

The Effects of Eugenol as an Anesthetic for an Insect: *Drosophila*, Adults, Larval Heart Rate, and Synaptic Transmission

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The examination of the active ingredient in clove oil, eugenol, as an anesthetic for use on *Drosophila melanogaster* was performed. *Drosophila melanogaster* adult flies were anesthetized with a simple flow through apparatus and they recovered to perform behavioral tests without any defects. Larvae did not become completely anesthetized even with longer exposure periods than adults; however, they did show reduced body wall movements and mouth hook movements. Application of 100ppm in physiological saline directly on the larval heart and neuromuscular junction reduced the heart rate and evoked synaptic transmission. No effect on the postsynaptic glutamate receptors was observed. It is likely eugenol blocks ionic sodium and calcium channels directly and can readily be reversed without any long term consequences in function. Thus, eugenol may serve as an alternative to CO₂ or cold as an anesthetic.

Keywords: *Drosophila*, anesthesia, neuromuscular junction, behavior, larva, heart rate, cardiac

Introduction

There is a need for an easily accessible, effective anesthetic for insects that gives rise to no long-term effects or damage. Insects are used in a number of experimental paradigms which, in some cases, involve sedating them for some time and preparing them for experimentation. Currently, various approaches are used, from exposure to carbon dioxide (CO₂), cold temperature, or specialty products such as FlyNap. There are concerns with these approaches for short and long term effects depending on the experimental conditions. CO₂ is the most commonly used approach to rapidly (i.e., a few seconds) paralyze and if needed, recover insects for later use (Badre *et al.* 2005). However, it is established that behaviors in adult *Drosophila melanogaster*, such as flying and climbing, can be affected for up to a week after a single CO₂ exposure (Bartholomew *et al.* 2015). There is a need to rapidly anaesthetize insects and have them recover without long term consequences. We address the potential use of eugenol vapors as a potential form of anesthesia for *D. melanogaster*.

Anesthesia by CO₂ and cold exposure requires a continuous exposure without it the animals will awaken quickly. The drastic drop in blood and cellular pH induced by the commonly used 100 % CO₂ exposure could have long term cellular consequences (Badre *et al.* 2005; Bierbower and Cooper, 2010). CO₂ can rapidly result in acidity of blood/hemolymph and the cytoplasm in cells from the reaction (CO₂+H₂O↔H₂CO₃ ↔ HCO₃⁻+H⁺) (Stone and Koopowitz, 1974). The enzyme carbonic anhydrase in cells rapidly catalyzes this reaction which is the probable reason intracellular pH drops so quickly (Baker and Honerjager, 1978). It is likely the low pH blocks the gap junctions in the insect and crustacean hearts which accounts for the cessation of heart rate upon exposure to CO₂ (Badre *et al.* 2005). The paralytic actions of CO₂ are not due to the lowered pH but from the blockage of the glutamatergic synapses at the neuromuscular junctions (NMJ) (Badre *et al.* 2005; Bierbower and Cooper, 2010).

As for cold exposure in the context of inducing a chill coma, one has to be careful to not damage tissue with freezing. The issue with adult flies and use of cold is due to moisture on the edges of the vials or surgical plate becoming wet with condensation. The moisture results in

the wings of the flies sticking to the surfaces. A walk-in cold room that is close to freezing and contains a dehumidifier could solve this issue; however, some may personally prefer not to work in these conditions. Also, a walk-in in cold room is not likely feasible for a classroom setting. Anesthesia by either CO₂ or chilling significantly delayed the time that it takes adults to start copulating (Barron, 2000). Insects use rapid cold hardening as a cellular mechanism for a quick response to cold allowing animals to survive longer bouts of cold (Lee *et al.* 1987). In fruit flies and flesh flies (commonly referred to as blow flies), this mechanism has been studied extensively (see review-Teets and Denlinger, 2013). The cellular mechanism involves rapid accumulation of cryoprotectants such as sorbitol and/or glycerol. In short exposures of cold (i.e., hours), these protective process can start to be manifested.

