Anoxia-Induced Changes in Action
Potential Propagation in a Non-Myelinated Axon

By

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ABSTRACT

Processing information in the nervous system is energetically expensive, constraining the ability of the system to survive disturbances caused by stress. While some organisms compensate for extreme changes in the abiotic features of their environment, the mechanisms underlying this are poorly understood. We used the locust Descending Contralateral Movement Detector (DCMD) neuron to study how the propagation characteristics of action potentials (APs) change following an acute energy stress in control and heat shock (HS) pre-treated animals. We also attempted to determine if Ca\(^{2+}\) is involved in the DCMD AP and the possible changes indicated above. Conduction velocity decreased over an hour of recording in all groups, except those with minimal dissections, and we observed an increase in AP half-width and a decrease in the slope of the rising phase of the AP over time. After HS pre-treatment the response to a standard looming stimulus was delayed, showed significantly fewer APs and a lower peak frequency compared to controls. Brief application of sodium azide (NaN\(_3\)) as an acute metabolic inhibitor did not subsequently affect DCMD’s conduction velocity or ability to fire at high frequencies during the recording period. There were no significant differences from control animals with extracellular Ca\(^{2+}\) manipulations; however we cannot conclude that Ca\(^{2+}\) does not contribute to DCMD’s AP because Na\(^+\) could have flowed through Ca\(^{2+}\) channels in the absence of extracellular Ca\(^{2+}\). Furthermore, examination of possible performance impairments with decreased Ca\(^{2+}\) currents, to indicate if Ca\(^{2+}\) current manipulation may account for the performance impairment, could not be conducted because no differences in AP characteristics were observed with Ca\(^{2+}\) manipulations. We suggest that the slowing of propagation in all
groups represents a response to energetic stress and that HS modifies neuronal properties in ways that can be interpreted as saving energy in case of future stressors.
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CHAPTER 1: INTRODUCTION

The threat of energy crises are apparent for any organism and can lead to drastic consequences, as most functions carried out by an organism require energy. An energy crisis can result from any event that increases the energy demand above the supply. Two common causes are hyperthermia, causing metabolic demand to increase, and hypoxia, causing energy supply to decrease. An example of the impact of these environmental stresses on survival comes from the impact of global warming on eelpout, a member of the ray-finned fish family Zoaridae, in the German Wadden Sea (Pörtner and Knust 2007). The effect of increased environmental temperatures has a two-fold impact on aquatic aerobic life because warming not only increases energy demand, but also decreases oxygen solubility and therefore can limit the production of ATP (Pörtner and Knust 2007). The end results of these increased temperatures are a higher mortality and decreased growth of eelpout in the hotter summers, as well as a decrease in abundance the following year, indicating a reduction in successful fertilization and reproduction in the hot year (Pörtner and Knust 2007).

Looking more closely at the impact of hotter summers on eelpout, one can see different levels of stress on the organism. First, when the pejus (‘turning worse’) temperature is surpassed, functions not immediately critical to survival are stopped, such as growth and reproduction, in order to conserve energy. If the critical temperature is then surpassed, most functions and activity are shut down in order to survive. Finally, a temperature is surpassed beyond which the fish can no longer survive (Pörtner and Knust 2007; Wang and Overgaard 2007). This example illustrates the drastic effects of energy stresses caused by changes in an organism’s environment, as well as how
organisms can attempt to compensate for such changes in order to save energy to survive.

Energy crises are not always caused by external factors, but can also be internal. An example in mammals is stroke, often caused by a blood clot blocking nutrient and oxygen supply to regions of the brain. This generates a hypoxic environment for the brain and creates an energy shortage for that region. This event has been associated with a phenomenon termed ‘spreading depression’, in which there is a major redistribution of ions across neuronal cell membranes, including a rapid increase in extracellular potassium, and a reduction of electrical activity, effectively depressing neural activity (Leão 1944; Leão 1986 as cited by Wu and Fisher 2000; Wu and Fisher 2000; Smith et al. 2006). This then spreads outwardly across other neuronal tissue (Leão 1944).

Spreading depression has also been characterized in the migratory locust, *Locusta migratoria*, evident following hyperthermia or hypoxia (Rodgers et al. 2007). Presumably this shut down of neuronal activity occurs in order to prevent neuronal tissue damage during extreme energy crises by drastically decreasing the immediate usage of energy.

The central nervous system (CNS) of organisms is a critically important organ and has a high energy demand. Energy shortages here can have a profound impact on organisms, as evident from the after-effects of stroke. Because energy supply to the brain is very important and inherently constitutes a large portion of the body’s energy, the design of the CNS is constrained such that energy usage must be as efficient as possible (Laughlin and Sejnowski 2003). Therefore, the CNS was designed to minimize energy usage by decreasing size, creating the shortest possible connections and being
organized in an efficient manner for communication, such as with topographic organization, as well as through communication by minimizing redundancy in signalling via mechanisms such as pruning and synaptic failures (Laughlin and Sejnowski 2003).

However, even though energy utilization is minimized in the CNS, it still constitutes a large portion of an animal’s energy budget, consuming on average 2 – 10% of resting energy (Laughlin and Sejnowski 2003). In addition, approximately half of the energy designated to the brain is used for signalling along axons and across synapses; the rest is used for maintenance and resting potentials (Laughlin and Sejnowski 2003). Research has been conducted to attempt to appreciate the amount of energy used for action potentials (APs) to travel along axons. Recent models consider three ionic flux phases of APs: Na$^+$ flux, K$^+$ flux, and a neutralized flux where both Na$^+$ and K$^+$ are crossing the membrane, thus neutralizing the charge distribution (Crotty et al. 2006). Estimates of energy usage in each of these phases for the squid giant axon are 4.2 nJ/cm of axon length, 4.1 nJ/cm and 8.4 nJ/cm, respectively (Crotty et al. 2006). Since this estimate comes from the Na$^+$/K$^+$ ATPase, only one of the single ion fluxes is considered for overall energy usage, which is estimated at 12.6 nJ/cm in the squid giant axon (Crotty et al. 2006).

Estimates can also be found in terms of ATP molecules utilized. In the squid it is estimated that $1.84 \times 10^{11}$ ATP molecules are used per cm$^2$ per AP, and in the cat $1.54 \times 10^{13}$ ATP molecules are used per cm$^2$ per AP, producing an average of $10^{12}$ ATP/cm$^2$ for each AP (Aiello and Bach-y-Rita 2000). Some systems have the ability to fire APs above 600Hz (Money et al. 2006), which would increase ATP usage considerably from the number quoted above not only from the large increase in the number of APs but also
from specialized currents needed to support this activity. In addition, these estimates do not consider ions other than Na\(^+\) and K\(^+\) and thus most likely underestimate the true cost of APs. Therefore, even though the CNS is efficient, it requires a great deal of energy and disruption of its supply can have dire effects.

Some animals have the ability to survive extreme energy stresses (e.g. locust, Robertson et al. 1996; fish, Jibb and Richards 2008; nematode, Anderson et al. 2009), and studying these organisms may help us to understand the compensation strategies that are evident in nature. One such animal that is able to survive extreme changes to its abiotic environment is the migratory locust (Robertson et al. 1996; Gray and Robertson 1998).

Locusts have the ability to adapt their CNS following minor, non-lethal stresses in order to survive future extreme stresses that would normally be lethal (Robertson et al. 1996; Gray and Robertson 1998). This is observed in many neural circuits in the locust, including the ventilation system, flight system and visual system (Robertson 2004). In the ventilation system, pre-treatment with heat shock (HS), cold shock or anoxia do not influence thermosensitivity of ventilator rhythm frequency or burst duration, but do significantly increase the chance of recovering the rhythm following hyperthermic failure (Newman et al. 2003). Time to recovery is also shorter in pre-stressed animals compared to control animals (Newman et al. 2003; Rodgers et al. 2007). In addition, control animals fail sooner than pre-treated animals at high temperatures (Newman et al. 2003). These results indicate that prior non-lethal stress results in thermoprotection of the ventilator motor pattern generator.

Similar results are found in the locust flight system, with pre-treatment increasing
the upper temperature limit for AP generation and a trend towards faster recovery following hyperthermic failure (Wu et al. 2002). Therefore, prior stress in this system also results in thermoprotection. Furthermore, the visual system shows thermoprotection, specifically in the Descending Contralateral Movement Detector (DCMD) neuron, a visual interneuron associated with escape behaviours that responds to the looming approach of objects (Rind and Simmons 1992; Gabbiani et al. 1999; Gray et al. 2001; Santer et al. 2005). In control locusts, as temperature is increased, spike count decreases, limiting the information coded in DCMD; however, in HS animals spike count remains constant, preserving information (Money et al. 2005; 2006). Instantaneous firing frequency increases in both control and HS animals in DCMD during a temperature ramp, however, HS animals show a greater increase, reaching higher peak frequencies (Money et al. 2005; 2006). Membrane excitability also increases following HS, which could improve conduction reliability in DCMD (Money et al. 2005). Single APs change following HS as well. Although in both control and HS locusts resting membrane potential (RMP) hyperpolarizes and AP amplitude decreases with increased temperature, in HS locusts RMP hyperpolarizes more and amplitude decreases less (Money et al. 2005). These changes in the DCMD AP in HS locusts work together to maintain activity at reliable levels during an increase in temperature (Money et al. 2005).

