



Association for Biology Laboratory Education

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The effects of eugenol as an anesthetic for an insect: *Drosophila*, adults, larvae, behaviors, larval heart rate and neural activity

by

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Abstract

The examination of the active ingredient in clove oil (i.e. eugenol) as an anesthetic for use on *Drosophila melanogaster* will be investigated. Adult fruit flies will be anesthetized with a simple flow through apparatus and the recovery will be performed. Behavioral tests to examine for alterations in behavior as well as to timing of recovery will be performed. Larvae will also be examined in a dose dependent manner to investigate the effect of eugenol. Behavioral assay with larvae will be performed with counts of body wall movements and mouth hook movements. Heart rate in intact larvae will also be measured after exposure to eugenol. Participations interested in direct application of 100ppm eugenol (mixed with physiological saline) directly on the larval heart and neuromuscular junction will be shown the dissections and experiments which can be performed. Eugenol can also be applied to other insects and invertebrates for comparisons. Injections and bath exposure of crayfish to eugenol will be demonstrated. This is a hands on mini-workshop. This is a true NGSS 3-dimensional student inquiry type of activity from engineering design to implementation and data analysis.

Note: This exercise is being developed to be published somewhere as an educational module but also as a research project. Thus, the intracellular measures of electrical

recordings are for the research component which will not be presented for the ABLE 2018 workshop due to equipment issues but the text remains in the write up to help illustrate the mechanism of action which might be useful for a classroom discussion.

A draft copy of a manuscript in progress is being provided. We would expect significant changes by the time of publication.

There is also a web site built for ABLE 2018 for the workshops presented on this web page:

<http://web.as.uky.edu/Biology/faculty/cooper/Teacher%20training.htm>

Binder Materials:

- a thorough introduction that provides sufficient background for those who might not be familiar with the material
- the student lab exercise
- instructor notes
- preparation instructions
- an equipment and materials list
- information about sources and suppliers for materials
- student evaluation feedback on the lab (if possible)

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Key words: *Drosophila*, anesthesia, neuromuscular junction, behavior, larva, cardiac

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Abstract

The examination of the active ingredient in clove oil (i.e. eugenol) as an anesthetic for use on *Drosophila melanogaster* was performed. Adults were anesthetized with a simple flow through apparatus and they recovered to perform behavioral tests without and 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 serves as an alternative to CO₂ or cold as an anesthetic.

Introduction

Insects are used in a number of experimental paradigms which in some cases involve sedating them for some time and preparing them for experimentation. Various approaches are used from exposure to CO₂ (carbon dioxide), to cold temperature as well as 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. However, it is established that behaviors in adult *Drosophila*, such as flying and climbing, can be affected 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 *Drosophila*.

FlyNap is composed of trimethylamine and is commonly used to anesthetize *Drosophila melanogaster* fruit flies for high school classes as well as research purposes. However, FlyNap has been shown to also have consequences by increasing the heart rate and altering 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 heart rate (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 on paralysis has not yet been elucidated as far as we know. In mammalian hippocampal neurons trimethylamine-HCl depolarizes the resting membrane potential and reduces input resistance by blocking potassium currents responsible for the afterhyperpolarizations during electrical activity. Not only are ionic currents altered by trimethylamine, but cytoplasmic pH which can result in neurons to exhibit an electrical bursting activity (Avakian and Kaltrikian, 1968; Kelly and Church, 2005)

Anesthesia by CO₂ and cold exposure requires continuous exposure otherwise 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 $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{HCO}_3^- + \text{H}^+$ (Stone and Koopowitz, 1974). The enzyme carbonic anhydrase in cells rapidly catalyzes this reaction which is likely the 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 while exposed to CO₂ (Badre et al, 2005). However, the paralytic action of CO₂ is due to blockage of the glutamatergic synapses at the neuromuscular junctions (Badre et al, 2005; Bierbower and Cooper, 2010).