FlyNap is composed of trimethylamine (N(CH₃)₃) and is commonly used to anesthetize *D. melanogaster* for high school classes as well as research purposes. However, FlyNap has been shown to also have consequences such as the heart rate and altered immune responses (Chen and Hillyer, 2013). Since trimethylamine is suggested to open gap junctions, it is not surprising there is a drastic effect on the HR in the myogenic heart of *Drosophila* (Medina-Ceja and Ventura-Mejía, 2010). The exact mechanism of how trimethylamine blocks neural and muscle function to result in paralysis has not yet been elucidated. In mammalian hippocampal neurons, trimethylamine-HCl depolarizes the resting membrane potential and reduces input resistance by blocking the potassium currents responsible for the after hyperpolarizations during electrical activity. Not only are ionic currents altered by trimethylamine, but also by cytoplasmic pH which can result in neurons exhibiting an electrical bursting activity (Avakian and Kaltrikian, 1968; Kelly and Church, 2005).

Clove oil has been used for many years as an essential oil for humans (Javahery *et al.* 2012) and even as an insect repellent (Maia and Moore, 2011). Some of the common uses include reducing pain or discomfort on the skin surface of humans. The active ingredient for the pain reducing effect of clove oil is eugenol (Davis *et al.* 2015). A mixture of eugenol and lidocaine is sold commercially as FLEMICAIN for use in humans as a dental anesthetic and for children to numb the pain of teething (Burgoyne *et al.*, 2010). The perfume industry also uses clove oil to be applied topically (Geier and Uter, 2015). Eugenol is commonly used as an anesthetic in fish (Grush, 2004). The action of eugenol on decreasing neural function is likely by blocking TTX-sensitive and TTX-insensitive voltage-gated Na⁺ channels (Park *et al.* 2006, 2009). Detailed experiments using crayfish have demonstrated a reduction in the amplitude of the action potential by intracellular recordings in the medial and lateral giant neurons within the ventral nerve cord (Ozeki, 1975). It was shown in a

crustacean that the glutamate receptors at the NMJ remain sensitive to glutamate while exposed to eugenol (Ozeki, 1975).

Other anesthetic compounds, such as sevoflurane, have been used on adult *Drosophila* without any long term consequences noted (MacMillan *et al.* 2017); however to obtain and use ULTANE® (sevoflurane) one needs health precautions in place due the rapid effects on humans and the potential hazards for use in a classroom school setting as well as the research laboratory (Brioni *et al.* 2017). Considering some concerns in using CO₂ and cold exposure for manipulative experiments in which one would like to make use of the flies after being anesthetized, we sought out to test eugenol as an anesthetic on larvae and adult *D. melanogaster* as well as to examine the effect on the myogenic heart and synaptic transmission at the NMJs in order to potentially confirm the suspected mechanism of action on neurons in *D. melanogaster*.

Materials

Fly Stocks

For all experiments wildtype *Canton S* (CS) *Drosophila melanogaster* were used (FBst0064349, Bloomington Fly Stock Center). The flies were held in a 12 h- light/dark cycle at 21-22°C and 75% humidity in vials containing cornmeal-agar-dextrose-yeast medium (Bloomington stock center recipe). The general maintenance for culturing *Drosophila* is described by Campos-Ortega & Hartenstein (1985). The flies have been isogenic in the lab for several years.

Eugenol Exposure of Adult Flies

In order to examine the administration of eugenol (4-Allyl-2-methoxyphenol, Sigma-Aldrich) as an anesthetic for adult flies, the volatile odorant was used. A similar flow through design described by Smith and Blumer (2017) was used with a precision air flow meter (LZM-6 model, Newegg.com Whittier, CA., USA) connected to an air pump (Fig. 1). Air pumps are readily available from pet stores for aerating aquatic tanks. The hose outlet from the flow meter was connected to the end of a plastic disposable transfer pipette (cat # 13-711-7, Fisher Scientific, <https://www.fishersci.com>). A small hole, just large enough for the cut end of the transfer pipette to fit, is made in the plastic 50 ml test tube (Sigma-Aldrich). Saran wrap is used to wrap the plastic connections. Holes were made in the lids of the 50 ml plastic test tubes with a soldering iron so that when the fine nylon mesh is wrapped over the tube the lid is able to be screwed on. Whatmann #1 filter paper (5.5 cm diameter) folded as a funnel and soaked with eugenol was placed in one vial close to the pump and healthy adult flies (approx. 10-12) in the other vial which is last in the series (Fig. 1A). Once the flow started the flies were observed until

flying ability was stopped. The vial containing the eugenol was removed and a cotton plug was placed in the open end. Subsequently, the time until the flies regained consciousness under fresh air was assed.

