.,* 2019), spiny lobsters, hermit crabs, and crabs (Rotllant *et al.,* 2023).

Previous studies have shown that several factors influence the efficacy of eugenol as an anesthetic in various animal species, including crustaceans. These factors include eugenol concentration, animal characteristics (growth stage, size, sex, maturity), and environmental conditions (temperature, salinity). And with increasing eugenol concentration, the time required for complete anesthesia in all crustacean species studied is significantly reduced and recovery time is increased (Rotllant *et al.,* 2023). The effectiveness of an anesthetic depends on the dose and other factors such as body weight and gender (Mirzargar and Seidger, 2023). The results of the study by Ghanawi *et al*. (2019) showed that concentrations of 375 and 500 µl/L of clove essential oil induced anesthesia and accelerated recovery time in red crabs. This was compared to 1200 mg/L in freshwater crayfish *Astacus Leptodactylus*. However, researchers or farmers who want to transport or handle freshwater crayfish for long periods of time may prefer minimal sedation and use lower concentrations (Cowing *et al.,* 2015). The effective concentration of an anesthetic depends on the species being tested. Li *et al.* (2018) reported that eugenol concentrations ranging from 100 to 500 µL/L were effective in anesthetizing *P. sinensis* shrimp and also noted that the most effective and safest dose of eugenol that caused anesthesia in *P. sinensis* was 200 µL/L.

Vartak and Singh (2006) tested different concentrations of clove oil on post larvae (15-125 mg/L) and young freshwater shrimp (*Macrobrachium rosenbergii*) (75-1000 mg/L) and concluded that clove oil was only suitable for post larvae of shrimp and a concentration of 15 mg/L was the appropriate dose for transport within 3 hours.

Parodi *et al*. (2012) reported that concentrations of 175 and 400 µL/L of clove oil caused rapid and profound anesthesia in post larval and juvenile white shrimp (*L. vannamei*), respectively, suggesting that higher concentrations are required for anesthesia of freshwater crayfish than shrimp. The results of Ghanawi *et al.* (2019) showed that the induction and recovery time from anesthesia increased with increasing size of red claw crabs. The reason for the increased induction and recovery time from anesthesia may be related to the fact that larger animals require less oxygen consumption relative to their body size compared to smaller animals. Since the absorption and elimination of clove oil or other anesthetic agents is influenced by oxygen consumption, the ratio of body volume to gill surface area, and the rate of gill blood flow, body size is expected to be inversely related to anesthetic efficiency. Therefore, in smaller crabs, the relative gill surface area is larger and the absorption and elimination of anesthetic is faster than in medium and larger crabs. Similar results have been reported for the herbivorous shrimp *P. sinensis* (Li *et al*., 2018; Ghanawi *et al.,* 2019). From a physiological perspective, behavioral testing is a valuable tool for assessing animal adaptation to rearing conditions and assessing stress levels (Zhu *et al.,* 2023). For fish, high concentrations of anesthetics increase movement distance and speed due to stimulation of the skin, gills, and gustatory-olfactory receptors (Aydin and Orhan, 2021). It has been shown that crabs, when anesthetized with ethanol, move farther and faster and show more frantic behavior than clove oil, which can be associated with limb amputation and indicate a higher level of stress. Therefore, clove oil can be considered a more desirable anesthetic compared to ethanol in crustaceans (Zhu *et al.,* 2023). In the present study, considering the optimal and appropriate time range for reaching stage III anesthesia and recovery from anesthesia at a concentration of 1200 mg/L of clove essential oil, this concentration was found to be the most appropriate concentration for anesthesia of freshwater crayfish (*Astacus leptodactylus*) with an average weight of 20 g for severe manipulation, marking, or laboratory sampling. However, if the goal is light anesthesia for transportation, lower doses are recommended in proportion to the distance of transportation.

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Conflict of interest
The authors declare that there are no conflicts of interest regarding the publication of this
paper.

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