As for cold exposure in inducing a chill coma, one has to be careful as not to damage tissue with freezing. We have had issues with adult flies and use of cold 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. Alternatively, a walk in cold room, close to freezing with a dehumidifier, could solve this issue; however, personally prefer

not to work in these conditions. After anesthesia by either CO₂ or chilling significantly delayed the time of adults to start copulating (Barron, 2000). Insects use rapid cold hardening as a 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, this mechanism has been studied extensively (see review-Teets and Denlinger, 2013). The cellular mechanism generally involves rapid accumulation of cryoprotectants such as sorbitol and/or glycerol. The short exposure of cold for hours turns on these process which can take a few hours to days to be manifested.

Clove oil has been used for many years as an essential oil on humans (Javahery et al., 2012) and even as an insect repellent (Maia and Moore, 2011). Some of the uses have been to reduce pain or discomfort on the skin surface of humans. The active ingredient for the pain reducing effect in 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 even 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 neuromuscular junction (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 a 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 the active ingredient (eugenol) as an anesthetic on adult *Drosophila melanogaster* as well as to examine the effect on the myogenic heart and synaptic transmission at the NMJs to potential confirm the suspected mechanism of action on neurons in *D. melanogaster*.

Methods and Material

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).

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. Two standard plastic vials (9.4 cm height, 2.4 cm top diameter and 2.25 cm bottom diameter), commonly used for culturing *Drosophila*, (Genesee Scientific, San Diego, CA 92126 USA) with cotton nets at one end were constructed and connected with tape (Figure 1A). Whatmann #1 filter paper (5.5 cm diameter) folded as a funnel and soaked with eugenol was placed in one vial and healthy adult flies (approx. 10-12) in the other vial (Figure 1A). To guarantee an even air flow, the vials were clamped into the fume hood with the air funneling from the chemical substance to the animals (Figure 1B). The air flow was measured at 27.43 meter/min without the 3 vials in place and when located under the slash the air flow directly in front of the tubes was 9.14 meter/min. To restrict airflow along the length of the slash and the frame, for focusing the flow by the vials, packing tape was used to block the air.

After initially assuring that all flies were viable and moving, the vials were placed under the slash of the fume hood and observed until no flying ability was observed. Subsequently, the time until regaining consciousness under fresh air was assed. The vial containing the eugenol was removed and a cotton plug was placed in the open end. About 1.5 h after the exposure, behavioral assays were implemented.

