2017

THE EFFECT OF COLD ON THE PHYSIOLOGY OF DROSOPHILA LARVA HEART AND ON SYNAPTIC TRANSMISSION AT CRAYFISH NEUROMUSCULAR JUNCTIONS

Yuechen Zhu
University of Kentucky, georgeznice@gmail.com
Digital Object Identifier: https://doi.org/10.13023/ETD.2017.408

STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained needed written permission statement(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine) which will be submitted to UKnowledge as Additional File.

I hereby grant to The University of Kentucky and its agents the irrevocable, non-exclusive, and royalty-free license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless an embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student's advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student's thesis including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

Yuechen Zhu, Student

Dr. Robin Lewis Cooper, Major Professor

Dr. David Westneat, Director of Graduate Studies

THE EFFECT OF COLD ON THE PHYSIOLOGY OF DROSOPHILA LARVA HEART AND ON SYNAPTIC TRANSMISSION AT CRAYFISH NEUROMUSCULAR JUNCTIONS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences
at the University of Kentucky

By
Yuechen Zhu

Lexington, Kentucky

Director: Dr. Robin Lewis Cooper, Associate Professor of Biology

Lexington, Kentucky

2017

ABSTRACT OF DISSERTATION

THE EFFECT OF COLD ON THE PHYSIOLOGY OF DROSOPHILA LARVA HEART AND ON SYNAPTIC TRANSMISSION AT CRAYFISH NEUROMUSCULAR JUNCTIONS

Ectothermic animals are susceptible to temperature changes such as cold shock with seasons. To survive through a cold shock, ectotherms have developed unique strategies. My interest is focusing on the physiological function of during cold shock and prolonged cold exposure in the fruit fly (Drosophila melanogaster) and crayfish (Procambarus clarkii). I used Drosophila melanogaster as a model system to investigate cardiac function in response to modulators (serotonin, acetylcholine, octopamine, dopamine and a cocktail of modulators) in acute cold shock and chronic cold shock conditions as possible mechanism to regulate heart rate in the cold. To examine if the dampened heart rate in the cold could still be enhanced by modulators or calcium loading, modulators and light-sensitive channelrhodopsin proteins were utilized to stimulate the heart. This light induced cardiac activation increased heart rate in all conditions, and potentially can be used for cardiac therapy in mammals. Also, the acute and chronic cold conditioned heart showed responsiveness to the above mention modulators. In examining how synaptic transmission is influenced by acute and chronic cold, the crayfish neuromuscular junction was used as a model. This is a good model as there are high and low output synapses to be investigated. The low output neuromuscular junction was enhanced in response to acute cold. The high output nmj increased in synaptic response to acute cold. In addressing chronic cold conditions, the nmj were physiologically assayed in their response to acute warm changes as well as influence of serotonin and octopamine. In chronic cold condition, the synaptic output was varied in enhanced and dampened responses to an acute warm environment. These junctions were enhanced in their synaptic output by serotonin and octopamine (100nM). In assessing, by HPLC assay, octopamine concentration increased in chronic cold crayfish. This suggests compensation in synaptic transmission in cold acclimation possibility via endocrine responses.

Keywords: serotonin, octopamine, heart rate, optogenetics, temperature, synaptic transmission

Yuechen Zhu

June 20, 2017

THE EFFECT OF COLD ON THE PHYSIOLOGY OF DROSOPHILA LARVA HEART AND ON SYNAPTIC TRANSMISSION AT CRAYFISH NEUROMUSCULAR JUNCTIONS

By
Yuechen Zhu

Dr. Robin Lewis Cooper

Director of Dissertation

Dr. David Westneat

Director of Graduate Studies

June 20, 2017

ACKNOWLEDGEMENTS

During my study in the PhD program, I have received enormous help from people around me. The following dissertation would not be possible without their help. First, I thank the people I worked with in the Cooper lab: Dr. Josh Titlow, Dr.Zana Majeed, Dr. Cole Malloy, and Dlovan Mahmood. During my study, they have given me countless help and mind exercises for my projects. I also thank for all undergraduates I worked with in these projects: Jacob Sifers, Emily Yocom, Henry Uradu, Angel Ho and Leo deCastro.

More than that, I also thank my committee members, respectively: Dr. Brian Rymond, Dr. Doug Harrison, Dr. Esther Dupont-Versteegden and outside examiner Dr. Francisco Andrade. Each of them have provided me great insight and recommendation on my studies and dissertation. Additionally, I want to thank Dr. Dupont for letting me use her laboratory to further my understanding in research. I also thank my parents for their support along my study.

Last and most importantly, I want to thank my great and inspiring mentor Dr. Cooper. It is my great fortune in my life that I can meet Dr. Cooper as my mentor during my study. Dr. Cooper helped me greatly in the research and life as I was adjusting to my course of life. His work ethic and determination in the research truly enlightened me how to succeed in the future.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

Introduction on the physiological changes associated with changes in environmental temperature with a focus on invertebrates

1.1 Cause of temperature fluctuations: daily and seasonal

A suitable temperature is the key for the emergence of life. Our earth has traveled around the sun for the past 4.6 billion years with a tilted axis. The tilted angle causes a difference of solar energy per unit surface on Northern and Southern Hemispheres annually. The axial tilt provided a fluctuation in temperature, which is thought to allow for proper ranges in temperatures to promote the evolution of life. About 1 billion years after the earth's formation, life started to appear in earth's primitive ocean, which could buffer extremes in changing temperatures. The rest of the story is legendary. The temperature fluctuations associated with the tilted earth are referred to as seasons.

1.2 Two strategies that animals use to adjust to temperature

Over the past 3.8 billion years, two main outcomes of evolution in dealing with temperature fluctuations have arisen in animals (i.e., ectothermy and endothermy). The endothermic animal maintains a core body temperature, regardless of changing ambient temperatures; whereas, the ectothermic animal's body temperature fluctuates with ambient temperatures. However, there can be behavioral modifications to regulate body temperature to ambient environments. With both types of animals, there are unique physiological processes that have evolved for the animals to survive. The physiological means for surviving high and low environmental temperatures have unique characteristics as well as some similarities. The range in temperatures that animals have evolved is relative. For example, an artic fish (*Myoxocephalus scorpioides*) has evolved to survive in a low range of temperatures (–2 °–8 °C) (Polar life, 2017), whereas a (*Poecilia reticulata*) fish in the tropics survives in a warmer range (25.5 °–27.8 °C) (Hargrove and Hargrove, 2006). Neither fish can live in the other's environment for any extended amount of time. Thus, when one examines the effect of warm or cold environments on an organism, it is done so with regard to the range in temperatures for the normal survival of that species.

1.3 Ectothermic and endothermic animals overwintering

In these studies, I have focused primarily on the physiological effects of cold environments. To adjust to a cold temperature, endothermic animals developed strategies that can mainly fall into two categories: leave or stay. Thus, the animals are hibernators or migrators. The ectothermic animal that experiences overwintering may use hibernation, or migration, or freeze tolerance. In my

study, which incorporates ectothermic animals, I focus on the effects of these animals being exposed to relatively cold environments for the model species in question.

For ectothermic animals, temperature is important as it not just affects their survival but is also a factor on their lifespan. The lifespan may be related to the metabolic rate of the animal, thus this can be varied with temperature. In general, the cold environment lowered metabolic rates. For example, lower ambient temperatures will lead to a longer lifespan in some *Drosophila* species (Loeb and Northrop, 1917; Ragland and Sohal, 1975; Speakman, 2005). Therefore, one of the outstanding advantages of ectothermic animals is their ability to endure decreasing temperatures and wait for a more suitable environment, by allowing the metabolic rate to decrease to conserve energy from having to try to maintain a constant temperature. Of course, there are ecological disadvantages to this approach such as predation.

1.4 Insects and crustaceans overwintering

As we mentioned above, some insects use migration as an attempt to completely avoid the cold environment as a strategy. For example, each year the butterfly (*Danaus plexippus*) migrates from north of the United States and Canada to southern aspects of the United States and northern Mexico (Brower, 1977; Altizer et al., 2015). However, other insects that do not migrate to avoid the harsh cold temperature have developed mainly two other strategies to adjust to freezing cold temperatures. One is freeze avoidance, and the other is freeze tolerance. Freeze avoidance is an approach to limit ice formation inside an organism, which prevents lethal freezing. Freeze tolerance is a way to survive below the freezing point. Researchers believe it is achieved through the animal's use of antifreeze proteins and/or raising intracellular osmolality to limit ice formation in the intercellular space. Therefore, ice formation can be limited.

Many Insect species also have a diapause stage to endure harsh winter weather. Diapause is a suspended development period in insects during unfavorable environmental conditions. Before entering a diapause stage, a series of mechanisms are activated to help allow the organism to cope with the cold environment. In this process, cryoprotectant substances like glycerol and glucose may be synthesized in large amounts. The membrane lipid composition may also change. Several protein chaperones can be produced that are similar to heat shock proteins. An example of this form of adaptation is the Gall fly (*Eurosta solidaginis*) (Lee et al, 1995).

Another mechanism some organisms use is rapid cold hardening (RCH). This mechanism is a quick response to cold that permits animals to survive longer bouts of cold (Lee et al, 1987). In fruit flies and flesh flies, this mechanism has been studied extensively (Teets and Denlinger, 2013). The cellular mechanism generally involves rapid accumulation of cryoprotectants such as sorbitol and/or glycerol. The functions of these chemicals are to stabilize the membrane

proteins in the cells and organelles. Sugars, such as trehalose, and amino acids, such as proline, are also recognized to function as cryoprotectants (Withers and King, 1979; Bhandal et al., 1985; Tsai et al., 2017).

Another example of RCH is the tiny Antarctic nematode, more commonly known as a round worm (*Panagrolaimus sp.* DAW1). This organism is originally from Antarctic coast at McMurdo Sound. It is considered one of the best examples of an organism with the ability to survive through freezing temperatures. The key to surviving freezing is by adapting cryoprotective dehydration, which eliminates all water content to prevent intracellular freezing (Thorne et al., 2017). In exploring gene expression patterns, researchers were able to show what biochemical pathways were still active in the nematodes while in a frozen state. Genetic expression analysis for *P.sp.* DAW1 undergoing freezing state shows during the freezing process there is high production of cuticle formation relevant genes and protease genes. This study highlighted the key genes enabling the organism to endure such an extreme physical state. This the first study of its kind to shed light on a possibly rare adaptation that could lead to new applications in understanding surviving cold exposure for long periods of time. Teets and Denlinger (2013) recent published a nice review of the various mechanisms of seasonal cold hardening and rapid cold hardening (RCH).

1.5 Focus of this study

In this dissertation, I focus primarily on the physiological conditions that occur with acute and chronic cold environmental effects on two model invertebrate preparations. These models are both arthropods (*Procambarus clarkii* and *Drosophila melanogaster*). Considering there are many species of crayfish and *Drosophila* as well as various other animal species that naturally live in different temperature ranges, I chose these two specific species that are convenient to work with. They also have some practical economic and research specific applications. In addition, these two species are naturally exposed to temperatures that occur in the range of 15 ^{o} to 30 ^{o}C (CABI, 2017) for the crayfish (*Procambarus clarkii*) and from 15 ^{o} to 30 ^{o}C (Maguire et al, 2014) for the fruit fly (*Drosophila melanogaster*). These temperatures can also be readily simulated for experimental purposes in a laboratory.

In using these two animal models, I have to also consider their behavioral abilities to avoid cold and hot environments. Both species show a behavioral preference to a range of temperatures. This crayfish species tends to prefer 21.8 ^{o}–25.9 ^{o}C (Tattersall et al., 2012) and *Drosophila* prefer near 18 ^{o}C (Dillon et al, 2009). In addition, the ability to seek altered thermal environments is quite different for crayfish and *Drosophila* and depending on the developmental stage. Juvenile crayfish, as well as adults, can basically swim (tail flip) or walk to a new location in a body of water. Both juvenile and adult crayfish can dig burrows, although a large crayfish is able to dig a deep burrow and overcome a harder substrate due to its strength. In addition, traveling on land by walking,

a larger crayfish is able to cover a larger distance in a shorter amount of time as compared to a smaller one. In considering body mass to surface area, a small crayfish is affected quicker in temperature throughout the entire body than a larger crayfish. As for *Drosophila*, the adult stage is able to fly to various open locations and even physical structures such as barns, houses and protected locations in leaf matter or within crevasses in trees or even in the ground. The larval forms of course are not able to travel as quickly as an adult and are thus subject to a solid media (dirt, detritus, fruit, and possible on the surface of an animal) for the most part. However, being buried in a medium or even in a decaying carcass can provide an altered temperature from the external environment. So, the surrounding air or water temperature or wind chill is not fully an indication of what the larvae or adult *Drosophila* or crayfish maybe experiencing. Thus, insects, which normally could not survive a cold exposure, can appear rapidly after cold weather due to their ability to be protected in some locations that do not obtain the same condition as air or ground surface temperature. Insects in general have also known to be captured in a jet stream or wind and travel 1000s of kilometers to new locations (Fang, 2010). Likewise, human transport of items from the water, fruit, and other items in the ballast of a ship is a well-established mechanism for the transport of insects and crustaceans to new non–native environments. These environments have differing seasonal temperatures than what they normally have experienced. Such transport can introduce invasive species to new environments (Hulme, 2009).

The manner in which ectothermic animals survive short or long cold exposures and still have their organs function during cold exposure or even after cold exposure without damage is an interest of mine. I am convinced that better understanding of other animals, such as invertebrates, that can handle cold exposure in short- and long-term conditions may help researchers use cold exposure to help humans for various therapies (i.e, cold induced coma, ice therapy for tissue injury, and nerve block) and medical breakthroughs such as organ preservation. As a start to undertaking the investigation of the function of organs during cold exposure, I focused on the heart of larval *Drosophila*. Chapter 2 of this dissertation focuses on a project investigating the effects of modulators on the heart of larval *Drosophila* exposed to acute and chronic cold conditions at a temperature of 10 ^{o}C. The modulators used in this study are serotonin (5-HT), acetylcholine (ACh), octopamine (OA), dopamine (DA), and a cocktail of these modulators (Zhu et al., 2016a). In a follow up study, I investigated the ability to drive the larval heart at 10 ^{o}C and at 20 ^{o}C with ionic manipulation through activating transgenetically expressed channel rhodopsin proteins with light (Zhu et al., 2016b), which is presented in Chapter 3 of this dissertation.

1.6 Temperature affects development in Drosophila

The thermal environment is critical for development in ectothermic animals. The developmental time is dependent on temperature. In a recent study, flies developed from larva to adults in 7.5 days at 29.5 ^{o}C or 49 days at 12 ^{o}C (Schou et al., 2017). If an insect is exposed slowly to cold conditions, various metabolic processes are able to function to help the organism survive and potentially continue to develop in the cold. In the *Drosophila* species *Chymomyza costata*, it was shown that sugars, polyols, and free amino acids are important in long-term cold acclimation. Sugars such as trehalose and glucose can serve as energy stores and as osmotic regulators to reduce freezing. Proline may also act as an osmotic regulator, which was also suggested to be the case for a beetle (*Alphitobius diaperinus)* and *Drosophila melanogaster* in cold conditioning (Koštál et al., 2012). In addition, an increase in the important polyol, glycerol, is correlated with cold acclimation to help maintain survival.

1.7 Crustaceans

In crustaceans, overwintering may occur with behavioral and physiological changes. In progressive and slow cold exposure, crustaceans such as crayfish may seek out a means to avoid the cold by burrowing. In spite of attempting to avoid the cold environment, the animals may still become colder. Various ecological factors are associated with cold, which can impact the behavior of crayfish through means such as reducing food availability, thermoclines from snow or ice melt, and alterations in diet and types of predation. In general, crayfish being exposed to colder environments over a winter results in reduced movement (i.e., a form of exercise) that by itself can have physiological consequences in maintaining skeletal muscle mass and neuronal function. Inactivity induced by cold is a compounding experimental factor and is difficult to control for the multivariable. Thus, measuring physiological mechanisms to account for the differences in acute and chronic cold exposure are complicated (e.g., comparing only the blood or hemolymph borne factors or gene expression and protein levels without measures of physiological function).

A previous study in using a prawn (*Macrobrachium rosenbergii*) native to warm environments (26 ^{o}–32 ^{o}C) (CABI, 2017) and the crayfish (*Procambarus clarkii*) was conducted to compare their cold temperature tolerance (lower than 10 ^{o}C). These animals were maintained at 21 ^{o}C and then rapidly exposed to 5 ^{o}C. Only the crayfish survived such a rapid change, whereas the prawns died within 2 hours. The prawn and crayfish were also conditioned to a gradual chronic cold exposure. The prawns all died at 11 ^{o}C by the second week; however, the crayfish survived well at 5^{o}C for 3 weeks (Chung et al., 2012). Additionally, all the crayfish were able to maintain responsiveness to outside stimuli at 5 ^{o}C. This clearly demonstrated that the nervous system (sensory, CNS, and motor nerves) as well as their neuromuscular junctions and muscles were functional at acute and chronic cold temperature exposures of 5 ^{o}C.

Procambarus clarkii (commonly referred as the red swamp crayfish) is a species native to the southern United States and northern Mexico. The territory of this species has expanded over the past decades. It can now be found at different temperature regions from the Great Lakes to the Scandinavian Peninsula. Historically, this species of crayfish has been an excellent neuroscience model for teaching in high schools and for food production. Thus, this species has spread around the world for various reasons with people in different locations raising them for these reasons. Also, this particular species has been used due to its ready availability and hardiness for shipping and housing in various locations from laboratories to outdoor environments.

The striking differences in the abilities of these two crustacean species in surviving a cold exposure are interesting. The differences could be a plethora of cellular, biophysical, and biochemical reasons. On the cellular level, the cell has to be able to sense the temperature to be able to respond to it. This cellular sensing mechanism can happen in DNA, RNA, and at the protein level. Thus, the response could involve transcription, translation, protein conformational changes, and bilipid membrane reconstructing.

1.8. Cellular sensing of temperature

On the cellular level, some cells have their own sensor and response mechanisms to lower temperatures. The responses can be complex as transcription, translation, protein refolding process, and membrane reconstructing. These may occur at various time scales and in additive and possible synergistic ways (Klinkert and Narberhaus, 2009).

The DNA acts as a thermosensor by its topology in three ways during cold shock. First, cold shock can cause increased negative supercoiling. Secondly, at low temperatures DNA bending will improve affinity for the RNA polymerase. Thirdly, DNA topology and binding affinity of histone-like nucleoid structuring protein (H-NS), a silencer of nucleoid proteins, is altered (Klinkert and Narberhaus, 2009; Phadtare and Severinov, 2010).

RNA is also considered well suited to have a role as a post-transcriptional intracellular thermosensor. The general mechanism is cis and trans conformational change. The cis acting RNA thermometers are based on the Shine-Dalgarno (SD) sequence and AUG start codon in 5'UTR of mRNA, which is base paired at low temperature. When temperature increases, this structure melts and allows binding to a ribosome (permitting the initiation of translation) (Narberhaus et al., 2006). In the cold shock group, the expression of cold shock protein is controlled by secondary structures in the 5'UTR. The trans acting RNA thermometer mechanism via sRNA forms a stable secondary structure that melts at higher temperatures. When the temperature increases, the unwound sRNA pairs with the anti-SD sequence in the 5'UTR region of mRNA to expose a translation initiation region (Lybercker and Samuels, 2007).

Proteins can also work as thermosensors, because their structures can be altered by temperature changes. Temperature changes can cause a protein to change from an active conformation to an inactive conformation or possibly induce disassembly of dimers (Hurme et al., 1997). These protein sensors can include transcriptional regulators, chemosensory proteins, chaperones, and proteases (Winter and Jakob, 2004). One example is RheA. RheA is a specific repressor of small heat shock gene hsp18, and it binds to the hsp18 promotor at low but not high temperatures. Therefore, at high temperatures the RheA repressor protein conformation changes and it no longer binds to the hsp18 promotor region, allowing the initiation of hsp18 transcription (Servant and Mazodier, 2001).

The membrane composition, particularly of the lipid component, changes in response to temperature changes. Such changes can take time as synthesis and incorporation into the bi-lipid membrane is a detailed process. The changes in lipid composition in response to temperature have been extensively studied in bacteria. There are various mechanisms used for cells to maintain fluidity of the membrane. The decreasing temperature can cause increasing proportions of unsaturated fatty acids like cis-vaccenic acid, which has a low melting point and high flexibility. Cis-vaccenic acid is synthesized from palmitoleic acid mediated by the cold shock activated enzyme β-ketoacyl-acyl carrier protein (ACP) synthase II. In some studies of bacteria, desaturases were shown to play an important role in altering the degree of saturation of fatty acids in membrane phospholipids (Aguilar et al., 1998). The reduction of temperature also caused a reduction in the average chain length of fatty acids (Suutari et al., 1997).

The major consequence of temperature at a cellular level can be a decrease in membrane fluidity affecting membrane associated cellular functions such as active transport and protein secretion, changes in nucleic acid structures, and reduced ribosome function leading to decreased translation and inefficient protein folding. One of the hallmarks of expression for cold stress in cells is the action of cold shock proteins. Cold shock proteins are a group of proteins that are thought to aid cells in survival through temperatures lower than their optimum temperature. Cold shock proteins contain RNA binding domains. A subgroup of RNA binding domains is also called the cold shock domain. These proteins have long conserved evolutionarily existence thoughout bacteria, plants, and animals. The cold shock proteins are considered to function as RNA chaperones and facilitate transcription and translation (Jiang et al., 1997). One example is CspA. It is the main cold shock protein in *E. coli*, and its homologs in other organisms act as RNA chaperones. CspA can melt the secondary structures in nucleic acids, which facilitates transcription and translation at low temperatures. The expression of CspA is first and most abundantly induced by cold shock. It can bind to ribosomes converting them to cold-adapted ribosomes that can translate non-cold–shock mRNAs (Jiang et al, 1997).

1. 9 The effect of cold on the nervous system in insects

In insects, the consequences of a lower temperature resulting in physiological changes in altering osmolality and ion composition can have an effect on neurons. The brain and large isolated nerves are protected from direct exchange of solutes with the hemolymph by the blood-brain barrier. This division helps to maintain a microenvironment that is different from the environment in the hemolymph. However, the ionic changes in the hemolymph or experimentally in a culture media can have a rapid effect on neuronal function. The changes of sodium, potassium, and calcium in the extracellular fluid around neurons can potentiate the local effects of cold exposure (MacMillan and Sinclair, 2011).

How a nervous system functions with temperature fluctuations is important in understanding the effects of chill coma and chill injury for mammals as well as invertebrates. In a study of semi-intact preparations from locusts, chilling was associated with rise in extracellular potassium in the nervous system (Rodgers et al., 2010). A rise in potassium generally depolarizes cells and alters their function. Neurons also are under influenced by circadian activity in terms of temperature cycles. Temperature fluctuation also can reset the circadian clocks by influencing certain sets of neurons as shown for the dorsally located neurons and lateral posterior neurons in *Drosophila melanogaster* (Miyasako et al., 2007). Synaptic sites in photoreceptor terminals of the house fly (*Musca domestica*) are known to be affected by temperature (Brandstater and Meinertzhagen, 1995). Synaptic sites in photoreceptor terminals can disappear within 2 to 3 hours during cold shock and reappear after 20 hours in recovery. Cold also has an effect, which is reversible, on epithelial glia cells in the organism by hypertrophy of the mitochondria.

1.10 The crayfish preparation for investigating the effect of cold

For the nervous system to be functional, the synaptic transmission is the critical step for constructing functional neuronal behavior. In the endothermic animal, the body temperature will remain at constant physiological temperature; the synaptic transmission is less being affected by the temperature changes. However, in my study I emphasize how synaptic transmission is being effected by cold temperature while still maintaining functionality in an ectothermic animal. In the early studies of neuroscience, temperature was known to alter synaptic transmission function in ectothermic animals (Katz and Miledi, 1965). Early studies on temperature effects of synaptic transmission focused on using the giant axon of the squid. The lowering the temperature also affected the calcium current in the presynaptic terminal. A lowering in temperature decreases presynaptic calcium current. In 1978, Llinás et al. found two main effects of temperature on the postsynaptic response. Decreasing from 15 ^{0}C to 5 ^{0}C increased synaptic delay and decreased peak amplitude of the excitatory postsynaptic potential (EPSP) in giant axon in squid. However, while the calcium current in the presynaptic terminal is being affected by temperature

change, it was not linked to be directly responsible for the EPSP amplitude changes in the postsynaptic terminal (Llinás et al., 1978). In a study by Weight and Erulkar (1976) they also demonstrated as temperature increases the EPSP amplitude increased for the squid synapses. However, this study failed to mention the initial acclimated temperature of squid.

Besides giant axon of the squid, electrophysiology studies have used the NMJ in other crustaceans to address the effects of cold and warm temperature on synaptic transmission in ectothermic animals. In crayfish, it was shown that as temperature increased there would be a decrease in synaptic delay and an increase in EPSP amplitude in the muscle fiber would occur (Charlton and Atwood, 1979). The EPSP of the closer muscle from crayfish (*Astacus leptodactylus*) which were acclimated to 12 ^{0}C showed an increase in the EPSP amplitude as temperature was increased up to 20 ^{0}C and then the EPSP amplitude dropped rapidly with higher temperatures. However, the tension of closer muscle increased as temperature decreased (Harri and Florey, 1976), which appears paradoxical since the EPSP amplitudes are smaller at cold temperature. The closer muscle from the Hawaiian ghost crab (*Ocypode ceratophthalma*) acclimated to betweem 26 ^{0}C to 27.5 ^{0}C (Florey and Hoyle, 1976) had the maximal EPSP amplitude and muscle tension between 22 ^{0}C and 28^{0}C. These parameters begin to decline below 22^{0}C or above 27^{0}C. Fischer and Florey (1981) demonstrated in the opener muscle of crayfish (*Astacus leptodactylus*) acclimated to 10 ^{0}C that the tension increased as temperature decreased. This study also reported that the membrane potential became more negative as temperature increased from 5 ^{0}C to 25^{0}C and that the EPSP amplitude increased as temperature decreased, which was different from other crayfish species (Charlton and Atwood, 1979). Harri and Florey (1978) examined the effect of acclimatization of crayfish (*Astacus Leptodactylus*) to 12 ^{0}C and 25^{0}C and showed that the EPSP amplitude was maximal at the acclimated temperatures of the whole animal. In addition, the muscle tension shifted toward the acclimated temperatures. These findings imply plasticity in synaptic function and muscle contraction to function best at the acclimated temperature of the whole animal for this particular species of crayfish. However, such physiological acclimatization to cold is not possible for other crustaceans such as the prawn.

A study by Stephens and Atwood (1982) on the Shore crab (*Pachygrapsus crassipes),* which hyperpolarized with increases in temperature and with increases in the EPSP amplitude is increased initially to a maximum value that is close to their acclimation temperatures. It decreases with higher temperature. In terms of tension development, the maximal muscle tension of the cold conditioned crab was at 8 ^{0}-16 ^{0}C and tension dropped as temperature increased. In the warm conditioned crab, the maximal muscle tension was at 13 ^{0}–20 ^{0}C and tension dropped as temperature increased.

Crayfish in general have been long used as a model to study physiological questions due to the ease in maintaining viable preparations of tissue in a

relative simple saline of basic salts. There are several advantages of using crayfish for investigating neurotransmission at the neuromuscular junction (NMJ). There are various NMJ preparations, which are easy to dissect and quantify synaptic transmission (Dudel et al. 1961a,b, 1963, 1965). The large size and identifiable individual motor neurons allow for experimental conditions not possible in other invertebrate preparations, which are better as genetic models such as *Drosophila melanogaster*. The innervation at the NMJs mimics the central type of synaptic physiology for mammals in that the postsynaptic EPSP are graded and glutamate is the neurotransmitter. The opener muscle in crayfish is one of the common NMJ preparations, since it is easy to dissect and the excitatory nerve is large enough to carry out intracellular recordings. The terminals of this nerve on the muscle allow one to use focal macropatch electrodes over defined regions of the terminals to quantify single vesicular events for quantal analysis. This NMJ preparation is viable in crayfish saline for hours while maintaining consistent synaptic responses. There is also a significant amount of background literature to draw from on the low output synapses in the innervation at the opener NMJ for comparative purposes. In addition, the high output NMJs of the abdominal extensor have been well characterized in this species of crayfish, but not in regard to alterations in synaptic transmission due to temperature changes.

In an effort to summarize the various studies in the literature using crustaceans and the squid, a composite in the findings is presented in Table 1.

Table 1.1 Summary of various studies on synaptic transmission with temperatures

Species	Acclimation	Muscle	EPSP amplitude as temp rises	Tension as temp rises	Membrane potential as temp rises	Citation
Crayfish (*Procambarus Clarkii*)	15 C	deep abdominal extensor muscles	Increase	N/A	N/A	Fredrich et al., 1994
Squid	N/A	Giant axon	Increase	N/A	N/A	Weight and

(*Loligo pealii*)						Erulkar, 1976
Crayfish (*Astacus Leptodactylus*)	12 C	Closer muscle third walking leg	Increase peak at 20 C then decrease	Decrease	Hyperpolarization	Harri and Florey, 1976
Hawaiian Ghost Crab (*Ocypode Ceraphthalm*)	26 C	Closer muscle third walking leg	Peak at 22 to 27 C. Decrease lower than 22 C or higher than 27 C	Same	Hyperpolarization	Florey and Hoyle, 1976
Crayfish (*Astacus leptodactylus)*	10 C	opener muscle of third walking legs	Decrease	Decrease	Hyperpolarization	Fischer and Florey, 1981
Crayfish (*Astacus Leptodactylus*)	12 C	Closer muscle of walking leg	Increase and peak at 12 C then decrease	Peak at around 12 C then decrease	Hyperpolarization	Harri and Florey, 1978
Crayfish (*Astacus Leptodactylus*)	25 C	Closer muscle of walking leg	Increase and peak at 25 C then decrease	Increase until around 25 C then decrease	Hyperpolarization	Harri and Florey, 1978
Shore crab (*Pachygrapsus crassipes)*	12 C	Stretcher muscle from walking leg	Increase and peak at 12 C then decrease	The maximal tension is lower shifted close to acclimation	Hyperpolarization	Stephens and Atwood, 1982

				temperature		
Shore crab (Pachygrapsus crassipes)	25 C	Stretcher muscle from walking leg	Increase and peak at 25 C then decrease	The maximal tension is higher shifted close to acclimation temperature	Hyperpolarization	Stephens and Atwood, 1982
Lobster (*Homarus Americanus*)	6 C	dactyl opener muscle	Decrease until around 6 C then increase and peak around 17 C then decrease	Decrease	Hyperpolarization	Hamilton et al., 2006

1.11 Mechanisms that can alter synaptic transmission during cold exposure

Synaptic efficacy is related to the vesicle fusion process and the number of quantal events is known to vary with temperature in crayfish (Johnson and Wernig, 1971). This is also the case in rodents. The relationship between vesicle pool depletion and the speed of endocytosis relative to exocytosis was shown to be slower at lower temperatures in the CNS of a rodent (Fernandez-Alfonson and Ryan, 2004). Not only the rate of vesicle recycling changed, but also the type of exocytosis was altered with changes in temperature. At 12 ^{0}C, the kiss-and-run endocytosis was responsible for up to 35% vesicle fusion. However, at 28 ^{0}C the percentage dropped by ½ (Zhang and Jackson, 2008).

