PROTEOMIC ANALYSIS OF THE HEAT SHOCK RESPONSE IN THE NERVOUS SYSTEM OF *LOCUSTA MIGRATORIA* 

by 

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Abstract

There is a thermal range for the operation of neural circuits beyond which nervous system function is compromised. Poikilotherms are particularly vulnerable to thermal stress, since their body temperature can fluctuate with ambient temperature. Animals that experience frequent hyperthermia have various coping mechanisms such as the thermoprotective effect of a prior exposure to sublethal temperatures (heat shock response). The molecular mechanisms of this thermoprotection have yet to be understood. This project studies the changes in protein expression in the nervous system of gregarious Locusta migratoria subjected to heat shock. For this purpose, proteins were extracted from metathoracic ganglia (MTG) by different methods and a proteomic map was subsequently obtained by 2-D gel electrophoresis which was compared between control (CON) and heat-shocked (HS) animals. Additionally, the localization pattern of Hsp70 was studied in the MTG of CON and HS gregarious locusts. Although 2-D gels showed changes in the amount of different isoforms of ATP-synthase β, the overall amount of this protein subunit was found to be unchanged. My experiments also revealed no significant change in the distribution of Hsp70 in the MTG of locusts caused by HS. However, new findings show that this protein is constitutively expressed at higher levels in perineurium, glia and tracheal cells than in neurons. In separate experiments, isolated locusts were also examined in order to measure any stress-associated increase of Hsp70 in the tissues of animals not previously exposed to crowding pressure. Quantitative western blots did not show a consistent change of the Hsp70 level in the MTG of isolated locusts following heat shock. Results of my research suggest that the change in the protein profile of the metathoracic ganglion following heat shock, if it exists, is subtle or occurs in very low-abundance proteins whose monitoring requires the application of special techniques. Alternatively, the thermoprotective effect of heat shock on the nervous system might
be promoted through other pathways which can change the protein activity at the post-translational level and may work independently from protein synthesis.
Acknowledgements

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<th>Description</th>
</tr>
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<tbody>
<tr>
<td>[K⁺]₀</td>
<td>Extracellular potassium concentration</td>
</tr>
<tr>
<td>AC.</td>
<td>Accession number</td>
</tr>
<tr>
<td>ACTD</td>
<td>Actinomycin D</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone fragment 18-39 human</td>
</tr>
<tr>
<td>AN</td>
<td>Anoxia</td>
</tr>
<tr>
<td>Angl</td>
<td>Angiopoietin1 human</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ASB-14</td>
<td>Amidosulfobetaine-14</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Cat. No.</td>
<td>Catalogue number</td>
</tr>
<tr>
<td>CBB</td>
<td>Coomassie brilliant blue</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>CHCA</td>
<td>α-cyano-4-hydroxycinnamic acid</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>Chloride ion</td>
</tr>
<tr>
<td>CON</td>
<td>Control</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>CSR</td>
<td>Cell stress response</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCMD</td>
<td>Descending contralateral movement detector</td>
</tr>
<tr>
<td>DHB</td>
<td>2,5-dihydroxybenzoic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E/C</td>
<td>Elytra/head capsule ratio</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>F/C</td>
<td>Femur/head capsule ratio</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GFP</td>
<td>([\text{Glu}^1])-Fibrinopeptide B human</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HS</td>
<td>Heat shock</td>
</tr>
<tr>
<td>Hsc70</td>
<td>Heat shock cognate 70</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IPG</td>
<td>Immobilized pH gradient</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>kD</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LC/MS/MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>ms</td>
<td>Millisecond</td>
</tr>
<tr>
<td>MTG</td>
<td>Metathoracic ganglion</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium ion</td>
</tr>
<tr>
<td>NCBI</td>
<td>National center for biotechnology information</td>
</tr>
<tr>
<td>OA</td>
<td>Octopamine</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>PBS+ Triton X-100</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>pmol</td>
<td>Picomole</td>
</tr>
<tr>
<td>ppm</td>
<td>Part per million</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>Q-TOF</td>
<td>Quadrupole-time-of-flight tandem mass spectrometry</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RMP</td>
<td>Resting membrane potential</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SB 3-10</td>
<td>Sulfobetaine 3-10</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>sHsps</td>
<td>Small heat shock proteins</td>
</tr>
<tr>
<td>TBP</td>
<td>Tributlyphosphine</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffer saline</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TEA</td>
<td>Tetraethylammonium</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction and Literature Review

Organisms face a variety of stresses in their environment such as ionizing radiation, osmotic stress, thermal shock, hypoxia and starvation. These stressors, if extreme, can imperil cell survival by damaging macromolecules including proteins, membrane lipids and DNA. Other threats arise from elevated amounts of reactive oxygen species (ROS) in the cell following stress. The cell stress response (CSR) consists of many interconnected events which repair damaged macromolecules, restore oxidative potential, direct the energy toward stress associated pathways and induce apoptosis (programmed cell death) in terminally damaged cells. Cells can often be protected against deleterious effects of stress by pre-exposure to sublethal doses of the same stress. Moreover, one form of stress can often protect the cells against other forms (cross-tolerance), since CSR is defined more by the damage occurring in cells than the nature of stress per se (Kultz, 2005).

The nervous system function is particularly vulnerable to high temperatures since it engages in a behavior-altering physiological response before the stress is potent enough to kill the cells (Robertson, 2004b). Unlike the cellular stress response which is widely studied, the nervous system response to stress and the adaptive mechanisms employed by animals to protect their neural functions in a stress-prone environment are less understood at the molecular level.

1.1 Classic heat shock response

It has been observed in many organisms that cells respond to stressors by increasing the expression of a set of highly conserved proteins, named heat shock proteins (Hsps). The response
was first discovered in *Drosophila* where a consistent puffing pattern was observed in the chromosomes of salivary glands after heat shock. This was the result of active transcription of certain genes in these regions of chromosomes and was called *heat shock response* (Ritossa, 1962). Later studies showed that those genes are also induced by other physiological insults and this response is strikingly conserved across species. The involvement of Hsps in the *heat shock response* is evidenced by three sets of observations (Parsell *et al.*, 1993):

1- The over-expression of Hsps following a stressful condition, in many organisms, is rapid and strong which is perceived as the emergency of response. For example, the striking increase in the synthesis of Hsp70 up to 1000-fold is reported in *Drosophila* cells after 45 minutes heat shock.

2- Although the temperatures at which Hsps are induced vary among organisms, they are correlated to the temperature limits for the survival of animals living in different environments.

3- Exogenous increase of these proteins is correlated with an improved resistance to hyperthermia as well as other overlapping environmental stresses.

Hsps assist unfolded proteins to regain their functional structure and as a result are called molecular chaperones. A short exposure to sub-lethal temperature is enough to induce synthesis of these chaperones which will then protect cells against more severe stresses in the future; this phenomenon is called *induced thermotolerance*. Hsps consist of a multigenic family of proteins which are named according to molecular size. Although Hsps have not been consistently classified in different literatures, Hsp100, Hsp90, Hsp70, Hsp60 (chaperonin) and Hsp40 as well as the small heat shock proteins (sHsps) are the most commonly mentioned ones. Ubiquitin and many other proteins whose involvement in heat shock response is more organism- or tissue-
specific and less characterized than the classical Hsps are occasionally discussed along with Hsps (Kultz, 2005; Jäättelä, 1999).

Hsp70 is the most widely studied Hsp and typically the most inducible one following hyperthermic stress. Both structurally and functionally, this protein is one of the most conserved proteins in nature as well (Daugaard et al., 2007). Eight members of this family have been characterized in mammals. Hsp70-5 and Hsp70-9 are localized to the lumen of the endoplasmic reticulum and the mitochondrial matrix respectively, whereas the other six Hsp70 proteins have been detected in both cytosol and nucleus. Among the latter group, Hsp70 1-a, Hsp70 1-b and Hsp70-6 are stress inducible ones whereas Hsp70-8 (Hsc70 or cognate Hsp70), Hsp70-1t and Hsp70-2 are constitutively expressed. Although there is remarkable similarity between Hsp70 members, different tasks have been speculated for each one after studying their mutants (Daugaard et al., 2007). In Drosophila melanogaster also, Hsp70 proteins belong to a multigenic family in which six copies code for inducible proteins and seven for constitutively expressed proteins. A dramatic increase in Hsp68 and Hsp70 levels has been observed in the tissues of this animal following whole body hyperthermia, while Hsc70 and Hsc72 are the most abundant Hsps in non-stressed animals (Lindquist et al., 1988). In addition to their protective effect during stressful conditions, many housekeeping activities have also been assigned to the Hsp70 family. Under normal conditions, they assist newly translated proteins in folding, they transport proteins between different cell compartments or across the cell membrane and they also facilitate proteolysis of damaged proteins (Bukau et al., 2006). The chaperonic activity of Hsp70 is found to be dependent on its interaction with other co-chaperones, the most important of which is Hsp40 (or DnaJ in bacteria). ATP hydrolysis, which is essential for Hsp70 interactions with its substrate, is enhanced through its cooperation with Hsp40 (Qiu et al., 2006).
Members of the Hsp90 family are abundant in normal temperatures as well as being inducible by different cellular stressors. In some organisms, such as Drosophila, these proteins are also developmentally regulated during ovogenesis. Different roles have been assigned to Hsp90. For example, it binds to kinases and keeps them soluble and inactive until they get situated at the membrane. This chaperone has also been isolated from steroid hormone receptors (in their inactive state); in other words, Hsp90 binds to the receptor complex and prevents its activation until the steroid hormone disrupts this attachment and facilitates the receptor/DNA binding which is necessary for gene transcription (Lindquist et al., 1988; Picard, 2002).

Hsp60 has been found in the cytoplasm of bacteria or in the matrix of chloroplasts and mitochondria. In bacteria, this protein is also called GroEL and in eukaryotic organisms chaperonin 60. Under normal conditions, Hsp60 binds to new proteins and catalyses their folding in the presence of ATP and Hsp10. A similar role has been identified for Hsp60 during high temperatures when it helps denatured proteins in the mitochondrion to regain their native folds. Hsp60 is found closely associated with some other Hsps, such as Hsp70. It has been assumed that Hsp70 binds to the proteins and keeps them unfolded until they are transferred to the mitochondrion and delivered to Hsp60 for correct folding (Parsell et al., 1993).