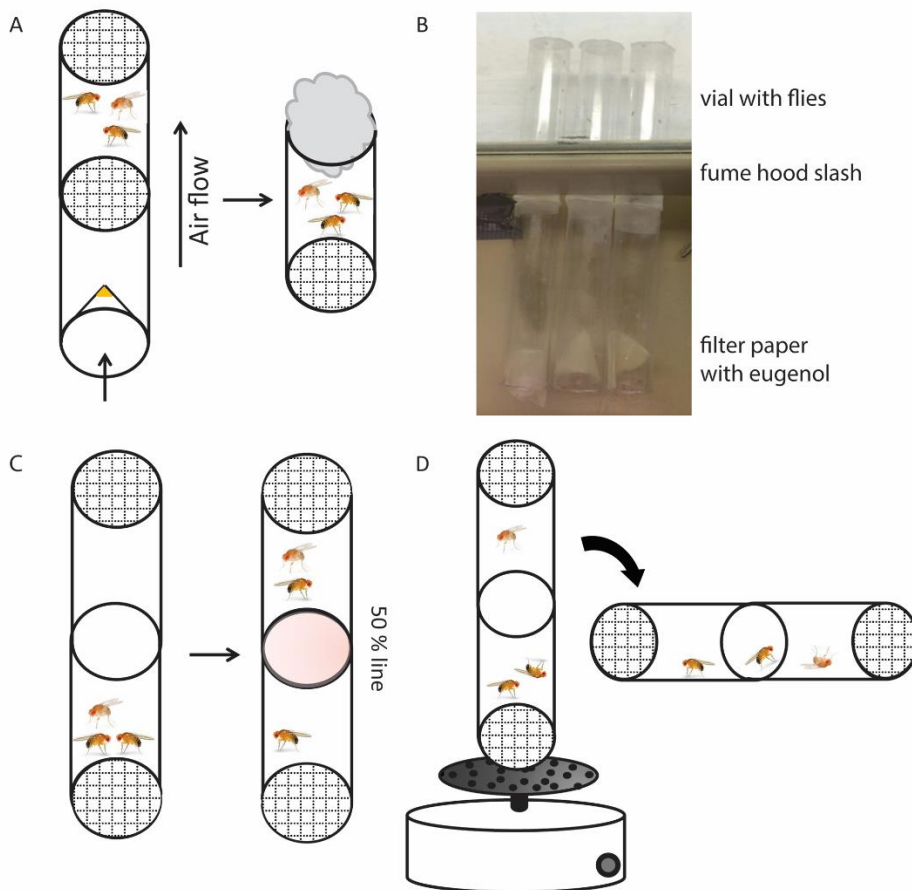


Figure 1: Schema of adult fly behavioral assays. A) Anaesthetizing flies via eugenol vapor exposure. B) Photo of the anaesthetizing set up in placed straddling the slash of the fume hood. C) Assessment of locomotion via a climbing assay. D) A vortex test righting assay.

Adult Fly Behavioral Assays

Evaluation of recovery to eugenol was accomplished using standardized behavioral assays in adult flies. A climbing assay was performed by placing flies in two fresh vials with the open ends facing each other and tapped together. All animals were tapped down to the bottom of the tube. The number of flies crossing one vial length (9.4 cm) were counted within 10 sec. This is a standardized behavioral test for adult *Drosophila* (Majeed et al., 2016).

Another standardized test to examine the righting reflex and coordination of flies is the “Vortex test”. The flies are placed in two connected tubes. 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 seconds was

observed. All behavioral assays were repeated and compared to control flies without eugenol exposure. Use of a vortex to mix flies is used in various ways (to stress flies- Fernandez et al., 2014; to induce traumatic brain injury- Barekat et al., 2016).

Larva Assays

As for the adult larvae being subjected to the vapor of eugenol, larvae were also examined. The early 3rd instars were transferred into a petri dish with 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 locomotion 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 were mixed with water). The larvae were left for one minute, and then the mouth hook movements (MHMs) were counted. The rate of mouth hook movement was counted by direct observation for 30 seconds and expressed as MHM.

NOTE for ABLE workshop: This approach maybe too difficult for a class room setting due to the fine dissection. In the next paragraph an alternative method is described.

To test the physiological effects of eugenol on larvae, the heart rate (HR) was analyzed using a semi-intact method (Cooper et al., 2009). Therefore, 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 saline devised to maintain the larvae heart rate for hours was used (deCastro et al., 2014). In general, the modified hemolymph-like 3 (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 implemented 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 assessing directly the heart contractions through before, during (1 min, 5 min and 10 min) and after (1 min and 2 min) eugenol exposure.

For the ABLE workshop a rapid approach to measure HR in the intact larvae:
We have a JOVE freely accessible movie in detailing how to do this method

Cooper, A.S., Rymond, K.E., Ward, M.A., Bocook, E.L. and Cooper, R.L. (2009) Monitoring heart function in larval *Drosophila melanogaster* for physiological studies. Journal of Visualized Experiments (JoVE) 32: <http://www.jove.com/video/1596/monitoring-heart-function-larval-drosophila-melanogaster-for>

Another approach 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 Vaseline (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.

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 eight.

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 or 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 with vapors of Eugenol by placing a soaked piece of paper close by their head or tail or both ends.
12. Finally, the heart rate can be observed by counting the number of pulses of the moving spiracles towards the caudal end in one minute.

This procedure is if one was able to use intracellular recordings. This does help to explain the actions of eugenol on motor neurons as a reference for ABLE workshop participants.

Electrophysiological Recordings

The impact of eugenol on the larval neuromuscular junction (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. 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. Subsequently, 100 ppm eugenol diluted in saline was exchanged with the bathing media while stimulating the motor neuron. In case of a rapid disappearance of the EPSPs, eugenol was washed out immediately. When 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 wash out was performed at least 3 times with fly saline to gain a returned of prominent amplitudes in the EPSPs.

Statistical Analysis

For statistical analysis and graphing, the program SigmaPlot (version 13.0) was used. Additionally to 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 ≤ 0.05 were considered statistical significant.