Most of the changes observed following pre-treatment are likely helpful during a subsequent stress to allow locusts to survive. Therefore, the CNS must be able to adapt and overcome energy stresses under certain circumstances. The phenomenon surrounding increased survival of HS animals is characterized by transcriptional activity
of genes (Morimoto 1993) that increases the level of HS proteins. The mechanisms of this in the locust have been addressed. A study by Armstrong and colleagues in 2006 examined the role octopamine (OA) has in producing HS thermoprotection. In that study, application of OA for longer than one hour or injection of OA four hours before the thermal stress produced similar thermoprotective effects to those observed after HS pre-treatment, having higher operation temperatures and faster recovery time. In addition, agonists of adenylyl cyclase, cAMP and protein kinase A (PKA) all induced thermotolerance similar to HS animals while antagonists reduced thermoprotection, indicating a pathway for the phenomenon. The effect of a minor stress could release OA into the CNS, which would then bind to receptors and activate adenylyl cyclase to produce cAMP from ATP. cAMP would then activate PKA, which would phosphorylate certain proteins to bring about the desired effect, and in this case at least one outcome would be protein synthesis, since it is required for OA induced thermotolerance (Armstrong et al. 2006). Therefore, OA is at least one molecule through which pre-treatment can induce thermoprotective effects and its pathway must contain both PKA activity and protein synthesis, and thus also contain adenylyl cyclase and cAMP. Application of serotonin has also shown thermoprotective effects similar to pre-treatment (Newman et al. 2003), indicating it could also play a role either in series or parallel with OA.

Another interesting requirement of the HS phenomena found during studies exposing the thoracic ganglia of locusts to a temperature ramp while maintaining brain temperature at room temperature is that the brain must undergo the extreme thermal stress in order for the thermoprotective effects to occur in the animal (Money et al.)
This indicates that there needs to be a direct temperature effect on processing centres in the brain in order to achieve HS-induced thermoprotection (Money et al. 2006).

Pre-treatment prepares the system to overcome future energy stresses and allows for better survival. However, it may also prepare the system for changes caused by the extreme acute stresses in order to further the animal’s ability to survive the stress by changing energy allocation and saving energy at a cost to performance. This adaptation is evident in pre-treated locusts that undergo two temperature ramps to failure (R.M. Robertson, unpublished). Action potential amplitude in DCMD, as well as the afterpotential, are changed following the first ramp such that the amplitude decreases and the afterhyperpolarization (AHP) increases, abolishing the afterdepolarization (ADP) observed at high temperatures (Figure 1). Both of these changes could be accounted for by the loss of depolarizing ionic currents across the membrane. This change is unlikely to be caused by damage due to the fact that it is not exacerbated by the second ramp (Figure 1). Thus, it seems energy consumption in the locust CNS is plastic such that when resources are plentiful performance can be optimal and efficient, and when resources become limited (i.e. during energy stresses) neuronal function can be down regulated in a controlled manner to save energy. This represents a theory of switching to an energy-save mode, allowing locusts to survive severe energy stresses at the cost of peak performance.

This theory can be applicable in the locust nervous system. From discussions above we see how energetically costly the functioning of a nervous system can be, with at least 50% of the energy supply to the CNS being used for communication and
Figure 1. Intracellular recording of DCMD during temperature ramps. Intracellular recordings from DCMD of the first temperature ramp (black) and second temperature ramp (red). A) and B) are at 22°C, and C) and D) are at 50°C. Obtained by R.M. Robertson.
computation (Laughlin and Sejnowski 2003), and of that 47% being used for APs (Attwell and Laughlin 2001). This leads to the major ATP cost in neural processing being the maintenance of ionic gradients (Sangrey et al. 2004; Crotty et al. 2006). Therefore, it is reasonable that plastic mechanisms would target processes that disturb or restore these gradients. It has been proposed in other research that reduced ionic currents could be a mechanism to save energy (Weckström and Laughlin 1995) and changes in $K^+$ conductance in locusts can be interpreted to be a mechanism involved in a coordinated physiological shift to protect systems and save energy (Ramirez et al. 1999).

However, adjusting these currents may result in the decrease of some measures of performance for neuronal communication. Better performance does cost the system more energy (Weckström and Laughlin 1995; Niven et al. 2007) and having plasticity in performance level could be a means to save energy for the system.

Conduction velocity is one measure of performance, and has been linked to energy demand and ion gradient restoration, such that AP velocity costs energy (Sangrey et al. 2004; Sangrey and Levy 2005; Crotty et al. 2006). Fewer APs may also be a means to save energy that will obviously decrease the amount of information conducted in the CNS. This is especially true in DCMD, where the frequency of APs in response to a looming stimulus code for information of how close the approaching object is to the locust (Gabbiani et al. 1999). The ability to fire at high frequencies can be energetically expensive because specialized currents would be needed throughout the course of an AP so that it can be of short duration and reset channels quickly. The duration of DCMD APs is short compared to other insect neurons, being 0.3 ms compared to approximately 1 to 3 ms for most (Bullock and Horridge 1965). More currents involved in an AP
means it will cost more, therefore shutting down some of the specialized current will still allow an AP to fire, but may not allow the system to fire at as high frequencies. Using the number of APs as a determinant of performance leads to measurements of AP count (or spike count) in a looming stimulus response, time to collision (TtC) of the first AP and peak instantaneous frequency since all of these measurements are dictated by when and how many APs fire. These measures are all coded for in the brain (O’Shea and Williams 1974; Rind 1984) however, and in our experiments they should only be influenced by the pre-treatment stress. To measure performance in terms of the ability to fire at high frequencies following an acute stress we consider the fidelity of transmission through sites of low safety factor, such as the DCMD axon travelling through the mesothoracic ganglion of the locust (Money et al. 2009), and measure the number of failed APs through this section of axon. This would indicate that a portion of the axon has been changed and can no longer support the high frequency firing coded for higher in the system.

Locusts provide a suitable model for studies of stress compensation because of their ability to adapt and survive extreme stresses, as well as the relatively straightforward methods to record from neural tissue. DCMD proves to be a good model neuron because of its importance in locust physiology. It is a visual interneuron connected to motoneurons in the thoracic ganglia (Simmons 1980) involved in the escape response of the locust (Pearson et al. 1980; Gabbiani et al. 1999; Gray et al. 2001; Santer et al. 2005; 2008). This means that DCMD must conduct information quickly and reliably, similar to the squid giant axon (Sangrey et al. 2004). For this to occur, DCMD must have a large diameter axon, being, in fact, the largest in the cord at
15-17 μm (O’Shea et al. 1974). In addition, DCMD lies on the dorsal-medial surface of the ventral cord (Figure 2). Thus, its location and size allow for relatively easy intracellular recording and discrimination of its signal in an extracellular recording since it produces the largest signal. The involvement of DCMD in escape behaviours also allows for measurements of performance. Conduction velocity is one measure of performance important in DCMD that can easily be measured. In addition, fidelity in DCMD is critical since it ‘tracks’ approaching objects (Gabbiani et al. 1999); this allows performance to be measured in terms of ability to fire at high frequencies and number of APs, since more APs can code more information about the ‘tracking’ of an object. Stimulating DCMD activity is also straight forward since it is a visual interneuron that responds to movements in the visual field, particularly objects on a collision course with the locust (looming stimuli; Rind and Simmons 1992; Gabbiani et al. 1999; Gray et al. 2001). Simulating an approaching object via a looming stimulus allows for a characteristic response in DCMD (Rind and Simmons 1997; Gabbiani et al. 1999) that can be analyzed.

The purpose of the studies described in this thesis was to determine if pre-treatment or acute energy stresses impair function, indicating a possible trade-off between energy conservation and functional ability. Our hypothesis was that stresses cause a prolonged change in DCMD performance that may be due to the loss of ionic currents contributing to the AP and may reflect an adaptive switch to an energy saving mode of firing. We set out to verify the presence of a prolonged impairment of DCMD performance following gradual and acute energy stressors by using HS and non-HS (NHS) locusts exposed to sodium azide (NaN₃) or standard locust saline. NaN₃ is a
Figure 2. Location of DCMD. DCMD axon in the ventral nerve cord revealed by intracellular injection of the fluorescent dye Lucifer Yellow. The scale bar represents 0.25 mm. Prepared and imaged by M.L. Anstey.
compound that blocks ATP production in the mitochondria by blocking electron transport through Complex IV (cytochrome c oxidase), drastically decreasing the energy supply of the tissues, therefore mimicking an anoxic stress. Having these four treatment groups allowed us to determine the potential impairment following either stress, as well as if pre-treatment is required to prepare the system for changes following the acute stress. We measured conduction delay, spike count, peak instantaneous frequency, TtC of first AP and the number of failed APs through the mesothoracic ganglion as indicators of DCMD performance.

