Effects of Heatshock on the Na^+/K^+-ATPase in *Locusta migratoria*

by

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Abstract

Most vertebrates suffer permanent damage after minutes of anoxia. Many insects however, have part of their life cycle in anoxia or constant hypoxia, such as during their egg-hatching phase, by living as deep burrowers, or at high altitudes. Insects are able to survive in anoxia from hours to days, or even months by developing various strategies through evolution. For example, the locusts (*Locusta migratoria*) enter a reversible coma during anoxia that is associated with an arrest of ventilation, and a reinitiation of ventilation when returned to normoxia. This coma is correlated with a surge in the concentration of extracellular potassium ions ($[K^+]_o$), and recovery from this reversible coma is dependent on re-establishing the functional $[K^+]_o$. Prior exposure to a sublethal heatshock (HS)-preconditioning grants locusts a temporary resilience to anoxia; however, the molecular mechanisms of this protection are still unclear.

This project investigated the effects of HS-preconditioning on locusts' ventilation, the total enzymatic activity of the Na^+/K^+-ATPase, as well as its distribution within the metathoracic ganglion and tested the hypothesis that HS-preconditioning alters locusts' ventilation and increases the totally Na^+/K^+-ATPase activity and its concentration within neuronal membranes. I recorded electromyograms of locusts' ventilatory motor patterns in the presence and absence of anoxic coma by placing a copper wire electrode on ventilatory muscles 161 or 173 in control and HS-preconditioned animals. In addition, I studied the enzymatic activity of the Na^+/K^+-ATPase using a pyruvate kinase/lactate dehydrogenase assay and the localization of the Na^+/K^+-ATPase using immunohistochemistry in control and HS-preconditioned locusts at different stages of coma. I found that the ventilatory cycle period was decreased and the ventilatory muscle burst duration was increased after recovery from anoxic coma in HS-preconditioned locusts. I also found that anoxia did not affect the activity or the localization of the Na^+/K^+-ATPase. However, HS-preconditioning increased the total activity of the Na^+/K^+-ATPase and the localization of the Na^+/K^+-ATPase within the neuronal membranes. From this project, I concluded that HS-preconditioning affected locusts' ventilatory motor pattern after recover from anoxia and increased the total activity and the neuronal membrane localization of the Na^+/K^+-ATPase.

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Table of Contents

List of Abbreviations

$[K^+]_o$	Extracellular potassium concentration
HS	Heatshock
NSERC	Natural Sciences and Engineering Research Council of Canada
EMG	Electromyography
CON	Control
Hsp	Heatshock proteins
PN	Perineurium
GL	Glial cell layer
CBG	Groups of cell bodies
JH	Juvenile hormone
E.R.	Endoplasmic reticulum
AQP 2	Aquaporin 2
AKAPs	Protein kinase A-anchoring proteins
PEP	Phosphoenolpyruvic acid
LDH	Lactate dehydrogenase
PMSF	Phenylmethanesulfonylfluoride
PK	Pyruvate kinase
TBST	Tris-Buffered-Saline-Triton® X-100
NGS	Normal goat serum
FITC	Fluorescein isothiocyanate
DAPI	4’,6-diamidino-2-phenylindole
FRET	Fluorescence resonance energy transfer

List of Figures

Chapter 1: Introduction and Literature Review

'Weather is a great metaphor for life – sometimes it's good, sometimes it's bad, and there's nothing much you can do about it but carry an umbrella' –Terri Guillemets.

Organisms thrive in favourable conditions but they also have to endure harsh environmental stresses. Through evolution, animals have adapted to the niches they occupy and they have also developed mechanisms to cope with bad weather and abiotic stresses. Some vertebrates hibernate to overcome freezing temperatures and limited food resources in the winter, some insects change their body orientation towards the sun to adjust their body temperature, and some amphibians can completely stop their metabolism and freeze during winter. Behavioural changes to overcome elemental stresses have been well described; however, there is still much to learn about the organisms' underlying biochemical mechanisms in coping with unfavourable conditions. In addition, it is well established that previous abiotic stress can affect the ability of an organism to cope with subsequent stresses. Once again, the biochemical mechanisms that underlie the preconditioning effects of a prior abiotic stress have on an animal are still unclear. Furthermore, there is still lack of knowledge on the results of having experienced a prior abiotic stress in terms of the organism's longevity after surviving the sequential stresses.

In this study, I investigated the effects of a heat stress (heatshock, HS) on the ventilatory motor pattern generation in locusts, as well as the effects of HS-preconditioning on the Na^+/K^+-ATPase in the metathoracic ganglion of locusts.

1.1 General background

The stress-induced arrest of ventilatory motor pattern generation in locusts is tightly correlated with an abrupt increase in the extracellular potassium concentration ($[K^+]_o$) in the metathoracic ganglion (Figure 1). The arrest of ventilation can be triggered by several metabolic stressors, such as anoxia, high temperature, and ATP-depletion (Rodgers *et al.*, 2007). These metabolic stressors compromise the activity of the Na^+/K^+-ATPase and result in accumulation of extracellular $[K^+]$.

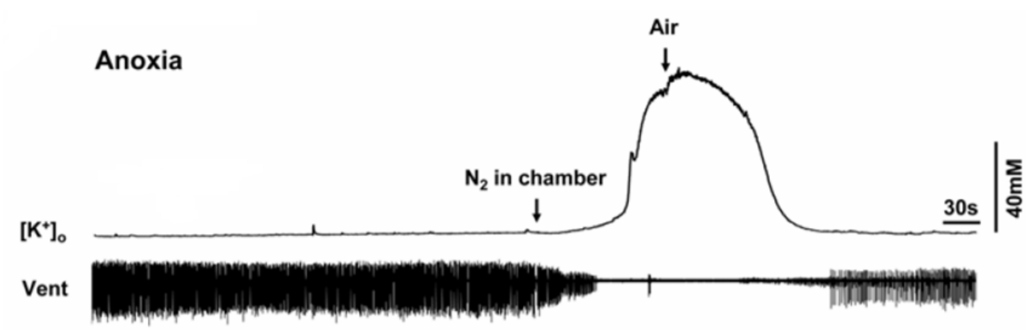

Figure 1: Effect of anoxia on ventilatory motor pattern generation. Top trace ($[K^+]_o$) - extracellular potassium concentration in a locust's metathoracic ganglion obtained using a potassium-sensitive electrode. Bottom trace (Vent) – electromyogram (EMG) recording of ventilatory motor activity. During anoxic stress (N_2 in chamber) motor patterning ceases. The arrest in the motor pattern is correlated with a surge of $[K^+]_o$ in the metathoracic ganglion. When oxygen becomes available again (Air), motor patterning recovers coincident with recovery of the baseline $[K^+]_o$ (From Rodgers *et al.*, 2007. Reprinted with permission).

Once the stressor is removed, these animals are able to restore their normal $[K^+]_o$, and recover their ventilatory motor patterning. Notably the arrest and recovery in neuronal function of the locust during stress shares the hallmarks of cortical spreading depression, which has been associated with pathologies such as migraine, stroke and epilepsy in mammals.

Exposure to a sublethal HS-pretreatment at 45 ^{o}C for 3 hours grants locusts a temporary resilience to spreading depression-like events induced during hyperthermia via a mechanism where HS-pretreatment delays the time to succumb and increases the rate of recovery from anoxia (Rodgers *et al.*, 2007). These changes result from a decreased rate of $[K^+]_o$ accumulation during and an increased rate of clearance following the exposure to the stress (Figure 2) which allowed HS-preconditioned locusts to maintain the proper $[K^+]_o$ longer and restore it faster after a $[K^+]_o$ disturbance. Consequently, HS-preconditioned locusts have a higher tolerance to hyperthermia and anoxia than control locusts.

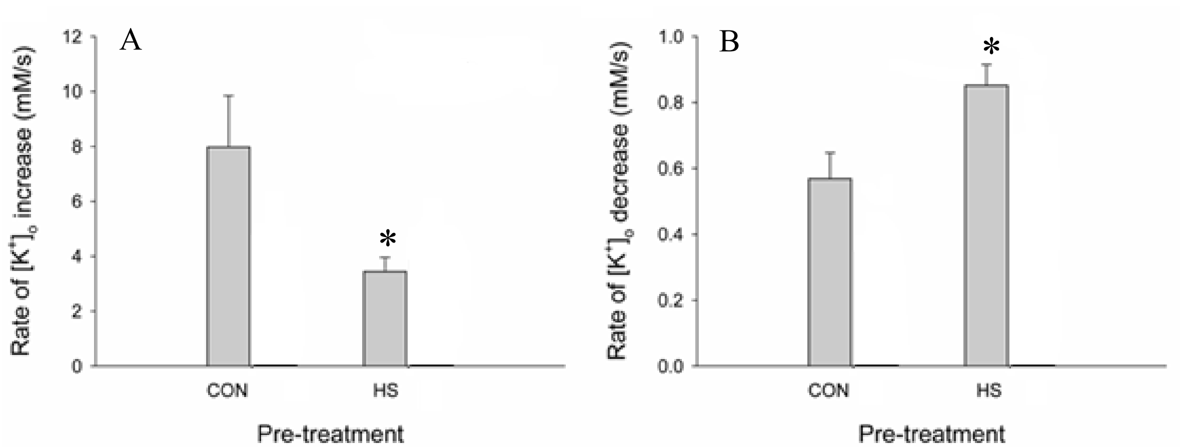

Figure 2: Effects of HS on $[K^+]_o$ dynamics during hyperthermic coma. A: HS-preconditioned animals (HS) were able to decrease the accumulation of $[K^+]_o$ in their metathoracic ganglion during hyperthermia compared with control animals (CON). B: Prior HS also increased the rate of $[K^+]_o$ clearance (From Rodgers *et al.*, 2007. Reprinted with permission).

It has been speculated that the Na^+/K^+-ATPase plays a role in this HS-induced thermal tolerance because ouabain, a Na^+/K^+-ATPase specific inhibitor, was able to remove this thermal protection. Thus, to elucidate the molecular mechanisms of the Na^+/K^+-ATPase underlying this thermal protection, Rodgers *et al.* measured the enzymatic activity of the ATPase and neuronal ATP levels in the metathoracic ganglion. However, they reported no significant difference in the total Na^+/K^+-ATPase activity and no significant difference in the neuronal ATP concentration between the control and HS-preconditioned groups (Rodgers *et al.*, 2007). This implied that the lower $[K^+]_o$ clearance rate observed in control locusts was not due to a lower Na^+/K^+-ATPase activity, or a lower concentration of ATP which could potentially limit the activity of the Na^+/K^+-ATPase and hinder the re-establishment of the $[K^+]_o$. However, a significantly lower ATP level was observed during anoxia in both control and HS-preconditioned animals compared to pre- and post-coma (Rodgers *et al.*, 2007). This suggests that the Na^+/K^+-ATPase could be affected during the coma to cause an accumulation of $[K^+]_o$. Nevertheless, there was no difference in the ATP levels between control and HS-preconditioned locusts at same stages of coma.

Thus, even though HS-preconditioning was able to induce an increase in the rate of $[K^+]_o$ clearance which resulted in thermal tolerance in locusts, the apparent increased rate was not due to an increase in the total Na^+/K^+-ATPase activity or a higher concentration of ATP in the metathoracic ganglion (Rodgers *et al.*, 2007). Consequently, the molecular mechanism underlying increased clearance of $[K^+]_o$ remains unclear.

1.2 Heatshock response

The heatshock (HS) response is a universal response that has been observed in every organism both *in vivo* and *in vitro* (Lindquist, 1986), and is characterized by increased transcription of a suite of chaperone proteins in response to thermal stress. For example, the HS response in fruit flies, *Drosophila melanogaster*, can be observed when the temperature has been elevated by 4 to

13 ^{o}C, and at the optimal range, heat-shock mRNAs are produced within few minutes and several thousand transcripts will be produced within an hour (Lindquist, 1980).

The HS response induces an increase in the production of a group of proteins called the HS proteins. Even though the concentrations of HS proteins are significantly increased during a HS response, their concentrations in unstressed cells are normally already higher than other proteins. The sequence of HS proteins are also highly conserved across kingdoms, even many of the differences in the genetic sequences that do occur, especially in the eukaryotes, are simply just conserved amino acid substitutions, for example, arginine – lysine, aspartic acid – glutamic acid, *etc* (Lindquist, 1986). HS proteins are demonstrated to be multi-purpose proteins such as their involvement in chaperone systems with other proteins to minimize the protein denaturation under abiotic stress. The role of HS proteins play in protein chaperone systems have been well documented, however, some of the detailed mechanisms by which HS proteins minimize denaturation and the role they play in restoration of other proteins' function are still to be discovered.

Using one of the most conserved heatshock proteins, the heatshock protein 70 (Hsp 70) as an example, the role of this protein in the folding of non-native proteins can be categorized into three related activities: prevention of aggregation, promotion of folding to the native state, and solubilisation and refolding of aggregated proteins. Of the three activities, the solubilisation and the refolding of aggregated proteins has been studied the most, and it includes the folding and assembly of newly synthesized proteins (Bukau *et al.*, 2000), refolding of incorrectly folded and aggregated proteins (Hartl and Hayer-Hartl, 2002), membrane translocation of proteins (Young *et al.*, 2003; Ryan and Pfanner, 2002; Pratt and Toft, 2003), and controlling the activity of regulator proteins (Neupert and Brunner, 2002).

Hsp 70 together with its co-chaperones of the J-domain protein family prevents the aggregation of non-native proteins by associating with the hydrophobic patches of the substrate proteins, protecting them from intermolecular interactions (Mayer and Bukau, 2005). In addition, it has been found that Hsp 70 can promote protein folding to the native state through two mechanisms. First, through repetitive substrate binding and release cycles, Hsp 70 can keep the free concentration of the substrate low enough to prevent aggregation while allowing free molecules to refold to the native state. In the second mechanism, the binding and release cycles initiate local unfolding in the non-native protein, helping the substrate to overcome the energetic barrier for refolding to the native state. However, the mechanism by which the Hsp 70 chaperone system aides in non-native folding intermediates to refold back to the native state remains unclear (Mayer and Bukau, 2005).

In summary, even though the rapid effects of the HS response in organisms, the conservation of the HS protein sequences, and the functions of HS proteins have been well investigated, there is still much to learn about the detailed mechanisms of how HS proteins chaperone protein structures and assist in the folding of non-native proteins back to their native state.

1.3 Adaptations to anoxia

The periodic anoxia or severe hypoxias such as during seasonal rainstorms, during floods or snowmelts, or at high altitudes are threats that insects would often face. Unlike mammals where merely minutes of anoxia can leave permanent damage on the organism, insects can survive in anoxic environments for hours to days, or even longer. Several theories have been proposed as to why insects have such an extraordinary ability to survive anoxia. One suggests that since many species of insects involve burrowing in some stage of their life, such as during their egg-hatching phase, or are simply deep burrowers, hypoxia and hypercapnia were strong selective forces in their evolution. Such selections maybe have focused on discontinuous gas exchange, which decreased the oxygen demand amongst arthropods (Lighton, 1998). In addition, those insects that live under hypoxia, such as high altitude insects, exhibit morphological changes, such as reduced wings, decreased flight capability, and smaller body size, which allowed those insects to reduce their oxygen requirements (Mani, 1968).

Studies have also shown that insects have much more versatile and robust primary metabolic requirements that allowed them to depress their metabolism (Hochachka *et al.*, 1993) or to enter a reversible coma state where they arrest their ventilation as observed in *Locusta migratoria* (Rodgers *et al,.* 2007), and a much more effective secondary metabolism which allowed them switch to anaerobic pathways during times of anoxia while still continuing the essential to metabolic activities (Wegener, 1993). More specifically, it has been demonstrated that *Cicindela togata* larvae are able to reduce their metabolic rates by 97% and have their anaerobic metabolic pathways to support the reduced metabolism (Hoback *et al.*, 2000). However, in other insects, such as the desert locust *Schistocerca gregaria* which can survive up to 8 hours of anoxia, the anoxia-induced metabolic depression is only about 6% relative to controls (Hochachka *et al.*, 1993; Wegener, 1993). In such insects, rather than relying mostly on reducing their metabolic demands, they have developed another pathway which allowed them to further harvest energy from one of the by-products of ATP hydrolysis, AMP. It has been illustrated in the flight muscle of *Locusta migratoria*, AMP as the main product from adenine nucleotide metabolism, increased by more than 20-fold. Nevertheless, some of the AMP was further deaminated to IMP and was again dephosphorylated to inosine, which in the process produced NADH. Consequently, after 3 hours of anoxia, less than 30% of the total adenine nucleotides were degraded (Weyel and Wegener, 1996). As a result, not only did this mechanism allow locusts to survive anoxia, this mechanism might have also contributed to the astonishing ability of locusts to recover from prolonged anoxia.

In conclusion, the ability of insects to decrease their metabolic demand, and recruit other pathways to harvest energy from adenine nucleotides allows them to not only survive but also thrive in ecological niches where they face daily hypoxia or anoxia.

1.4 Locusts

To study the HS-induced abiotic stress tolerance, I used adult male gregarious *Locusta migratoria migratorioides* due to their substantially larger central nervous system compared to many other insects such as fruit flies, *Drosophila melanogaster*. In addition, when comparing *L. migratoria* to mammals such as laboratory rats, the life cycle of locusts are significantly shorter and locusts are also much easier to raise. Furthermore, the central nervous system of *L. migratoria* has been well studied and classified, and their stress responses have been described.

The locusts used in my project were raised in crowded colonies prior to my experiments. To eliminate variation in thermal tolerance due to sex differences, only gregarious adult males were used during this project. Also most of the previous studies have been conducted on males, and females locusts were left for breeding.

The tissue of interest of my project is the metathoracic ganglion in locusts. This is because the metathoracic ganglion contains one of the many central pattern generators in locusts which are responsible for generating behaviours, such as the ventilatory motor pattern. Since the HS-preconditioned locusts were more resilient in delaying the stress-induced ventilatory arrest and more competent at recovering their ventilation after stress, we expected the molecular difference between the control and HS-preconditioned locusts would occur within their metathoracic ganglia.