Results

First, the physiological alterations due to eugenol inhalation was tested. Each vial was maintained in the air flow until a 100% of the flies were inactive. Some vials the flies were inactive within 8 minutes and the longest was 25 minutes. This approach of varied exposure time was used as this would be what one might experience in a teaching or a research laboratory. Therefore, some flies within a vial might be exposed to longer duration of eugenol vapors. The time it took for each vial to reach a 100% of the occupant to show inactivity is illustrated in Figure 2. After the adults showed inactivity from being exposed they were monitored for observable recovery. It took 2 minutes for the first flies to start moving and by 40 minutes all animals revealed some locomotion/flying ability. They all recovered within 140 minutes to appear normal (Figure 2). The vial labeled # 6 in Figure 2 was exposed the longest to the vapors but the flies recovered quicker than for vials exposed with even less time

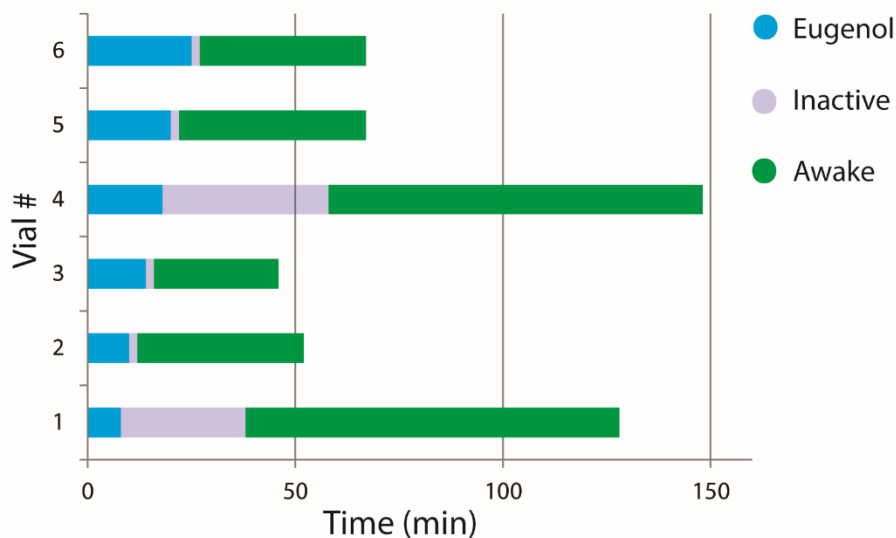


Figure 2: Representation of the time required to anesthetize 100% of all the flies within a vial and the time it took for them to start and fully recover. The exposure time to the vapors of eugenol varied between each vial, but as soon as all the flies did not move and appeared to fall sideways, the vial was removed from the airflow and vapors of eugenol. (N= 10-12 flies per vial).

To assess functional recovery, the flies were examined for their ability to climb and rapidly respond to a strong sensory stimulus. The climbing ability of the adults was very

robust after 1.5 hours from removing the eugenol vapors. Controls were obtained from the same stock of adults housed in the same conditions but not exposed to eugenol (Figure 3A). There is no significant difference between the groups. Similarly, controls and ones exposed to vapors of eugenol were examined with the vortex assay after 1.5 hours (Figure 3B). Flies rapidly righted themselves within 1 second. One fly appeared to have died in each of the groups which was likely due to the harsh vortex environment.