A second set of experiments was conducted in order to determine if the loss of a particular ion could account for the presumed impairment in DCMD performance. The candidate ion in this study was Ca\(^{2+}\). Calcium currents have been shown to be present in locust neurons, including thoracic ganglia neurons (Laurent et al. 1993; Pearson et al. 1993; Bickmeyer et al. 1994), such as dorsal unpaired medial (DUM) neurons (Heidel and Pflüger 2006), as well as insect neurons operating in a similar fashion to those found in locusts (Wicher and Penzlin 1997). Calcium has also been shown to be a component in APs of some neurons (Wicher and Penzlin 1997; Höger et al. 2005). Therefore, it is possible that Ca\(^{2+}\) has a component in the DCMD AP and since Ca\(^{2+}\) works to depolarize the cell, the loss of this current could account for a decreased AP amplitude (Figure 1). In addition, Ca\(^{2+}\)-dependent Cl\(^{-}\) channels may account for the ADP in DCMD APs observed at higher temperatures (Money et al. 2005) and the loss of Ca\(^{2+}\) would thus cause the loss of the ADP and increase in AHP, as shown in Figure 1. These changes relate to performance since AP amplitude is linked to conduction velocity via the space constant and the ADP may be a specialized current to allow high frequency firing in
DCMD. The experiments were used to determine if DCMD has a Ca\(^{2+}\) component in its AP and to examine if the loss of this component could account for changes in AP characteristics observed following an energy stress, as well as the performance impairments hypothesized to be found.

This work will represent progress towards understanding plastic features of the nervous system in response to abiotic changes in an organism’s environment. Moreover, it will demonstrate a level of phenotypic plasticity of neural systems that was previously unappreciated and support the idea that neural circuits exist in a dynamic harmony with the environment by using abiotic information for continuous adaptive neuromodulation. The anticipated results could also strengthen the idea that there is a trade-off in neurons of energy conservation for performance during times of energy stress, as well as provide a possible mechanism for this trade-off. Furthermore, understanding this phenomenon can broaden our understanding of many disorders of the nervous system that disrupt nutrient and energy supplies, such as that evident during and after stroke.
CHAPTER 2: METHODS

2.1 Animals

Locusts (*Locusta migratoria*) were housed in crowded colonies in the Department of Biology at Queen’s University on a 12:12 light:dark cycle. The temperature in the colony was 27 ± 1°C during light and 23 ± 1°C during dark with a humidity of 23 ± 1%. Animals were cared for in accordance with the Canadian Council of Animal Care and the University Animal Care Committee and fed wheat seedlings, and a dry mixture of oats, bran, skim milk powder and torula yeast, supplemented with sliced carrots. Each cage was lit with a 40 W incandescent light bulb until two weeks post final moult when an equivalent wattage fluorescent bulb was used for lighting. This was to prevent the animals from overheating themselves in the cages by occupying space in the immediate vicinity of the bulb. Adult locusts were used for the experiments three to six weeks following their final moult.

Animals for the *Conduction Delay in an Intact Animal* data set were cared for the same as above, except a 40 W incandescent light bulb was used for their entire life.

2.2 Animal Preparation

A semi-intact preparation, as described previously (Robertson and Pearson 1982), was used to expose the ventral nerve cord for recordings (Figures 3 and 4). Briefly, appendages and wings were removed to limit movement during experiments. The dorsal part of the pronotum was removed and a dorsal midline incision was made from the 3rd abdominal segment to the head. The locusts were then pinned open and their gut removed, along with fat and connective tissues that cover the ventral nerve cord. The nerve roots exiting the lateral portion of the mesothoracic ganglion were cut to ensure
the salines flowed into the ganglion to reach DCMD. The meso- and metathoracic ganglia were then mounted on a metal plate for accessibility and to decrease disturbances during recordings due to muscle twitches and other movements. Preparations were grounded using a silver wire inserted into the abdominal cavity of the locusts. Recording electrodes were then placed to record the right DCMD neuron.

2.2.1 Performance Impairment

The data set of performance impairment experiments had four treatment groups: NHS/control (Con.), NHS/Na$_3$N$_3$, HS/Con. and HS/Na$_3$N$_3$. The first portion of the two-part treatment title refers to the presence (HS) or absence (NHS) of HS pre-treatment, and the second part of the title refers to the presence (Na$_3$N$_3$) or absence (Con.) of Na$_3$N$_3$ application. Because of the HS procedures, animals were collected four to eight hours before dissection. For the HS groups, locusts were placed in an oven for 3 hours at 45°C in a well ventilated 1L container. The locusts were then left for 1 hour or more at room temperature (23 ± 1°C) to cool and settle. NHS locusts were collected in similar 1L containers and left at room temperature for 4 hours or more. The dissection was then conducted and extracellular suction electrodes placed, one anterior and one posterior to the mesothoracic ganglia (Figure 3A). The preparation was left to sit for 20 minutes to allow the locusts to settle from manipulations and for the suction electrodes to form a good seal. Initial recordings were then taken and in Na$_3$N$_3$ groups the saline flow was switched to apply Na$_3$N$_3$ for 10 minutes, at which time the saline was switched back to standard locust saline and recordings were taken every 5 minutes for 55 minutes. For non-Na$_3$N$_3$ groups, following the initial recordings, 15 minutes were allowed to elapse to account for the time of exposure to Na$_3$N$_3$ and the first 5 minutes of washout in the other
Figure 3. Experimental protocol for performance impairment experiments. A) Locust preparation; saline was superfused through the locust thorax and abdomen and extracellular suction electrodes placed anterior and posterior to the mesothoracic ganglion on the right connective. Hand waving was used to stimulate DCMD in the first data set and a looming stimulus (shown) was used in the second data set. B) Timeline of experiments. Four groups were studied: NHS/Con. (top timeline with no HS), NHS/NaN\textsubscript{3} (bottom timeline with no HS), HS/Con. (top timeline with HS pretreatment), and HS/NaN\textsubscript{3} (bottom timeline with HS pretreatment). C) Example recording for how conduction delay was measured from the two recording electrodes. E) Example of a looming response in DCMD from the anterior and posterior suction electrodes and a graph of how the looming stimulus expanded in the locust visual field over time. The grey line represents when the last frame of the stimulus occurred.
groups, and recordings were taken every 5 minutes for 55 minutes (Figure 3B). Activation of DCMD was achieved by a replicable looming stimulus (see below).

Suction electrodes were used in all performance impairment experiments for recording electrical activity in DCMD. Recordings were amplified using an A-M Systems Differential AC Amplifier model 1700 (Carlsborg, WA, USA) and digitized to a computer using an Axon Instruments Digidata 1322A or 1440A (Sunnyvale, CA, USA). Software from Molecular Devices (Sunnyvale, CA, USA), pClamp v9.0, was used to record data. We examined conduction delay, TtC of first AP, peak instantaneous frequency, spike count, and number of failed APs as measures of performance.

2.2.2 Conduction Delay in an Intact Animal

In order to determine why conduction delay increased over time (see Results) another data set was collected. There were four treatment groups in this data set: a control group, a group exposed to glucose for the entire experiment, a group exposed to Compound C for the final half of the experiment, and a group that underwent a dissection to leave the locust almost entirely intact (called an intact preparation here) named the ventral group because a ventral dissection was utilized.

Glucose experiments were used to determine if a decrease in energy resources caused by the locusts’ hemolymph being washed away in the semi-intact preparation could cause the increase in conduction delay. The experimental design was the same as mentioned above for NHS/Con. animals, except that the locusts were kept at room temperature for only 2 hours before dissection and recordings were taken at t = 5 and 10 min. Animals were exposed to glucose saline for the entire experiment.

Compound C was used to investigate if an energy depletion signaling pathway is
involved in the increase in conduction delay. Low energy availability in a cell is signaled by high ratios of AMP:ATP and this signal activates AMP-activated protein kinase (AMPK) (Hardie and Carling 1997; Jibb and Richards 2008). Compound C inhibits AMPK, causing its activation to be blocked. These experiments were conducted the same as the glucose experiments, except that the locusts were exposed to standard locust saline until \( t = 30 \) min., after which Compound C saline was switched into the preparation.

A new control group was also collected that was exposed to standard locust saline for the 70 minutes of the experiment.

Another experimental group was collected to ensure the increase in conduction delay was a side effect of the semi-intact preparation. In this dissection the locust was pinned down ventral side up and two windows were cut in the exoskeleton above the ventral nerve cord between the subesophageal ganglion and prothoracic ganglion and the pro- and mesothoracic ganglia. Hook electrodes were then used to record the dorsal surface of the connectives of the nerve cord utilizing the same equipment as with suction electrodes. Vaseline was placed around the hook electrodes and the preparation was left for 20 minutes. Recordings were then made with the looming stimulus every 5 minutes for 70 minutes, as in the other data sets. This preparation allowed the hemolymph to remain in the locust and the trachea to remain intact during experiments.