A standardized test to examine the righting reflex and coordination of flies is the “Vortex test”. Vortexing occurred for 10 sec with a 45 degree slant of the vial on a vortex (Fisher, Vortex Genie 2, cat # 12-812 at a level of the start of 4th level in speed). Afterwards the vials were immediately adjusted horizontally and the ability of the flies to rise and walk or fly within the first couple of seconds was observed. All behavioral assays were repeated and compared to control flies without eugenol exposure. This assay of a vortex with adult flies is commonly used to stress flies (Fernandez *et al.*, 2014) or to induce traumatic brain injury with longer times or repeated trails (Barekat *et al.* 2016).

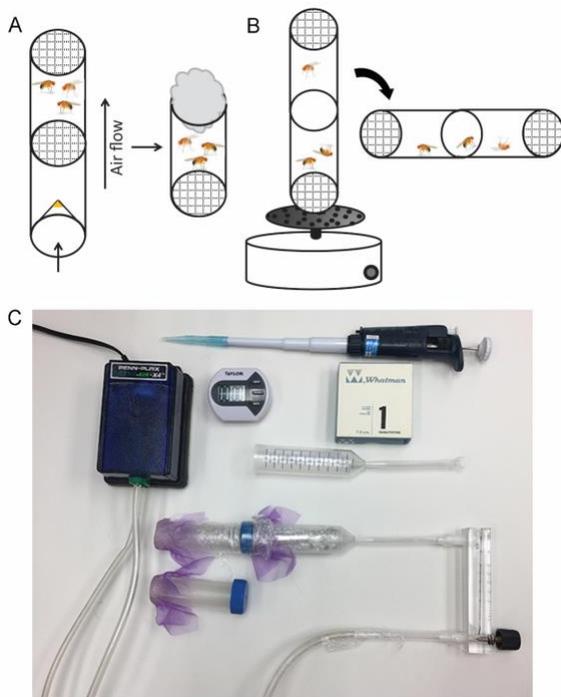


Figure 1. Schema of adult fly behavioral assays. A) Anaesthetizing flies via eugenol vapor exposure. B) A vortex test righting assay. C) Supplies used for the flow through system. Air pump tubing is connected to the flow meter. The outlet is connected to the cut end of the plastic bulb end of the transfer pipette. The other end of the pipette is placed in a tight fitting hole in the 50 ml test tube. The nylon mesh prevents the flies from coming in direct contact with the filter paper soaked with eugenol in the first test tube. The flies are placed in the second test tube in series.

Larva Assays

Early 3rd instars were transferred into a petri dish filled with agar (1 % agarose gel and 33 % apple juice) to induce crawling (5 larvae per dish). A filter paper (approx.

2.5 cm) was drenched in 99% eugenol and attached to the inner surface of the lid. After 2 h of exposure, the mouth hook movements (MHM) and the body wall contractions (BWC) were assessed after transferring the larvae to a new agar-apple juice dish. The BWC were used as a measure of locomotion per minute and compared to control larvae which were not exposed to eugenol. Individual larvae were placed inside a Petri dish that contained yeast solution (a few dried yeast granules mixed with water). The larvae were left for one minute, and then the mouth hook movements (MHMs) were counted. The rate of MHM was determined by direct observation for 30 seconds.

Heart Rate Assay

To test the physiological effects of eugenol on larvae, the heart rate (HR) was analyzed using a semi-intact method (Cooper *et al.* 2009). Third instar larvae were pinned on the dorsal side and dissected in a drop of saline on a glass plate (Zhu *et al.* 2016). A modified hemolymph-like 3 (HL3) saline was devised to maintain the larvae HR for hours (deCastro *et al.* 2014). In general, the HL3 saline (Stewart *et al.* 1994) contains [in mMol/l] 70 NaCl, 5 KCl, 20 MgCl₂, 10 NaHCO₃, 1 CaCl₂, 5 trehalose, 115 sucrose, 25 N,N-bis-(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES). For better visualization of the HR, a midline incision on the ventral side of the larvae was performed and the internal organs close to the skin removed (Desai-Shah *et al.* 2010). Subsequently, the animal was exposed to 100 ppm eugenol diluted in saline for 10 min. The HR was measured in beats per minute (BPM) by visually counting contractions. Measures were taken before and during exposure at 1 min, 5 min and 10 min time points. Rates were measured again 1 min and 2 min after the eugenol exposure was removed.