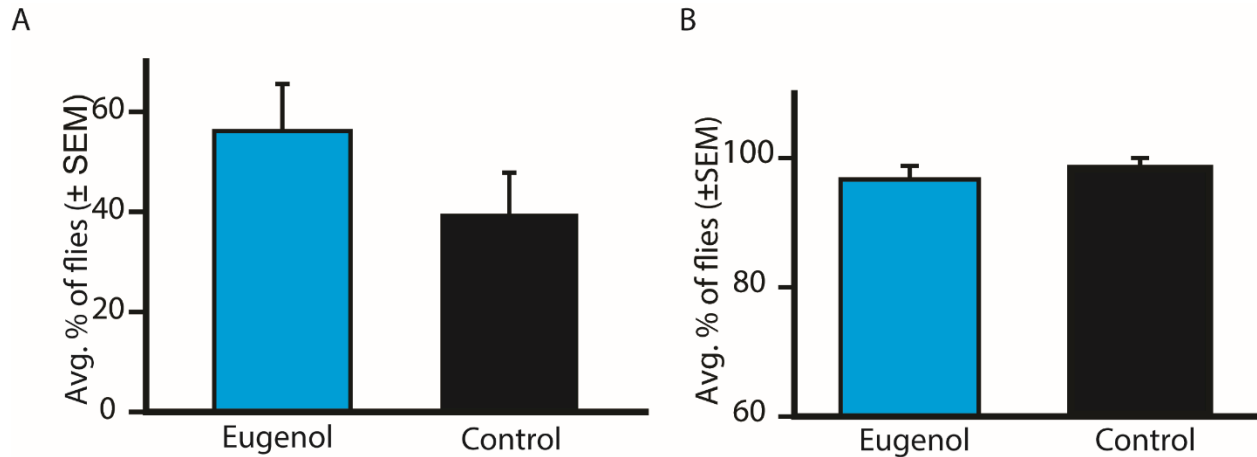
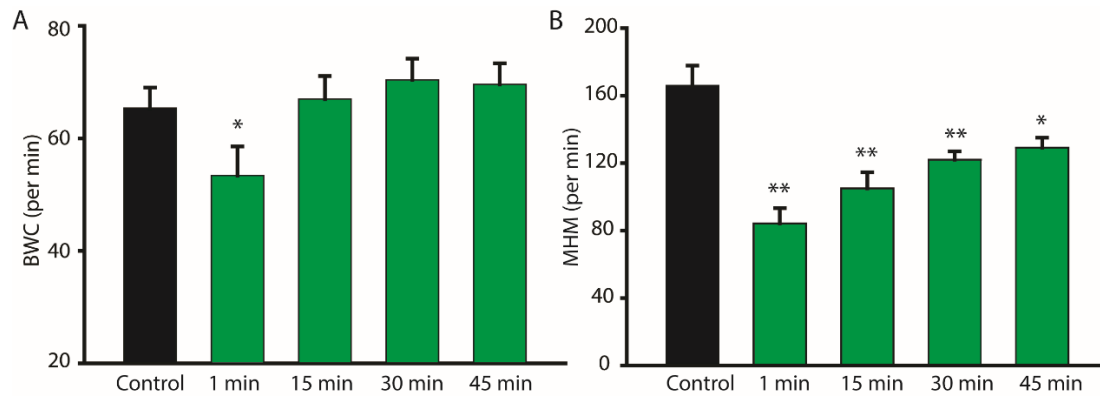


Figure 3: Assessment of behavioral alterations due to eugenol in adult flies. A) The average % of flies from each of the 6 vials for eugenol vapor exposure and 6 vials of controls passing the length of one tube during 10 sec. B) The average % of flies from each of the 6 vials for each condition which could right themselves and move after the vortex test (vortex for 10 sec) (mean ± SEM). No significant differences between treated and non-treated groups were present ($P>0.05$).

The eugenol vapors exposed to larvae had a longer lasting effect in altering behaviors than for adults. Despite being exposed for 2 hours in the dish with concentrated eugenol vapors the larvae appeared to not to be affected as their nerve showed paralysis complete cessation of movements. After rapidly transferring the larvae to fresh apple juice-agar dishes, with no lids, and quantifying body wall and mouth hook movements it was apparent there were significant reductions in both measures (Figure 4; One-tailed ANOVA).



*Figure 4: 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).*

In determining if larval heart rate would be impacted by direct exposure to eugenol, it 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 bathing eugenol saline with fresh saline without eugenol the rate quickly started to return to basal levels. In 2 of the 6 preparations the rates were nearly the same as initial values and 4 of the 6 still slightly reduced after 2 minutes (Figure 5).