1.5 General locust thoracic ganglia and the metathoracic ganglion

Locusts have several ganglia distributed along their ventral nerve cord. Listed starting anteriorly, the locusts have a brain, a suboesophageal ganglion, and three thoracic ganglia, named as the prothoracic ganglion, the mesothoracic ganglion and the metathoracic ganglion. In addition, locusts also have eleven abdominal ganglia, the three anterior ganglia numbered as abdominal 1 (A1), abdominal 2 (A2), and abdominal (A3) are fused with the metathoracic ganglion during embryogenesis forming the metathoracic ganglionic mass. The last four non-fused ganglia are the abdominal 4 (A4), abdominal 5 (A5), abdominal 6 (A6), and abdominal 7 (A7). The terminal abdominal ganglion, is another fusion which contains another four neuromeres, A8 – A11. The three thoracic ganglia control different functions and are structurally different. However, they do share common basic features.

All of the three thoracic ganglia are enveloped in a connective tissue sheath, called the neural lamella, under which there is a thin layer of cellular perineurium (PN). Beneath the PN lies the glial cell layer (GL), where groups of cell bodies (somata) (CBG) of the neurons can be found. CBG lie mostly ventrally and laterally, but a few are located dorsally. The very central cores of the three ganglia (GC) are composed of mainly neuron processes (nerve fibres), and the processes of the glial cells that enclose them. The central core of a ganglion has a clear basic framework of nerve tracts formed by fibres groups running together in bundles. Between these tracts there are finer fibrous areas, called the neuropil (Tyrer and Gregory, 1982).

The neuropils are the regions where the vast majority of synaptic contacts occur between branches of neurons. Only a very few synaptic contacts between neurons occur in the tracts and no synapses are made on the cell bodies. The production of behaviour, which is the measure of the output from these ganglia, results from the complex network interactions of neurons having branches in different regions of the neuropil.

In addition to the neural components, there are tracheae supplying oxygen to the suboesophageal ganglion, the three thoracic ganglia, and to the other CNS ganglia. Specifically, there are four tracheae entering each mesothoracic and metathoracic ganglion. These tracheae then branch to form an extensive network of tracheoles that ensure no neurons are more than 5 µm from a site of gaseous exchange.

Anterior to the metathoracic ganglion, two tracheae, the left and the right ventral longitudinal tracheae are joined together by a trachea about 200 µm in diameter. This trachea lies ventral to the paired connectives between the meso- and metathoracic ganglia. From this cross-link, a pair of tracheae 100-150 µm in diameter runs posteriorly over the surface of the ganglion. Branches of these tracheae, medial tracheae, enter the ventral surface of the metathoracic ganglion approximately 150 µm to the left and to the right of the midline and some 350 µm from the anterior edge. Other branches supply air sacs that invest the ventral surface of the ganglion and smaller branches that run over the surface of the ganglion and the nerve roots.

The metathoracic ganglion is the most structurally complicated ganglion amongst the three thoracic ganglia in locust. At the end of the metathoracic ganglion before the ventral nerve cord, there are three abdominal segment neuromeres, A1, A2 and A3. All of the three abdominal neuromeres have both an anterior and a posterior median nerve. However, of the three pairs of abdominal neuromeres, the most anterior set of the neuromere, A1, has only one pair of (ventral) peripheral nerves, while the other two sets of neuromeres also each have one pair of the dorsal and ventral nerves. These three abdominal neuromeres are fused posteriorly onto the metathoracic T3 neuromere. The metathoracic T3 neuromere possesses six paired peripheral nerves and one posterior median nerve. Both the mesothoracic ganglion and the metathoracic ganglion are responsible for ventilatory and motor flight pattern generation.

The metathoracic ganglionic mass is considered as the neural site for the central pattern generator for ventilation in the locust because only the frequency of ventilation is reduced after the removal of the brain, and by removing the subsequent suboesophageal, pro- and mesothoracic ganglia there is little further deterioration in the ventilation. In addition, an isolated metathoracic ganglionic mass is able to produce a ventilatory rhythm that has the same structure as from an intact locust (Burrows, 1996). However, the suboesophageal, pro- and mesothoracic ganglia are not able to generate a ventilatory rhythm in isolation. Furthermore, the abdominal ganglia 4 to 8 were able to produce a ventilatory rhythm in isolation, however, the frequency is always low and the pattern is abnormal. Therefore, it is been speculated that the ventilatory motor pattern in the locusts is produced by the brain, the abdominal ganglia 4 to 8, and in conjoint with the metathoracic ganglionic mass.

1.6 Ventilatory muscles

The ventilatory mechanism in insects is significantly different than the mammalian mechanism. Unlike mammals which need constant breathing, some insects, like the locusts, can breathe very slowly or even have intermittent ventilation with pauses lasting up to 30 minutes during resting phase. But similar to mammals, insects can also significantly increase their ventilation during intense activities.

In addition, the negative pressure created for inhalation in mammals is created by the flexing the diaphragm. Oxygen is then delivered along with other nutrients from the digestive system via the circulatory system to rest of the body. In insects however, oxygen is delivered to rest of their body via the tracheal system, whereas the haemolymph, which is similar to the vertebrate blood, conveys nutrients to the insect's body but plays a minor role in gas exchange. The negative pressure required for inhalation in insects is created by the pumping motion initiated by their abdominal muscles and the air is allowed into insects' bodies through spiracles.

Specifically in locusts, the abdominal pump creates strong expiration through abdominal muscle contraction. However, the weaker inhalation also requires activity from abdominal muscles in addition to the elastic recoil. Moreover, even though the locust abdomen contains 11 segments, only the middle section is actively responsible for ventilation. This is because the first 2 segments in locusts lack dorso-ventral inspiratory muscles and segments 9 – 11 are modified to bear the genitalia.

The two muscles, muscles 161 and 173, that were studied in this project are both responsible for expiration. The muscle 161, located in the abdominal segment 2 (A2), together with muscle 162 is controlled by the dorso-ventral nerve in the A2. And the muscle 173 is located in the abdominal segment 3 (A3), controls expiration along with the controlling the dorsal longitudinal muscle in the A3.

1.7 Na^+/K^+-ATPase

The Na^+/K^+-ATPase is an important membrane-bound active ion-transporter protein that is responsible for maintaining the sodium and potassium gradients across cellular membranes and used for generating and conducting action potentials in neurons. The Na^+/K^+-ATPase, also known as the sodium-potassium pump, is expressed in all multicellular organisms (Shenk and Steele, 1993). It is a heterodimer that contains α- and β-subunits. The α-subunit is the catalytic subunit of this enzyme with a size of approximately 100kDa (Fagan and Saier, 1994). In mammals, it has been noted that the ATPase is composed of at least three α-subunits and three β-subunits (Horisberger *et al.*, 1991). Grishin *et al.* (1994) have also reported expressions of three different tissue-specific isoforms of the α-subunit in vertebrates (Grishin *et al.*, 1994). However, there is no evidence indicating the expression of different isoforms in insects (Emery *et al.*, 1995).

The Na^+/K^+-ATPase belongs to the P-type ion-motive ATPase family which also contains H^+, H^+/K^+, Ca^{2+}, and heavy metal pumps (Palmgren and Axelsen, 1998). The active transport of ions across cell membranes accomplished by this ATPase is the greatest single energy-consuming process in most cells (Clausen, 1986). Depending on the tissue type, Na^+/K^+-ATPases consume between 5 – 40% of the cellular energy expenditure.

The key roles of this ATPase include but are not limited to regulating cell volume, providing ion gradients to initiate action potential in neurons, to facilitate solute movements, and acting as a physiological regulator. In cardiac muscles, the Na^+/K^+-ATPase can even act as an indirect regulator of the myocardial contraction (Blaustien, 1977).

Furthermore, the Na^+ and K^+ ion-gradients created by the ATPase across cellular membranes are also critical to the functionality of many cell types, especially to sensory, muscle and nerve cells.

The Na^+/K^+-ATPase assists neurons to sustain their Na^+ and K^+ homeostasis by shuffling Na^+ and K^+ ions across the plasma membranes. For each molecule of ATP hydrolyzed, this ion-transporter protein exports three Na^+ ions from the cytoplasm to the extracellular space for every two K^+ ions imported into the cell.

Due to the vital role that the Na^+/K^+-ATPase plays in sustaining action potentials and maintaining Na^+ and K^+ homeostasis across cellular membranes, the activity of this ATPase is targeted and affected by multiple regulatory mechanisms to ensure its correct functioning. Failure in the regulatory mechanisms leads to deleterious effects, such as, in the case of ion pump failure due to an ATP limitation, the dissipation of potential difference across the membranes leading to multiple degenerative events and eventually resulting in cell death (Hochachka, 1986).

Since the Na^+/K^+-ATPase is vital in sustaining action potentials, cells have developed various overlapping mechanisms to failsafe the regulation of the Na^+/K^+-ATPase. For example, the activity of the ATPase can be enhanced by its substrates, or inhibited by cardiac glycosides and their related steroid units (Hootman and Ernst, 1988; Petris *et al.*, 1996).

In addition, the Na^+/K^+-ATPase enzyme and substrate affinity also can vary according to the cellular environment. Ferrandi *et al.* (1999) demonstrated that the addition of cytoskeletal proteins can increase the affinity of Na^+/K^+-ATPase for ATP. Furthermore, various hormones, such as insulin, and other signalling molecules can positively or negatively affect the activity of the protein (Ahl and Brown, 1991; Davey *et al.*, 1993; Grinstein and Erlij, 1974). Moreover, the activity of the ATPase is also subjected to regulation by reversible protein phosphorylation (McMullen and Storey, 2008), a phenomenon that is well documented as a response to anoxia exposure in anoxia-tolerant vertebrates (Hochachka *et al.,* 1996).

This reversible Na^+/K^+-ATPase phosphorylation is also a conserved hypometabolism mechanism under other conditions such as estivation and hibernation. Last but not least, hormonal control of the Na^+/K^+-ATPase has also been demonstrated in insect species. For example, the infusion of juvenile hormone (JH) III and methoprene altered the total activity of the Na^+/K^+-ATPase in *Musca domestica* and *Locusta migratoria* (Davey *et al.*, 1993; Gong *et al.*, 1994). However, it often remains unclear as to whether hormones are affecting the total amount of Na^+/K^+-ATPase protein or altering the activity of the existing protein.

In conclusion, there are numerous mechanisms regulating the activity of this protein. These regulatory mechanisms through various routes, such as reversible phosphorylation, substrate-induced activity enhancement, and hormonal control, ensure the proper functioning of this enzyme in maintaining the Na^+ and K^+ ion-gradients, a duty that is critical to an organism's survival.

1.8 Trafficking

Protein trafficking is an important feature of the eukaryotic cells. Trafficking or translocation, involves the intracellular shuttling of molecules packed in transporter vesicles. Trafficking allows cells to store away unnecessary or excess proteins in vesicles, but rapidly deploy them when a demand emerges. It has been noted that the effects of trafficking can often be seen within half an hour or less (Petris *et al.*, 1996). Through trafficking, a cell can greatly reduce its protein turn-over rates; consequently, the cell efficiently reduces its energy expenditure while performing all of its functions in a timely fashion.

Protein trafficking is a common phenomenon that has been described across many species. For example, in humans, copper pumps are trafficked to the cell membrane from the endoplasmic reticulum (E.R.) in the presence of excessive cellular copper ions (Petris *et al.*, 1996). Recently, trafficking has also been demonstrated to be regulated by environmental cues. For example, in electric fishes, voltage-gated sodium channels are trafficked according to the circadian cycle onto the excitable membranes of electrogenic cells before conducting weak electric field for communication and navigation at night and are stored away in vesicles to avoid detection by predators during the day (Markham *et al.*, 2009).

Moreover, using Aquaporin 2 (AQP2), a water channel that is important in body water homoeostasis regulation, as an example to illustrate some of the regulatory mechanisms for trafficking, it has been shown that the stimulation of vasopressin, an antidiuretic hormone, will cause AQP2 to be translocated from intracellular storage vesicles to the apical plasma membrane of kidney renal collecting-duct cells, rendering the cell permeable to water and causes water reabsorption (Marples *et al.*, 1995; Nielsen *et al.*, 1995; Sabolic *et al.*, 1995; Yamamoto *et al.*, 1995). On the other hand, the withdrawal of vasopressin triggers the endocytosis of AQP2-containing vesicles and restores the water-impermeable state of the kidney apical membrane.

The translocation of AQP2 requires the activation of PKA which leads to phosphorylation of AQP2 at Ser^{256} (Fushimi *et al.*, 1997; Katsura *et al.*, 1997; Nishimoto *et al.*, 1999; Zelenina *et al.*, 2000).

However, Valenti *et al.* (2000) noted that a Ser/Thr phosphatase inhibitor, okadaic acid, induced the translocation of AQP2, even in the presence of a specific PKA inhibitor, H89. Thus, they concluded that the AQP2 translocation may involve a mechanism which is independent of PKA phosphorylation in renal collecting-duct cells. Nevertheless, the activity of PKA is thought to be regulated and mediated by AKAPs (protein kinase A-anchoring proteins) which tether PKA to specific sites and limit its access to a subset of substrates (Colledge and Scott, 1999; Smith and Scott, 2002; Tasken and Aandall, 2004). This prevents PKA from binding with other proteins and is essential for the localization of PKA to AQP2 in a timely, specific and spatially effective fashion.

Furthermore, in addition to PKA phosphorylation sites on AQP2, other kinase sites such as PKG, PKC, protein kinase CK2 are also present in the AQP2 sequence (Procino *et al.*, 2003). Moreover, Bouley *et al.* (2000) have demonstrated that nitric oxide and atrial natriuretic factor stimulate the insertion of AQP2 in renal epithelial cells through a cGMP-dependent pathway. From these studies, it is obvious that the activity of an important protein such AQP2 can be precisely controlled by trafficking, and that trafficking itself is under regulations by other mechanisms.

One of the important organelles used during trafficking is the Golgi apparatus. It plays two central roles in many intracellular trafficking events; they comprise protein synthesis and delivery, as well as internalizing molecular processes via endocytic pathways (Rothman, 1994; Lowe and Kreis, 1998; Pfeffer, 1999; Nichols and Pelham, 1998, Glick and Malotra, 1998; Allan and Balch, 1999). By studying the AQP2 protein, researchers have found that Ser^{256} is also a substrate for Golgi CK2. It has been illustrated that the phosphorylation of AQP2 by Golgi CK2 is required for Golgi transition (van Balkom *et al.*, 2002). The Golgi apparatus is also responsible for the internalization of molecules through an endocytic pathway. Researchers have found that applications of cardiotonic steroids, such as ouabain, can regulate the plasmalemmal Na^+/K^+-ATPase through endocytosis and decrease the membrane concentration of the ATPase (Liu *et al.*, 2004 and 2005). This is particularly interesting because since ouabain was able to eliminate the thermal protection provided by HS-preconditioning, this endocytosis pathway could be one of the mechanisms that explain this phenomenon.

The cellular trafficking mechanism is often accomplished via motor proteins following the cellular tracks laid by cytoskeletal proteins. In more depth, proteins appear to be trafficked by different types of cytoskeleton tracks depending on the phase of the cell cycle (Geldner *et al.*, 2001). For example, PIN1, an auxin efflux carrier candidate protein seems to traffick along an actin-dependent pathway to and from the plasmalemmal membrane during interphase. However, it is trafficked along a microtubule-dependent pathway to the cell plate during cytokinesis.

Due to the importance of trafficking, the translocation of cellular proteins is also regulated by multiple mechanisms. For example, brefeldin A, a chemical that blocks membrane trafficking from the endoplasmic reticulum to the Golgi, also inhibited protein synthesis (Lippincott-Schwartz *et al.,* 1989). Specifically, brefeldin A inhibits the formation of a specific type of vesicular carrier that participates in anterograde/retrograde membrane transport in the endoplasmic reticulum and the Golgi apparatus. This proved that protein synthesis is not often completed within one single organelle, for example, the ribosomes, and their translocation is a necessary step in protein synthesis. Another study has also found that besides protein synthesis, progression of murine oocyte maturation possibly also requires functional membrane trafficking, resulting in either the modification of proteins at the Golgi level, or the delivery of these proteins to appropriate post-Golgi sites (Moreno *et al*., 2002). These studies showed that trafficking is not only responsible for the translocation of proteins, but it might also have many downstream effects such as protein synthesis.

Furthermore, a multi-protein hsp90/hsp70-based chaperone machinery is believed to regulate cellular trafficking. Hsp90 is a highly conserved and essential stress protein that is expressed in all eukaryotic cells. Being a HS protein, hsp90 is still one of the most abundant proteins in unstressed cells.

The hsp90 performs cellular housekeeping functions by regulating the activity, the turnover and the trafficking of a variety of proteins (Pratt and Toft, 2003). As a reflection of the growing interested in hsp90, many more studies have been conducted to examine the role of chaperones in receptor trafficking through the cytoplasm (Pratt *et al*., 1999; Pratt *et al.*, 2001) and within the nucleus (DeFranco, 2000). This rapid hsp90-dependent trafficking occurs along cytoskeletal tracts, and a variety of observations support the idea that the steroid receptors traffick through the cytoplasm to the nucleus when they are in this hsp90/hsp70 heterocomplex. However, the mechanism behind the hsp90-binding immunophilins is unclear. This finding is also particularly interesting because if the HS-induced redistribution of the Na^+/K^+-ATPase hypothesis was true, this hsp90/hsp70-based chaperone machinery could be one of the mechanisms behind the thermal protection provided by HS-preconditioning that involved the Na^+/K^+-ATPase.

More interestingly, there has been speculation that protein trafficking systems such as the Hsp90/Hsp70 system would have been evolved and developed prior to neurons and eukaryotic cells. This is because simple cellular diffusion is usually not sufficient as a method of substance transportation within eukaryotic cells. Thus, substance movements had to be achieved via a specialized trafficking mechanism rather than through diffusion (Pratt and Toft, 2003). Consequently, in eukaryotic organisms that possess a nervous system, the function of hsp90 in protein trafficking might eventually have become essential for the survival of that organism.