At the ABLÉ workshop, a rapid approach to measure HR in the intact larvae was used.

Another approach to measuring HR is to restrain the larva to one location by using double stick tape on a glass slide and placing the ventral side of the larva to the tape (Baker *et al.* 1999). However this approach does not work well if the tape gets wet when feeding the larvae. To avoid the tape getting wet one can use petroleum jelly (injected out of a small needle around the base of the larvae and around the tape edge). Here one can feed larva over time without having to chase the larvae into the focus plane or while it is moving on a dish. If one wishes to free the larvae the tape can be moistened and it loses its adhesiveness to the animal. There is a freely accessible movie in detailing how to use this method (Cooper *et al.* 2009).

1. Take a clean slide and place a cover slip at one end of it.
2. Put a small strip of double stick tape on a part of the slide. Locate your *Drosophila* larva and remove it from the test tube.
3. Place the larva in a Petri dish and rinse it with a small amount of water to remove any excess food.
4. Soak up remaining food with the corner of a small tissue or paper towel.
5. Gently pick up larva with tweezers and place it on your slide on the opposite end from your cover slip.
6. Place the slide under the microscope and adjust your lens on the larva. The larva should be on its stomach with its back facing upwards. You can distinguish between the two sides of the larva because their backs feature two 'racing stripes' which are the trachea. The stomach has faint horizontal grooves running along it with very fine black hairs.
7. If the larva is facing the incorrect way, simply turn the right way by gently flipping it over with your tweezers.
8. Under the microscope, double check to make sure the larva is still in the correct position. If it has turned over, see step seven.
9. Now, with the tweezers used to handle larva, pick up the larva and place it gently on the fresh patch of tape. Make sure the black mouth hooks are located over the edge of the tape on the glass and neither they nor the brown spiracles come in contact with the tape.
10. Carefully press down on the larva to flatten it out.
11. Now that the larva is in place, you can administer the substances which you wish to test them, such as with vapors of Eugenol by placing a soaked piece of paper close by their head or tail or both e
12. The heart rate can be observed by counting the number of pulses of the moving spiracles towards the caudal end.

The following procedure is if one was able to use intracellular recordings. This helps to explain the actions of eugenol on motor neurons.

Electrophysiological Recordings

The impact of eugenol on the larval NMJ was examined using electrophysiological intracellular recordings in 3rd instar larvae. Similar to the dissection of the larvae for HR analysis, the animals were dissected and prepared in a recording dish (Ruffner *et al.* 1999). After the removal of the internal organs, the segmental nerve of the 3rd segment was isolated and drawn into a suction electrode filled with saline. The saline used is described above for the HR assay.

To guarantee an efficient intracellular recording, microelectrodes with a resistance of 40 megaOhm were used to stab and record from muscle fiber 6 or 7. The segmental nerve was stimulated consistently at 0.5 Hz. Prior to eugenol exposure, the excitatory postsynaptic potentials (EPSP) and minis were recorded for at least 2 min for a baseline measure. A 100 ppm eugenol diluted in saline was exchanged with the original saline while stimulating the motor neuron. After the amplitude of the evoked responses slowly declined within the 10 min exposure, the media was exchanged back to normal saline without eugenol. The saline washout was performed at least 3 times with fly saline to gain a return of prominent amplitudes in the EPSPs.

Statistical Analysis

For statistical analysis and graphing, the program SigmaPlot (version 13.0) was used. Additionally with the raw data, mean values \pm standard error of the mean (SEM) are displayed. For adult fly and larval behavioral assays, an unpaired T-test was performed. The HR alterations during various stages in eugenol exposure were compared via the paired T-test. P-values \leq 0.05 were considered to be statistically significant.

Notes for the Instructor

Results

In examining the physiological alterations due to eugenol inhalation, each vial was maintained in the air flow until a 100% of the flies were inactive. The time it took for each vial to reach a 100% of the occupants to show inactivity is illustrated in Figure 2. After the adults showed inactivity from being exposed they were monitored for observable recovery. The time it took for the flies to recover in each vial is also shown (Fig. 2). The values for each of the seven discrete trials is shown as well as the mean (\pm -SEM) for all seven trials.

