

Effects of clove oil (eugenol) on proprioceptive neurons, heart rate, and behavior in model crustaceans

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Clove oil contains eugenol as an active ingredient and is used as a topical anesthetic in mammals to remedy pain and to anesthetize fish and other seafood for short periods; however, the exact mechanism of action of eugenol is not fully understood. We examined use of eugenol as a reversible anesthetic in crustaceans by examining its effect on sensory and motor neurons in the Red Swamp crayfish (*Procambarus clarkii*), Blue crab (*Callinectes sapidus*) and Whiteleg shrimp (*Litopenaeus vannamei*) with electrophysiological recordings. The neurogenic heart rate in the three species was also monitored along with behaviors and responsiveness to sensory stimuli. The activity of the primary proprioceptive neurons was reduced at 200 ppm and ceased at 400 ppm for both crayfish (i.e., muscle receptor organ) and crab (i.e., leg PD organ) preparations when exposed to saline containing eugenol. Flushing out eugenol resulted in recovery in the majority of the preparations within five to ten minutes. Administering eugenol to crayfish and crabs both systemically and through environmental exposure resulted in the animals becoming lethargic. Direct injection into the hemolymph was quicker to decrease reflexes and sensory perception, but heart rate was still maintained. Eugenol at a circulating level of 400 ppm decreased electromyogram activity in the claw muscle of crabs. Surprisingly, this study found no change in heart rate despite administering eugenol into the hemolymph to reach 400 ppm in crabs or crayfish but heart rate in shrimp preparations decreased. Our results demonstrate the feasibility of eugenol as a short-term anesthetic for crustaceans to decrease stress during handling or transportation, considering its effectiveness at decreasing sensory input and the quick recovery of upon removal of eugenol. A neurophysiology course took this project on as an authentic course-based undergraduate research experience (ACURE).

Abbreviations: ASC-acid sensitive stretch activated channels; CO-chordotonal organs; MRO-muscle receptor organ; PD-propodite-dactylopodite joint; SACs-stretch activated ion channels; SARs-stretch activated receptors; sec-second.

Keywords: Proprioception, sensory, invertebrate, pharmacology, clove oil, eugenol

Introduction

Clove Oil

Clove oil has been used for many home remedial therapies, such as aiding toothache pain, eliminating acne, reducing

gum disease, and improving blood circulation. Given that eugenol is the active ingredient of clove oil (Markowitz et. al, 1992) and that the mechanism of action is not readily known, we chose to study more on the potential mechanisms of action by using

crustaceans. In addition, eugenol is already used in humans for use in relieving dental pain, without noticeable problems (Park et al., 2006). We examined if eugenol might be a means to anesthetize edible crustaceans and have them recover and potentially serve other purposes in transport or surgical needs. A number of studies to date mention ways to anesthetize crustaceans but lack direct measures of neural activity of other physiological measures such as cardiac function, sensory activity and sensory to motor reflexes. In this study, we used these various physiological measure to examine the effects of eugenol.

To anesthetize edible crustaceans for more immediate human consumption, a series of different anesthetizing to euthanizing protocols was recently examined using lobster and crayfish while monitoring neural activity (Fregin and Bickmeyer, 2016). Traditional methods with exposure to chilling, heating, carbon dioxide, MgCl₂ and electric stunning were studied, and it was demonstrated that slow heating the environment or boiling the animals was the quickest means to silence neural activity. However, in such conditions, the animals would undergo permanent neurological damage, which is not ideal in all situations. Temporarily paralyzing crustaceans does not imply they are anesthetized but rather that they cannot respond with observable behaviors to external stimuli. Prior studies chilling crayfish and lobsters to 0 °C indicated that cardiac function can be depressed but some crustaceans (crayfish) continued to show an alteration in the heart rate to sensory stimulation or direct neuronal activity (Bierbower and Cooper, 2010, 2013; Chung et al., 2012; Fregin and Bickmeyer, 2016). The exposure to CO₂ not only blocks synaptic transmission at the neuromuscular junction in crustaceans and insects, but also severely reduces the body pH (Badre et al.,

2005; Bierbower and Cooper, 2010, 2013). Blocking synaptic transmission at the glutamatergic neuromuscular junction produces paralytic responses. In addition, CO₂ can stop cardiac function in crustaceans and insects which is assumed to be due to block of gap junctions in the heart induced by the low pH, but sensory and CNS activity remain functional (Badre et al., 2005; Bierbower and Cooper, 2010; Cooper et al., 2009). Thus, direct measures of neuronal function are required to avoid a misunderstanding in the anesthetization of an animal. A better understanding in the actions of the anesthetizing agents can occur with direct sensory nerve recordings as well as sensory-CNS-motor and sensory-CNS-cardiac measures.

Eugenol is commonly used to anesthetize fish for placing monitoring tags or surgical purposes (Javahery et al., 2012). These surgical procedures in crustaceans include implanting electrodes for monitoring biological functions such as heart rate (HR), ventilatory rate (VR), electromyograms (EMGs) (Cooper et al., 1998; Listerman et al., 2000; Shuranova et al., 2003) or removing limbs for studying regeneration (Cooper, 1998). For example, when implanting electrodes in shore crabs, *Carcinus maenas*, the HR appears high for up to three days after handling stress (Wilkens et al., 1985). In order to study some species, transport to a research facility is necessary; however, the resulting stress can sometimes compromise the animal (Cooper and Cooper, 2004). Some crustaceans are very sensitive to stressors, such as cave crustaceans (Li and Cooper, 2001; Kellie et al., 2001) and shrimp, alike to mammals, possess an autonomic-like nervous system to survive in the wild. (Shuranova et al., 2006). A light anesthetic to be used in transportation of the animals might help to prevent social aggression and damage to the crustacean, which is used for human

food consumption or for research purposes (Coyle et al., 2004; Schapker et al., 2002). In some conditions, a live healthy crustacean specimen is needed to display to customers prior to being processed (Aram et al., 1999). Therefore, finding an effective anesthetic would be beneficial to the commercial industry as well as research purposes.

With cultured mammalian cells and nerve recordings in rodents, eugenol may lead to the inactivation of voltage gated sodium channels and calcium channels. This action could account for lessening pain and decreased overall neural activity (Ohkubo and Kitamura, 1997; Huang et al., 2012; Seo et al., 2013; Lee et al., 2015). The actions of eugenol on invertebrates has not been extensively studied, but a few key studies with a land snail (*Caucasotachea atrolabiata*) and crayfish (*Procambarus clarkii*) suggest eugenol acts via a dose dependent blockage of voltage gated sodium and calcium channels with excitation of neurons also found at certain dosages (Ozeki 1975; Vatanparast et al., 2017). We examined the effects of eugenol on various physiological measures (heart rate-HR, ventilatory rate-VR, and electromyogram-EMG) and stimuli induced changes in HR as well as behavioral responses (eye withdrawal and tail flip) for three different crustaceans commonly used for food as well as for research purposes. The Red Swamp crayfish (*Procambarus clarkii*), Blue crab (*Callinectes sapidus*) and Whiteleg shrimp (*Litopenaeus vannamei*) each have different natural environments and are commercially important for aquaculture and fishers. Thus, examining the effect of eugenol on basic physiological functions is important to understand, particularly as it is a common technique used on edible crustaceans in industry.

