Investigating Effects of eugenol on nerve conduction and synaptic transmission at neuromuscular junction in an amphibian

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Eugenol is a natural anesthetic. This study provides insights into how eugenol affects neuro-muscular communication in a vertebrate to address its anesthetic property. Eugenol's effects at the neuromuscular junction (NMJ) have not been well studied. This study was to determine if eugenol affects the activity of cholinergic synapses at the frog pectoris NMJ. Second, this study set out to determine if eugenol could block evoked compound action potentials (CAP) in the frog sciatic nerve at 0.05 mM. This study also provides a novel procedure for isolating the frog cutaneous pectoris muscle. This thin surface muscle is ideal to use in studying synaptic responses. The results showed evoked excitatory junction potentials at the frog pectoris NMJ were depressed during exposure to eugenol, however, quantal events were not. This indicated that the nicotinic acetylcholine receptors on the muscle fibers were not blocked by eugenol. Eugenol also depressed conduction of compound action potentials along the frog sciatic nerve. These results indicate eugenol likely targets membrane proteins associated with electrical signaling. In summary, this study successfully demonstrated use of the sciatic nerve and cutaneous pectoris muscle for examining the effects of eugenol on neural-muscular function and could potential be extended for use as an anesthetic.

Abbreviations: neuromuscular junction (NMJ), excitatory junction potentials (EJPs); miniature excitatory junction potentials (mEJPs); compound action potentials (CAP).

Keywords: Anesthetic, neuromuscular junction, synaptic transmission, clove oil, eugenol

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Introduction

Amphibians, particularly frogs, are sometimes used as vertebrate models for research and for teaching instead of higher vertebrates as part of the three R's (replace, reduce, and revise). While using such model vertebrates, humane animal use and care guidelines often require using tricaine methanesulfonate (MS-222) for anesthesia and euthanasia unless otherwise necessitated (Leary, 2013), however, there are times when alternative types of anesthetics would be warranted. For example, frogs are sometimes used in teaching to demonstrate properties of the vertebrate nervous system, skeletal muscles, and cardiac function. Frogs use the same type of neurotransmitter receptors at the neuromuscular junction as mammals and the nerves function in a very similar manner. Use of alternative anesthetics during handling, euthanasia, or after euthanasia could help elucidate particular traits or features of the different tissues or systems being studied or taught as well as to better clarify how these anesthetics work.

Eugenol is an anesthetic approved in proprietary form for use on food fish in countries such as Chile, Australia, and Norway (AQUI-S® 2018). It is also sold for human use in the form of clove oil, as an essential oil (Javahery et al., 2012). Although it has not yet been approved by the United States Food and Drug Administration (FDA), it carries a *Generally Regarded as Safe* (*GRAS*) designation by the US Food and Drug Administration. A mixture of eugenol and lidocaine is commercially marketed as FLEMICAINE for a dental anesthetic to numb the pain of teething in children (Burgoyne et al., 2010; Park et al., 2006). The wide spread use of eugenol has stimulated research into the mechanism of action of its anesthetic properties. Its *GRAS* designation by the FDA further makes it an ideal anesthetic for use in teaching laboratories. Despite interest in eugenol's potential as an anesthetic, research is somewhat limited. The limitations may be due to the side effects associated with high concentrations. It is known that high concentrations retard liver function and can be potentially lethal (Hartnoll et al., 1993).

Further study of this anesthetic could help uncover unique mechanisms of action in research fields, as well as be used as the basis of authentic course-based undergraduate research experiences (CUREs) for students. For example, how does eugenol anesthesia work in different types of excitable cells, physiologically?

In the nervous system, research has suggested that eugenol's mechanism of action is likely through blocking tetrodotoxin- (TTX-) sensitive and TTX-insensitive voltage-gated sodium channels in vertebrate (Park et al., 2006, 2009) and invertebrate preparations (i.e. crayfish, crab, shrimp and insect; Ozeki, 1975; Weineck et al., 2019; Wycoff et al., 2018). Voltage-gated sodium channels are used by neurons and muscle cells to conduct electrical signals (action potentials) along the plasma membranes during a communication event. Thus, inhibition of voltage-gated sodium channels should decrease the ability of the affected cells or regions along the cell's membrane to send action potentials or impulses. In frog sciatic nerve preparations, researchers observed decreased peak compound action potentials and decreased signal conduction when exposed to 0.5 mM for 20 min (Tomohiro et al., 2013). However, it is not clear how other concentrations or exposure times might affect the neural activity. Much work remains to be done to determine which types of neurons or muscle cells are affected in such a manner, which types of voltage-gated channels are most affected, and how action potential properties are affected during exposure to eugenol.

Communication events among neurons and between neurons and other cells are mediated by synapses. In vertebrates for example, somatic motor neurons communicate with skeletal muscle fibers through cholinergic-type synapses whereby the pre-synaptic neuron releases acetylcholine (Ach) onto the post-synaptic muscle fiber's plasma membrane. However, studies in the effects eugenol have on synaptic transmission at cholinergic synapses in mammals and amphibians are lacking. A few studies have investigated eugenol's actions on glutamatergic synapses at the neuromuscular junctions (NMJs) in *Drosophila* and in crayfish (Ozeki, 1975; Weineck et al., 2019; Wycoff et al., 2018). The muscle fibers at these invertebrate NMJs remained sensitive to glutamate from presynaptic spontaneous vesicular fusion events, but evoked events were blocked when exposed to eugenol. Such findings suggest an action on the nerve and not on the postsynaptic receptors by eugenol. It is not clear whether exposure to eugenol triggers

the same events in vertebrate animals at the cholinergic-type synapses.

This study was to determine if the cholinergic synapses at the frog NMJ of skeletal muscle remained responsive to spontaneous vesicular fusion events as shown in other studies with glutamatergic synapses, and if there is a reduced ability to evoke synaptic transmission when neuromuscular junction is exposed to as little 0.05 mM eugenol. Changes to the activity of the cholinergic synapses were determined by measuring excitatory junction potentials (EJPs) elicited by the motor nerve as well as the spontaneous quantal events or miniature excitatory junction potentials (mEJPs). Secondly, this study was conducted to determine if eugenol would block evoked compound action potentials (CAP) in nerves of the frog with as little as 0.05 mM. In crustaceans (i.e., crab and crayfish) exposure to 400 ppm (2.4 mM) silenced nerve activity within 2 min of exposure; however, 200 ppm (1.2 mM) did reduce activity but did not consistently stop neural activity in all preparations within the same time period (Wycoff et al., 2018).