2.2.3 \( \text{Ca}^{2+} \) Manipulations

In experiments exploring calcium’s role in the DCMD AP, locusts were collected two to eight hours prior to dissection. The locusts were then dissected and electrodes placed around the mesothoracic ganglion as shown in Figure 4A, with a suction
Figure 4. Experimental protocol for Ca\textsuperscript{2+} manipulation experiments. A) Locust preparation; saline was superfused through the locust thorax and abdomen, extracellular suction electrodes were placed anterior to the meso- and metathoracic ganglia and an intracellular microelectrode was placed posterior to the mesothoracic ganglion on the right connective. A looming stimulus (shown) was used in the second data set. B) Timeline of experiments. Four groups were studied: Controls (top timeline), Nominally zero-Ca\textsuperscript{2+} (bottom timeline), EGTA (bottom timeline), and high-Ca\textsuperscript{2+} (bottom timeline). C) Measurements recorded for data analysis. The RMP (arrow), rise slope of the AP (s), half-width of the AP (d), AP amplitude (a), and AHP amplitude (AHP) were analyzed.
electrode anterior to the mesothoracic ganglion, intracellular electrode posterior to the
ganglion and another suction electrode posterior to that. The preparation was left for 10
minutes to allow the animal to settle and recordings stabilize. There were four treatment
groups in this data set with varying degrees of manipulation to the extracellular Ca\(^{2+}\)
concentration: a control group, a nominally zero-Ca\(^{2+}\) group, a zero-Ca\(^{2+}\) group (termed
the EGTA group) and a high-Ca\(^{2+}\) group. In the control group an initial recording was
taken, 10 minutes following that an ‘exposure’ recording was taken and 15 minutes later
a ‘washout’ recording was obtained (Figure 4B). In the other three groups the initial
signal was recorded and immediately following that the saline being supplied to the
locusts was switched to the respective manipulated saline. The manipulated saline was
superfused over the ganglia for 10 minutes and the exposure recording was obtained.
Standard locust saline was then superfused through the locusts for 10 minutes and the
washout recording was taken (Figure 4B). The 5 minute discrepancy between control
and manipulated treatments accounts for the time to switch salines in the manipulated
groups. During recordings five consecutive looming stimuli were presented to the locust
followed by approximately 30 seconds of random visual stimuli. Only the first AP of
the first loom response was used in analyses in this data set.

For intracellular electrode manipulation an Aus Jena grease plate
micromanipulator was used, and an A-M Systems Neuroprobe Amplifier model 1600
(Carlsborg, WA, USA) was used to amplify the signal. A Grass AM8 Audio Monitor
(West Warwick, RI, USA) was utilized to listen to the intracellular electrode signal in
order to help locate DCMD. Intracellular electrodes were pulled to a resistance of 20 –
60 MΩ and backfilled with 1 M KAc.
2.3 Salines

Standard locust saline consisting of (in mM): 147 NaCl, 10 KCl, 4 CaCl₂, 3 NaOH, and 10 HEPES buffer (pH 7.2) was used in all control treatments, as well as initial and all recovery or washout recordings. Salines were superfused over the thoracic and abdominal cavities of the locust using a Peri-Star peristaltic pump (World Precision Instruments Inc., Sarasota, FL, USA). Salines entered into the anterior body cavity, flowed posteriorly over the ganglia and were pumped out of the body cavity from the abdomen.

2.3.1 Performance Impairment

For acute energy stress NaN₃ was used as a model, which disrupts ATP production in mitochondria causing a chemically induced anoxia state. The NaN₃ saline was composed of (in mM): 147 NaCl, 10 KCl, 4 CaCl₂, 3 NaOH, 10 HEPES buffer and 1 NaN₃ (pH 7.2).

2.3.2 Conduction Delay in an Intact Animal

In these experiments no flow through system was used to administer the salines, rather the thoracic and abdominal cavities were filled with saline that stayed in the locust. Most often the saline would slowly leak out of the preparation; therefore the saline in the animals was supplemented after every 10 minutes during the experiments.

The glucose saline consisted of standard locust saline plus 1 mM glucose. The Compound C saline was made of standard locust saline and 1 x 10⁻⁴ M Compound C.

2.3.3 Ca²⁺ Manipulations

Three salines were utilized to manipulate the extracellular Ca²⁺ concentration of the locusts’ nervous system, two with a decreased Ca²⁺ concentration and one with an
increased concentration. The nominally zero-Ca\textsuperscript{2+} saline contained only contaminant levels of Ca\textsuperscript{2+} (Sakakibara et al. 2004) and was made of (in mM): 147 NaCl, 10 KCl, 4 MgCl\textsubscript{2}, 3 NaOH, and 10 HEPES buffer (pH 7.2). The EGTA saline was composed of the same ingredients as above with the addition of 0.5 mM EGTA (pH 7.2 ± 0.2). The high-Ca\textsuperscript{2+} saline contained (in mM): 147 NaCl, 10 KCl, 8 CaCl\textsubscript{2}, 3 NaOH, and 10 HEPES buffer (pH 7.2).

2.4 Looming Stimulus

The looming stimulus used in this study was a computer-generated video produced using Macromedia Flash Player 7.0 (Adobe). The video image was an exponentially expanding black disk on a white background. The computer running the program had an ATI Radeon 256 MB video board and the video was displayed by a Sharp Notevision XG-C55X digital projector (Mississauga, Ont., Can.) onto a rear projector screen 8 cm from the locusts’ left eye. The image had an apparent velocity of 1 m/s on a collision trajectory towards the locusts’ left eye, 90° to the body axis. The first frame had an angular size of 1.3° and the final image size was 46°. The expansion of the image on the locust retina can be seen in Figure 3D. A sound output was attached to the first and last image frame and used as inputs to data acquisition to calculate TtC. Because the stimulus was constant from one presentation to another, we were able to measure DCMD response characteristics.

2.5 Analyses

All values were obtained using ClampFit (part of the pClamp programs) and displayed graphically using SigmaPlot 11.0 (San Jose, CA, USA).
2.5.1 Performance Impairment

With the use of a repeatable stimulus we were able to characterize the response of DCMD in order to gain understanding into performance changes. One looming stimulus was presented every five minutes to obtain the data. We measured conduction delay between the two recording electrodes for the first AP of the response (Figure 3C), as well as TtC of first AP, spike count and peak instantaneous frequency of response (Figure 3D). Given that DCMD almost always shows an on-response to the stimulus used (Figure 3D) we determined the first AP to be the first spike that was associated with a gradual increase in firing frequency, and not the APs that were related to the on-response or short bursts of activity (2 – 8 APs) that were not associated with the loom response and were most likely caused by uncontrolled visual stimuli. Because TtC of first AP, spike count and peak instantaneous frequency are coded for in the brain (O’Shea and Williams 1974; Rind 1984) and therefore should only affect the HS groups, another measurement was used to examine the effects of NaN\textsubscript{3} and that was the number of failed APs through the mesothoracic ganglion. This was done by determining the number of APs that were present in the anterior electrode but not in the posterior electrode. This allowed us to examine if the portion of the DCMD axon over which NaN\textsubscript{3} was applied was changed by not being able to support the firing frequency coded for in the brain. Conduction delay was measured in terms of relative change from the initial recording to account for slight difference in electrode placement even though an effort was made to place the electrode in the same position within each locust.

2.5.2 Conduction Delay in an Intact Animal

One looming stimulus was presented every 5 minutes for the duration of the
experiments in order to obtain data. In all groups, conduction delay was measured using the first AP of the looming response.

2.5.3 Ca\textsuperscript{2+} Manipulations

The first AP of the first loom response was used to measure RMP, AP amplitude, AHP amplitude, AP half-width and AP rise slope (Figure 4C). Data for this section was expressed as relative change from the initial recording in order to better show the change over time between the treatment groups. The data for decreased Ca\textsuperscript{2+} manipulations were collected first, along with control data, and the three treatments were compared. The finding of a lack of Ca\textsuperscript{2+} contribution (see Results) led us to decide not to fully pursue high-Ca\textsuperscript{2+} manipulation experiments. However, a small sample size (n = 5) was collected, interspersed with control locusts, in order to complete the entire data set and to determine if it was worthwhile to pursue a more acceptable sample size. Comparisons were made between the control and high-Ca\textsuperscript{2+} groups with no observed effect of the increased extracellular Ca\textsuperscript{2+} concentration (see Results) and therefore no further data were collected. Also, because there was no difference between controls and high-Ca\textsuperscript{2+} group, no comparison was made between low-Ca\textsuperscript{2+} groups and the high-Ca\textsuperscript{2+} group. Furthermore, because of the lack of significant effects found in this section, no performance measurements were made on the data collected.