Primary Sensory Function

Crab legs contain sensory structures that monitor joint movements. These chordotonal organs (CO) offer unique properties as the sensory endings are embedded in an elastic strand with cell bodies and endings that are relatively exposed when the preparation is dissected and placed in a saline bath. In this study, we focused on the propodite-dactylopodite (PD) chordotonal organ, as it is the most readily accessible of the chordotonal organs within the leg and the stimulus is easier to be reproduced among each preparation (Dayaram et al., 2017). In addition, some neurons within the PD organ are dynamically sensitive and only fire during the initial displacement. Other neurons are static position-sensitive and are recruited at various displacement positions. The static position-sensitive neurons show a mild accommodation over time (Hartman and Boettiger, 1967; Cooper and Hartman, 1999; Cooper 2008; Dayaram et al., 2017). Thus, the effects of eugenol on the two different neuronal types can be easily investigated. The crayfish muscle receptor organ (MRO) is more complex, as the sensory endings are embedded within muscle fibers. When the muscle fibers are stretched as the abdomen flexes or extends, the sensory endings are displayed and open stretch activated ion channels (Kuffler 1954). The cell bodies and axons are well exposed in the dissected preparations to compounds during exchange of the bathing saline (Cooper et al., 2003). There are two types of sensory neurons, each associated to their own distinct muscle fiber. One MRO is referred to as the rapidly adapting receptor and the other, the slow adapting neuron (see Rydqvist et al., 2007 for a review).

These two organs (PD and MRO) provide proprioceptive input to the animals similar to muscle spindles for mammals (Whitear 1960; Burgess et al., 1982; Bewick

and Banks 2015). The motor axons in these animal models also have the same basic properties as those in all animals (Hodgkin and Huxley, 1952; Atwood 1982), so they too can serve as a model for nerve activity in vertebrates as well as humans with exposure to eugenol. Many pharmacological agents such as sodium channel blockers (TTX) and potassium blockers (4-AP, TEA) work just as effectively in crustaceans as in mammals (Huang et al., 1990; Lin 2012, 2013).

Heart Rate and EMG Measures

In addition to using primary sensory function to assess how eugenol may work, a neural circuit that alters the heart rate was used as another bioindex. By stimulating the animals with tactical or visual input, there is normally a response detected in a change of the HR. The hearts of these three crustaceans are driven by neural control (i.e., neurogenic) allowing a sensory-CNS-cardiac neural circuit to be driven before and during exposure to eugenol to examine if central control on the heart is compromised (Alexandrowicz, 1932; Yamagishi and Hirose, 1997; Yamagishi et al., 1997; Wilkens, 1985). The known sympathetic-like response in defense posturing increases in HR and VR for crustaceans has been studied extensively (Bethe, 1897; Huxley, 1880; McMahan, 1995; Miyazaki et al., 1985; Shuranova et al., 2006; Wiersma, 1961). It was shown that crayfish and lobster rapidly increase their HR with defense posturing or a tactile touch or even slight vibratory disturbances in the surrounding water environment (Listerman et al., 2000; Yazawa and Katsuyama, 2001).

In considering motor units and the potential effect of eugenol on neuromuscular control, the EMGs of the closer muscle within the chelipeds in crabs and crayfish were monitored. The activity of the closer muscle is readily obtained with EMG

recordings as it is the largest muscle in the chelipeds and easily accessible for monitoring. In addition, there is a known sensory-CNS-motor reflex by stimulating the “teeth” of the cheliped with tactile stimuli resulting in closing of the claw (Eckert 1959; Wiens and Gerstein, 1976).

We postulated that the heart rates of these animals, as well as the motor and sensory activity within the limbs of the animals would cease when exposed to eugenol. We also predicted cessation of any sensory-CNS-cardiac or sensory-CNS-motor to closer muscle circuit activity, which would result in the animal becoming lethargic. The purpose of this study was to examine the effects of eugenol on primary sensory neurons as well as reflexes in the intact animal, which affect HR and muscle activity. It was also of interest if the effects of eugenol on the neurons and whole animal could be reversed.

Methods

Animals

Experiments were performed using Red Swamp crayfish (*Procambarus clarkia*; Atchafalaya Biological Supply, Raceland, LA, USA), Blue crab (*Callinectes sapidus*; food distribution center Atlanta, GA, delivered to and bought from Yu Yu Asian Supermarket in Lexington, KY, USA), and Whiteleg shrimp (*Litopenaeus vannamei*; Aquaculture Research Center, Frankfort, KY, USA). Six animals were used in each condition.

Throughout the study, mid-sized crayfish measuring 6-10 cm in body length and 12.5-25 g in body weight were used. Each animal was housed in individual standardized plastic containers with weekly exchanged dry fish food and oxygenated water (20-21°C).

To optimize the health of the Blue crab, they were accommodated in a seawater aquarium prior to use for three to five days. All experiments were implemented in female adults with a carapace width (from point to point) of 10-15 cm and a body weight of 140-225 g. The crabs were fed with frozen squid and the water temperature was maintained between 20-21°C. These crabs were caught from the wild and most likely were two to three years old.

Shrimp at the Kentucky State University were housed prior to experiments for several months in oxygenized water at 21-22°C and fed with commercial fish food pellets (salinity 15.2 ppt; O₂ at 7.35-7.7 mg/l). For detoxification of ammonium ions, bacteria and algae were cultured in the holding tanks (McRae et al., 1999).

Studies in Belize used six shrimp raised in outdoor open ponds in a large-scale aquaculture farm, Belize Aquaculture Ltd. They were transferred to an open window laboratory ranging in water temperatures from 30-31 °C. Shrimp with a postorbital carapace length of 20-35 mm were used from Kentucky State University and 25-38 mm from Belize.

Electrophysiological recordings of proprioceptive sensory nerves

Procedure for dissection and preparation of the crab PD organ can be found in detail in Dayaram et al. (2017). Briefly, the blue crabs were obtained and checked for response to stimuli prior to autotomizing the first or second leg and placing it in a Sylgard-lined dish with crab saline. The PD nerve was then exposed and pulled into a suction electrode for recording. During the experiment, the dactyl was moved from a flexed position to an open position and then released. The movements were evoked with a wooden dowel to displace the segments with rates of movement at 1s. This

was repeated after a rest period with another movement of 1s from a flexed position to an extended position and held in the extended position statically for at least 10s. The number of neurons producing spikes was used to calculate firing frequency. The saline used was the accepted composition, described earlier (Majeed et al., 2013; Leksrisawat et al., 2010). Crab saline: solution (in mM: 470 NaCl, 7.9 KCl, 15.0 CaCl₂·2H₂O, 6.98 MgCl₂·6H₂O, 11.0 dextrose, 5 HEPES acid and 5 HEPES base adjusted to pH 7.4). All bathing and experimental solutions were kept at the experimental room temperature of 21°C.

Details of the crayfish MRO preparation can be found in Dayaram et al. (2017) and Leksrisawat et al. (2010). The dissected crayfish abdomen was placed in a Sylgard-lined dish filled with crayfish saline. The MRO was moved using a wooden dowel from a relaxed position to a stretched position. An insect dissecting pin was used to mark the displacement range, and each displacement was marked on the computer recording file. The displacement rates were the same as for the crab PD organ. The crayfish saline was a modified Van Harreveld's solution (in mM: 205 NaCl, 5.3 KCl, 13.5 CaCl₂·2H₂O, 2.45 MgCl₂·6H₂O, and 5 HEPES adjusted to pH 7.4). The concentrations of eugenol for the various preparations are stated in the Results.