In conclusion, the trafficking of protein is a cellular process that can be accurately regulated by many mechanisms such as HS-protein chaperone machineries, reversible phosphorylation, environmental cues, and many organelles and proteins such as the Golgi apparatus and the cytoskeletal tracks. Furthermore, protein trafficking has proven to be a multi-functional process that is not only responsible for the translocation of proteins; it also has many downstream effects on other essential functions such as protein synthesis.

1.9 Research objectives and experimental outline

The goal of this research project is to elucidate the effects of HS-preconditioning on locusts' ventilatory motor pattern generation, and the molecular mechanism of the Na^+/K^+-ATPase underlying the decrease in the rate of accumulation and the increase in the rate of $[K^+]_o$ clearance observed in HS-preconditioned locusts.

From my electrophysiology experiments, I expected to see a significantly longer ventilatory muscle burst duration or a significantly shorter muscle cycle period in HS-preconditioned locusts which might have allowed them to inspire more air and in turn allowed them a faster recovery from this reversible coma. The ventilatory EMGs of control and HS-preconditioned animals were recorded by placing a copper wire electrode on the ventilatory muscles 161 or 173. Ventilatory motor burst duration and cycle period were then analyzed to characterize the locusts' ventilatory motor pattern. Sodium azide, which is an electron transport chain inhibitor, was bath-applied to study the responses of control and HS-preconditioned locusts under and recovering from an anoxia stressor.

In addition, two types of biochemical assays, immunohistochemistry and spectrophotometry, were utilized to study the effects of HS-preconditioning on the localization and the total enzymatic activity of the Na^+/K^+-ATPase. During my spectrophotometry experiments, I estimated the total Na^+/K^+-ATPase protein activity from the metathoracic ganglion in control and HS-preconditioned locusts via a pyruvate kinase/lactate dehydrogenase assay by measuring the decrease of NADH to test the hypothesis that HS-preconditioning increased the activity of the

Na^+/K^+-ATPase. In addition, data were collected using animals that were exposed to anoxic coma through water immersion or sodium azide bath-application to study the effects of different stressors on the activity of the Na^+/K^+-ATPase. Lastly, data were also collected before, during and after coma to study the effects of coma on the activity of the ATPase (Figure 3).

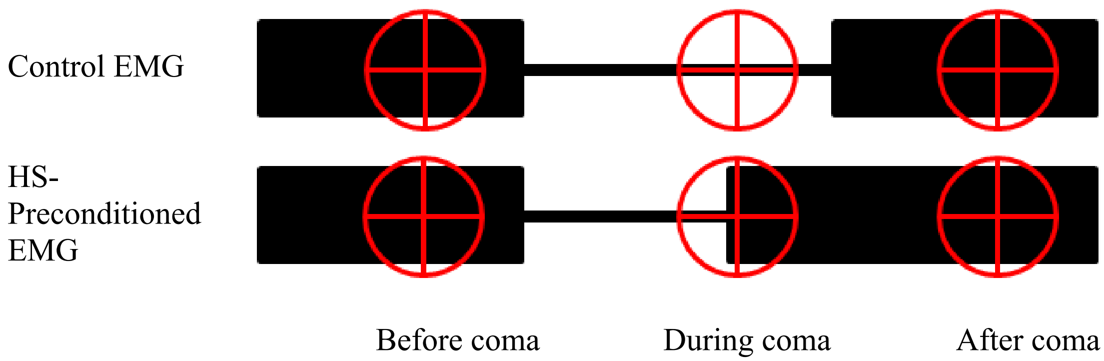

Figure 3: Schematic illustrations demonstrating the time of data collection relative to arrest and recovery of ventilatory motor patterning. Black bars represent the ventilation motor pattern and black lines represent arrests of ventilation during coma. Red crosses indicate time points for tissue collection: before coma, during coma at the time when HS-preconditioned locusts are just recovering from coma while control animals were under coma, and after coma.

Lastly, immunohistochemistry was used to test the hypothesis that HS-preconditioning increased the concentration of the Na^+/K^+-ATPase in neuronal membranes and the hypothesis that anoxic coma induced by sodium azide also caused a difference in the localization of the ATPase.

In these experiments, the metathoracic ganglia from control and HS-preconditioned animals before coma, during coma, and after coma were sectioned at 4 µm and a Na^+/K^+-ATPase α-subunit 5 specific antibody (developed by Fambrough *et al.*, and used by Fambrough *et al.* in *D. melanogaster*, Rarey *et al.* in rats, Eskelinen *et al.* in chicken, and *etc.*) was used to tag the Na^+/K^+-ATPase. The ATPase was then visualized using a fluorescent secondary antibody.

If the HS-induced effect hypothesis is correct, I expect to see a significantly higher ATPase concentration in the neuronal membranes of HS-preconditioned animals which would help explain the higher $[K^+]_o$ clearance previously observed in HS-preconditioned animals; and if the effect of coma on the ATPase distribution hypothesis was true, we expected to see a different neuronal ATPase distribution between samples collected before, during and after coma.

Chapter 2: Material and Methods

2.1 Animals

Locusta migratoria migratorioides were raised in a crowded colony maintained in the Biosciences Complex located at Queen's University. The animals were reared under a 12h:12h dark:light circadian regime. Each cage was individually lit with a 40W incandescent light bulb to simulate the sun for light and warmth. The temperature of the room that contains the cages was maintained at $25 \pm 1\ ^{o}C$. All animals were provided daily with fresh wheat grass, carrot slices or tomato pieces, and an *ad libitum* mixture of one part skim milk powder, one part torula yeast, and 13 parts bran by volume. During my experiments, age-appropriate adult animals were randomly selected from our colony and brought up to our laboratory in a ventilated plastic container.

2.2 Experimental treatments

2.2.1 Treatment group assignment

All of the locusts in this project were randomly but equally distributed to either the control or HS-preconditioned treatment group. The animals in these two treatment groups were then again randomly assigned into two subgroups that were composed of animals that either had or had not been exposed to a reversible coma induced by stressor (temporary anoxia).

2.2.2 HS-preconditioning

Adult male locusts between 4 to 6 weeks old were randomly selected from the colony and kept in a ventilated plastic container until the experiments. HS-preconditioned locusts were placed in a humid 45 ^{o}C incubation chamber for three hours. Animals were then allowed to recover from this sub-lethal preconditioning for an hour at room temperature before experiments.

No manipulations were applied to control animals. During the time when HS-preconditioning animals were under HS, control locusts were left in a container without feeding to eliminate any variables that the 4 hours and the environment outside of the colony might have on the animals.

2.2.3 Stressors

In both control and HS-preconditioned locusts, half of the animals were exposed a reversible coma via two different types of anoxia treatments, natural anoxia or chemical anoxia. Chemical anoxia was induced in locusts to study the effects of reversible coma on the locust. Natural anoxia experiments were conducted to determine whether chemical anoxia could be assumed to have the same effects as natural anoxia on the metathoracic ganglion Na^+/K^+-ATPase in the locust.

Once the stressors were removed, all of the animals were able to restore their ventilatory motor pattern. The times it took for those animals to recovery ventilations were then recorded using a stopwatch in natural anoxia experiments and calculated from their ventilatory motor pattern EMG recordings in chemical anoxia experiments.

a) Natural anoxia

Natural anoxia was induced in intact locusts through a 30-minute water immersion. During water immersions, locusts were kept in a plastic cage which was immersed in room temperature distilled water. All of the animals entered a reversible coma during this 30-minute water immersion. After the water immersions, the plastic cage was removed from the water and allowed to drain.

b) Chemical anoxia

Chemical anoxia was induced in semi-intact locust preparations by bath-applying 2 mL of 1 mM sodium azide into the thoracic cavity. Once the locusts had arrested their ventilation, sodium azide was washed out using standard isotonic locust saline.

2.3 Dissection and tissue collection

In semi-intact preparations, the legs, wings and the pronotum of male *Locusta migratoria* were removed. The animals were then dorsally dissected from the anterior of the thorax to the posterior of the abdomen to expose their internal organs. The animals were pinned onto a corkboard dorsal side up to gain access to their metathoracic ganglia. Once the gut and fat bodies were removed, the metathoracic ganglion and ventilatory muscles 161 and 173 of the locusts became clearly visible; the animal was then ready for experiments.

During the experiments, a fresh flow of standard isotonic locust saline (147 mM NaCl, 10 mM KCl, 4 mM $CaCl_2$, 3mM NaOH, 10 mM HEPES dissolved in distilled water, pH 7.2) was constantly bath applied to the animal from the anterior of their thorax and drained from the posterior of their abdomen using a Peri-Star peristaltic pump (World Precision Instruments Inc., Sarasota, FL, USA) at a rate of 10 mL per minute to prevent the internal organs from desiccation (Figure 4).

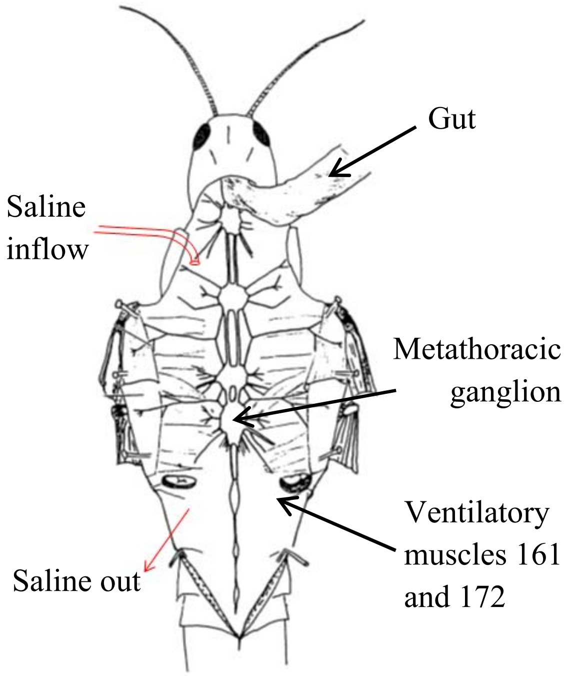

Figure 4: Dorsal view of a dissected locust exposing its metathoracic ganglion and ventilatory muscles. Standard isotonic locust saline was bath applied to the animal posterior to the head and drained posterior to the thoracic region (modified from Money, Anstey and Robertson, 2005).

All experiments and tissue collection were performed during the day. The locusts were not fed during the time when they were kept in the ventilated container before experiments or during HS-preconditioning. In addition, all of the animal dissections, and tissue collections were completed within 5 minutes to minimize the stress imposed on the animal.

2.4 Electrophysiology

Electromyograms (EMG) were recorded by placing a copper wire EMG electrode on ventilatory muscles 161 or 173 (Figure 5). The copper wire (tip area - 0.32 mm^2) is insulated by polypropylene coating except at the tip. Ventilatory motor patterns were first amplified by a Grass P15 AC Preamplifier. The signals were then digitized using a MiniDigi A1 Two-Channel Acquisition System (Axon Instruments), and finally saved onto a computer using AxoScope 9.0

(Molecular Devices Inc). After the EMG was recorded, the animal's ventilatory muscle burst duration and period were calculated using IGOR (WaveMetrics).

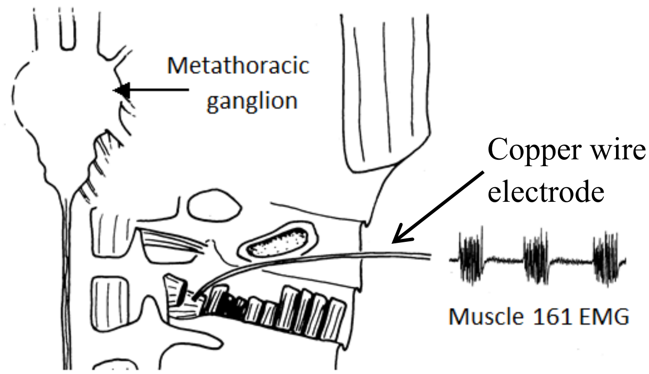

Figure 5: Dorsal view of the placement of a copper wire electrode for ventilatory EMG collection. Ventilatory EMG of the locust was placed by placing a copper wire electrode on ventilatory muscles 161 or 173 of a dorsally dissected locust (modified from Newman *et al.*, 2003).

2.5 Na^+/K^+-ATPase enzymatic activity assay

The Na^+/K^+-ATPase enzymatic activity assays were performed on the locust metathoracic ganglion. The ganglia were kept individually in a plastic 1 mL microtube (manufactured by Diamed Lab Supplies Inc.) and flash frozen in liquid nitrogen immediately after tissue dissection. The tissues were then stored in a –80°C freezer until they were thawed for the enzymatic activity assay. The total Na^+/K^+-ATPase activity was estimated through the difference of the total metathoracic ganglia ATP metabolism in the presence or absence of ouabain, a Na^+/K^+-ATPase specific inhibitor, which will either eliminate or include the ATPase activity in a pyruvate kinase/lactate dehydrogenase assay.

During the pyruvate kinase/lactate dehydrogenase assay (Figure 6), the Na^+/K^+-ATPase enzymatic activity was estimated through the disappearance of NADH, which was measured using a spectrophotometer at 340 nm. This assay was initiated by the hydrolysis of ATP into ADP catalyzed by the Na^+/K^+-ATPase. The pyruvate kinase can then cleave the phosphoenolpyruvate (PEP) into pyruvate. The resultant pyruvate was then reduced by the lactate dehydrogenase (LDH) into lactate, while the LDH oxidizes the NADH into NAD^+ and hence decreasing the concentration of NADH. Therefore, the difference in the rate of NADH consumption in the presence and absence of ouabain reflects the activity of the Na^+/K^+-ATPase.

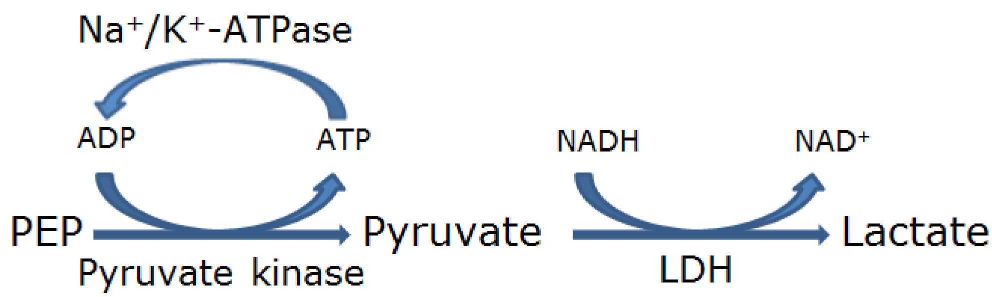

Figure 6: The pyruvate kinase/lactate dehydrogenase assay. The hydrolysis of ATP to ADP catalyzed by the Na^+/K^+-ATPase initiated the pyruvate kinase/lactate dehydrogenase assay. The enzymatic activity of the Na^+/K^+-ATPase was assessed through the disappearance of the NADH using a spectrophotometer.

Prior to the enzymatic assays, each ganglion was homogenized in the presence of 300 µL of lysis buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole and 0.1% deoxycholate, pH 7.3) and phosphorylation and dephosphorylation inhibitors (10mM EGTA, 50mM NaF, 10mM tetrasodium pyrophosphate, 1mM phospho-serine, 1mM phospho-threonine, and 1mM phospho-tyrosine) which prevented any Na^+/K^+-ATPase phosphorylation status changes that might affect the enzymatic assays. After homogenization, 1 % in homogenate volume of 100 mM phenylmethanesulfonylfluoride (PMSF) (dissolved in methanol) was also added to the homogenate to deactivate any serine proteases that might be present in the homogenate.

During the Na^+/K^+-ATPase enzymatic activity assay, the ATPase activity was determined by spectrophotometrically measuring the metathoracic homogenate (30 μL) for 10 minutes at 340 nm for the production of NADH. In addition to the tissue homogenate, the following solutions were also added in a sequential order: 228 μL of assay buffer (50 mM imidazole, 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$), 3 μL each of 0.2 mM NADH, 0.5 mM of PEP, 10 U/mL of LDH, and 10 U/mL of pyruvate kinase (PK), 15 μL of either ouabain (0.5 mM) or assay buffer, and lastly 15 μL of ATP (3 mM). Each sample was distributed amongst 6 wells on a 96 well plate, 3 of which were examined in the presence of ouabain, and 3 in the absence of ouabain which was replaced with assay buffer. The first 10 minutes of the linear portion of the absorbance reading was used to assess the activity of the Na^+/K^+-ATPase.

Once the enzymatic assay was initiated by the addition of ATP, the absorbance was recorded for 10 minutes, the difference between the slopes of the samples with and without ouabain then gave a value for only the Na^+/K^+-ATPase activity. This value was then normalized to the total protein amount within the sample, which was determined for each homogenate though a standard protocol based on the Bradford method (Bradford, 1976) where the quantity of the Na^+/K^+-ATPase was assessed through protein-dye binding.

2.6 Immunohistochemistry

2.6.1. Paraffin wax embedding and sectioning techniques

The three coma groups examined during the immunohistochemistry experiments were also before coma, during coma, and after coma. 24 adult male locusts were randomly assigned to the three groups to study the effects of anoxic coma on the trafficking of the Na^+/K^+-ATPase. Both control and HS-preconditioned animals were randomly assigned to these three coma groups to study whether there was an effect of coma on the trafficking of the ATPase.

After the metathoracic ganglia were dissected, the tissues were immediately preserved by fixing in a 4% formaldehyde solution buffered in standard isotonic locust saline at room temperature for 24 to 48 hours. Once the tissues were fixed, they were then dehydrated and the cellular fluid was replaced with anhydrous ethyl alcohol (ethanol, Commercial Alcohols Inc.) by sequential washes of 80% and 90% ethanol for half an hour each. Lastly, the tissues were washed twice using 100% ethanol for half an hour each.

Cellular lipids were then replaced by xylene (Sigma-Aldrich®) with two washes of 100% xylene for half an hour each to aid the penetration of Paraffin into the tissue. Finally, the tissues were preserved in Paraffin by incubating in Paraffin wax (Sigma-Aldrich®) twice at $60^{o}C$ for 30 minutes each.