To assess functional recovery, the same adult flies anesthetized with eugenol were examined for their ability right themselves after being shaken in the tube placed on a vortex for 10 seconds. The vortex assay was performed after 1.5 hours. The eugenol flies were able to rapidly right themselves within 1 second as well as flies not exposed to eugenol. No differences in the groups were noted.

The larvae exposed to eugenol vapors had a longer lasting effect in altering behaviors than the adults. Despite being exposed for 2 hours in the dish with concentrated eugenol vapors the larvae appeared to not be drastically affected as they did not show complete cessation of movements. However, they did slow down. After rapidly transferring the larvae to fresh apple juice-agar dishes with no lids and quantifying BWCs and MHMs, it was apparent there were significant reductions in both measures (Fig. 3; One-tailed ANOVA).

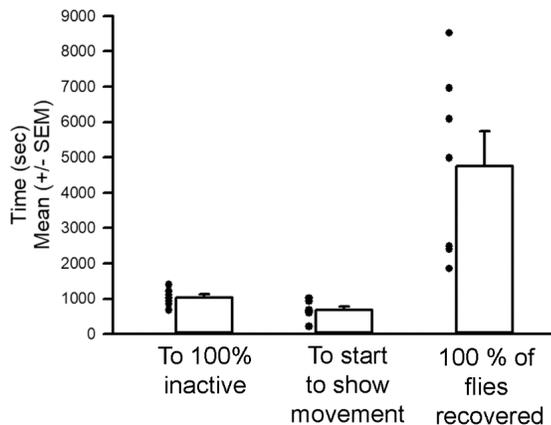


Figure 2. Representation of the time required to anesthetize 100% of all the flies within a tube and the time it took for the first fly in a tube to start showing recovery as well as for all the flies to fully recover. As soon as all the flies did not move and appeared to fall sideways, the vial was removed from the airflow and vapors of eugenol and were then observed for recovery. (N= 10-12 flies per trial, 7 trials with different flies).

To determine if larval heart rate would be impacted by direct exposure to eugenol, the heart rate was measured while bathed in a saline without eugenol and then with a 100 ppm eugenol. The decline in the heart rate was rapid (within the first minute) and by the 10th minute the rate was nearly zero for 2 of the 6 preparations. Upon exchanging the eugenol saline for fresh saline without eugenol the rate quickly started to return to baseline levels. In 2 of the 6 preparations the rates were nearly the same as initial values and 4 of the 6 larvae still had slightly reduced rates after 2 minutes (Fig. 4).

Since the vapors of eugenol anesthetized the adult flies and slowed the larval locomotion as well as MHM, we set to examine the direct action of eugenol on synaptic transmission at the NMJ in larvae. These larval body wall muscles are ideal for examining the effects on synaptic transmission because they produced graded postsynaptic responses. If there is a reduction in the amplitude due to reducing the number of open voltage gated sodium channels in the motor neuron then the number or duration of the voltage gated calcium channels in the presynaptic terminal may be reduced. This would lead to fewer synaptic vesicles fusing with the presynaptic terminal membrane and a smaller postsynaptic EPSP. One can also observe the occurrence of the spontaneous (non-evoked) vesicle fusion events (minis) to determine if the postsynaptic receptors are being blocked