The Hsp100 family includes Hsps bigger than 100 kD (including Hsp110 in eukaryotes or Hsp104 in yeast) whose expression is increased under stressful conditions. This family, which is not as broadly studied as the other Hsps, has a low expression under normal physiological conditions. Therefore, yeast mutants that lack a functional Hsp104 can grow normally at room temperature, however these mutants are especially sensitive to extreme high temperatures (e.g. 50°C) (Parsell et al., 1993; Craig et al., 1993).
Some small heat shock proteins (sHsps), ranging between 15 and 30 kD, have also been characterized. These proteins tend to form large oligomers under normal conditions. During stress such as heat shock, these oligomers dissociate and exchange their subunits with the substrate, which could be an aggregate-prone protein. The resultant Hsp/substrate complex is stable for hours and can prevent partially denatured proteins from irreversibly aggregating. During recovery time these small Hsps collaborate with the other chaperones such as Hsp104 and Hsp70 to refold denatured proteins in an ATP-dependent process (Nakamoto et al., 2007).

1.2 The effect of hyperthermia on nervous system function

Temperature affects numerous biological processes. The mathematical relationship between temperature and a chemical reaction is predicted by the Arrhenius equation:

\[ K_{T_f} = K_{T_i} e^{-\mu / R(T_f - T_i)} \]

in which \( K \) is the constant of the reaction at a certain temperature (T), \( \mu \) is the activation energy and \( R \) is the universal gas constant. Another relevant term, often used in neurophysiological studies, is \( Q_{10} \) which is the temperature coefficient:

\[ Q_{10} = \left( \frac{R_{T_2}}{R_{T_1}} \right)^{10/(T_2-T_1)} \]

According to this equation, \( Q_{10} \) is equal to the change in reaction rate (\( R_{T_2}/R_{T_1} \)) when temperature increases by 10°C (\( T_2-T_1=10 \)). Thus, different components of neural circuit function are affected by temperature with a specific \( Q_{10} \) (Janssen, 1992). Some of these features will be discussed here. Yet due to the complexity of nervous system, prediction of the collective response is not always straightforward.
The relationship between the temperature and resting membrane potential (RMP) is predicted in Nernst equation (assuming that K\(^+\), Na\(^+\) and Cl\(^-\) are the only involved ions):

\[
E = \frac{RT}{F} \ln \frac{[K]_o + [Na]_o + [Cl]_o}{[K]_i + [Na]_i + [Cl]_o}
\]

In this equation, E, R, T and F are the membrane potential, the activation energy, the temperature, and the Faraday constant, respectively. In addition, [ ]\(_o\) and [ ]\(_i\) indicate the concentration of ions outside and inside of the cell, in turn. A linear correlation between temperature and resting membrane potential is predicted by this equation, when all other factors are unchanged. In other words, increasing the temperature will proportionally increase the membrane potential and result in its hyperpolarization. In the Goldman version of the equation, membrane permeability for different ions is also considered. Membrane permeability itself is temperature-dependent in a manner which is different for various ions. The response of the membrane over time could be also different when it is held at a certain temperature. Likewise, the relationship between RMP and temperature is found different in animals which are acclimated to various environments (Janssen, 1992).

Conduction velocity, or the speed of action potential propagation in the neuron, is also directly affected by the temperature. This correlation is the result of both the change in ion kinetics and the cable properties of the nerve fiber. Increased conduction velocity is counted as one of the main factors which is responsible for the increased speed of physical behaviors at high temperature (e.g. increased motor pattern rhythm frequency in the flight system of locusts (Xu et al., 1996)).

Moreover, the efficiency of synaptic transmission is temperature-dependent with a Q\(_{10}\) between 2 and 8. The increased rate of synaptic transmission at higher temperatures could be
attributed to the increased rate of neurotransmitter release and its diffusion in the synaptic cleft as well as a faster postsynaptic response (Janssen, 1992).

There is no doubt that neural circuits have lower and upper temperature limits for their operation. When these thresholds are exceeded, some components of nervous system function or a combination of them will be perturbed. The effect of hyperthermia on the action potential clearly reflects some of these harmful effects:

Action potential generation and propagation in excitable cells is predicted by the Hodgkin-Huxley model (Hodgkin et al., 1952). Modified versions of this equation also include the effect of temperature on the ion conduction through the membrane. These models have been used to simulate action potential behavior at different temperatures. According to these studies, action potential duration and amplitude decrease with increasing temperature, which results in miniature action potentials (Cocherová, 2002). In vivo experiments have also confirmed these predictions (Figure 1; Money et al., 2005). This phenomenon, in other words, happens since the K⁺ ion currents increase during hyperthermia with a bigger coefficient compared to the Na⁺ currents. Thus the repolarization of membrane, which is a consequence of K⁺ channel activation, begins when the membrane is not fully depolarized and continues with a steep slope over time (Robertson, 2004b).

The temporal effect of this phenomenon is an extracellular build-up of K⁺ ions which happens when animal remains at a sub-lethal high temperature. Action potential failure in the locust’s ventilatory motorneurons is found to be correlated with the loss of K⁺ gradient across the membrane (Figure 2; Rodgers et al., 2007). Neural circuit failure happens at temperatures which are still not lethal to the cells and tissues. For example, action potentials in flight motorneurons of the locust fail at about 42°C while these animals can survive temperatures up to 50°C (Wu et al.,
Figure 1. Increasing the temperature in neurons significantly decreases action potential duration and amplitude (Money et al. 2005).
Ventilatory motor pattern (vent) failure following stress is associated with an abrupt surge in extracellular K$^+$ ([K$^+$]$_o$). Ventilatory motor pattern is restored when [K$^+$]$_o$ returns to its baseline. A. Experiments have been done with increasing the temperature of superfusing saline on metathoracic ganglion (MTG). B. MTG has been subjected to anoxia by blowing N2 over it. Reoxygenation of ganglion is correlated with the [K$^+$]$_o$ clearance (Rodgers et al., 2007).
2001; Uvarov, 1966). A similar $[K^+]_o$ surge has been recorded when the locust ganglion is exposed to pure nitrogen until the ventilatory motor pattern fails. Once the ganglion was reoxygenated, the $[K^+]_o$ baseline was restored (Rodgers et al., 2007).

Meanwhile, it should be remembered that sometimes action potentials may respond to the hyperthermia in a slightly different way. For example, in locust flight motorneurons, resting membrane potential is depolarized when the animal stays at high temperature and the amplitude of the action potential does not change, whereas the duration of action potential increases according to the prediction. It has been speculated that high temperatures cause membrane phospholipids to dissociate and in such a condition, membrane potential cannot be maintained. This could also be due to the $Na^+/K^+$ ATPase inactivation at high temperatures (Wu et al., 2001). The inconsistent effect of temperature on action potential amplitude is also reflected in the results of the other studies (Janssen, 1992).

In summary, temperature increase influences nervous system function in a variety of aspects including resting membrane potential, action potential generation and propagation, and synaptic transmission. Stability of these components is prerequisite of a coordinated and rapid response. Many animals with thermoregulatory mechanisms can adjust their internal body temperature, but for the animals that cannot avoid these fluctuations, application of alternative strategies seems necessary to rescue their nervous system operation.

1.3 *Locusta migratoria*

1.3.1 Biology

Locusts (e.g. *Locusta migratoria*) belong to the superfamily of Acridoidea which is the biggest superfamily in the order Orthoptera. While locusts could be considered as one form of grasshoppers, the major distinguishing factor between two groups is their behavior. Once reared
in a crowded condition, locusts have a great tendency to aggregate; however, such a behavior is not normally observed in other grasshoppers. Another important feature of the locusts is that they are able to migrate during the day time while they form huge swarms containing millions of individuals (Chapman, 1976).

*Locusta migratoria* or the African Migratory locust occupies the widest geographical zone compared to the other Acridoidea. This animal is native to the semiarid regions of the world including Sub-Saharan Africa, China, Japan, the Philippines and is occasionally found in Europe. It has never been reported in the Americas (reviewed by Uvarov, 1966; Chapman, 1976). The ambient temperature in the areas they live may exceed 40°C during the hot summer days while the thermal income of the animal might be even higher due to direct and diffuse radiation from sun. These animals utilize many behavioral strategies to maintain their internal body temperature in a permissive range for their enzyme activities. For example, they avoid flying when the air temperature is above 40°C because the metabolic heat produced from their flight muscles will elevate the thoracic temperature about 10°C above the ambient temperature. In such conditions they prefer to shelter in cooler microhabitats in their environment or to alter their posture in order to reduce their heat absorption area. One example of these orientations is “crouching” when the substrate has a more favorable temperature than the environment. In this posture, the ventral surface of the body is closely attached to the ground for the maximum heat conductance between the body and the substrate. Even with the application of such thermoregulatory behaviors, the internal temperature of the animal could still rise frequently. This may specially happen when animals encounter high temperatures during their long distance migrations without any chance to land, for example when they are flying above the water. Therefore, they must have evolved some intrinsic mechanisms as well as inducible thermotolerance strategies for protecting their tissues and physiological responses.
1.3.2 The importance of L. migratoria in the neurophysiological studies of heat shock

*L. migratoria* has been recognized as a suitable model for neurophysiological studies. Insects have a relatively simple nervous system with robust behavioral responses. These behaviors and their underlying circuits which can be easily manipulated and monitored, still share many features with those from other evolutionary distant organisms such as mammals. Scientists were originally motivated to study the locust due to its importance as a devastating agricultural pest. It was later that neurobiologists discovered the benefits of this insect for their own particular studies; for example, locusts can be easily reared in the laboratory and their five larval instars provide an excellent tool for studying developmental neurobiology. Since then, many of the neurons in the locust nervous system have been identified and their associated behaviors have been characterized. Therefore, the great database formed over the years has been always encouraging for the continued neurobiological studies with this animal (Burrows, 1996).

Furthermore, locusts naturally inhabit areas in which the ambient temperature can easily go above 40°C during the summer (Uvarov, 1966). This could be particularly challenging for poikilotherms, since they have limited control on their body temperature and they can only take advantage of some behavioral strategies to maintain their internal temperature within a permissive range. It is expected, therefore, that the tissues of these animals experience hyperthermia many times during their lives and locusts must have developed thermoprotective mechanisms to rescue their cells as well as their physiological responses from the detrimental effects of heat.