Before the tissues were incubated with antibodies, a Leica Reichert-Jung 820-II Histocut Microtome was used to section those Paraffinized metathoracic ganglia into 4 μm thick slices. The slices were then floated in a tray that contained distilled water heated at ~58^{o}C on a slide warmer (manufactured by Chicago Surgical and Electrical Co., model number 26000) to stretch out the sections. Once the sections were flattened out, they were mounted onto poly-L-lysine slides (purchased from Sigma-Aldrich®). The mounted slides were then allowed to air dry at room temperature for 30 minutes and oven-dried in a 45 ^{o}C incubation chamber overnight.

2.6.2. Immunohistochemistry protocol

Before immunohistochemistry, the mounted slides were first deparaffined by washing in xylene (purchased from Sigma-Aldrich®) for 30 minutes in room temperature. The slides were also rehydrated by washing sequentially in 100%, 95%, 80% and 50% ethanol for 10 minutes each at room temperature. The slides were then rinsed once in distilled water, and Tris-Buffered-Saline-Triton® X-100 (TBST) buffer (20mM Tris, 137mM NaCl, 0.10% Triton® X-100, pH 7.5) for 5 minutes each. To unmask the antigen, the Na^{+}/K^{+}-ATPase, the slides were washed at 95^{o}C for 30 minutes in a Tris-EDTA-Triton® X-100 buffer bath (10mM Tris, 1mM EDTA, 0.05% Triton® X-100, pH9.0), promoting the antigen-antibody binding ability. Lastly, the slides were rinsed three times using TBST buffer for 5 minutes each, and then background blocked in 5% normal goat serum (NGS) (Sigma-Aldrich®) diluted using the TBST buffer for an hour in room temperature.

Once the slides were ready for staining, the Na^+/K^+-ATPase was tagged by incubating the slides with a primary mouse monoclonal antibody raised against chicken's α5 subunit of the Na^+/K^+-ATPase (purchased from Developmental Studies Hybridoma Bank at the University of Iowa) at a 5% dilution (TBST buffer and 5% NGS as a diluent) for 48 hours at 4^{o}C. The excess primary antibodies were then washed off using three washes of the TBST buffer for 5 minutes each. The localization of the Na^+/K^+-ATPase was then visualized by incubating in a Goat Anti-Mouse IgG (whole molecule) – fluorescein isothiocyanate (FITC) conjugated secondary antibody (Sigma-Aldrich®) as the secondary antibody diluted to a 1:100 ratio (TBST buffer and 5% NGS as the diluent) for 24 hours at 4^{o}C.

After staining using antibodies, the metathoracic ganglion slides were further labelled to stain the cellular DNA using 4',6-diamidino-2-phenylindole (DAPI) for 5 minutes under room temperature at a concentration of 0.1 μg DAPI/mL diluted by distilled water. DAPI binds onto the minor groove of DNA, and it has a maximum absorption wavelength at 358nm, and maximum emission wavelength at 461nm. After DAPI staining, the slides were rinsed twice for 5 minutes each using the TBST buffer. The slides were then finally sealed using SlowFade Gold Antifade kit (Sigma-Aldrich®) to minimize the fluorescence fading.

The slides were then examined under an Axioplan 2 Imaging Microscope (Carl Zeiss MicroImaging LLC), and images were taken using a Leica DC 500 digital camera

(Spectronic Camspec Ltd.), and saved using Openlab 4.0.1 (developed by Improvision PerkinElmer). Data from each ganglion was collected within the same session using the same gain and settings to avoid discrepancies in fluorescence intensity. The images were then later analyzed using ZEN 2009 Light Edition (Carl Zeiss MicroImaging LLC).

2.6.3. Negative and specificity controls

To test the binding specificity of the secondary antibody, negative controls were prepared by omitting the primary antibody incubation. Consequently, the negative control slides only displayed dim background fluorescence under the fluorescent microscope indicating binding specificity of the FITC antibody to the primary antibody.

2.7 Analysis and statistical tests

2.7.1. Analysis

a) Electrophysiology

From the raw ventilatory motor pattern recording, the muscle burst durations and cycle periods were analyzed using IGOR (Figure 7). Ten muscle burst durations and periods were selected and averaged at 10 min intervals for each animal from both control and

HS-preconditioned normoxia subgroups. Similarly, for the control and HS-preconditioned anoxia subgroups, 10 peaks were selected and averaged. However, the ventilatory motor patterns were analyzed at 5 min intervals in anoxia groups to obtain a better resolution on the physiological changes occurred within the animals.

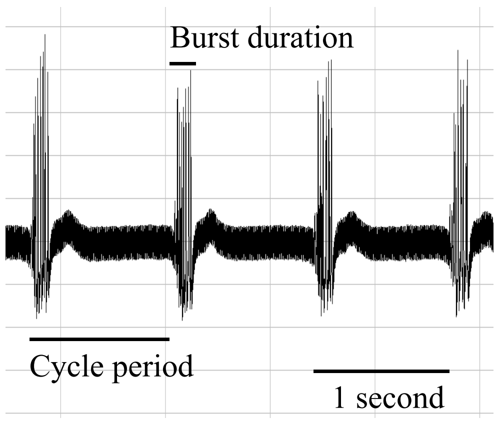

Figure 7: Sample ventilatory motor pattern EMG illustrating the muscle burst duration and period. The ventilatory muscle burst duration and cycle period were the two characteristics utilized in my experiments to describe the ventilation of locusts.

Once all the EMGs were analyzed, the data were then averaged again for all of the animals (n = 10) within each of the 4 subgroups (total n = 40). All of the EMGs were also standardized to their each individual pre-coma ventilator rhythms for those subgroups that have encountered sodium azide to eliminate the difference between animals. Lastly, the respective standard errors for each subgroup were calculated, and statistical analyses were performed using SigmaPlot 11.0.

b) Na^+/K^+-ATPase assay

To study whether different stressors would have different effects on the activity of the Na^+/K^+-ATPase, two different anoxic stressors, sodium azide bath-application and water immersion were used. 24 locusts (12 control and 12 HS-preconditioned locusts) were randomly assigned to the water immersion treatment and 27 animals (14 control and 13 HS-preconditioned locusts) were assigned to the sodium azide treatment. The metathoracic ganglion of each animal was collected after the animal recovered from its respective stressor.

The effects of HS-preconditioning and coma on the activity of the Na^+/K^+-ATPase activity were also studied by randomly assigning 48 locusts to control and HS-preconditioned groups. The metathoracic ganglia from both control and HS-preconditioned locusts were randomly collected but evenly distributed to before coma (n = 8), during coma (n = 8), and after coma groups (n = 8).

The Na^+/K^+-ATPase activity values were determined by measuring the activity difference of the metathoracic ganglia in the presence or absence of ouabain. Each Na^+/K^+-ATPase activity value was standardized to their total protein value to eliminate any differences due to ganglion size, and the data were presented before they were adjusted for the NADH molar extinction coefficient, which is an intrinsic property that describes the strength that NADH absorbs light at a given wavelength.

c) Immunohistochemistry

After the immunofluorescent images were taken and saved, the intensity of the neuronal membranes and cytoplasmic FITC stain which represented the concentration of the Na^+/K^+-ATPase was analyzed using ZEN 2009 Light Edition (Zeiss). The intensity of the each nuclear FITC stain was also analyzed as a background control since the nuclei were presumably absent of Na^{+}/K^{+}-ATPase and any fluorescence observed would be background staining.

Three intact cells that best represented the slide were selected from each metathoracic ganglion to determine the localization of the Na^{+}/K^{+}-ATPase. The intensities of the membranes, nuclei, and cytoplasmic FITC stains from each cell were analyzed at three different locations within the cell to minimize fluorescent variables. The fluorescence intensity reading was recorded at the pixel of the cursor's location and then averaged for each of the three individual coma groups (n = 3), including both control and HS-preconditioned animals (total n = 18). The membrane and cytoplasmic FITC intensities were also standardized to the respective nuclear fluorescence intensity of each cell to control for background fluorescence.

2.7.2. Statistical tests

All statistical analyses were conducted using SigmaPlot 11.0 (Systat Software Inc) and reported in the Appendices. Two Way Repeated Measures ANOVA was used to assess the effects of HS-preconditioning and time on the ventilatory motor burst duration and period, where Factor A was either control or HS-preconditioning and Factor B was time. The data were either the ventilatory motor burst duration or period.

Two Way ANOVA was used to assess the effects of HS-preconditioning and either the stressor or stages of a coma on the activity of the Na^+/K^+-ATPase. In these statistical tests, Factor A was either control or preconditioning, or water immersion or sodium azide, Factor B was the different groups of a coma, and the data was the activity of the ATPase.

Lastly, Two Way ANOVA was used to assess the effects of HS-preconditioning and the three different stages of a coma on the localization of the Na^+/K^+-ATPase where Factor A was either control or HS-precondition, Factor B was the three coma groups, and data was the FITC fluorescence intensity implicating the localization of the ATPase.

Mann-Whitney tests were used as the non-parametric test when the normality test failed and a confidence interval of 95% was considered to assess the significance of differences in all the tests. Data were plotted using SigmaPlot and error bars represented the standard error of means.

Chapter 3: Results

3.1 Electrophysiology

From the ventilatory muscle burst EMG experiments, I first found that HS-preconditioned locusts took a significantly shorter amount of time to recovery from anoxic coma than control animals. My results showed that the time it took for the control ($n = 10$) and HS-preconditioned ($n = 10$) locusts to start ventilating after sodium azide induced coma were 15.3 ± 1.1 minutes and 12.1 ± 1.4 minutes respectively ($P < 0.05$, Figure 8).

In addition, the data of the ventilatory motor pattern under normoxia suggested that there was no significant difference in the expiratory muscle burst duration and cycle period between control and HS-preconditioned locusts (Figure 9; Two Way RM ANOVA, factors: treatment (control vs. HS-preconditioning) and time, normalized duration: Unadjusted $P = 0.293$, normalized period: Unadjusted $P = 0.47$, $n = 20$). However, the duration of the experiment had a significant effect on the animals' ventilatory muscle burst duration and cycle period, such that the normalized ventilatory muscle burst duration increased significantly to 1.65 ± 0.23 and 1.49 ± 0.20 of their original values (HS-preconditioned and control animals). Furthermore, the cycle period of the locusts was shown to be significantly increased after 30 minutes of the experiment to 1.46 ± 0.07 and 1.46 ± 0.10 of their original values for HS-preconditioned and control animals (Two Way RM ANOVA, factors: treatment (control vs. HS-preconditioning) and time, normalized duration, 30 minutes vs. HS-preconditioned initial: Unadjusted $P = 0.002$; normalized period, 30 minutes vs. control initial: Unadjusted P = 0.001). Nevertheless, the effect of the duration of the experiment was consistent between control and HS-preconditioned locusts (Two Way RM ANOVA, factors: treatment (control vs. HS-preconditioning) and time, normalized duration for control vs. HS-preconditioned animals at 30 minutes: Unadjusted P = 0.52; normalized period for control vs. HS-preconditioned animals at 30 minutes: Unadjusted P = 0.997) (Figure 9).

The data for the ventilatory motor pattern under anoxia indicated that there was a significantly shorter normalized ventilatory cycle period in HS-preconditioned locusts (3.56 ± 0.58) compared to control animals (6.75 ± 1.4) immediately after recovery from sodium azide-induced anoxia (One Way ANOVA: factor treatment (control vs. HS-preconditioning), Unadjusted $P < 0.001$). Furthermore, a significantly different normalized ventilatory muscle burst duration was also observed between control and HS-preconditioned locusts after their recovery from anoxia (Two RM Way ANOVA, factors: treatment (control vs. HS-preconditioning) and time, Unadjusted P = 0.049). Moreover, a significantly different normalized ventilatory muscle burst duration was observed in both groups of animals 5 minutes after recovering from anoxia (Two Way RM ANOVA, factors: treatment (control vs. HS-preconditioning) and time, 5 minutes vs. Baseline: Unadjusted $P < 0.001$, 5 minutes vs. 0 minutes: Unadjusted P = 0.001). Nevertheless, there was no significant difference in the normalized ventilatory muscle burst duration between before anoxia and immediately recoverying from anoxia (Two Way ANOVA, factors: treatment (control vs. HS-preconditioning) and time, Baseline vs. 0 minutes: Unadjusted P = 0.26) (Figure 10). However, both control and HS-preconditioned locusts displayed a significantly longer ventilatory muscle burst cycle period immediately after their recovery from anoxia than their baseline values (Two Way ANOVA, factors: treatment (control vs. HS-preconditioning) and time, 0 minutes vs. Baseline: Unadjusted $P < 0.001$).

Eventhough a significantly longer ventilatory muscle burst period was observed immediately after recovery from anoxia, the locusts were able to partially attenuate this difference by moderately decreasing the increased ventilatory burst period (Two Way ANOVA, factors: treatment (control vs. HS-preconditioning) and time, 5 minutes vs. 0 minutes: Unadjusted $P = 0.004$) (Figure 10). However, the animals were only able to partially attenuate because there was still a significant difference in the ventilatory burst period between the anoxia group (2.78 ± 0.47) and the normoxia group (1.46 ± 0.09) when comparing the normalized ventilatory muscle burst cycle period of these two groups of animals at 30 minutes of the experiment (the 30-minute anoxia group was calculated by combining the the recovery time which was 12.1 ± 1.4 minutes and the 20 minutes after recovered from anoxia) (Two Way ANOVA , factors: treatment (control vs. HS-preconditioning) and time: Unadjusted $P < 0.001$; Figure 11).

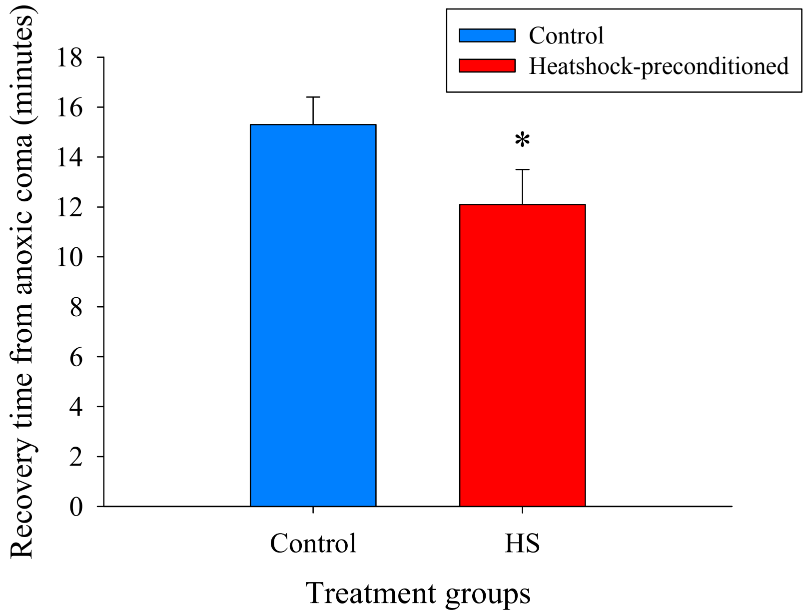

Figure 8: HS-preconditioned locusts recover faster from anoxic coma. The HS-preconditioned locusts were able to recover significantly faster from anoxic coma than control locusts, which is indicated by the asterisk, ($\bar{x} \pm$ SE). (HS-preconditioned locusts will be illustrated in red, control locusts will be illustrated in blue, and error bars will represent standard error for rest of the thesis.)

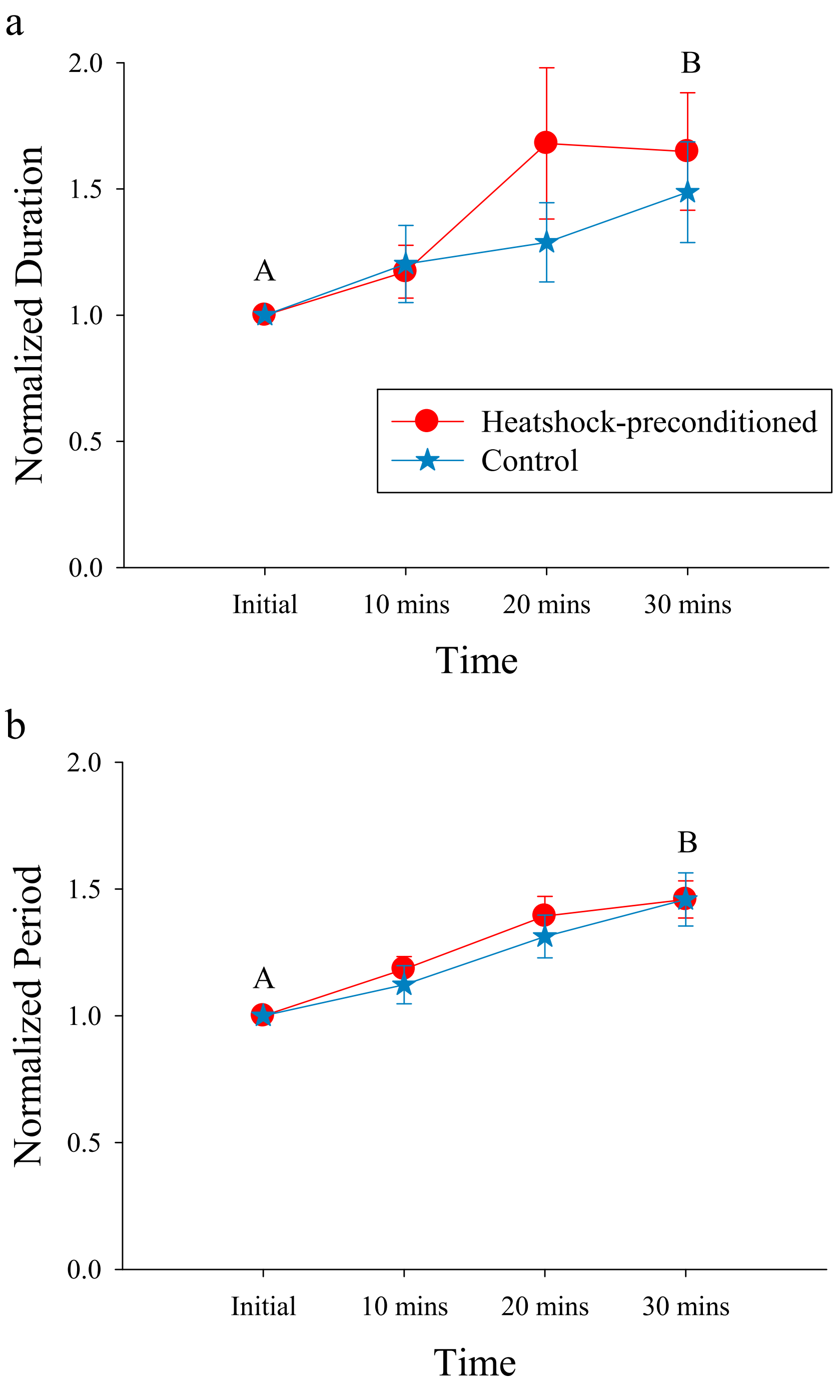

Figure 9: Normalized ventilatory motor pattern of control and HS-preconditioned locusts under normoxia. a and b: no significant difference in the normalized duration and period were observed between control and HS-preconditioned locusts. However, there was a significant difference in normalized duration and period at 30 minutes of the experiments compared to the initial ventilatory motor pattern which was indicated by the capitalized letters, ($\bar{x} \pm$ SE).