2.6 Statistics

Two-Way Repeated Measures ANOVAs (2-Way RM ANOVA) were conducted for all comparisons in this study using SigmaPlot 11.0 (San Jose, CA, USA) and had $\alpha = 0.05$. Where significances were found pairwise multiple comparison procedures were performed (Holm-Sidak method; $\alpha \leq 0.05$ depending on corrections for multiple tests).
CHAPTER 3: RESULTS

3.1 Performance Impairment

HS locusts showed a smaller and delayed response to a looming stimulus (Figure 5). This was evident in the analysis of TtC of the first AP, peak instantaneous frequency and spike count over 70 minutes. There was a significant difference between the four treatment groups in terms of when the DCMD loom response started (2-Way RM ANOVA, df = 3, p = 0.026) with the NHS/NaN3 response having a larger TtC of first AP than HS/NaN3 (Holm-Sidak method, p = 0.005; Figure 5B). There was also a trend for NHS/NaN3 to be significantly larger than HS/Con. but failed significance when corrected for multiple comparisons (Holm-Sidak method, α = 0.01, p = 0.018). There was no change in the timing of the first AP over the 70 minutes in any treatment (2-Way RM ANOVA, df = 12, p > 0.05). A similar relationship was found within the four treatment groups with respect to peak instantaneous frequency. There was a significant difference between the groups (2-Way RM ANOVA, df = 3, p = 0.014) with NHS/NaN3 reaching higher frequencies than HS/NaN3 (Holm-Sidak method, p = 0.004; Figure 5C). Trends toward significance occurred between NHS/Con. and HS/Azide (Holm-Sidak method, α = 0.01, p = 0.012), and NHS/NaN3 and HS/Con. (Holm-Sidak method, α = 0.013, p = 0.049) with the NHS/- groups reaching higher instantaneous frequencies. There was no change over time in the peak frequency reached in each loom response within each group (2-Way RM ANOVA, df = 12, p > 0.05). Spike count also showed a significant difference between the treatment groups (2-Way RM ANOVA, df = 3, p = 0.047; Figure 5D). The loom response of NHS/NaN3 had more APs than that of HS/NaN3 (Holm-Sidak method, p = 0.009). There was a trend toward NHS/NaN3 having a
Figure 5. HS animals have a delayed and smaller response to a looming stimulus. A) Example recording from the anterior suction electrode of a NHS/NaN$_3$ and a HS/NaN$_3$ locust. The bottom graph shows the expansion of the looming stimulus in the locust’s visual field over time. The grey line represents when the last frame of the stimulus occurred. B) TtC of the first spike in each loom response of the anterior suction electrode in the four treatments. HS/- animals had a lower TtC of first spike than NHS/- animals. C) Peak instantaneous frequency of the loom response in the anterior suction electrode in the four treatments. NHS/- locusts reached higher peak frequencies than HS/- locusts. D) Spike count of the loom response in the anterior suction electrode in the four treatments. HS groups had a lower spike count than control groups in response to the same stimulus, but all groups showed a decrease in spike count over time. In all graphs the 15 min. data points corresponds to the equivalent of 5 min. washout in standard locust saline; washout continued for 55 min. Error bars represent standard error.
higher spike count than HS/Con. (Holm-Sidak method, $\alpha = 0.01, p = 0.025$). In addition, spike count decreased for all groups over time (2-Way RM ANOVA, df = 12, $p < 0.001$; Figure 5D) with most early measurements being greater than the last three measurements (Holm-Sidak method, p-values < 0.001).

To determine the local effects of energy stress on DCMD performance the number of APs that failed to conduct through the mesothoracic ganglion was measured. There was no difference between the treatment groups or over time in the number of failed APs (2-Way RM ANOVA, df = 3, 12, p-values > 0.05; data not shown), with all mean values being less than 1.

There was no significant effect of the pre-treatment or NaN$_3$ application on conduction delay; all groups followed a statistically similar trend (2-Way RM ANOVA, df = 3, $p > 0.05$; Figure 6A). Relative conduction delay increased over time in all groups (2-Way RM ANOVA, df = 12, $p < 0.001$; Figure 6A), with most times before $t = 40$ being significantly faster than after $t = 40$ (Holm-Sidak method, p-values < 0.001). These results were duplicated in a data set without HS locusts (2-Way RM ANOVA, df = 1, $p > 0.05$; df = 11, $p < 0.001$; Figure 6B). In addition, despite a trend for NaN$_3$ groups to have faster conduction following NaN$_3$ application no significance was found (Holm-Sidak Method, $t = 20$: NHS/Con. vs. NHS/NaN$_3$, $\alpha = 0.009, p = 0.04$; HS/Con. vs. NHS/NaN$_3$, $\alpha = 0.01, p = 0.044$; $t = 25$: NHS/Con. vs. NHS/NaN$_3$, $\alpha = 0.01, p = 0.026$; HS/Con. vs. NHS/NaN$_3$, $\alpha = 0.009, p = 0.019$; $t = 30$: HS/Con. vs. NHS/NaN$_3$, $\alpha = 0.009, p = 0.03$; Figure 6A).

### 3.2 Conduction Delay in an Intact Animal

In order to determine why an increase in conduction delay was observed in the
Figure 6. Conduction delay increased equally over time in all treatments. Conduction delay normalized to the change from initial measurement. A) Data examining the effect of both pre-treatment and acute stress. Conduction delay increased over time for all groups equally. The 15 min. data points correspond to the equivalent of 5 min. washout in standard locust saline; washout continued for 55 min. B) Data from a duplicate data set examining only the effect of the acute stress. A similar relationship was found in this data set, in addition to observing that conduction delay increases the most during the first 15 min. of recording. The 10 min. data point in the NaN\textsubscript{3} plot corresponds to 5 min. of washout in standard locust saline; washout continued for 45 min. Error bars represent standard error.
performance impairment data set, a new data set was collected to control for the semi-intact preparation, the control being a ventral dissection, as well as to determine if the increase in conduction delay could be overcome with Compound C or glucose in the dorsal dissection. The observed increase in conduction delay in DCMD is due to the semi-intact preparation. A significant interaction was found between differences in the four treatment groups, control, Compound C, glucose and ventral, and time (2-Way RM ANOVA, df = 42, p ≤ 0.001) with the ventral group having a constant conduction delay (Holm-Sidak method, p-values ≥ 0.05) and the other three groups showing equal increases in conduction delay over time (Figure 7). The ventral group has significantly smaller changes in relative conduction delay compared to the control and glucose groups from t = 20 to t = 70 min. and compared to the Compound C group from t = 40 to t = 70 (Holm-Sidak method, p-values ≤ 0.005). The Compound C group also showed a significantly lower increase in conduction delay compared to controls at t = 70 (Holm-Sidak method, p = 0.006) and a trend towards significance at t = 65 (Holm-Sidak method, α = 0.017, p = 0.031).

3.3 Ca\textsuperscript{2+} Manipulations

Mean values for all measurements can be found in Table 1. For all graphical depictions, data are shown in terms of relative change from initial recordings.