Due to the mentioned reasons, this animal has been extensively used for investigating the thermoprotective effects of a prior stress on nervous system function. *L. migratoria* was also used in the current research in order to make the results comparable with the previous work.
1.3.3 Induced thermotolerance in *L. migratoria* by a prior stress

Heat shock pretreatments with various temperatures and durations have been examined for characterizing the treatment with the maximum thermotolerance conferred to 3-week-old locusts. According to these experiments, animals that have experienced a 45°C temperature for three hours are almost 100% thermotolerant once exposed to 50°C temperature (Whyard *et al.*, 1986).

In another study, thermotolerance of the adults at different ages have been compared one hour after heat shock (45°C, 3 hours). Except for the 1-day-old animals which were assumed to have higher intrinsic thermotolerance, this pretreatment could significantly improve survival of the animals at the otherwise lethal 53°C temperature. This study also showed that, after one week, heat shocked animals are still more thermotolerant than the control group. The over-expression of Hsps has been suggested as the cause of this long term effect (Robertson *et al.*, 1996).

1.3.4 Phase polymorphism

Locust phase polymorphism was described for the first time by Sir Boris Uvarov (Uvarov, 1928). He suggested that what was recognized as two different species at that time was, in fact, two phases of the same species under different population densities. In an environment which provides enough food or space for every individual, locusts tend to have a solitarious life similar to grasshoppers. However, once these food or habitat resources get scarce, animals have to share those with each other. In other words, they have to concentrate in certain areas which could be those with better sunlight or more food. Density increase triggers aggregation behavior in locusts which is the most immediate implication of the gregarious phase happening within minutes from the time individuals come together. Yet, the complete gregarization occurs if animals live in the crowded condition for generations. Similarly, animals are transformed from gregarious to solitarious when they have access to a vast and uniform environment (Uvarov, 1966).
The most conspicuous difference between solitarious and gregarious animals is in the colouration of both the juvenile instars (hoppers) and adults. The solitarious hoppers have a green or brown colour depending on the colour of their background; this prevents them from being easily detected by their predators, whereas the gregarious hoppers have noticeable patterns of black and yellow which help them to find each other in the bands. The green colour persists in the solitarious adults while the gregarious adults have a straw colour turning into yellow in certain areas by aging. Among the other morphological characteristics of solitarious animals is their arched pronotum with an obvious crest in the middle; this is replaced by a flat or concave pronotum in gregarious animals. There are also some morphometric changes associated with the phase transformation. The solitarious males are much smaller than gregarious ones; this pattern is reversed in females (Figure 3). Meanwhile, the hind femur in solitarious animals is longer than in gregarious locusts, which gives them a greater jumping ability. The femur size is normalized to the maximum head width (F/C) and used as a morphometric index of phase change. The converse is true for the normalized size of elytra (E/C) which is bigger in gregarious animals and is correlated with their greater flight ability (Uvarov, 1966).

Gregarious animals are significantly more active than solitarious ones, associated with their aggregating nature; the average distance walked by a gregarious fourth instar in 20 min is about four times longer than the solitarious counterpart. Different behaviors predictably necessitate different metabolisms. As an example, gregarious animals have a notably larger amount of fat bodies which provides them with an extra source of energy during their long migrations. Phase transformation in locusts is also characterized by reproductive, developmental and physiological changes; however, discussing all of them is not within the scope of the current document (see the review by Uvarov, 1966).

The most effective method of transforming solitarious animals to gregarious is found to be a
Figure 3. Some of the morphological differences between two phases of *L. migratoria*. a. Solitary (S) and gregarious (G) males. b. Solitary and gregarious females (Uvarov, 1966). Solitarious animals have a convex pronotum which is transformed into a concave pronotum in gregarious animals. Solitarious males are much smaller than gregarious ones; this pattern is reversed in females. Phase transformation also causes some changes in colouration which are not shown in this picture.
tactile stimulus on the outward surface of hind femur. It is believed that this area of body is not normally self-touched by the animal, however, it is easily stimulated in the presence of other locusts (Simpson et al., 2001). To a lesser extent, animals could be driven gregarious by olfactory stimuli which include pheromones produced by the crowd. Visual stimuli which come from the other locusts living nearby cannot gregarize the animal unless they are combined with the olfactory cues (Simpson et al., 1999).

The locust phase polymorphism is a complex phenomenon which is not completely unraveled at the molecular level but it is found to be accompanied with a widespread change in the neurotransmitters and neuromodulators. For example, following 4 h crowding, previously solitary hoppers display a nine fold increase of serotonin in their metathoracic ganglion which coincides with rapid behavioral modifications (Rogers et al., 2004). The involvement of [His\(^7\)]-corazonine\(^1\) in this phase transition and body colourations associated with it is also well-evidenced (Pener, 1991; Tanaka, 2006). The phase characteristics are epigenetically transmitted to future generations; it has been demonstrated that the females have a memory of the extent of gregariousness they have experienced during their life. They can predict the kind of life whose offspring may face based on this memory which will itself determine the amount of the gregarizing factor released in their egg foam. Therefore, some of the phase specific features such as migration tendency take a few generations to change while some others may happen within hours (Simpson et al., 2008). This makes the phase polymorphism in locusts a continuous phenomenon rather than a binary one, which means there are also intermediate states between extreme solitary and extreme gregarious. These intermediate forms are sometimes called

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\(^1\) Corazonine is a neurohormone synthesized in insects. [His\(^7\)]-corazonine is the isoform of the hormone isolated from cockroaches and certain locusts including *L. migratoria* (Predel et al., 2007).
congregans and dissocians (depending on the phase they have segregated from) and display a combination of features from both phases (Kennedy, 1956).

1.4 Thermoprotective effect of a prior stress on the nervous system function

As it has been already discussed, increasing the temperature could disrupt nervous system function even before proteins are denatured and cells die. Obviously, the first could be as life threatening as the latter, since some of the animal’s vital behaviors such as ventilation and predator avoidance will be impaired. Developing mechanisms to protect nervous system operation during increased temperatures is particularly important for the animals inhabiting areas with extreme temperatures. Of course, being a poikilotherm, with a limited control on body temperature, adds to this need. Aside from the possible intrinsic mechanisms which make these animals thermotolerant, neural circuit function could become more resistant to hyperthermia by a previous exposure to stress. The thermoprotective effect of a prior stress including heat shock, anoxic coma and cold shock on the locust’s nervous system have been mainly investigated by three different models: ventilatory motor pattern, flight neuromuscular circuitries, and the activity of the descending contralateral movement detector (DCMD) which is a visual interneuron responding to looming stimuli (Robertson, 2004a). These three components of the nervous system are involved in the vital behaviors of animal including predator avoidance. Thus, developing some thermoprotective mechanisms to maintain these activities at high temperatures seems necessary for the animal.

According to the studies of Newman et al. (2003), heat-shocked locusts can sustain their ventilatory rhythm at 47°C for twice as long as control animals. They are also able to recover
their ventilatory motor pattern three times faster than the control animals when they return to normal temperature.

The studies conducted on the flight system of locusts show that heat shock increases failure temperature of the tethered flight by about 6 C°. According to these studies, central flight pattern rhythm is increased when temperature goes up; however, heat shock can significantly decrease the slope of this correlation and make the circuit more robust to the hyperthermia (Robertson et al., 1996). Heat shock can also render the parameters of excitatory postsynaptic potentials in the flight motorneurons less sensitive to high temperature. These parameters include the amplitude, latency, conduction velocity and synaptic delay. Therefore, the thermostability of the flight motor rhythm following heat shock could be partly attributed to the thermoprotection of the circuit synapses (Dawson-Scully et al., 1998). According to Wu et al. (2001), induced thermotolerance in the flight system is also correlated with a more robust action potential. In other words, heat shock increases the upper temperature limit of action potential generation in flight motor neurons. The duration of action potentials in these neurons is also increased by a previous heat shock which will compensate the predicted reduction in the action potential duration occurring at high temperatures.

Finally, the firing frequency of the visual interneuron (DCMD) in HS animals in response to a looming target is found fairly stable for a wide range of temperatures. For example, the first spike before collision, which is delayed at high temperatures in CON animals, appears relatively earlier in HS animals (Figure 4; Money et al., 2005).
Figure 4. A delay in first spike appearance, increased spike frequency, decreased amplitude, and hyperpolarized resting membrane potential are the characteristics of an intracellular recording from DCMD (responding to a looming stimulus) following a temperature increase. In HS animals, these features are more stable against hyperthermia. In these experiments, stimulus approached the animal with a certain speed and stopped at 100 ms before predicted collision. The angular size of looming target increases by its movement (Money et al., 2005).
1.5 Underlying mechanisms of heat shock response in the nervous system of *L. migratoria*

The molecular basis of the heat shock response in the nervous system is not fully uncovered. However, the most consistent results show that this stress-mediated thermoprotection is tightly correlated with a decreased K⁺ conductance across the neural membrane (Rodgers *et al.*, 2007; Wu *et al.*, 2001; Ramirez *et al.*, 1999). The extracellular K⁺ ([K⁺]₀) build-up in the metathoracic ganglion which is associated with increasing temperature, is delayed in stress-pretreated animals. The rate of [K⁺]₀ clearance is also found significantly faster in HS animals than the control group, which corresponds with a shorter recovery time in stress-preconditioned animals (Rodgers *et al.*, 2007). The involvement of K⁺ channels in stabilizing K⁺ homeostasis in heat shocked animals has been tested by bath application of tetraethylammonium (TEA), a K⁺ channel blocker, on the MTG. According to the results obtained from these studies, K⁺ channel blockade mimics the protective effect of heat shock on the nervous system (Rodgers *et al.*, 2007; Wu *et al.*, 2001). These results are also supported by another study in which the outward currents of potassium ions in neuronal somata have been examined. In these experiments, when temperature was increased from 38°C to 42°C, the amplitude of K⁺ efflux in MTG slices from HS animals was markedly lower than control animals (Ramirez *et al.*, 1999). Therefore, modulation of K⁺ current through the cell membrane could be considered as one of the mechanisms underlying heat shock response in the neural circuits. This could be promoted by the regulation of both K⁺ channels and/or Na⁺/K⁺ ATPase, although there is not enough evidence yet for involvement of the latter in heat shock induced protection of the nervous system (Rodgers *et al.*, 2007).