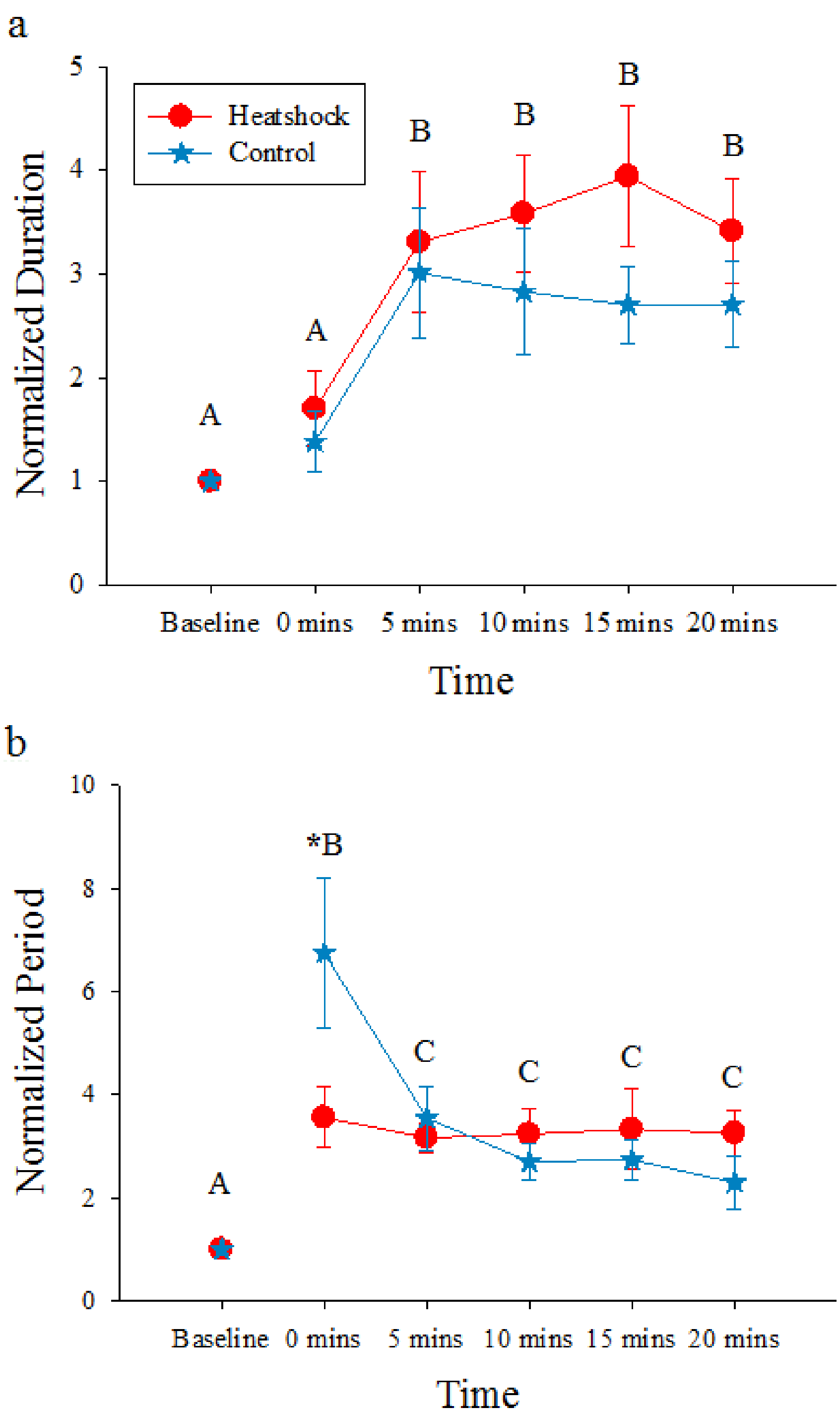

Figure 10: Normalized ventilatory motor pattern of control and HS-preconditioned locusts after anoxia. a: A significantly longer ventilatory duration was observed after 5 minutes of recovery from anoxia in both groups of animals as indicated by the letters. b: There is a significant difference in the ventilatory muscle burst period immediately (0 mins) after anoxic recovery between the two groups of locusts as denoted by the asterisk; also, a significantly longer cycle period was observed in both groups of animals immediately after recovery (0 mins), however, the animals were able to partially attenuate this difference, which were indicated by the letters ($\bar{x} \pm$ SE).

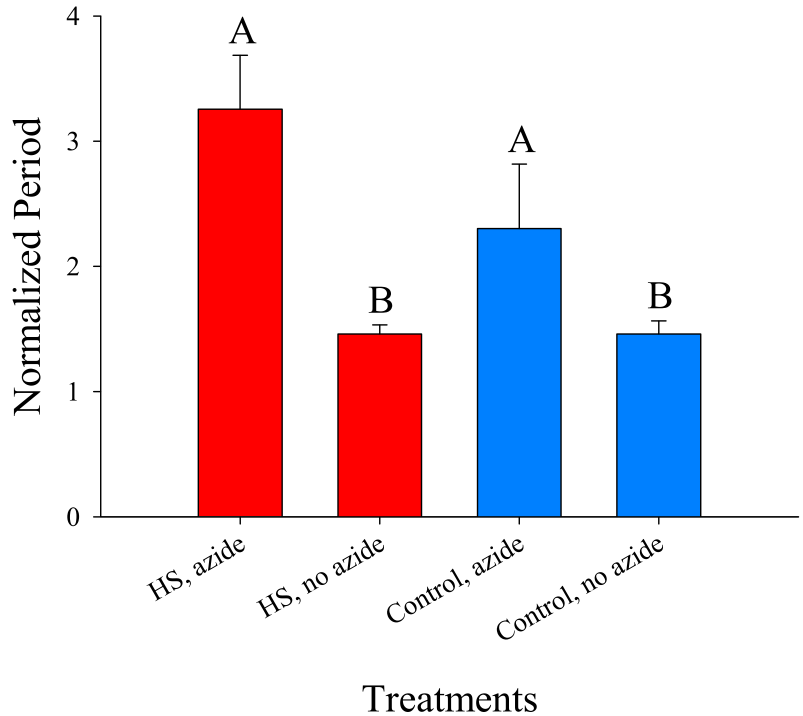

Figure 11: Normalized ventilatory muscle burst cycle period of control and HS-preconditioned locusts in normoxia and anoxia at 30 minutes of the experiment. No significant difference was found between control and HS-preconditioned animals in their normalized cycle period in normoxia (no azide) and anoxia (azide) (HS, azide vs. control, azide; and HS, no azide vs. control, no azide) which was indicated by the capitalized B's; however, a significant difference in the normalized period was observed between normoxia (no azide) and anoxia (azide) for both 2 groups of animals (control, azide vs. control, no azide; and HS, azide vs. HS, no azide) which was indicated by the capitalized A's and B's.

3.2 Na^+/K^+-ATPase activity assay

During my enzymatic activity assays, I investigated the total enzymatic activity of the Na^+/K^+-ATPase in control and HS-preconditioned animals under natural and chemical anoxia, as well as before, during and after coma. From these experiments, I found that there was no significant difference in the total enzymatic activity of the Na^+/K^+-ATPase in natural anoxia where the coma was induced by water immersion between chemical anoxia where the coma was induced by sodium azide bath-application (One Way ANOVA, factor: treatment (anoxic coma vs. natural coma): $P = 0.28$, $n = 51$) (Figure 12).

In addition, there was no significant difference in the total activity of the ATPase before, during, and after sodium azide-induced anoxic coma (Two Way ANOVA, factors: treatment (control vs. HS-preconditioning) and stages of coma (before, during, and after coma), the effects of coma: $P = 0.140$, $n = 48$). However, HS-preconditioning significantly increased the total Na^+/K^+-ATPase enzymatic activity (Two Way ANOVA: Unadjusted $P < 0.001$) (Figure 13).

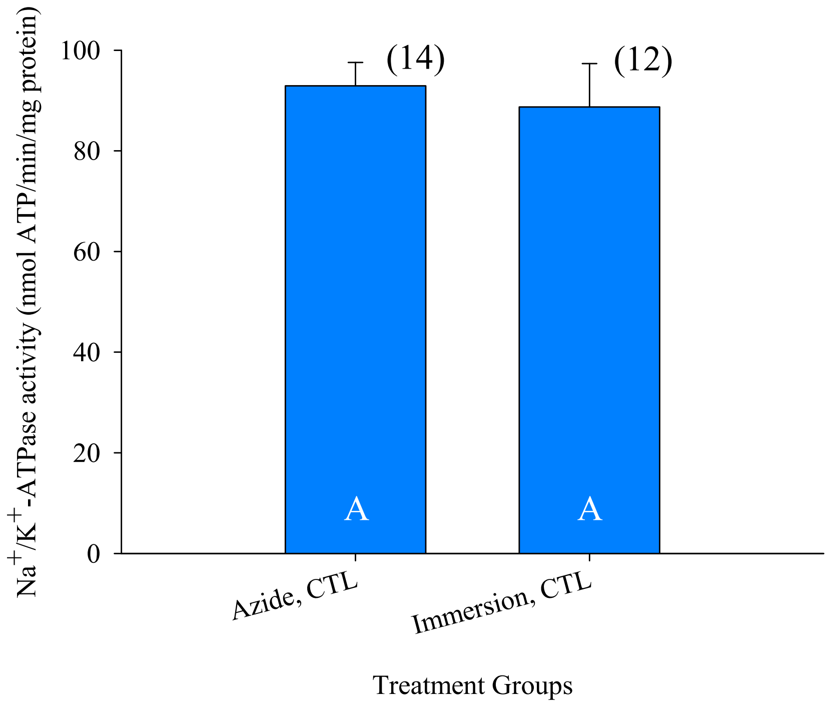

Figure 12: Activity of the Na$^+$/K$^+$-ATPase in control and HS-preconditioned animals under natural and chemical coma. No significant difference in the total activity of the Na$^+$/K$^+$-ATPase was observed in control (CTL) animals that were either under natural anoxia (Immersion) or chemical anoxia (Azide) as indicated by the letters, ($\bar{x} \pm$ SE).

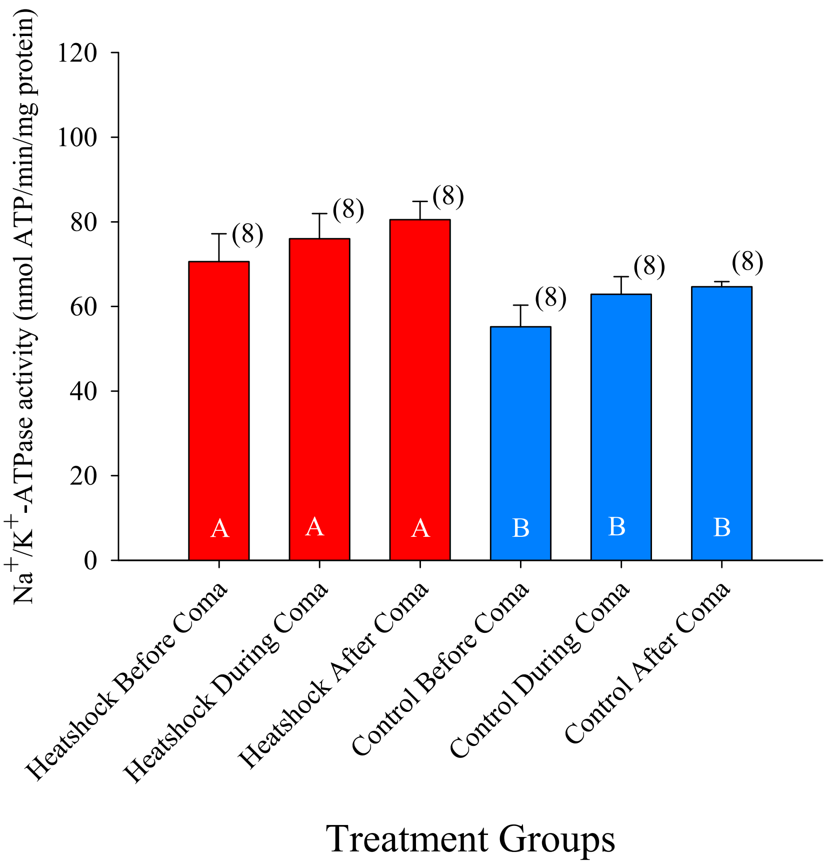

Figure 13: Activity of the Na^+/K^+-ATPase in control and HS-preconditioned animals before, during, and after chemical anoxic coma. No significant difference in the activity of the Na^+/K^+-ATPase was observed before, during or after sodium azide-induced chemical coma. However, there was a significantly higher Na^+/K^+-ATPase activity in HS-preconditioned animals which was indicated by the letters, ($\bar{x} \pm$ SE).

3.3 Immunohistochemistry for the localization of the Na^+/K^+-ATPase

The localization of the Na^+/K^+-ATPase was assessed via the ATPase fluorescence antibody staining intensity (Figure 14). The specificity of the secondary antibody was first confirmed in negative controls where the primary antibody was omitted, resulting in only faint background fluorescence (Figure 15). During the Na^+/K^+-ATPase antibody fluorescence analysis, only the cell cluster from the first abdominal ganglion of the metathoracic ganglionic mass was analyzed to minimize variables caused by different cells types (Figure 16). DAPI staining was used to confirm that the fluorescence intensity that was being analyzed was actually cells rather than artificial staining patterns (Figure 17).

During data analysis, three intact cells from separate locations that best represented the ganglion were randomly chosen from the cell cluster within the abdominal neuromere 1. The fluorescence staining intensity values were then recorded from 3 different locations of the cursor (intensity detection area = 1 pixel) on the cellular membranes as well as from the cytoplasm. Lastly, the membrane and cytoplasm fluorescence intensity values were normalized to their respective cellular nuclear intensity to eliminate staining variances that were caused by background fluorescence, or by the differences amongst animals (Figure 18).

When comparing the cytoplasmic Na^+/K^+-ATPase antibody fluorescence intensities, the data indicated that there was no significant difference between the control and HS-preconditioned locusts in their ATPase antibody fluorescence (Two Way ANOVA, factors: treatment (control vs. HS-preconditioning) and stages of coma (before, during, and after coma), $P = 0.13$, $n = 18$)

(Figure 19). In addition, no significant difference in the cytoplasmic ATPase antibody fluorescence was observed before, during, and after chemical anoxic coma in the two groups of animals (Two Way ANOVA, factors: treatment (control vs. HS-preconditioning) and stages of coma (before, during, and after coma), $P = 0.75$). Similarly, no significant difference was observed in the membrane Na^+/K^+-ATPase antibody fluorescence intensity before, during, or after anoxic coma (Two Way ANOVA, factors: treatment (control vs. HS-preconditioning) and stages of coma (before, during, and after coma), $P = 0.63$) (Figure 20).

However, when comparing the membrane Na^+/K^+-ATPase antibody fluorescence intensity with the cytoplasmic ATPase antibody intensity, a significantly higher membrane ATPase antibody fluorescence was observed in both control and HS-preconditioned animals (Two Way ANOVA, factors: treatment (control vs. HS-preconditioning) vs. location (membranes vs. cytoplasm), control membrane vs. control cytoplasm: Unadjusted $P = 0.013$; HS membrane vs. HS cytoplasm: Unadjusted $P < 0.001$) (Figure 21). Furthermore, the membrane of the HS-preconditioned animals exhibited an even higher Na^+/K^+-ATPase antibody florescence intensity compared to control locusts (Two Way ANOVA, factors treatment (control vs. HS-preconditioning) vs. location (membrane vs. cytoplasm): Unadjusted $P <0.001$).