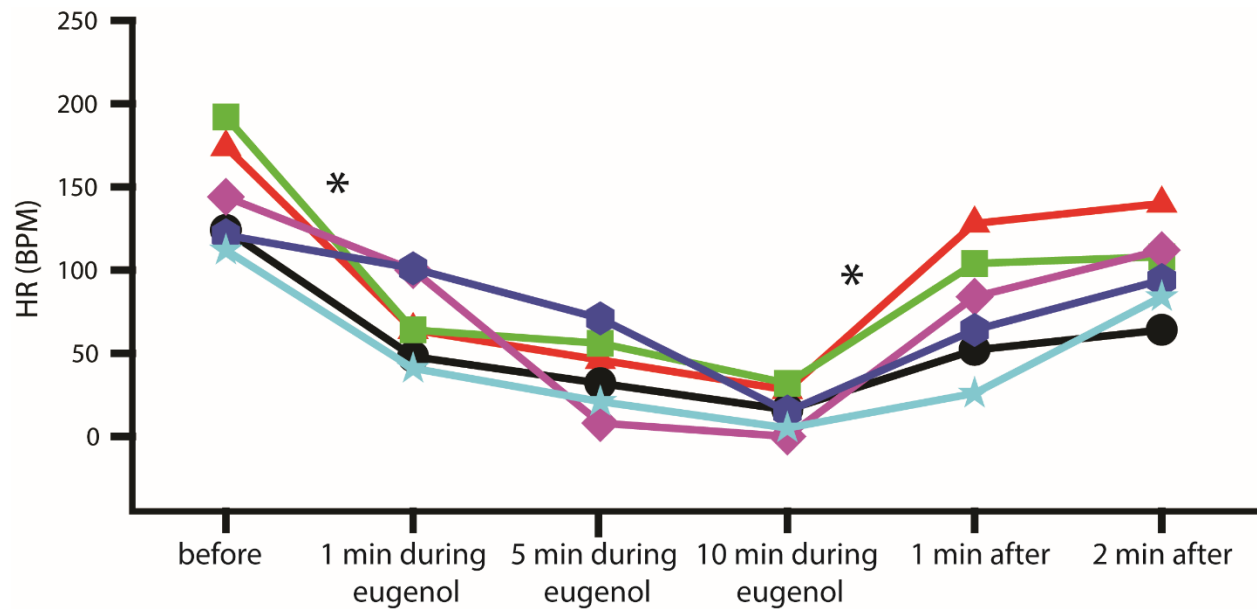


Figure 5: Physiological effects of eugenol on the larval heart rate (HR). Illustration of the beats per minute (BPM) before, during and after direct exposure to 100 ppm of eugenol dissolved in the saline bathing the exposed preparations. Each line represents a single larvae followed over time during 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).*

Since the vapors of eugenol anesthetized the adult flies and slowed the larval locomotion as well as mouth hook movements, we set to examine the direct action eugenol on synaptic transmission at the neuromuscular junction (NMJ) in larvae. These non-spiking larval body wall muscles are ideal to examine the effects on synaptic transmission. If there is a reduction in the action potential amplitude of the motor neuron then fewer synaptic vesicles will fuse within the presynaptic terminal and a smaller graded postsynaptic EPSP is observed. One can also observe the occurrence of the spontaneous vesicle fusion events to determine if the postsynaptic receptors are being blocked by eugenol to account for a reduction in the evoked EPSPs. This phenomenon is nicely shown in Figure 6 in which the EPSP in saline (Figure 6A) and during saline containing 100 ppm eugenol (Figure 6B). Two evoked EPSPs are shown in Figure 6B with drastically reduced amplitudes. After exchanging the bathing media to fresh saline without eugenol the evoked EPSP starts to recover (Figure 6C).