Changes to the evoked CAPs were measured by changes in the amplitudes of the CAPs before vs. during exposure to eugenol. A third purpose of this study was to provide a novel and detailed procedure for isolating the frog cutaneous pectoris muscle for teaching purposes since this thin surface muscle is ideal to use for electrophysiological measures of synaptic responses to local anesthetics. Combined, this study is designed to be useful for researchers, instructors and students who may wish to use eugenol as a compound for studying physiological concepts and to be able to isolate the cutaneous pectoris muscle for various teaching and experimental purposes.

Methods

Model Animal

Adult, unsexed Northern leopard frogs (*Lithobates pipiens*) were obtained from a commercial supplier (Carolina Biological, Burlington, NC) in August 2018. Frogs were housed communally and fed daily with live crickets *ad lib* for up to two months before use. On the day of use, each frog was euthanized using blunt force trauma to the head immediately followed by pithing of the central nervous system at the base of the skull, as an adjunctive method. Frog animal care was approved by our institutional animal care and use committee (IACUC Protocol No. 2014-1295).

Saline

Freshly isolated frog tissues were maintained during dissection and during nerve end recordings using standard frog saline (Ringer's solution) composed of (mM): NaCl 0.11; KCl 0.33; $CaCl_2$-$2H_2O$ 0.21; Glucose 0.11; HEPES 1.0. Saline was adjusted to a pH of 7.4 using NaOH. For NMJ recordings, a modified frog saline was prepared by reducing the $CaCl_2$-$2H_2O$ to 0.105 M and adding $MgCl_2$ at 8.0 mM. This NMJ saline was necessary to reduce twitching of the muscle fibers when the nerve was electrically stimulated. Eugenol was diluted to 200 ppm or 0.05 mM eugenol in the Ringer's solutions used. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Dissections

Cutaneous pectoris muscle

Each freshly euthanized frog was pinned ventral side up in a Sylgard-lined dish (Figure 1A) so that the cutaneous pectoris muscle could be isolated. The cutaneous pectoris muscle is attached to the skin on the ventral side of the thorax horizontally in-line to the front legs. At the point of attachment to the skin, each single layer of muscle bundles makes a slight depression which can be observed by moving the skin in a caudal motion (Figure 1B). This anatomical feature can be used to locate the muscles beneath the skin.

Two different approaches can be taken to excise the skin, muscle, and xiphoid caudal attachment of the muscle. One approach is to remove the left and right cutaneous pectoris muscles together with the skin and a large section of the xiphoid attachment. A slightly more time-consuming approach is to remove one side at a time. This second approach provides more freedom to move the tissue away from the lateral side when needing to locate the nerve to the muscle. Both approaches are described below.

To remove the left and right muscle together (dissection approach 1), the skin was cut outlining the region to be removed (Figure 2A). The skin was then cut free of the connective tissue along the lateral sides (Figure 2B). The anterior attachments of the muscle to the skin were then located by carefully peeling back the skin in an anterior-to-posterior direction (Figure 3A). Next, connective tissue between the cutaneous pectoris muscle and underlying muscle was carefully teased apart with fine scissors or tweezers (Figure 3B).

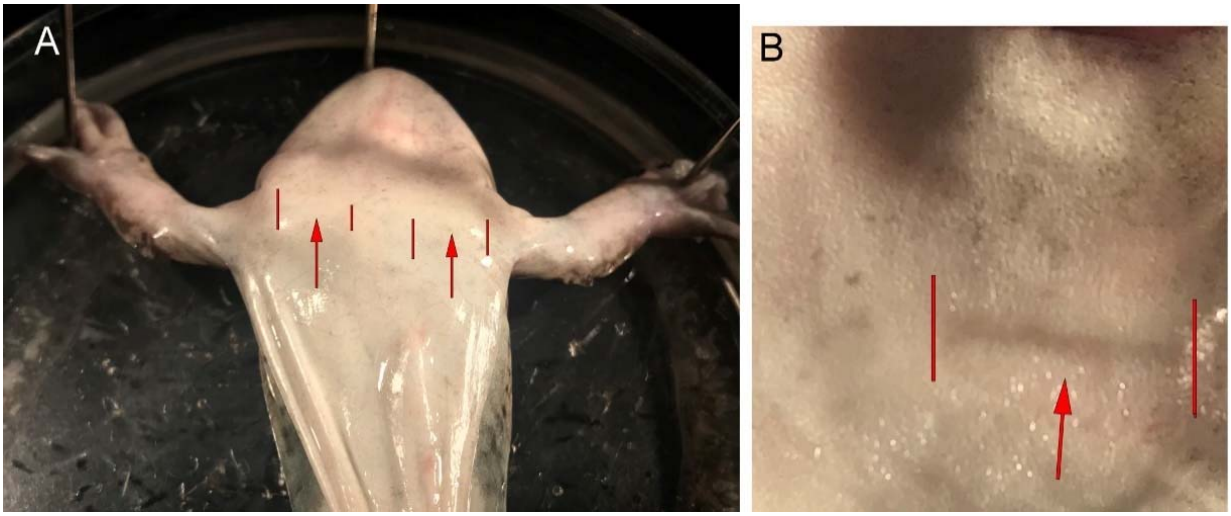

Figure 1: The location of the cutaneous pectoris muscle attachment to the skin. The dimpling of the skin in the horizontal plane between the two arms is the attachment point of the fibers to the skin. (A) The arrows between the lines indicates where the attachment occurs. (B) An enlarged view of the dimpling on the left cutaneous pectoris muscle. Lines demark the end of the lateral and medial attachments of the distal region of the muscles.