The exact mechanisms through which heat shock protects K⁺ homeostasis or some other possible pathways working in parallel to safeguard nervous system function during high
temperatures are not clear. There is evidence though for the involvement of the cytoskeleton in the heat shock response. Bath applying MTG with concanavalin A (a cytoskeleton stabilizer) can mimic the thermoprotective effects of heat shock in the ventilatory pattern generation which includes higher failure temperature and shortened recovery time. The converse effect has been observed by administering colchicine (cytoskeleton destabilizer) which abolishes the protective effect of heat shock (Garlick et al., 2007). Similar experiments on the flight neuromuscular junctions also support mediation of cytoskeletal elements in the heat shock response (Klose et al., 2004). It has been thought that stress preconditioning increases the level of Hsps in the neuron and thus protects the nervous system through interaction of these proteins with cytoskeleton. Similar to the results obtained for the cytoskeleton, bath application of some neuromodulators such as serotonin and octopamine (an invertebrate neuropeptide similar to norepinephrine in vertebrates) on the MTG imitates the effect of heat shock on ventilatory pattern generation (Newman et al., 2003; Armstrong et al., 2006). These observations are in accordance with the elevated amounts of octopamine in the hemolymph of locusts following stress (Davenport et al., 1984). The thermoprotective effect of this neurohormone on the nervous system of locusts is mediated by a cAMP/PKA pathway which is found to be dependent on protein synthesis. Hsps have been assumed the first candidates for being up-regulated during octopamine treatment; however, there is not enough evidence for that (Armstrong et al., 2006). Serotonin, on the other hand, has not been measured in locusts following heat shock; however this neurotransmitter is a well-established $K^+$ channel modulator which decreases the $K^+$ conductance in neurons through channel phosphorylation (Camardo et al., 1983).
1.6 Heat shock proteins and the thermoprotection of nervous system operation

The thermoprotective effect of Hsp70 on the nervous system function has been investigated by incubation of mouse brain slices in a solution containing Hsp70. This exogenous Hsp70 protected synaptic transmission against detrimental effects of hyperthermia, which is similar to the heat shock response; however, the amount of endogenous Hsp70 in brain slices did not change following heat shock (Kelty et al., 2002). It has been suggested that heat shock may confer its thermoprotective effect on the nervous system through some Hsp–independent pathways. Other evidence also exists for the thermoprotective effect of Hsp70 on synaptic performance; *Drosophila* larvae over-expressing Hsp70 display an appreciably improved presynaptic performance at elevated temperatures which could be compared with the effect of heat shock. However, the endogenous levels of hsp70 in the nervous system of wild type larvae, following hyperthermia, has not been measured (Karunanithi et al., 2002).

The first attempt to study heat shock response in *L. migratoria* at the protein level dates back to 1986 (Whyard et al., 1986). In that study, fat bodies of mature locusts were extracted and cultured in vitro. Newly synthesized proteins were labeled by adding $^{35}$S-methionine to the culture for 30 minutes before tissues were harvested and proteins extracted. The results of this study show that the synthesis of six putative Hsps increases remarkably at high temperatures, whereas the expression of many others decreases. The proteins whose synthesis had increased were not identified; however, they were tentatively named Hsp83, Hsp73, Hsp68, Hsp42, Hsp28 and Hsp24 according to their position on the SDS gel (Whyard et al., 1986).

More recently, the gene encoding a member of Hsp70 family has been cloned from *L. migratoria*. For that purpose, a cDNA fragment of Hsp70 had been amplified using partially degenerate oligonucleotide primers designed based on inducible Hsp70 sequences obtained from
different organisms. The resulting cDNA fragment was cloned and sequenced and once the entire cDNA sequence was obtained, the corresponding genomic fragment was amplified. The cloned gene has been assumed to encode an inducible Hsp70, since its transcript in the tissues increases following heat shock, although modestly (Qin et al., 2003).

Sequence information obtained for the locust Hsp70 has been used to design forward and reverse primers for real-time PCR experiments. Using this approach, Hsp70 change at the transcript level has been examined in different tissues of *L. migratoria* following various stressors. Metathoracic and mesothoracic ganglia, muscle and fat bodies have been the tissues tested and heat shock, anoxia, LPS injection, hunger and extreme high temperature (53°C) were the treatments examined separately. Similar experiments have also been done by octopamine injection into the hemocoel based on the results obtained by Armstrong et al. (2006). The results of real-time PCR show that the change in Hsp70 transcript following stress in the tissues of *L. migratoria* is always less than 2-fold and is not consistent (Figure 5; Shoemaker et al., unpublished data). Hsp70 level in the fat bodies and ganglion of locusts has also been quantified by ELISA which yielded a modest (~2 fold) increase following heat shock (Qin et al., 2003). Therefore, the results of real-time PCR and ELISA differ from those obtained by Whyard et al. (1986). This discrepancy could be caused by a high constitutive level of Hsps in the tissues of *L. migratoria* which would not have interfered in the experiments of Whyard et al. (1986) where only the newly synthesized proteins have been detected. Alternatively, it may have originated from the difference between in vitro and in vivo responses.
Figure 5. Real-time PCR results for changes of Hsp70 transcription in three different tissues (ganglion, muscle and fat bodies) of *L. migratoria* following various stressors; heat shock (HS); lipopolisacharide injection (LPS); anoxia; octopamine (OA); hunger; and an extreme temperature (53°C). The Hsp70 level does not change more than 2-fold (dashed line) due to any of these treatments and the response is not consistent. Two to five animals were used for each group. Where error bars are not shown, it means that there has been only one replicate (Shoemaker *et al.*, unpublished data).
1.7 Research objectives & experimental outline

1.7.1 2-D gel electrophoresis

In vitro studies show increased synthesis of several Hsps in the tissues of *L. migratoria* at high temperatures, which is also expected according to the classic heat shock response (Whyard *et al.*, 1986). In addition, some heat shock mimetic treatments such as octopamine injection into the hemocoel are rendered ineffective by the protein synthesis blockade (Armstrong *et al.*, 2006). However, other studies aimed at measuring the Hsp70 transcript in tissues of *L. migratoria* after a whole body hyperthermia, report a non-significant change (Qin *et al.*, 2003; Shoemaker *et al.*, unpublished data). Therefore, in the current study, a proteomic approach was taken to study protein expression changes in metathoracic ganglion following heat shock in a broader spectrum. Both Hsps and other proteins which were down- or up-regulated could be identified in this way and pathways underlying the heat shock response in the locust nervous system could be discovered. For this purpose, 2-D gel electrophoresis was utilized to compare the protein profiles of the metathoracic ganglion under control conditions and following heat shock.

2-D gel electrophoresis followed by mass-spectrometry analyses has also been applied in other studies to examine protein expression in the insect tissues during different developmental or pathological conditions. For example, Stadler *et al.* (2002) has examined different methods to extract proteins from different tissues of Australian locust. 2-D gels in that study also displayed significant differences between the tissues of parasitized and non-parasitized locusts; two Hsp70 members were among the differentially expressed proteins. In another study, 2-D gels have been employed to compare protein expression in the brain of diapausing and non-diapausing pupa of flesh fly. Several proteins, including Hsp70, were found to be differently expressed during the two conditions (Li *et al.*, 2007). I took advantage of such studies to design proteomic approaches
applied in the current research. Various protein preparation methods were examined to obtain the optimum results.

1.7.2 Measuring Hsp70 increase in the tissues of isolated locusts following stress

It was speculated that gregarious locusts have already encountered some level of stress by living in a crowded colony and having to compete for the limited food resources; therefore, the constitutive level of Hsps including Hsp70 in their tissues is high (Wang et al., 2007). Solitarious or even isolated animals, on the other hand, due to the lower level of Hsps in their tissues were expected to be less thermotolerant and heat shock was predicted to induce a more robust over-expression of Hsps in their tissues. In other words, measuring Hsp70 increase in the tissues of isolated animals was planned as one of the possible approaches to eliminate the effect of high background level of Hsp70 which has been assumed to interfere with previous experiments (Qin et al., 2003). Based on this hypothesis, a number of animals were reared under isolated conditions and their Hsp70 response was examined by quantitative western blots following stress (heat shock and anoxia, separately).

1.7.3 Studying localization pattern of Hsp70 in MTG following heat shock

Although other studies have reported no significant change in the level of Hsp70 in locust ganglion following heat shock, this does not eliminate the possibility of an Hsp70 increase in a small group of cells in the MTG; a change which would remain undetectable in the total protein extracts of the tissue. As a supplementary study along with the results of previous work, thin sections of ganglion, CON or HS, were subjected to immunohistochemistry with Hsp70 antibody, to compare.
Chapter 2
Materials and Methods

2.1 Animals

2.1.1 Gregarious locusts

Animals were taken from a crowded colony of *Locusta migratoria migratorioides* maintained in the Department of Biology at Queen’s University. Locusts were reared under a 12h:12h (light: dark) circadian regime at room temperature (25±1°C) and humidity of 23±1%. Animals also received radiant heat from a 40 W light bulb installed in each cage. Locusts were fed fresh wheat seedlings and a dry mix of wheat bran, torula yeast and milk powder. To control for age, animals used in this study were collected immediately following final ecdysis and raised in a separate cage for 3 weeks. Only adult males were used in these experiments. In this document, “locusts” refers to the animals in gregarious phase unless otherwise specified.

2.1.2 Isolated locusts

Locusts were isolated by collecting 1st instar nymphs from the gregarious-phase colony. Each individual was housed in a separate container (~0.5L) and reared together in a separate room from migratory animals. Locusts were on a 12h:12h light: dark cycle at room temperature (25±1°C). Incandescent light bulbs (100 W) were installed approximately 60 cm above animals’ containers to provide them with enough light and radiant heat needed for development. These animals were fed the same diet as the animals in the migratory colony. Their containers were cleaned every other day while minimizing the mechanical stimuli caused by handling the animals. Males which were three weeks past the adult molt were used. The femur length (F) and head capsule width (C) were measured with calipers with 0.1 mm precision (Cat. No. 134150000,
Scienceware) after animals had been dissected. The F/C ratio, as the index of phase transformation, was calculated for isolated animals as well as a number of gregarious locusts.

2.2 Experimental treatments

2.2.1 Heat shock

Animals were heat shocked in a perforated plastic container in a humid incubator (45°C) for 3 hours while CON group was kept in a similar container at 22±1°C. After this period, animals recovered at room temperature for 1 hour and then dissected (Robertson et al., 1996). Solitary animals were kept in individual containers while being subjected to heat shock. Recovery time was increased to 8, 12 and 24 hours in some experiments mentioned in results.