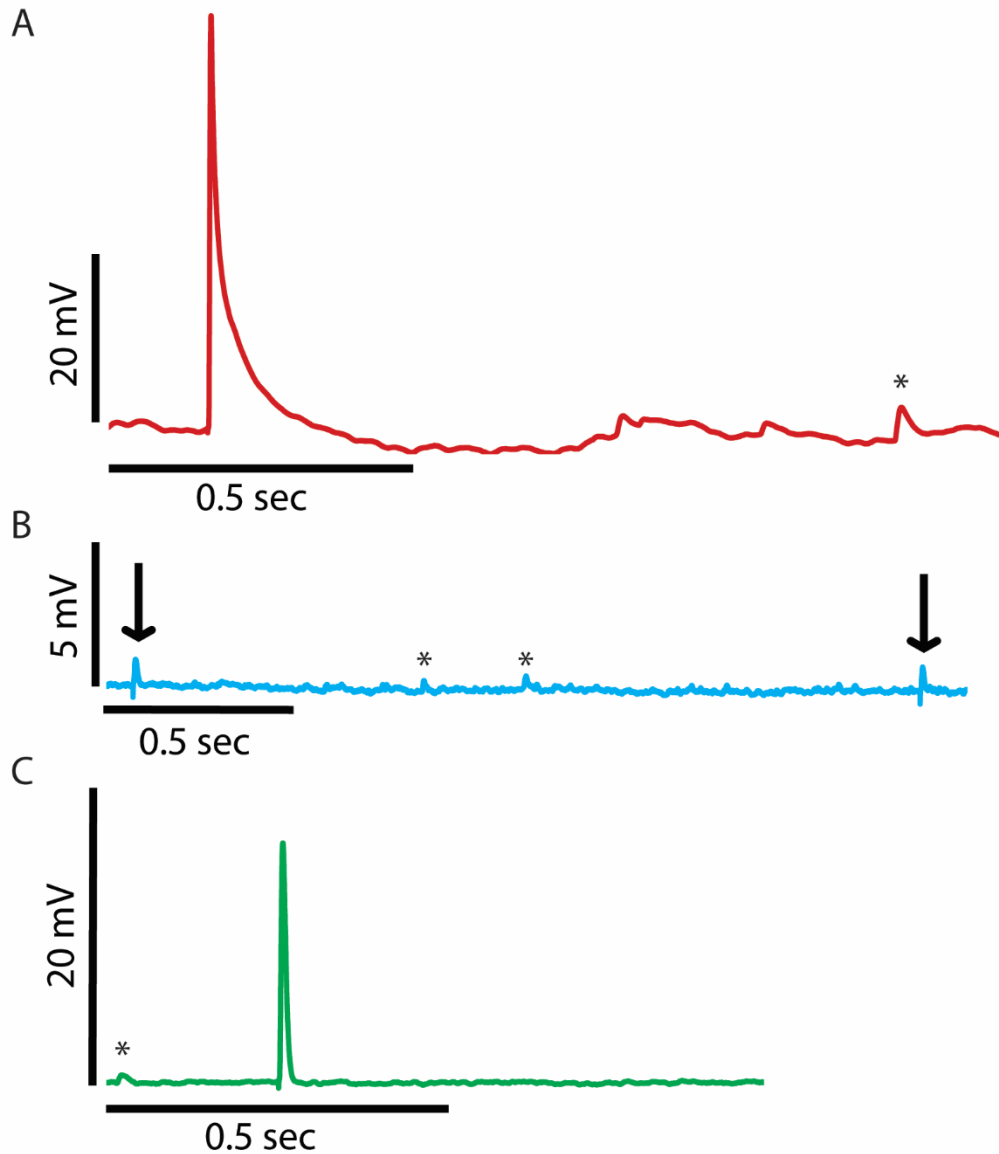


Figure 6: Reversible and gradual effects of eugenol on amplitude of the evoked EPSPs at the larval neuromuscular junction. A) Before, B) during and C) after direct exposure to 100 ppm of eugenol on a preparation. The time scale in B is condensed so that two evoked events can be observed (arrows). Note 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.

In another representative preparation the effect of eugenol (100 ppm) was more sudden in the reduction of the evoked EPSP amplitude (Figure 7A). The segmental nerve was

continuously being stimulated at 0.5 Hz throughout the experiment. Note that the spontaneous (i.e. minis) occur throughout the exposure to eugenol as noted by asterisks in Figures 7B, C and D. The boxes shown in Figure 7A represent the expanded traces shown in Figures 7B, C and D.