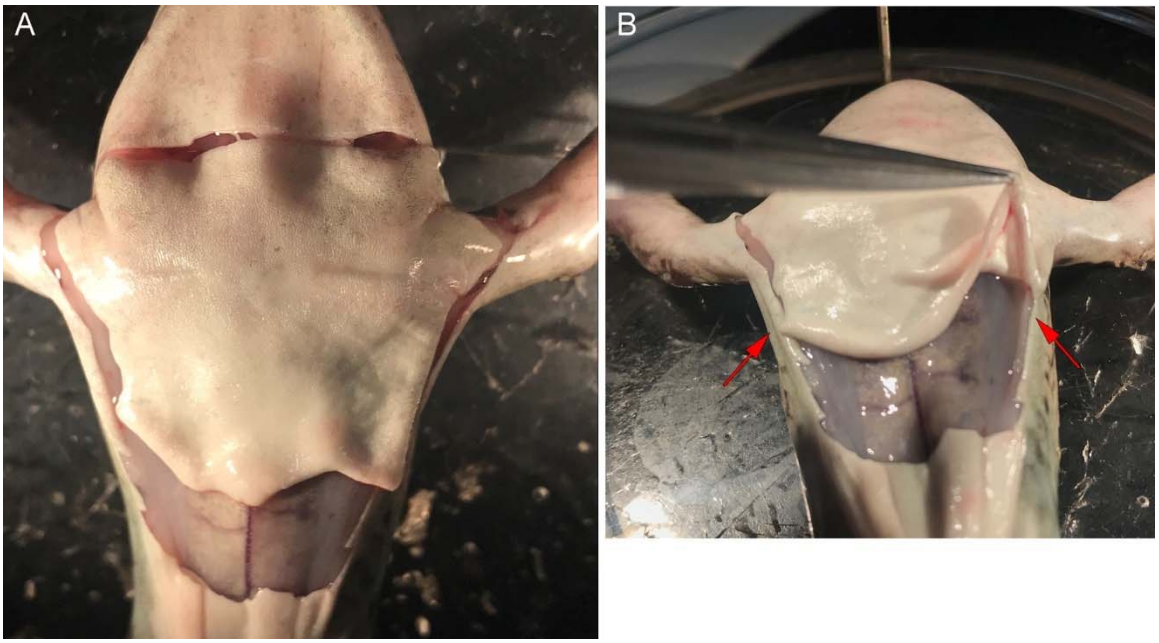

Figure 2: The outline of the dissection to remove the cutaneous pectoris muscle. (A) The skin to cut around the region to be excised from the body. (B) Illustrating the connective tissue which needs to be cut along the lateral edges of the skin to be removed. The red arrows indicate the connective tissue attachment points.

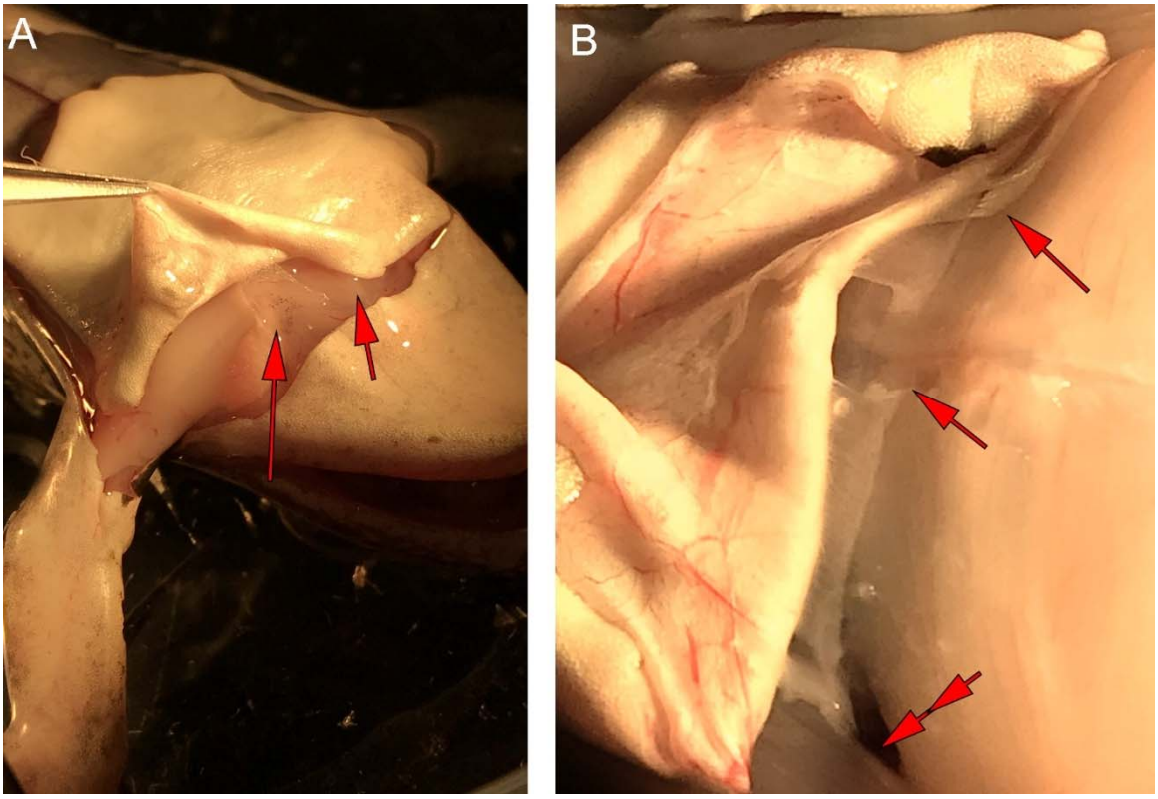

Figure 3: Isolating the anterior aspect of the cutaneous pectoris muscles. (A) The fascia and connective tissue needs to be careful cut away from the cutaneous pectoris muscles without pulling on the cutaneous pectoris muscles. Single head red arrows show fascia and connective tissue attachment points. (B) Enlarged view illustrating the connective tissue attachments. The double headed arrow illustrates the lateral muscle which is to be transected as to expose the brachial nerve.