In the crustaceans, the motor nerve is classified (in vertebrates) as a tonic or phasic motor neuron due to its association with the phenotypic muscles. The phasic motor neurons and muscles are generally associated with fast-twitch muscles and producing large EPSP. In the crayfish, the abdominal fast-twitch muscle is mainly responsible for tail flips related to escape functions. The opener muscle in the walking leg and chelipeds in crayfish is innervated with a tonic-like motor nerve; however, the innervations produce regional differences and are associated with variation in muscle phenotype (Mykles et al., 2002).

The associated tonic NMJs have a low mean quantal content but can facilitate with increased stimulation frequencies and are generally fatigue resistant. This is due to a low number of primed and docked synaptic vesicles, with a large number in the pool of reserve vesicles in the presynaptic terminal. Whereas, the phasic NMJs produce large EPSP and fatigue relatively rapid as compared to tonic NMJs. The rationale for the differences in tonic NMJs is that there are more primed and docked synaptic vesicles in terminals of phasic neurons, with a lower number of reserved vesicles in the reserve pool. Thus, more vesicles fuse at phasic NMJs and are not able to recycle fast enough with high frequency stimulation. This results in a decrease in the EPSP amplitudes, which relates to fatigue in the phasic motor units (Atwood and Cooper, 1995, 1996; Logsdon et al., 2006; Wu and Cooper, 2010, 2012)

There are numerous possibilities as to how cold exposure can alter synaptic transmission from effects such as recruiting the motor nerves, electrical conduction, depolarization of the presynaptic terminal, and Ca^{2+} handling; to vesicle dynamics as well as the postsynaptic response of the glutamate receptors in depolarization of the muscle fibers. The effect of muscle contraction and force development is also a factor for the net output of the motor unit function.

The motor unit and muscle may maintain or offset the effects of cold by modulation to maintain an ability to heighten synaptic responses and muscle function. Modulation may occur by changes in ionic composition of the hemolymph as well as peptides and hormones, such as biogenetic amines like 5-HT and OA (Friedrich et al., 1994; Colton and Freeman, 1974). Modulators such as 5-HT,OA, and DA are well known within crustaceans and insects as being important for having effects on neuronal, cardiac, GI, respiratory, and skeletal muscle function (review by Shuranova et al., 2006; Strawn et al., 2000; Listerman et al., 2000; Cooper et al. 2011a, b). It is known that DA, 5-HT and OA rise in the hemolymph during exercise in crabs (Sneedon et al., 2000). OA is also known to increase in insects with locomotive activity (Goosey and Candy, 1980; David and Coulon, 1985). These modulators are generally thought to serve as a stress response in invertebrates (Pagé et al., 2007; Even et al., 2012). However, many aspects of how modulation alters the neuromuscular junction transmission with the effect of variation in temperature are not clear yet. Little is known about how temperature may alter the sensitivity of neuromuscular systems to endogenous neuromodulatory hormones. It was demonstrated on the *Drosophila* larvae heart that 5-HT has a strong excitatory effect in increasing the larval heart rate for acute and chronic cold exposed larvae (Zhu et al., 2016a). One paper addressing a modulatory action demonstrated in crayfish (*Procambarus clarkii*) that the neuropeptide DF_2 modulation is temperature- dependent (Friedrich et al., 1994). The authors suggest that DF_2 helps the crayfish to compensate for its loss in synapse efficacy. The DF_2 increased the EPSP amplitude by 300% at 5 ^{0}C and at 20 ^{0}C it increased the EPSP amplitude by 50%. Hamilton et al. (2006) demonstrated 5-HT modulation of the lobster opener muscle with cold. This study reported that 5-HT increased the EPSP amplitude at 2 ^{0}C by 30% and increased the tension by 800%, while the control had no tension development at 2 ^{0}C. This

study also reported that 5-HT increases both EPSP amplitude and size of neural evoked relaxations. In addition, they reported 5-HT increases resting muscle tension in cold (2 ^{0}C) temperature. Thus, modulation of evoked contractions by 5-HT is temperature dependent and even resting muscle tension is 5-HT dependent. However, this earlier study did not report if 5-HT increases in the hemolymph by cold acclimatization.

Thus, in parallel with my studies in addressing the effects of cold on synaptic transmission at the crayfish NMJ, I sought out to examine the effects of exposure to 5-HT in addition to the changes in temperature.

1.12 Specific aims

The main objectives of this dissertation are:

1. To investigate the role of modulators on larval heart function, specifically 5-HT, OA, DA and ACh, with acute cold exposed and chronically conditioned (10 days) cold (10 ^{o}C) *Drosophila melanogaster* larvae.

2. To investigate the action of Ca^{2+} influx through optogenetics on the larval heart in *Drosophila melanogaster* in cold temperature (10 ^{o}C).

3. Characterize acute cold and chronic cold exposure effects on the crayfish neuromuscular junction

4. Determine if neuromodulators play a role in acute and chronic cold exposure in the model crayfish species.

CHAPTER 2

Modulatory effects on Drosophila larva hearts: Room temperature, acute and chronic cold stress

This chapter is published in the Journal of comparative Physiology B (Zhu et al., 2016) J Comp Physiol B Oct;186(7):829-41. DOI 10.1007/s00360-016-0997-x

Author contribution: Yuechen Zhu, Emily Yocom, Jacob Sifers, Henry Uradu and Robin Cooper collected data. Data analysis were performed by Yuechen Zhu and Emily Yocom. Manuscript were written by Robin Cooper and Yuechen Zhu.

ABSTRACT

Ectothermic animals are susceptible to temperature changes such as cold shock with seasons. To survive through a cold shock or season, ectotherms have developed unique strategies. Our interest is focusing on the modulation of physiological functions during cold shock and prolonged cold exposure in the fruit fly. We use *Drosophila melanogaster* as a model system to investigate cardiac function in response to modulators (5-HT-serotonin, Ach-acetylcholine, OA-octopamine, DA-dopamine and a cocktail of modulators) in acute cold shock and chronic cold shock conditions. Semi-intact larvae are used to provide direct access to the modulators of known concentration in a defined saline. The results show that 10 µM 5HT is the only modulator which maintains heart rate for larva raised at 21^{o}C and then exposed to acute cold shock (10^{o}C). The modulators 1 µM OA, 10 µM 5HT, 1 mM Ach, 10 µM Ach and a cocktail of modulators (at 10 µM) increased heart rate significantly in larvae which were cold conditioned (10^{o}C for 10 days). HPLC analysis indicated both OA and 5-HT decreased in chronic cold conditioning. The larvae maintain heart function in the cold which may be contributed to by low circulating levels of modulators. The larval heart responds better to 5-HT, OA, and Ach in conditioned cold than for acute cold, suggesting some acclimation to cold.

INTRODUCTION

The unique ability of many invertebrates to survive during environmental temperature changes are a result of various behavioral and physiological strategies, such as burrowing for physical protection, changing the osmolarity of the hemolymph, altering expression of heat or cold shock proteins and production of antifreeze proteins for cold exposure. An index for adaptability to cold in insects is how well they can tolerate cold. For example, a measure which indicates at what temperature there is no longer a response to stimuli or when a loss of motor unit function occurs. At such a point when physiological function stops is termed the critical thermal minimum (Andersen et al., 2015). This measure was recently used for various species of *Drosophila* to determine how well they were be able to handle cold stress (Andersen et al., 2015). Some

insects, like the ghost moth, apply a strategy to compensate by increasing their metabolism rather than decreasing metabolism (Zhu et al. 2016b). This enhanced metabolism could potentially indicate an increase production of neuromodulators/modulators during long term cold exposure. However, few studies report on regulation of biogenic amines, peptides, or neuromodulators/modulators in cold acclimated organisms in relation to physiological conditions. Investigating the heritability of heart rate in different strains of *Drosophila* may help to point to particular physiological factors to explain the underlying mechanism (Jennings, et al. 2009). Andersen et al., (2015) examined various *Drosophila* species which evolved in different temperate and tropical regions and had shown the temperate species had a lower critical thermal minimum than tropical species. To better understand the physiological mechanisms to respond to such stress, genomic and metabolomics approaches are being readily implemented (Colinet et al. 2012). *Drosophila* species offer experimental advantages but they are also limited in addressing extreme cold or prolonged freezing acclimation as they have not been shown to survive freezing conditions (Chen and Walker 1994). However, the ability of some species of *Drosophila* to survive short bouts of cold likely contributes to the wide ecological distribution and numerous species present worldwide.

Repeating a cold exposure with *D. melanogaster* has been shown to enhance survival. This rapid cold-hardening (RCH) ability suggests an expression of cryoprotectant substances and/or physiological changes in the animal. However, neither Hsp70, glycerol or carbohydrate cryoprotectants showed an increase in these experimental conditions for *D. melanogaster* while other insects show significant increases in cryoprotectants with cold shock (Kelty and Lee 1999, 2001). Repeated cold exposure reduces apoptotic occurrences in *Drosophila* suggesting cellular protection by reducing the triggers for apoptosis as a potential mechanism for RCH (Yi et al. 2007). RCH treatment of *D. melanogaster* does produce elevated levels (50–100% increase) of glucose and trehalose. This increase in the osmolality of the hemolymph reduces freezing in the whole animal and likely accounts for prolonging survival in freezing conditions (Overgaard et al. 2007). Slight changes in the amino acid levels are also observed but may not account for survival with RCH (Overgaard et al. 2007). As mentioned in Overgaard et al. (2007), subtle changes in various cryoprotectants, osmolality and modifications in the bi-lipid membrane structure together are the major factors for conditioning (Overgaard et al. 2005, 2006; Lee et al. 2006; Michaud and Denlinger 2006) as these approaches are common for various invertebrate species for cold and dehydration exposure (Burton et al. 1988; Goto and Kimura,1998; Feder and Hofmann, 1999; Bennett et al. 1997; Bayley and Holmstrup 1999; Bayley et al. 2001; Overgaard et al. 2007; Tomčala et al. 2006).

Little attention has been given to neuromodulators/modulators with cold exposure in invertebrates. Modulators, such as serotonin (5HT), octopamine (OA), dopamine (DA), and acetylcholine (Ach) as well as peptides, can rapidly alter physiological function in slowing or enhancing physiological processes depending on the target tissue in most all invertebrates investigated. Very small changes in these neuromodulators within synaptic clefts at sites of neuronal communication have substantial effects on the neuronal function, altering behavior and release of hormones involved with cold acclimation. Small changes in the level of released modulators within the CNS of small organisms, such as a *Drosophila* larvae, are hard to distinguish. Examining excised brains for analysis does not allow one to know the minuscule released amounts within synaptic clefts as compared to the whole brain. However, modulators released into the hemolymph for distribution within the whole body are feasible, considering one can readily pool hemolymph samples. Modulators such as 5-HT, OA, and DA are well established within crustaceans and insects as being key compounds altering neuronal, cardiac, GI, ventilatory, and skeletal muscle function (review by Shuranova et al. 2006; Strawn et al. 2000; Listerman et al. 2000; Cooper et al. 2011a, b). It is known that DA, 5-HT and OA rise in the hemolymph during exercise in crabs (Sneedon et al. 2000). OA is also known to increase in insects with locomotive activity (Goosey and Candy 1980; David et al. 1985). These modulators are generally thought to serve as a stress response in invertebrates (Pagé et al. 2007; Even et al. 2012). OA is known to increase in the hemolymph of locust with heat stress (Davenport and Evans 1984) and there is suggestive evidence that 5-HT may as well increase with heat in locust; however, direct 5-HT measures in hemolymph are lacking (Newman et al. 2003). OA and the precursors for DA and 5-HT all rise in concentration with heat and vibratory stress in the American cockroach (Hirashima and Eto 1993). Even though El-Kholy et al., (2015) did not identify mRNA expression for OA receptors in larval heart of *D. melanogaster* by RT-PCR we still examined the possibility of action by OA on the larval hearts.

To initiate studies on rapid and long-term acclimation to cold and the potential role of modulators in *D. melanogaster* we choose to use a bioindex of cardiac function. *D. melanogaster* utilizes an open vascular system. The heart is a dorsal vessel with the anterior aspect of the tube serving as an aorta. The larval heart of *D. melanogaster* is myogenic and bathed in hemolymph; thus, any modulator can have direct effect on the entire heart (Rizki 1978; Dowse et. al 1995; Gu and Singh, 1995; Johnson et al. 1998). The larval *D. melanogaster* heart has been used to investigate the electrophysiological properties of cardiomyocytes in normal and mutant larvae by recording cardiomyocyte action potentials (Lalevée et al. 2006; Desai-Shah et al. 2010) and is a good model to study the role of ions in generation of heart beat (Johnson et al. 1998; Desai-Shah et al. 2010) as well as the effects of modulators (i.e., 5-HT, DA, Ach). With the advent of a recently modified saline which maintains exposed heart for hours (de Castro et al. 2014) it is now possible to advance to more detailed electrophysiological and cellular studies in the mechanisms regulated the ionotropic and chronotropic actions in the larval heart of this model organism

as well as posing questions on the role of modulators during environmental stressors.

Pharmacological and genetic studies in identifying receptor subtypes on the larval heart have revealed putative 5-HT2 (Johnson, et al. 1997, 2002; Majeed et al. 2014), type-1 DA and type-2 DA (Tiitlow et al. 2013), as well as both nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs) (Malloy et al. 2015). All three modulators (DA, 5-HT and Ach) show a dose response effect in altering the heart rate (HR). High doses can decrease HR; however, at 10 µM all three modulators produce increases in HR without any further increases in HR at higher concentrations. Preliminary trials with acute exposure of intact *D. melanogaster* larvae from 21^{o}C to 10^{o}C substantially slowed the HR; however larvae raised at 10^{o}C had a higher HR than the acutely exposed larvae. This suggested to us regulation beyond cryoprotectant substances and/or hemolymph osmolality changes in maintaining cardiac function as part of acclimation to the 10^{o}C. Recently we have shown that using optogenetics and expression of light sensitive channelrhodopsin protein (ChR2.XXL) that cold conditioned larval hearts are able to substantially increase HR, this implies the cold is not physically restricting the ability of the contractile units from functioning (Zhu et al., 2016a).

The purpose of this study was to investigate the role of modulators, specifically 5-HT, OA, DA and Ach, on acute cold exposed and chronically conditioned (10 days) cold (10^{o}C) larvae. The significance of the findings helps to establish a potential role of these modulators in allowing *D. melanogaster* to function with seasonal changes in the natural environments. In addition, the role of modulators may open novel investigations into the mechanisms of maintaining physiological functions with RCH in other insects and invertebrates with cold exposure.

MATERIALS AND METHODS

Fly rearing and stocks

Wild type *Canton S* (CS) flies were used for HR analyses using the semi-intact method and for intact larvae by the “ant farm technique” (Cooper et al. 2009). In brief, the semi-intact method is performed by pinning the third instar larvae ventral side up on a glass plate and dissected in a droplet of saline (shown as a movie, Cooper et al. 2009). The *Drosophila* heart is very sensitive to pH (Gu and Sing 1995); therefore, the saline is adjusted to pH 7.1 and maintained with the high concentration of buffer as described in de Castro et al. (2014). An illustration of the preparation used can be found in Desai-Shah et al. (2010). The third instar larvae were opened by an incision in the ventral midline and the internal organs were washed aside by saline in order to expose the intact heart to various solutions.

The ant farm technique is a procedure to record intact freely moving larvae. This technique consists of two glass plates (microscope slides; 75 x 25 mm; J. Melvin Freed Brand) narrowly spaced (1 to 1.5 mm) apart by a thin layer of

larvae food (e.g. moist corn meal- a modified version of Lewis, 1960) so that the larvae are able to be visualized within one plane of focus. Spacers commonly used for gel electrophoreses plates (mini gel Bio-Rad; Life Science Research, Hercules, CA 94547, USA) work very well since they can be purchased with varying thickness for use with 1st , 2nd or 3rd instar larvae. Also an option is to use a solid plastic of a given thickness and cut out the region to use as the crawling space. Slightly tilting the platform at 20 to 45 degrees causes the larvae to remain, the majority of the time, with their head pointed downward and their tail containing the spiracles out above the food or within an air passage in the food layer. In this configuration the larvae tend not to crawl throughout the food, but instead they only slightly move around in the 2D plane eating so it is relatively easy to count HR. White light is projected from the underside of the microscope stage with a mirror so that it can be moved accordingly for the best contrast of the heart or the two trachea which move while the heart contracts. A microscope (adjustable zoom 0.67 to 4.5; World Precision Instrument; Model 501379) is used. A 2X base objective and tube objective 0.5X is used to gain enough spatial resolution and magnification to cover a 1cm by 0.5 cm rectangle. A mounted camera through a trinocular mount is used (Mintron, MTV; World Precision Instrument) and the HR is counted on a TV screen.

This CS strain has been isogenic in the lab for several years and was originally obtained from Bloomington Fly Stock. In order to obtain staged larvae, the flies were held at 21^{o}C in a 12 hour light/dark incubator before being tested. All animals were maintained in vials partially filled with a cornmeal-agar-dextrose-yeast medium. The general maintenance is described in Campos-Ortega and Hartenstein (1985).

Pharmacology

Acetylcholine, serotonin hydrochloride [5-hydroxy-tryptamine (5-HT)], octopamine, dopamine-HCl and the salts for making the saline were purchased from Sigma-Aldrich (St. Louis MO, USA). Fly saline, modified Hemolymph-like 3 (HL3) (Stewart et al. 1994) containing: (in mmol/L) 70 NaCl, 5 KCl, 20 $MgCl_2$, 10 $NaHCO_3$, 1 $CaCl_2$, 5 trehalose, 115 sucrose, 25 N,N-Bis-(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES) was used with the following modifications: pH was decreased from 7.2 to 7.1 and BES buffer was increased from 5.0 mmol/L to 25.0 mmol/L to maintain a stable pH (de Castro et al., 2014).

Heart rate measurement

Because heart performance is very sensitive to pH change, the pH was tightly regulated and adjusted as needed. Modulators were applied at various concentrations as indicated in the Results. The preparations were left for 1 min in saline after dissection, and then heart beats were counted for the following minute. The difference in the HR before and after application of drugs was used to measure the effects of the various compounds or temperature.

Experimental conditions

In this study, cold is referring to 10^{o}C., and room temperature and warm refers to 21^{o}C. Larvae raised at 21^{o}C until the early 2nd instar age and then placed in a 10^{o}C cold room for 10 days were considered cold conditioned. The larvae did molt to a 3rd instar but they were generally smaller than 3rd instars raised at 21^{o}C. Larvae raised in room temperature until the 3rd instar for experiments were considered as room temperature conditioned larvae. The instar stages were determined by using the assay of mouth hook development (Alpatov 1929; Okada 1963). For room temperature conditioned with acute cold exposure with or without modulators, larvae were dissected and the HR was measured at 21^{o}C with saline and then exchanged to a 10^{o}C saline or a cold saline containing various modulators in a cold room. For cold conditioned larvae and cold exposure with or without modulators, larvae were dissected and the HR was measured in a 10^{o}C cold room immersed in 10^{o}C saline, then the saline was exchange to cold saline (as a control for exchanging saline) or to a cold saline containing modulators of interest. Whole intact larval experiments were performed with the ant farm techniques and exposing the animals to the various temperatures. A thermal probe was placed in the corn meal containing the larvae within the ant farm apparatus to ascertain the correct temperatures.

The mechanical disturbance and time effects on HR

The mechanical disturbance with exchanging the saline plays an important role in altering HR in a semi-intact open preparation (Majeed et al., 2013). Also, HR will slightly decrease over the time in the modified saline used. To account for the mechanical disturbance with exchanging the bathing medium and duration of time effect, a set of control groups were conducted for the same temperature conditions and time frame by exchanging saline with saline only. In the experimental groups, first the HRs were measured in the temperature the larvae were raised and then the saline was exchanged to the temperature of interest and maintained at that temperature for the duration of the experiment.

Four different temperature conditions were examined: (1) Room temperature conditioned larvae exposed to acute cold. (2) Room temperature conditioned larvae exposed to acute cold saline containing one or more modulators. (3) Cold conditioned larvae exposed to cold saline. (4) Cold conditioned larvae exposed to saline containing one or more modulators.

HPLC analysis

The quantification of the 5-HT level in third instar larvae were accomplished through high pressure liquid chromatography (HPLC). The hemolymph was obtained from third instars either raised at 21^{o}C or raised from 2nd instar for 10 days at 10^{o}C. The third instar larvae were removed from food, washed with water and dried off. To collect the hemolymph, cuticle was nicked directly above the caudal end of the heart after they were lifted into air and a small hole was cut using fine scissors. The larvae were placed into the 1.5 ml Eppendorf tube containing 80 µl of HPLC solution. The larvae were left to sit for 5 min, vortexed lightly for 2 sec and then lightly spun down. The larvae were removed with a

fine insect dissection pin. The remaining solution in the tube was immediately frozen and stored at -80^{o}C until HPLC could be performed. The measures were provided per 50 larvae. Given that a single 3rd instar larvae contains approximately 250 nl of hemolymph, the provided values were divided by 50 and concentration estimates of per larvae were based on the MW of the compound of interest. Example: 1000 ng/ml reported would be 20ng/ml per larvae and for 5-HT this would be approximately 114 nM/larvae. A commercial facility was used to analyze the samples (Center for Microelectrode Technology CenMeT and Parkinson's Disease Translational Center of Excellence, University of Kentucky Medical Center, Lexington, KY 40536-0298).

Statistical analysis

All data are expressed as mean $\pm$ SEM. The rank sum pairwise test was used to compare the difference of HR after exchanging solution with saline containing chemicals or temperature. An ANOVA was used to examine the before and after data as a repeated measures and if the data sets were normally distributed data. A Tukey's test was used as a post hoc test following the ANOVA to compare the relative changes of HRs for all the compounds within an experimental paradigm to determine significant differences. This analysis was performed with Sigma Stat software. P of $\leq$ 0.05 is considered as statistically significant. The number of asterisks are considered as P $\leq$ 0.05 (*), P $\leq$ 0.02 (**), and P$\leq$ 0.001 (***).

Results

Room temperature conditioned larvae: acute cold exposure

When intact early 3rd instar larvae, which were conditioned to 21^{o}C, are exposed to rapid cold (10^{o}C) a drastic decrease in HR occurred. All 10 out of 10 larvae decreased HR within a minute (P<0.001; rank sum test; **Figure 2.1A**). The average decrease is 60%. To determine the effect of acute cold exposure on semi-intact dissected larvae, we measured HR before and after exposing cold saline to 21^{o}C raised larvae. Third stage larvae were dissected at 21^{o}C and the HR obtained. Afterwards, the preparations were transferred to a cold room and the saline exchanged to cold saline. The HRs during the cold exposure decreased substantially (**Figure 2.1B**; P<0.001; rank sum test). To examine the effect of cold conditioned larvae to warm exposure, embryos were allowed to develop to late 2nd instars at 21^{o}C and then were placed for 10 days in 10^{o}C prior to being dissected in the cold. The HR was measured in the cold and then the preparations were removed from the cold room. The saline was exchanged to a saline at 21^{o}C without any modulators present. In all cases the HR increased sustainably with exposure to the warm saline (**Figure 2.1C**; P<0.001; rank sum test).

Room temperature conditioned larvae: acute cold exposure with modulators

The effect of modulators on HR for 21^{o}C conditioned larvae were examined at various concentrations (1 uM OA, 10 uM OA, 10 uM 5HT, 10 uM Ach, 10 uM DA, 1 uM DA and a cocktail solution). The modulators were directly exposed to the cardiac tissue when exchanging the bathing media. The effects of the modulators showed varied responses in altering the HR. The acute cold exposure to saline containing OA (10 µM) produced a greater decrease in HR than cold saline on its own (**Figure 2.2A**, $P<0.05$, non-parametric rank sum). Since some of the preparations completely stopped with 10 µM, a lower concentration of 1 µM was used with the same experimental paradigm (**Figure 2.2B**). Only 1 of the 10 preparations stopped beating with the lower concentration (**Figure 2.2B**, $P<0.05$, non-parametric rank sum). Also one preparation showed an increase but 9 of 10, preparations decreased. However, 10 µM 5-HT exposure for acute cold prevented a decrease in the rate for most preparations (**Figure 2.2C**). Only 1 preparation had a large drop in HR. Exposure to Ach (10 µM) in the acute cold produced a decrease in HR with 2 out of the 10 having mild increase in HR (**Figure 2.2D**, $P<0.05$, non-parametric rank sum). Exposure to DA (10 µM) in the acute cold behaved similar to OA with the majority of preparations completely stopping (7 out of 11 preparations) (**Figure 2.2E**, $P<0.05$, non-parametric rank sum). Since some preparations went into cardiac arrest with 10 µM DA, we also examined if similar effects would occur at a lower concentration. With 1 µM DA, 4 out of 10 preparations stopped while the remaining 6 preparations showed a decrease to a similar extent as for the 10 µM (**Figure 2.2F**, $P<0.05$, non-parametric rank sum). We also tested a cocktail of modulators where we generally knew the effect at a given concentration. We used a cocktail of cold solution with each modulator at 10 µM (OA, DA, Ach, and 5-HT). All 10 larvae showed a decrease in HR and 2 completely stopped (**Figure 2.3A**). The acute cold cocktail had a significant change in decreasing HR ($P<0.05$, non-parametric rank sum). Since preparations showed a large variation in initial HRs, a percent change from 21^{o}C to cold or cold and modulators, were compared (**Figure 2.3B**). The only group different from cold saline exposure itself is the 10 µM 5-HT since the rates increased instead of decreasing. The parametric statistical analysis did not indicate differences from exchanging saline alone to the other modulators as the data is not normally distributed with HR stopping for a number of preparations with DA and OA.

Cold conditioned larvae: Influence of modulators

The cold conditioned larvae showed no effect of exchanging the saline with cold saline to account for mechanical disturbance. Such saline disturbance does alter HR for larvae maintained at 21^{o}C (de Castro et al., 2014 figure 2; Majeed et al., 2013 figure 1, 2014 figure 1). There is a significant difference in the effect of a bath change on HR with room temperature conditioned larvae on acute cold exposure as compared to saline exchange in cold conditioned larvae with a cold saline exchange ($P<0.05$, Mann-Whitney; **Figure 2.4A**).

Modulators effect on cold conditioned larvae was also tested at different concentrations (10 uM OA, 1 uM OA, 10 uM 5-HT, 10 uM DA and cocktail solution). In all 6 preparations examined, OA at 10 µM stopped the heart (**Figure 2.4B**, P<0.05, non-parametric rank sum). With a reduced concentration of OA (1 µM) only 1 preparation out of 11 showed a decrease with most having a mild increase in HR (**Figure 2.4C**, P<0.05, non-parametric rank sum). However, 5-HT at 10 µM produced substantial increases in HR with cold conditioning (**Figure 2.4D**, P<0.05, non-parametric rank sum) as well as Ach at 10 µM (**Figure 2.4E**, P<0.05, non-parametric rank sum). DA was not as consistent in its effect at 10 µM with 6 out of 10 preparations increasing HR and decreasing in others (**Figure 2.4F**). As for the RT to acute cold exposure, a cocktail of the modulators (OA, DA, Ach and 5-HT, each at 10 µM) was examined for the cold conditioned larvae. In all cases, 10 out of 10 preparations, there was a substantial increase in HR (**Figure 2.5A**, P<0.05, non-parametric rank sum). Since there is a large variation in initial HRs a percent change normalizes the trends for ease in comparisons to the effect of the modulators. It is readily apparent 10 µM OA showing an opposite effects from the other modulators at the same concentration. The exchange of saline on cold conditioned larvae did not produce any notable effect (**Figure 2.5B**). In comparing the percent changes for the various modulators, 1 µM for OA and 1 mM for Ach is also shown in the graph for comparisons. The greatest increases in HR are with 5-HT and the cocktail. It is interesting to note the inhibitory effect of OA at 10 µM was overridden with the cocktail containing the other modulators. Even the negligible effect of DA did not appear to dampen the effect of the cocktail.

A recent publication by (El-Kholy et al., 2015) appeared in which OA receptors were not able to be identified in larval heart by RT-PCR utilizing receptor gene specific primer pair. However, a tyramine receptor (TyrR) was shown to be expressed in the heart. So we also assayed tyramine at 10 µM for acute cold and cold conditioned larvae. In 9 of 11 preparations, HR decreased with acute cold exposure (P<0.05, non-parametric rank sum) and 8 of 10 preparations HR increased in cold conditioned larvae (**Figure 2.6A, B**). The percent change for acute cold is -39% $\pm$12% and the percent change for cold conditioned is 72% $\pm$ 39%.

HPLC analysis of hemolymph

The quantification of OA and 5-HT in 3rd instar larva hemolymph showed different concentrations by HPLC analysis. The average value of 5-HT in room temperature raised larva is 957 ng/ml or 109 nM per larva based on three samples. Meanwhile, the 5-HT level of larvae raised in the 10^{0}C deceased to 116 ng/ml or 13 nM. The overall OA level is substantially higher than the 5-HT, and OA value is decreased from 21^{o}C raised larva 15,719 ng/ml or 2,052 nM to cold conditioned larva 2,808 ng/ml or 367 nM. To convert these values to concentration per larva, the volume of hemolymph was measured to approximately 250 nl. The results indicated a drop in the circulating levels of OA and 5-HT over 10 days of conditioning in the cold as compared to larvae maintained at 21^{o}C (**Figure 2.7**).

Discussion

In this study, it was demonstrated that acute and chronic exposure to cold (21ºC to 10ºC) reduced the HR in larval *D. melanogaster*. However, when the hearts were directly exposed to 5-HT upon acute or chronic cold exposure the HRs showed a substantial increase. Other known modulators (DA, Ach, OA) did not have a pronounced effect on reducing the cold response on HR; however, at room temperature all four modulators combined (5-HT, DA, Ach, OA) have a significant effect in increasing HR (Malloy et al. 2015; Majeed et al. 2014; Titlow et al. 2013; Zornik et al. 1999). In addition, OA alone or DA alone at 10 µM produces marked increases in HR at room temperature (Majeed et al. 2014; Titlow et al. 2013); however, at 10ºC these compounds produce a cessation of HR in a number of trials. Lower concentration of OA or DA (1 µM) did not cause the cold hearts to stop. The HPLC results show a decrease in OA in cold conditions which may in fact help to keep the hearts functioning if higher concentrations reduce the rate. These studies do indicate a differential degree in the responsiveness to modulators in altering HR to acute and chronic cold exposure. Addressing the potential effects of modulators in offsetting the physical effect of cold shock and longer-term cold exposure are novel results as well as the effects of a modulator cocktail. It appears that effects of the modulators are not additive when combined in a cocktail.

The rationale to focus on these four modulators specifically is that these are commonly investigated to assess modulation on neuronal and heart function in insects and crustaceans. In addition, recent pharmacological and genetic manipulations have putatively identified the receptor subtypes for dopamine as type-1 DA and type-2 DA (Tiitlow et al. 2013), and for serotonin 5-HT2 receptors as the main subtype (Majeed et al. 2014) on the larval heart. There appears to be both nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs) (Malloy et al. 2015) on the larval heart and in this study we present data that the larval hearts are sensitive to octopamine when provided directly to the heart in a defined saline.