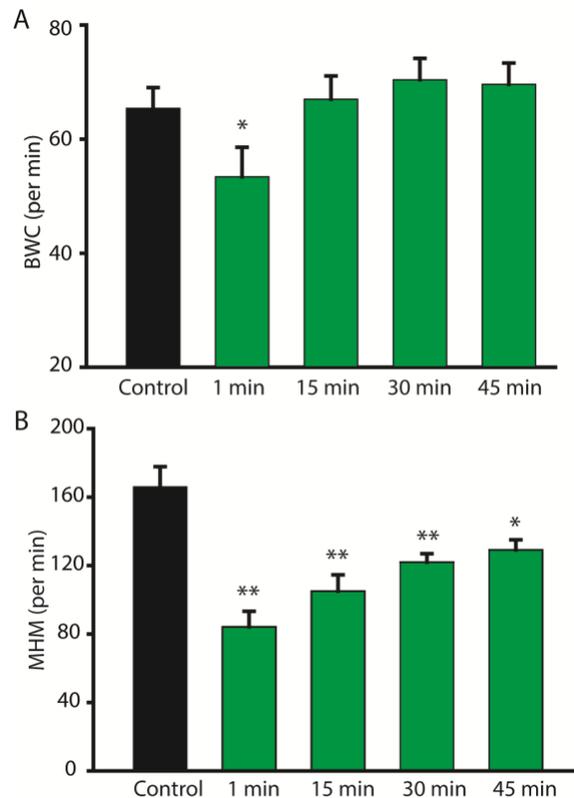


Figure 3. Assessment of changes in larval locomotion and mouth hook movements after exposure to vapors of eugenol. The number of body wall contractions (BWC) and mouth hooks per minute in 3rd instar after 2 hours of vapor exposure in comparison to larvae not exposed to the vapors (control). Mean \pm SEM. (In comparison to control, ** $p \leq 0.001$, * $p \leq 0.05$) (N = 10 per group).

by eugenol, which could also account for a reduction in the evoked excitatory postsynaptic potential EPSP. The altered evoked synaptic transmission is shown in Figure 5 in which the EPSP in saline (Fig. 5A) and during saline containing 100 ppm eugenol (Fig. 5B) is shown. Two evoked EPSPs are shown in Figure 5B with drastically reduced amplitudes. After exchanging the bathing media to fresh saline without eugenol, the evoked EPSP starts to recover (Fig. 5C).

In another representative preparation, the effect of eugenol (100 ppm) was more sudden in the reduction of the evoked EPSP amplitude (Fig. 6A). The segmental nerve was continuously being stimulated at 0.5 Hz throughout the experiment. Note the minis occur throughout the exposure to eugenol as noted by asterisks in Figures 6B, C and D. The boxes shown in Figure 6A represent the expanded traces shown in Figures 6B, C and D.

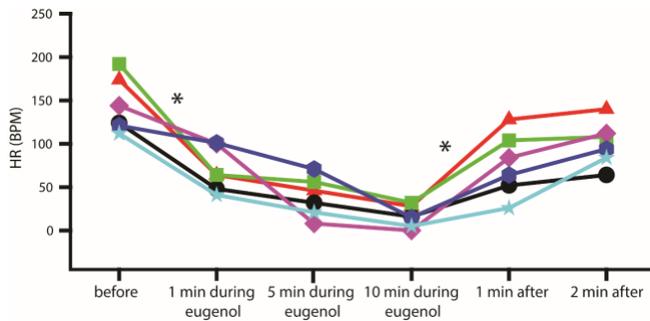


Figure 4. Physiological effects of eugenol on the larval heart rate (HR). Illustration of the beats per minute (BPM) before, during and after direct exposure to 100 ppm of eugenol dissolved in the saline bathing the exposed preparations. Each line represents a single larvae followed over time during the experimental paradigm. There is a significant decline in HR upon exposure to saline tainted with eugenol and a significant rise in heart rate after removing the tainted saline by replacing it with fresh saline. (* $p \leq 0.05$, sign test).

Discussion

We have demonstrated the potential mechanism of action of the active ingredient of clove oil (eugenol) which produces a lethargic to paralytic effect in *Drosophila* by reducing the amplitude of action potential to below the threshold of activating the voltage gated calcium channels in the presynaptic terminal. The postsynaptic receptivity is not affected by eugenol as spontaneous quantal events are still present while evoked EPSPs are drastically reduced or absent. The heart rate in larvae is almost completely stopped after a short time of exposure to 100 ppm eugenol. Synaptic transmission as well as the heart rate quickly recovers after the removal of the compound without any obvious consequences in behavioral function.

In a study by Ozeki (1975), in which action potentials were directly measured with intracellular electrodes of the large axons in the crayfish ventral nerve cord, the amplitudes were decreased with 50 ppm of eugenol. Ozeki (1975) also reported that the conduction velocity of the axons were reduced during exposure to eugenol. In the nerve of an amphibian (bull frog) 100 ppm eugenol blocks nerve conduction (Kozam, 1977). In sensory nerves of rodents eugenol was shown to block sodium currents from recordings made with whole-cell patch-clamp (Park *et al.* 2006). Both TTX-sensitive and TTX-insensitive voltage-gated Na^+ channels in these sensory neurons are blocked by eugenol (Park *et al.* 2006, 2009). It has also been shown that eugenol blocks high-voltage-activated calcium channel sensory nerves of rodents. The block was measured in both capsaicin-sensitive and capsaicin-insensitive dental primary afferent