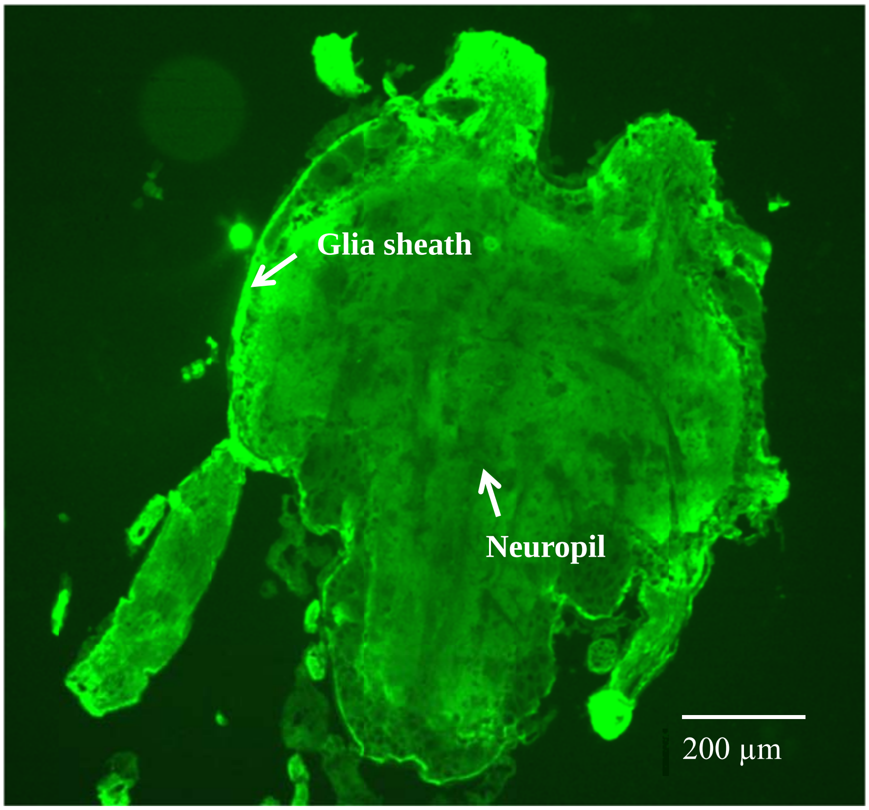

Figure 14: Example of an immunohistochemistry image of the metathoracic ganglion. The intense fluorescence of the glia sheath and neuropil from the metathoracic ganglion, where ion exchange and neuronal connection occurs is seen.

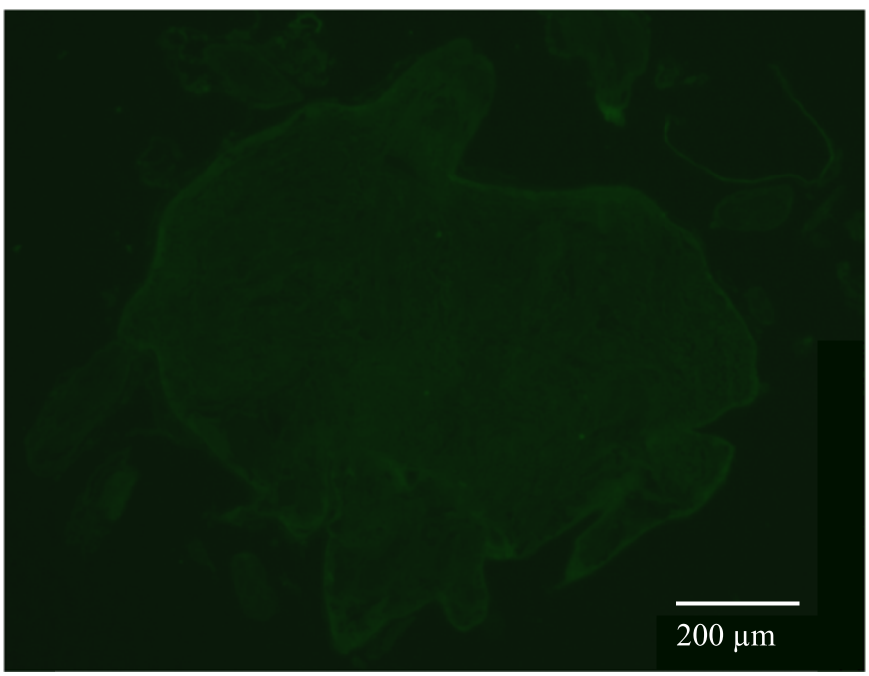

Figure 15: Example of a negative control immunohistochemistry image of the metathoracic ganglion. In negative controls where the primary antibody was omitted, the immunohistochemistry images only exhibited faint background fluorescence.

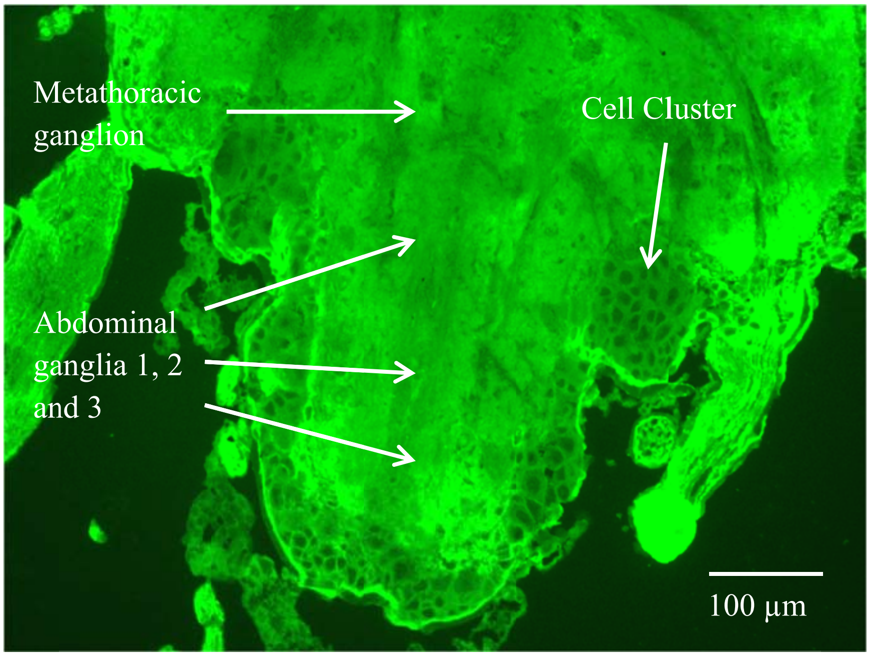

Figure 16: Example of an immunohistochemistry image of the metathoracic ganglion focusing on the posterior portion of the metathoracic ganglion and the 3 fused abdominal ganglia, used during analysis. This is a zoomed-in image from Figure 14. Only the cell cluster from the first abdominal ganglion of the metathoracic ganglionic mass was assessed. This was to eliminate ATPase concentration differences caused by cellular functions and locations.

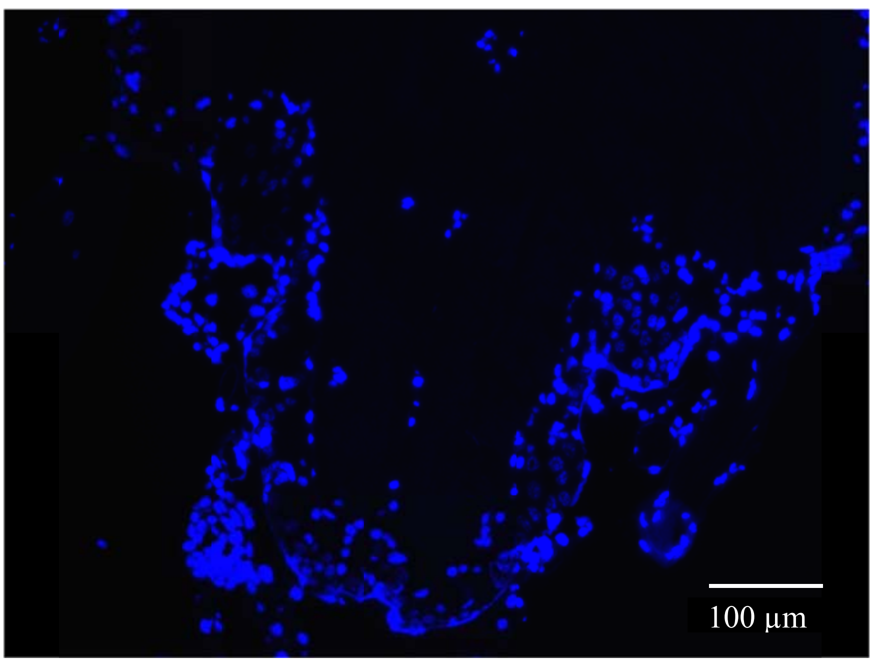

Figure 17: Example of a DAPI staining image focusing on the same posterior portion of the metathoracic ganglion and the 3 abdominal ganglia. DAPI stain was used to help identify and confirm the location and the nuclei of the cells.

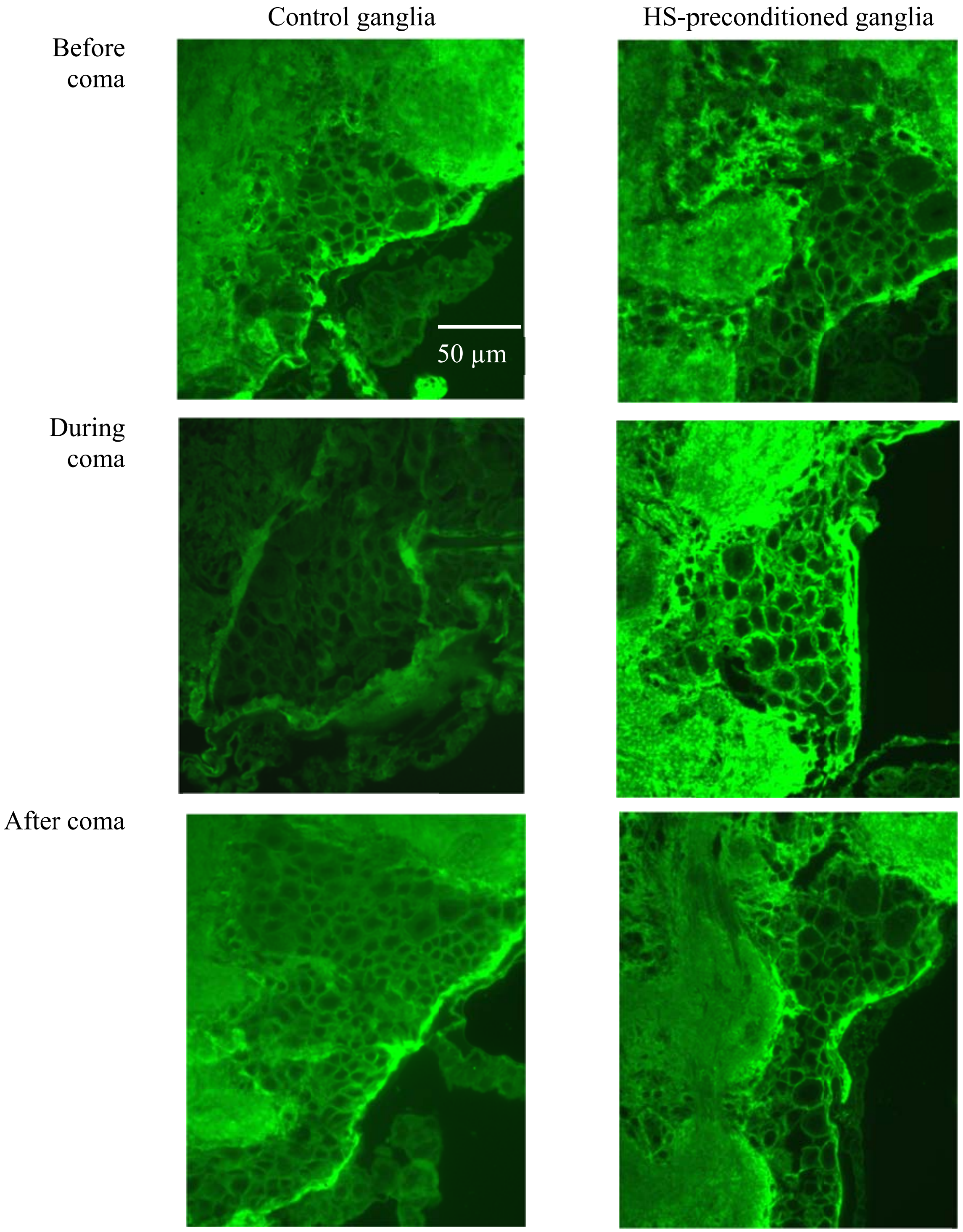

Figure 18: Immunohistochemistry images illustrating the Na^{+}/K^{+}-ATPase antibody fluorescence. These images illustrate the ATPase antibody in control and HS-preconditioned animals before, during, and after anoxia coma. The Na^{+}/K^{+}-ATPase antibody fluorescence from the cellular membranes and cytoplasm is clearly visible.

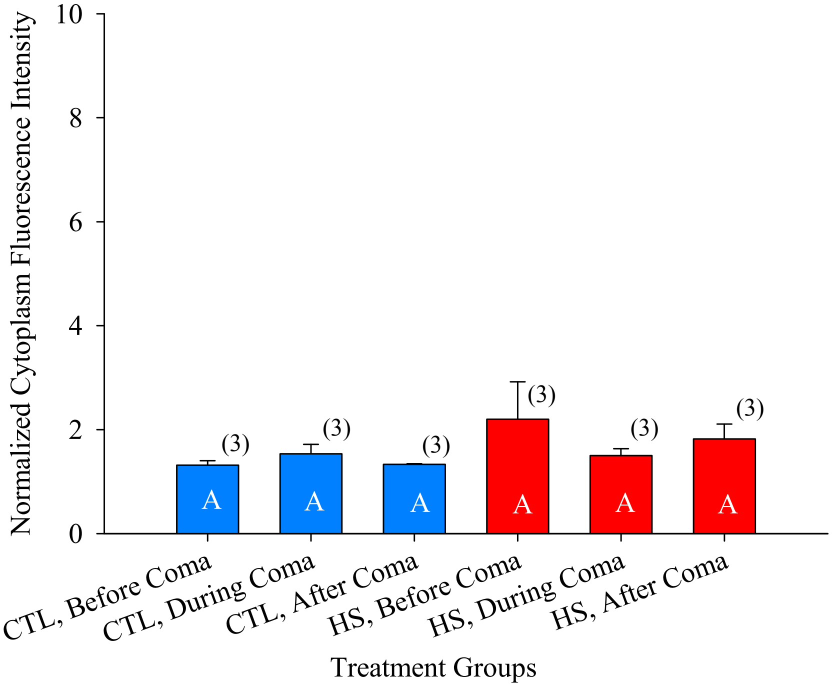

Figure 19: Neuronal cytoplasmic fluorescence intensity of the Na^+/K^+-ATPase antibody in control and HS-preconditioned animals before, during, and after coma. No significant difference was observed in the normalized cytoplasmic ATPase antibody fluorescence between control and HS-preconditioned animals. In addition, there was no significant difference in the normalized cytoplasmic ATPase antibody fluorescence before, during, or after anoxic coma as illustrated by the letters, ($\bar{x} \pm$ SE).

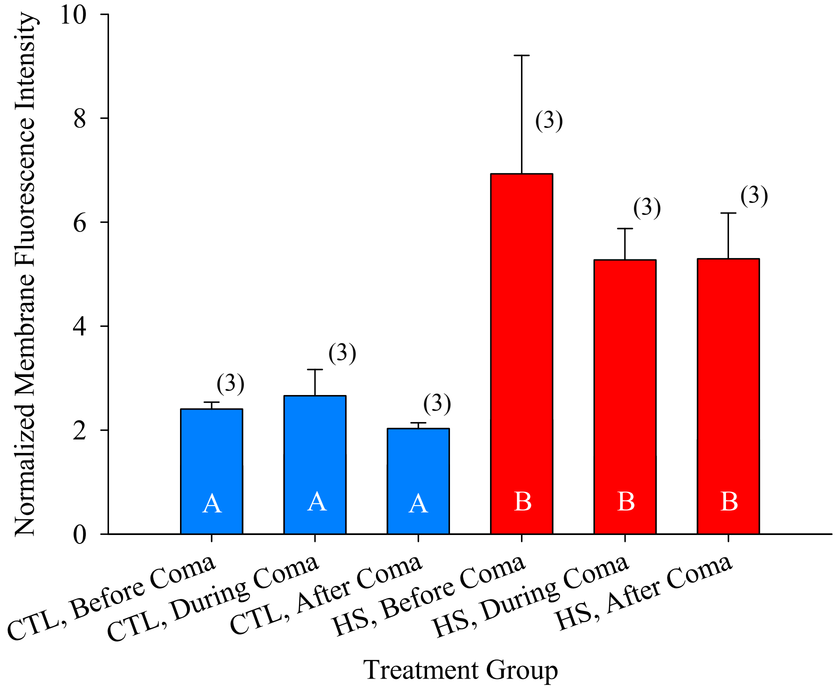

Figure 20: Neuronal membrane fluorescence intensity of the Na^+/K^+-ATPase antibody in control and HS-preconditioned animals before, during, and after coma. No significant difference was observed before, during, or after anoxic coma in the ATPase antibody fluorescence in the neuronal membrane between control and HS-preconditioned locusts. However, there was a significant difference in the membrane ATPase antibody fluorescence between control and HS-preconditioned animals which was shown using different letters, ($\bar{x} \pm$ SE).

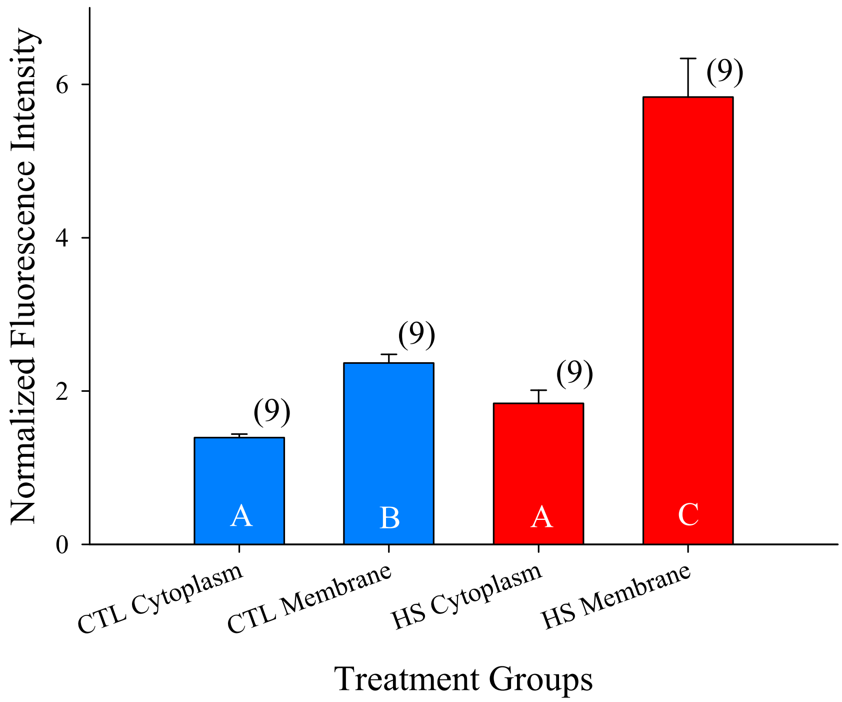

Figure 21: Neuronal membrane and cytoplasm fluorescence intensities of the Na^+/K^+-ATPase antibody in the control and HS-preconditioned locusts. No significant difference was observed in the cytoplasmic ATPase antibody intensity between control and HS-preconditioned animals which were shown using capitalized A's. However, the membranes exhibited a significantly higher ATPase antibody fluorescence in control and HS-preconditioned animals as shown using B and C. Furthermore, the membrane ATPase antibody fluorescence intensity was further increased in HS-preconditioned animals compared to control animals as illustrated by capitalized C, ($\bar{x} \pm$ SE).