2.2.2 Anoxia

Animals were exposed to a pure nitrogen atmosphere at room temperature (22±1°C) for 2 hours and subsequently allowed to recover at normal atmosphere for 1 hour (Wu et al., 2002). Control animals were kept in a normal atmosphere for 3 hours.

2.3 Dissection

Metathoracic ganglia were excised by cutting the connectives midway between the MTG, mesothoracic ganglion and abdominal ganglion (A4) as well as all other nerve roots. MTG were then rinsed with cold standard locust saline (in mM: 147 NaCl, 10 KCl, 4 CaCl2, 3 NaOH, 10 HEPES buffer, pH 7.2) and blotted dry on a clean lint-free wipe. The centrifuge tubes containing the ganglia were kept in liquid nitrogen during the dissection procedure and then stored at -80°C for no longer than two weeks. For some analyses mentioned in the text, a portion from one of the
biggest dorsolongitudinal muscles in thorax (m112), numbered according to Albrecht (1953), was also dissected.

2.4 2-D gel electrophoresis

2.4.1 Sample preparation

2.4.1.1 Total protein extraction

A lysis buffer composed of 7 M urea, 2 M thiourea, 1% CHAPS, 1% ASB-14 and 1% Triton X-100 was chosen after it proved to be more efficient in protein extraction than another lysis buffer containing 7 M urea, 2 M thiourea and 4% CHAPS (Appendix 1). TCA/acetone precipitation was also examined as an approach to remove possible contaminants such as salts and polysaccharides. This method resulted in fewer spots on the gel and no improvement in the resolution; thus it was not further employed (Appendix 2, 3). Sixty µl of lysis buffer was used per ganglion while 5 metathoracic ganglia were used in each experiment. Cell disruption was facilitated by sonication in an ice-bath for 20 min and plunging the tissue through a hypodermic 21G11/2 needle until no large particles were visible. Samples were then centrifuged for 20 min at 21000 xg and the supernatant was used immediately or stored at -20º C for subsequent analyses in less than two days.

In an effort to decrease the horizontal streaks on the gels, proteins were also extracted using the ReadyPrep 2-D clean-up kit (Cat. No. 163-2130, Bio-Rad) designed to wash away contaminants such as ionic detergents, salts, nucleic acids and lipids (Li et al., 2007). Protein samples extracted by the lysis buffer, described in the last paragraph, were cleaned up according to the kit manual instructions. This procedure did not have a significant effect on the quality of the gels; therefore it was not used in the next experiments.
2.4.1.2 Sequential protein extraction

With this method, proteins were extracted in different phases according to their differential solubility. Given the higher amount of total protein fractionated by this procedure, low abundance proteins have a better chance of being visualized. In addition, separating proteins in different phases reduces sample complexity and results in a better resolution of spots on the gel. The three different lysis buffers provided in the sequential extraction kit (Cat. No. 163-2100, Bio-Rad) have different detergent and chaotropic natures (Thorén et al., 2002). The first reagent, which is a 40 mM trisbase solution, extracts highly soluble (hydrophilic) proteins such as cytosolic proteins. The second reagent contains 8 M urea, 4% CHAPS, 40 mM tris, 0.2% 3-10 ampholyte and 2 mM tributylphosphine (TBP). Urea applies its effect by disrupting the hydrogenic and ionic bonds between amino acid residues and CHAPS is a zwitterionic detergent acting as a surfactant without imposing an extra charge on the protein (Gorg et al., 2004). The second buffer, therefore, has an intermediate potential for solubilizing hydrophobic molecules. Finally the third solution is composed of 5 M urea, 2 M thiourea, 2% CHAPS, 2% SB 3-10, 40 mM Tris, 2 mM TBP and 0.2% Bio-Lyte 3-10. Thiourea is known for its exclusive power in solubilizing hydrophobic membrane proteins and is commonly used in combination with urea. Also the sulfobetaine (SB) 3-10 included in this buffer is a stronger zwitterionic detergent than CHAPS for extracting the membrane proteins (Gorg et al., 2004). The last buffer, as a result, is designed to extract the remaining proteins which have not been solubilized in the first two buffers. Sample preparation was performed according to the instruction manual of the kit. Twenty MTG were all suspended in 60 μl reagent 1 and proteins were solubilized using ice-bath sonication as well as aspiration through a 21G11/2 needle.
2.4.2 Determining total protein concentration of the samples

Total protein concentration of the samples was determined using the Bio-Rad protein assay (Cat. No. 500-0002) which is based on the method of Bradford. Bovine serum albumin (BSA) provided with the kit was used as the standard protein and a stock solution of 10 mg/ml was prepared. This stock was diluted with ultrapure water to make 70, 50, 35, 25, 12.5 µg/ml concentrations. Samples were diluted 50, 100, 200 and 400 times to ensure at least one concentration is in the linear range of the assay. All substances used in the 2-D gel lysis buffer or RIPA buffer had a compatible concentration using these dilutions. All standards and samples were loaded in duplicates while 40 µl of dye concentrate was mixed with 160 µl of sample or standards in each well. Absorbance was measured at 595 nm in an ELx800 Universal Microplate Reader (BioTek Instruments, Inc.) and a standard curve generated based on the control (BSA) was used to interpolate the concentration of the samples.

2.4.3 Isoelectric focusing (IEF)

ReadyStrip IPG strips from Bio-Rad were used to separate proteins according to their isoelectric point (pI). Seven centimeter strips (Cat. No. 163-2000, 163-2004) were used in the initial experiments when the protocol was becoming optimized for either total or sequential protein extracts. In order to find the best range of pH covering most of the proteins in the sample, proteins were first run on a 7 cm strip, pH 3-10. Since proteins were mainly focused between pH 5 and 8, 17 cm strips, pH 5-8 (Cat. No. 163-2011) were used in all of the subsequent experiments. According to the manufacturer’s recommendations for 7 cm strips, an appropriate amount of sample containing 50 µg protein and for 17 cm strips, a volume of sample containing 100 µg protein was loaded on each strip. Strips were actively rehydrated overnight in rehydration buffer containing the protein sample (150 µl and 350 µl for 7 cm and 17 cm strips, respectively). The rehydration buffer was composed of the same lysis buffer used to extract the proteins plus 2 mM
TBP and 0.2% ampholyte 3-10. Before adding to the strips, samples were incubated in rehydration buffer at room temperature for 4 hours. They were then centrifuged at 21000 xg for 20 min and supernatant was used to rehydrate the strips. Isoelectric focusing (IEF) was conducted in a Protean IEF Cell (Cat. No.165-4001, Bio-Rad) for 15 minutes at 250 V, 6 hours at 500 V, 2 hours at 2000 V and for a total of 100000 Vh at 10000 V. In an effort to remove possible ionic impurities, 250 V the initial step was also extended into 2 hours which did not yield a better isoelectric focusing. IEF for both CON and HS samples were performed simultaneously and in the same unit.

2.4.4 SDS-PAGE

Immediately after isoelectric focusing, IPG strips were incubated in the first equilibration buffer containing 6 M urea, 2% SDS, 50 mM Tris-HCl pH 8.8, 20% glycerol supplemented with 2% DTT for 15 min followed by incubation in the second equilibration buffer supplemented with 5% iodoacetamide for another 15 min. IPG strips (one 17 cm or two 7 cm) were mounted on a large format polyacrylamide gel (180×160×1 mm). For 12% gels, 40 ml of 30% Duracryl 37.5:1 (Cat. No. 80-0148, Genomic Solutions), 25 ml 1.5 M Tris-HCl, pH 8.8 and 1 ml 10% SDS were mixed. This solution was made up to 100 ml by adding ultrapure water and degassed for 30 minutes. Fifty microlitres TEMED and 500 μl 10% APS were added to the solution immediately before pouring. The same protocol was followed to make 7.5% gels, except that only 25 ml of 30% Duracryl was used. Ten microlitres of Low Range SDS-PAGE Standards (Cat. No. 161-0304, Bio-Rad), diluted (1:20) in the SDS loading buffer, was always loaded beside the IEF strip as a protein ladder. Electrophoresis was carried out in a Protean II xi Cell (Cat. No. 165-1811, Bio-Rad) at 20° C and 50 V overnight followed by a constant current of 24 mA per gel until the bromophenol blue reached the bottom of the gel. The concentrated running buffer was composed of 25 mM Tris, 192 mM glycine and 0.1% SDS which was five times diluted by distilled water.
before use. After completion of electrophoresis, gels were fixed in a solution of 40% methanol and 10% acetic acid for 2 hours. SDS-PAGE for both CON and HS samples were run side by side.

**2.4.5 Silver staining**

In order to minimize the background staining, gels were washed with distilled water three times (each for 15 minutes) and left in an additional wash overnight. Gels were sensitized with 0.02% sodium thiosulphate for 2 min then rinsed with two washes of distilled water for 1 min each. This was followed by 40 min incubation in 0.1% silver nitrate on a rocking shaker at room temperature. Gels were then twice washed with water (each for 1 minutes) and developed in a solution of 0.04% formalin and 2% sodium carbonate until the desired staining was achieved. Development was stopped by transferring the gels to 5% acetic acid replaced by 1% acetic acid after 1 hour and gels remained in this solution until analysis. Both CON and HS gels were stained at the same time to eliminate errors caused by technique deviations.

**2.4.6 2-D gel image analysis**

Gels were scanned in a GS-800 Calibrated Densitometer (Bio-Rad) with 500 dpi resolution and analyzed using PDQuest 2-D Gel Analysis Software V7.1.0 (Bio-Rad). Automated spot detection by PDQuest was followed by manual corrections to add undetected spots and remove incorrect detections. In order to compare gels, a MatchSet was created in which a synthetic image called “master” represented all the information from the members. Automated spot matching between gels was carried out by manual matching of a few spots as the landmarks. To compensate for the non expression-related variations, spot quantities were normalized based on the total quantity in valid spots on the gel and shown in ppm. CON and HS gels were assigned to different replicate groups and three different analysis sets were created: a qualitative analysis set.
including all the spots present in one replicate group and absent from the other, a quantitative set
including all the spots whose average quantity had changed above two fold in HS group
compared to the CON and a statistical analysis set including the spots whose change is significant
based on student’s t-test while the significance level was set at 0.05. A Boolean analysis set was
then built from the union of these three analysis sets. The latter was used to create a quantity table
of the spots on each individual gel which was used in further statistical analyses.