After the anterior region of both cutaneous pectoris muscles were freed from the connective tissue, the nerve innervating the muscle was located and cut from the brachial nerve in the base of the arms. Next, the muscle attached from the base of the arm to the lateral side of the frog was cut as shown in the Figure 3B (double arrow heads) and Figure 4. Anatomically, the nerve to the cutaneous pectoris muscles branches off the large nerve bundle which proceeds to the arm. The nerve was transected at the branch point, and the connective tissue was then cut to free the nerve so it was still attached to the muscle but free of any connective tissue holding it to the body.

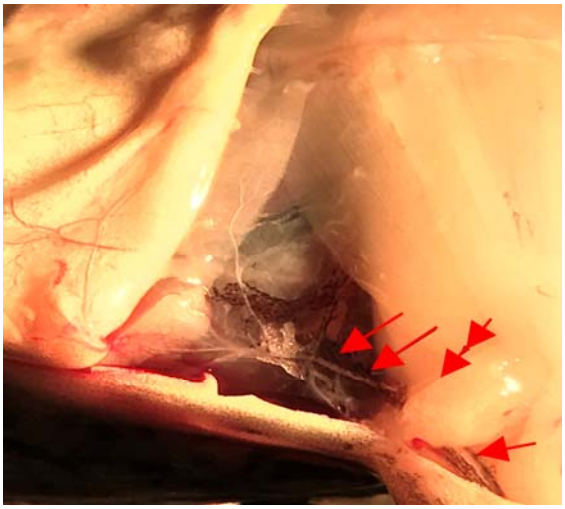

Figure 4: Isolating the nerve to the cutaneous pectoris muscle. After transecting the lateral muscle under the base of the arm the nerve bundle (arrows) to the cutaneous pectoris muscle is readily observed as well as the brachial nerve to the arm. The double headed arrow illustrates the lateral muscle which was transected as to expose the brachial nerve.

The same procedure was performed on the other side of the animal. Care was taken not to pull on the cutaneous pectoris muscle fibers so as to avoid tearing off the skin or the xiphoid cartilage. With the anterior region of both sides of the cutaneous pectoris muscle free of the body, the posterior and lateral regions of the xiphoid were trimmed away from the body. This was performed by moving the flap of skin and cutaneous pectoris muscles toward the ventral midline and cutting the tissue and muscle under the cutaneous pectoris muscle, and then cutting across the sternum (Figure 5). The same procedure was performed on the other side until both cutaneous pectoris muscles and the xiphoid with a short piece of the sternum was free and could be removed and placed in a dish with normal Ringer. After placing in a Sylgard-lined dish, the preparation was pinned out with the ventral side of the muscle facing the observer. Again, care was taken not to overstretch the muscle and not to pin or pinch the nerve bundle (Figure 6).

Figure 5: Excising the cutaneous pectoris muscle from the body. The underlying muscle, xiphoid and sternum is cut under the cutaneous pectoris muscles on both sides as shown by the series of arrows.

The muscle preparation was then split into two halves so only one preparation would be in a dish for physiological recordings (Figure 7).

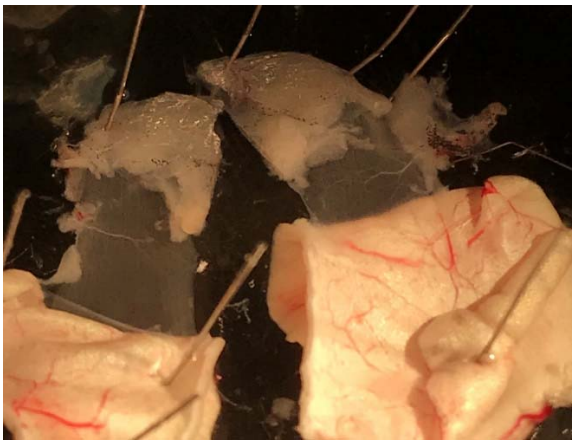

Figure 7: The isolation of each cutaneous pectoris muscle. The xiphoid and sternum are cut along the midline to provide two preparations to be used.

The second approach to removing the cutaneous pectoris muscle fibers was to remove one side at a time. This was approached in the same manner as described above by outlining the region to be excised from the body by cutting the skin (Figure 8A). However, rather than cutting around the entire perimeter of the pectoral muscles, the cut was made only on the midline. The midline was carefully cut so as not to damage the cutaneous pectoris muscle on either side. Also, when cutting the xiphoid and sternum, care was taken to ensure the cut along the midline did not damage the attachment points of the cutaneous pectoris muscle (Figure 8B). The isolated side of the cutaneous pectoris muscle was then transferred to a Sylgard-lined dish and pinned while being bathed in normal Ringer (Figure 8C).