Increases or decreases in extracellular Ca\textsuperscript{2+} concentration had little effect on AP characteristics (Figures 8 to 11). With regard to decreases in extracellular Ca\textsuperscript{2+} concentration, RMP changed over time (2-Way RM ANOVA, df = 2, p = 0.025), with a significant hyperpolarization upon the ‘exposure’ recording, followed by a partial recovery (Holm-Sidak method, p = 0.007 for initial vs. exposure; Figure 8A\textsubscript{1} and B).
Figure 7. Conduction delay remains constant with an intact preparation. Conduction delay normalized to the change from initial measurement. Data examining the effect of both glucose, Compound C and an intact preparation on conduction delay changes over time. Conduction delay increased over time for control, glucose and Compound C groups equally, but remained constant in the ventral preparation group. The blue arrows indicate when Compound C was added to the preparation. Error bars represent standard error.
Table 1. Absolute values for Ca\(^{2+}\) manipulations experiments. Data is expressed as mean ± standard error.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Phase</th>
<th>High-Ca(^{2+}) (n = 5)</th>
<th>Control (n = 12)</th>
<th>Nominally Zero-Ca(^{2+}) (n = 9)</th>
<th>EGTA (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP (mV)</td>
<td>Initial</td>
<td>-57.2 ± 3.3</td>
<td>-57.9 ± 2.1</td>
<td>-58.6 ± 1.8</td>
<td>-59.7 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>Exposure</td>
<td>-57.0 ± 2.7</td>
<td>-59.0 ± 2.9</td>
<td>-64.6 ± 2.4</td>
<td>-60.8 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Washout</td>
<td>-56.2 ± 2.8</td>
<td>-58.5 ± 3.4</td>
<td>-61.2 ± 2.2</td>
<td>-60.4 ± 1.4</td>
</tr>
<tr>
<td>AP Amplitude (mV)</td>
<td>Initial</td>
<td>92.1 ± 2.5</td>
<td>95.7 ± 1.7</td>
<td>97.8 ± 2.1</td>
<td>89.7 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>Exposure</td>
<td>94.2 ± 2.1</td>
<td>95.0 ± 1.5</td>
<td>97.6 ± 2.1</td>
<td>87.4 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>Washout</td>
<td>88.8 ± 3.2</td>
<td>90.4 ± 1.9</td>
<td>94.0 ± 2.1</td>
<td>91.8 ± 2.3</td>
</tr>
<tr>
<td>AHP Amplitude (mV)</td>
<td>Initial</td>
<td>-4.5 ± 0.9</td>
<td>-5.0 ± 0.5</td>
<td>-4.1 ± 0.3</td>
<td>-3.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Exposure</td>
<td>-3.9 ± 0.6</td>
<td>-3.9 ± 0.3</td>
<td>-4.8 ± 0.5</td>
<td>-3.9 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Washout</td>
<td>-4.2 ± 0.8</td>
<td>-3.9 ± 0.4</td>
<td>-4.4 ± 0.6</td>
<td>-4.1 ± 0.4</td>
</tr>
<tr>
<td>AP Rise Slope (mV/ms)</td>
<td>Initial</td>
<td>475.3 ± 51.2</td>
<td>463.1 ± 26.9</td>
<td>483.8 ± 41.6</td>
<td>410.0 ± 21.2</td>
</tr>
<tr>
<td></td>
<td>Exposure</td>
<td>433.0 ± 44.5</td>
<td>403.7 ± 18.1</td>
<td>468.4 ± 34.1</td>
<td>373.7 ± 29.4</td>
</tr>
<tr>
<td></td>
<td>Washout</td>
<td>302.1 ± 20.5</td>
<td>300.4 ± 11.8</td>
<td>323.4 ± 12.5</td>
<td>322.3 ± 17.9</td>
</tr>
<tr>
<td>AP Half-Width (ms)</td>
<td>Initial</td>
<td>0.336 ± 0.005</td>
<td>0.337 ± 0.008</td>
<td>0.359 ± 0.023</td>
<td>0.360 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>Exposure</td>
<td>0.339 ± 0.015</td>
<td>0.338 ± 0.009</td>
<td>0.382 ± 0.019</td>
<td>0.382 ± 0.015</td>
</tr>
<tr>
<td></td>
<td>Washout</td>
<td>0.325 ± 0.031</td>
<td>0.364 ± 0.010×10(^{-4})</td>
<td>0.374 ± 0.028</td>
<td>0.394 ± 0.019</td>
</tr>
</tbody>
</table>
Figure 8. Decreased extracellular Ca\textsuperscript{2+} has little impact on AP characteristics. A) Overlaid intracellular recordings illustrating the change in RMP (I) and amplitude (II) over time. B) The RMP hyperpolarized during the Exposure recording and partially recovered in the Washout recording for all groups, but was mostly caused by the nominally zero-Ca\textsuperscript{2+} (N-Ca\textsuperscript{2+}) group. C) There is an interaction between the treatment and time for the relative change in AP amplitude. Controls decreased over time, the nominally zero-Ca\textsuperscript{2+} group showed no change and the EGTA group decreased initially then increased passed initial levels. D) There were no differences in the relative change of the AHP amplitude in any treatment or over time. Error bars represent standard error. The letters and asterisk on the graphs represent where significances lie following ANOVAs and post hoc tests.
There was no significant difference in how the RMP changed for each group with decreases in extracellular \( \text{Ca}^{2+} \) concentrations (2-Way RM ANOVA, \( df = 2, \ p > 0.05 \)). However, the effect of time was driven by the nominally zero-\( \text{Ca}^{2+} \) group with that group alone being the only one to show significance over time (Holm-Sidak method, \( p = 0.002 \)) and there being a trend towards significance after corrections for multiple comparison of the nominally zero-\( \text{Ca}^{2+} \) group being more hyperpolarized compared to control (Holm-Sidak method, \( \alpha = 0.017, \ p = 0.018 \)) and EGTA (Holm-Sidak method, \( \alpha = 0.025, \ p = 0.033 \)) groups at the exposure recording. There were no differences between treatments (2-Way RM ANOVA, \( df = 2, \ p > 0.05 \)) or over time (2-Way RM ANOVA, \( df = 2, \ p > 0.05 \)) with changes to RMP when the extracellular \( \text{Ca}^{2+} \) concentration was increased (Figure 9A).

Changes in AP amplitude showed a significant interaction between treatment group and time when extracellular \( \text{Ca}^{2+} \) concentration was decreased (2-Way RM ANOVA, \( df = 4, \ p = 0.005 \); Figure 8C). Here, control locusts showed a significant decrease in amplitude during the final recording (Holm-Sidak method, \( p \)-values \( \leq 0.01 \)), whereas the EGTA group showed a decrease then an increase past initial levels for the second and third phases, respectively (Holm-Sidak method, \( p = 0.012 \) for exposure vs. washout). There were no changes over time in the nominally zero-\( \text{Ca}^{2+} \) group (Holm-Sidak method, \( p > 0.05 \)). In the washout phase, both control and nominally zero-\( \text{Ca}^{2+} \) groups had a significantly smaller AP amplitude than the EGTA group (Holm-Sidak method, \( p \)-values \( \leq 0.015 \)). The high-\( \text{Ca}^{2+} \) group followed the same trend as the control group for changes in AP amplitude over time (2-Way RM ANOVA, time: \( df = 2, \ p = 0.005 \); treatment: \( df = 1, \ p > 0.05 \); Figure 9B). There were no differences over time or
Figure 9. Increased extracellular Ca\textsuperscript{2+} has no impact on AP characteristics. A) There were no changes in RMP in either treatment over time. B) There was an overall decrease in the normalized AP amplitude during the Washout recording. C) There were no differences in the relative change of the AHP amplitude in the treatments over time. Error bars represent standard error. The letters on the graphs represent where significances lie following ANOVAs and post hoc tests.
between treatments in AHP amplitude with decreased (2-Way RM ANOVA, df = 2, p-values > 0.05; Figure 8D) or increased (2-Way RM ANOVA, df = 1, 2, p-values > 0.05; Figure 9C) extracellular Ca\(^{2+}\) concentration.

There was a significant decrease in the rising phase of DCMD’s AP over time in both the decreased (2-Way RM ANOVA, df = 2, p < 0.001; Holm-Sidak method, p-values ≤ 0.015; Figure 10A and B) and increased (2-Way RM ANOVA, df = 2, p > 0.001; Holm-Sidak method, p-values < 0.01; Figure 11A) extracellular Ca\(^{2+}\) concentration data sets, but no differences between treatments (2-Way RM ANOVA, df = 2, 1, p-values > 0.05). A significant increase in the AP half-width was also found over time when compared in the decreased extracellular Ca\(^{2+}\) concentration data set (2-Way RM ANOVA, df = 2, p = 0.006; Holm-Sidak method, p = 0.002; Figure 10C and D), with no difference between the treatment groups (2-Way RM ANOVA, df = 2, p > 0.05). When extracellular Ca\(^{2+}\) concentration was increased, no differences were found in AP half-width between the high-Ca\(^{2+}\) group and the control group or over time (2-Way RM ANOVA, df = 1, 2, p-values > 0.05; Figure 11B).
Figure 10. Decreased extracellular Ca\(^{2+}\) has no impact on the AP rising phase or half-width. A) Overlaid recording of APs illustrating the change in AP rise slope over time. B) The rising phase of the AP decreased over time equally in all groups: control, nominally zero-Ca\(^{2+}\) (N-Ca\(^{2+}\)) and EGTA. C) Overlaid recordings of APs illustrating the change in AP half-width over time. D) There was an increase in the AP half-width over time for all treatments. Error bars represent standard error. The letters on the graphs represent where significances lie following ANOVAs and post hoc tests.
Figure 11. Increased extracellular Ca\(^{2+}\) has no impact on the AP rising phase or half-width. A) There was a decrease in the AP rise slope over time for all treatments. B) There were no changes in the AP half-width over time for either treatment. Error bars represent standard error. The letters on the graphs represent where significances lie following ANOVAs and post hoc tests.
CHAPTER 4: DISCUSSION

Results show that HS locusts have a different DCMD loom response than control locusts. The response in HS locusts had fewer APs, reaching a lower peak frequency and starting closer to the estimated time of collision than the control locust response to the same looming stimulus (Figure 5). This indicates a decrease in performance in response to a moderate thermal energy stress. However, HS and control locusts showed no change in DCMD loom response following an extreme, acute energy stress of NaN₃ application. There were no additional APs failing to travel through the mesothoracic ganglion in locusts exposed to NaN₃ compared to locusts continually exposed to standard locust saline.

Interestingly, there were no differences found between treatments when examining changes in conduction delay through the mesothoracic ganglion following energy stresses; however, all four groups showed an increase in conduction delay over time, representing a decrease in conduction velocity (Figure 6). In addition, in another data set AP half-width increased and the rising phase of the AP decreased over time, which may contribute to the slowing of conduction.

Other measurements of AP characteristics and how they change with extracellular Ca²⁺ concentration manipulations, as observed with AP rise slope and half-width, showed changes over time, but no consistent trend between treatments. The RMP hyperpolarized during the exposure phase, and was followed by a partial recovery. AP amplitude decreased over time in control locusts; in the EGTA group the amplitude was greatest in the washout phase of the experiment, and in the nominally zero-Ca²⁺ group no change was observed. The AHP amplitude showed no difference over time or
between conditions.

Despite our results not following what we expected in some cases, they can still be interpreted to indicate a plastic nervous system that adjusts energy consumption to suit environmental conditions in a trade-off with performance.