2.5 In gel tryptic digestion

Spots were excised from four or five gels by a pipet tip. A blank piece of gel was used as the
negative control to show the possible protein contaminations in the gel. Some pieces of a
reference gel containing 2 pmol of bovine serum albumin (BSA) were also processed in parallel
as a positive control. Gel pieces were silver-destained in a freshly prepared solution of 30 mM
potassium ferricyanide and 100 mM sodium thiosulfate, and then twice washed with solutions of
50 mM ammonium bicarbonate and 100% acetonitrile, alternately. Proteins were reduced by
incubating the gels in a solution of 10 mM DTT at room temperature for one hour. This solution
was then replaced by 55 mM iodoacetamide in which samples were incubated for half an hour in
the dark. Gel pieces were again subjected to three alternate washes with solutions of 40 mM
ammonium bicarbonate and 100% acetonitrile. Twenty µl of Sequencing Grade Modified
Trypsin (Cat. No. V5111, Promega) with a concentration of 20 ng/µl was added to each tube and
incubated on ice for 15 minutes. This enzyme solution was discarded and gels were subsequently
incubated in 20 µl of 40 mM ammonium bicarbonate at 37º C overnight. Peptides were extracted
once using 100 µl of 2% acetonitrile and 1% formic acid and then twice in 100 µl of 50%
acetonitrile and 1% acid formic for 5 minutes each with sonication. The total of 320 µl extract
from each sample was concentrated to 10 µl in a SpeedVac centrifuge (Cat. No. SPD121P,
Savant Instruments) and desalinized using OMIX C18 Pipette Tips (Cat. No. A5700310K, Varian) according to the manufacturer’s instruction.

2.6 Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS)

Digested proteins were spotted on a 100-well MALDI target plate using two different matrices: α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB), both from Sigma-Aldrich (Cat. No. C2020, G5254). For CHCA, a solution of 0.4% matrix in 50% acetonitrile, 0.1%TFA was prepared. This solution (0.5 µl) was spotted on the plate as the first layer and air dried. Then 0.5 µl of the same solution mixed 1:1 with sample was spotted as the second layer and air dried. For DHB, the first layer was composed of 20 mg/ml DHB in a solution of 70% acetonitrile and 0.1%TFA; the second layer was made of 30 mg/ml DHB in a solution of 30% acetonitrile and 0.1%TFA solution. Sample (0.5 µl) was separately spotted on the second layer. An external calibration was applied with each matrix using a standard four-peptide mix which consisted of Renin, ACTH, GFP and Ang1 (Cat. No. R8129, A0673, F3261 and A9650, Sigma). A stock solution of 20 pmol/µl was stored at -20ºC which was 100 times diluted in 50% acetonitrile before use. When spots were completely dry, the plate was inserted in a Voyager-DE PRO Biospectrometry Workstation (Applied Biosystems).

The mass spectra of the proteins were analyzed using Data Explorer V4.0 while an internal calibration, based on the trypsin autolysis peaks, was also conducted. Monoisotopic masses in the spectrum were used to perform a search in SwissProt database using Mascot Peptide Mass Fingerprint search engine. One trypsin missed cleavage was allowed and mass tolerance was set at 50 ppm. Carbamidomethyl (C) and oxidation (M) were considered as the fixed and variable modifications, respectively. The accuracy of protein match was assessed at p< 0.05.
2.7 Quadrupole-time-of-flight tandem mass spectrometry (Q-TOF LC/MS/MS)

Q-TOF LC/MS/MS analysis was performed along with MALDI-TOF, whenever mentioned in the text, to increase the accuracy of protein identification by providing further information about its amino acid sequence. The remaining sample from MALDI-TOF experiments, which was not less than 8 μl, was used in these analyses. The spectra were acquired with a Waters Q-TOF Global Ultima instrument which was operated in data-dependent acquisition mode using MassLynx 4.0. The inlet chromatography was performed on a Water's CapLC-XE using a Dionex Pepmap C18 column. The linear gradient was 5% acetonitrile to 40% acetonitrile over 40 minutes. The data was processed under MassLynx 4.0 and searched in Swissprot database through Mascot MS/MS Ion search engine. One missed cleavage was allowed and carbamidomethyl (C) and oxidation (M) were considered as the fixed and variable modifications, respectively. Error tolerances were set at 100 ppm and 0.2 Da for the parent mass and fragment mass, respectively.

2.8 Western blot

2.8.1 Sample preparation

Tissues from three or four animals were used for each replicate. RIPA buffer used to extract proteins was composed of 50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS while the pH was adjusted to 8.0 and the buffer was stored at 4° C. Triton X-100 (1.5%) and protease inhibitor (2%) (Cat. No. P-2714, Sigma-Aldrich) were freshly added to the buffer before use. Ten or fifty microlitres of the extraction buffer were used for each ganglion or piece of muscle, respectively. Tissues were homogenized by an electrical homogenizer and incubated on ice for 45 minutes. The lysate was then centrifuged at 21000 xg for 10 minutes and
the supernatant was stored at -20º C for not longer than two days. Protein concentration of the samples was determined according to the Bio-Rad protein assay explained before.

2.8.2 Immunoblotting

Proteins were denatured in SDS loading buffer (12.5 mM Tris-HCl (pH6.8), 4% glycerol, 0.4% SDS, 1% β-Mercaptoethanol (freshly added) and 0.1% bromophenol blue) at 100º C for 5 minutes. For ATP synthase β studies, serial dilutions of 16, 8, 4 and 2 µg of the total protein for both CON and HS samples were loaded on a 12% SDS-PAGE in order to obtain the optimum band intensity. To quantify Hsp70, these numbers were changed into 80 and 40 µg loaded on a 7.5% SDS-PAGE. Both 12% and 7.5% resolving gels were prepared by 30% acrylamide/bis 37.5:1 (Cat. No. 161-0158 Bio-Rad) according to the same protocol used for 2-D gels. A 4% stacking gel was composed of 0.9 ml stacking gel buffer (0.5 M Tris-HCl pH 6.8), 0.625 ml 30% acrylamide, 37 µl 10% SDS, 50 µl 10% ammonium persulfate, 5 µl TEMED and 2.31 ml ultrapure water in the final volume of 4 ml. Prestained SDS-Page Standard Broad Range (Cat. No. 161-0318, Bio-Rad) was used as the molecular weight marker. Proteins were separated in the SDS gel using a Bio-Rad Mini-PROTEAN 3 Cell (Cat. No. 165-3301) at 150 v for 1 h and then transferred from gel to the nitrocellulose membrane (Cat. No. RPN203D, Amersham Biosciences) for 1 hour at 100 V in a transfer buffer composed of 25 mM Tris, 192 mM glycine and 10% methanol. Membrane was blocked in Blotto solution (5% non-fat dry milk and 0.05% Tween-20 in TBS) for 1 hour at room temperature. It was then incubated in a solution of polyclonal anti-ATP synthase β, produced against a conserved peptide in the sequence of this protein across different species (1:2000; Cat. No. AS05 085, Agrisera) or polyclonal anti-Hsp70, against recombinant human Hsp72 (1:5000; Cat. No. ADISPA-812 Stressgen) in Blotto, at 4ºC overnight.

2 Tris buffer saline (TBS) was composed of 150 mM NaCl, 50 mM Tris, pH 8.0.
After several washes in TBS-T (TBS, 0.1% Tween-20), membranes were incubated in HRP-conjugated goat polyclonal anti-rabbit IgG (1:10000; Cat. No. DC03L, Calbiochem) at room temperature for 1 hour. β-actin was considered as the internal loading control and was recognized by the primary antibody against a peptide containing amino acid residues 20-33 in the actin sequence, (1:10000; Cat. No. A5060, Sigma-Aldrich). The specific bands were visualized by Immobilon Western Chemiluminescent HRP Substrate (Cat. No. WBKLS0100, Millipore). X-ray films were scanned in an HP Scanjet 3670 (Hewlett-Packard Co.) with 200 dpi resolution and images were analyzed in Adobe Photoshop 7.0 (Adobe Systems Inc.). The band intensities for ATP-synthase β or Hsp70 were normalized to the actin band in the same lane while the background intensity had been subtracted from all the values.

2.9 Immunohistochemistry

Metathoracic ganglia were fixed immediately upon excision in 4% paraformaldehyde at 4°C overnight and cryoprotected for 48 hours in a solution containing 30% sucrose in PBS\(^3\). Ganglia were then embedded in cryomatrix (Cat. No. 6769006, Thermo Scientific) and frozen in chilled 2-methylbutane. Serial cryosections (10 μm-thick) were prepared from the tissue horizontally by a Shandon Cryotome (Thermo Fisher Scientific Inc.) and mounted on Superfrost-Plus slides (Cat. No. 12-550-15, Fisher Scientific). Sections were stored at –20º C until use.

For immunostaining, sections were subjected to antigen retrieval by three washes in 0.25% Triton X-100 in PBS (PBS-T), each for 10 min. Following this stage, tissues were blocked in a solution of 10% normal goat serum (Cat. No. G9023, Sigma Aldrich) and PBS-T for 1 h at room

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\(^3\) Phosphate buffer saline (PBS) was composed of 9 mM NaH\(_2\)PO\(_4\), H\(_2\)O, 12 mM Na\(_2\)HPO\(_4\), 150 mM NaCl, pH 7.4.
temperature. Sections were then transferred to a solution of polyclonal anti-Hsp70, against recombinant human Hsp72 (1:500; SPA-812, Stressgen) and 3% normal goat serum in PBS-T. After incubation in primary antibody for 48 h at room temperature, slides were three times rinsed in PBS-T, each for 10 min and then incubated in a solution of FITC-conjugated goat polyclonal anti-rabbit IgG (1:300; Cat. No. F9887 Sigma Aldrich) and 3% normal goat serum in PBS-T for 2 h, at room temperature. Ten microlitres of DAPI (Cat. No. D8417 Sigma-Aldrich) were added to the slides prior to the coverslip mounting. Working concentration of DAPI (1:10000) was prepared by diluting the stock solution (20 mg/ml) in ultrapure water and it was stored at 4°C. CON and HS slides were immunostained at the same time to control for day to day technique deviations. A control slide was always processed without primary antibody to verify non-specific signals which are not originated from antibody-target binding. Another control was applied by replacing the primary antibody with a pre-immune rabbit serum in the same concentration; this approach allows non-specific fluorescence caused by the other serum IgGs to be monitored.