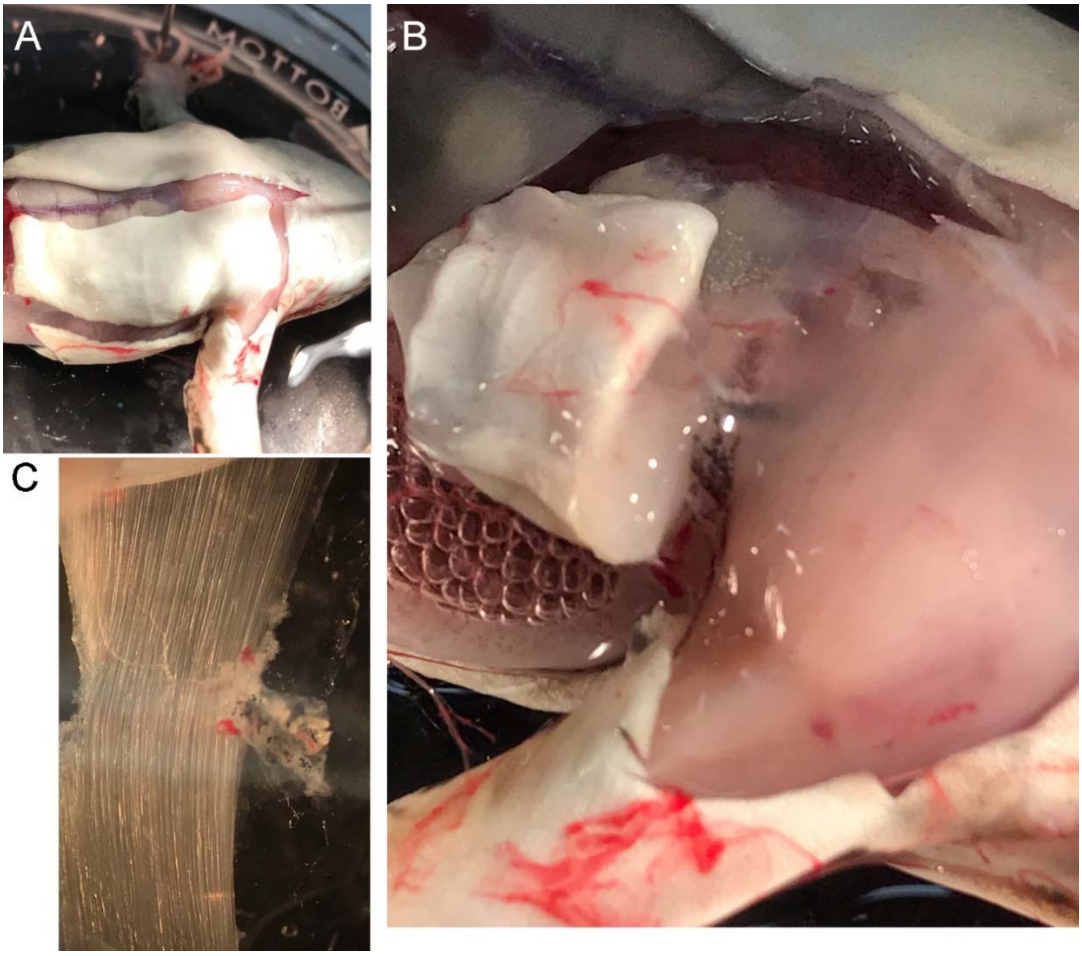

Figure 8: Excising one cutaneous pectoris muscle. (A) An outline of the skin cuts demarks the region to be excised. (B) The xiphoid and sternum are cut along the midline and underlying lateral muscles to expose the nerve and cutaneous pectoris muscle. The fascia and connective tissue is cut while peeling the skin in a posterior direction. (C) The isolated cutaneous pectoris muscle and nerve is pinned in a dish for physiological measures.

Sciatic nerve

The sciatic nerve was removed using standard procedures. Special care was taken not to pull on the nerve during the dissection and to be careful at the knee joint to cut through the tendons without pinching the nerve. This allowed us to isolate a longer viable length of the nerve, which could be excised at the ankle compared to the more traditional procedure of stopping at the knee. Also, if the nerve can be followed to the vertebra where it exists in the spinal cord, a longer proximal length of the nerve might be obtained as compared to cutting the nerve at the proximal end of the hindlimb.

Electrophysiological recordings

Synaptic measures at the NMJ

Evoked and spontaneous synaptic responses of the cutaneous pectoris muscle were measured using glass microelectrodes filled with 3 M KCl (30-40 MegaOhm resistance). It was necessary to pierce the individual muscle fibers in a parallel fashion as the electrodes did dislodge easily with the perpendicular approach.

Recordings were collected and analyzed using LabChart and LabScope

software (AD Instruments) with a PowerLab/4s A/D converter. The excitatory junction potentials (EJPs) elicited by the motor nerve as well as the spontaneous quantal events or miniature excitatory junction potentials (mEJPs) were recorded with a 1 × LU head stage and an Axoclamp 2A amplifier (Molecular Devices, Sunnyvale, CA, USA). The motor nerve was stimulated with the use of suction electrodes made from glass pipettes fitted with plastic tips to record extracellular signals from the cut nerves (details of making the suction electrodes is provided in Baierlein et al., 2011). The transected end of the nerve was suctioned into the tip of the electrode after prefilling the tip with saline. After the nerve ending was suctioned into the tip, a small amount of clear petroleum jelly was placed around the tip of the electrode to provide a tight fit for the nerve and lumen of the electrode. The tight fit allowed for a lower voltage to be used to stimulate the nerve. The conceptual details for this stimulating electrode are explained in Baierlein et al., (2011). The motor nerve was then stimulated through the suction electrode via an S88 Stimulator (Astro-Med, Inc., USA) at a frequency of once every 10 s. This allowed for evoked EJPs and mEJPs to both be recorded before and during eugenol application. The acquisition rate was 20 kHz. The recoding arrangement is illustrated in Figure 9.

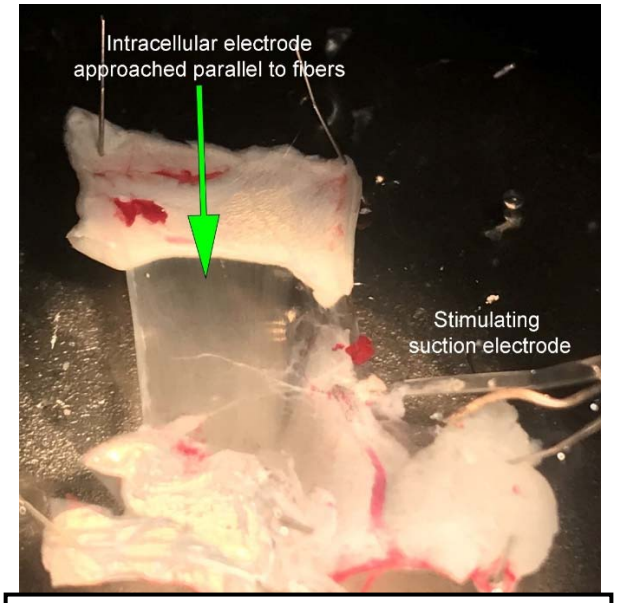

Figure 9: Set up to stimulate the nerve and obtain intracellular recordings of EJPs and mEJPs.