Heart rate and muscle activity: HR & EMG

For electrophysiological experiments, electromyograms (EMG) and electrocardiograms (ECG) were obtained. The preparation of the recording wires consisted of insulated stainless steel wires (diameter 0.005 inches/0.008 inches with coating; A-M Systems, Carlsburg, WA). The insulation was burned off the ends with a flame to provide a good connection with the recording devices.

To obtain an optimal HR measure, two holes were formed on the dorsal carapace directly over the heart using a fine-point scalpel. Small holes, just the thickness of the wires, cause minimal loss of hemolymph and a higher probability of the wires remaining in place during fixation. The insulated steel wires were placed into the carapace, spanning the heart to facilitate an accurate impedance measure (UFI, model 2991; Listerman et al., 2001). To eliminate the risk of damaging internal organs, special attention was made to insert only a short portion of wire (1-2 mm). After placing the wire in the optimal position, the fixation was ensured via a small drop of glue (cyanoacrylate ester) and accelerator (HobbyTown USA, Lexington, KY). The advantage of using fast drying glue is the reduction of stress in the animal, since especially shrimp seem to be very susceptible to die during the placement of recording wires. However, only small volumes of accelerator were utilized, as it is toxic and can result in death. The impedance detector, which measures the dynamic resistance between the two wires, was linked to a PowerLab/4SP interface (AD Instruments) and calibrated with the PowerLab Chart software version 5.5.6 (AD Instruments, Australia). The acquisition rate was set on 10 kHz. The calculation of the HR was accomplished by direct counts of each beat over 15s intervals and transformed onto beats per minute (BPM).

For an optimal EMG signal, the insulation (~0.5 mm) was removed exposing the tip of the wires. These were inserted into holes made in the cuticle on one or the other chelipeds. The wires spanned the closer muscle in a rostral-caudal arrangement. A third wire located in the carpopodite region of the same limb served as a ground (Bradacs et al., 1997; Cooper et al., 1998). Similar to the ECG, the holes in the cuticle were formed and wires prepared, inserted, and fixed in the

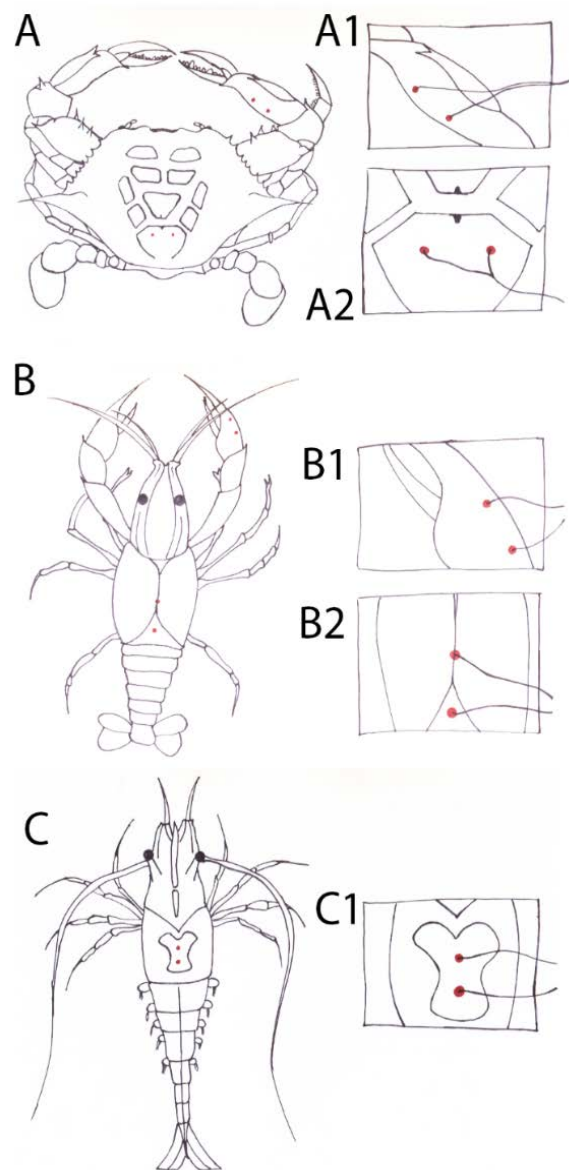


Figure 1: Placement of recording leads for measuring heart rate in an electrocardiogram (ECG) and skeletal muscle activity in an electromyogram (EMG) for the crab (A), crayfish (B) and shrimp (C). The 2 differential EMG leads to record the EMG activity of the closer muscle in the chela were placed ventrally in the propodite segment. A third lead, not shown, was placed under the cuticle in any of the more proximal segments to serve as a ground lead (A1). The ECG leads for the crab spanned the heart laterally for best ratio in signal to noise recordings (A2). As for the EMG recordings in the crab, similar lead placements were made as for the crayfish (B1). The ECG leads for the crayfish were placed in an anterior-posterior arrangement for obtaining the best signals (B2). Only ECG recordings were obtained in the shrimp and a similar placement of the leads as for the crayfish were made (C1).

respective area spanning the closer muscle in its central region. A Grass AC preamplifier (P15; Grass Instruments) amplified the potentials, which were acquired digitally with a PowerLab/4SP interface (AD Instruments) and calibrated with the PowerLab Chart software version 5.5.6 (AD Instruments, Australia) to measure HR. To elicit responses in the EMG signal, the crabs and crayfish were teased using a wooden pencil placed near the chelipeds for the animals to clamp down on the object. The placement of the ECG and EMG leads used are depicted in Figure 1.

Eugenol

The eugenol in this experiment came from a stock solution. This solution was diluted down to 200 ppm and 400 ppm for use in the experiments. These concentrations were deemed the low and high dose of eugenol, respectively.

Behaviors

Visual observational studies were made for crayfish and shrimp when the tail was taped with a glass rod with minimal visual disturbance of the animals. The behaviors to note were if the animal showed a tail flip or some responsiveness such as trying to move away from the stimulus. An animal unaffected by eugenol would always show this responsiveness to the stimuli. In addition, a slight touch on the eye was made for crayfish, shrimp, and crabs to note if an eyestalk withdrawal occurred. If the animal was unable to display an escape behavior or an eyestalk withdrawal, it was deemed unresponsive.

The responsiveness of the sensory-CNS-cardiac ganglion circuit on HR was also assessed by using a wooden pencil to tap on the dorsal carapace between the eyes of the three species. A note was made on the recording file for HR when any stimulus

occurred so it could be correlated to with a response in the HR. If no change in HR occurred simultaneously with the stimulus, then the ganglion circuit was deemed unresponsive.

Statistical analysis

All data are expressed as an average value along with the standard error of the mean (i.e., \pm SEM). The rank sum pairwise test or a sign test was used to compare the differences in HR or EMG activity of a behavior before and after exchanging saline with the solution containing compounds. This analysis was performed with Sigma Stat software version 13.1. Probability of ≤ 0.05 is considered as statistically significant.

Results

Crab proprioceptor

The PD proprioceptive sensory organ of the distal joint in the walking legs detects the dynamic movements and static positions of the PD joint (Figure 2). The rapid 1s displacement from 90-degrees to 0-degrees produces a high frequency and recruitment of various neurons producing different amplitude spikes in the nerve recording (Figure 2, left panel). With this 90-degree bend on the extracellular nerve, spikes are easily recorded. The return movement is not measured with precise timing, as it is just to reset the joint into the position for the next displacement. To measure the responses from the static position sensitive neurons the joint is again rapidly moved, within 1s, from the 90-degree to the 0-degree and held for 10s at the 0-degree position (Figure 2, right panel). Representative recordings are shown for the 1s and 10s paradigms during saline exposure and the responses after 2 min of exposure to eugenol (400 ppm) as well as after three exchanges of the bath to fresh saline.