Chapter 4: Discussion

Locusts enter a reversible coma that is associated with a rapid increase in the $[K^+]_o$ in their metathoracic ganglion during abiotic stresses. Recovery from this reversible coma is dependent on restoring the disrupted $[K^+]_o$ back to its baseline level, and a prior sub-lethal HS-preconditioning is able to grant locusts a temporary resilience to abiotic stresses such as hyperthermia or anoxia. This temporary HS-induced protection is accomplished through a mechanism which prolongs the functional $[K^+]_o$ by decreasing the rate of accumulation, and increasing the rate of clearance of the high $[K^+]_o$ during such reversible coma events (Rodgers *et al.*, 2007). However, the molecular mechanism which facilitates this temporary HS-induced protective mechanism is still unclear.

By studying the ventilatory motor pattern of control and HS-preconditioned locusts during normoxia, the duration of the electrophysiology experiments has found to significantly increase the ventilatory muscle burst duration and cycle period of the locust. In addition, the ventilatory muscle burst duration and the cycle period were also found to be significantly increased after chemical anoxia. However, the locusts were able to partially attenuate the increased ventilatory cycle period during recovery after anoxia but they were not able to attenuate the increased ventilatory muscle burst duration. Furthermore, by comparing the ventilatory motor pattern of control and HS-preconditioned locusts after recovery from anoxic coma, HS-preconditioned animals exhibited a longer ventilatory muscle burst duration and a lower ventilatory muscle burst cycle period.

Furthermore, from studying the total activity of the Na^+/K^+-ATPase, I concluded that there was no significant difference in the activity of ATPase between a natural coma induced by water immersion and a chemical coma that is induced by sodium azide. In addition, there was no significant difference on the total activity of the Na^+/K^+-ATPase before, during, and after anoxia coma. However, a significantly higher total Na^+/K^+-ATPase activity was observed in HS-preconditioned locusts.

Lastly, the Na^+/K^+-ATPase immunohistochemistry experiments suggested that there was no significant difference on the localization of the Na^+/K^+-ATPase before, during, and after coma. In addition, no significant difference on the localization of the Na^+/K^+-ATPase in the neuronal cytoplasm was observed between control and HS-preconditioned locusts. However, the localization experiments confirmed that the membrane Na^+/K^+-ATPase concentration was significantly greater than the cytoplasmic concentration. Moreover, a significantly greater membrane Na^+/K^+-ATPase concentration was observed in HS-preconditioned locusts than in control animals.

4.1 The duration of the experiment and HS-preconditioning affected the ventilatory motor pattern in locusts

The electrophysiology experiments illustrated that there was no significant difference in the ventilatory muscle burst duration and cycle period between control and HS-preconditioned animals during normoxia. However, the same data indicated that there was a significantly longer ventilatory muscle burst duration and cycle period at 30 minutes of the experiments compared to their initial ventilatory motor pattern in both groups of animals under normoxia.

This finding suggested that the ventilatory experiments or the semi-intact preparation utilized for this series of experiment could have affected the animals' ventilatory muscle burst duration and period. This effect could be caused by the damaged tracheal system after the semi-intact dissection, which would hinder the locusts' ability to obtain oxygen. In addition, since inspiration relies on elastic recoil created by the expiratory muscles in addition to the weak contractions from inspiratory muscles, this longer expiratory muscle burst duration could increase the magnitude of the elastic recoil and allowing the locust to inspire more oxygen.

In addition, this difference in the ventilatory motor pattern observed during normoxia could also be a result of a different ion concentration between the standard isotonic locust saline used during the experiments and the haemolymph of the locust. It has been found that the changes within extracellular volume caused by solute concentration, and different potassium concentrations in the standard isotonic locust saline can affect the ventilatory motor pattern of the locusts (Witiuk and Robertson, 2009; DePass and Robertson, 2011). Nevertheless, this increase in the ventilatory muscle burst duration and cycle period in normoxia equally affected control and HS-preconditioned locusts; hence, it is safe to compare the normalized ventilatory motor pattern between control and HS-preconditioned animals.

During the second portion of the electrophysiology experiments, I studied the ventilatory muscle pattern after recovery from anoxia. From these experiments, I found that both control and HS-preconditioned animals had significantly longer ventilatory muscle burst durations after recovery from anoxic coma. This longer ventilatory muscle burst duration, which could be a mechanism to increase the magnitude of the elastic recoil for inspiration, could be explained by a mechanism that has been observed in other insects where the metabolic rates were increased to above normoxic values after recovery from anoxia to pay off the 'oxygen debt' (Hoback *et al.*, 1998).

Moreover, anoxic coma also significantly increased the ventilatory cycle period in both control and HS-preconditioned locusts. This increase in the cycle period was significantly exacerbated in control animals immediately after their recovery from coma. However, the animals from both groups were able to partially attenuate this effect after 5 minutes recovery from coma since there was still a significantly longer cycle period after coma recovery after factoring in the effect of the semi-intact preparation which also increased the ventilatory cycle period. This attenuation suggests that both groups of animals attempted to compensate for their 'oxygen debt' by restoring their increased ventilatory muscle burst cycle period back to the baseline frequency. However, there was a difference between control and HS-preconditioned locusts in their ventilatory muscle burst cycle period attenuation. The attenuation did not occur in control locusts until 5 minutes after recovery, while HS-preconditioned started this restoration immediately after their recovery. This improved ventilatory muscle burst attenuation observed in HS-preconditioned animals could be a result of the additional HS proteins, produced from the prior sub-lethal HS-preconditioning, which might have increased the production rate of ventilatory pattern generating proteins by assisting in the folding of such proteins (Bukau *et al.*, 2000; Hartl and Hayer-Hartl, 2002) which would help HS-preconditioned locusts to pay off their 'oxygen debt' that they accumulated during anoxia.

Furthermore, the increase in the ventilatory muscle burst duration and cycle period after recovering from abiotic stress observed in this project has also been reported in previous experiments where after recovery from hyperthermia, the locusts also adopted longer ventilatory burst duration, and increased ventilatory burst period or lower ventilatory frequency (Newman *et al.*, 2003). Thus, it could be speculated that such an alternation in the ventilatory motor pattern might be a mechanism which allows the animals to emerge from coma and to attenuate the deleterious effects of an abiotic stress.

In conclusion, this series of the project implied that a sub-lethal HS-preconditioning could initiate a protective mechanism against anoxic coma by altering locusts' ventilatory motor pattern. More specifically, HS allowed locusts to have a more stable ventilatory muscle burst cycle period and a longer ventilatory muscle burst duration after recovery from anoxic coma. These two changes could be considered protective because they could allow HS-preconditioned locusts to obtain more air which consequently, would allow them to recover faster from anoxic coma.

4.2 Activity of the Na^+/K^+-ATPase was significantly higher after HS-preconditioning

Through the pyruvate kinase/lactate dehydrogenase assay, the total Na^+/K^+-ATPase activity experiments first confirmed that there was no significant difference in the protein activity when exposing the locusts to natural or chemical anoxic comas. This finding was expected because both comas inhibit the activity of the electron transport chain by preventing the oxygen/hydrogen coupling achieved by the ATP synthase, and therefore, equally halting aerobic respiration via a similar mechanism. Consequently, these experiments suggest that the effects of chemical and natural coma on restricting aerobic respiration through disrupting the electron transport chain are interchangeable, and a chemical coma can be used to simulate a natural coma. Nevertheless, only chemical comas were induced in the locusts throughout my project because chemical coma induced a more rapid response from the locusts and it can be applied more easily in a semi-intact electrophysiology experiment.

In addition, the Na^+/K^+-ATPase enzymatic activity assays also indicated that there was no significant difference in the protein activity before, during, and after coma in control and HS-preconditioned locusts. However, there was a significantly higher Na^+/K^+-ATPase activity after HS-preconditioning compared to control animals. This increase in the enzymatic activity could be a result of an increase in the affinity of the Na^+/K^+-ATPase for its substrates caused by the addition of cytoskeletal proteins (Ferrandi *et al.* 1999) that were produced during the prior HS-preconditioning. The increased Na^+/K^+-ATPase enzymatic activity after HS-preconditioning could cause this slower rate of accumulation and the increased rate of $[K^+]_o$ clearance observed in HS-preconditioned locusts.

Furthermore, even though a previous study has found no significant difference in the activity of the Na^+/K^+-ATPase between control and HS-preconditioned locusts (Rodgers *et al.*, 2007), my experiments demonstrated a significantly higher enzymatic activity in the ATPase after HS-preconditioning. This could be a result of the larger sample size I used in my experiments. By including the ATPase activity data from during and after anoxia coma groups, the Two Way ANOVA was able to indicate a significantly higher ATPase activity in the HS-preconditioned group.

In summary, from my Na^+/K^+-ATPase activity assays, I have confirmed that there was no significant difference between chemical and natural anoxic coma on the activity of the protein. However, there was a significantly higher Na^+/K^+-ATPase enzymatic from HS-preconditioned locusts than control animals.

4.3 Neuronal membranes contained a significantly higher concentration of the Na^+/K^+-ATPase than the cytoplasm, and the membrane ATPase concentration was significantly increased after HS-preconditioning

During my localization experiments, I used a Na^+/K^+-ATPase specific antibody to tag the protein, and a FITC secondary antibody to visualize the distribution of the ATPase. From these experiments, I have first confirmed the specificity of the secondary antibody, and from the rest of the immunohistochemistry images, I also noticed the intense Na^+/K^+-ATPase antibody fluorescence from the glial sheath, and the neuropil where high Na^+/K^+-ATPase concentration is expected to sustain ion homeostasis, and to conduct action potentials.

After analyzing the fluorescence intensity, as a control assessment, I confirmed that there was no significant difference in the cytoplasmic Na^+/K^+-ATPase antibody-fluorescence intensity from the metathoracic ganglia between control and HS-preconditioned locusts. However, there was a significantly higher ATPase antibody-fluorescence intensity within the neuronal membranes compared to the cytoplasmic intensity for both control and HS-preconditioned animals. This implies that there is no difference in the cytoplasmic ATP concentration between control and HS-preconditioned locusts but there is a higher membrane ATPase concentration in both groups of animals. This was anticipated because the Na^+/K^+-ATPase is an essential neuronal membrane protein that shuttles sodium and potassium ions across cellular membranes, if the Na^+/K^+-ATPase was a mechanism that improved the ability of HS-preconditioned animals in sustaining the correct $[K^+]_o$, the difference occur within the neuronal membranes.

Sequentially, after analysing the neuronal membrane antibody-fluorescence, even though the data suggested that there was no significant difference in the distribution of ATPase before, during, and after anoxic coma in control and HS-preconditioned locusts, a significantly higher Na^+/K^+-ATPase concentration within the neuronal membranes of the HS-preconditioned animals was found compared to the membrane Na^+/K^+-ATPase concentration of the control animals. This could be a result of a trafficking of the Na^+/K^+-ATPase which could be facilitated by a multi-protein hsp90/hsp70-based chaperone machinery (Pratt and Toft, 2003) that became abundant after the prior HS-preconditioning, where the concentration of the locust Hsp70 was shown to increase after a HS-preconditioning (Qin *et al.*, 2003). Thus, the decreased rate of accumulation and the faster $[K^+]_o$ clearance observed in HS-preconditioned locusts could be a result of a higher Na^+/K^+-ATPase concentration within the neuronal membranes, which will aid the restoration of the disrupted $[K^+]_o$ after an anoxic coma.

In summary, my immunohistochemistry experiments proposed that there was no significant difference in the cytoplasmic ATP distribution between control and HS-preconditioned animals. In addition, anoxia coma also did not cause a significant redistribution of the neuronal Na^+/K^+-ATPase in control and HS-preconditioned locusts. However, there was a significantly increased membrane Na^+/K^+-ATPase concentration from the HS-preconditioned animals compared to the control animals, which could be the result of a trafficking of the Na^+/K^+-ATPase.

4.4 Future directions

From my electrophysiology experiments, I proposed that the change in the ventilatory motor pattern during normoxia could be a result of different ion concentrations between the standard isotonic locust saline and the haemolymph of the locust. Alternatively, damaging the tracheal system during dissection could have also hindered locusts' ability to obtain oxygen, and forced them to compensate for the lack of oxygen with a different ventilatory motor pattern. To test the first hypothesis, we can investigate whether changes occurred in the $[K^+]_o$ within the metathoracic ganglion throughout a semi-intact electrophysiology experiment; since a nonisotonic locust saline would alter the ventilatory motor pattern of the locust. Also, to investigate whether hypoxia due to damaged tracheal system was the underlying cause for the different motor pattern, we can vary the oxygen concentrations in a closed-chamber and observe whether that had any effects on the ventilatory motor pattern over time.

In addition, based on the Na^+/K^+-ATPase activity and the localization data from this project, many other experiments can be conducted in the future to extend our knowledge of the role the Na^+/K^+-ATPase plays under abiotic stresses after a HS-preconditioning.

This project illustrated there was an increase in the total Na^+/K^+-ATPase activity after a sub-lethal HS-preconditioning; however, the actual mechanism behind this increase in the total activity is still unclear. Nevertheless, it has been demonstrated that the activity the Na^+/K^+-ATPase could be regulated by juvenile hormones, reversible protein phosphorylation, and its substrates (Gong *et al.*, 1994; McMullen and Storey, 2008; Hootman and Ernst, 1988). Thus future experiments could investigate the actual mechanism that was responsible for this increase in the total Na^+/K^+-ATPase after a HS-preconditioning. In addition, it has also been shown that HS proteins can form chaperone machinery complexes to regulate protein trafficking (Pratt and Toft, 2003). Therefore, the increased membrane concentration of the Na^+/K^+-ATPase after HS-preconditioning could be the result of an increased abundance in HS-chaperone protein machineries. This would increase the trafficking of the Na^+/K^+-ATPase onto neuronal membranes, and consequently increasing the total Na^+/K^+-ATPase activity.

To verify whether HS proteins were involved in this trafficking event, fluorescence resonance energy transfer (FRET) techniques could be utilized to observe if more HS proteins were interacted with the Na^+/K^+-ATPase after a HS-preconditioning to aid the trafficking of the ATPase. Furthermore, FRET could also be used to study whether cytoskeletal proteins are involved in the trafficking of the ATPase or in the increase in the activity of the Na^+/K^+-ATPase (Ferrandi *et al.* 1999).

Moreover, paraffin wax sectioning has proven to be a useful technique to study the localization and the trafficking of the Na^+/K^+-ATPase, however it might even more interesting to observe the trafficking of the Na^+/K^+-ATPase real-time in *in vitro* preparations using cell cultures. *in vitro* observations would allow us to study the Na^+/K^+-ATPase localization and its trafficking mechanism in much greater details and accuracies. For example, details that would be readily observed from *in vitro* preparations, such as a change in the trafficking rate, would not possibly be observed from paraffin sections. In addition, since paraffin slides would require tissues to be fixed, dehydrated, rehydrated, washed in xylene, and sectioned, all of which are invasive to the tissue, *in vivo* preparations would allow tissues to function in a more physiologically relevant state.

Last but not least, it could be interesting to investigate whether other ion channels are also involved in the locusts during the clearance of the $[K^+]_o$ following a reversible coma, and to see whether their functionality could also be affected by a prior HS-preconditioning. For example, it is known that the glia cells within the glia sheath of neurons play an important role in ion exchange. Also, different gap junctions would be targeted by various mechanisms to regulate ion homeostasis.

In summary, even though this project demonstrated that the ventilatory motor pattern, the activity and the localization of the Na^+/K^+-ATPase of the locust are altered after a prior HS-preconditioning, it is still unclear as to through which pathways did the HS-preconditioning alter that improved the physiological and molecular performance of the locust. Therefore, by conducting further studies in these areas, we could reveal such HS-induced mechanisms that would change the functioning of the locust and help us to better understand the effects of HS-preconditioning would have on locusts.

4.5 Conclusion

It is impossible to live in a world without facing climatic elements. However, organisms have evolved different mechanisms to cope with the forever changing environment. Locusts are able to enter a reversible coma during hyperthermia and anoxia, and recover from this coma once the stressor is past (Rodgers *et al.*, 2007). Exposing locusts to a prior HS-preconditioning has been shown to allow the locust to become more resilient to the subsequent hyperthermia or anoxia. This makes an important adaptive mechanism for the locust because it allowed the organism to survive the more severe abiotic stress after experiencing a prior sub-lethal stress.

In addition, this project demonstrated that HS-preconditioning was also able to significantly change the ventilatory motor pattern of the locusts by increasing the ventilatory muscle burst duration and decreasing the ventilatory muscle cycle periods, both of which could allow the animals to inspire more oxygen. Furthermore, my project has also illustrated that there is also an increase in the total Na^+/K^+-ATPase activity and an increased membrane Na^+/K^+-ATPase concentration after a HS-preconditioning which would allow the neurons from HS-preconditioned animals to become more competent and more efficient in maintaining the $[K^+]_o$ and in restoring the $[K^+]_o$ baseline level.

In conclusion, my project has revealed some of the mechanisms that the locust has developed through fine tuning the activity and localization of the Na^+/K^+-ATPase to allow them to cope with abiotic stresses. In the end, even though there is not much one can do about bad weathers except to carry an umbrella; through evolution, organisms have nevertheless developed various mechanisms to cope with those undesirable, however unavoidable, inclement weathers.