Compound action potential measures of the sciatic nerve

Suction electrodes were also used to stimulate the motor nerve of the cutaneous pectoris muscle and were similar to those described for sciatic nerve stimulation above. Compound action potential responses were collected by recording through the suction electrode and connecting it to a P-15 amplifier (Grass Instruments, Astro-Med, West Warwick, RI) in conjunction with a PowerLab/4s A/D converter and Lab Chart 7 and Scope 5 software (ADI Instruments, Colorado Springs, CO). Details of instrumentation set-up of equipment for use of suction electrodes for stimulating nerve bundles and recording compound action potential can be found in Robinson et al. (2011). Signals were recorded at a 20 kHz sampling rate. The recoding arrangement is illustrated in Figure 10.

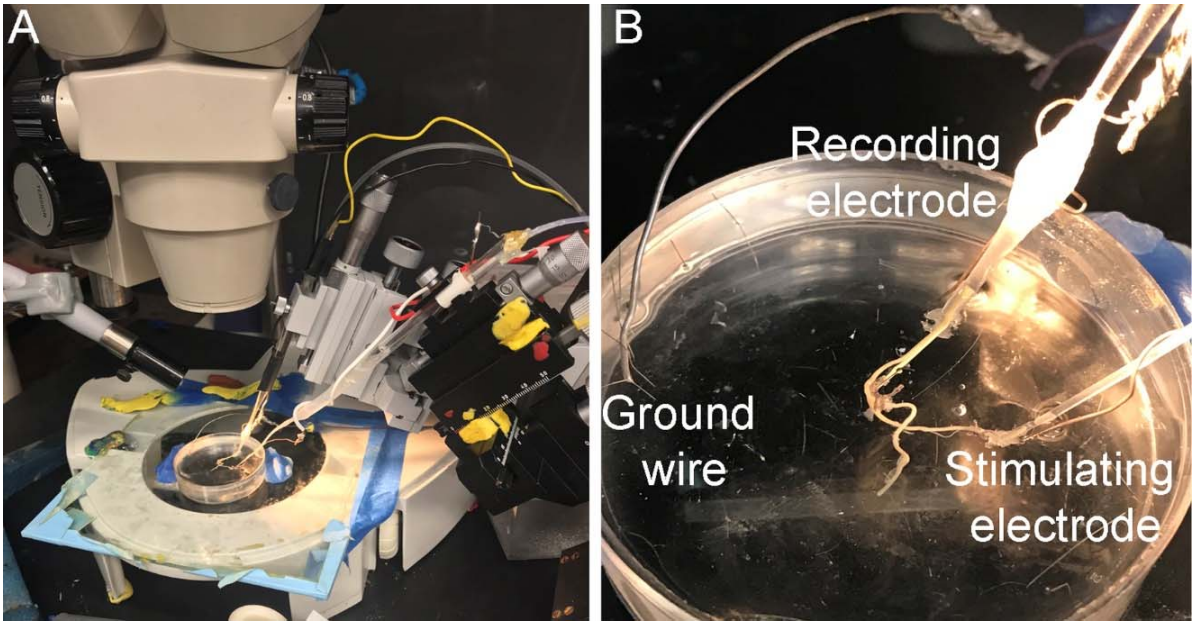

Figure 10: Set up to record compound action potentials from the sciatic nerve. (A) The recording dish filled with normal Ringer and the two electrodes with micromanipulators. (B) The recording and stimulating electrodes in place with Vaseline plugs around the nerve and the lumen of the electrodes. A ground wire is attached to the Faraday cage around the recording set up.

Statistical analysis

Data was analyzed using spreadsheet software (MS Excel). The sign test was used to identify significant differences between EJP and CAP responses before versus after exchanging control saline with the saline containing 0.05 mM eugenol. This concentration was used for comparison with the study by Tomohiro et al., (2013) in which eugenol and other compounds were tested on frog nerves. An alpha level of 0.05 was used to determine significance of the statistical test.

Results

Synaptic responses at the NMJ

Evoked EJPs were depressed in the NMJ saline; however, it was observed that the muscle still twitched slightly in this saline (*z*-value is 2.45. The P= 0.014; N=6 non-parametric Sign test). In some cases, the evoked EJP varied from a graded response to an action potential as seen in Figure 11A. Upon exposure to 0.05 mM eugenol, the EJPs amplitudes gradually decreased (Figure 11 B). The opposite response occurred when the eugenol-NMJ saline was replaced with the NMJ saline without eugenol. The EJPs gradually increased in amplitude upon washout of eugenol by the NMJ saline (Figure 12A). However, after 20 min recovery with multiple washouts (minimum 3) none of tissue preparations responses fully recovered to pre-exposure levels (n=6). As the amplitudes of the EJPs would recover so would the slight twitching of the muscle after the evoked EJPs as seen in the downward deflection after the EJPs due to an artifact of moving the tip of the intracellular electrode (Figure 12B).

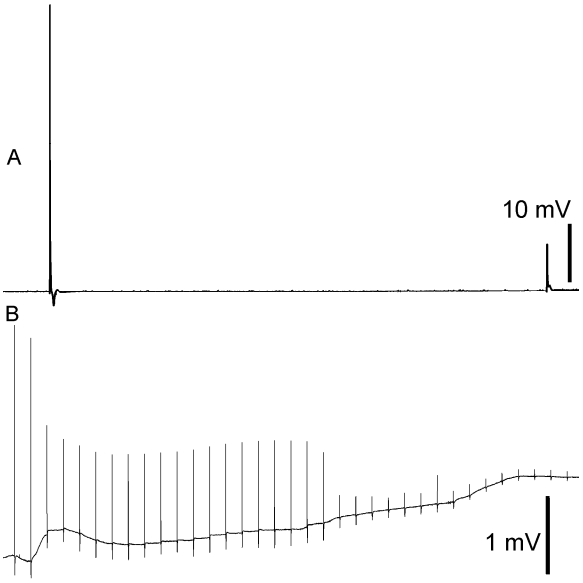

Figure 11: The evoked excitatory junction potentials (EJPs) in northern leopard frog pectoris nerve-muscle preparation before and during exposure to 0.05 mM eugenol in NMJ saline. The NMJ saline suppressed twitch of the muscle without preventing EJP activity, thereby allowing the EJP activity to be observed using microcapillary electrodes. (A) Recording shows the evoked EJPs at the threshold of producing an action potential in the muscle as compared to a subthreshold EJP. The subthreshold EJPs occurred after exchanging the saline from standard frog saline to the modified NMJ saline. The two evoked EJPs are occurring within 10 seconds of each other. (B) The decrement of the evoked EJPs upon exposure to 0.05 mM eugenol in NMJ saline. Each EJP is occurring every 10 seconds.