4.1 Trade-Off Theory

4.1.1 Pre-Treatment

There was a difference observed between HS/- and NHS/- groups, with HS/- groups tending to have delayed and smaller responses to a looming stimulus (Figure 5). This means that HS locusts fired fewer APs and did not reach as high a peak frequency as control locusts did. Typically, experiments examining changes induced by a pre-treatment focus on the changes in the ability of the locust to survive subsequent normally lethal stresses, with pre-treated locusts being able to maintain neuronal function longer, recover from neuronal shut down sooner and have a higher probability of survival (Robertson et al. 1996; Gray and Robertson 1998; Wu et al. 2002; Newman et al. 2003; Money et al. 2005; 2006; Armstrong et al. 2006). However, some studies have looked at pre-treatment with HS and how it influences AP firing in DCMD. In these studies it was found that at 25°C there were no differences between HS and control locusts in terms of spike count, firing frequency and TtC of first AP (Money et al. 2005; 2006). The discrepancies between the present and previous studies may arise because in previous studies measurements were taking during a temperature ramp to failure, whereas the present study had a constant temperature and simply measured changes over time. Since previous studies did not make multiple recordings at one temperature over time they would not be able to find the difference observed in the current study. Our
study allowed for a trend in differences to be found over time even though at certain
time points the different groups may have been similar in a measurement due to variance
in the animals (for example see t = 70 min. with peak frequency measurements; Figure
5C). In addition, a different looming stimulus was utilized in each of the studies, with
the present study having a larger final subtended angle for the stimulus (Figure 3D).
Since DCMD tracks the stimulus as it simulates approach by increasing its activity
(Gabbiani et al. 1999), DCMD fired more in the current study than in the previous ones.
This could draw out differences by having more activity to examine.

The changes observed in the present study suggest that the HS locusts are saving
energy compared to control locusts. Since fewer APs are being generated, ionic
gradients do not have to be re-established as often, thus saving energy in DCMD. In
addition, higher performance in neurons is associated with increased energy usage
(Weckström and Laughlin 1995; Niven et al. 2007) and the ability to fire at high
frequencies is a form of higher performance. Since more currents would be involved in
the AP in order to reset the components for the next AP, more energy is subsequently
required. An example of this is the ADP in DCMD APs (Money et al. 2005), which
could be caused by additional Cl\textsuperscript{-} flux across the membrane. This current would require
more energy for regenerating its gradient, and thus the ability to fire APs in rapid
succession, as observed in DCMD with firing over 600 Hz (Money et al. 2006), can be
energetically expensive. The observed decrease in peak firing frequency in HS locusts
may indicate the loss or shut down of an ionic current, which saves DCMD energy not
only by firing fewer APs, but also by having fewer currents.

These changes can be linked to better survival in DCMD function upon exposure
to normally lethal stresses (Robertson et al. 1996; Gray and Robertson 1998; Money et al. 2005; 2006). With exposure to a temperature ramp to failure following HS pre-treatment, HS locusts maintain function and do so without as much degradation of the response compared to control locusts (Gray and Robertson 1998; Money et al. 2005; 2006). Maintenance of DCMD function can be achieved because fewer APs are generated for example, and thus energy is saved, allowing for the saved energy to possibly be utilized in other aspects of the HS response to enable better function and survival at high temperatures; whereas control locusts would not have this saved energy for redistribution, leading to a deterioration of the DCMD response and earlier failure in activity than HS locusts (Gray and Robertson 1998; Money et al. 2005; 2006). Having this ‘free’ energy could also account for the quicker recovery in HS locusts and their higher probability of survival because the energy could be used in some aspect of the HS response to help the system prepare for recovery and overcome the stress.

The ability to maintain function comes at a price to the locust however, and that price is a decrease in performance of DCMD. The decrease in the number of APs fired during a loom response indicates that not as much information can be coded for by the locusts. For example, in terms of the TtC of the first AP, since HS locusts signal detection of the looming stimulus later in its approach compared to control locusts, HS locusts will likely have less time to evade the object in nature. This may not be a large issue for DCMD however, since during flight it has been shown that DCMD is linked to a last-ditch gliding behaviour to avoid predators if steering manoeuvres could not remove the stimuli from the visual field (Gray et al. 2001; Santer et al. 2005). Losing the first AP in the loom response could still limit the tracking of the object in DCMD
and decrease the time of preparation for the last chance behaviour. Furthermore, if this change can be translated to other neurons, such as those that are involved in flight manoeuvring, it could cause a greater chance of predation on the locust because manoeuvres would be initiated later in the approach. In addition, firing frequency was previously discussed in terms of performance level. With a decreased ability to fire at high frequencies, energy is saved, however, performance is lost. Not as much information is able to be coded for in DCMD for the tracking of the looming objects, which could lead to changes in behaviour. The trade-off of energy for performance observed here equals a better chance to survive the imminent threat of a lethal energy crisis for the chance of being less able to detect and evade predators.

Therefore, in terms of pre-treatment and gradual minor stresses that can induce long-term changes in neuronal function there seems to be an ability to adjust performance and energy utilization in order to reserve energy for future functioning. The locust CNS, at least in DCMD, has the ability to reconfigure the functional design of neuronal signalling in response to environmental cues with respect to energy/information processing trade-offs in the long term.

4.1.2 Acute Stress

There were no differences in AP conduction caused by the application of NaN$_3$. For TtC of first AP, peak instantaneous frequency and spike count this was expected since these characteristics are determined in the brain (O’Shea and Williams 1974; Rind 1984) and NaN$_3$ was not applied there. However, the number of AP failures through the mesothoracic ganglion and AP conduction delay could be affected locally on the DCMD axon and were expected to change with NaN$_3$ application. It was thought that the
number of failures would increase following NaN$_3$ application because the axon would undergo changes that would not allow it to support high frequency firing that axonal areas not affected by NaN$_3$ would normally be able to. Additionally, it was expected that conduction delay would increase following NaN$_3$ application, but no significant differences were found, indicating no effect of NaN$_3$. However, a trend was observed in the conduction delay data where the NaN$_3$ group at t = 15 returned to delay levels similar to control groups at t = 5 (Figure 6B). It seems as though at t = 10 or 15 (Figure 6B and A, respectively) the DCMD axon was recovering from NaN$_3$ induced failure, but not yet fully recovered causing delay to be higher. Following a full recovery these locusts were ‘reset’ to control levels at t = 5 (Figure 6B). This could indicate that NaN$_3$ simply shut down activity and, upon recovery, returned activity to the same level as before failure, avoiding the effect of time because no activity was induced to drain energy stores. This leads to one possible reason for why no long-term effects of NaN$_3$ application were observed; NaN$_3$ may act too quickly in shutting down the system and ‘freeze it in time’ so that no changes can be induced by the neuron.

Overall, there was no effect caused by NaN$_3$ on DCMD performance. A possible explanation for this is that NaN$_3$ is too rapid in shutting down neuronal activity, as stated above, thus not providing sufficient time for changes to be undertaken. In research using a temperature ramp to failure, changes in AP characteristics are observed that are maintained following recovery (Robertson unpublished; Figure 1) indicating that prolonged changes do occur that could affect performance. With a temperature ramp the locusts are gradually exposed to higher temperatures over five minutes; however, with NaN$_3$ exposure activity generally ceases within two minutes with no gradual effect. The
rapid shut down of activity in a cell could prevent changes from being induced that are required for the switch to an energy conservation mode. The fact that activity returns to near pre-exposure levels indicates that no damage was incurred by DCMD, and could thus signify that a modification of the axon was omitted. Therefore, the possible presence of a trade-off mechanism in response to acute stressors is still viable. In order to confirm the presence of the trade-off, another stressor other than NaN$_3$ may have to be used to give sufficient time to induce the proposed trade-off.

4.1.3 Experimental Considerations

It was found that conduction delay increased throughout the experiments regardless of the treatment, indicating that DCMD was changing throughout the experiments, which leads to another possible reason why NaN$_3$ had no observable effect. The increase in conduction delay could be related to the decrease in the rising phase of the AP and increase in the AP half-width observed in all groups of the Ca$^{2+}$ manipulation data set. The change in half-width is questionable as to whether it is biologically relevant since the change is so small (Figure 10C), but the decrease in the AP rise slope is applicable (Figure 10A). The change in the rising phase of the AP can be related to the slowing of APs since it would take longer for adjacent regions of the axon to reach threshold with a shallower sloped AP. We believe that this increase in conduction delay may be related to a decrease in energy supplies and deteriorating preparation over time because some of the trachea are severed and hemolymph washed out, limiting the supply of oxygen and nutrients. The dissection will prevent certain organs and tissues in the locust from resupplying their energy stores. With a decrease in energy supply, DCMD could be undergoing a shift to a new energy utilization mode in
terms of restoring ionic gradients even within control locusts. If DCMD is already undergoing a shift in energy consumption before the application of NaN$_3$, it is possible that a switch that would normally be caused by NaN$_3$ would be masked. This could account for the lack of significant findings surrounding NaN$_3$ application.