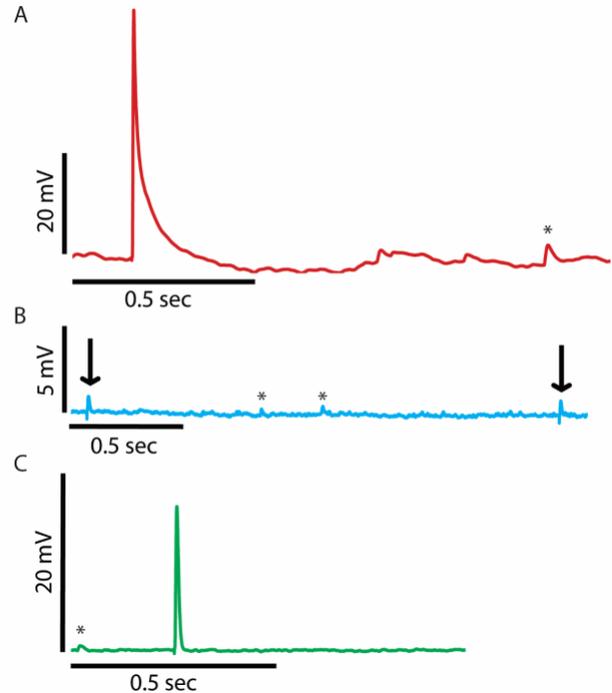


Figure 5. Reversible and gradual effects of eugenol on amplitude of the evoked EPSPs at the larval neuromuscular junction. A) Before, B) during and C) after direct exposure to 100 ppm of eugenol on a preparation. The time scale in B is condensed so that two evoked events can be observed (arrows). Note the gradual reduction (B) and partial recovery in the amplitude of the EPSP (C). The segmental nerve was continuously being stimulated at 0.5 Hz throughout the experiment.

neurons suggesting that the calcium channels block are not the vanilloid 1 (TRPV1) channels. It appears the human N-type calcium channels are the calcium channel subtype blocked by eugenol (Lee *et al.* 2005). In *Drosophila* the motor nerve terminals express voltage-gated calcium channels containing $\alpha 1$ subunits encoded by Ca_v2 family genes (Badre and Cooper, 2008; Gu *et al.* 2009; Xing *et al.* 2005). The neurons in *Drosophila* do exhibit TTX-sensitive voltage-gated Na^+ channels (Feng *et al.* 1995; O'Dowd and Aldrich, 1988). So it is feasible that both channels maybe blocked in the motor neurons of *Drosophila* by eugenol. The shapes of the spontaneous quantal events do not appear to show any differences. Therefore, there is no direct effect on the glutamate receptors. The narrower evoked EPSP while being exposed or recovering from the eugenol is likely due to the reduction in evoked vesicle fusion events within the presynaptic terminals

As for the reduction in the heart rate, the effect of eugenol is direct on the heart since the pacemaker region of the larvae heart is not innervated (Johnstone and Cooper, 2006). The larval *Drosophila* heart is myogenic and the