A previous study had shown that larval hearts are not sensitive to octopamine at concentrations less than 10 µM. However, this study was performed by injection into the hemolymph of the whole animal while restrained (Zornik et al.1999). A recent study examining the expression profiles of OA and tyramine receptors only found TyrRs present in the larval heart and not OA receptors (El-Kholy et al. 2015). So, the responses we observed in acute exposures to cold and cold conditioned flies to OA 10 µM warrants discussion. Zornik et al. (1999) did report at 100 µM an 86% increase over a baseline HR in larvae and responses to lower levels in injected pupa for OA. Raw data is not presented in this earlier study; thus, we cannot know what the rate was changing from with the saline injections used to obtain the percent changes for the modulators. We predict the injection and restraint of the larvae likely induces some stress where modulators may already be released into the hemolymph. We report baseline values for the effect of the saline to saline exchanges to compare to the effects of mechanical disturbances on the hearts. It was reported that just exchanging

the bathing fluid over the heart will alter HR with usually a 10 to 15% increase in the rate (Becnel et al. 2013; Majeed et al. 2014; Titlow et al., 2013; de Castro et al. 2014). On the other hand, rapidly dissecting the larvae and washing out the hemolymph to expose the heart to a defined salt solution is not as natural as in vivo preparations but it does allow multiple variables to be reduced to defined conditions for comparison. Since we know 5-HT, OA, DA all work on the larval CNS to excite neural circuits and enhance motor unit activity this could have substantial effects in intact larvae for hormonal release and even contracted and relaxed states of body wall muscles (Dasari and Cooper 2004). As for the lack in expression of OA receptors on the larval heart, it does appear that there is little if any GFP expressed in the transgenic lines in which the presumptive promoter for all OA receptors is utilized (El-Kholy et al. 2015). Since tyramine receptor expression is observed in the larval heart, potentially OA is activating tyramine receptors and initiating the effects we observed. OA and tyramine are similar in chemical structure as tyramine is a precursor to OA (El-Kholy et al. 2015).The interesting point is that OA at 10 µM causes the heart to stop upon exposure in the cold; whereas, the lower concentration did not show as prominent response any different than just a saline exchange for the acute cold saline exposure. Using the same concentration of tyramine and OA (10 µM), OA showed a larger degree of change than tyramine. Both compounds decreased HR in acute cold exposure. If OA and tyramine were binding to the same receptors it would appear OA produced a greater response. Since OA is also similar in chemical structure to DA it may also be possible that OA is binding to DA receptors and inducing a response. However, OA actions are greater than that shown for DA at the same concentration for acute cold. Only OA, DA, and tyramine at 10 µM showed a strong trend to decrease HR with acute cold, whereas Ach and 5-HT did not. However, Ach and 5-HT did reduce the inhibitory effect of the cold exposure. So, even if OA is activating specific OA or tyramine receptors on the heart, the mechanism responsible for stopping the heart is not known. Note that at warmer temperatures (21ºC) OA and DA increases HR within the defined saline. Since OA receptors are G-coupled, maybe the Gαq subunit is activated but in cold this action is suppressed and Gαi-coupled receptors are activated. This remains to be examined with further pharmacological studies by blocking the potential Gαq activation of phospholipase Cβ (PLC) with OA at 21ºC like it was performed in addressing the mechanism of 5-HT's action on the heart (Johnson, et al. 2002; Becnel et al. 2011, 2013; Majeed et al. 2014) and suppress Gαi in cold conditioned or acutely exposed hearts.

Addressing the many potential biogenic amines, catecholamines, peptides and other substances known to be in insect and crustacean hemolymph which alter cardiac function in a one by one fashion will take a long time and may not be informative enough of the true physiology in the whole animal but at the same time one is stuck with compounding indirect effects by addressing these actions within the animal. As Marder (2012) pointed out in a review of modulation in neural circuits in invertebrates, it is highly unlikely any one modulator is working in isolation. This is likely true for the cardiac function for insect hearts as well.

At least in the early larval stages the heart is devoid of direct neural innervation (Johnstone and Cooper 2006); thus, in the isolated in situ early 3rd instar studies with bathing media of known composition being exchanged, the indirect effects of any substances being released by stimulating neurohumoral actions is minimized. Cocktails of modulators in known quantities can be examined for their effects on the heart to compare to actions of individual modulators as we have approached in this study. The interesting finding here is that 5-HT appears to be the dominate modulator in reducing the effect of cold shock and long term cold conditioning by itself. Also, as a cocktail the excitatory effect of 5-HT overcame the inhibitory action caused by DA and OA at 10 µM for acute cold exposure. The timing in exposing tissue to individual modulators when applying them in a sequential series can be problematic as noted in a crustacean study of modulation of synaptic transmission at the neuromuscular junction in applying OA and then 5-HT as compared to 5-HT then OA (Djokaj et al. 2001). As noted, there are numerous experimental paradigms to mimic in vivo exposure of modulators with still keeping track of the introduced variables.

The results of the HPLC revealed that cold conditioned decreased the level of OA and 5-HT drastically in the hemolymph. The reduction in 5-HT and OA was counterintuitive in view that 5-HT increased HR at 21ºC. The reduction may well be due to a reduced production or release as compared to warmer conditions. As the modulators turnover there may be a reduction in biochemical syntheses and release into the hemolymph in the cold resulting in the lower levels measured. It is unlikely that an increase in the degradation is taking place in the cold since overall metabolism is likely decreased. Potentially even receptor expression levels could be altered. However, to address these specifically points, more detailed experimentation is required. Measures in other invertebrates in non-stressful temperatures via HPLC revealed for the honey bee is about 17.5 nM 5-HT (French et al. 2014) whereas the 5-HT concentration in tobacco cutworm is in the range of 15-25 nM (Ikemoto et al., 1993). In *Aplysia* OA is around 100 nM (Levenson et al. 1999). It would be of interest for comparison if future studies would address the changes in the levels of modulators with acute and chronic cold conditions.

The different magnitude of action in altering HR for the modulators during acute cold as compared to chronic cold (10 days) warrant further studies in the potential mechanisms. The cold stress is likely to cause many physiological responses which would account for the differences; however, a likely scenario is the altered levels of endogenous modulators and/or altered expression of receptors as well as even differential expression in the subtypes for a given modulator. The paradigm we used of rapid cold shock and maintenance at 10ºC prior to examining the effects of modulators might not have allowed the same physiological responses as those responsible for RCH. If we gradually reduced the temperature or pulsed the cold shock a few times prior to holding the larva in the cold, the pulsing of cold shocks in a gradual manner may allow the animal to have periods of higher metabolism to conduct physiological alterations in preparation for the cold as noted in other studies on survival with cold exposure

(Czajka and Lee 1990). It would also be of interest to examine the effects of diet in natural habitats for insects in response to cold shock which as far as we are aware has not been addressed fully. This could be examined well in laboratory conditions and even the responsiveness to modulators for larvae or adults cultured in various media. Differences in diets in the natural environment might also account for survival among insect species to cold exposure. It is known different *Drosophila* species are noted for varying abilities to acclimatize to cold (Graham et al. 2012; Vesala et al. 2012). What cellular responses might be driving this ability is the key to understanding the mechanisms. The effect of temperature on HR with exposure to deuterium oxide revealed that exposure to deuterium oxide reduced the changes in HR with temperature (White et al., 1992). The mechanism by which this occurs is still unknown. Recovering from cold upon re-exposure to higher temperatures may likely involve regulation of endocrine factors (Terhazaz et al., 2015). There are a number of remaining studies needing to be addressed in relation to cardiac function in *Drosophila*. Identifying hormonal changes in the hemolymph with various environmental conditions and repetitive exposures may explain selective acclimation. It would be interesting to take the hemolymph of cold conditioned larvae and apply to acute cold exposed hearts to determine if HR is increased or not. In addition, examining the irregularity in beats would be good to assess over time for a better representation of the effects of various conditions (Jennings et al., 2009). The rates over the time counted in this study did vary depending on the condition; thus only an average rates are reported.

A recent study on crayfish *(Procambarus clarkii)* and prawn *(Macrobrachium rosenbergii*), which both are native to warm waters, showed marked difference in behavior and cardiac physiology when exposed to cold abruptly as well as in a gradual manner over weeks (Chung et al. 2012). With an acute temperature change (21^{o}C to 5^{o}C) the prawns died within 2 hrs; whereas, crayfish were still alive and responsive to touch on their telson for days. Monitoring the HR indicated the prawns stopped any alteration in the HR in conjunction with the lack of notable behavioral changes to stimuli; whereas crayfish which only showed slight behavioral responses still produced significant alteration in HR when physically disturbed. Such hardiness in this species of crayfish may well account for their wide distribution in North America and invasiveness after being introduced in Europe and Asia (Ackefors 1999; Nyström 1999; García-Arberas et al. 2009).

With knowing that epigenetic factors are tied to environmental stressors and that RCH is more pronounced with repetitive exposures, it would also be of interest if there is a correlation to account for selective protein expression within a species but also among *Drosophila* species exposed to the same stressors (Vesala et al. 2012). In addition, being able to pin point potential receptors or cellular processes which may signal cold and produce hormonal changes as a response is a target of interest to potentially understand the cascade of events to cold conditioning. Interestingly, some HSP increase with cold and heat exposure and may serve the same function in stability of particular proteins. It

is of interest to note that changing cold saline with cold saline in cold conditioned larvae did not produce an increase in HR as known to occur with larvae raised at 21°C and exchanged with 21°C saline (Majeed et al., 2014; Titlow et al. 2013, deCastro et al. 2014; Malloy et al. 2015). The small increase in HR at 21°C with exchanging the saline is likely a response due to stimulating stretch-activated ion channels known to be on the heart in mammals (Baumgaertner et al. 2012) and are present in *Drosophila* sensory neurons (Coste et al. 2012). Considering the increase in HR does not occur with cold conditioned larvae when the saline is exchanged could indicate the channels are too rigid in the cold and that the possible accessory proteins may not be maintaining the structural integrity of the channel to sense the mechanical movements. It may also be possible that the cold conditioning has activated the expression of chaperonin HSP which stabilize the membrane and prevent mechanical deformation and dampen the effect of stretch activated channels (Kayukawa and Ishikawa 2009). We are now addressing these possibilities in a follow up study (abstract, deCastro et al. 2015).

While our methods likely do not reflect natural conditions, our data do indicate that neuromodulators can affect cardiac function of cold conditioned larvae. Thus, subsequent experiments will focus on testing the in vivo function (RNAi approaches) of these neuromodulators during the course of cold conditioning. Maintaining HR in the cold is essential for circulating nutrients/cryoprotectants, immune function and since we demonstrated that modulators do have activity at low temperature suggests they may be ecologically important.

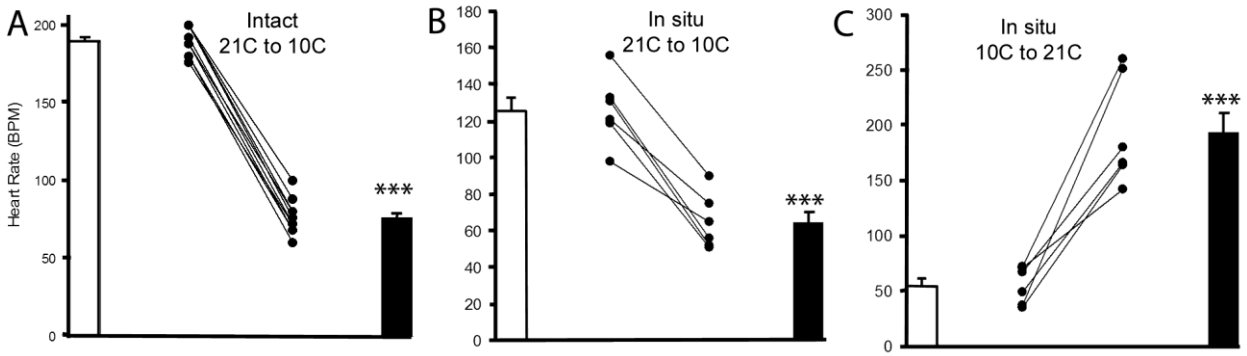

Figure. 2.1: (**A**) Change in HR in intact early 3rd instar larvae raised at RT (21ºC) and cold shocked. The heart rate was counted in whole larvae for 1 minute and then exposed to 10ºC for 1 minute before counting the rate in the next minute. In 10 out of 10 larvae the HR substantially declined. (**B**) Change in HR of in-situ hearts exposed directly to saline in early 3rd instar larvae raised at RT and cold shocked. HRs were obtained after dissection and exposed to saline at RT and then exposed to saline at 10ºC in a 10ºC cold room. The hearts were allowed to adjust for 1 minute prior to counting the rate. (**C**) The HR of in-situ hearts was measured in the cold and then the preparations were removed from the cold room. The saline was exchanged to a saline at 21ºC without any modulators present. P<0.001; rank sum test). The mean (+/- SEM) of the rates before (open) and after (closed) the change in temperature are shown as bar graphs.

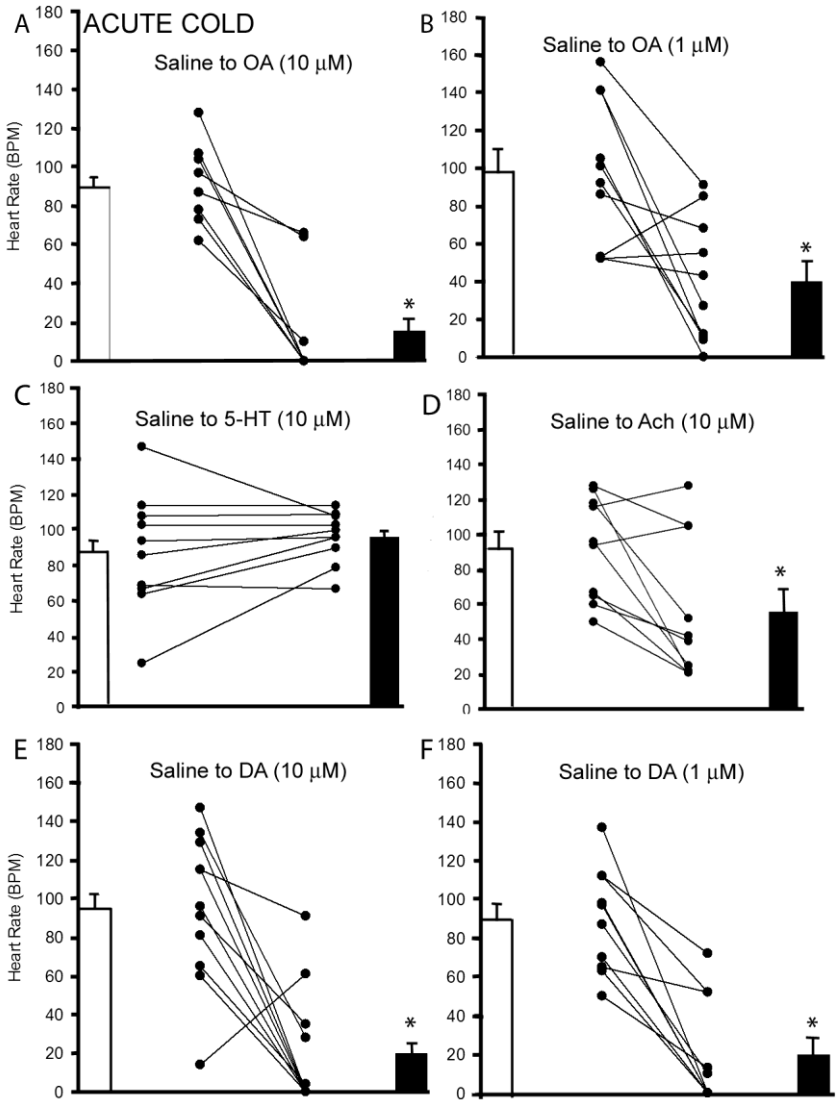

Figure. 2.2: Change in HR of in-situ hearts exposed directly to cold and octopamine (OA). The 3rd instar larvae were dissected and exposed to saline at RT followed by a saline exchange containing OA exchange in a 10°C cold room. The hearts were allowed to adjust for 1 minute prior to counting the rate. (**A**) In 8 out of 8 larvae the HR substantially declined for the 10 µM OA and 8 out of 10 declined for the 1 µM OA (**B**). (**C**) Saline (21°C) exchanged to one containing 5-HT in a 10°C cold room resulted in 4 out of 10 larvae to increase and 2 out of 10 larvae to decrease while 4 out of the 10 did not show substantial change for the 10 µM 5-HT. (**D**) Saline exchange to cold saline containing Ach (10 µM) resulted in 8 out of 10 larvae to decrease HR. (**E**) Saline exchange to cold saline containing DA (10 µM) resulted in 9 out of 11 larvae to substantially decrease and 9 out of 10 to decline for exposure to 1 µM DA (**F**). The mean (+/- SEM) of the rates before (open) and after (closed) the change in temperature and exposure to the compounds are shown as bar graphs.

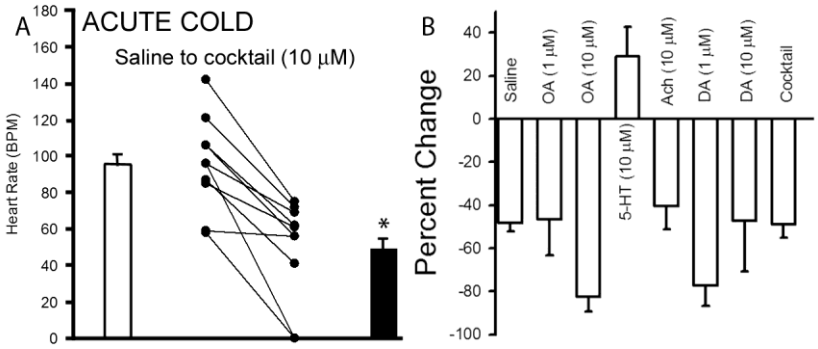

Figure 2.3: Change in HR of in-situ hearts exposed directly to cold and a cocktail of modulators each at 10 μM octopamine (OA), serotonin (5-HT), dopamine (DA) and acetylcholine (Ach). Larvae were raised at 21^{o}C. The 3rd instar larvae were dissected and exposed to saline at RT. This was followed by an exchange to cold saline containing the modulator cocktail in a 10^{o}C cold room (**A**). A comparison in the percent change of each individual modulator and the cocktail for the acute cold shock is shown in (**B**) for comparison. The percent change in rate was determined for each individual larvae and then averaged (mean $\pm$ SEM).

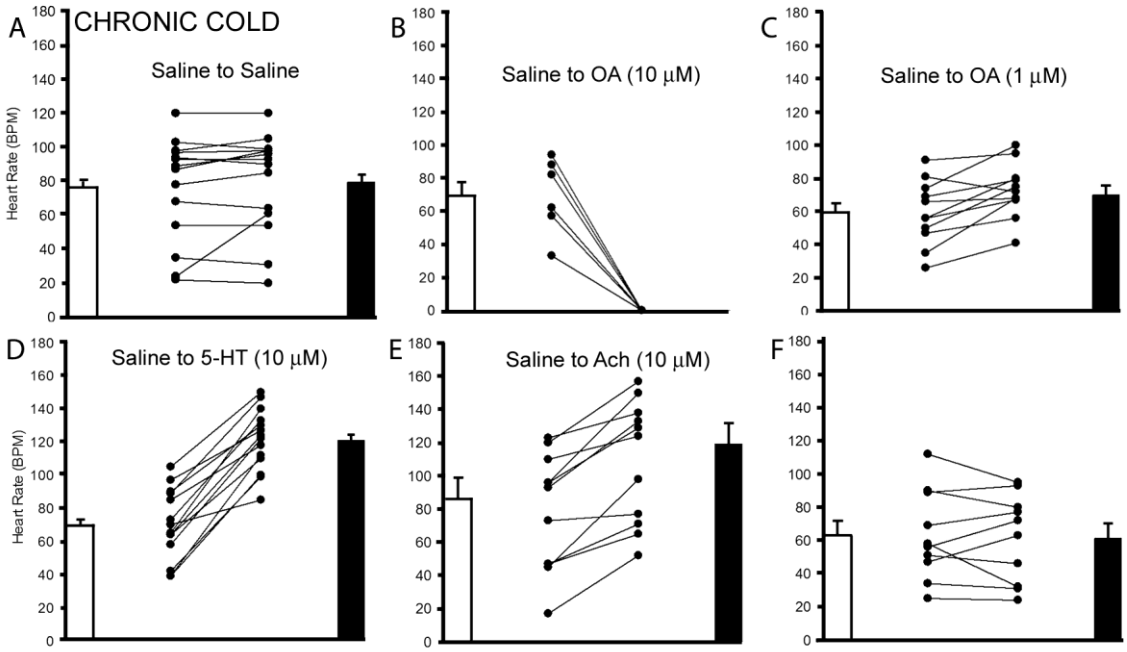

Figure. 2.4: Change in HR of in-situ hearts in cold conditioned larvae. Larvae were raised at 10ºC from 2nd instar for 10 days and then dissected in early 3rd instar stage. HRs were obtained after 1 minute following the dissection which occurred in a 10ºC cold room. The hearts were allowed to adjust for 1 minute prior to counting the rate after saline only exchange or with saline containing a modulator. The saline to saline only exchange was to control for any movement effects on the heart by exchanging the saline. No overall significant differences occurred for exchanging the saline (**A**). One out 14 larvae showed an increase. (**B**) Exposure to octopamine (OA) for cold conditioned larvae resulted in 6 out of 6 larvae substantially decrease HR and even stop during a 10 µM but 10 out of 11 larval HRs substantially increased with a 1 µM OA exposure (**C**). (**D**) Cold conditioned hearts exposed to serotonin (5-HT, 10 µM) resulted in 14 out of 14 larvae to substantially increase HR. (**E**) Hearts exposed to acetylcholine (Ach, 10 µM) resulted in 11 out of 11 to substantially increase in rate. (**F**) Dopamine (DA, 10 µM) exposure resulted in 6 out of 10 larvae to increase their HR.

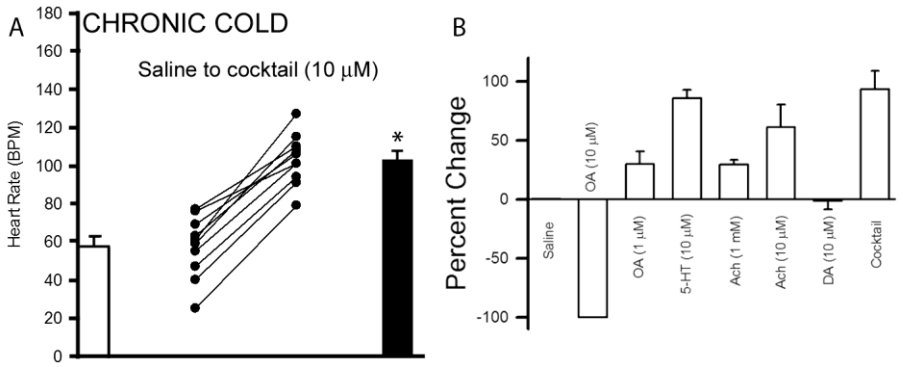

Figure. 2.5: Change in HR of cold conditioned larvae where the in-situ hearts are exposed directly to a cocktail of modulators each at 10 μM octopamine (OA), serotonin (5-HT), dopamine (DA) and acetylcholine (Ach). All 10 out of 10 larvae substantially increased their HR (**A**). A comparison in the percent change of each individual modulator and the cocktail for the chronic cold exposured larvae is shown in **B** for comparison. The percent change in rate was determined for each individual larvae and then averaged (mean ± SEM).

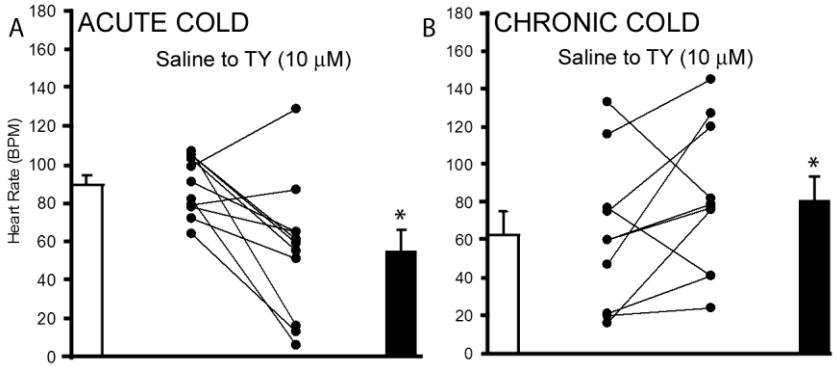

Figure. 2.6: The effect of tyramine on acute cold shock and cold conditioned hearts. (**A**) Tyramine at 10 µM on acute cold exposure hearts produced a decrease in HR even more so than cold saline exposure alone. (**B**) Tyramine on cold conditioned hearts produced for the most part an increase in HR (8 out of 10 preparations).

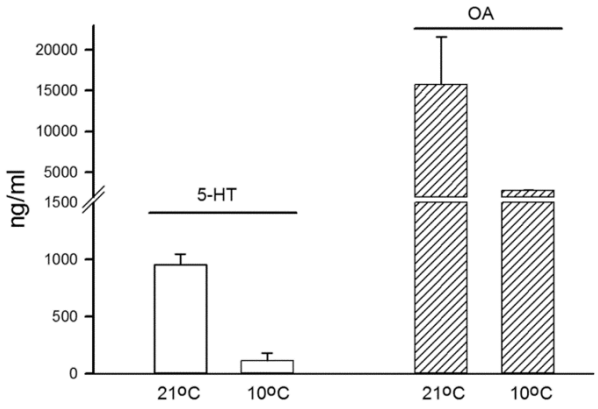

Figure. 2.7: Analysis in the changes in the concentrations of 5-HT and OA within the hemolymph of larvae raised at 21ºC and larvae conditioned to cold (10ºC) for 10 days. Each sample contained pooled hemolymph from 50 larvae and 3 samples in each conditioned were analyzed (mean +/- SEM).

CHAPTER 3

Optogenetic stimulation of Drosophila heart rate at different temperatures and Ca2+ concentrations

This chapter is published in the Journal of Physiol Rep (Zhu et al., 2016) Feb; 4(3): e12695 doi: 10.14814/phy2.12695

Author contribution: Yue Chen Zhu, Henry Uradu, and Robin L. Cooper collected data. Data analysis were performed by Yuechen Zhu and Robin Cooper. Fly lines are provided by Zana Majeed. Manuscript were written by Robin Cooper and Yuechen Zhu.

Abstract

Optogenetics is a revolutionary technique that enables non-invasive activation of electrically excitable cells. In mammals, heart rate has traditionally been modulated with pharmacological agents or direct stimulation of cardiac tissue with electrodes. However implanted wires have been known to cause physical damage and damage from electrical currents. Here we describe a proof of concept to optically drive cardiac function in a model organism, *Drosophila melanogaster*. We expressed the light sensitive channelrhodopsin protein ChR2.XXL in larval *Drosophila* hearts and examined light-induced activation of cardiac tissue. After demonstrating optical stimulation of larval heart rate the approach was tested at low temperature and low calcium levels to simulate mammalian heart transplant conditions. Optical activation of ChR2.XXL substantially increased heart rate in all conditions. We have developed a system that can be instrumental in characterizing the physiology of optogenetically controlled cardiac function with an intact heart.

Introduction

Pharmacology is the front line treatment for intervening in cardiac pathologies that impair heart rate. In addition or in combination with drug applications, direct electrical control of the heart through pacemakers is also widely practiced. Pacing of human hearts with electrical stimulating pacemakers or implantable cardioverter defibrillators - which are placed on or nearby cardiac tissue - have saved and prolonged many lives. However in some cases, these implanted devices can cause irritation and damage to cardiac tissue over time, requiring additional surgeries (Kaye et al., 1975; Stefanidis et al., 2009; Wolk et al., 2013). Novel approaches to control cardiac function are now possible with gene therapy or a combination of gene therapy and regulated gene expression (Lee et al., 1999), although there are various results that warrant caution regarding the safety and efficacy of gene therapy (Giacca and Baker, 2011; Jessup et al., 2011). Clearly more research is needed to enhance the tools and approaches to gene therapy in cardiac tissue to make effective, customized treatments a reality.

One of the gene therapy interventions being considered in cardiac medicine is optogenetics. Light-controlled pacemaker cells could be an effective alternative to implanted electrode pacemakers. Basic research in cardiac optogenetics is needed to gather information about the physiology of optically stimulated heart tissue. Fruit flies are an attractive model system to efficiently gather molecular data on the use of optogenetics on the heart. *Drosophila melanogaster* has been used extensively to investigate cardiac function in response to chemical modulators (Majeed et al. 2014; Titlow et al. 2013; Malloy et al. 2015), environmental changes (stress, temperature), (de Castro et al. 2014; Zhu et al., 2015) as well as developmental abnormalities and cellular regulation of Ca^{2+} dynamics (Becnel et al. 2013; Johnson et al. 1998; Desai-Shah et al. 2010). Although the anatomy of the larval heart tube in *Drosophila* is quite different from the chambered heart of vertebrates, the key molecular mechanisms in heart development and function are shared to a large degree between the two (Bodmer et al. 1998). In addition, the physiological parameters of cardiac output, rate, and duration of systole and diastole between *Drosophila* and vertebrates are quite comparable (Choma et al. 2011). The larval *Drosophila* heart is myogenic and pacing is not regulated by direct neural connections. The vast collection of genetically engineered lines for controlling cellular activity and manipulating gene expression make *Drosophila* a powerful model system (Jones, 2009; Yoshihara and Ito, 2012). The use of optogenetics in *Drosophila* has already been optimized for altering specific behaviors by driving channelrhodopsins in identifiable sets of neurons (Titlow et al., 2015; Owald et al., 2015).

Here we demonstrate that heart rate can be elevated in the *Drosophila* larva heart using the light sensitive channel rhodopsineChR2.XXL, a hypersensitive variant of ChR2 (Alex et al., 2015). Our approach allowed us to examine the effects of ion exchange and bath temperature on the efficacy of optical stimulation. The motivation for testing different conditions was to assess the plausibility of using optogenetics in conditions suitable for human heart transplants. The immersion saline used to maintain tissue viability for human transplants, called “Celsior” (Michel et al., 2014), has low Ca^{2+} concentration and is used at low temperatures, conditions which could impair the channelrhodopsin cation channel. Thus to explore the potential for using light as a pacemaker in mammalian heart transplant conditions, we investigated optogenetic stimulation of the heart over a range of bath temperatures and Ca^{2+}concentraitons.

Methods and Methods

Drosophila lines

The filial 1 (F1) generations were obtained by crossing females of the recently created ChR2 line (which is very sensitive to light) called y^1 w^{1118}; PBac{UAS-

ChR2.XXL}VK00018 (BDSC stock # 58374) (Dawydow et al., 2014) with male 24B-Gal4 (III) (BDSC stock # 1767). These background strains were also examined for the effect of light on the heart.

Preparation of fly food supplemented with ATR

All trans retinal (ATR; Sigma-Aldrich, St. Loius, MO, USA) was diluted in 50 ml of standard fly food to a final concentration of 200µM and protected from light with aluminum foil. For control experiments, larvae were cultured in food that only contained the solvent (100 µl of absolute ethanol in 50 ml of fly food).

Heart rate measurement and experimental conditions

Larvae were dissected in a modified hemolymph-like 3 (HL3) solution (Stewart et al. 1994) containing: (in mmol/L) 70 NaCl, 5 KCl, 20 $MgCl_2$, 10 $NaHCO_3$, 1 (or 0.5mM) $CaCl_2$, 5 trehalose, 115 sucrose, 25 N,N-Bis-(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES). Heart rate in *Drosophila* larvae is very sensitive to pH changes, thus, the saline requires substantial buffering to reduce drift in pH. The following modifications to the HL3 saline were made: a decrease in pH from 7.2 to 7.1, and increase in BES buffer from 5.0 mmol/L to 25.0 mmol/L to maintain a stable pH (de Castro et al., 2014).