Previous studies have not reported any deterioration of signals over time with this preparation (Robertson and Pearson 1982; Gabbiani et al. 1999; Money et al. 2005; 2006). However, we show, through an increase in conduction delay, that the preparation is changing over time, most likely related to a decrease in nutrient and energy supplies caused by the semi-intact preparation. Locusts that were left intact during experiments did not show a change in conduction delay over time (Figure 7), indicating that the disruption caused by the semi-intact preparation accounts for the change. The ventral dissection maintains both the hemolymph and trachea system, allowing for the maintenance of the nutrient and energy supply to the CNS and, therefore, neuronal function to remain the same.

To determine if this could be overcome in the semi-intact preparation, locusts were exposed to a glucose saline or a saline containing Compound C. Glucose was used to replenish nutrient supplies in the locusts to attempt to fill the role of the hemolymph. Compound C was used to block the stress response pathway and determine if the increased conduction delay could be quickly reversed. Compound C is a chemical that acts on AMPK in an inhibitory fashion. AMPK is activated when there are high cellular ratios of AMP:ATP, indicating low energy availability (Hardie and Carling 1997; Jibb and Richards 2008). Thus, Compound C works to shut down a the energy stress response of the cell and return cellular functioning to levels similar to when resources
did not limit functioning. However, both of these groups showed no difference from control locusts and had an increasing conduction delay over time. Since this is the first time the degradation of the preparation has been examined to the authors’ knowledge, it is possible that the dose of glucose and Compound C were too low to cause measurable effects. A trend can be observed with Compound C treatment slowing the increase in conduction delay and a higher concentration may be able to exaggerate the effect. In the future a dose response should be generated in order to determine if these compounds could be used to overcome preparation deterioration. Alternatively, for Compound C, the chemical may act on a slower time scale than the experiment could allow for since the Compound C group showed a faster conduction velocity than controls after 40 minutes exposure to Compound C. Future work could examine if there is an increase in conduction delay and, if present, its extent with constant exposure to Compound C, similar to the current experiments with glucose.

4.2 Ca$^{2+}$ Contribution to DCMD AP

The current study was not conclusive on the contribution, or lack thereof, of Ca$^{2+}$ in the DCMD AP. Upon examining AP characteristics, no significant differences between conditions with respect to RMP, AHP amplitude, AP rise slope and AP half-width were found (Figures 8 to 11). However, the change in AP amplitude over time depended on what treatment was used (Figure 8C) when examining a decrease in extracellular Ca$^{2+}$ concentration, and it seems as though the nominally zero-Ca$^{2+}$ treatment group was driving the observed change in RMP over time (Figure 8B). These later findings lead us to be unable to conclusively state that there is no Ca$^{2+}$ component in the DCMD AP. In addition, many other neurons show Ca$^{2+}$ components in their APs
in locusts and other insects (Pearson et al. 1993; Wicher and Penzlin 1997; Höger et al. 2005; Heidel and Pflüger 2006). Thus, it is still possible that DCMD has a Ca\(^{2+}\) component. If this is the case, a potential explanation for the lack of findings in the current study is that the Ca\(^{2+}\) channels allowed Na\(^+\) to flow across the membrane because little or no Ca\(^{2+}\) was in the extracellular fluid (Hess et al. 1986). To avoid this confound in the future, Ca\(^{2+}\) channels could be blocked using ions, such as Ni\(^{2+}\) or Cd\(^{2+}\), known to inhibit voltage-gated Ca\(^{2+}\) channels in insects (Pearson et al. 1993; Höger et al. 2005; Heidel and Pflüger 2006) instead of decreasing the extracellular concentration of Ca\(^{2+}\).

The lack of findings with increased extracellular Ca\(^{2+}\) concentration may be due to the increase not being enough to significantly affect the electrochemical gradient for Ca\(^{2+}\). Little literature could be found on the effects of increased extracellular Ca\(^{2+}\) on insect neuronal APs to help determine how much to increase the extracellular Ca\(^{2+}\) concentration in the current work. Most literature that was found worked largely by stopping Ca\(^{2+}\) flux with channel blockers or decreased Ca\(^{2+}\) concentration and chelators. Manipulations in vertebrate nervous systems were also found, however insect voltage-gated Ca\(^{2+}\) currents are different than typically found in vertebrates, having thresholds between -50 mV and -30 mV, and peaking between -10 mV and +10 mV, as well as having different pharmacological influences (Pearson et al. 1993; Wicher and Penzlin 1997). Therefore, since little guidance could be found, we attempted to double the Ca\(^{2+}\) concentration as a starting point for this work. One study did find that an incremental increase in extracellular Ca\(^{2+}\) concentration from 0 mM to 2 mM to 5 mM did incrementally increase the maximum conductance of the Ca\(^{2+}\) current of cultured DUM neurons from the cockroach (Defaix and Lapied 2005), indicating that a doubling of the
concentration may show effects. However, no change in AP characteristics was observed in the current study with increased extracellular Ca\textsuperscript{2+} concentration, possibly because the presumed increase in conductance was not enough to significantly affect DCMDs AP over its short duration.

Future work could generate a dose response curve for increased extracellular Ca\textsuperscript{2+} in order to determine when an effect would be found, if Ca\textsuperscript{2+} channel blockage is found to change the DCMD AP. Alternatively, the lack of findings may be explained by Ca\textsuperscript{2+} channels not being present in DCMD, although, we do not believe this to be true given the experiments with decreased extracellular Ca\textsuperscript{2+} concentrations and theories surrounding those findings.

Since the current study is not conclusive in support of a role for Ca\textsuperscript{2+} in the DCMD AP, it still remains to be shown if manipulation of Ca\textsuperscript{2+} currents in DCMD can account for changes in AP characteristics following energy stressors (e.g. Figure 1). More research needs to be conducted with Ca\textsuperscript{2+} channel blockers to attempt to mimic the observed changes after energy crises and, furthermore, to determine if those Ca\textsuperscript{2+} current manipulations modulate the performance in DCMD.

4.3 Conclusions

Experiments investigating performance changes following pre-treatment show that performance is decreased in HS animals. It is possible that this change is part of a dynamic equilibrium in locust neurons that allow them to be plastic in their performance and energy consumption depending on if resources are limiting or not. If this is true, nervous systems are able to limit their use of energy in a trade-off with performance in order to conserve or redistribute energy in case the organism encounters future energy
stresses. This phenomenon was not found following an acute stress of anoxia induced by NaN\textsubscript{3}; however, NaN\textsubscript{3} may act too quickly to allow the switching mechanisms for energy conservation to take place. Alternatively, the switch may have already occurred for acute stressors due to the locust encountering an energy stress as a side effect from the dissection. Therefore, it is still possible that these adaptations can occur following an extreme acute energy stress.

The mechanism of action for the changes in performance remain unknown since the Ca\textsuperscript{2+} manipulation experiments showed no significant differences between groups. Ca\textsuperscript{2+} flux is still a possibility for accounting for the observed and proposed changes, and additional experiments need to be conducted in order to determine calcium’s role in DCMD and the potential trade-off mechanism. Future work to address this should be experiments with Ca\textsuperscript{2+} channel blockers and, if the proposed AP characteristic changes occur, whether these changes impair performance.

Future work also needs to examine acute stresses. Some possible models to replace NaN\textsubscript{3} application are temperature ramps to failure, a proven working model (Klose \textit{et al.} 2004; Money \textit{et al.} 2005; 2006; Armstrong \textit{et al.} 2006; Rodgers \textit{et al.} 2007), or Compound C and AICAR, an activator of AMPK, experiments to directly interact with the energy stress response. With these manipulations performance can be examined in order to start to understand if the energy/performance trade-off theory is also applicable in response to an acute stress. In addition, the impact of the preparation on conduction velocity needs to be overcome to help determine a proper model to look at the effects of acute stressors. When a more thorough understanding has been reached with the proposed work, energy consumption models can be created for the DCMD AP
similar to models in other neurons (Aiello and Bach-y-Rita 2000; Sangrey et al. 2004; Crotty et al. 2006). After this, an understanding of the possible amount of energy saved by the DCMD neuron can be known and related to the performance impairments to be applied to the theory.

Understanding this phenomenon in locusts and their robust ability to survive extreme environmental energy stresses can build on our understanding of many disorders of the nervous system that disrupt nutrient and energy supplies. Some instances where this knowledge can help are in migraine, seizure and stroke research, since spreading depression has been linked to these events (Wu and Fisher 2000; Smith et al. 2006) and occurs in both mammals and locusts during extreme energy stresses (Rodgers et al. 2007). Understanding how the locust CNS is able to overcome and survive these stressors could help in understanding how mammalian nervous systems may be able to do the same.


APPENDIX 1: ABBREVIATIONS

2-Way RM ANOVA – 2-Way Repeated Measures ANOVA.
ADP – after-depolarization.
AHP – after-hyperpolarization.
AMPK – AMP-activated protein kinase.
AP – action potential.
CNS – central nervous system.
DCMD – descending contralateral movement detector.
DUM – dorsal unpaired medial.
HS – heat shock.
NaN₃ – sodium azide.
OA – octopamine.
RMP – resting membrane potential.
TtC – time to collision.