Seconds after exposure to eugenol , evoked EJP amplitudes rapidly depressed to the point of not being able to observe any evoked responses and only the stimulus artifact was visible.

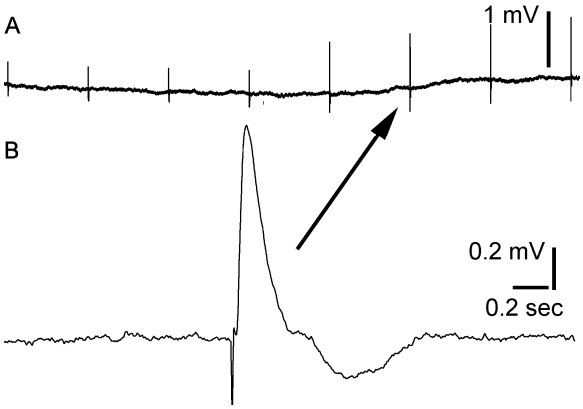

Figure 12: Recordings show evoked excitatory junction potential (EJP) recovery in northern leopard frog pectoris nerve-muscle prep following 0.05 mM eugenol exposure. (A) Recording shows how triple washout of the neuromuscular junction prep with NMJ saline without eugenol resulted in increased EJPs after eugenol exposure. Evoked EJPs were stimulated every 10 seconds. (B) An enlarged view of single evoked EJP illustrated from the trace above.

Exposure to 0.05 mM eugenol resulted in rapid depression of EJPs, but spontaneous quantal events (or mini excitatory junction potentials, mEJPs) were still visible with magnification of the baseline recording. Many mEJPs were detected along the baseline between the evoked stimuli (Figure 13).

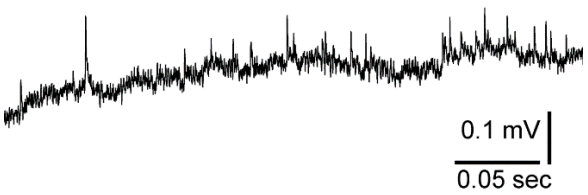

Figure 13: Recording shows many spontaneous quantal events or mini excitatory junction potentials (mEJPs) in northern leopard frog pectoris nerve-muscle prep during exposure to 0.05 mM eugenol. The mEJPs were visible during eugenol exposure when the larger excitatory junction potentials could not be evoked.

Compound action potential responses of the sciatic nerve

Maximal CAP amplitude was determined for each sciatic nerve preparation before exposure to eugenol by incrementally increasing the stimulus voltage until the resulting CAP did not further increase (Figure 14).

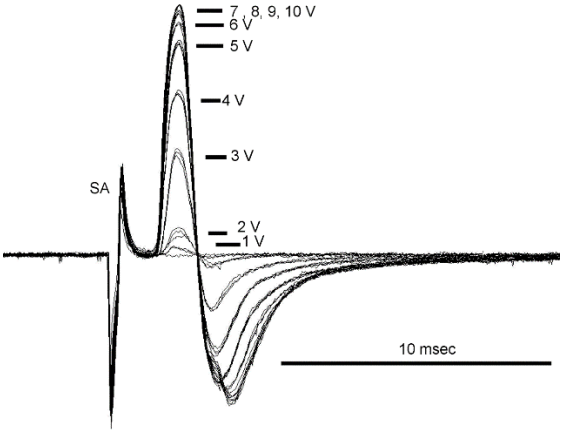

Figure 14: Recording shows the amplitude of the compound action potentials to incremental increases of stimulating voltage in the northern leopard frog sciatic nerve. The sciatic nerve was stimulated using a suction electrode at increasing voltages, beginning with 0V (not shown) through 10 V, until the amplitude no longer changed. The minimal stimulus voltage needed to evoke the maximal CAP amplitude was then to determine CAP amplitude changes before versus during eugenol exposure.

The sciatic nerve CAP amplitudes rapidly decreased within 2 min of exposure to 0.05 mM eugenol (Figure 15; *z*-value is 2.45. The P= 0.014; N=6 non-parametric Sign test). The timing in reducing the amplitudes to almost an undetectable level occurred within 6 to 10 min in a static bath containing 0.05 mM eugenol. After the amplitude of the CAP was reduced to the baseline, the eugenol was removed and replaced with NMJ saline without eugenol. Evoked CAP responses gradually returned after about 5 min and multiple washouts using standard saline without eugenol.

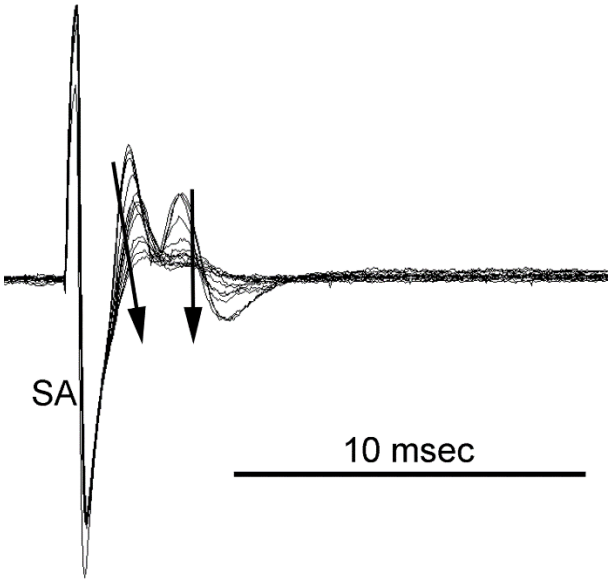

Figure 15: Recording shows changes to the amplitude of the compound action potential in the northern leopard frog sciatic nerve during exposure to 0.05 mM eugenol. Repeated recordings have been overlaid to demonstrate how the CAP amplitude decreased (indicated by the arrows) over a 6-minute exposure period. Note that multiple wave forms were visible within the CAP event; this is indicative of various neurons of differing conduction velocities firing during the CAP.