Larvae were either raised at room temperature 21^{o}C or placed at 10^{o}C as 2nd instar for 10 days prior to experimentation (herein referred to as cold conditioned). When the larvae reached a 3rd instar stage they were used. Cold conditioned larvae were dissected in a 10^{o}C cold room and tested in 10^{o}C saline. Room temperature larvae were dissected at 21^{o}C and exposed to saline at the same temperature. A detailed description of the dissection protocol is shown in video format (Cooper et al., 2009). In brief, larvae were slit along the mid-ventral longitudinal axis and pinned flat. The internal organs and GI tract were removed and care was given not to damage either the trachea or heart. The brain was left intact as the anterior end of the heart tube is attached to the connective tissue around the brain. With the heart exposed, fresh saline was applied and the contraction rate was counted at the most caudal end. Heart rate (HR) was counted after allowing the preparation to remain still for 1 minute and counted in the subsequent minute under dim white light. For optogenetic experiments, a continuous beam of blue light (470nm wavelength) from a high intensity LED was focused on the specimen through a 10x ocular objective while the HR was counted (Titlow et al., 2014).

Statistical analysis

All data are expressed as raw values or mean ± SEM. A paired t-test (before and after) or a rank sum pairwise test was used to analyze changes in HR after changing bath conditions or stimulating with blue light. On the bar charts of the raw data for HR, a star with a T (*T) indicates a significant difference $p<0.05$ for the T-test. Horizontal lines above the bar charts indicate significant differences ($p<0.05$) among the groups by a Dunn's Test of rank differences. Since the

groups are not normally distributed (a number of zeros in some groups) and having different sample sizes a Dunn's Test of rank differences appears to be a feasible approach.

Results

Extracellular calcium levels regulate heart rate in cold-conditioned larvae

Heart transplants from mammalian donors are kept at low temperature in a low calcium buffer to reduce metabolic damage and increase viability. To develop an assay system that simulates those conditions we cold conditioned *Drosophila* larvae to 10^{o}C and analyzed heart function with the tissue bathed in 10^{o}C physiological saline at different calcium concentrations (Figure 3.1). Larval hemolymph has a Ca^{2+} concentration between 1.0 and 1.5 mM (Stewart et al., 1994). We investigated heart function at lower Ca^{2+} concentrations to compare with the mammalian Celsior buffer, which has a Ca^{2+} of 0.25mM. At 10^{o}C some hearts did not beat in saline with 0.5 mM [Ca^{2+}], but began beating after calcium levels were increased to 1.0 mM. Increasing [Ca^{2+}] from 0.5 to 1.0mM caused HR to increase in all six experiments tested (Figure 3.1). Therefore these calcium conditions were used for subsequent experiments.

Optogenetic stimulation increases larval heart rate

To determine if optogenetics could be used to stimulate heart contractions we expressed ChR2.XXL (24B-Gal4>Chr2.XXL) in heart tissue and counted HRs before and during continuous exposure to blue light. In each of the 10 preparations tested, continuous optical stimulation caused the HR to increase (Figure 3.2; 1.0mM Ca^{2+}, +ATR). The effect of optical stimulation was substantially reduced in low calcium saline (Figure 3.2; 0.5mM, +ATR), with only 3/9 preparations showing an increase in HR. Hearts that weren't beating in 0.5mM Ca^{2+} began to beat rapidly during exposure to blue light.

As a negative control, HRs were recorded in larvae that weren't pre-treated with the ATR cofactor. During optical stimulation in these larvae, HR increased for 8/11 and 6/11 specimens in normal and low calcium conditions respectively (Figure 3.2; 1.0mM Ca^{2+} -ATR and 0.5mM Ca^{2+} -ATR). This result suggests that there could be a low level of ChR2.XXL activity in the heart even without the co-factor. As an additional negative control we tested genetically related strains that were fed ATR but did not express ChR2.XXL (24B-Gal4/+ and UAS-ChR2.XXL/+). Optical stimulation had negligible effects on HR in these strains, i.e., during stimulation the average HR for these lines changed from 159 ± 8 BPM to 155 ± 5 BPM (24B-Gal4/+) and from 189 ± 12B PM to 194 ± 15 BPM (UAS-ChR2.XXL/+) respectively. The absence of a response in these lines combined with the robust response to optical stimulation at normal calcium levels when ChR2.XXL was expressed in the heart tissue shows that optogenetic stimulation reliably elevates HR in the dissected larva preparation.

Optogenetic heart stimulation is effective in cold-conditioned larvae

To further test the possibility of using this assay in conditions that simulate mammalian heart transplants, we characterized the efficacy of optogenetic heart stimulation in cold-conditioned larvae. Not unexpectedly, the effect of optical stimulation on larval HR was slightly reduced at 10°C but HR increased in 10/13 preparations tested (Figure 3.3A; 1.0mM Ca^{2+}, + ATR). As in the room temperature experiments, optical stimulation of HR was calcium-dependent. HR increased in fewer preparations and exhibited a smaller percent change at 0.5mM Ca^{2+} than at 1mM Ca^{2+} (Figure 3.3B). Also similar to room temperature experiments we observed an optical response in the absence of ATR, again suggesting that ChR2.XXL could be active without the co-factor. Nonetheless, these results show that ChR2.XXL is functional at low temperatures in the heart and that they can be activated under low calcium conditions.

Discussion

The possibility of optically stimulating cardiac tissue in lieu of electrical stimulation provides alternative approaches for regulation of cardiac function within an animal as well as regulation of isolated tissue. In this study, we illustrate the potential for optogenetics to be used in conditions suitable for human heart transplants and/or mammalian cardiac tissue cell culture. We demonstrated the use of optogenetics in various conditions to gain a better understanding of the chemical and physical limits of optically driving the cardiac tissue to pace. Activity of the Gal4 transcriptional activator decreases when flies are raised at low temperature (Duffy, 2002). The current study shows that even at low temperature, the channelrhodopsin-XXL is robustly activated in the cardiac tissue. Ultimately these experiments show that the ChR2.XXL channelrhodopsin is functional down to 10°C and 0.5mM Ca^{2+}.

Exposed hearts in filleted larval *Drosophila* preparations with defined saline were used so we could control the ionic media and rapidly shift the temperature without additional variables of hormones or other unknown hemolymph borne factors within intact larvae. In the dissected cardiac preparations, the heart tube is flooded with saline magnitudes of volume greater than the volume of hemolymph in the intact larva; and the saline is exchanged after the dissection. So any light induced stress which may induce the release of hormones within the larvae would have negligible effects on the heart. The controlled concentration of calcium in the saline, temperature, and the ATR food supplement all showed significant effects depending on the experimental conditions.

An important consideration in optogenetic stimulation of the heart and other tissues is photosensitivity of the channelrhodopsins. Larvae expressing the ChR2.XXL were so sensitive to light that HR was elevated during exposure to a dim fiber optic white light source. After dark conditioning and then immediately counting HR under dim white light, the HR increases rapidly and reaches a steady state. That is why larvae pre-treated with ATR exhibited higher baseline HRs than un-treated controls for both calcium conditions and at both

temperatures. On top of this background sensitivity to unfiltered light, we still observed significant effects for 470nm light on HR. We are currently developing a high throughput approach to electrically monitor HR in the absence of light to eliminate this problem and to determine the wavelength specificity for channelrhodopsins expressed in larval heart tissue. The hypersensitive ChR2.XXL channel may prove to be more efficacious in translational science to intact mammals than previous versions of ChR2 due to its increased photosensitivity. In activating mammalian cardiac tissue in vivo, light would likely need to penetrate fluid bathing the heart either within the pericardial sac and within the cardiac chambers. The light would be dampened in the fluid, thus higher photosensitivity may be necessary.

Direct optogenetic pacing of the *Drosophila* heart in larvae, pupa and adult stages was recently reported by Alex et al. (2015). The current study complements their non-invasive approach by enabling direct access to the tissue for pharmacology and ion exchange. This adds another excellent tool to the fly genetic toolbox which can be used to study various physiological aspects of heart rate at low temperatures.

Acknowledgements:

This work was funded by personal funds (RLC) and in part by University of Kentucky Department of Biology. ZRM supported by Higher Committee for Education Development (HCED) scholarship in Iraq. We thank Dr. Maya Guglin, (MD, PhD, Professor of Medicine, Director, Mechanical Assisted Circulation, Gill Heart Institute, University of Kentucky) for her comments on the study and Dr. Joshua Titlow (PhD, Department of Biochemistry, University of Oxford, United Kingdom) for editorial review and assistance.

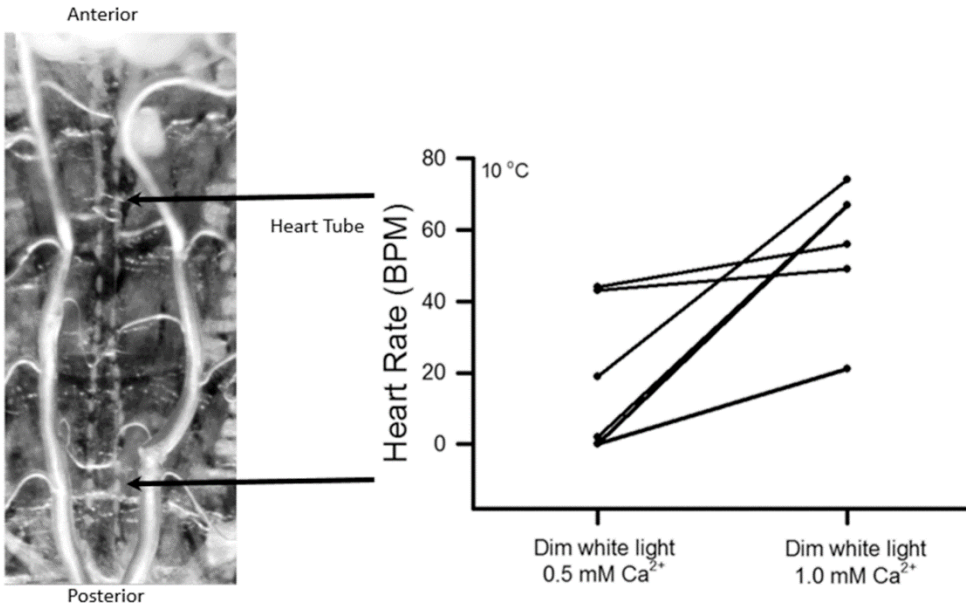

Figure 3.1: Heart rate (HR) is regulated by extracellular calcium concentration in cold conditioned larvae (10^{o}C). (A) The larva heart tube is located between the two trachea in a dissected preparation. The posterior region is the true heart where the rates are measured. (B) Changing the buffer from 0.5 to 1.0 mM Ca^{2+} increased HR in all preparations ($p<0.05$, non – parametric analysis). These are the control background strain UAS-ChR2.XXL fed ATR

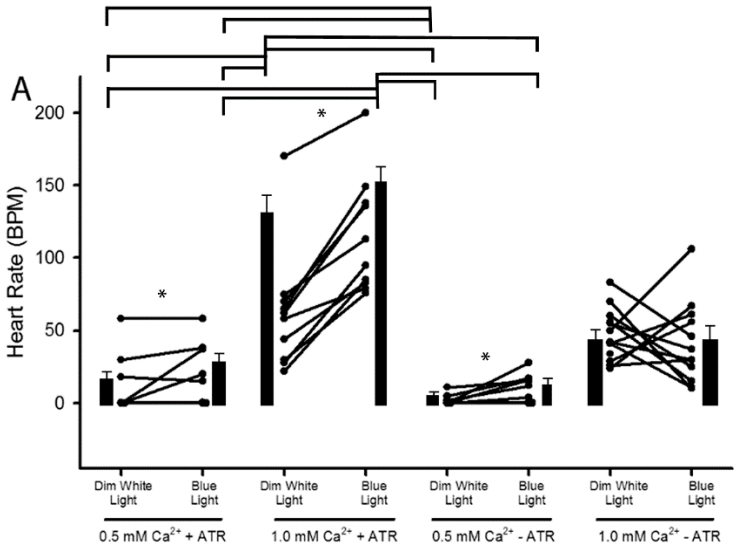

Figure 3.2: Optogenetic stimulation of larval heart rate (HR) at room temperature (21^{o}C). HRs measured in individual larvae in 0.5 mM and 1.0 mM Ca^{2+} with or without food supplemented with ATR. The only condition which did not show a significant increase in HR is 1.0 mM Ca^{2+} without ATR (-ATR) (star is significant, non-parametric $P<0.05$). The mean HR (+/-SEM) for each condition is also shown (solid bars). Horizontal lines above the bar charts indicate significant differences ($P<0.05$) among the groups by a Dunn's Test of rank differences.

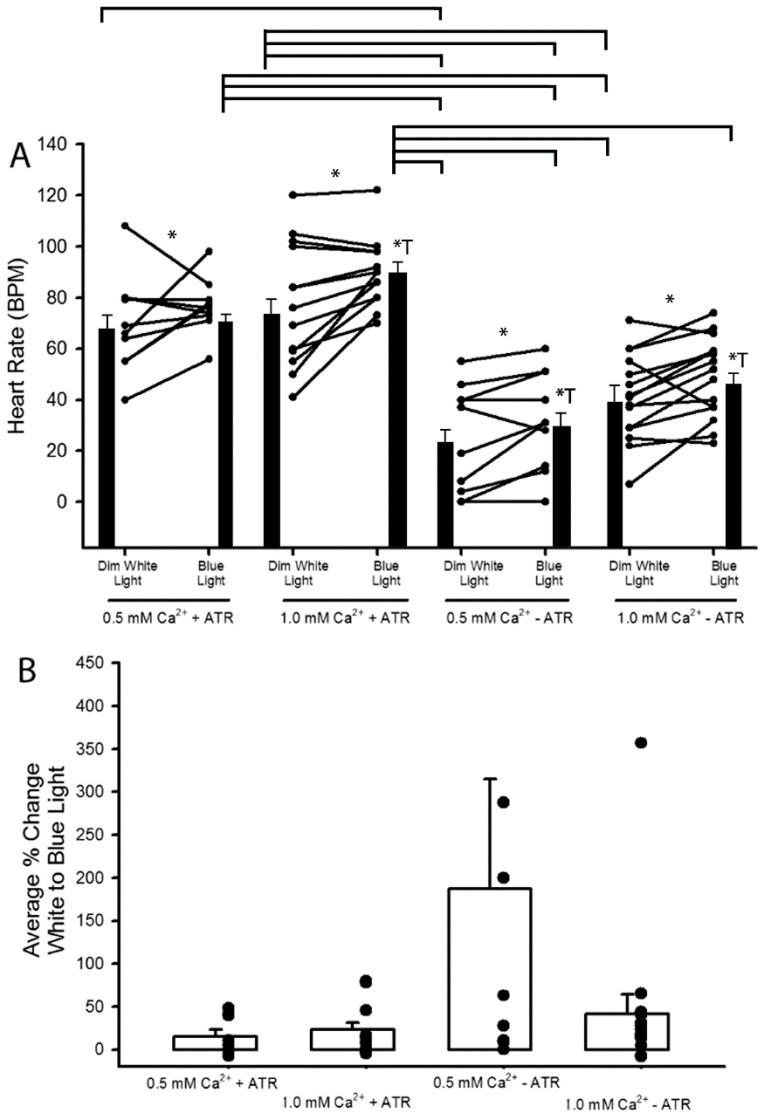

Figure 3.3: Optogenetic stimulation of heart rate (HR) in cold conditioned larvae (10ºC). (A) HRs measured in individual larvae in 0.5 mM and 1.0 mM Ca^{2+} with or without food supplemented with ATR. All conditions show a significant increase in HR (star is significant, non-parametric $P<0.05$); stars with a T are significant with measures as a before and after effect performed with a T-test. All are significant except 0.5 mM Ca^{2+} with ATR). The mean HR (+/-SEM) for each condition is also shown (solid bars). Horizontal lines above the bar charts indicate significant differences ($P<0.05$) among the groups by a Dunn's Test of rank differences. (B) The percent change before and after focused blue light for each condition in (A) revealed a greater change for preparations not fed ATR (-ATR).

CHAPTER 4

This chapter is a draft of a manuscript which about to be submitted for peer review. Leo deCastro was a high school student who helped to initiate this project. Yuechen and Robin did the experiments and wrote the manuscript. Leo reviewed the study and made significant editorial comments.

The effect of temperature changes and exposure to modulators on synaptic transmission at crayfish tonic and phasic neuromuscular junctions

By Yuechen Zhu[1], Leo de Castro[1,2], Robin Lewis Cooper[1]

1. Department of Biology, University of Kentucky, Lexington, KY 40506-0225
2. Massachusetts Institute of Technology, Electrical Engineering and Computer Science (EECS), address is 50 Vassar St, Cambridge, MA 02142.

Figures: 11 Tables: 1

Abbreviated title: Effects of temperature change on neuromuscular transmission

Correspondence to Dr. Cooper, 675 Rose St., Dept. of Biology, Univ. of KY, Lexington, KY, USA 40506-0225 Email: RLCOOP1@UKY.EDU

Abstract

Ectothermic animals are susceptible to extreme temperature changes, such as cold shock in winter. To survive through varied temperatures, ectotherms have developed unique strategies. This study focuses on the synaptic transmission functions in cold temperature, as it is one vital component for ectothermic animals' survival. In examining how synaptic transmission is influenced by acute (20^{o}C to 10^{o}C) and chronic (10^{o}C) cold, the crayfish (*Procambarus clarkii*) neuromuscular junctions (NMJ) were used as a model. To simulate chronic cold conditions, crayfish were acclimated at 15^{o}C for one week and then 10^{o}C for one week. They were then used to examine the synaptic properties associated with the low output nerve terminals on the opener muscle in the walking legs and the high output innervation on the abdominal deep extensor muscle. The opener NMJs increased in amplitude of excitatory postsynaptic potential (EPSP) with acute warm (20^{o}C) exposure after being acclimated to cold; however, the deep extensor muscles showed varied changes in EPSP amplitude. Both NMJs are enhanced with exposure to the modulators serotonin or octopamine. The membrane resistance of the muscles decreased 33 percent and the resting membrane potential hyperpolarized upon warm exposure. HPLC analysis of hemolymph indicated that octopamine increased for cold conditioning. These results suggest modulation is possibly a key for synaptic transmission to remain functional at low temperatures.

Key words: invertebrates, crustacean, adaptation, modulation, synaptic transmission

Highlight: Shift from 20^{o}C to 10^{o}C increased input resistance and depolarized muscle without a rise in EPSP amplitudes.

Acclimated 10^{o}C animals exposed to 20^{o}C increased EPSP amplitude, and decreased input resistance and hyperpolarized membrane potential.

Octopamine increased with 10^{o}C acclimation.

INTRODUCTION

The effect of temperature on neuronal function has been of interest for years, as there are fundamental questions about how various ectothermic animals can adapt to extreme temperatures and, in some cases, live in conditions where other ectotherms cannot (Somero et al., '96; Chung et al., 2012; Veselý et al., 2015). There is also an interest in understanding how cold affects neural tissue when inducing abnormal cold conditions in endotherms (humans) for therapeutic reasons (Mourvillier et al., 2013; Jin et al., 2014). Intact neural systems within whole organisms are too complex to address all the various factors induced by temperature changes since each cell may vary in terms of properties of the membrane, ion channels, synaptic actions, and biochemical cellular responses, which may alter the excitability of neurons. The focus of this study is on the effects of acute and moderate conditioning to cold on synaptic transmission at neuromuscular junctions (NMJs). We choose to use a robust crustacean species (*Procambarus clarkii*) which can live in nature in nearly freezing conditions for short periods (i.e., minutes) and in warm seasonal conditions (40^{o}C). The crayfish (*Procambarus clarkii*) is a successful ectothermic species which can adjust to different environments. It is native to the subtropical climate but is able to live across different temperature zones (Veselý et al., 2015).

In addressing the fundamental synaptic alterations in defined synaptic NMJ preparations, a better understanding can be gained of how complex neural networks in intact nervous systems function with temperature changes (Katz and Kuffler, '46; Katz, '49). NMJs have served as models for years in addressing synaptic function; however, there are also limitations in using the model to address neuron to neuron synaptic communication. The crayfish NMJs are unique as synaptic models since the evoked responses are graded in synaptic strength and can show synaptic facilitation and/or depression. The motor neurons utilize glutamate or GABA as a neurotransmitter, which the postsynaptic muscle must integrate much like a dendrite of a postsynaptic neuron (Atwood, '82; Atwood and Cooper, '96; see review by Titlow and Cooper, 2017).

Two synaptic neuromuscular junctions were chosen to investigate the effect of rapid and acclimated cold temperatures, since they differ in their synaptic efficacy, one being a tonic, low-output and the other a phasic, high-output preparation; moreover, there are previous studies on the properties of these

two model crustacean preparations for comparison (Atwood, '76; Atwood and Cooper, '96). In this study, the tonic NMJ consisted of using the excitatory innervation of the most distal muscle fibers on the opener muscle of the first and second walking legs (Mykles et al., 2002), whereas, for the phasic NMJ, we made use of the deep lateral extensor muscle (DEL1) in the adjoining segment to the segmental root being stimulated (Nguyen and Atwood, '90; Cooper et al., '98). This muscle is used by the animal for rapid escape behavior by tail flips and swimming backwards. Both of these preparations have been investigated in relation to the muscle phenotype and innervation characteristics. Use of the opener muscle for studying synaptic transmission has been reviewed (Cooper and Cooper, 2009). Use of the most distal muscle fibers in the preparation provides a reference for consistency among preparations (Cooper et al., '95b). The innervation on these fibers is low output and fatigue resistant but has pronounced facilitation (Crider and Cooper, '99, 2000). The innervation of the DEL1 muscle is of high output, is fatigue prone, and shows enhanced synaptic plasticity and altered responsiveness to biogenic amine modulation based on motor nerve activity (Cooper et al., '98; Griffis et al., 2000).

When ectothermic animals are exposed to low temperature, this may cause chill injury, as physical properties of molecules and rate processes will be affected. It is suggested that this chill injury happens in four different ways: temperature effect on the structure of membrane lipids and membrane associated proteins; different temperature effects on weak chemical bonds, consequently affecting protein structure and function; temperature effect on ion permittivity and ion channel activity; and the effect on rates of biological processes (Storey and Storey, '88). Chill injury can lead to death. One of the most critical issues is the disruption to electrochemical gradients of ions, which leads to loss of neural activity and neuromuscular coordination, and cessation of respiratory movements (Friedlander et al., '76; Hayward et al., 2014).

Modulators are important factors for inducing physiological changes for arthropods to respond to different environments. Modulators like serotonin (5-HT), octopamine (OA), and dopamine (DA) are well established in crustaceans and insects to alter neuronal, cardiac, GI, ventilatory, and skeletal muscle function (Listerman et al., 2000; Strawn et al., 2000; review by Shuranova et al., 2006; Cooper et al., 2011a,b). Octopamine produces long-lasting contracture in exoskeletal muscles in lobsters and can have direct effects on muscle (Evans et al., '75). 5-HT also enhances synapse output and muscle tension generation in a temperature-dependent manner in lobsters (Hamilton et al., 2007). Peptides can also influence NMJ function. The peptide DF2 (DRNFLRFamide) is known to enhance synapse efficacy in crustaceans (Friedrich et al., '94).

As previous studies have shown, 5-HT and OA enhance synaptic transmission on the opener and DEL1 muscles in the crayfish; however, the studies did not focus on actions of the modulators in temperatures colder than 20^{o}C (Djokaj et al., 2001) and did not examined crayfish acclimated at lower temperatures. Both modulators are known to have a wide range of effects in crustaceans (see review by Shuranova et al., 2006). Considering that 5-HT and OA rapidly alter synaptic transmission centrally and at NMJs, it would not be surprising for

thermal stress to result in acute changes and potentially long-term effects in influence synaptic efficacy. 5-HT and OA have both been shown to alter social behaviors in crayfish (Livingston et al., ’80; McRae, ’96; Huber and Delago, ’98; Listerman et al., 2000) and are known to be rapidly altered due to whole animal activity in crabs (Sneddon et al., 2000) and insects (Hirashima and Eto, ’93).

The purpose of this study is to understand how these two model tonic and phasic NMJs function with acute and relatively chronic cold conditioning, as well as the responsiveness to modulation of two commonly assessed biogenic amines (5-HT and OA) in the cold acclimated crayfish.

MATERIALS AND METHODS

Husbandry

These experiments were all performed using *Procambarus clarkii* crayfish measuring 6–8cm in body length (obtained from Atchafalaya Biological Supply Co., Raceland, LA, USA). Each animal was individually stored in an aquatic facility and was fed commercial fish food pellets (Aquadine)—marketed as “shrimp and plankton sticks: sinking mini sticks”—for at least two weeks prior to experimentation. All the animals were fed equal portions at equal time intervals. The experimental paradigm was designed in two conditions: one where crayfish were maintained at room temperature (20–21^{o}C) and another group which underwent cold conditioning by being exposed to 15^{o}C for one week and then one week at 10^{o}C prior to experimentation (Fig. 1). Modulators were exposed to the dissected preparations at the acclimated temperature.

Dissection

The dissection to expose and selectively stimulate the exciter motor neuron of the opener muscle is described in textual and video format (Cooper and Cooper, 2009). In brief, the excitor neuron is isolated from the inhibitor neuron and stimulated in the meropodite segment. The most distal fibers of the opener were always used for recording synaptic responses. The stimulation paradigm consisted of providing a train of 30 pulses at 40 Hz with ten seconds between trains. An average of 10 to 20 trains in each of the conditions was used for measures of EPSPs (Fig. 2A).

The dissection procedure for exposing the phasic abdominal muscle (DEL1) is also described in text and video format (Baierlein et al., 2011; Robinson et al., 2011). The animals were sacrificed in less than five seconds by rapid decapitation followed by removal of the abdomen. The intracellular muscle recordings were taken from the most medial DEL1 muscle fibers in the abdominal segment most distal to the segmental nerve being stimulated. These fibers are innervated in part by a descending branch of a single motor neuron that also innervates the DEL1 muscle of the segment in which the segmental nerve was stimulated (Parnas and Atwood, ’66; Lnenicka and Atwood, ’89; Mercier and Atwood, ’89; Sohn et al., 2000). Using the more distal segmental DEL1 muscle allowed the recordings to be made from a single identified motor neuron without the possibility of other phasic motor neurons compounding the excitatory responses, as the DEL1 and DEL2 muscles within a segment are innervated by multiple excitatory phasic types of motor neuron

(Fig 2B). In order not to fatigue the NMJ, the segmental nerve was stimulated at 0.5 Hz for 10 to 20 trains in the various conditions so that an average amplitude in the EPSPs could be measured.

Electrophysiology

Dissected preparations were maintained in crayfish saline, a modified van Harreveld's solution (in mM: 205 NaCl, 5.3 KCl, 13.5 $CaCl_2 \cdot 2H_2O$, 2.45 $MgCl_2 \cdot 6H_2O$, and 5 HEPES adjusted to pH 7.4). All intracellular recordings were made with a sharp glass electrode filled with 3.0M KCl, and both recorded and analyzed via Scope and LabChart software (ADInstruments). The amplitudes of the excitatory postsynaptic potentials (EPSPs) elicited by the motor nerve were monitored. Intracellular responses were recorded with a 1 x LU head stage and an Axoclamp 2A amplifier (Molecular Devices, Sunnyvale, CA, USA). Membrane resistance in muscle cells was obtained by delivering pulses of current and measuring the voltage changes in a plateau of the response (~200msec). Current-voltage plots were constructed and the membrane resistance was determined from the slope of the best fit linear curve. For the room temperature experiments, recordings were made in a standard laboratory; however, for the cold conditioned experiments, electrophysiological recordings were made in a walk-in incubator where the crayfish were conditioned at 10^{o}C.

All chemicals were obtained from SIGMA-Aldrich (St. Louis, MO, USA). Serotonin (5-HT) and octopamine (OA) were made into a stock of 1mM in aliquots with crayfish saline. On the day of experimentation, the stock aliquot was diluted to 100nM in crayfish saline.

Statistical analysis

All data are expressed as means (±SEM). SigmaPlot (version 13.0) was employed for statistical analysis. T-test (paired) was used in some experiments to compare the percentage change before and after altering the temperature or the effect of cold conditioning. As specified in the results section, a t-test (unpaired) was used to compare between treatments in cases where there were two groups but not paired. In some cases, a non-parametric analysis (Wilcoxon signed-rank) in the direction of change within each individual was used to compare EPSP amplitude differences based on treatments. $P<0.05$ is considered statistically significant.

RESULTS

Acute exposures to changes in temperature and modulators for low-output NMJs

The effect of rapid exposure (<1 min) to 10^{o}C from an acclimated 20^{o}C of the isolated opener NMJ produced an increase in the 30th EPSP amplitude of the 40 Hz stimulus train in five of the six preparations and a decrease in one preparation (Figures 3A and 3B). The facilitation index mimicked the increases in EPSP amplitude with five of the six preparations showing an increase for the 30th/10th index (Fig. 3C). The facilitation index for earlier pulses within the stimulus train were not as consistent in response to acute temperature change.

The $20^{th}/10^{th}$ facilitation index showed mixed results, with four preparations increasing in facilitation and two showing a reduction in the colder temperature.

The crayfish acclimated to 10ºC resulted in six out of seven preparations presenting an increase in the 30^{th} EPSP amplitude (Fig. 4A). This is quite different from the acute change from 20ºC to 10ºC; the 10ºC to 20ºC resulted in only two of the seven preparations increasing the $30^{th}/10^{th}$ facilitation index (Fig. 4B) with mixed results for the facilitation of the earlier EPSPs within the train ($20^{th}/10^{th}$).

The effect of 5-HT (100nM) and OA (100nM) on the opener NMJs was consistent in enhancing the amplitudes of the 30^{th} EPSP within the stimulus train for every preparation of the 10ºC acclimated crayfish (Fig. 5A and 5C). However, the facilitation indices resulted in mixed responses for the $30^{th}/10^{th}$ and $20^{th}/10^{th}$ for both 5-HT and OA exposure (Fig. 5B and 5D). Like for the 10ºC acclimated crayfish, the 20ºC acclimated animals all resulted in increases in the amplitude of the 30^{th} EPSP of the stimulus train for both 5-HT (100nM) and OA (100nM) (*P>0.05; Fig. 6A and 6C). The facilitation index for the $30^{th}/10^{th}$ and $20^{th}/10^{th}$ with 5-HT exposure resulted in five of the six preparations decreasing (Fig. 6B). The results were not as consistent for exposure to OA for the facilitation indices (Fig. 6D).

Acute exposure to changes in temperature and modulators for high-output NMJs

The phasic (high-output) NMJs of the DEL1 produced an increase in the EPSP amplitude in four of the six preparations upon acute exposure to 10ºC for the 20ºC acclimated crayfish (Fig. 7A and 7B). The results were varied not only in the initial amplitude of the EPSPs, but also in the degree of change upon exposure. Similarly, mixed results occurred for the preparations of 10ºC acclimated crayfish acutely exposed to 20ºC saline (Fig. 7C).

As observed for the low-output tonic NMJs, the high-output phasic NMJs all resulted in increases in the amplitude of EPSP upon exposure to 5-HT (100nM) for crayfish acclimated at both 10ºC and 20ºC (*$P < 0.05$; Fig. 8A and 8C). However, one preparation in each of the 10ºC (one out of six preparations) and 20ºC (one out of seven preparations) acclimated crayfish resulted in a decrease in EPSP amplitude during exposure to OA (100nM) (Fig. 8B and 8D).

Muscle fiber resting membrane potential, input resistance, and OA concentration with changes in temperature

The resting membrane potentials obtained from all the tonic and phasic preparations used in this study were compared at the acclimated temperatures of 10ºC and 20ºC. Both preparations were significantly depolarized at 10ºC as compared to 20ºC (tonic: N=16, 19, unpaired t-test, one tail, $P < 0.05$; phasic: N=19, 20, unpaired t-test, two tail, $P < 0.05$).