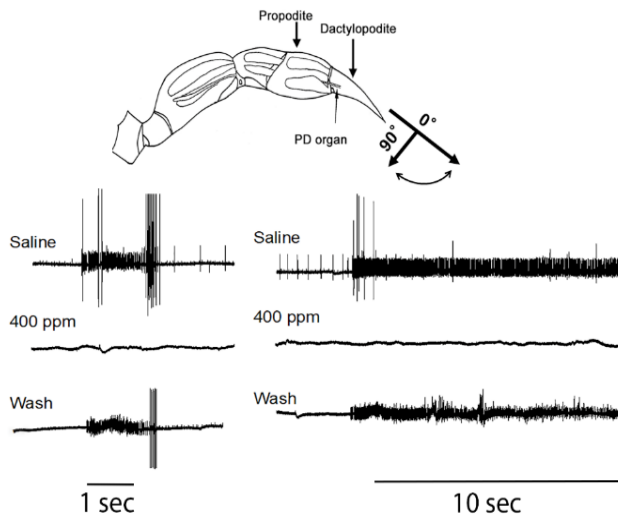


Figure 2: The anatomical location and range of displacements with representative neural activity for the PD organ in the crab walking leg. An insect dissecting pin is placed at the 0-degree location so the distal segment is moved to the same position. Rates of displacement for the crab joint were 1s and 10s, with the joint held in a static position for 10s. The joint was held initially at 90-degree and fully extended to 0-degree. Representative firing activity while bathed in saline and saline with 400 ppm eugenol followed by washing the recording dish three times with fresh saline. Note that the neural activity is completely silenced when exposed to 400 ppm eugenol. Anatomical drawing is the same shown in Dayaram et al. (2017).

In examining the effect of a low and high concentration of eugenol, a 200 ppm and a 400 ppm was tested on the exposed PD organ. Exchanging the bathing saline with saline containing 200 ppm eugenol and waiting 2 min decreased the neural responses. Some preparations had one or two spikes and others had higher activity during the 1 or 10s movement paradigms. To avoid any long-term damage to the preparations from exposure to eugenol, the saline bath is rapidly exchanged three times with fresh saline. After a few minutes, the responses began to return for the displacements. The timing of the responses with repetitive movements during saline, eugenol (200 ppm) and saline wash out applications for one preparation is shown in Figure 3 (top panel). Each preparation varied in time to regain function

with repetitive movements after returning to fresh saline, but all preparations showed some activity within 5 min (Figure 3; $N = 7$, $p < 0.05$ non-parametric sign test). The 200 ppm exposure for 2 min did not silence the activity in each preparation but did decrease activity in varying magnitudes for the 1s and 10s paradigms.

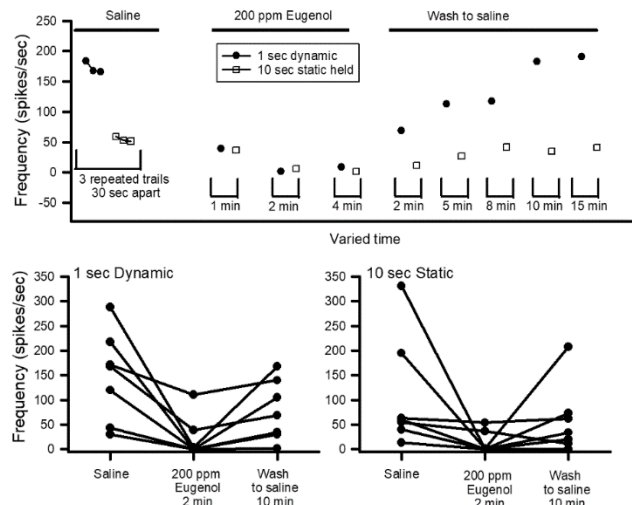


Figure 3: Neural activity of the PD nerve for joint displacements over time while exposed to eugenol followed by recovery of activity from washing the preparation with fresh saline. The spike frequency for 1s displacements and the activity over the 10s when the joint is held at 0-degrees are plotted together for each of the representative times. Three repetitive trials were conducted in saline and then the saline in the recording dish was exchanged with saline containing 200 ppm eugenol. After 4 min, the bathing solution was exchanged with three washes of fresh saline and the displacements trials were repeated. Quantitative measures made after 2 min of eugenol exposure and after 10 min following washing the recording dish with fresh saline were used for each of the seven preparations for both the 1s displacement and for the 10s while the joint is held at 0-degrees for 10s. Note that 200 ppm eugenol depressed activity more so in some preparations.

The 400 ppm exposure silenced the neural activity of both the dynamic sensitive neurons and the static sensitive neurons within 2 min for each preparation (Figure 4 top panel; $N=6$, $p<0.05$ Non-parametric sign test). In comparing the effects of the 200 ppm and 400 ppm exposures, the average percent change in activity from saline to eugenol

exposure at the 2 min exposure time are shown for both the 1s and 10s stimulation paradigms (Figure 4, bottom panel). A percent change is used for comparisons as each preparation produces a different initial frequency of spike activity in saline.

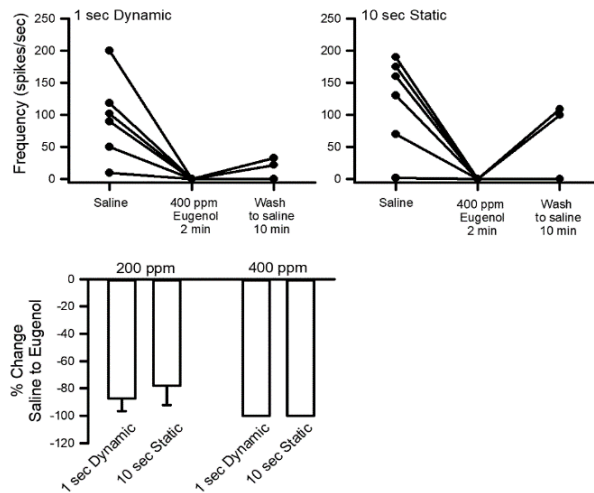


Figure 4: Neural activity of the PD nerve for joint displacements over time while exposed to eugenol at 400 ppm followed by recovery of activity from washing the preparation with fresh saline. The spike frequency for 1s displacements (top left panel) and the activity over the 10s (top right panel) when the joint is held at 0-degrees are plotted together for each of the representative times. Quantitative measures made after 2 min of eugenol exposure and after 10 min following washing the recording dish with fresh saline were used for each of the seven preparations for both the 1s displacement and for the 10s while the joint is held at 0-degrees for 10 sec (Bottom panel). Note that 200 ppm eugenol depressed activity to about 80 percent of the starting activity where 400 ppm essentially silenced the activity for 1s displacement and the 10s static held position.