Images were taken by a fluorescent Leitz microscope (Leica Microsystems) and a CCD Sensicam camera and analyzed using SlideBook version 4.2 (Intelligent Imaging Innovation). Images from CON and HS slides were captured using the same exposure time and then analyzed within the same intensity range. Quantitative analyses were carried out by manually selecting the regions of interest and comparing their mean intensities between CON and HS tissues.

2.10 Statistical tests

All statistical analyses were performed with SigmaPlot 9.01 (Systat Software, Inc.). Two-tail student t-tests were used to assess intensity differences between CON and HS groups of 2-D gels. Mann-Whitney test was used as the non-parametric test when the normality test failed and it is mentioned in the text. Two-tail t-tests were also used to compare CON and HS data in
immunohistochemistry experiments while multiple comparison of the Hsp70 expression in various parts of metathoracic ganglion was performed using one-way ANOVA followed by Holm-Sidak test. F/C measurements for gregarious and solitarious animals were also evaluated by unpaired t-tests. Differential expression of proteins measured by western blots was analyzed by paired t-test to compensate for variations in the band intensity of target protein over actin during repeated experiments. A confidence interval of 95% was considered to assess the significance of differences in all the tests. Data were plotted using Microsoft Excel. Error bars represent standard error of means.
Chapter 3

Results

3.1 2-D gel electrophoresis

3.1.1 The total protein extracted from metathoracic ganglion

Total protein extracted from MTG was initially examined in a broad pH range (3-10). Based on preliminary experiments this range was then narrowed into 5-8 to increase the spot resolution for all the proteins in the sample (Appendix 2). Likewise, for monitoring proteins with a reasonably broad range of molecular weight, proteins were separated on 12% acrylamide gels, in the second dimension. The five replicates of these gels provided enough information about the expression of proteins with a lower molecular weight than ~55 kD. This protocol was to be further optimized for proteins bigger than this range which were buried in the streaks.

Quantitative analysis for these gels revealed that there were only two spots whose intensity was significantly different between CON and HS gels (P=0.032 for spot 1 according to Mann–Whitney test and P=0.028 for spot 2 according to unpaired t-test; Figure 6, Appendix 4). These spots were excised from the gels but following enzymatic digestion and extraction from the gels, insufficient protein was obtained for identification by MALDI-TOF analyses and the acquired spectra did not contain any peaks. Whether the observed change in the intensity of spots is associated with heat shock or not, cannot be certainly concluded from the current results, since both spots are among the faintest ones on the gel and their intensity is close to the lower limit of the silver staining dynamic range (Smales et al., 2003).
Figure 6. The protein map obtained from metathoracic ganglion of *L. migratoria* by 2-D gel electrophoresis. The total protein content of tissue was extracted and separated using IPG Strips (17 cm, pH 5-8) and 12% SDS-PAGE. The top image represents one of the five replicates (HS). Two spots with a differential pattern of expression in CON and HS gels are marked on the picture and shown in the individual replicates as well. (P=0.032, P=0.028; Mann–Whitney and unpaired t-tests respectively).
These results suggested that the possible proteomic changes following heat shock might happen in very low-abundance proteins. In order to detect such proteins, an approach was required to intensify their corresponding spots on the gel. This issue could be addressed by one of the sample fractionation methods. One of the commonly used procedures for this purpose is splitting the sample according to the solubility of its proteins. This approach will be discussed with more detail, in the next section.

Moreover, 12% gels did not provide the desired resolution for proteins bigger than ~55 kD. To overcome this issue, a 7.5% acrylamide gel was used to separate proteins between ~35 and 100 kD. Better resolution of spots above ~55 kD was achieved using these gels, although the optimum spot quality was still not fully obtained (Appendix 5). Two replicates of these gels provided partial information about the expression of proteins bigger than ~55 kD. Unpaired t-tests on the resultant data showed that heat shock does not induce a significant change in the expression of this group of proteins. Gels were also visually inspected, in order to find possible differences which were not reported significant by the statistical tests.

3.1.2 The sequential extraction of proteins from MTG

Based on the need to resolve low-abundance proteins, the tissue homogenates were sequentially extracted using lysis buffers with different solubilizing capacities. Regardless of the nature of proteins extracted with each buffer, this method increases the total loading capacity of 2-D gels by splitting the sample between three IPG strips and effectively amplifies spots with the low intensity. Additionally, the number of proteins in each lysate is narrowed down by this approach and a lower complexity of sample will normally result in less streakiness on the gels.

The three protein fractions resulting from this method were separated using 17 cm IPG strips and 12% SDS PAGE (Appendix 6). Results of protein concentration assays and subsequent 2-D gels showed that nearly three quarters of the proteins were extracted in the first fraction, while the
majority of the remaining proteins were extracted by the second buffer and very few proteins were found in the third extract. Since there were not many spots on the gels resulting from the third extract and the existing spots were not obviously different between CON and HS gels, this extract was examined only once. Studying three replicates of the first extract also did not yield any significant difference between CON and HS groups (unpaired t-test). However, in three replicates of the second extract, five spots showed an increased intensity in HS gels (Figure 7, appendix 7). Although this difference between CON and HS gels was not statistically significant (unpaired student t-test), further steps were taken to identify all of these proteins.

Protein content of the mentioned spots was identified by MALDI-TOF technique following the protein digestion and extraction from the gels. These spots were all found to have the same protein which was subunit β of ATP-synthase (Appendix 8, 9; Mowse scores: 118, 139, 129, 60 and 116 and sequence coverages: 23%, 26%, 24%, 10% and 20% for spots 1, 2, 3, 4 and 5, respectively). Spots 1, 2 and 3 appeared on the gel with a molecular weight around 55 kD which is the expected size of ATP-synthase β. However, spots 4 and 5 are located at ~ 40 kD on the gels. While this spot multiplicity in the horizontal dimension could be result of various post-translational modifications (PTMs), a big decline in the molecular weight, similar to the one observed here, is usually caused by protein degradation. In order to confirm that the proteins located in the two molecular weight levels are all ATP-synthase β or its derivatives, the digested samples from representative spots in each level (spots 2, 5) were also subjected to LC/MS/MS analysis. The amino acid sequences of a few peptides in each sample digest were determined by LC/MS/MS, based on which both proteins were again identified as ATP-synthase β (Appendix 10; Mowse scores: 134 and 53, sequence coverages: 7.2%, 2.3% for spots 2 and 5, respectively).
Figure 7. Proteins were extracted in three different fractions according to their solubility properties (Bio-Rad sequential extraction kit). The protein extract used to prepare current gel is obtained by the second lysis buffer in the kit containing 8M urea and 4% CHAPS. This lysis buffer is meant to extract proteins with an intermediate solubility. Five spots appeared with an increased intensity in HS gels compared to CON ones. Image subsets and graphs on the right show the spot intensity in individual replicates. These changes were not statistically significant (unpaired t-test). The gel image on the left is one of the three replicates (HS).
3.1.3 Quantitative western blot for ATP-synthase β

ATP-synthase β could have been over-expressed at high temperatures to fulfill the energy demand in the cells during stressful conditions. However, spot multiplicity in the 2-D gels did not allow precise estimation of the overall protein level (even some other unidentified spots on the gels might contain ATP-synthase β). To investigate this issue, quantitative western blot was employed. Although the full sequence of ATP-synthase β in L. migratoria is unknown, the sequence from D. melanogaster had the highest score for matching the MALDI-TOF spectrum.

The first antibody tested was a monoclonal anti-ATP-synthase β (Cat. No. ab5432, abcam) which was raised against intact rat mitochondrion. Despite there being 91% identity between the protein sequences obtained for rat (AC. P10719, Swiss-Prot) and fruit fly (AC. Q05825, Swiss-Prot), this ATP-synthase β antibody failed to recognize the protein from L. migratoria (Appendix 11).

The second antibody examined was a polyclonal antibody against a recombinant peptide containing a highly conserved part of the sequence. Two bands were detected by this antibody on western blots of the locust ganglion extract: one band at around 55 kD (the expected MW of ATP-synthase β) and the other one about 30 kD (Figure 8). The smaller band could be a product of protein degradation or antibody cross-reactivity with a different protein. Since a similar protein degradation was also observed in 2-D gels and the aim was to measure any possible change in the protein level, both bands were taken into account. Therefore, the intensity of individual bands as well as the overall intensity in both bands were measured and normalized to the β-actin.

According to the results of three replicates, assessed by paired t-test, the overall level of ATP-synthase β does not change appreciably following heat shock.

In addition, the current antibody was used for immunoblotting a 2-D gel (from the second sequential extract), in order to detect all of the possible spots containing the epitope. The antibody recognized only three spots with the higher molecular weight (~55 kD; Appendix 12). The
ATP-synthase β (~55kD) was immunoblotted using a polyclonal primary (against a recombinant peptide conserved across the protein from different species) and a goat HRP-conjugated anti-rabbit secondary antibodies. Approximately 8 and 4 µg of the total protein for both CON and HS samples were loaded on a 12% gel side by side. The antibody detected two bands at around 55 and 30 kD. The gel image demonstrated is a representative of three replicates. The intensity of putative ATP-synthase β bands was normalized to the β-actin band visualized by a rabbit anti-β-actin (against a peptide covering amino acids 20-33). Each band’s intensity in addition to the overall intensity of both bands was considered in quantitative analysis. Numbers in the table represent the ratio of protein expression in HS tissues to the CON ones. Paired t-test analyses show that no significant change has happened in the protein level in metathoracic ganglion due to heat shock.
MALDI spectra from the five studied spots show that the two lower spots do not have the peptide peak nearest to the C-terminal, while this peak is present in the spectra from all of the bigger proteins (Appendix 8, 9). According to the information from antibody supplier, the peptide sequence used to produce the antibody is located after amino acid 368 which includes the mentioned peak sequence. These two sets of data confirm that the spots 4 and 5 both contain a partial sequence of the protein and can be a product of protein degradation. However the pattern of protein degradation in 2-D gels and western blots seemed to be different, since the smaller band detected in western blots was located at around 30 kD and the lower spots in 2-D gels were at about 40 kD. The dissimilarity observed could be due to the different sample preparations or simply because of the different protein migration patterns on an SDS gel while performing western blots or 2-D gels.