The input resistance of the distal muscle fibers from the walking leg opener muscle preparation was determined upon acute changes in temperature for

10ºC and 20ºC acclimated crayfish (Fig. 9). The 20ºC acclimated crayfish all produced an increase in input resistance upon exposure to 10ºC ($P < 0.05$; signed rank, N=6). The 10ºC acclimated crayfish all produced a decrease in input resistance upon 20ºC exposure saline ($P < 0.05$; signed rank, N=5). The wide range in values is likely due to different sized muscle fibers among the different preparations; hence the reason for examining changes for given fibers while acutely exposed to a change in temperature. It is interesting to note that even though relatively similar sized crayfish and legs were used for the experiments, the 20ºC conditioned crayfish resulted in a higher input resistance then the acutely exposed 10ºC preparations ($P < 0.05$; t-test).

Analysis of the amount of OA in samples of hemolymph obtained from six crayfish acclimated at either 10ºC or 20ºC revealed that the 10ºC crayfish contained a higher level of OA than the 20ºC acclimated crayfish ($P < 0.05$; paired t-test) (Fig. 10). The resting membrane potential of the opener muscle depolarizes as temperature is lowered in the 20ºC acclimated crayfish ($P=0.063$; signed rank test) and the membrane hyperpolarizes as temperature rises in the 10ºC acclimated crayfish ($P < 0.05$; signed rank t-test) (Table 1). The Q10 values for the resting membrane potentials also varied depending on the direction of temperature change. For the opener muscle starting at 20ºC and being exposed to 10ºC, the value is 1.37. However, starting at 10ºC and going to 20ºC, the value is 2.50. The directional shifts are even larger for the DEL1 muscle, with 20ºC to 10ºC at 3.26 and 10ºC to 20ºC producing a 1.21 Q10 value.

DISCUSSION

In this study, it was demonstrated that a temperature shift from 10ºC to 20ºC increases EPSP amplitudes in the tonic preparation. However, the temperature shift (20ºC to 10ºC or 10ºC to 20ºC) in phasic preparation did not have a consistent effect on EPSP amplitudes. When tonic and phasic preparations were exposed to 5-HT, EPSP amplitudes showed a substantial increase in both 10ºC and 20ºC acclimated crayfish. Exposure to OA in the tonic preparations also resulted in an increase in EPSP amplitudes in both 10ºC and 20ºC acclimated crayfish. However, exposure to OA resulted in a consistent increase in the phasic preparations in 10ºC, while at 20ºC the acclimated crayfish showed varied results. Membrane input resistance increased and resting membrane potential depolarized in temperature changes from 20ºC to 10ºC. HPLC analysis showed an increase of OA concentration in the hemolymph of 10ºC acclimated crayfish. This increased OA may help crayfish neuromuscular junctions remain functional at low temperature. These studies showed different responsiveness to modulators in enhancing neuromuscular junction transmission in cold conditions.

If given a choice, some crustaceans show a behavioral preference for a thermal environment (Hobbs, '81; Forward, '90; Payette and McGaw, 2003). However, the environment can change rapidly enough that an animal may not have a chance to move to a more suitable condition, causing acute exposure to the new conditions. Thus, survival requires the ability to change physiologically in one aspect and also to behave well enough to forage and avoid predation.

Crustaceans not able to undergo diapause (Hand, 2016) but needing a functional nervous and muscular system for survival would need the ability to maintain cellular responses. The adaptive physiological processes are unique depending on the crustacean species. *Macrobrachium rosenbergii*, commonly referred to as the Malaysian prawn, will die when rapidly or slowly exposed to 10^{o}C (Chung et al., 2012), whereas *Procambarus clarkii* are able to survive and respond to sensory stimuli. The heart and neural function ceases in this prawn species at 10^{o}C, which is likely the reason the animal dies. The small freshwater crustaceans *Niphargus rhenorhodanensis* and *Niphargus virei* (both cave crustaceans) and a close relative to these two cave species, *Gammarus fossarum* (surface crustacean), are known to survive to -2°C (Issartel et al., 2005). The estuarine crab (*Hemigrapsus crenulatus) is found in nature* within cold environments of 2.5°C (Cumillaf et al., 2016). Even though various crustaceans are known to occur in cold environments and have biochemical differences which likely account for their survival, the physiological reactions to acute changes in temperature have not been investigated. A limited ability to survive changes in temperature may be a reason for the local endemic nature of some species as compared to hardy invasive species' ability to survive a cold environment as well as rapid changes in temperature (Veselý et al., 2015). However, animals may choose ecological niches which do not expose them to harsh environmental conditions to survive (Jakobs, 2015).

There are various aspects to consider in conditioning to cold, such as cold hardening, as compared to the effects of an acute change within a few minutes of cold exposure. The effects on changes in osmolality of the hemolymph, gene expression, transcriptome differences, amino acid composition, and concentrations of sugars for cold hardening and longer-term cold exposures have been intensively investigated in insects (Czajka and Lee, '90; Overgaard et al., 2007; Colinet et al., 2010; Teets et al., 2012; MacMillan et al., 2016) and bacteria (Phadtare and Severinov, 2010). In rapid exposure to cold the cell membrane does not have time to alter composition in fluidity; thus, ion channels, pumps, and receptors need to function in a potentially more rigid environment when going from 20^{o}C to 10^{o}C. The physiological function of the proteins may be quite different when the membrane has time to undergo changes in fluidity and cold shock proteins have had time to be expressed. The ability of animal survival with gradual exposure to cold as compared to rapid change is well known in insects, but little attention has been given to the physiological differences in synaptic transmission of animals to function in rapid (< five minutes), as compared to hours or days of, cold exposure.

In an intact animal, initiating a movement involves a neural command and activation of descending neural circuits to then activate motor neurons. The synaptic connections and electric flow along axons, as well as muscle contraction, all involve moving proteins as ion channels, ion binding proteins, vesicular docking-fusion complexes, receptors, pumps, exchangers and proteins associated with muscle contraction and relaxation. The effect of temperature on neural circuits has just recently been investigated in the crustacean stomatogastric nervous system for preparations acclimated to

different temperatures (Marder et al., 2015). The function of NMJs and the development of muscle tension in crustacean preparations (crabs and crayfish) are also known to be influenced differently if the intact animal is acclimated to differing temperatures. Muscle fibers in the claws of stone crabs and blue crabs show cold acclimation and have a higher input resistance at 8°C than non-cold acclimated crabs. This higher input resistance resulted in a broadening of EPSPs which enhanced EPSP summation, muscle fiber depolarization, and muscle force (Fischer and Florey, '81; Hamilton et al., 2007).

Studies in differing species of crayfish (*Astacus leptodactylus*, *Procambarus clarkii)* and crab (*Ocypode ceratophthalma*, *Carcinus maenas, Cancer pagurus*) all show a reduction (more hyperpolarized) in membrane potential with acutely increasing temperature. The relative change is within 1 mV to 1.3 mV /1^{o}C change (Florey and Hoyle, ’76; Harri and Florey, ’77, 1979; Hyde et al., 2015). We observed a similar hyperpolarization of the muscle membrane potential from 20 to 10^{o}C. The rationale for the change has not been explicitly addressed in prior reports, but it is likely to do with the more negative equilibrium potential of K^+ (E_K), since the membrane is more permeable to K^+ and the resting potential is driven mostly by the E_K.

With a more negative E_K, one would also assume a large EPSP would result in the muscle due to a larger driving gradient to E_{Ca} and E_{Na} for glutamate receptor-induced depolarization of crustacean muscle. Crayfish skeletal muscle uses voltage gated Ca^{2+} channels in the plasma membrane as a contribution to the EPSP and muscle contraction. However, in crabs and crayfish it has been shown that the amplitude of EPSP decreases with increasing temperature past the conditioned temperature of the animal (Florey and Hoyle, ’76; Harri and Florey, ’77, ’79; Hyde et al., 2015). The amplitudes of the EPSP are varied and have been shown to be generally optimal at the temperature in which the animal is conditioned. This maximum amplitude in the EPSP for an acclimated temperature is more commonly apparent for the tonic NMJs than for the phasic NMJs (Harri and Florey, ’79). However in our studies, the 20^{o}C conditioned animals showed a slight enhancement in the EPSP amplitude with acute exposure to 10^{o}C, whereas the 10^{o}C conditioned animals showed an increase with acute exposure to 20^{o}C. The effect of cooling on increasing input resistance and depolarizing the resting membrane potential is well documented in crustacean muscles for crabs and crayfish (Harri and Florey, ’77, 1979; Fisher and Florey ’81; Blundon, ’89). Depolarization of the presynaptic terminal can also be delayed at lower temperatures compared to high temperatures due to slower calcium ion movement, as shown for squid axons (Charlton and Atwood, ’79); however, this has not been directly assessed in crayfish nerve terminals.

No mechanistic explanations have yet accounted for the differences observed in the maximum EPSP responses at tonic and phasic NMJs in acclimated animals. In speculating, potentially the tonic NMJs, being more metabolically active, undergo a rapid change to homeostatically regulate synaptic efficacy. Perhaps if the acclimation periods in the experimental studies were extended, the phasic NMJs may also show a shift in optimal amplitude of EPSP to the

conditioned temperature. However, there might just be intrinsic differences which do not allow the phasic NMJs to shift the EPSP amplitude. In natural settings, crayfish show seasonal differences in phasic EPSP amplitudes. In the winter, the phasic NMJs produce larger EPSPs which are depression prone, as compared to summer crayfish, which are more active and have smaller amplitude EPSP for phasic NMJs, but are also fatigue resistant (Lnenicka, '93; Lnenicka and Zhao, '93). Activity dependence changes were also induced in laboratory conditions with electrical conditioning of the phasic motor neurons (Cooper et al., '98). Thus, the phasic NMJs are dynamic enough to change their synaptic output with evoked activity, but adjusting to acclimated temperature for a maximal response must involve different processes. The seasonal effects are apparently species-dependent, as the crab *Cancer pagurus* does not show seasonal effects as compared to *Carcinus maenas* in regard to muscle physiology (Hyde et al., '15), but both crabs *Menippe mercenaria* (Say) and *Callinectes sapidus* (Rathbun) show changes with seasonal temperature changes, suggesting different adaptive advantages (Blundon, 1989). However, it is difficult to separate the direct effect of temperature from activity-dependent change, as the behavior of the animal changes with environmental temperature.

The muscle plasma membrane and ion channels on the muscle membrane also contribute to the degree of synaptic strength. The ability of the plasma membrane to make changes in its composition likely accounts for the differences in membrane resistance for acute changes in temperature for non-acclimated and acclimated animals (Chapelle, '77, '78; Chapelle et al. '79). The increase in input resistance for 20^{o}C acclimated crayfish exposed to acute saline change at 10^{o}C would suggest that leak channels do not allow ions to pass as readily. In comparison, 10^{o}C conditioned animals showed a decrease in input resistance in acute exposure to 20^{o}C. The increased input resistance at 10^{o}C would in part account for some of the increase in the amplitude of EPSPs 10^{o}C. The acclimated 10^{o}C animals had a slightly lower input resistance on average, which may account for changes in the composition of the membrane. The potential changes which can occur are likely similar to those well documented for insects as reviewed in Teets and Denlinger (2013).

Considering how rapidly hormones can act on altering synaptic transmission and muscle tension, it is realistic to entertain the possibility that rapid cold stress exposure may result in altered release of biogenic amines or peptides to potentially compensate for altered synaptic function. We are not aware of any reports in which biogenic amines or peptides have been measured in the hemolymph of insects or crustaceans during exposure to cold, except for Zhu et al. ('16). Zhu et al. showed that OA decreased in cold-acclimated (10^{o}C) larval *Drosophila* as compared to 20^{o}C. We are also not aware of any reports on changes in biogenic amine levels in cold conditions as compared to warmer temperatures for a crustacean. The increase in OA for the cold-conditioned crayfish may enhance the skeletal muscle to produce and increase force of contraction, since it is known to do so in insects and crustaceans in warmer temperatures (Fischer and Florey, '87). In addition, the increase in EPSP amplitude on exposure to OA for the 10^{o}C acclimated crayfish indicates that the

preparations could respond to an even greater concentration in the intact system. It is unlikely that the effect would have dissipated within five minutes of preparing the preparation for electrophysiological recording and exposure to 100nM of OA. The larger increases in EPSP amplitude in the isolated preparations for 20^{o}C acclimated crayfish may be indicative of the lower concentration in the intact animal, thus resulting in an increased sensitivity when exposed. In our HPLC analysis, we were not able to detect any differences in 5-HT levels among the different temperatures; however, the isolated NMJs responded well to exposure to 5-HT for both the 10^{o}C and 20^{o}C acclimated crayfish. As for OA, the animal is likely able to respond to higher levels of 5-HT within the hemolymph in the intact system if it is released in the animal under stressful conditions. In previous studies, 5-HT and OA displayed a strong ability to enhance presynaptic and postsynaptic processes at the neuromuscular junction in crustaceans. Octopamine is able to increase contractile force in the crustacean skeletal muscle (Fischer and Florey, '87) as well as alter muscle force and synaptic structure in insects (Koon and Budnik, 2012). It was shown for the isolated pyloric muscle in lobster that a decrease in its rhythmicity occurred at warmer temperatures; however, in the presence of dopamine, the activity was maintained (Thuma et al., 2013). The authors suggested that within the animal dopamine may be responsible for the animal maintaining its similar consumption of food in the warm as in the cold. So in this case, an increase in a modulator is helping the NMJs and animal to function better in a warm environment.

For the 10^{o}C acclimated crayfish, we noticed that in 40 Hz stimulating pulses to the opener motor nerve, with a facilitating train, the 30th EPSP was almost undetectable when bathed in 10^{o}C saline. However, upon adding 5-HT or OA, the amplitude of the EPSP increased. Initially, it appeared as if the nerve was not being stimulated since the EPSPs were so small in saline alone. However, before removing the leg, the animal was able to close the claw in the walking leg robustly when disturbed. It would be interesting to test the force of contraction of the claw in the first pair of walking legs in intact animals in the two acclimated temperatures to see if force generation is different. At 20^{o}C, the same stimulus train always revealed large amplitude EPSP by the 30th pulse within the train. So, despite the lower EPSP amplitude in cold, the force of muscle generation may be similar, since OA is increased in the 10^{o}C acclimated crayfish. As shown in larval *Drosophila*, the shapes of the evoked EPSP are not affected by OA despite an increase in force generation in the muscle. Subsequent studies in cold acclimated crayfish and effects on muscle tension development with OA exposure would potentially answer this question. However, upon acclimating the animals to cold, the animal will raise the endogenous OA levels. A pharmacological means of blocking the synthase of OA in 20^{o}C and 10^{o}C acclimated crayfish and then examining the isolated leg's ability to generate force when exposed might be feasible, assuming the receptor expression level would not be differentially regulated.

When changing the stimulation pulse from 40 Hz to 60 Hz, the EPSP amplitudes will increase, even if barely detectable at 10^{o}C. This may suggest that lower temperature disrupts the calcium channels in the presynaptic terminal to cause less, or significantly delay, calcium influx. Therefore, with longer trains of stimulation, more calcium could accumulate in the presynaptic terminals to result in more vesicle fusion events (Cooper et al., 1995; Desai-Shah et al., 2008). We had assumed that the nerve may have failed in being evoked at colder temperature, as known for squid axons (Weight and Erulkar, '76; Prosser and Nelson, '81), but upon a higher stimulation frequency or with the addition of 5-HT or OA the EPSPs would increase to be measurable. Thus, we knew that the axon was indeed being recruited. It was noted in an earlier study (Jacobs and Atwood, '81) using crayfish in which differences in long-term facilitation was being investigated for cold exposed animals, as well as isolated NMJ preparations, that synaptic responses are more pronounced within the animal.

Gaining a better understanding of the general effects on synaptic physiology of acute and longer-term cold exposure will potentially aid in applications of therapeutic hypothermia used for humans (Shankaran, 2009; Yenari and Han, 2012; Sadaka et al., 2013). In hibernating animals, which lower their body temperature, a cold induced protein shows promise in protecting neurons from damage and aids in synapse reformation in the rewarming of hibernating mammals (Peretti et al., 2015). It is known that synapses can fade away with cold and can reappear not only in hibernating mammals (Peretti et al., 2015), but also in arthropods (Brandstatrer and Meinertzhagen, '95).

In the cold environment, the ability of the proteins to function could be decreased due to improper or ridged folding. It is estimated that at some chemical mediated synapses there are over 2,000 different proteins, based on proteomic studies (Dieterich and Kreutz, 2016). Therefore many aspects of synaptic transmission on the presynaptic and postsynaptic sides could be affected by temperature changes. In the presynaptic terminal, the voltage gated calcium, sodium, and potassium channels, mono-cation leak channels, ion exchangers/pumps (PMCA-plasma membrane calcium pump, NCX-Na-Ca exchanger and Na/K ATP dependent pump), proteins involved with ATP production in mitochondria, proteins involved with vesicle recycle, and SNARE complex may all be affected by temperature. In the postsynaptic terminal, the glutamate receptors and mono-cation leak channels which would alter the membrane resistance and resting membrane potential could also be affected by temperature.

In highlighting the various factors to consider at the NMJ with changes in temperature, a generalized model is provided as a building block to work from to address the potential mechanisms (Fig. 11). In the acute temperature shift from 20^{o}C to 10^{o}C, a delayed or prolonged voltage gated calcium entry would have a direct effect on synaptic transmission. This would likely cause a heightened EPSP amplitude. The direct effect of cold temperature is expected to alter the equilibrium potential for potassium by producing a more positive value, based on the Nernst equation, and may account in part for the depolarized resting membrane potential. Also, with decreased temperature, we

note that membrane resistance increases in the muscle fiber, which would also suggest some leak channels might be closed or reduced in ion permeability. If a similar phenomenon occurs in the nerve terminal, the voltage gated sodium channel will face smaller driving gradients but, with much higher membrane resistance, would tend to result in larger action potential. A larger action potential could cause more calcium entry and more priming for vesicle fusion. On the other hand, the function of mitochondria could be compromised, resulting in reduced ATP production and therefore reduced function of PMCA and other ATP-dependent steps. A decease in the function of PMCA would result in an increase in residual calcium. Therefore, in the 20^{o}C to 10^{o}C change, we expect to see higher EPSP based primarily on action on the presynaptic terminal; however, this was not observed. Despite increased membrane resistance in the muscle fiber, the amplitudes of the EPSPs were not largely influenced, which then draws attention to the responsiveness of the glutamate receptors or alterations in the transmission from the presynaptic terminal. However, the more depolarized resting membrane potential will reduce the driving gradient for EPSP. Considering acclimated exposure to 10^{o}C accentuated the reduced amplitude of EPSP with the acute responses, there did not appear to be a homeostatic compensation in these biophysical properties in excised preparations. The rate of vesicle recycling through a rapid or endosomal path would likely be slowed in colder temperatures. The docked vesicles and reserve vesicles to be recruited to synapse within the trains of stimuli may well have varying kinetics of fusion, which will impact the summation of the graded EPSP amplitudes.

In our study, acute and chronic cold exposure caused depolarized resting membrane potential. These depolarized resting membrane potentials could be explained with several factors. The Na/K pump Q10 is around 2.4 in sea urchin with temperatures (Leong and Manahan, ’97). Also, several papers (MacMillian and Sinclair, 2011; Andersen et al, 2013; MacMillan et al, 2015) indicates that in insect cold exposure, potassium will leak towards the hemolymph cause a progressive increase of extracellular potassium. The reduction of Na/K pump and increased potassium extracellular concentrations can lead to depolarization of membrane potential. This may explain the depolarization that we observed in the 20^{o}C to 10^{o}C acute cold exposure. More than that, with different temperatures acclimated crustaceans there are changes with potassium ion concentrations in the hemolymph with temperatures. In Legarra et al,. ’84 paper, the potassium concentrations are decreased with lower temperatures in *Procambrus clarkii* crayfish. Wong and Freeman (’75) also showed potassium concentration in hemolymph is decreased with lower temperature. Combine lowered extracellular potassium concentrations with decreased Na/K pump function in lower temperature, it is observed that resting membrane potential was still depolarized in our study with chronic cold exposure. It may indicate that decreased Na/K is playing a bigger effect compare to possible lowered potassium concentrations in the chronic cold exposure crayfish.

In the temperature shift from 10^{o}C to 20^{o}C, we do observe a significant increase in the EPSP amplitude, which may be expected with a larger driving gradient from a more negative resting membrane potential; but one would not expect an increased EPSP amplitude with a reduced input resistance. This indicates that these biophysical components involved in synaptic transmission might be more in tune for 20^{o}C, as it is more closely aligned to the optimal temperature range of this species of crayfish.

In this model, there are components that are contradictory to each other's expected function in the temperature shift. The synaptic transmission efficacy is an integration of all these components. The actual mechanism of neuromuscular junction response to different temperatures is more complicated than the biophysical model we proposed and generally considered. Perhaps now, adding the potential effects of a modulator such as OA and drawing from findings in other invertebrates that muscle force is increased with OA (Evans et al., '75; Ormerod et al., 2013) and synaptic responses are increased (Fischer and Florey,'87; Djokaj et al., 2001), this could account for in vivo abilities of the NMJs to remain active.

In addressing some additional potential mechanisms to account for the synaptic efficacy changes with acute cold exposure, it would be of interest to know if the presynaptic terminal is loaded with more evoked Ca^{2+} influx due to a potentially larger driving gradient for the voltage gated Ca^{2+} channels, or if there are direct effects on the channels. This issue might potentially be addressed with Ca^{2+} sensitive indicators or quantal analysis by focal macro patch electrodes placed over segments of the nerve terminal (Cooper et al., '95). An analysis of the quantal events to determine if changes in the amplitude and/or decay time occur with acute temperature changes would aid in determining the contribution of the single quantal events to the compound evoked EPSP. Additionally, it would be of interest to know if the modulators induced different cellular responses related to Ca^{2+} influx in the presynaptic motor nerve terminals for cold vs warm acclimated preparations, as well as if different effects occur in tonic as compared to phasic motor nerve terminals.

ACKNOWLEDGEMENTS

We thank Ms. Elena Piana (Sea Farms Limited, Redditch, Worcestershire, United Kingdom B98 0RE.) and Ms. Shannon Conlin (Swansea University, United Kingdom) for editorial suggestions.

Table 4.1 Resting membrane potential (RP) with temperature shifts from 10°C to 20°C and 20°C to 10°C in tonic opener muscle preparations.

10°C to 20°C			20°C to 10°C		
	10°C RP (mV)	20°C RP (mV)		20°C RP (mV)	10°C RP (mV)
Prep 1	-60	-72	Prep 1	-81	-71
Prep 2	-65	-79	Prep 2	-63	-57
Prep 3	-72	-78	Prep 3	-75	-64
Prep 4	-67	-70	Prep 4	-87	-60
Prep 5	-75	-92	Prep 5	-82	-70
Prep 6	-62	-74	Prep 6	-95	-85
Average percent change (+/-SEM)	-16 (+/-0.03)		Average percent change	12 (+/-0.05)	

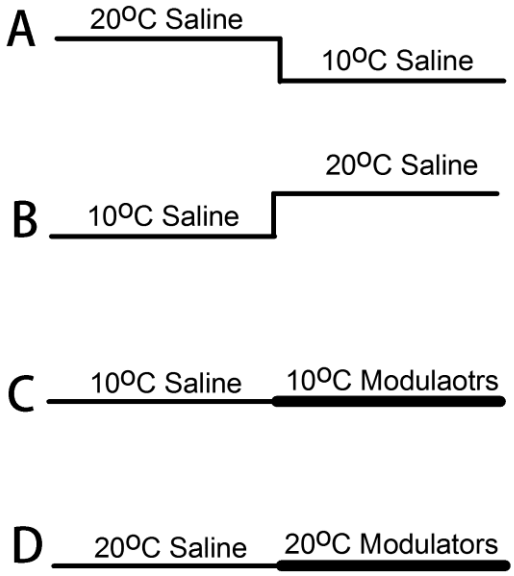

Figure 4.1 The methodological paradigm for acute and chronic conditioning. The acute cold (A) and chronic cold (B) paradigms and application of modulators (C and D) under chronic conditions are depicted at the various temperatures. Inducing a chronic 10°C condition, the 20°C animals were placed at 15°C for one week and then one week at 10°C before being used for experiments.

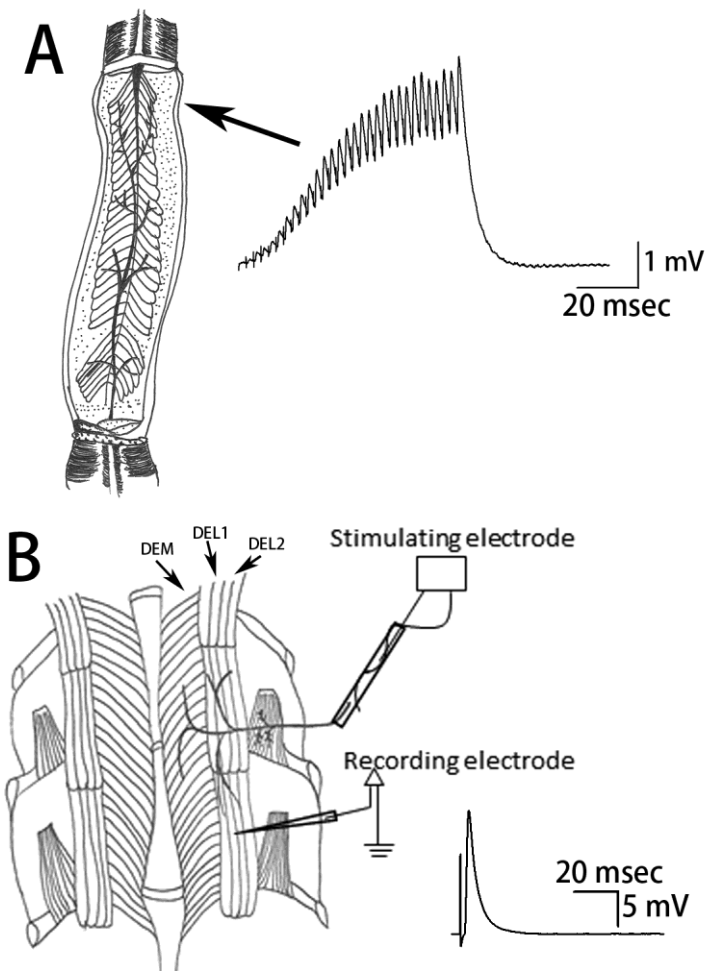

Figure 4.2 The walking leg opener and abdomen neuromuscular preparations. (A) The low output tonic-like neuromuscular junctions on the opener muscle produce excitatory postsynaptic potential (EPSP) which rapidly facilitates within a 30 pulse train of 40 Hz stimulation. The 10^{th}, 15^{th}, 20^{th}, and 30^{th} amplitudes of the EPSP are used to index synaptic efficacy and facilitation. The ventral view looking dorsally at the opener muscle is schematically illustrated. The most distal muscle fiber in each preparation was consistently used to minimize variation among preparations. (B) The high output phasic-like neuromuscular junctions on the deep abdominal lateral extensor muscle (DEL1) produce large EPSP with single evoked stimuli. The DEL1 muscle fiber in the segment below the one being stimulated is used since only one motor neuron innervates these fibers from the more anterior segment. The stimulus artifact is recorded, followed by the large amplitude phasic EPSP. The amplitude of the EPSP is used to index the effects of temperature and/or modulation.

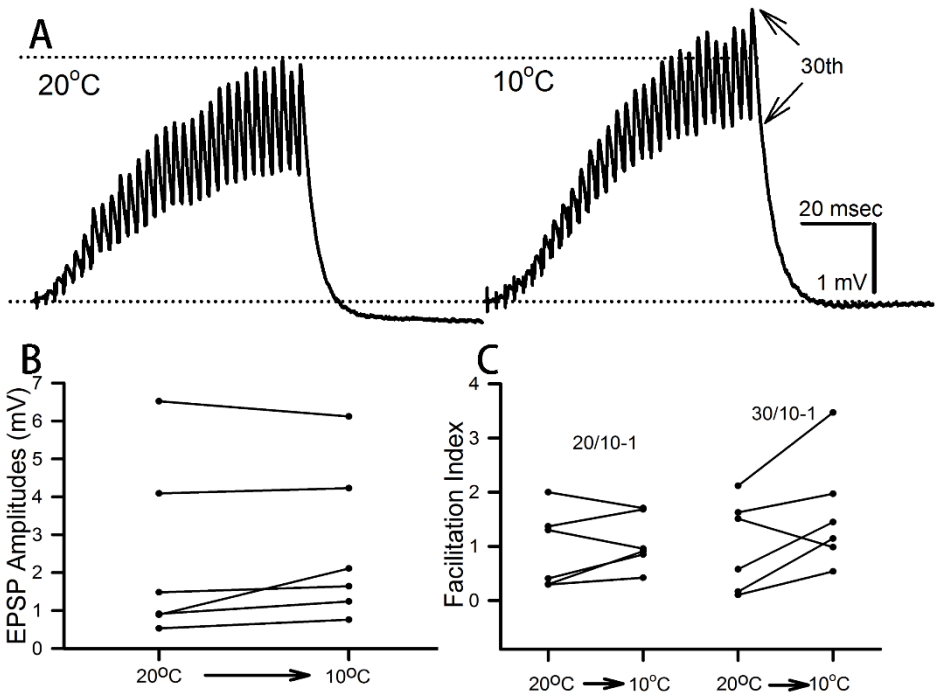

Figure 4.3 The effect of acute cold exposure on the tonic-like NMJs. (A) Representative EPSP traces of the changes which occur when exposing a preparation conditioned at 20°C to 10°C. Measurement of the EPSP amplitude is made from the trough preceding the event to the peak of the event, as illustrated for the 30th event within the stimulus train. (B) The amplitude of the 30th EPSP from the 40 Hz stimulation trains before and during the acute cold exposure is shown for each preparation. (C) The facilitation index (FI) for each preparation is illustrated. The indices are shown for 20th/10th and 30th/20th EPSPs to indicate the degree in change in facilitation due to the acute cold exposure. The FI is determined by the 30th or 20th EPSP amplitude divided by the 10th EPSP amplitude and subtracting a unit of one to ensure that, if no facilitation occurred, the FI would be zero.

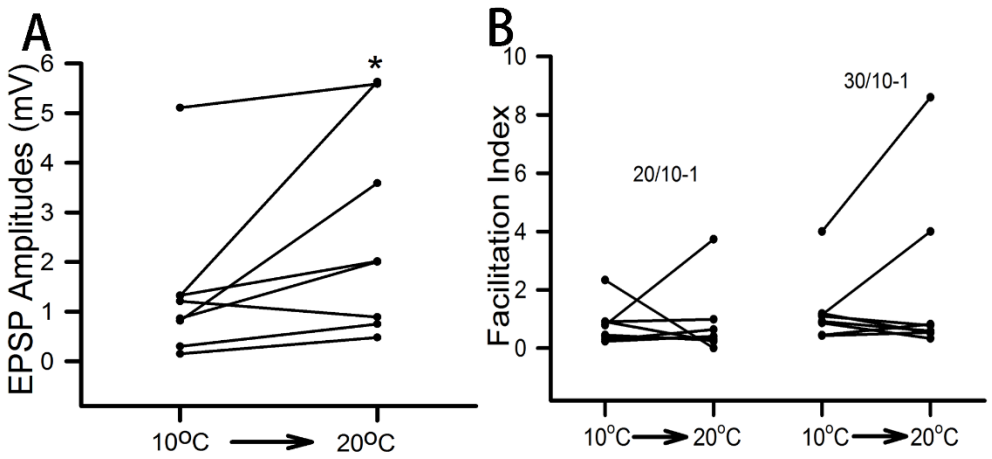

Figure 4.4 The effect of chronic cold conditioning at 10°C on the tonic NMJs when exposed to 20°C. (A) The amplitude of the 30th EPSP from the 40 Hz stimulation trains during the acute cold exposure and after the exposure to 20°C is shown for each preparation. (B) The facilitation index (FI) for each preparation is illustrated. The indices are shown for 30th/10th and 20th/10th EPSPs to indicate the degree in change in facilitation due to the change in cold conditioning to a warm exposure.