Crayfish Proprioceptor

The neurons of crayfish proprioception in the abdomen, like that of the PD organ in the crab, are fully exposed to the bathing saline and produce spikes in the extracellular nerve recording upon bending of the joint in which each neuron monitors. Since the segmental nerve only measures the two neurons (i.e., a dynamic movement sensitive one and a static sensitive one), fewer variations in the amplitudes of spikes are recorded for the MRO preparations as compared to the crab PD organ (Figure 5). The two sensory neurons have their sensory endings embedded within a single associated muscle fiber for each neuron. The muscle fibers span the joint in which they monitor, and they reside directly under the dorsal cuticle. From a ventral view, looking dorsally, they reside between the DEL1 muscle fibers and the cuticle (Figure 5, top panel). With the joint being moved rapidly in 1s to a set position or rapidly moved in 1s and held for 10s, both types of neurons are able to be monitored. Because both 200 ppm and 400 ppm produced reliable responses with the crab PD organ, the same concentrations were examined for the crayfish preparations. Representative responses are shown for both dynamic sensitive neuron and static sensitive neuron prior to eugenol exposure, during exposure (400 ppm) and after three washes of the preparations with fresh saline (Figure 5, bottom left and right panels).

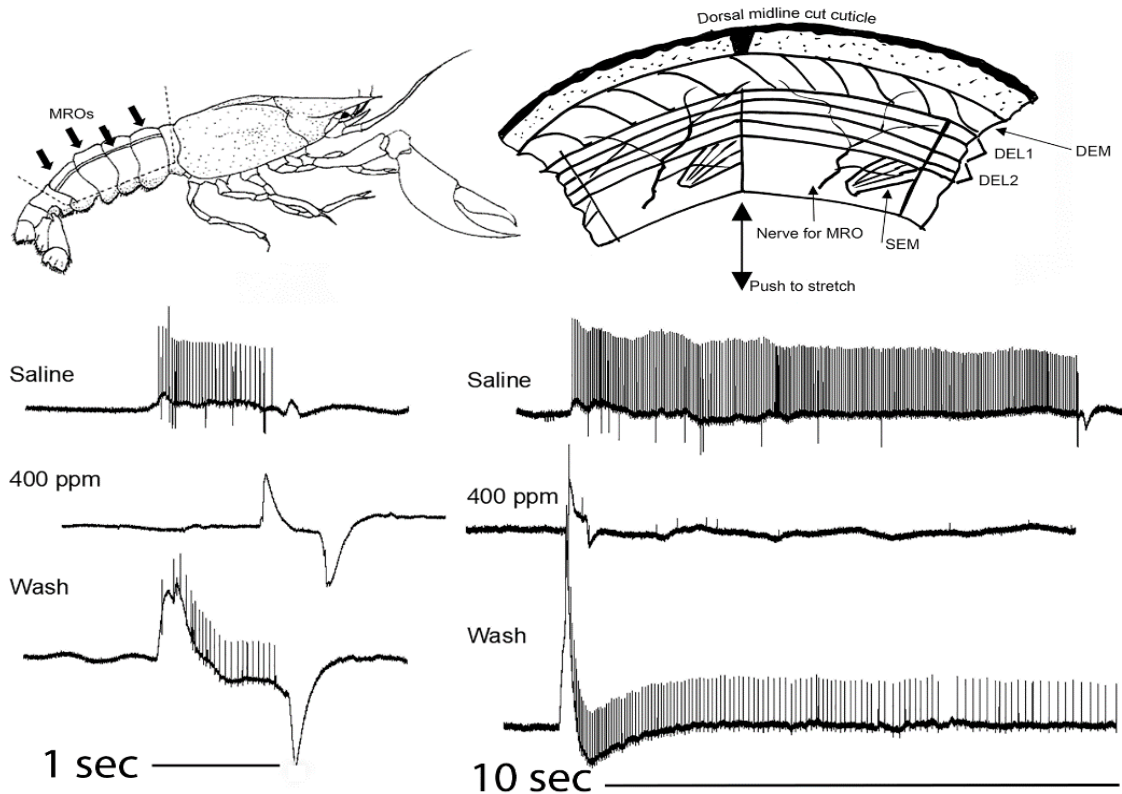


Figure 5: The anatomical location and range of displacements with representative neural activity for the MRO organ in the crayfish abdomen. An insect dissecting pin was placed at a set location for maximal displacement of bending the joint to the same position. Rates of displacement for the segment were 1s and 10s held in a static position. The joint was held initially at an extended position and then bent to the maximal flexion. Representative firing activity while bathed in saline and saline with 400 ppm eugenol was followed by washing the recording dish three times with fresh saline. Note that the neural activity is depressed when exposed to 400 ppm eugenol. The large movements in the baseline were due to recording artifacts in movements of the cuticle touching the suction electrode which does not impede measuring the spike activity. Anatomical drawing is the same shown in Dayaram et al. (2017). The particular muscles identified: deep extensor medial (DEM) muscles have a spiral fiber pattern; DEL1 is the first lateral group followed by the DEL2 muscles; the superficial extensor medial muscle (SEM) lies directly dorsal to DEL2. The two MRO muscles are more dorsal to the DEL1.

As for the PD organs of the crab, exposure to 400 ppm on the MRO preparations completely silenced the neural activity of both neuronal types within 2 min for 5 preparations for the 1s movements. One preparation still had minimal activity (i.e., 7 spikes) during the eugenol exposure; however, it was the one preparation with the most robust activity prior to eugenol exposure as compared to the other preparations (Figure 6, left panel).

All preparations demonstrated a substantial reduction in activity during for the 1s paradigm and complete inactivity during the 10s static held position ($N=6$, $p<0.05$ Non-parametric sign test). Every preparation showed a pronounced recovery with exchanging the bathing saline 3 times with fresh saline and repetitive movements over a 10 min period.

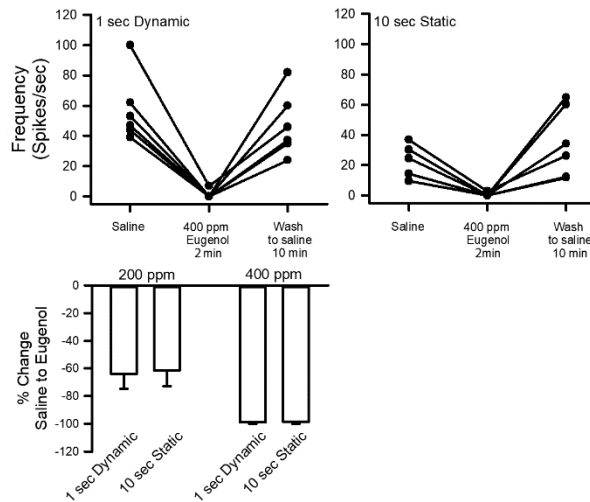


Figure 6: Neural activity of the MRO for joint displacements over time while exposed to eugenol at 400 ppm (top) followed by recovery of activity from washing the preparation with fresh saline. The spike frequency for 1s displacements (left panel) and the activity over the static position for 10s (right panel) when the joint is held in an extended position. Quantitative measures made for both 200 ppm and 400 ppm after 2 min of eugenol exposure and after 10 min following washing the recording dish with fresh saline were used for each of the six preparations during both displacement paradigms (bottom). Note that both 200 and 400 ppm eugenol substantially depressed activity in all preparations.