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Appendix 1

Two Way Repeated Measures ANOVA (Two Factor Repetition) for Ventilatory Muscle Burst Duration under Normoxia

The difference in the mean values among the different levels of Treatment (HS/CTL) is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Time. There is not a statistically significant difference ($P = 0.489$).

The difference in the mean values among the different levels of Time is greater than would be expected by chance after allowing for effects of differences in Treatment (HS/CTL). There is a statistically significant difference ($P = <0.001$). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Treatment (HS/CTL) does not depend on what level of Time is present. There is not a statistically significant interaction between Treatment (HS/CTL) and Time. ($P = 0.303$)

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Treatment (HS/CTL)**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
HS vs. Control	0.131	0.721	0.489	0.050	No

Comparisons for factor: **Time**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
30 mins vs. Initial	0.568	4.230	<0.001	0.009	Yes
20 mins vs. Initial	0.485	3.610	0.001	0.010	Yes
30 mins vs. 10 mins	0.380	2.833	0.009	0.013	Yes
20 mins vs. 10 mins	0.297	2.213	0.036	0.017	No
10 mins vs. Initial	0.188	1.398	0.174	0.025	No
30 mins vs. 20 mins	0.0832	0.620	0.541	0.050	No

Comparisons for factor: **Time within HS**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
20 mins vs. Initial	0.680	3.765	<0.001	0.009	Yes
30 mins vs. Initial	0.648	3.588	<0.001	0.010	Yes
20 mins vs. 10 mins	0.508	2.811	0.007	0.013	Yes

30 mins vs. 10 mins	0.476	2.634	0.011	0.017	Yes
10 mins vs. Initial	0.172	0.954	0.344	0.025	No
20 mins vs. 30 mins	0.0320	0.177	0.860	0.050	No

Comparisons for factor: **Time within Control**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
30 mins vs. Initial	0.487	2.696	0.009	0.009	No
20 mins vs. Initial	0.289	1.598	0.116	0.010	No
30 mins vs. 10 mins	0.284	1.574	0.121	0.013	No
10 mins vs. Initial	0.203	1.122	0.267	0.017	No
30 mins vs. 20 mins	0.198	1.098	0.277	0.025	No
20 mins vs. 10 mins	0.0860	0.476	0.636	0.050	No

Comparisons for factor: **Treatment (HS/CTL) within Initial**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
HS vs. Control	0.000	0.000	1.000	0.050	No

Comparisons for factor: **Treatment (HS/CTL) within 10 mins**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
Control vs. HS	0.0304	0.130	0.898	0.050	No

Comparisons for factor: **Treatment (HS/CTL) within 20 mins**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
HS vs. Control	0.392	1.673	0.109	0.050	No

Comparisons for factor: **Treatment (HS/CTL) within 30 mins**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
HS vs. Control	0.161	0.689	0.498	0.050	No

Appendix 2

Two Way Repeated Measures ANOVA (Two Factor Repetition) for Ventilatory Muscle Burst Cycle Period under Normoxia

The difference in the mean values among the different levels of Treatment (HS/CTL) is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Time. There is not a statistically significant difference (P = 0.641).

The difference in the mean values among the different levels of Time is greater than would be expected by chance after allowing for effects of differences in Treatment (HS/CTL). There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Treatment (HS/CTL) does not depend on what level of Time is present. There is not a statistically significant interaction between Treatment (HS/CTL) and Time. (P = 0.800)

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Treatment (HS/CTL)**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
HS vs. Control	0.0354	0.483	0.641	0.050	No

Comparisons for factor: **Time**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
30 mins vs. Initial	0.459	8.202	<0.001	0.009	Yes
20 mins vs. Initial	0.353	6.308	<0.001	0.010	Yes
30 mins vs. 10 mins	0.306	5.464	<0.001	0.013	Yes
20 mins vs. 10 mins	0.200	3.571	0.001	0.017	Yes
10 mins vs. Initial	0.153	2.737	0.011	0.025	Yes
30 mins vs. 20 mins	0.106	1.894	0.069	0.050	No

Comparisons for factor: **Time within HS**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
30 mins vs. Initial	0.459	6.055	<0.001	0.009	Yes
20 mins vs. Initial	0.393	5.189	<0.001	0.010	Yes
30 mins vs. 10 mins	0.275	3.627	<0.001	0.013	Yes
20 mins vs. 10 mins	0.209	2.761	0.008	0.017	Yes

10 mins vs. Initial	0.184	2.428	0.019	0.025	Yes
30 mins vs. 20 mins	0.0656	0.866	0.390	0.050	No

Comparisons for factor: **Time within Control**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
30 mins vs. Initial	0.459	6.060	<0.001	0.009	Yes
30 mins vs. 10 mins	0.337	4.445	<0.001	0.010	Yes
20 mins vs. Initial	0.313	4.129	<0.001	0.013	Yes
20 mins vs. 10 mins	0.190	2.514	0.015	0.017	Yes
30 mins vs. 20 mins	0.146	1.931	0.059	0.025	No
10 mins vs. Initial	0.122	1.615	0.112	0.050	No

Comparisons for factor: **Treatment (HS/CTL) within Initial**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
HS vs. Control	0.000	0.000	1.000	0.050	No

Comparisons for factor: **Treatment (HS/CTL) within 10 mins**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
HS vs. Control	0.0616	0.639	0.529	0.050	No

Comparisons for factor: **Treatment (HS/CTL) within 20 mins**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
HS vs. Control	0.0804	0.834	0.413	0.050	No

Comparisons for factor: **Treatment (HS/CTL) within 30 mins**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
Control vs. HS	0.000346	0.00359	0.997	0.050	No

Appendix 3

Two Way Repeated Measures ANOVA (Two Factor Repetition) for Ventilatory Muscle Burst Duration after Anoxia

The difference in the mean values among the different levels of Treatment (HS/CTL) is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Time. There is not a statistically significant difference ($P = 0.145$).

The difference in the mean values among the different levels of Time is greater than would be expected by chance after allowing for effects of differences in Treatment (HS/CTL). There is a statistically significant difference ($P = <0.001$). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Treatment (HS/CTL) does not depend on what level of Time is present. There is not a statistically significant interaction between Treatment (HS/CTL) and Time. ($P = 0.709$)

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Treatment (HS/CTL)**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
HS vs. Control	0.552	1.596	0.145	0.050	No

Comparisons for factor: **Time**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
15 mins vs. Baseline	2.321	6.264	<0.001	0.003	Yes
10 mins vs. Baseline	2.204	5.949	<0.001	0.004	Yes
5 mins vs. Baseline	2.159	5.827	<0.001	0.004	Yes
20 mins vs. Baseline	2.058	5.554	<0.001	0.004	Yes
15 mins vs. 0 mins	1.781	4.806	<0.001	0.005	Yes
10 mins vs. 0 mins	1.664	4.491	<0.001	0.005	Yes
5 mins vs. 0 mins	1.619	4.368	<0.001	0.006	Yes
20 mins vs. 0 mins	1.518	4.096	<0.001	0.006	Yes
0 mins vs. Baseline	0.540	1.458	0.152	0.007	No
15 mins vs. 20 mins	0.263	0.710	0.482	0.009	No
15 mins vs. 5 mins	0.162	0.437	0.664	0.010	No
10 mins vs. 20 mins	0.146	0.395	0.695	0.013	No
15 mins vs. 10 mins	0.117	0.315	0.754	0.017	No

5 mins vs. 20 mins	0.101	0.273	0.786	0.025	No
10 mins vs. 5 mins	0.0453	0.122	0.903	0.050	No

Comparisons for factor: **Time within HS**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
15 mins vs. Baseline	2.941	5.359	<0.001	0.003	Yes
10 mins vs. Baseline	2.581	4.703	<0.001	0.004	Yes
20 mins vs. Baseline	2.411	4.394	<0.001	0.004	Yes
5 mins vs. Baseline	2.306	4.203	<0.001	0.004	Yes
15 mins vs. 0 mins	2.241	4.083	<0.001	0.005	Yes
10 mins vs. 0 mins	1.881	3.427	<0.001	0.005	Yes
20 mins vs. 0 mins	1.711	3.118	0.002	0.006	Yes
5 mins vs. 0 mins	1.606	2.927	0.004	0.006	Yes
0 mins vs. Baseline	0.700	1.276	0.205	0.007	No
15 mins vs. 5 mins	0.635	1.156	0.251	0.009	No
15 mins vs. 20 mins	0.529	0.965	0.337	0.010	No
15 mins vs. 10 mins	0.360	0.656	0.513	0.013	No
10 mins vs. 5 mins	0.274	0.500	0.618	0.017	No
10 mins vs. 20 mins	0.169	0.308	0.758	0.025	No
20 mins vs. 5 mins	0.105	0.192	0.848	0.050	No

Comparisons for factor: **Time within Control**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
5 mins vs. Baseline	2.011	3.665	<0.001	0.003	Yes
10 mins vs. Baseline	1.828	3.330	0.001	0.004	Yes
20 mins vs. Baseline	1.704	3.106	0.003	0.004	Yes
15 mins vs. Baseline	1.701	3.099	0.003	0.004	Yes
5 mins vs. 0 mins	1.631	2.972	0.004	0.005	Yes
10 mins vs. 0 mins	1.447	2.637	0.010	0.005	No
20 mins vs. 0 mins	1.324	2.412	0.018	0.006	No
15 mins vs. 0 mins	1.320	2.406	0.018	0.006	No
0 mins vs. Baseline	0.380	0.693	0.490	0.007	No
5 mins vs. 15 mins	0.311	0.566	0.573	0.009	No
5 mins vs. 20 mins	0.307	0.560	0.577	0.010	No
5 mins vs. 10 mins	0.184	0.335	0.738	0.013	No
10 mins vs. 15 mins	0.127	0.231	0.818	0.017	No
10 mins vs. 20 mins	0.123	0.225	0.823	0.025	No
20 mins vs. 15 mins	0.00349	0.00636	0.995	0.050	No

Comparisons for factor: **Treatment (HS/CTL) within Baseline**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
HS vs. Control	0.000	0.000	1.000	0.050	No

Comparisons for factor: **Treatment (HS/CTL) within 0 mins**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
HS vs. Control	0.320	0.510	0.612	0.050	No

Comparisons for factor: **Treatment (HS/CTL) within 5 mins**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
HS vs. Control	0.295	0.470	0.640	0.050	No

Comparisons for factor: **Treatment (HS/CTL) within 10 mins**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
HS vs. Control	0.753	1.202	0.235	0.050	No

Comparisons for factor: **Treatment (HS/CTL) within 15 mins**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
HS vs. Control	1.240	1.978	0.054	0.050	No

Comparisons for factor: **Treatment (HS/CTL) within 20 mins**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
HS vs. Control	0.707	1.128	0.265	0.050	No

Appendix 4

Two Way Repeated Measures ANOVA (Two Factor Repetition) for Ventilatory Muscle Burst Cycle Period after Anoxia

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of Treatment (HS/CTL) depends on what level of Time is present. There is a statistically significant interaction between Treatment (HS/CTL) and Time. (P = 0.011)

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Treatment (HS/CTL)**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
Control vs. HS	0.248	0.572	0.581	0.050	No

Comparisons for factor: **Time**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
0 mins vs. Baseline	4.155	7.787	<0.001	0.003	Yes
0 mins vs. 20 mins	2.376	4.453	<0.001	0.004	Yes
5 mins vs. Baseline	2.358	4.419	<0.001	0.004	Yes
0 mins vs. 10 mins	2.183	4.091	<0.001	0.004	Yes
0 mins vs. 15 mins	2.118	3.969	<0.001	0.005	Yes
15 mins vs. Baseline	2.037	3.818	<0.001	0.005	Yes
10 mins vs. Baseline	1.972	3.696	<0.001	0.006	Yes
0 mins vs. 5 mins	1.797	3.368	0.002	0.006	Yes
20 mins vs. Baseline	1.779	3.334	0.002	0.007	Yes
5 mins vs. 20 mins	0.579	1.085	0.284	0.009	No
5 mins vs. 10 mins	0.386	0.723	0.473	0.010	No
5 mins vs. 15 mins	0.321	0.601	0.551	0.013	No
15 mins vs. 20 mins	0.258	0.484	0.631	0.017	No
10 mins vs. 20 mins	0.193	0.362	0.719	0.025	No
15 mins vs. 10 mins	0.0651	0.122	0.903	0.050	No

Comparisons for factor: **Time within HS**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
0 mins vs. Baseline	2.562	3.242	0.002	0.003	Yes

15 mins vs. Baseline	2.334	2.954	0.004	0.004	No
20 mins vs. Baseline	2.255	2.854	0.005	0.004	No
10 mins vs. Baseline	2.239	2.834	0.006	0.004	No
5 mins vs. Baseline	2.166	2.742	0.007	0.005	No
0 mins vs. 5 mins	0.395	0.500	0.618	0.005	No
0 mins vs. 10 mins	0.323	0.408	0.684	0.006	No
0 mins vs. 20 mins	0.307	0.388	0.699	0.006	No
0 mins vs. 15 mins	0.227	0.288	0.774	0.007	No
15 mins vs. 5 mins	0.168	0.213	0.832	0.009	No
15 mins vs. 10 mins	0.0952	0.121	0.904	0.010	No
20 mins vs. 5 mins	0.0887	0.112	0.911	0.013	No
15 mins vs. 20 mins	0.0793	0.100	0.920	0.017	No
10 mins vs. 5 mins	0.0728	0.0921	0.927	0.025	No
20 mins vs. 10 mins	0.0159	0.0201	0.984	0.050	No

Comparisons for factor: **Time within Control**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
0 mins vs. Baseline	5.749	7.276	<0.001	0.003	Yes
0 mins vs. 20 mins	4.446	5.627	<0.001	0.004	Yes
0 mins vs. 10 mins	4.044	5.118	<0.001	0.004	Yes
0 mins vs. 15 mins	4.009	5.073	<0.001	0.004	Yes
0 mins vs. 5 mins	3.199	4.049	<0.001	0.005	Yes
5 mins vs. Baseline	2.550	3.227	0.002	0.005	Yes
15 mins vs. Baseline	1.740	2.202	0.030	0.006	No
10 mins vs. Baseline	1.705	2.158	0.034	0.006	No
20 mins vs. Baseline	1.303	1.649	0.103	0.007	No
5 mins vs. 20 mins	1.247	1.578	0.118	0.009	No
5 mins vs. 10 mins	0.845	1.069	0.288	0.010	No
5 mins vs. 15 mins	0.810	1.025	0.308	0.013	No
15 mins vs. 20 mins	0.437	0.553	0.581	0.017	No
10 mins vs. 20 mins	0.402	0.509	0.612	0.025	No
15 mins vs. 10 mins	0.0350	0.0443	0.965	0.050	No

Comparisons for factor: **Treatment (HS/CTL) within Baseline**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
HS vs. Control	0.000	0.000	1.000	0.050	No

Comparisons for factor: **Treatment (HS/CTL) within 0 mins**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
Control vs. HS	3.187	3.669	<0.001	0.050	Yes

Comparisons for factor: **Treatment (HS/CTL) within 5 mins**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
Control vs. HS	0.384	0.442	0.661	0.050	No

Comparisons for factor: **Treatment (HS/CTL) within 10 mins**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
HS vs. Control	0.534	0.615	0.542	0.050	No

Comparisons for factor: **Treatment (HS/CTL) within 15 mins**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
HS vs. Control	0.594	0.684	0.497	0.050	No

Comparisons for factor: **Treatment (HS/CTL) within 20 mins**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
HS vs. Control	0.952	1.096	0.278	0.050	No

Appendix 5

Two Way Analysis of Variance for the Na^+/P^+-ATPase Enzymatic Activity

The difference in the mean values among the different levels of Treatment is greater than would be expected by chance after allowing for effects of differences in Coma. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Coma is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Treatment. There is not a statistically significant difference (P = 0.140).

The effect of different levels of Treatment does not depend on what level of Coma is present. There is not a statistically significant interaction between Treatment and Coma. (P = 0.955)

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Treatment**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
Heatshock vs. Control	14.788	3.718	<0.001	0.050	Yes

Comparisons for factor: **Coma**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
After Coma vs. Before Coma	9.693	1.990	0.053	0.017	No
During Coma vs. Before Coma	6.556	1.346	0.186	0.025	No
After Coma vs. During Coma	3.136	0.644	0.523	0.050	No

Comparisons for factor: **Coma within Heatshock**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
After Coma vs. Before Coma	9.912	1.439	0.158	0.017	No
During Coma vs. Before Coma	5.404	0.784	0.437	0.025	No
After Coma vs. During Coma	4.508	0.654	0.516	0.050	No

Comparisons for factor: **Coma within Control**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
After Coma vs. Before Coma	9.474	1.375	0.176	0.017	No
During Coma vs. Before Coma	7.709	1.119	0.270	0.025	No

After Coma vs. During Coma	1.765	0.256	0.799	0.050	No

Comparisons for factor: **Treatment within Before Coma**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
Heatshock vs. Control	15.410	2.237	0.031	0.050	Yes

Comparisons for factor: **Treatment within During Coma**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
Heatshock vs. Control	13.105	1.902	0.064	0.050	No

Comparisons for factor: **Treatment within After Coma**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
Heatshock vs. Control	15.849	2.300	0.026	0.050	Yes

Appendix 6

Two Way Analysis of Variance for the Na^+/K^+-ATPase Immunofluorescence Intensity

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of Treatment depends on what level of Location is present. There is a statistically significant interaction between Treatment and Location. (P = <0.001)

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Location within Control**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
membrane vs. cytoplasm	0.972	2.517	0.013	0.050	Yes

Comparisons for factor: **Location within HS**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
membrane vs. cytoplasm	3.995	10.343	<0.001	0.050	Yes

Comparisons for factor: **Treatment within cytoplasm**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
HS vs. Control	0.446	1.154	0.251	0.050	No

Comparisons for factor: **Treatment within membrane**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
HS vs. Control	3.468	8.980	<0.001	0.050	Yes