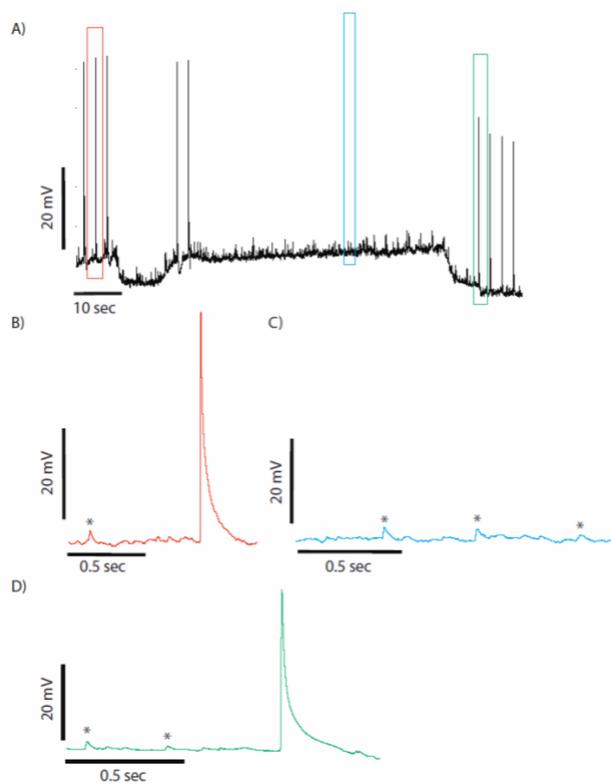


Figure 6. Reversible effects of eugenol on the evoked EPSPs and sparing the postsynaptic glutamate receptors at the larval neuromuscular junction. A) Overview of an extend recording of evoked and spontaneous EPSPs before during and after exposure to eugenol (100 ppM). B) A spontaneous quantal event (mini) is shown occurring before an evoked EPSP prior to treatment with eugenol. C) During eugenol exposure the evoked events are absent and only spontaneous events are observed. D) After the tainted eugenol saline is replaced with fresh saline a recovery of evoked EPSPs start to appear while spontaneous event continually occur. The segmental nerve was continuously being stimulated at 0.5 Hz throughout the experiment.

the rate is readily altered by biogenic amines, catecholamines and peptides which likely have their influence on the ion channels on the plasma membrane (Dasari and Cooper 2006; Majeed *et al.* 2014; Malloy *et al.* 2016; Titlow *et al.* 2013; Zornik *et al.* 1999). The effect of eugenol on the heart rate and the evoked synaptic transmission is rapidly reversed upon exchanging the bathing media. The rapid physiological effects are likely targeted to the extracellular surface of ionic channels in the membrane and not due to altered structure in bi-lipid membrane or within the cytoplasm.

The varied recovery times for the intact adults and larvae may well be due to individual variation in metabolic rate and status of the animal. The adults showed a complete cessation of movement with the exposure to vapors whereas the larvae, even after 2 hours, did not completely

stop crawling or eating. The adults took some time to recovery but showed no adverse effects to climbing or righting themselves with vigorous shaking.

The larvae recovered quicker in regards to body wall contractions as compared to mouth hook movements. Considering the rate in which the mouth hooks rapidly move perhaps the effect is more rapid and this is potentially a more sensitive measure for subtle changes in neuronal function as well as time for recovery.

There are other types of adult behavioral assays instructors may like to use for a classroom setting to examine the effects of eugenol or even other suspected anesthetic agents. Evaluation of recovery to an agent could be accomplished using standardized behavioral assay in climbing (Majeed *et al.* 2016). For larvae the attraction to particular odorants can be used (Badre and Cooper, 2008).

The use of eugenol may gain some momentum in research and teaching laboratories utilizing insects due to its relative ease of use and the feasibility of recovery time. The commonly used approaches with CO₂ or cooling are easily applied but have some difficulties which may be alleviated with the use of eugenol. Eugenol is well established for use in fish surgeries, tagging and experimentation (Davis *et al.* 2015; Javahery *et al.* 2012). We are currently investigating the role for the use in crustaceans and addressing physiological consequences (Wycoff *et al.* 2018).

There remains several questions to be answered in subsequent investigations. For instance, how long of an exposure is needed to result in death of the adults and larvae? Would potentially direct contact of the intact adults and larvae with eugenol accentuate the effects and would it be more difficult to reverse effects? Also, various dilutions and exposure times remain to be examined.

Finally, in considering the use of clove oil or eugenol for teaching in a classroom setting, some precautions need to be discussed. Students should not directly breathe the vapors. Nitrile gloves are recommended and to be changed if contacted with the oil. If the oil is in contact with the skin, wash with soap and water as soon as possible.

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Acknowledgments

Supported by: Deutscher Akademischer Austausch Dienst (DAAD) German Academic Exchange Service, RISE - Program (Research Internships in Science and Engineering) (KW); Personal funds (RLC). We thank Ms. Shannon Conlin from Sea Farms Limited, Worchestershire United Kingdom, for introducing us to the use of eugenol.

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Citing This Article

Weineck K, Stanback A, and Cooper RL. 2019. The effects of eugenol as an anesthetic for an insect: *Drosophila*, adults, larval heart rate, and synaptic transmission. Article 54 In: McMahon K, editor. *Tested studies for laboratory teaching*. Volume 40. Proceedings of the 40th Conference of the Association for Biology Laboratory Education (ABLE). <http://www.ableweb.org/volumes/vol-40/?art=54>

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