Heart rate and skeletal muscle activity with systemic eugenol injections

Direct exposure of sensory neurons to eugenol in a recording dish produced a rapid effect. To examine whether similar effects on neurons occurred within the animal upon a systemic injection, the EMG activity of the closer muscle in the large chela of the crab and crayfish was monitored. Since the hearts in all three species are neurogenic, we used the heart rate as an additional bioindex to the effect of eugenol. To reduce the number of animals needed to be examined each one was fitted with leads to monitor EMG as well as ECG activity as shown in Figure 1. The systemic concentration was estimated to be 400 ppm from a bolus injection of a concentrated stock into the open circulation. The hemolymph was rapidly exchanged

throughout the body in all three species with high heart rates and giving the injection over a 30s period. The EMG and ECG measures were made intermittently for each species. Given the differing conditions and locations in which the measures were made (i.e., a teaching lab with a set class time and an aquaculture facility) as well as the stress of the shrimp to handling the precise periods used for analysis were not the same for each species, but they are comparable.

The signals measured from the crab heart are more robust than for the crayfish or shrimp. This was likely due to the larger and stronger heart tissue producing a stronger signal. As shown in crayfish (Schapker et al., 2002; Shuranova et al., 2006) a tactile stimulus (i.e. a tap with wooden rod) on the carapace between eye stalks produces a pause in the heart rate which is usually followed by an increase in the rate for a few minutes. This is apparent in Figure 7 in a representative ECG trace of the crab prior to eugenol injection. The EMG recording of the closer muscle showed pronounced responses when the animal grips on the wooden rod and produces a gripping force. Rubbing on the teeth of the chela produced the reflexive action to the motor neurons to produce the gripping response. Thus, a sensory-CNS-motor circuit was recruited by the rubbing action on the teeth (Eckert 1959; Wiens and

Gerstein, 1976). The EMG activity is also very apparent in the representative trace shown in Figure 7 for a crab prior to eugenol injection. After 20 min post-injection, the heart rate was still robust with some alteration because of tactical stimulation. However, the EMG signals were dampened and the crabs in general do not pinch the rod

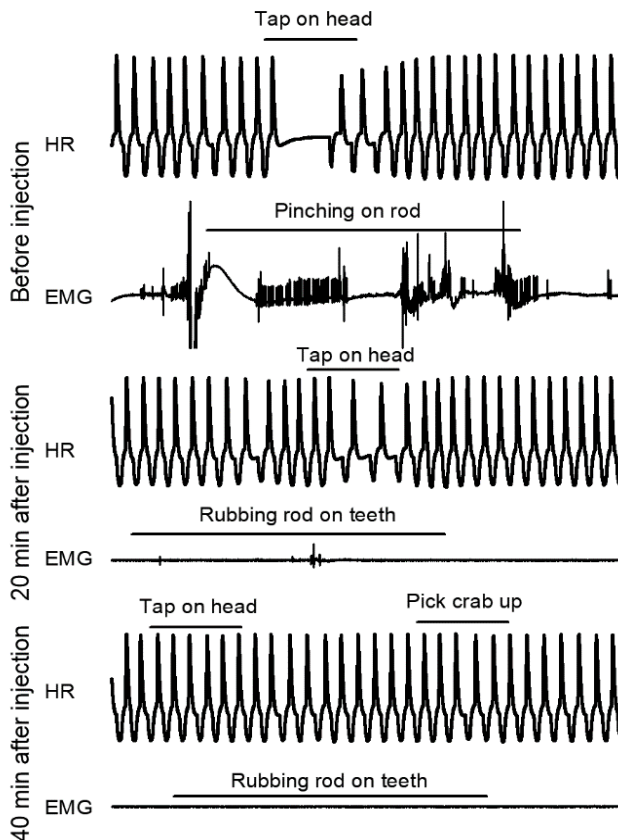


Figure 7: Representative heart rate (HR) and skeletal muscle activity (EMG) obtained in a crab before and during exposure to eugenol from systemic injection (~400 ppm estimated circulating concentration) over time. Heart rate normally shows a slight pause in a beat or two when the animal is tapped with a glass rod on the dorsal carapace (top panel). The pause was a result of activity from a sensory-CNS-cardiac ganglion reflex, which was dampened over time with exposure to eugenol at 20 min and 40 min. The activity in the closer muscle was stimulated to occur by placing a small wooden rod in the jaws of the chela and rubbing on the teeth. This stimulated a sensory-CNS-motor nerve circuit, which was also dampened over time from exposure to eugenol at 20 min and 40 min. Note the crab did not alter HR with being picked up out of the tank after 40 min of exposure. This was the same time point the animal is lethargic with dangling limbs when picked up. Each trace was a recording over a 20s period.

with force. After 40 min, the ECG is still strong and all five animals tested produced minor if any alteration in the ECG traces with the same tactile stimulation. This was not the case for the EMG activity with rubbing on the

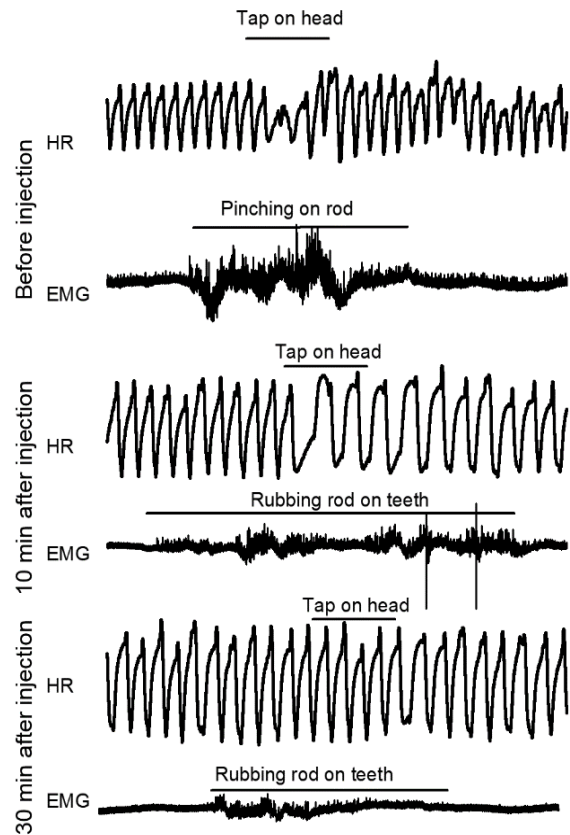


Figure 8: Representative heart rate (HR) and skeletal muscle activity (EMG) obtained in a crayfish before and during exposure to eugenol from systemic injection (~400 ppm estimated circulating concentration) over time. Heart rate normally shows a slight pause in a beat or two when the animal was tapped with a glass rod on the dorsal carapace (top panel). The pause was a result of activity from a sensory-CNS-cardiac ganglion reflex, which was dampened over time with exposure to eugenol at 10 min and 30 min. The activity in the closer muscle was stimulated to occur by placing a small wooden rod in the jaws of the chela and rubbing on the teeth. This simulated a sensory-CNS-motor nerve circuit, which is also dampened over time from exposure to eugenol at 10 min and 30 min. This particular crayfish depicted was the only one, which displaced some EMG activity after 30 min and was shown to illustrate the activity was substantially dampened in background activity before rubbing on the teeth as well as during stimulation as compared to prior eugenol injection. After 30 min, the animal was lethargic with dangling limbs when picked up. Each trace was a recording over a 20s period.

teeth of the chela. All the animals appeared to be in a passive stance with the chela closed and unresponsive to prodding in trying to open the chela to place the rod. Upon rubbing the teeth none of the five crabs produced activity which could be detected (N=5, statistical significant at $p < 0.05$ Non-parametric sign test). The crabs were lethargic after 40 min, as they did not move when prodded and when picked up out of the water, their limbs dangled without any movement.