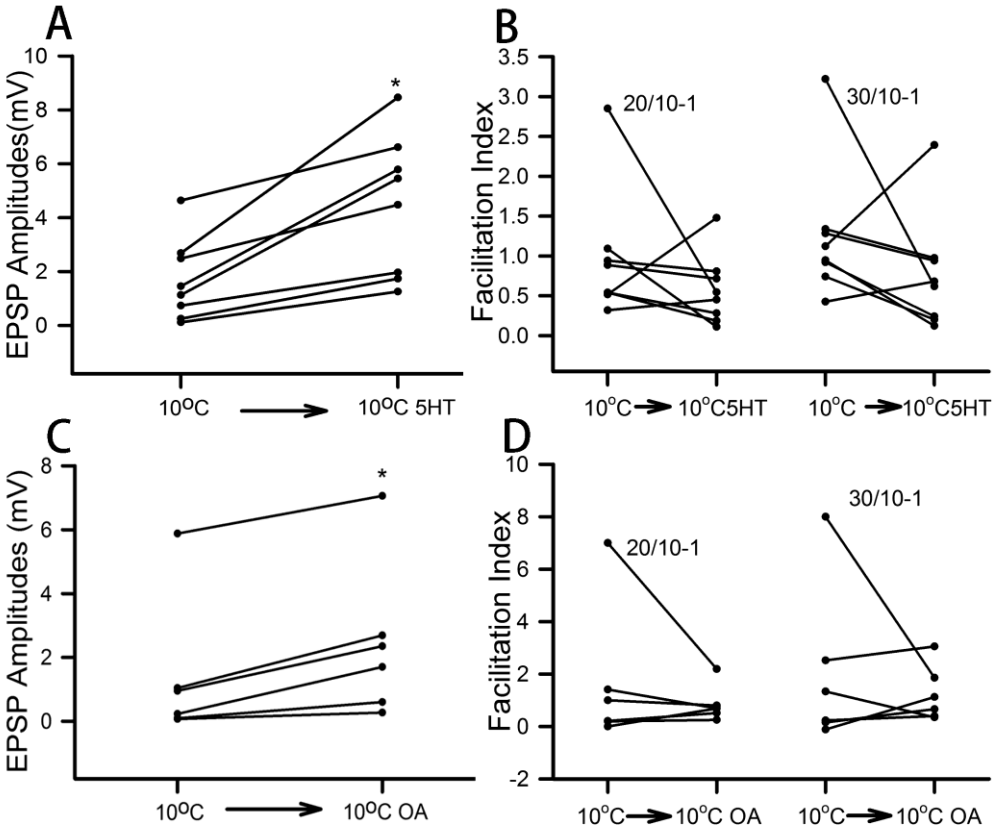

Figure 4.5 The effect of serotonin (5-HT) or octopamine (OA) on the synaptic efficacy of chronic cold 10°C acclimated preparations. The amplitude of the 30th EPSP from the 40 Hz stimulation trains during acute cold exposure is shown for each preparation before and during exposure to 5-HT (A) and OA (C). The facilitation index (FI) for each preparation is illustrated before and during exposure to 5-HT (B) and OA (D). The indices are shown for 30th/10th and 20th/10th EPSPs to indicate the degree in change in facilitation due to exposure to the modulators (P>0.05 signed rank test).

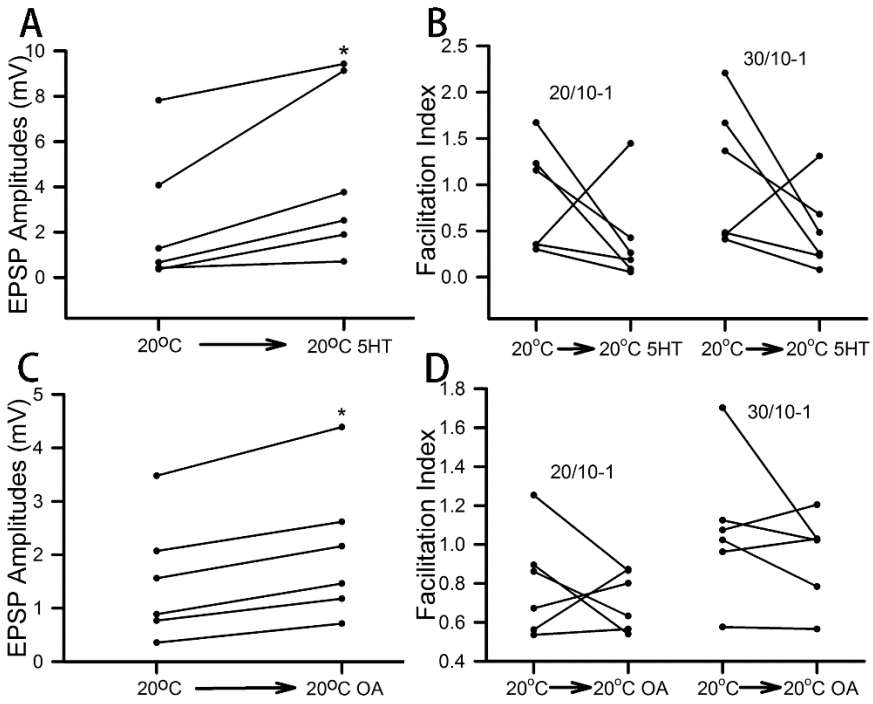

Figure 4.6 The effect of serotonin (5-HT) or octopamine (OA) on the synaptic efficacy of 20°C acclimated tonic NMJs. The amplitude of the 30th EPSP from the 40Hz stimulation trains is shown before and during the exposure to 5-HT (A) and OA (C). The facilitation index (FI) for each preparation is illustrated before and during exposure to 5-HT (B) and OA (D).

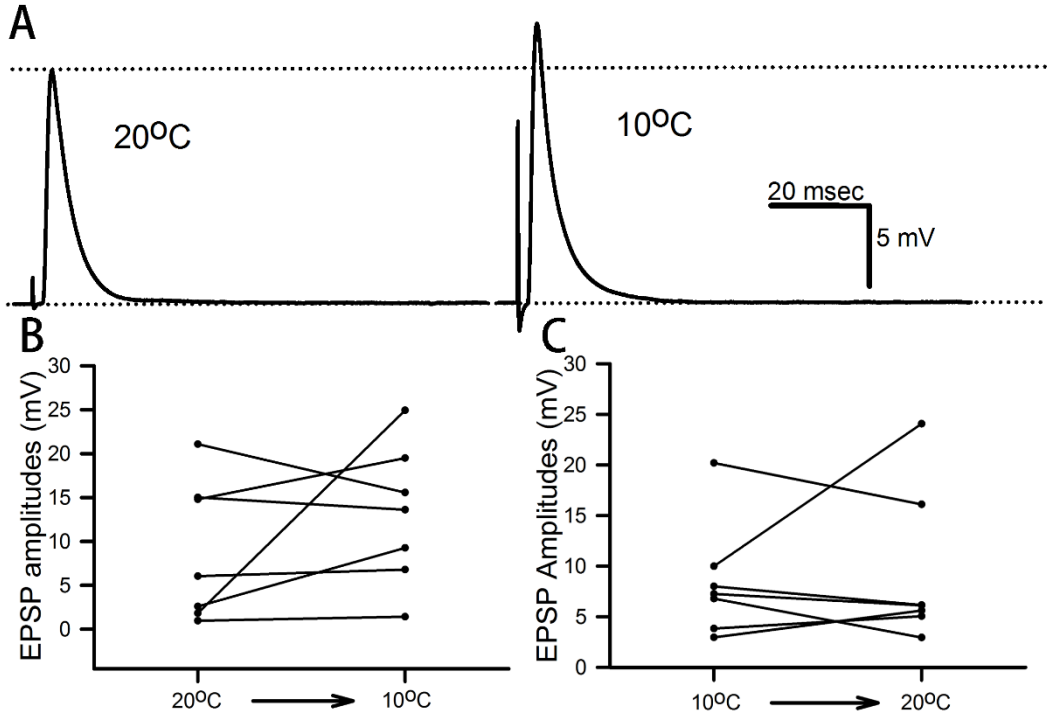

Figure 4.7 The effect of acute cold exposure on the phasic NMJs. (A) Representative EPSP trace when exposing a preparation conditioned at 20°C to 10°C. (B) The amplitude of the EPSP before and during the acute cold exposure is shown for each preparation. (C) The amplitude of the EPSP before and during acute exposure of preparations conditioned at 10°C to 20°C.

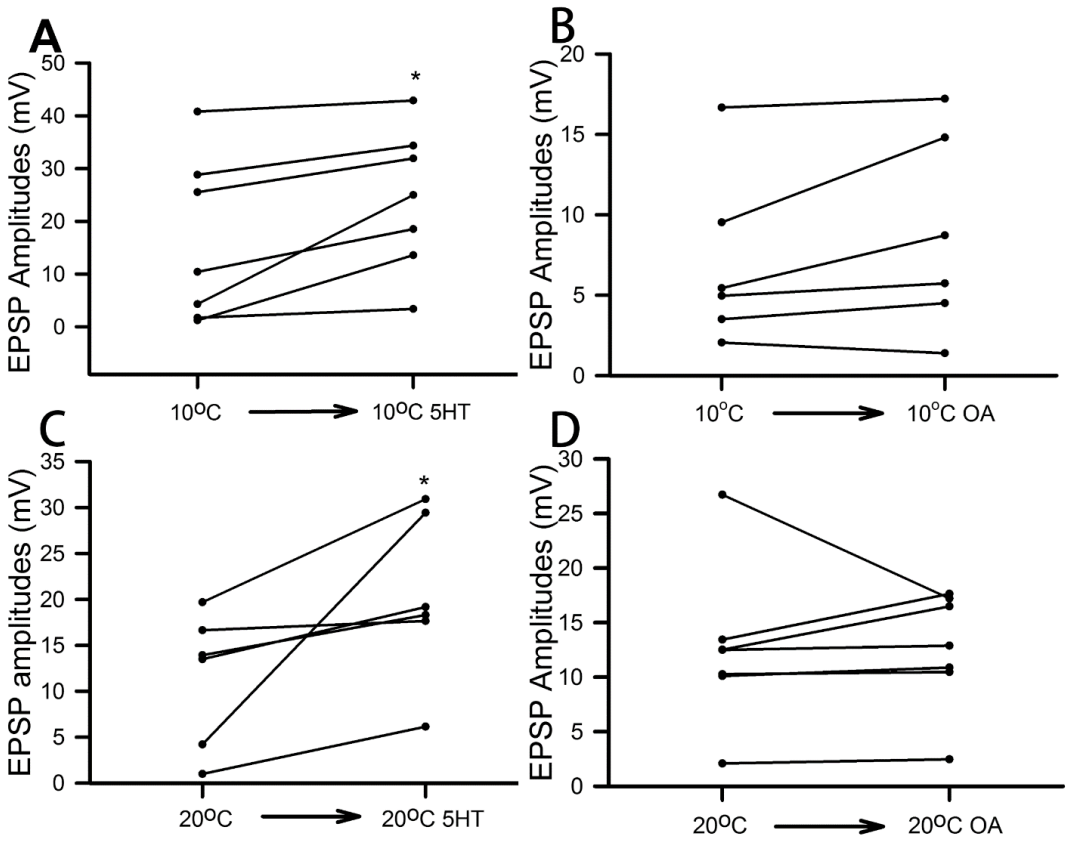

Figure 4.8 Comparison of the effect of 5-HT or OA on the EPSP amplitudes for phasic NMJs for crayfish acclimated at 20°C or 10°C temperatures. Crayfish were acclimated at 10°C and exposed to 5-HT (100nM) (A) or octopamine (100nM) (B). Similarly, crayfish were acclimated at 20^0C and exposed to 5-HT (100nM) (C) or octopamine (100nM) (D).

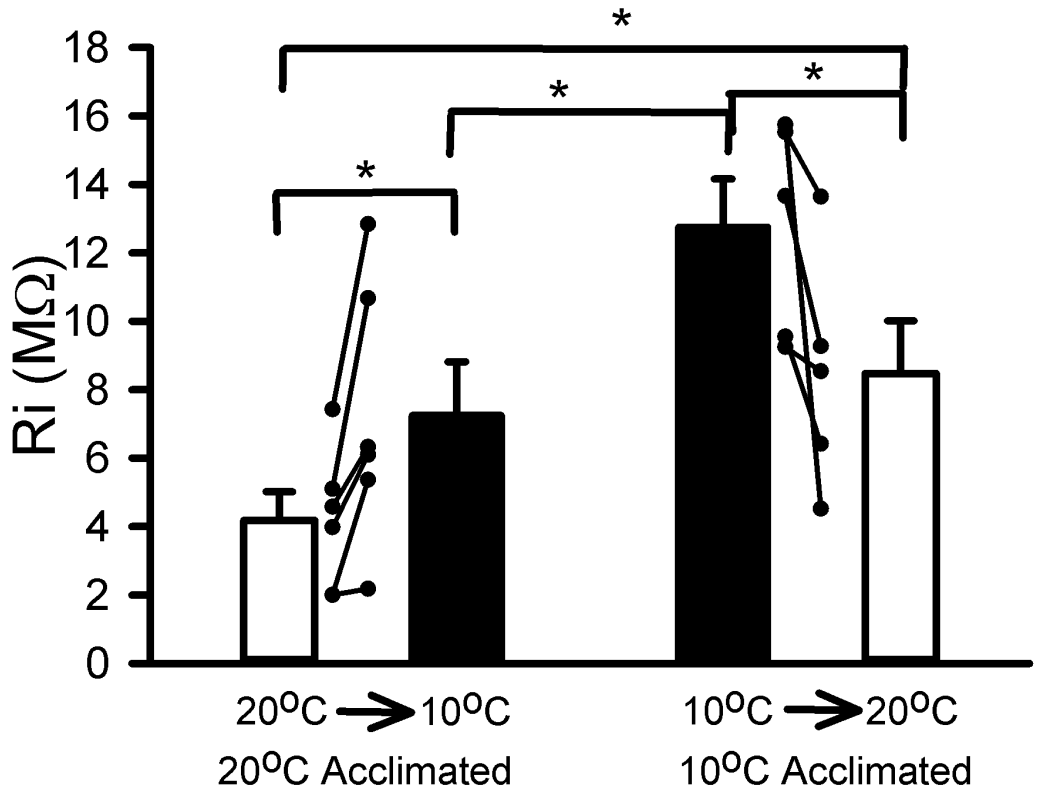

Figure 4.9 Muscle fiber input resistance for muscle conditioned at 20°C and exposed to an acute change to 10°C (A), as compared to a muscle chronically conditioned at 10°C and exposed acutely to 20°C (B). Individual preparations are shown, along with an averaged response (+/-SEM). ($P < 0.05$, signed rank test).

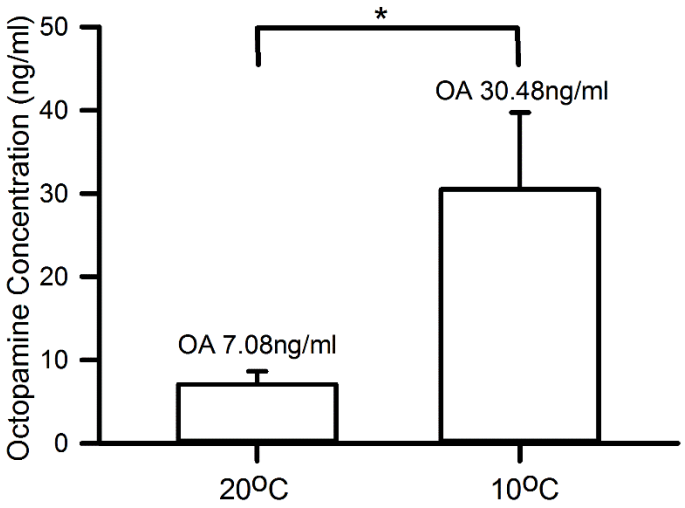

Figure 4.10 HPLC analysis of octopamine within the hemolymph of crayfish maintained at 20°C and acclimated to 10°C. Each sample was from individual crayfish at each temperature condition and then averaged. Six crayfish were used for each conditioned (mean +/- SEM) ($P < 0.05$, T-test).

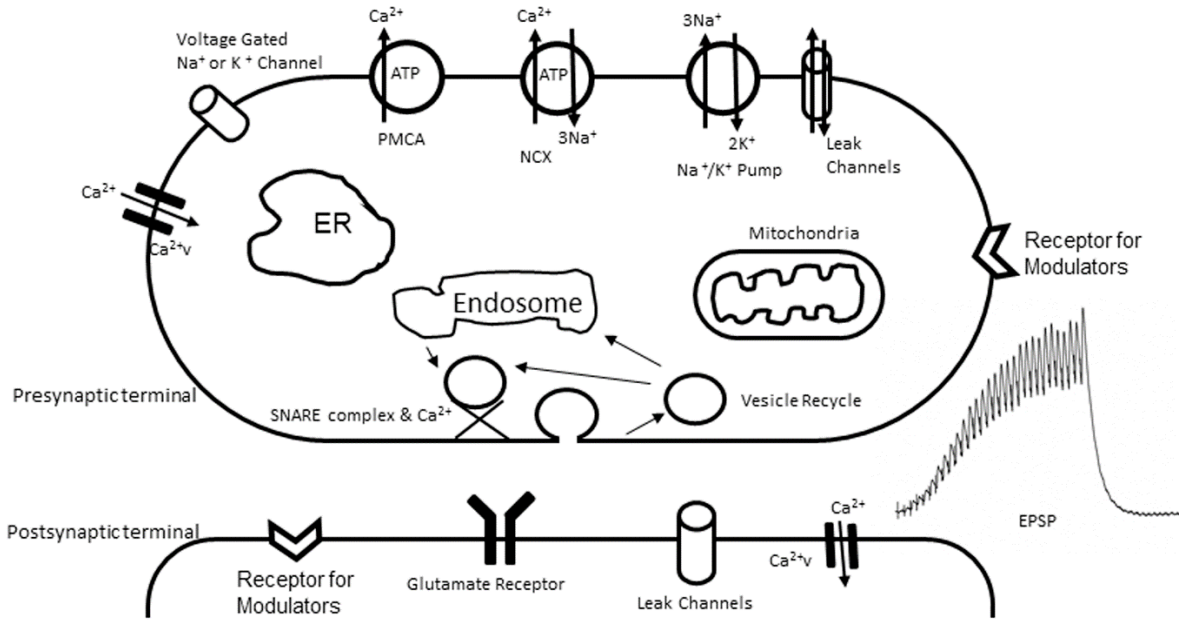

Figure 4.11 Model to explain the potential actions of changes in temperature on presynaptic and postsynaptic targets at crayfish NMJ. Highlighted are a few key potential proteins and organelles which may be altered by temperature changes and which could have acute and long-term consequences on synaptic transmission, resting membrane potential, and input resistance.

Abbreviations: Plasma membrane calcium pump (PMCA), sodium calcium exchanger (NCX), endoplasmic reticulum (ER). See text for more details.

CHAPTER 5: OVERVIEW

In this study, I set out to study how ectothermic animals respond to altered temperatures from warm to cold and cold to warm. Generally ectothermic animals lack the ability to generate internal heat to maintain body temperature but yet can survive across different temperature zones. This ability has been an interest in the science community for many years and understanding the mechanisms has been tackled by examining the various abilities of the organisms in anatomically and physiologically differences as well as metabolically and differences in the molecular function of proteins. However, little attention has been given to how hormonal modulation might play a role in response to low temperature exposures for the cardiac and the nervous systems

In my studies, I emphasized on how cold influenced cardiac and neural function in two ectothermic animals which are both arthropods. The two animal models I used are *Drosophila melanogaster* and *Procambarus clarkii*. I used heart rate (HR) as an assay in larval *D. melanogaster* to examine the effect of acute exposure to low temperature as well as larvae developed in the cold to acute warm exposure. The hypothesis being tested was that cold acclimated *D. melanogaster* would have a higher HR then acutely exposed larva to cold due to changes in hormonal levels. I had assumed 5-HT, dopamine and octopamine would increase in the hemolymph for the cold acclimated larvae due to the fact that it was shown in earlier reports that these compounds increase heart rate on their own (Johnson, et al. 1997, 2002; Majeed et al. 2014; Tiitlow et al. 2013). In addition, since HR is dramatically slowed with acute cold exposure I wanted to test if I was able to depolarize the myocytes with optogenetic approaches. The effects of hormonal or optogenetic control is better assessed in *D. melanogaster* larvae as compared to adults since the heart is easy to visualize and it is myogenic. Only in very late stages in the 3rd instar and adults is the heart neurally innervated (Johnstone and Cooper, 2006). However, recently our lab has shown that HR in larval *D. melanogaster* can be regulated by optogentically driven hormonal release from the CNS (Malloy et al., 2017).

The changes in heart rates for larval *D. melanogaster* in response to different modulators and temperatures are presented in Chapter 2. The ability of optogenetically driving heart rates in low temperature is the focus of Chapter 3.

In Chapter 2 the studies were performed by exposing intact larvae to cold and warm temperatures and dissected in-situ exposure to saline and saltines containing various concentration of modulators. The heart rates decreased after exposure to low temperature (10^{o}C) in room temperature (20^{o}C-21^{o}C) acclimated flies. The heart rate did increase with modulators (octopamine, acetylcholine, dopamine, and 5-HT) in the room temperature. However, heart rate was not increased with octopamine, acetylcholine and dopamine in the cold acclimated larvae. This study was the first of its kind in demonstrating that modulators have different effects on HR at the different temperatures in *D. melanogaster*. The receptor subtypes of 5-HT, dopamine and acetylcholine have been investigated by pharmacological and genetic means for larvae at room temperature (Majeed et al. 2013, 2014; Tiitlow et al. 2013; Malloy et al. 2015). However, the function of receptors in low temperatures has not been studied. There may be differential regulation in expression of receptor subtypes

at different temperatures for helping the animal to acclimate. Another significant finding I discovered while acclimating larva to cold temperatures was that 5-HT and octopamine decreased in the hemolymph. This was a novel and unexpected finding as I hypothesized an increase in both of these modulators with cold acclimation.

Since I did not report in detail in the early chapter how the samples were processed for HPLC I add this brief description. For obtaining hemolymph for HPLC analysis we placed 50 larvae on a dish and then dried the larvae with tissue paper. We nicked the larvae tail with a scissor and emerge all larvae into 1.5 ml Eppendorf tube with 80 µl HPLC preparation solution. The Eppendorf tube was placed on a vortex for 2 seconds, then centrifuge for 10 sec (5,000 RPM). The larvae were removed with a needle and the solution was frozen at -80^{o}C. The samples were submitted to a HPLC facility on dry ice for analysis.

We also re-measured the volume of hemolymph in the 10^{o}C conditioned larva by using microscale on a slide. The larvae is considered as cylinder shape to calculate the volume of larva ($\mathrm{Volume} = \pi \mathrm{R}^2 \times \mathrm{length}$). Because the tail and mouth are cone shape, this measure will overestimate the volume of larva. Based on samples, the difference of body volume between 20^{o}C conditioned third stage larva and 10^{o}C conditioned third stage larva is small. In average, the 10^{o}C conditioned larva is 11% larger than 20^{o}C conditioned larva in terms of their body volume. The hemolymph volume was previously determined by utilizing micropipette when larval hemolymph was collected after being centrifuged from the combined 50 larvae. An estimate per larvae was then obtained. This method provided an estimate of the hemolymph being about 25% of the total body volume. Therefore, the adjusted values for 5HT and OA concentration in 10^{o}C conditioned larva are respectively 105.45ng/ml and 2552.72ng/ml. However, the differences of body size between 10^{o}C conditioned larva and 20^{o}C conditioned larva is statistical insignificant (P=0.894, Whitney Rank Sum Test, n=12). The variability between different temperatures conditioned larva body size, based on the estimated cylinder shape, is negligible.

There may be sex differences in the modulators for larvae. We did not account for the sex in our HPLC analysis. A large sample size could minimize the any difference of OA and 5-HT in the hemolymph for the sexes at different temperatures. There is no report on sex differences in the modulators within hemolymph for larval stages.

Some of the variability we have observed might be caused by temperature-dependent larva sizes. In general, cold climate produces large individuals and warm climate produces small individuals (Bochadanovits and De, 2003). However, temperature differences in body size differences was not statistically significant in our experimental design.

The variability among preparations in recording HRs for dissected larvae is a topic which should be addressed. There are various reasons to potentially account for the wide differences in HR. One largest contributing factor is likely the dissection technique. Sometimes the heart might be in a more relaxed or stretch position during the experiment. In addition, the heart might be damaged from the dissection resulting in abnormal beating.

In chapter 3, with using optogenetics to excite larval hearts, I demonstrated that the heart could physically contract at low temperatures and increase in the rate if the cells were depolarized. Thus, I had shown that the endogenous pacemaker activity was a limiting factor in reducing HR with cold exposure. Since no reports have occurred in measuring the electrical events in cold exposed larval hearts, it was unknown if the heart was pacing but the myocytes were limiting the ability to contract in the cold. In addition, I showed that increasing the [Ca^{2+}] in the bathing media increased HR when the heart was excited by activating the expressed channel rhodopsin proteins. This also helped to demonstrate that increased ion flux into the myocytes was limiting the HR again pointing to the reduced function of the ionic fluxes associated with regulating the pacemaker activity in the cold.

In broadening my quest in examining how cold influences physiological processes, I also studied the effects of acute and acclimated cold exposure on synaptic transmission at the crayfish neuromuscular junction (NMJ). After starting this project, I became intrigued to examine low output (tonic) and high output (phasic) NMJs in the crayfish as well as the effect of 5-HT and octopamine on synaptic function in cold conditioned crayfish. I expected a large reduction in the synaptic responses for the high output NMJs as compared to the low output NMJs with acute exposure to cold. Over time with acclimation to the cold I hypothesized that the synaptic responses would be enhanced due to disuse but that the responses would fatigue rapidly for high output NMJs and that low output NMJs would not be as largely affected. I based this hypothesis on the earlier reports where it was demonstrated that crayfish from the field, caught in the winter and summer, had differences in the amplitudes in the excitatory postsynaptic potentials (EPSPs) (Lnenicka, ’93; Lnenicka and Zhao, ’93). In addition, it was shown in laboratory conditions that electrically conditioned phasic motor neurons, with pulses of activity over a period of days, reduced the EPSP amplitudes neurons (Cooper et al., ’98) as shown for crayfish caught in the summer months when they are behaviorally more active (Lnenicka, ’93; Lnenicka and Zhao, ’93). In addition, the research I conducted with larval fruit flies (chapters 2 and 3) kept my interest in the effects of modulators in physiological function with animals adapted or acclimated to cold temperatures. Upon researching the literature there really are only two significant studies which have examined the effects of modulators on synaptic function at NMJs in crustaceans (Friedrich et al., 1994; Hamilton et al., 2007). It was shown that 5-HT enhances synapse output and the generation of muscle tension in a temperature dependent manner in lobsters (Hamilton et al., 2007). Given that the lobsters used in this study were already naturally cold conditioned to around 10^{o}C there was not as large of differences in the temperature drop to 2^{o}C. However, it was demonstrated that the most robust responses in synaptic transmission and tension were closely matched to the temperature the lobsters were acclimated to and that 5-HT enhanced synaptic transmission in the cold as well as warmer acclimated temperatures (2 to 20^{o}C). However, 5-HT as well as other modulators were not measured in the hemolymph of the lobsters in the various temperatures in that study nor are there any reports I could find of any measures in modulators for crustaceans in which temperature was varied. Thus, my study is novel in the respect that I obtained measures for crayfish acclimated to cold as well as warm temperatures to address the possibility that modulators maybe altered in the

hemolymph as an effect of varied temperature exposures. One other study did address the effect of temperature on neural circuits within the stomatogastric nervous system, of a lobster, acclimated to different temperatures and the effect of dopamine (Thuma et al. 2013). This study demonstrated that dopamine was able to increase muscle force over temperatures from 9^{o}C to 15^{o}C and remove the effects of temperature dependence on synaptic transmission. Again, an important note is that dopamine was not measured in the hemolymph of the animals exposed to the different temperatures in this earlier study.

There was a lot of variability in the synaptic responses at the NMJs in the initial amplitudes of the EPSPs and even the responses to temperature changes or exposure to modulators. I cannot account for why there are such variations, but some reasons can be due to the health of the crayfish caught from the wild. Compromised health from parasitism or infections is something I could not control for in these studies. In addition, the life history, and the ability to handle the stress of being housed in laboratory conditions may vary for each individual crayfish. Slight changes in modulators released into the hemolymph could have longer lasting effects then the time for dissection and measurements. So removing a limb or decapitation could have a hormonal effect which I was not able to regulate. I did my best to minimize handling differences among the crayfish and would always pick healthy looking and behaving crayfish and ones between molt cycles in order to minimize inducing variability.

My contribution to the field of synaptic physiology and the effects of acute and acclimated cold as well as cold acclimated NMJs to warm is that synaptic transmission was measured for low and high output synapses in these different environmental conditions as well as examining the effects of octopamine on synaptic function which I showed to be enhanced in the hemolymph in cold acclimated crayfish. In order to compile results, I obtained in this study of the crayfish NMJ, a model is presented and discussed in Chapter 4. The significance of these studies broadens our understanding in the effects of modulators during cold exposure in ectothermic animals which are not native to cold temperature. Modulation has an important role in regulating an animal's physiological response. In our lab we extensively studied the importance modulators have on *Drosophila* cardiac function: Dopamine (Titlow et al. 2013), serotonin (Majeed et al. 2014), acetylcholine (Malloy et al. 2015). However, studies are lacking on how modulators may function for ectothermic animals during acute and chronic cold exposures. My study filled a gap on this topic demonstrating that in the cold environment ectothermic animals may use these modulators as a strategy to help them cope with cold temperatures. Especially, I showed octopamine and serotonin can enhance neuromuscular junction transmission efficacy in a cold environment. During my study, I also showed that channelrhodopsin can be activated at 10^{o}C in the *Drosophila* cardiac tissue. This was the first study to validate channelrhodopsin is can properly functional at low temperature. This offers a new way to study *Drosophila* cardiac function or potentially other tissues during cold exposure. Also, the results may provide more insights on the effects for the widely used hypothermia treatments on neural activity in humans (Shankaran, 2009; Yenari and Han, 2012; Sadaka et al., 2013).

These studies have taught me how to address scientific questions and to learn the techniques required to address questions. Working out conditions with the channels rhodopsins took some time to master and how to measure the heart rates in dissected preparations while examining the HR before and during light exposure while working in a cold room incubator was a task. In addition, electrophysiological measures in a cold room with electrical noise and vibration issues were other factors which needed to be worked out prior to being able to collect any feasible responses. In obtaining EPSPs without movement artifacts interfering for the twitching phasic preparations involved just the right placement of the intracellular electrodes as well as the correct stretching and binding of the abdomen so as not to lose the recording and to minimize spurious electrical artifacts from bending the intracellular electrode.