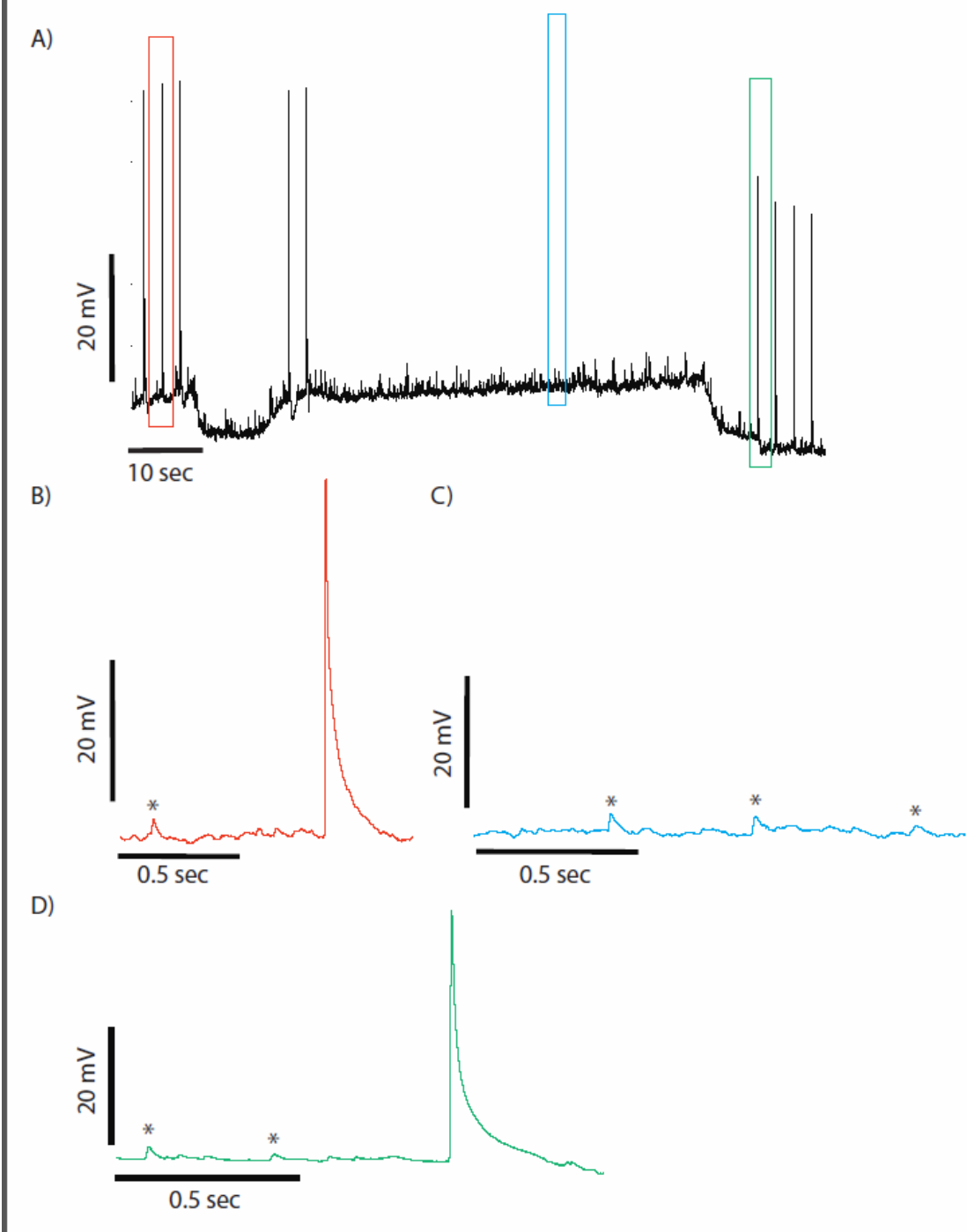


Figure 7: Reversible effects of eugenol on the evoked EPSPs and sparing the postsynaptic glutamate receptors at the larval neuromuscular junction. A) Overview of an extend recording of evoked and spontaneous EPSPs before during and after exposure to eugenol (100 ppM). B) A spontaneous quantal event (mini) 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.

Discussion

We have demonstrated the potential mechanism of action of the active ingredient of clove oil (i.e., eugenol) which produces a lethargic to paralytic effect in *Drosophila* as reducing the amplitude of action potentials 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 with a short time of being directly exposed 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 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 recording 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 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 directly 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 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). Remarkable the effect of eugenol on the heart rate and the evoked synaptic transmission is rapidly reversed upon exchanging the bathing media. Thus, 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 potentially a more sensitive measure for subtle changes in neuronal function as well as time for recovery.

The use of eugenol may gain some momentum in research and teaching laboratories utilizing insects since it is relatively easy to use and the recovery time is feasible. 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.

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. In considering the use of clove oil or eugenol for teaching in a classroom setting some precautions need to be discussed.

References

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