The eyes were also touched to look for an eyestalk withdrawal, of which the animal showed no obvious response. In close visual observation of the scaphognathite in the prebranchial chamber, which serves as a ventilatory organ, was still moving and appeared to function in producing a gill current (Schapker et al., 2002). The crabs were left in fresh seawater and checked for survival and responsiveness after 24 hours. They all survived and were as active as freshly obtained crabs. Most had pulled off the EMG leads or mangled the leads indicating their restored activity levels.

The crayfish showed a similar trend as the crabs in maintaining cardiac function after an estimated 400 ppm systemic exposure to eugenol. Also like the crabs, the responsiveness to a tactile stimulation was reduced after prolonged exposure (Figure 8, at 30 min). The EMG activity was reduced after 10 min and was completely absent in four of the five crayfish tested after 30 min. The one that showed a slight responsiveness in the EMG with rubbing on the teeth of the chela is shown in Figure 8 (bottom trace). Given that all 5 crayfish had substantially reduced EMG activity after 30 min and that the animals were also lethargic when picked up, the systemic injection had a significant effect on crayfish (N=5, $p < 0.05$ Non-parametric sign test). Since there was a reduction in EMG induced activity from a

sensory-CNS-motor reflex induced by rubbing on the teeth, it may be expected that the ECG would not show alterations with the tap on the carapace. However, the HR was robust all through the 30 min of monitoring after eugenol injection (Figure 9). Movements of the scaphognathite are more easily seen in the crayfish as compared to the crabs. It was still moving as for the crabs even after the 30 min exposure when the limbs were limp. Three crayfish were examined for eyestalk withdrawal with touch to the tip of the eye and no responsiveness was observed. All the crayfish were alive after 24 hours although not as lively in reaching pre-injection activity as for the crabs. They appeared to have some residual effect of the eugenol exposure but were able to raise their chela and walk around in their holding tanks.

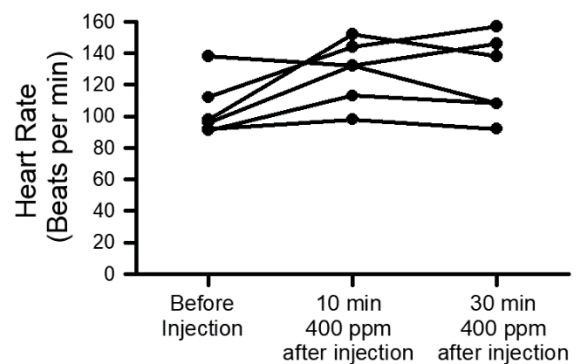


Figure 9: The heart rate (HR) recorded in crayfish before and at two times after systemic injection of eugenol. Five of the six crayfish injected showed an increase in HR after 10 min of injection and three of the six still had higher rates 40 min post-injection. Only one of six crayfish demonstrated a reduction in HR after 30 min from the time before the eugenol injection.

The shrimp proved to be more of a challenge to monitor HR as the ECG traces showed more fluctuation and artifacts due to the movements of the shrimp. This was likely due to the fact the shrimp have large swimmerets as compared to the crayfish and beat them continuously while being held in the recording chambers. The beating swimmerets resulted in body movements of the shrimp and baseline movements of the

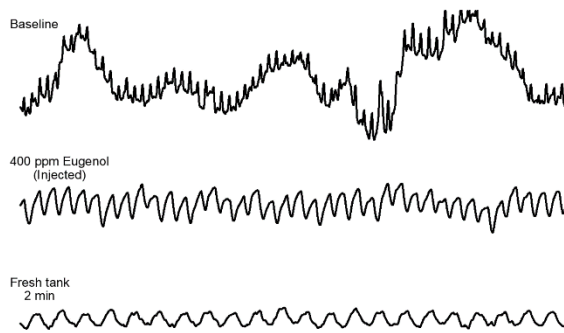


Figure 10: Representative heart rate (HR) obtained in a shrimp before and during exposure to eugenol from systemic injection (~400 ppm estimated circulating concentration) over time. Shrimp displayed lethargic behavior as quick as 2 min after injection. The HR after 2 min of eugenol injection showed a reduction. In some cases, the rates continued to decrease after the animals were placed in another holding tank with freshly exchanged sea water. Each trace was recorded over a 20s period.

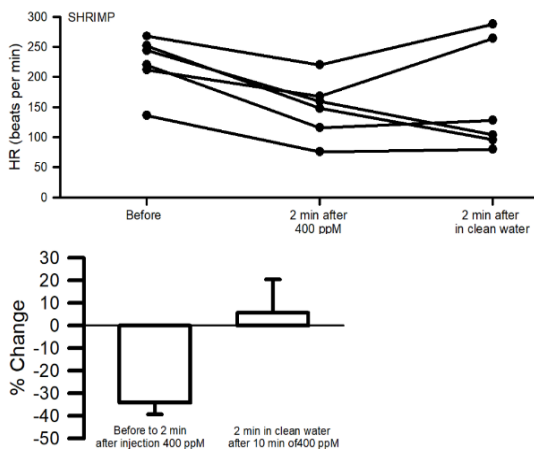


Figure 11: Heart rates (HR) obtained for six shrimp before, during exposure to eugenol from systemic injection and after being placed in new holding tank with clean seawater. Shrimp displayed lethargic behavior as quick as 2 min after injection. The HR after 2 min of eugenol injection showed a reduction in all six-shrimp tested. In two of the six cases, the rates increased after the animals were placed in a new tank with fresh sea water. Two of the six continued to decrease substantially. On average there is about a 35% reduction of HR after two min without any significant trends after being placed in clean water, as there was substantial variation among the animals.

ECG trace; however, the individual heart beat cycles are readily visible (Figure 10). The shrimp appear to be more susceptible to handling stress than the crabs or crayfish as during the placement of the lead for recording the HR some shrimp died. The shrimp also did not appear to show any pauses in the ECG to tactile stimulation on the carapace. This may be due to the high stress already occurring in the animals, as basal rates were in the range of 150 to 250 beats a min (Figure 11). The high basal HR may also be the reason each animal examined showed a reduction in rate after 2 min post-injection of an estimated 400 ppm circulating eugenol (N=6, $p < 0.05$ Non-parametric sign test). Since the shrimp were so susceptible to stress and given their HR were so high, they were only monitored for 2 min prior to being placed in a fresh container. They were placed in a fresh container in order to avoid re-exposing them to eugenol from excretion or diffusion out of the gills. In three shrimp, the HR increased again and in two, the rates continued to drop with one not showing any change in the recovery tank. In the following 5 to 10 min of monitoring, the shrimp did not die. They were not monitored individually over the next 24 hours due the leads impeding their need to swim. The scaphognathite in the shrimp are easily seen through the transparent cuticle. This organ continued to beat the entire time while exposed to eugenol. However, swimmeret activity stopped completely within the 2 min post-injection as well as any effort to tail flip when pinched on the telson. Due the very narrow and thin chela of the shrimp as well as their high stress of being handled, EMG activity of the closer muscle was not attempted. In preliminary studies in Belize with Whiteleg shrimp (*Litopenaeus vannamei*), two animals were bathed in 200 ppm eugenol and were inactive within 20 minutes (30.5 °C). Upon being placed back in holding tanks overnight with fresh seawater they recovered fully. At the

Aquaculture facility at Kentucky State University (21-22°C) shrimp were inactive to tail pinches within 6 to 12 min (inactive times were 6, 10.36, 10.5, 10, 12, 12 min) when bathed in 200 ppm seawater and when kept in the eugenol bath for 1 hour all six died.