Discussion

This study demonstrated that exposure of eugenol at 0.05 mM reduced and blocked evoked synaptic transmission at the neuromuscular junction of the frog pectoris muscle but not the receptivity to spontaneous quantal events. This indicated that the nicotinic acetylcholine receptors on the muscle fibers were not blocked by eugenol. Thus, the reduced evoked responses are likely due to the lack of a sufficient amplitude in the action potential to open voltage-gated calcium channels within the presynaptic nerve terminal. It is not likely that the fusion machinery of vesicular fusion is inhibited since spontaneous quantal events still occurred in the presence of eugenol. We did initially try 0.2 mM eugenol on three preparations and the evoked EJP depressed within 30 s. However, after 20 min of

extensive washouts with eugenol-free NMJ saline, the evoked EJPs did not return. Thus, the eugenol concentration was reduced to 0.05 mM which allowed for both rapid depression of neural activity during exposure and relatively rapid recovery following eugenol washout. Since we assumed the EJPs were reduced and inhibited by the action of eugenol on preventing the initiation or blocking of conduction of action potentials along the motor nerve, the effect of eugenol on the compound action potential of the sciatic nerve preparation was examined.

The results supported the notion that the amplitude of the action potentials is reduced and eventually is not able to be evoked during exposure to eugenol. The timing in reducing the amplitudes to almost an undetectable level occurred within 6 to 10 min in a static bath containing 0.05 mM eugenol, indicating a rapid onset of neural depression. These results were comparable to those reported by Tomorhiro et al., (2013) wherein they reported a 40% reduction in the CAPs of the frog sciatic nerve after 20 min exposure to 0.5 mM eugenol. The 10X difference in effective concentration of eugenol between our study and that of Tomohiro et al. may be related to the way in which the eugenol containing salines were prepared. We used a slightly different standard frog saline containing less sodium and potassium, additional glucose, and no phosphate. The reason is that glucose is likely needed as an energy source and that this composition is well tested for frog NMJ preparations to reduce twitching of the muscle fibers (Rotshenker, 1979). Such differences may have changed the responsiveness of the nerve-muscle prep to the eugenol treatment. In either case, CAP amplitudes decreased when exposed to eugenol.

After complete depression of the CAPs in our study, the nerve was stimulated every 10 sec at a voltage to induce a maximum amplitude in the compound action potential and even increased in voltage (up to 10 V) to try to evoke responses with no avail. After replacement of the eugenol saline with standard saline without eugenol, CAP responses were more difficult to recover from the depression by eugenol for the sciatic nerve preparation as compared to the NMJ preparation of the cutaneous pectoris muscle. Even with repeated washouts with standard frog saline without eugenol, the responses of the sciatic nerve stimulation were very small after 10 and 20 min. The nerves never regained the initial amplitude of the compound action potential after 20 min of recovery. Possibly with a longer recovery time and more extensive bath exchanges a full recovery might occur as demonstrated during the study by Tomohiro et al., (2013). During recovery from eugenol in the Tomohiro et al. study, complete recovery of the frog sciatic nerve CAP was observed after approximately 20 to 30 min.

Past studies on the action of eugenol on a land snail (*Caucasotachea atrolabiata*) and crayfish (*Procambarus clarkii*) suggest eugenol acts via a dose-dependent blockage of voltage-gated sodium and calcium channels (Ohkubo and Kitamura, 1997; Ozeki, 1975; Vatanparast et al., 2017). Sodium current recordings with whole-cell patch-clamp in sensory neurons of rodents are blocked with eugenol (Park et al., 2006). In addition, it is suggested that the human N-type calcium channels are blocked by eugenol (Lee et al., 2005). The possibility that the voltage-gated calcium channels are blocked in the presynaptic terminal of the frog motor neurons would also explain the inability to evoke EJPs but considering the compound action potential of the sciatic nerve was blocked would indicate this also occurs for the motor neurons to the cutaneous pectoris muscle. Possibly both types of

voltage-gated sodium and calcium channels are inhibited by eugenol at the frog NMJ. Calcium imaging of the nerve terminals with focal electrical depolarization or depolarization with high potassium exposure in the presence of eugenol would provide a clue to if the voltage-gated calcium channels were inhibited. It also appeared the threshold of the nerve was not reduced but rather it was not able to be recruited since even higher stimulus voltages did not recruit the nerve to fire after the nerve was depressed by eugenol. The limitation for research studies is that the responses do not always recovery to baseline conditions prior to eugenol application. High concentration and longer exposures may reduce recovery to a greater degree.

Given that intracellular recording from axons of a motor neuron in a crustacean revealed a slow decrement in the amplitude of the action potential without narrowing the width would suggest that voltage-gated potassium channels were not targeted by eugenol (Wycoff et al., 2018). The findings at the frog NMJ are also paralleled at the larval *Drosophila melanogaster* NMJs (Weineck et al., 2019).

This study successfully utilized the frog cutaneous pectoris muscle as a model for vertebrate NMJ research and possible for teaching purposes. This study demonstrated the use of the sciatic nerve and cutaneous pectoris muscle for examining the effects of anesthesia on one of the many aspects of neural function. Future studies and CUREs based research could investigate the effects of eugenol at different concentrations and times of exposure and recovery. It would be of interest if higher concentrations and exposure times might be damaging to the NMJ, nerves, and muscles as to learn more about the potential effects of eugenol. Additionally, this study has provided a detailed protocol for dissection and isolation of the cutaneous pectoris muscle for future studies and for teaching purposes in physiological measures of synaptic transmission at NMJs of the frog. The protocols would likely be applicable to toads and various other species of frogs.

There are numerous iterations of experiments students can try on these preparations for gaining more insight to the actions of eugenol and its anesthetic properties. Altering the temperature during or prior to exposure may enhance or dampen the effects and the onset of effects as well as recovery from eugenol.

Endnotes

The protocols presented and suggestive nature for future experimentations by undergraduate students in a physiology course could be conducted also by a team of students. Such an approach is 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).

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