Working with fellow graduate students and helping to mentor undergraduates was a joy and very beneficial for my studies. Many undergraduates over the years help to contribute to data collection and analysis. Their contributions are mentioned in the beginning of chapters 2 and 3.

Future directions

As with most any research new questions arise from addressing the initial questions. There are numerous future studies which could help to address some of the mechanisms behind the observations I have made in these various studies.

In addressing more detailed understanding in findings obtained with the *Drosophila* heart studies, it would be interesting to know if the receptor subtypes for the modulators are differentially expressed on the myocytes as compared to warmer temperatures. I only investigated a few of the 30 or so modulators known to be in the hemolymph of invertebrates, so it would be interesting to know the role of other biogenic amines and peptides on the function of the larval heart and if they may also vary in concentration in the hemolymph with environmental temperature changes. There are studies which demonstrated that some tissues in insects vary in the Ca^{2+} handling in cells with cold exposure but no studies have examined cardiac tissues. So it would be interesting to know if myocytes might try to compensate with higher resting or driven Ca^{2+} fluxes in acclimating to the cold.

In Chapter 4 Figure11, we purposed a generalized model to address the potential mechanisms for neuromuscular junction to compensate for cold exposure. This model provides a building block to work from to address the potential mechanisms. In the acute temperature shift from 20^{o}C to 10^{o}C, a delayed or prolonged voltage gated calcium entry would have a direct effect on synaptic transmission. This would likely cause a heightened EPSP amplitude. As for future studies in synaptic transmission with the crayfish preparations, it would be interesting to know if acute cold reduced the Ca^{2+} influx with evoked release and if Ca^{2+} lingered longer in the nerve terminal. I would expect this to be the case but then other factors could also contribute to reduced transmission in the cold due to reduced ability of vesicle fusion or sensing intracellular Ca^{2+}. Despite increased membrane resistance in the muscle fiber,

the amplitudes of EPSPs were not changed to a large degree. This leads our attention to the responsiveness of the glutamate receptors or alternations in the transmission from the presynaptic terminal. In the model, we also purposed that cold temperature could affect the vesicle fusion in the presynaptic terminal. The rate of vesicle recycling through a rapid or endosomal path would likely be slowed in colder temperatures. Possibly this could be addressed with quantal analysis by electrophysiology and even vesicle associated vital dyes (i.e. FM1-43) and imaging with confocal microscopy. In addition, I would like to know if there are differences in the skeletal muscle to generate force with acute cold and acclimated cold conditions for low output and high output NMJs as well as if the modulators 5-HT and OA produce differential effects in force generation during acute and cold acclimated conditions.

Conclusion

This work provides important understanding of how modulators affect ectothermic animal cold response and adaptation in model organisms. The focus has been on *Drosophila* cardiac function and in synaptic transmission at crayfish high- and low-output neuromuscular junctions. I believe my work sheds light upon further understanding the fundamentals in how the nervous system function in the low temperature for ectothermic animals.

Chapter 1 References:

Aguilar PS, Cronan JE Jr, de Mendoza D. 1998. A Bacillus subtilis gene induced by cold shock encodes a membrane phospholipid desaturase. J Bacteriol 180(8):2194–2200.

Altizer S, Hobson K, Davis A, de Roode J, Wassenaar L. 2015. Do healthy monarchs migrate farther? Tracking natal origins of parasitized vs. uninfected monarch butterflies overwintering in Mexico. PLoS One 10(11)

Atwood H, Cooper R. 1995. Functional and structural parallels in Crustacean and *Drosophila* Neuromuscular systems. Amer Zool 35:556-565.

Atwood H, Cooper R. 1996. Synaptic diversity and differentiation: crustacean neuromuscular junctions. Invert Neurosci 1:291-307.

Bhandal I, Hauptmann R, Widholm J. 1985. Trehalose as cryoprotectant for the freeze preservation of carrot and tocabacco cells. Plant Physiol 78:430-432.

Brandstater J, Meinetzhagen I. 1995. The rapid assembly of synaptic sites in photoreceptor terminals of the fly's optic lobe recovering from cold shock. PNAS 92:2677-2681.

Brower L. 1977. Monarch Migration. Natural History. 85:40-53.

CABI DATABASE. 2017. *Procambarus clarkii* (red swamp crayfish). http://www.cabi.org/isc/datasheet/67878. Accessed March 15, 2017

CABI DATABASE. 2017. Macrobrachium rosenbergii (giant freshwater prawn). http://www.cabi.org/isc/datasheet/96269. Accessed March 15, 2017.

Charlton MP, Atwood HL. 1979. Synaptic transmission: temperature sensitivities of Ca entry in presynaptic terminals. Brain Res 170:543-546.

Chung YS, Cooper RM, Graff J, Cooper RL. 2012. The acute and chronic effect of low temperature on survival, heart rate and neural function in crayfish (*Procambarus clarkii*) and prawn *(Macrobrachium rosenbergii*) species. Open J Mol Integrat Physiol 2(3):75-86.

Colton CK, Freeman AR. 1974. Dual response of lobster muscle fibers to L-glutamate. Comp Biochem Physiol 51C:275-284.

Cooper AS, Leksrisawat B, Gilberts AB, Mercier AJ, Cooper RL. 2011a. Physiological experimentations with the crayfish hindgut: A student laboratory exercise. JoVE URL: http://www.jove.com/video/2324

Cooper RM, Finucane HS, Adami M, Cooper RL. 2011b. Heart and ventilator measures in crayfish during copulation. OJMIP 1:6-42.

David J, Coulon J. 1985. Octopamine in invertebrates and vertebrates. A review. Prog Neurobiol 24:141-185.

Dillon M, Wang G, Garrity P, Huey R. 2009. Review: Thermal preference in Drosophila. J Therm Biol 34(3):109-119.

Dudel J. 1963. Presynaptic inhibition of the excitatory nerve terminal in the neuromuscular junction of the crayfish. Pflügers Arch ges Physiol 277:537-557.

Dudel J. 1965. The mechanism of presynaptic inhibition at the crayfish neuromuscular junction. Pflügers Arch 284:66-80.

Dudel J, Kuffler SW. 1961a. The quantal nature of transmission and spontaneous miniature potentials at the crayfish neuromuscular junction. J. Physiol. (Lond). 155:529-552.

Dudel J, Kuffler SW. 1961b. Presynaptic inhibition at the crayfish neuromuscular junction. J Physiol 155:543-562.

Even N, Devaud JM. Barron A. 2012. General stress responses in the honey bee. Insects 3(4): 1271-1298.

Fang J. 2010. Moth catch the wind to speed migration. Nature. doi:10.1038/news.2010.54

Fernández-Alfonso T, Ryan RA. 2004. The kinetics of synaptic vesicle pool depletion at cns synaptic terminals. Neuron 41(6):943–953.

Fischer L, Florey E. 1987. Octopamine action on the contractile system of crustacean skeletal muscle. Comp Biochem Physiol C 88:335-342.

Friedrich RW, Quigley PA, Srivastava M, Skerrett M, Mercier AJ. 1994. Temperature dependence of synaptic modulation by a FMRFamide-related neuropeptide in crayfish. Neurosci Let 169:56-58.

Florey E, Hoyle G. 1976. The effects of temperature on a nerve muscle system of the Hawaiian ghost crab, *Ocypode ceratophthalma* (Pallas). J Comp Physiol 110(1):51-64.

Goosey M, Candy D. 1980. The D-octopamine content of the haemolymph of the locust, *Schistocerca Americana gregaria* and its elevation during flight. 10(4):393- 397.

Hamilton JL, Edwards CR, Holt SR, Worden MK. 2006. Temperature dependent modulation of lobster neuromuscular properties by serotonin. J Exp Biol 210:1025-1035.

Hargrove M, Hargrove M. 2006. Freshwater Aquariums for Dummies (2nd ed.). Hoboken: Wiley. p99.

Harri M, Florey E. 1976. The effects of temperature on a neuromuscular system of the Crayfish, *Astacus leptodactylus*. J Comp Physiol 117:47-61.

Harri M, Florey E. 1978. The effect of acclimation temperature on a neuromuscular system of the crayfish *Astacus Leptodactylus*. J Exp Biol 78:281-293.

Hulme P. 2009. Trade, transport and trouble: managing invasive species pathways in an era of globalization. J Applied Eco 46(1):10-18.

Hurme R, Berndt KD., Normark SJ., Rhen M. 1997. A proteinaceous gene regulatory thermometer in Salmonella. Cell 90:55–64.

Jiang W, Hou Y, Inouye M. 1997. CspA, the major cold-shock protein of *Escherichia coli*, is an RNA chaperone. J Biol Chem 272(1):196–202.

Johonson EW, Wernig A. 1971. The binomial nature of transmitter release at the crayfish neuromuscular junction. J Physiol 218:757-767.

Katz B, Miledi R. 1965. The effect of temperature on the synaptic delay at the neuromuscular junction. J Physiol 181(3):656-670.

Klinkert B, Narberhaus F. 2009. Microbial thermosensors. Cell Mol Life Sci 66:2661-2676.

Koštál V, Šimek P, Zahradníčková H, Cimlová J, Štětina T. 2012. Conversion of the chill susceptible fruit fly larva (Drosophila melanogaster) to a freeze tolerant organism. Proc Natl Acad Sci USA 109:3270–3274.

Lee RE Jr, Chen CP, Denlinger DL. 1987. A rapid cold-hardening process in insects. 238(4832):1415-1417.

Lee RE, Dommel RA, Joplin KH, Denlinger DL. 1995. Cryobiology of the freeze-tolerant gall fly Eurosta solidaginis: overwintering energetics and heat shock proteins. Clin Res 5:61-67.

Listerman L, Deskins J, Bradacs H, Cooper RL. 2000. Measures of heart rate during social interactions in crayfish and effects of 5-HT. Comp Biochem Physiol A .125:251-264.

Llinás R, Walton K., Sugimori M. 1978. Voltage-clamp study of effects of temperature on synaptic transmission in squid. Biol Bull 155:454 (Abstract).

Loeb J, Northrop JH. 1917. On the influence of food and temperature upon the duration of life. J Biol Chem 32:103 -121.

Logsdon S, Johnstone AFM, Viele K, Cooper RL. 2006. The regulation of synaptic vesicles pools within motor nerve terminals during short-term facilitation and neuromodulation. Journal of Applied Physiol 100:662-671.

Lybecker C, Samuels D. (2007) Temperature-induced regulation of RpoS by a small RNA in *Borrelia burgdorferi*. Mol Microbiol 64:1075–1089.

MacMillan H, Sinclair B. 2011. The role of the gut in insect chilling injury: cold-induced disruption of osmoregulation in the fall field cricket, *Gryllus pennsylvanicus*. J Exp Bio 214:726-734.

Maguire SE, Schmidt PS, Sehgal A. 2014. Natural Populations of Drosophila melanogaster reveal features of an uncharacterized circadian property: The lower temperature limit of rhythmicity. J Biol Rhythms 29(3):167-180.

Miyasako Y, Umerzaki Y, Tomioka K. 2007. Separate sets of cerebral clock neurons are responsible for light and temperature entrainment of Drosophila circadian locomotor rhythms. J Biol Rhythms 22(2):115-126.

Mykles DL, Medler SA, Koenders A, Cooper RL. 2002. Myofibrillar protein isoform expression is correlated with synaptic efficacy in slow fibres of the claw and leg opener muscles of crayfish and lobster. J Exp Biol 205 (4):513-522.

Narberhaus F, Waldminghaus T, Chowdhury S. 2006. RNA thermometers. FEMS Microbiol Rev 30(1):3-16.

Page M, Hailes W, Cooper RL. 2007. Modification of the tail flip escape response in crayfish by neuromodulation and behavioral state with and without descending CNS input. Int J Zool Res 3:132-144.

Phadtare S, Severinov K. 2010. RNA remodeling and gene regulation by cold shock proteins. RNA Biol 7(6):788-795.

Polar life. 2017 Arctic Sculpin, *Myoxocephalus scorpioides*. Canada's Polar Life. Accessed March 17th, 2017

Ragland SS, Sohal RS. 1975. Ambient temperature, physical activity and aging in the housefly. Exp Gerontol 10:279 -289.

Rodgers CI, Armstrong GAB, Robertson RM. 2010. Coma in response to environmental stress in the locust: a model for cortical spreading depression. J Insect Physiol 56:980-990.

Schou M, Kristensen T, Pedersen A, Karlsson G, Loeschcke V, Malmendal A. 2017. Metabolic and functional characterization of effects of developmental temperature in *Drosophila melanogaster*. AJP 312(2):R211-R222.

Servant P, Mazodier P. 2001. Negative regulation of the heat shock response in Streptomyces. Arch Microbiol 176:237–242

Shuranova ZP, Burmistrov YM, Strawn JP, Cooper RL. 2006. Evidence for an Autonomic Nervous System in Decapod Crustaceans. Int J Zool Res 2(3):242-283.

Smith BR, Wojtowicz JM, Atwood HL. 1991. Maximum likelihood estimation of non-uniform transmitter release probabilities at the crayfish neuromuscular junction. J Theor Biol150(4):457-472.

Sneddon LU, Taylor AC, Huntingford FA, Watson DG. 2000. Agonistic behavior and biogenic amines in shore crabs *Carcinus maenas*. J Exp Biol 203:537-545.

Speakman J. 2005. Body size, energy metabolism and lifespan. J Exp Biol (208):1717-1730.

Stephens PJ, Atwood HL. 1982. Thermal acclimation in a crustacean neuromuscular system. J Exp Biol 98:39-47.

Strawn JR, Neckameyer WS, Cooper RL. 2000. The effects of 5-HT on sensory neurons, central, and motor neurons driving the abdominal superficial flexor muscles in the crayfish. Comp Biochem Physiol B 127:533-550.

Suutari M, Rintamäki A, Laakso S. 1997. Membrane phospholipids in temperature adaptation of Candida utilis: alterations in fatty acid chain length and unsaturation. J Lipid Res 38(4):790-794.

Tattersall GJ, Luebbert JP, LePine OK, Ormerod KG, Mercier AJ. 2012. Thermal games in crayfish depend on establishment of social hierarchies. J Exp Biol 215:1892–1904.

Teets N, Denlinger D. 2013. Physiological mechanisms of seasonal and rapid cold-hardening in insects. Physiol Entomol 38(2):105-116

Thorne M, Seybold A, Marshall C, Wharton D. 2017. Molecular snapshot of an intracellular freezing event in an Antarctic nematode. Cryobiol doi: 10.1016/j.cryobiol.2017.01.003. [Epub ahead of print]

Tsai S, Chong G, Meng P, Lin C. 2017. Sugars as supplemental cryoprotectants for marine organisms. Rev Aquaculture. http://dx.doi.org/10.1111/raq.12195

Weight F, Erulkar SD. 1976. Synaptic transmission and effects of temperature at the squid giant synapse. Nature 261:720-722.

Winter J, Jakob U. 2004. Beyond transcription—new mechanisms for the regulation of molecular chaperones. Crit Rev Biochem Mol Biol 39:297–317.

Withers L, King P. 1979. Proline: A novel cryoprotectant for the freeze preservation of cultured cells of *Zea mays* L. Plant Physiol 64:675-678.

Wu WH, Cooper RL. 2010. Physiological recordings of high and low output NMJs on the Crayfish leg extensor muscle. J Vis Exp JoVE 45:2319 http://www.jove.com/index/details.stp?id=2319

Wu WH, Cooper RL. 2012. The regulation and packaging of synaptic vesicles as related to recruitment within glutamatergic synapses. Neurosci 225:185–198.

Zhang Z, Jackson MB. 2008. Temperature Dependence of Fusion Kinetics and Fusion Pores in Ca2+-triggered Exocytosis from PC12 Cells. J Gen Physiol 131(2):117–124.

Zhu Y, Yocom E, Sifers J, Uradu H, Cooper RL. 2016a. Modulatory effects on Drosophila larva hearts: Room temperature, acute and chronic cold stress. J Comp Physiol A. 186(7):829-841.

Zhu Y, Uradu H, Majeed ZR, Cooper RL. 2016b. Optogenetic drive of *Drosophila* heart rate at different temperatures and Ca2+ concentrations. Physiol Rep 4(3):e12695.

Chapter 2 References:

Ackefors H. 1999. The positive effects of established crayfish introductions in Europe. In:Gherardi F, Holdich DM (ed)Crayfish in Europe as Alien Species — How to Make the Best of a Bad Situation? CRC Press PP49-61

Alpatov WW. 1929. Growth and variation of the larvae of *Drosophila melanogaster.* J Exp Zool 52(3):407-437

Andersen JL, MacMillan HA, Overgaard J .2015. Temperate Drosophila preserve cardiac function at low temperature. J Insect Physiol 77:26-32. doi: 10.1016/j.jinsphys.2015.03.016

Baumgartner U, Greffrath W, Treede RD .2012. Contact heat and cold, mechanical, electrical and chemical stimuli to elicit small fiber-evoked potentials: Merits and limitations for basic science and clinical use. Neurophysiol Clin 42(5):267-280. doi:10.1016/j.neucli.2012.06.002

Bayley M, Holmstrup M. 1999. Water vapor absorption in arthropods by accumulation of myoinositol and glucose. Sci 285(5435):1909-1911

Bayley M, Petersen SO, Knigge T, Köhler H, Holmstrup M .2001. Drought acclimation confers cold tolerance in the soil collembolan Folsomia candida. J Insect Physiol 47(10):1197-1204

Becnel J, Johnson O, Luo J, Nassel DR, Nichols CD .2011. The serotonin 5-HT7Dro receptor is expressed in the brain of *Drosophila*, and is essential for normal courtship and mating. PloS One 6(6):e20800. doi:10.1371/journal.pone.0020800

Becnel J, Johnson O, Majeed ZR, Tran V, Yu B, Roth BL, Cooper RL, Kerut EK, Nichols CD .2013. DREADDs in *Drosophila*: a pharmacogenetic approach for controlling behavior, neuronal signaling, and physiology in the fly. Cell Rep 4 (5):1049-1059. doi:10.1016/j.celrep.2013.08.003

Bennett VA, Pruitt NL, Lee RE .1997. Seasonal changes in fatty acid composition associated with cold-hardening in third instar larvae of *Eurosta solidaginis*. J Comp Physiol B 167, 249–255

Burton V, Mitchell HK, Young P, Petersen NS .1988. Heat shock protection against cold stress of *Drosophila melanogaster*. Mol Cell Biol 8:3550–3552

Campos-Ortega JA, Hartenstein V. 1985. The embryonic Drosophila melanogaster. Berlin: Springer-Verlag.

Chen CP, Walker VK .1994. Cold-shock and chilling tolerance in Drosophila. J Insect Physiol 40:661-669

Chung YS, Cooper RM, Graff J, Cooper RL .2012. The acute and chronic effect of low temperature on survival, heart rate and neural function in crayfish (*Procambarus clarkii*) and prawn *(Macrobrachium rosenbergii*) species. Open J Mol Integrat Physiol 2(3):75-86. doi:10.4236/ojmip.2012.23011

Colinet H, Larvor V, Laparie M, Renault D .2012. Exploring the plastic response to cold acclimation through metabolomics. Functional Ecol 26(3):711-722. http://dx.doi.org/10.1111/j.1365-2435.2012.01985.x

Cooper RM, Finucane HS, Adami M, Cooper RL .2011a. Heart and ventilatory measures in crayfish during copulation. Open J Mol Integrat Physiol 1(3):36-42. doi:10.4236/ojmip.2011.13006

Cooper, AS, Leksrisawat B, Mercier AJ, Gilberts AB, Cooper RL .2011b. Physiological experimentations with the crayfish hindgut: A student laboratory exercise. JoVE.47:http://www.jove.com/details.php?id=2324 doi: 10.3791/2324

Cooper AS, Rymond KE, Ward MA, Bocook EL, Cooper RL .2009. Monitoring heart function in larval *Drosophila melanogaster* for physiological studies. J Vis Exp 33:E1596, doi:10.3791/1596

Coste B, Xiao BL, Santos JS, Syeda R, Grandl J, Spencer KS, Kim SE, Schmidt M, Mathur J, Dubin AE, Montal M, Patapoutian A .2012. Piezo proteins are pore-forming subunits of mechanically activated channels. Nature 483(7388):176-U172. doi:10.1038/nature10812

Czajka MC, Lee RE, Jr. .1990. A rapid cold-hardening response protecting against cold shock injury in *Drosophila melanogaster*. J Exp Biol 148:245-254

Dasari S, Cooper RL .2004. Modulation of sensory–CNS–motor circuits by serotonin, octopamine, and dopamine in semi-intact *Drosophila* larva. Neurosci Res 48(2):221-227. doi:10.1016/j.neures.2003.10.005

Davenport AP, Evans PD .1984. Stress-induced changes in the octopamine levels of insect haemolymph. Insect Biochem 14 (2):135-143

David J, Coulon J .1985. Octopamine in invertebrates and vertebrates a review. Prog in Neurobiol 24:141-185

de Castro C, Titlow J, Majeed ZR, Cooper RL .2014. Analysis of various physiological salines for heart rate, CNS function, and synaptic transmission at neuromuscular junctions in Drosophila melanogaster larvae. J Comp Physiol A Neuroethol Sens Neural Behav Physiol. 200: 83-92. doi: 10.1007/s00359-013-0864-0

de Castro C, Titlow J, Majeed ZR, Vaughn M, King K, Cooper RL .2015. Maintaining the *Drosophila* larval heart in situ: Modulators and stretch activated channels. The 9th International Congress of Comparative Physiology and Biochemistry. August 23-28, (Abstract).

Desai-Shah M, Papoy AR, Ward M, Cooper RL .2010. Roles of the sarcoplasmic/endoplasmic reticulum Ca2+-ATPase, plasma membrane Ca2+-ATPase and Na+/Ca2+ exchanger in regulation of heart rate in larval *Drosophila*. The Open Physiol J 3:16-36

Djokaj S, Cooper RL, Rathmayer W .2001. Presynaptic effects of octopamine, serotonin, and cocktails of the two modulators on neuromuscular transmission in crustaceans. J Comp Physiol A 187(2):145-154 DOI 10.1007/s003590100187

Dowse H, Ringo J, Power J, Johnson E, Kinney K, White L .1995. A congenital heart defect in *Drosophila* caused by an action-potential mutation. J Neurogenetics 10 (3):153-168. doi:10.3109/01677069509083461

El-Kholy S, Stephano F, Li Y, Bhandari A, Fink C, Roeder T .2015. Expression analysis of octopamine and tyramine receptors in *Drosophila*. Cell Tissue Res doi:10.1007/s00441-015-2137-4

Even N, Devaud J-M, Barron A. 2012. General stress responses in the honey bee. Insects 3(4):1271-1298. doi:10.3390/insects3041271

Feder M E, Hofmann GE. 1999. Heat-shock proteins, molecular chaperones and the stress response: Evolutionary and ecological physiology. Annu Rev Physiol 61:243–283

French AS, Simcock KL, Rolke D, Gartside SE, Blenau W, Wright GA. 2014. The role of serotonin in feeding and gut contractions in the honeybee. J Insect Physiol 61: 8–15

García-Arberas L, Rallo A, Antón A. 2009. The future of the indigenous freshwater crayfish Austropota-mobius italicus in Basque Country streams: Is it possible to survive being an inconvenient species? Knowledge and Management of Aquatic Ecosystems 19:394-395.

Goosey M, Candy D. 1980. The D-octopamine content of the haemolymph of the locust, *Schistocerca Americana gregaria* and its elevation during flight. Insect Biochem10(4):393-397

Goto SG, Kimura MT. 1998. Heat- and cold-shock responses and temperature adaptations in subtropical and temperate species of *Drosophila*. J Insect Physiol 44:1233–1239

Graham AM, Merrill JD, McGaugh SE, Noor MA. 2012. Geographic selection in the small heat shock gene complex differentiating populations of *Drosophila pseudoobscura*. J Hered 103 (3):400-407. doi:10.1093/jhered/esr150

Gu GG, Singh S. 1995. Pharmacological analysis of heartbeat in *Drosophila*. J Neurobiol 28 (3):269-280. doi:10.1002/neu.480280302

Hirashima A, Eto M. 1993. Effect of stress on levels of octopamine, dopamine and serotonin in the American cockroach. Comp Biochem Physiol 105C (2):279-284

Ikemoto Y, Kawaii S, Mizutani J. 1993. Microdialysis for the analysis of insect haemolymph. Biosci Biotech Biochem 57 (3):402-404

Jennings T, Ringo J, Dowse H. 2009. The relationship of heart function to temperature in Drosophila melanogaster and its heritability. J Exp Zool 311A: 689-696

Johnson E, Ringo J, Bray N, Dowse H. 1998. Genetic and pharmacological identification of ion channels central to the *Drosophila* cardiac pacemaker. J Neurogenet 12(1):1-24

Johnson E, Sherry T, Ringo J, Dowse H. 2002. Modulation of the cardiac pacemaker of Drosophila: Cellular Mechanisms. J Comp Physiol B 172:227-236

Johnson E, Ringo J, Dowse H. 1997. Modulation of Drosophila heartbeat by neurotransmitters. J Comp Physiol B 167:89-97.

Johnstone AF, Cooper RL. 2006. Direct innervation of the *Drosophila melanogaster* larval aorta. Brain Res 1083(1):159-163. doi:10.1016/j.brainres.2006.02.007

Kayukawa T, Ishikawa Y. 2009. Chaperonin contributes to cold hardiness of the onion maggot *Delia antiqua* through repression of depolymerization of actin at low temperatures. PLoS One 4(12):e8277. doi:10.1371/journal.pone.0008277.

Kelty JD, Lee RE Jr. 1999. Induction of rapid cold hardening by cooling at ecologically relevant rates in *Drosophila melanogaster*. J Insect Physiol 45(8):719-726

Kelty JD, Lee RE Jr. 2001. Rapid cold-hardening of *Drosophila Melanogaster* (Diptera: Drosophilidae) during ecologically based thermoperiodic cycles. J Exp Biol 204:1659-1666

Lalevee N, Monier B, Senatore S, Perrin L, Semeriva M. 2006. Control of cardiac rhythm by ORK1, a *Drosophila* two-pore domain potassium channel. Curr Biol 16 (15):1502-1508. doi:10.1016/j.cub.2006.05.064

Lee RE Jr, Damodaran K, Yi S, Lorigan GA. 2006. Rapid cold-hardening increases membrane fluidity and cold tolerance of insect cells. Cryobiol 52:459-463

Levenson J, Byrne JH, Eskin A. 1999. Levels of Serotonin in the Hemolymph of Aplysia are Modulated by Light/Dark Cycles and Sensitization Training. J Neurosci 19(18):8094–8103

Lewis EB. 1960. A new standard food medium. Drosophila Inform Serv 34:117

Listerman L, Deskins J, Bradacs H, Cooper RL. 2000. Measures of heart rate during social interactions in crayfish and effects of 5-HT. Comp Biochem Physiol A125:251-264

Malloy C, Ritter K, Robinson J, Cooper RL. 2015. Pharmacological identification of cholinergic receptor subtypes on *Drosophila melanogaster* larval heart. J Comp Physiol B 186(1):45-57

Majeed ZR, Nichols CD, Cooper RL. 2013. 5-HT stimulation of heart rate in *Drosophila* does not act through cAMP as revealed by pharmacogenetics. J Appl Physiol 115 (11):1656-1665. doi:10.1152/japplphysiol.00849.2013

Majeed ZR, Stacy A, Cooper RL .2014. Pharmacological identification of serotonin receptor subtypes on the *Drosophila* larval heart. J Comp Physiol B 184(2):205-219

Marder E. 2012. Neuromodulation of neuronal circuits: Back to the future. Neuron 76 (1):1-11. doi:10.1016/j.neuron.2012.09.010

Michaud MR, Denlinger DL. 2006. Oleic acid is elevated in cell membranes during rapid cold-hardening and pupal diapause in the flesh fly, Sarcophaga crassipalpis. J Insect Physiol 52(10):1073-1082

Newman AEM, Foerster M, Shoemaker KL, Robertson RM. 2003. Stress-induced thermotolerance of ventilator motor pattern generation in the locust, *Locusta migratoria*. J Insect Physiol 49:1039-1047

Nyström P. 1999. Ecological impact of introduced and native crayfish on freshwater communities: European perspectives. In: Gherardi F, Holdich DM (ed) Crayfish in Europe as Alien Species —How to Make the Best of a Bad Situation? CRC Press PP 63-85

Okada T. 1963. Caenogenetic differentiation of mouth hooks in Drosophilid larvae. Evolution 17:84-98

Overgaard J, Malmendal A, Sørensen JG, Bundy JG, Loeschcke V, Nielsen NC, Holmstrup M. 2007. Metabolomic profiling of rapid cold hardening and cold shock in *Drosophila melanogaster*. J Insect Physiol 53(12):1218-1232

Overgaard J, Sørensen JG, Petersen SO, Loeschcke V, Holmstrup M. 2005. Change in membrane lipid composition following rapid cold hardening in *Drosophila melanogaster*. J Insect Physiol 51(11):1178-1182

Overgaard J, Sørensen JG, Petersen SO, Loeschcke V, Holmstrup M. 2006. Reorganization of membrane lipids during fast and slow cold hardening in *Drosophila melanogaster*. Physiol. Entomol. 31, 328-335

Pagé MP, Hailes W, Cooper RL. 2007. Modification of the tail flip escape response in crayfish by neuromodulation and behavioral state with and without descending CNS input. Int J Zool Res 3:132-144

Rizki TM. 1978. The circulatory system and associated cells and tissues. In: Ashburner M, Wright TRF (eds) The genetics and biology of *Drosophila*. Vol. 2b. Academic Press.

Shuranova ZP, Burmistrov YM, Strawn JP, Cooper RL. 2006. Evidence for an Autonomic Nervous System in Decapod Crustaceans. Int J Zool Res 2(3):242-283

Sneddon LU, Taylor AC, Huntingford FA, Watson DG. 2000. Agonistic behavior and biogenic amines in shore crabs *Carcinus maenas*. J Exp Biol 203:537-545

Strawn JR, Neckameyer WS, Cooper RL. 2000. The effects of 5-HT on sensory neurons, central, and motor neurons driving the abdominal superficial flexor muscles in the crayfish. Comp Biochem and Physiol B 127:533-550

Stewart BA, Atwood HL, Renger JJ, Wang J, Wu CF. 1994. Improved stability of *Drosophila* larval neuromuscular preparation in hemolymph-like physiological solutions. J Comp Physiol A 175:179–191

Terhzaz S, Teets NM, Cabrero P, Henderson L, Ritchie MG, Nachman RJ, Dow JA, Denlinger DL, Davies SA. 2015. Insect capa neuropeptides impact desiccation and cold tolerance. Proc Natl Acad Sci USA. 112(9):2882-2887. doi: 10.1073/pnas.1501518112

Titlow JS, Rufer JM, King KE, Cooper RL. 2013. Pharmacological analysis of dopamine modulation in the *Drosophila melanogaster* larval heart. Physiol Rep 1 (2):e00020. doi:10.1002/phy2.20

Tomcala A, Tollarová M, Overgaard J, Simek P, Kostál, V. 2006. Seasonal acquisition of chill tolerance and restructuring of membrane glycerophospholipids in an overwintering insect: triggering by low temperature, desiccation and diapause progression. J Exp Biol 209:4102-4114

Vesala L, Salminen TS, Laiho A, Hoikkala A, Kankare M. 2012. Cold tolerance and cold-induced modulation of gene expression in two *Drosophila virilis* group species with different distributions. Insect Mol Biol 21(1):107-118. doi:10.1111/j.1365-2583.2011.01119.x

White LA, Ringo JM, Dowse HB. 1992. Effects of deuterium oxide and temperature on heart rate in Drosophila melanogaster. J Comp Physiol B 162(3):278-283

Yi SX, Moore CW, Lee RE Jr. 2007. Rapid cold-hardening protects *Drosophila melanogaster* from cold-induced apoptosis. Apoptosis 12(7):1183-1193

Zhu YC, Uradu, H, Majeed, Z, Cooper, RL. 2016a. Optogenetic stimulation of Drosophila heart rate at different temperatures and Ca2+ concentrations. Physiological Reports 4(3):e12695

Zhu W, Zhang H, Li X, Meng Q, Shu R, Wang M, Zhou G, Wang H, Miao L, Zhang J, Qin Q. 2016b. Cold adaptation mechanisms in the ghost moth Hepialus xiaojinensis: Metabolic regulation and thermal compensation. J Insect Physiol 85:76-85

Zornik E, Paisley K, Nichols R. 1999. Neural transmitters and a peptide modulate *Drosophila* heart rate. Peptides 20(1):45-51

Chapter 3 References:

Alex A, Li A, Tanzi RE & Zhou C. 2015. Optogenetic pacing in Drosophila melanogaster. Science Advances.1 (9), e1500639 doi:10.1126/sciadv.1500639

Becnel J, Johnson O, Majeed ZR, Tran V, Yu B, Roth BL, Cooper RL, Kerut EK & Nichols CD. 2013. DREADDs in Drosophila: a pharmacogenetic approach for controlling behavior, neuronal signaling, and physiology in the fly. Cell Rep. 4(5):1049-1059. doi:10.1016/j.celrep.2013.08.003.