Discussion

In this study, we reported the effect of direct exposure of eugenol on various neurological responses in model crab, crayfish, and shrimp species. We predicted eugenol exposure would result in a cessation or dampening of heart rate, motor nerve activity, and sensory nerve activity. Additionally, we expected the animals to exhibit a lethargic behavior due to a cessation of the sensory-CNS-cardiac or sensory-CNS-motor circuit. Our experiments found a reversible decrease in neural activity at both 200 and 400 ppm. With a wash of fresh saline, both the crab PD organ and crayfish MRO returned to normal function. However, 400 ppm of eugenol failed to cause any decrease in HR in any animal, contrary to our predictions. While the stress of handling the animals may have caused the HR to increase, the eugenol still failed to cause a significant decrease in HR, even though the animals' hearts were neurogenic.

An exposure of 200 ppm had varied effects with some preparations showing a marked depression of neural activity while others a moderate effect. At 400 ppm, neural activity was basically silenced within 2 min of exposure but the responses were able to be recovered in 5 to 10 minutes upon removal of eugenol and exchanging the bathing solution a least three times with fresh saline.

Systemic injections within the hemolymph of crab and crayfish to an estimated 400 ppm resulted in decreased skeletal muscle activity and an inactivity within 30 to 40 minutes. Shrimp, however,

showed reduced swimmeret activity within 2 minutes of injection along with a decreased HR. Shrimp were more active during basal conditions and had a higher heart rate prior to injection of eugenol than crab and crayfish. Additionally, HR was not altered with the induction of a sensory-CNS-heart reflex during eugenol exposure, suggesting a dampened sensory input. Crabs, crayfish and shrimp maintained a high cardiac function despite the loss of limb function and sensory-CNS-motor neuron or sensory-CNS-cardiac drive. Only shrimp showed a slight reduction in HR with eugenol exposure. In addition, all three species maintained ventilatory function with the beating of scaphognathites during eugenol exposure while limb skeletal muscles were not active.

Both types of proprioceptors, the crayfish MRO and crab PD organ had rapid decreased activity; however, the sensory endings of the MRO are embedded within muscle fibers whereas the PD organ are embedded in a collagen-elastin chordotonal strand. The effect of eugenol may influence relaxation of the muscle fibers differently than effects on the chordotonal strand. The movements of the joints could be sensed differently for the transduction on the stretch-activated channels on the sensory endings. If the main mechanism of action of eugenol is on the electrical conduction or production of action potentials in the neurons, the differences in subtypes of the stretch activated channels might not be of concern.

Considering the saline as well as the hemolymph for the marine crab and shrimp is a much higher osmolarity, almost twice, as compared to the saline and hemolymph of the freshwater crayfish, there may be differences in the solubility and dissociation of eugenol. The interaction of salts with eugenol is not known. All our studies were around room temperature of 21-22 °C except for studies in Belize where the room temperature was 30.5

°C in a research shelter. The volatile nature of eugenol would likely penetrate tissue more readily in warm temperatures but also it may be excreted more rapidly from the body. Considering the metabolic activity differences in the heterothermic crustaceans, the degree and duration in the effects of eugenol on the animal would likely be different under different environmental temperatures. This could be addressed in future studies, investigating the duration and effects of eugenol in differing environments. For example, the temperature the animals are kept in during long-distance transportation versus the temperature in a crustacean-rearing facility.

With systemic injection of eugenol into the open circulation of these crustaceans, the binding on tissues and absorption into the various tissues likely reduces the free level of eugenol circulating. The levels of eugenol detected in muscle of shrimp (*Litopenaeus vannamei*) were examined, and the extent to which the circulating levels were decreased would depend on the total mass for any given individual (Li et al., 2016). However, the effects of dampening the rapid sensory-CNS-motor nerve reflex of the chela closing for crab and crayfish within 5 to 10 min would indicate the paralyzing effects are present. The lethargic nature of the shrimp within 2 min suggest a quicker action on neural tissue in this animal.

Longer exposure or maintaining the animals in the same small holding tanks with excreted eugenol re-circulating may result in a longer suppression of activity and eventually depress of the ventilatory and cardiac function. Excretion rates of eugenol in crustaceans has not been determined. More thorough studies of dosages and excretion rates have been conducted in fish (Kildea et al., 2004; Zhao et al., 2017). The short exposures used in our study were sufficient to allow the animals to recover over a short

term. Exposure of eugenol at 600 and 900 $\mu\text{L/L}$ in the aquaria for *Nephrops norvegicus* (i.e., Norway lobster) produced anesthesia in about 7 min and 5 min respectively with a rapid recovery in about 10 min and these studies were conducted in cold water (Cowing, et al., 2015). The animals in our study were not followed for longer periods to know if long-term consequences occurred due to the exposure; however, further investigation could be performed to determine any potential long-term consequences. As with various species of fish, the dosage of eugenol to reach an effective level of anesthesia will vary for different species of crustaceans (Coyle et al., 2004; Zhao et al., 2017).

Eugenol appears to be relatively safe at low levels in mammals as well as for fish although there may be side effects if overused and nerves are silenced for long periods. When off target tissues are compromised, the side effects can be varied. A high dosage can decrease liver function and be potentially lethal (Hartnoll et al., 1993). At the level that produced decreased sensory responsiveness in the three crustaceans used in this study, the heart and ventilatory organs were still functional indicating that not all the nerves were silenced under these conditions. The muscles used to beat the scaphognathites are innervated by several motor nerves and were apparently not depressed, and nor is neurotransmission at these glutamatergic neuromuscular junctions. Likewise, the hearts in these crustaceans are neurogenic and require neural input to contract (McMahon, 1995; Ransdell et al., 2013). There are various speculations to why these neurons were spared from a more protective glial sheath wrapping to variation in ionic channels present on this motor neurons as compared to those of the limb muscles or sensory neurons. Additionally, since these motor neurons are driven by a central pattern

generator or the cardiac pacemaker ganglion possibly there is a feedback homeostatic regulation to increase the motor nerve activity to keep these essential organs functional. Until direct measures of the nerves are made or isolated preparations can be studied, this phenomenon remains unresolved.

The approach of injection of eugenol to induce rapid anesthesia of crabs and crayfish for experimental purposes such as placing tags or reducing stress for transport appears to be feasible as sensory input is reduced, while the anesthetic effect is completely reversible. Since shrimp are easily stressed, a slight cooling of the water and then injection of eugenol might be an approach to reduce handling stress. Cooling may even result in a lower concentration of eugenol to have an effect in reducing neural activity for crabs and crayfish. The precise mechanism of action in reducing neuronal activity is not fully addressed but results to date indicate a blockage of voltage-gating sodium and calcium channels (Ozeki 1975; Vatanparast et al., 2017). However, it would be of interest to examine directly whether there is an effect of eugenol on stretch activated channels used for proprioception and mechanical deformation of sensory endings as well as differing sensitivities on sensory and motor neurons.

Endnotes

Many of the authors were students in a neurophysiology lab based class who addressed authentic scientific based questions in regards to the topic of examining how eugenol would affect sensory and central nervous system functions. This course project is part of a new trend in teaching science to undergraduates (Linn et al., 2015). Course-based undergraduate research experiences (CUREs) are relatively new and an approach being adopted by science

educators in high schools and colleges (Bakshi et al., 2016). Our hope is to present a new acronym for authentic course-based undergraduate research experience (ACURE).

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