Bodmer R & Venkatesh TV. 1998. Heart development in *Drosophila* and vertebrates: conservation of molecular mechanisms. Dev Genet. 22(3):181-186.

Choma MA, Suter MJ, Vakoc BJ, Bouma BE, Tearney GJ. 2011. Physiological homology between Drosophila melanogaster and vertebrate cardiovascular systems. Dis Model Mech. 4(3):411-420. doi: 10.1242/dmm.005231

Cooper AS, Rymond KE, Ward MA, Bocook EL & Cooper RL. 2009. Monitoring heart function in larval *Drosophila melanogaster* for physiological studies. J Vis Exp 33:E1596, doi:10.3791/1596

Dawydow A, Gueta R, Ljaschenko D, Ullrich S, Hermann M, Ehmann N, Gao S, Fiala A, Langenhan T, Nagel G, Kittel RJ. 2014. Channelrhodopsin-2-XXL, a powerful optogenetic tool for low-light applications. Proc Natl Acad Sci USA. 111(38):13972-13977. doi:10.1073/pnas.1408269111.

de Castro C, Titlow J, Majeed ZR, Cooper RL. 2014. Analysis of various physiological salines for heart rate, CNS function, and synaptic transmission at neuromuscular junctions in Drosophila melanogaster larvae. J Comp Physiol A Neuroethol Sens Neural Behav Physiol. 200(1):83-92. doi: 10.1007/s00359-013-0864-0.

Desai-Shah M, Papoy AR, Ward M, Cooper RL. 2010. Roles of the Sarcoplasmic/Endoplasmic reticulum Ca2+-ATPase, plasma membrane Ca2+-ATPase and Na+/Ca2+ exchanger in regulation of heart rate in larval Drosophila. The Open Physiology Journal. 3:16-36.

Duffy JB. 2002. GAL4 system in Drosophila: a fly geneticist's Swiss army knife. Genesis 34(1-2):1-15.

Giacca M, Baker AH. 2011. Heartening results: The CUPID gene therapy trial for heart failure. Molecular Therapy 19(7): 1181–1182. doi:10.1038/mt.2011.123.

Jessup M, Greenberg B, Mancini D, Cappola T, Pauly DF, Jaski B, Yaroshinsky A, Zsebo KM, Dittrich H, Hajjar RJ. 2011. Calcium upregulation by percutaneous administration of gene therapy in cardiac disease (CUPID) investigators. Circulation 124(3):304-13. doi: 10.1161/CIRCULATIONAHA.111.022889.

Johnson E, Ringo J, Bray N, Dowse H. 1998. Genetic and pharmacological identification of ion channels central to the *Drosophila* cardiac pacemaker. J Neurogenet. 12(1):1-24.

Jones WD. 2009. The expanding reach of the GAL4/UAS system into the behavioral neurobiology of *Drosophila*. BMB Rep. 42(11):705-12. Review.PMID:19944010

Kaye D, Frankl W, Arditi LI. 1975. Probable postcardiotomy syndrome following implantation of a transvenous pacemaker: Report of the first case. Heart J 90(5):627-630.

Lee LY, Zhou X, Polce DR, El-Sawy T, Patel SR, Thakker GD, Narumi K, Crystal RG, Rosengart TK. 1999. Exogenous control of cardiac gene therapy: evidence of regulated myocardial transgene expression after adenovirus and adeno-associated virus transfer of expression cassettes containing corticosteroid response element promoters. J Thorac Cardiovasc Surg. 118(1):26-35.

Majeed ZR, Stacy A, Cooper RL. 2014. Pharmacological and genetic identification of serotonin receptor subtypes on Drosophila larval heart and aorta. J Comp Physiol B. 184(2):205-219. doi: 10.1007/s00360-013-0795-7.

Malloy CA, Ritter K, Robinson J, English C, Cooper RL. 2015. Pharmacological identification of cholinergic receptor subtypes on Drosophila melanogaster larval heart. J Comp Physiol B. 2015 Oct 5. [Epub ahead of print]. PMID: 26438517

Michel SG, La Muraglia GM 2nd, Madariaga ML, Titus JS, Selig MK, Farkash EA, Allan JS, Anderson LM, Madsen JC. 2014. Preservation of donor hearts using hypothermic oxygenated perfusion. Ann Transplant. 19:409-416. doi: 10.12659/AOT.890797.

Owald D, Lin S, Waddell S. 2015. Light, heat, action: neural control of fruit fly behavior. Philos Trans R Soc Lond B Biol Sci. 370(1677):20140211. doi: 10.1098/rstb.2014.0211 PMCID: PMC4528823

Stefanidis AS, Margos PN, Kotsakis AA, Papasteriadis EG. 2009. Three-dimensional echocardiographic documentation of pacemaker lead perforation presenting as acute pericarditis. Hellenic J Cardiol 50(4):335-337.

Titlow JS, Anderson H, Cooper RL. 2014. Lights and larvae: Using optogenetics to teach recombinant DNA and neurobiology. The Science Teacher, National Science Teacher Association, NSTA. 81(6):3-9.

Titlow JS, Rufer JM, King KE, Cooper RL. 2013. Pharmacological analysis of dopamine modulation in the *Drosophila melanogaster* larval heart. Physiol Rep. 1(2):e00020. doi: 10.1002/phy2.20.

Titlow JS, Johnson BR, Pulver SR. 2015. Light activated escape circuits: A behavior and neurophysiology lab module using Drosophila optogenetics. J Undergrad Neurosci Educ. 13(3):A166-73. eCollection 2015.

Stewart BA, Atwood HL, Renger JJ, Wang J, Wu CF. 1994. Improved stability of *Drosophila* larval neuromuscular preparations in hemolymph-like physiological solutions. J Comp Physiol A-Sensory Neural and Behavioral Physiology 175(2):179-191. doi:10.1007/bf00215114

Wolk B, Dandes E, Martinez F, Helguera M, Pinski SL, Kirsch J. 2013. Postcardiac injury syndrome following transvenous pacer or defibrillator insertion: CT imaging and review of the literature. Curr Probl Diagn Radiol. 42(4):141-148. doi: 10.1067/j.cpradiol.2012.12.001. Review.PMID:23795993

Yoshihara M, Ito K. 2012. Acute genetic manipulation of neuronal activity for the functional dissection of neural circuits-a dream come true for the pioneers of behavioral genetics. J Neurogenet. 26(1):43-52. doi: 10.3109/01677063.2012.663429.

Zhu Y-C, Yocum E, Sifers J, Uradu H, Cooper RL. 2015. Modulatory effects on Drosophila larva hearts in room temperature, acute and chronic cold stress. (In revision, Journal of Comparative Physiology - B).

Chapter 4 References:

Andersen JL. Findsen A. Overgaard J. 2013. Feeding impairs chill coma recovery in the migratory locust (*Locusta migratoria*). J. Insect Physiol 59 (10): 1041-1048.

Atwood HL. 1976. Organization and synaptic physiology of crustacean neuromuscular systems, Prog Neurobiol 7:291–391.

Atwood HL. 1982. Synapses and Neurotransmitters, in The Biology of Crustacea. Academic Press: San Diego pp.105-150.

Atwood HL and Cooper RL. 1996. Synaptic diversity and differentiation: Crustacean neuromuscular junctions. Invert Neurosci 1:291-307.

Atwood, HL and Cooper RL. 1996. Synaptic diversity and differentiation: Crustacean neuromuscular junctions. Invert Neurosci 1:291-307.

Baierlein B, Thurow AL, Atwood HL, Cooper RL. 2011. Membrane potentials, synaptic responses, neuronal circuitry, neuromodulation and muscle histology using the crayfish: Student laboratory exercises. JoVE 47.

Blundon JA. 1989. Effects of temperature and thermal history on neuromuscular properties of two crustacean species. J Comp Physiol B 158:689-696.

Brandstatrer JH and Meinertzhagen IA. 1995. The rapid assembly of synaptic sites in photoreceptor terminals of the fly's optic lobe recovering from cold shock. PNAS 92:2677-2681.
Chapelle S. 1977. Lipid composition of tissue of marine crustaceans. Biochem System Eco 5:241-248.

Chapelle S. 1978. The influence of acclimation temperature on the fatty acid composition of aquatic crustacean (*Carcinus maenai*). J Exp Zool 304:337-346.

Chapelle S, Zwinclestein G, Meister R, Brjchon R. 1979. The influence of acclimation temperature on the phiospholipid metabolism of an aquatic crustacean (*Carcinus Maenas*). J Exp Zool 310:371-380.

Charlton MP and Atwood HL. 1979. Synaptic transmission: temperature sensitivities of Ca entry in presynaptic terminals. Brain Res 170:543-546.

Chung YS, Cooper RM, Graff J, Cooper RL. 2012. The acute and chronic effect of low temperature on survival, heart rate and neural function in crayfish *(Procambarus clarkii)* and prawn *(Macrobrachium rosenbergii)* species. Open J Mol Integr Physiol 2:75-86.

Colinet H, Lee SF, Hoffmann A. 2010. Temporal expression of heat shock genes during cold stress and recovery from chill coma in adult Drosophila melanogaster. FEBS J 277:174-185.

Cooper AS, and Cooper RL. 2009. Historical view and demonstration of physiology at the NMJ at the crayfish opener muscle. JoVE. 33. http://www.jove.com/index/details.stp?id=1595; doi: 10.3791/1595.

Cooper AS, Leksrisawat B, Mercier AJ, Gilberts AB, Cooper RL. 2011a. Physiological experimentations with the crayfish hindgut: A student laboratory exercise. JoVE. https://www.jove.com/video/2324/physiological-experimentation-with-crayfish-hindgut-student

Cooper RM, Finucane HS, Adami M, Cooper RL. 2011b. Heart and ventilator measures in crayfish during copulation. OJMIP 1:36-42.

Cooper RL, Marin L, Atwood, HL. 1995. Synaptic differentiation of a single motor neuron: Conjoint definition of transmitter release, presynaptic calcium signals and ultrastructure. J Neurosci 15:4209–4222.

Cooper RL, Warren WM, Ashby HE. 1998. Activity of phasic motor neurons partially transforms the neuronal and muscle phenotype to a tonic-like state. Muscle Nerve 21:921-931.

Crider ME and Cooper R.L. (1999) The importance of the stimulation paradigm in determining facilitation and effects of neuromodulation. Brain Research 842: 324-331.

Crider ME and Cooper RL. 2000. Differentially facilitation of high- and low-output nerve terminals from a single motor neuron. J Appl Physiol 88:987-996.

Cumillaf, JP, Blanc J, Paschke K, Gebauer P, Díaz F, Re D, Chimal ME, Vásquez J, Rosas, C. 2016. Thermal biology of the sub-polar–temperate estuarine crab *Hemigrapsus crenulatus* (Crustacea: Decapoda: Varunidae). Biol Open 5:220–228.

Czajka MC and Lee R Jr. 1990.A rapid cold-hardening response protecting against cold shock injury in Drosophila melanogaster. J Exp Biol 148:245-254.

Desai-Shah M, Viele K, Sparks G, Nadolski J, Hayden B, Srinivasan VK, Cooper RL. 2008. Assessment of synaptic function during short-term facilitation in motor nerve terminals in the crayfish. Open Neurosci J 2:24-35.

Dieterich DC and Kreutz MR. 2016. Proteomics of the synapse - A quantitative approach to neuronal plasticity. Mol Cell Proteomics 15(2):368-381.

Djokaj S, Cooper RL, Rathmayer W. 2001. Effects of octopamine, serotonin, and cocktails of the two modulators on synaptic transmission at crustacean neuromuscular junctions. J Comp Physiol A 187:145-154.

Evans PD, Talamo BR, Karvitz EA. 1975. Octopamine neurons: morphology, release of Octopamine and possible physiological role. Brain Res 90:340-347.

Fischer L and Florey E. 1981 Temperature effects on Neuromuscular transmission (Opener muscle of Crayfish, *Astacus Leptodactylus).* J Exp Biol 94:251-268

Fischer L and Florey E. 1987. Octopamine action on the contractile system of crustacean skeletal muscle. Comp Biochem Physiol C 88:335-342

Florey E and Hoyle G. 1976. The effects of temperature on a nerve-muscle system of the Hawaiian ghost crab, *Ocypode ceratophthalma* (Pallas). J Comp Physiol 110:51-64

Forward RB. 1990. Behavioural responses of crustacean larvae to rates of temperature change. Biol Bull 178:195–204.

Friedlander MJ, Kotchabhakdi N, Prosser CL. 1976. Effects of cold and heat on behavior and cerebellar function in goldfish. J Comp Physiol 112:19-45.

Friedrich RW, Quigley PA, Srivastava M, Skerrett M, Mercier AJ. 1994. Temperature dependence of synaptic modulation by FMRF amide-related neuropeptide in crayfish. Neurosci Lett 169:56-58

Griffis B, Bonner P, Cooper RL. 2000. Sensitivity of transformed (phasic to tonic) motor neurons to the neuromodulator 5-HT. Comp Biochem Physiol A 127:495-504.

Harri M and Florey E. 1976. The effects of temperature on a neuromuscular system of the crayfish, *Astacus leptodactylus*. J Comp Physiol 117:47-61

Harri M and Florey E. 1979. The effects of acclimation temperature on a neuromuscular system of the crayfish, *Astacus leptodactylus*. J Exp Biol 78:281-293.

Hamilton JL. Edwards CR. Holt SR. Worden MK. 2007. Temperature dependent modulation of lobster neuromuscular properties by serotonin. J of Exp Biol 210:1025-1035

Hand SC, Denlinger DL, Podrabsky JE, Roy R. 2016. Mechanisms of animal diapause: Recent developments from nematodes, crustaceans, insects, and fish. Am J Physiol Regul Integr Comp Physiol 310:R1193–R1211.

Hayward S, Manso B, Cossins A. 2014. Molecular basis of chill resistance adaptations in poikilothermic animals. J Exp Biol 217:6-15

Hirashima A and Eto M. 1993. Effect of stress on levels of octopamine, dopamine and serotonin in the American cockroach (*Periplaneta americana L*.). Comp Biochem Physiol C 105:279-284.

Hobbs HH. 1981. The crayfishes of Georgia. Smith Cont Zool 318:1–549.

Huber R, Delago A. 1998. Serotonin alters decisions to withdraw in fighting crayfish, *Astacus astacus*: the motivational concept revisited. J Comp Physiol 182:573–583.

Hyde D, Pearson T, Qari S, Bowler K. 2015. Adaptive considerations of temperature dependence of neuromuscular function in two species of summer- and winter-caught Crab (*Carcinus maenas and Cancer pagurus*). J Comp Physiol B 185:629-636 .

Issartel J, Renault D, Voituron Y, Bouchereau A, Vernon P, Hervant F. 2005. Metabolic responses to cold in subterranean crustaceans. J Exp Biol 208:2923-2929.

Jacobs, JR and Atwood HL. 1981. Effects of thermal history on long term neuromuscular facilitation in intact crayfish and isolated claw preparations. J Comp Physiol A 143(1):53-60.

Jakobs R, Gariepy TD, Sinclair BJ. 2015. Adult plasticity of cold tolerance in a continental-temperate population of Drosophila suzukii. J Insect Physiol 79:1-9.

Jin SW, Choi YH, Lee DH. 2014. Utilization of rapid infusion system with cold saline in the induction of therapeutic hypothermia. J Int Med Res 42:744-749.

Katz B. 1949. Neuro-muscular transmission in invertebrates. Biol Rev 24:1-20.

Katz B and Kuffler SW. 1946. Excitation of the nerve-muscle system in crustacea. Proc R Soc Lond B Biol Science133:374-389.

Koon AC and Budnik V. 2012. Inhibitory Control of Synaptic and Behavioral Plasticity by Octopaminergic Signaling. J Neurosci 32:6312–6322.

Legarra I. Andreu-Moliner ES. Montoro R. Nunez-Cachaza A. 1984. Effect of temperature on the concentration of Ca2+, Mg2+, Na+ and K+ in the hemolymph and urine of Procambarus clarkii, Girard. Revista Espanola de Fisiologia 41:325-330.

Leong PKK and Manahan DT. 1997. Metabolic importance of Na+/K+-ATPase activity during sea urchin development. J of Exp Biol 200:2881-2892.

Listerman L. Deskins J. Bradacs H, Cooper RL. 2000. Measures of heart rate during social interactions in crayfish and effects of 5-HT. Comp Biochem and Physiol A.125:251-264.

Livingston MS, Harris-Warrick RM, Kravitz EA. 1980. Serotonin and octopamine produce opposite postures in lobsters. Science 208:76–79.

Lnenicka GA, 1993. Seasonal differences in motor terminals. Comp Biochem Physiol 104:423–429.

Lnenicka GA and Atwood HL 1989. Impulse activity of a crayfish motoneuron regulates its neuromuscular synaptic properties. J Neurophysiol 61:91–96.

Lnenicka GA, Zhao Y. 1993. Seasonal differences in the physiology and morphology of crayfish motor terminals. J Neurobiol 22: 561–569.

MacMillan HA. Baatrup E. Overgaard J. 2015. Concurrent effects of cold and hyperkalaemia cause insect chilling injury. Pro R soc B 282:20151483

MacMillan HA, Knee JM, Dennis AB, Udaka H, Marshall KE, Merritt TJ, Sinclair BJ. 2016. Cold acclimation wholly reorganizes the Drosophila melanogaster transcriptome and metabolome. Sci Rep 6:28999.

MacMillan HA, Sinclair BJ. 2011 The role of the gut in insect chilling injury: cold-induced disruption of osmoregulation in the fall field cricket, *Gryllus pennsylvanicus*. J Exp Biol 214: 726– 734.

Marder E. Haddad SA. Goeritz ML. Rosenbaum P. Kispersky T. 2015. How can motor systems retain performance over a wide temperature range? Lessons from the crustacean stomatogastric nervous system. J Comp Physiol A 201:851–856.

McRae T. 1996. On the postural effects induced in female Cherax destructor (Clark) by serotonin and octopamine. Freshwater Crayfish 11: 293–298.

Mercier AJ and Atwood HL. 1989. Long-term adaptation of a phasic extensor motoneurone in crayfish. J Exp Biol 145:9–22.

Mourvillier B, Tubach F, van de Beek D, Garot D, Pichon N, Georges H, Lefevre LM, Bollaert PE, Boulain T, Luis D, Cariou A, Girardie P, Chelha R, Megarbane B, Delahaye A, Chalumeau-Lemoine L, Legriel S, Beuret P, Brivet F, Bruel C, Camou F, Chatellier D, Chillet P, Clair B, Constantin JM, Duguet A, Galliot R, Bayle F, Hyvernat H, Ouchenir K, Plantefeve G, Quenot JP, Richecoeur J, Schwebel C, Sirodot M, Esposito-Farèse M, Le Tulzo Y, Wolff M. 2013. Induced hypothermia in severe bacterial meningitis: a randomized clinical trial. JAMA 310:2174-2183.

Mykles DL, Medler SA, Koenders A, Cooper RL. 2002. Myofibrillar protein isoform expression is correlated with synaptic efficacy in slow fibres of the claw and leg opener muscles of crayfish and lobster. J Exp Biol 205: 513-522.

Nguyen PV and Atwood HL. 1990. Expression of long-term adaptation of synaptic transmission requires a critical period of protein synthesis. J Neurosci 10:1099–1109.

Ormerod KG, Hadden JK, Deady LD, Mercier AJ, Krans JL. 2013. Action of octopamine and tyramine on muscles of Drosophila melanogaster larvae. J Neurophysiol 110(8):1984-1996.

Overgaard J, Malmendal A, Sørensen JG, Bundy JG, Loeschcke V, Nielsen NC, Holmstrup M. 2007.Metabolomic profiling of rapid cold hardening and cold shock in Drosophila melanogaster. J Insect Physiol 53:1218-1232.

Pamas I and Atwood HL. 1966. Phasic and tonic neuromuscular systems in the abdominal extensor muscles of the crayfish and the rock lobster. Comp Biochem Physiol 18:701-723.

Payette AL, McGaw IJ. 2003. Thermoregulatory behavior of the crayfish Procambarus clarki in a burrow environment. Comp Biochem Physiol A 136:539–556.

Peretti D, Bastide A, Radford H, Verity N, Molloy C, Martin MG, Moreno JA, Steinert JR, Smith T, Dinsdale D, Willis AE, Mallucci GR. 2015. RBM3 mediates structural plasticity and protective effects of cooling in neurodegeneration. Nature 518:236- 239.

Phadtare S. and Severinov K. 2010. RNA remodeling and gene regulation by cold shock proteins. RNA Biol 7:788-795.

Prosser, CL and Nelson, DO. .1981. The role of nervous systems in temperature adaptation of poikilotherms. Ann Rev Physiol 43:281-300.

Robinson MM, Martin JM, Atwood HL, Cooper RL. 2011. Modeling biological membranes with circuit boards and measuring conduction velocity in axons: Student laboratory exercises. JoVE:47 http://www.jove.com/video/2325 doi:10.3791/2325

Sadaka F, Veremakis C, Lakshmanan R, Palagiri A. 2013. Therapeutic Hypothermia in Traumatic Brain Injury. In Sadaka F, editor. Therapeutic hypothermia in Brain Injury. INTECH Press.

Shankaran S. Neonatal Encephalopahty: Treatment with hypothermia. J Neurotrauma 26:437-443.

Shuranova ZP, Burmistrov YM, Strawn JR, Cooper RL. 2006. Evidence for an Autonomic Nervous System in Decapod Crustaceans. Int J Zool Res 2:242-283.

Sneddon LU, Taylor AC, Huntingford FA, Watson DG. 2000. Agonistic behavior and biogenic amines in shore crabs Carcinus maenas. J Exp Biol 203:537–545.

Sohn J, Mykles DL, Cooper RL. 2000. The anatomical, physiological and biochemical characterization of muscles associated with the articulating membrane in the dorsal surface of the crayfish abdomen. J Exp Zool 287:353-377.

Somero GN, Dahlhoff E, Lin JJ. 1996. Stenotherms and eurytherms: mechanisms establishing thermal optima and tolerance ranges. In: Johnston

I.A, Bennett A.F, editors. Animals and temperature: phenotypic and evolutionary adaptation. Cambridge, UK. Cambridge University Press. p 53–57.

Storey KB and Storey JM. 1988. Freeze tolerance in animals. Physiological Reviews 68:27-84.

Strawn JR, Neckameyer WS, Cooper RL. 2000. The effects of 5-HT on sensory neurons, central, and motor neurons driving the abdominal superficial flexor muscles in the crayfish. Comp Biochem and Physiol B 127:533-550.

Teets NM and Denlinger DL. 2013. Physiological mechanisms of seasonal and rapid cold-hardening in insects. Physiol Entomol 38:105-116.

Teets NM, Peyton JT, Ragland GJ, Colinet H, Renault D, Hahn DA, Denlinger DL. 2012. Combined transcriptomic and metabolomic approach uncovers molecular mechanisms of cold tolerance in a temperate flesh fly. Physiol Genomics 44:764-777.

Thuma JB, Hobbs KH, Burstein HJ, Seiter NS, Hooper SL. 2013. Temperature sensitivity of the pyloric neuromuscular system and its modulation by dopamine. PLOS One 8(6):e67930.

Titlow JS and Cooper RL. 2017. Glutamatergic synthesis, recycling and receptor pharmacology at pharmacology at *Drosophila* and crustacean neuromuscular junctions. Walz W, editor. Biochemical approaches to glutamatergic neurotransmission. Springer Press.

Weight F F and Erulkar D. 1 976. Synaptic transmission and effects of temperature at squid giant synapses. Nature 261:720-722.

Wong TM and Freeman RFH. 1975. Seasonal and thermal effects on the concentration of the haemolymph in the New Zealand freshwater crayfish Paranephrops Zealandicus white. Comp Biochem Physiol 55A:17-22

Veselý L, Buřič M, Kouba A. 2015. A. Hardy exotics species in temperate zone: can “warm water” crayfish invaders establish regardless of low temperatures? Sci Rep 5:16340

Yenari MA and Han HS. 2012. Neuroprotective mechanisms of hypothermia in brain ischaemia. Nature Rev Neurosci 13: 267–278.

Zhu YC, Yocom E, Sifers J, Uradu H, Cooper RL. 2016. Modulatory effects on Drosophila larva hearts: room temperature, acute and chronic cold stress. J Comp Physiol B 186:829-841.

Chapter 5 References:

Bochdanovits Z, De Jong G. 2003. Temperature dependent larval resource allocation shaping adult body size in *Drosophila melanogaster.* J Evol Biol 16:1159-1167

Cooper RL, Warren WM, Ashby HE. 1998. Activity of phasic motor neurons partially transforms the neuronal and muscle phenotype to a tonic-like state. Muscle & Nerve 21:921-931.

Friedrich RW, Quigley PA, Srivastava M, Skerrett M, Mercier AJ. 1994. Temperature dependence of synaptic modulation by a FMRFamide-related neuropeptide in crayfish. Neurosci Let 169:56-58.

Hamilton JL, Edwards CR, Holt SR, Worden MK. 2007. Temperature dependent modulation of lobster neuromuscular properties by serotonin. J Exp Biol 210:1025-1035.

Johnson E, Ringo J, Dowse H. 1997. Modulation of Drosophila heartbeat by neurotransmitters. J Comp Physiol B 167:89–97.

Johnson E, Sherry T, Ringo J, Dowse H. 2002. Modulation of the cardiac pacemaker of Drosophila: cellular mechanisms. J Comp Physiol B 172:227–236.

Johnstone AF, Cooper RL. 2006. Direct innervation of the Drosophila melanogaster larval aorta. Brain Res 1083(1):159-163.

Lnenicka GA, 1993. Seasonal differences in motor terminals. Comp Biochem Physiol 104:423–429.

Lnenicka GA, Zhao Y. 1993. Seasonal differences in the physiology and morphology of crayfish motor terminals. J Neurobiol 22: 561–569.

Malloy C, Sifers J, Mikos A, Samadi A, Omar A, Hermanns C, Cooper RL. 2017. Using optogenetics to assess neuroendocrine modulation of heart rate in Drosophila melanogaster larvae. Journal of Comparative Physiology A. (In Press)

Malloy C, Ritter K, Robinson J, Cooper RL. 2015. Pharmacological identification of cholinergic receptor subtypes on Drosophila melanogaster larval heart. J Comp Physiol B 186(1):45-57.

Majeed ZR, Nichols CD, Cooper RL. 2013. 5-HT stimulation of heart rate in Drosophila does not act through cAMP as revealed by pharmacogenetics. J Appl Physiol 115 (11):1656-1665.

Majeed ZR, Stacy A, Cooper RL. 2014. Pharmacological identification of serotonin receptor subtypes on the Drosophila larval heart. J Comp Physiol B 184(2):205-219.

Sadaka F, Veremakis C, Lakshmanan R, Palagiri A. 2013. Therapeutic Hypothermia in Traumatic Brain Injury. In Sadaka F, editor. Therapeutic hypothermia in Brain Injury. INTECH Press.

Shankaran S. 2009. Neonatal Encephalopahty: Treatment with hypothermia. J Neurotrauma 26:437-443.

Thuma JB, Hobbs KH, Burstein HJ, Seiter NS. Hooper SL. 2013. Temperature sensitivity of the pyloric neuromuscular system and its modulation by dopamine. PLoS ONE. 2013;8:e67930.

Titlow JS, Rufer J, King K, Cooper RL. 2013. Pharmacological analysis of dopamine modulation in the Drosophila melanogaster larval heart. Physiological Reports. 1 (2), 2013, e00020.

Yenari MA, Han HS. 2012. Neuroprotective mechanisms of hypothermia in brain ischaemia. Nature Rev Neurosci 13: 267–278.

Curriculum Vitae

Yuechen Zhu

EDUCATION

Ph.D Candidate, Biology August 2013–Present

Department of Biology, University of Kentucky

Lexington, KY

Anticipated graduation date August 2017

Bachelor of Science, Biology May 2013

College of Biological Science, University of Minnesota-Twin cities

Minneapolis, MN

RESEARCH EXPERIENCE

Undergraduate Research Assistant September 2010 – June 2013

Biothermodynamics and Biomechanics Lab, Department of Mechanical Engineering

Minneapolis, MN

University of Minnesota-Twin Cities, Minneapolis, MN

Graduate Research Assistant August 2013- Present

Department of Biology

University of Kentucky, Lexington, KY

TEACHING EXPERIENCE

Graduate Teaching Assistant August 2013–May 2017

Department of Biology

University of Kentucky, Lexington, KY

HONOR

Undergraduate Research Opportunity program (UROP) Minneapolis, MN 2012

3th Place in graduate student presentation 100th Annual Meeting of Kentucky Academy of Science Lexington, KY 2015

University of Kentucky Mini Grant Funding winner Lexington, KY 2016

SCHOLARSHIP

Journal Publications

Anirudh Sharma, **Yuechen Zhu**, ShengShee Thor, Bethanie Stadler, Allison Hubel, "Magnetic barcode nanowires for osteosarcoma cell control, detection and separation," *IEEE Transactions on Magnetics, Vol. 49, No.1, January 2013*

Anirudh Sharma, Gregory Orlowski, **Yuechen Zhu**, Daniel Shore, Seung Yeon Kim, Michael DiVito, Allison Hubel, Bethanie Stadler "Inducing cells to disperse nickel nanowires via integrin-mediated responses" Nanotechnology. 2015 Mar 27;26(13):135102. Epub 2015 Mar 12.

Yuechen Zhu, Emily Yocom, Jacob Sifers, Henry Uradu & Robin L. Cooper. "Modulatory effects on Drosophila larva hearts: room temperature, acute and chronic cold stress" Journal of Comparative Physiology *B* 186(7):829-841 May 2016

Yuechen Zhu, Henry Uradu, Zana R. Majeed, Robin L. Cooper. Optogenetic stimulation of Drosophila heart rate at different temperatures and Ca2+ concentrations. Physiological Report 4(3):e12695 January, 2016.

Yuechen Zhu, Leo deCastro, Robin Cooper. The effect of chronic and acute cold temperature on synaptic transmission at crayfish neuromuscular junction. (In manuscript) 2017