3.1.4 Proteomic analysis of the metathoracic ganglion following longer recovery times from heat shock

Sometimes a change in protein expression starts during heat shock and accumulates over time (Diller, 2006). In other words, while some proteomic changes are not detectable after one hour recovery, they may become significant after a few hours. To investigate this matter, tissues were examined after longer recovery times including 8, 12 and 24 hours. Total protein extracted from these tissues, as well as the control and 2 h-recovery tissues, were separated on 17 cm IPG strips (pH5-8) and 12% SDS pages. Samples were all processed in parallel in order to have more reproducible 2-D gels. There were no obvious differences among the first replicates of these gels, and therefore, experiments were not repeated (data not shown).
3.2 Studying the effect of stress on Hsp70 expression in the tissues of isolated *L. migratoria*

The lack of a significant heat shock response in *L. migratoria* at the protein expression level could be attributed to a high constitutive level of heat shock related proteins in the tissues of studied animals. Living in crowded conditions could be one reason for having a constant high level of Hsps. In fact, other studies show that population density is a determinant factor for Hsp synthesis in the tissues of *L. migratoria* (Wang *et al.*, 2007). Therefore, one may hypothesize that the environmental stresses such as heat shock or hypoxia will change the protein profile of the tissues in solitarious or isolated locusts more profoundly than the gregarious animals. To investigate this issue, a colony of isolated animals was reared by separating 1st instar hoppers from the gregarious colony. These animals, which could be considered as dissocians (the transient form segregated from gregarious phase), were separated as soon as they hatched. This procedure has been shown to have the same effect on the animal’s morphometrics as the more invasive procedure of washing and separating the eggs (Maciver, unpublished data).

Isolated hoppers raised in this condition were brown and when they grew up as adults, they developed some level of green pigmentation in the areas of their body which are normally yellow in the gregarious animals (Figure 9). These green areas were still not as large and intense as the ones observed in the extreme solitaries. The pronotum, with its high convex crest, had noticeably transformed toward solitarious. F/C ratio as an index which is widely used to characterize phase state in the locusts was also used to evaluate solitarious state of the animals (Hoste *et al.*, 2002). A significant shift was observed on the F/C of both isolated males and females compared to their gregarious parents (unpaired t-test, P<0.001; Figure 10). Comparing these numbers with the
Figure 9. Gregarious nymphs have a conspicuous pattern of yellow and black, whereas the solitarious hoppers vary from brown to green depending on the colour of local vegetation. The latter also has a higher crest on its pronotum. Gregarious adults have a straw colour while a yellowish pigmentation develops by aging in certain areas of their body, especially in the males. These areas have a green colour in solitarious adults. Isolating the animals immediately after they hatch drives their morphology noticeably toward solitarious (solitarious hopper and adult, image courtesy of Stephen Simpson and Gabriel Miller, respectively).
Figure 10. F/C ratio is one of the most reliable indices for phase change in locusts. Isolated males (n=27) and females (n=10) had a significantly higher F/C than the animals reared in crowded colony; n (males) = 10, n (females) =10 (unpaired t-test, p≤0.01). Published values are obtained from Uvarov (1966). Exp.; experimental, Pub.; published

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<th>Male</th>
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<tr>
<td>Isolated</td>
<td>3.46</td>
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<tr>
<td>Exp. gregarious</td>
<td>3.06</td>
<td>2.98</td>
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<tr>
<td>Pub. solitarious</td>
<td>2.93</td>
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<td>Pub. gregarious</td>
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* indicates statistically significant difference from the isolated group.
published F/C for solitarious animals shows that the current procedure has driven animals’ morphology significantly toward solitarious. In another analysis, current F/C values were compared with the published numbers for isolated locusts that were raised by separating and washing the eggs (Hoste et al., 2002). These analyses prove both procedures equally efficient to change the morphometrical indices in male and female locusts (exp.; 3.46±0.01, 3.25±0.03, pub.; 3.35±0.15, 3.15±0.16).

The expression of Hsp70 in both ganglion and muscle of the gregarious and isolated animals was examined by quantitative western blots (Figure 11). Animals were subjected to heat shock or anoxia which have been both reported to have the same thermoprotective effect on the nervous system function of L. migratoria (Newman et al., 2003). None of these treatments changed the level of Hsp70 significantly in the metathoracic ganglion or muscle of gregarious locusts. These results are consistent with the ones obtained by previous studies in which Hsp70 transcript and protein have been measured via real-time PCR and ELISA (Shoemaker et al., unpublished data; Qin et al., 2003). Likewise, Hsp70 level never changed beyond 1.5 times in ganglion or muscle of the isolated locusts following either anoxia or heat shock (three replicates were performed with each treatment.). This change was not significant according to the paired t-test and could be caused by biological or non-biological variations not associated with the treatment.

3.3 Comparing localization pattern of Hsp70 in the metathoracic ganglion of control and heat shocked locusts

Previous studies show that unlike many other organisms, the level of Hsp70 transcript or protein in the MTG of L. migratoria does not change appreciably following heat shock (Qin et
Figure 11. Hsp70 level in the ganglion and muscle of isolated and gregarious locusts was measured by western blots following heat shock or anoxia (AN), separately. Forty µg of total protein was loaded per lane. Hsp70 and β-actin were detected by polyclonal antibodies against recombinant human Hsp70 and a conserved peptide in the N-terminal of actin, respectively. An HRP-conjugated anti-rabbit IgG was used as the secondary. Data were normalized to the β-actin level. Images shown represent one of the two (gregarious) or three (isolated) replicates. Statistical analysis show that the difference in Hsp70 expression between tissues from experimental and control animals are not significant (paired t-test). Figures in the table indicate the change in Hsp70 level compared to control.
Similar results were obtained for the whole protein profile of the tissue studied by 2-D gel electrophoresis in the current research. However, these results do not rule out the possibility of local, although small, protein changes in the ganglion following heat shock. This issue was investigated for Hsp70, as an example, by doing immunohistochemistry (IHC) on horizontal sections from metathoracic ganglion. In these sections, neuron cell bodies were located at the periphery and were most concentrated near the base of connectives to the mesothoracic or abdominal ganglia. In the more ventral sections, the number of neurons in the posterior end was increased, due to the larger population of neuron cell bodies in the ventral surface of ganglion, particularly in the posterior half (Tyrer et al., 1982).

When sections were stained by hematoxylin and eosin, the cell bodies of the neurons could be easily distinguished from glial cells by their large and darkly stained cytoplasm which was full of Nissl bodies (Figure 12). In contrast, their nuclei were lightly coloured compared to the glial and tracheal cells. Glial cells were scattered between the neuron cell bodies and separated them from the core of the ganglion which was mainly composed of neural processes and synapses. The entire ganglion is surrounded by a perilemma which consists of neural lamella on the outside and the cellular perineurium inside. The former was not always visible in the sections. The core of the ganglion which is mostly occupied by axonal tracts, commissure and neuropile was poorly stained. The ganglion has also a great supply of tracheae which penetrate the perineurium and are frequently seen in different areas of MTG.

Since monoclonal antibodies are normally preferable for doing immunohistochemistry, a monoclonal anti-Hsp70 (Cat. No. C92F3A-5, Stressgen) was first tested on western blots. This antibody was raised against the native human Hsp70 protein. The translated cDNA sequence from *L. migratoria* (AC. AAP57537, Protein) and human Hsp70 sequence (AC. P08107, Swiss-Prot) have 86% identity; however, this antibody could not recognize the protein from locusts in
Figure 12. Horizontal section of metathoracic ganglion stained with hematoxylin & eosin and viewed at 50X, 200X (a, b). In a typical middle section of the tissue, neuronal cell bodies (ne) are on the periphery; mainly gathered at the four corners. Each is distinguished by its pale nucleus and darkly stained cytoplasm full of Nissl bodies. Glial cells (gl) are mainly scattered between neuronal cell bodies and occasionally seen in the core of the ganglion (cg). The whole ganglion as well as the nerves and connectives are wrapped in a fibrous perineurium (pe). Tracheae (tr) supply the ganglion by penetrating into the organ as deep as its core.
western blot. Subsequently, a polyclonal antibody against a recombinant human Hsp70 (inducible) was examined which resulted in a clear band at ~70 kD (Appendix 13). This antibody was used for the subsequent immunostainings.

In the sections stained with Hsp70 antibody, the highest immunofluorescence belonged to the cytoplasm of perineurial and tracheal cells. The faintest signal which was very close to the background intensity came from the nuclei of the neurons and glial cells; therefore, the fluorescent intensity in neuron nuclei (which was also easily distinguished from their cytoplasm) was used for normalizing the intensity in the other areas. These experiments also showed that there was an appreciably higher level of Hsp70 in the cytoplasm of glial cells compared to the neurons, whereas Hsp70 was more localized in the membrane of neurons. In the core of the ganglion which was, in general, darkly stained with the Hsp70 antibody, there was an irregular distribution of brighter spots. This pattern could be due to the higher concentration of protein in the membranes of the neural processes (Figure 13).

Two controls were used for the immunohistochemistry experiments in this study. In the first control, it was confirmed that the observed signal did not originate from non-specific binding of secondary antibody to the tissue or the autofluorescence in MTG. For this purpose, the primary antibody was omitted from one slide while the other steps were still the same. The other control was performed to ensure that the immunofluorescence was not caused by other non-specific IgGs in the serum containing the primary antibody. In order to do that, primary antibody in one slide was replaced by a pre-immune rabbit serum. These two control slides were then analyzed with the same exposure time and within the same intensity range as the slides receiving the anti-Hsp70. No fluorescence signal was observed in the control slides when studied in this condition.

Immunostained slides prepared from CON and HS tissues were visually analyzed under equal conditions as explained above. According to these analyses, heat shock did not induce an
Figure 13. Immunostaining of MTG sections with polyclonal anti-Hsp70 (against recombinant human Hsp72) and FITC-conjugated polyclonal goat anti-rabbit. The highest immunoreactivity was observed in perineurium as opposed to the neuron nuclei with the lowest intensity (a,b). The cytoplasm of glial cells and tracheal epithelium also contained higher amounts of cross-reacting protein than the neurons (c,d). In neurons Hsp70 was more localized in the cell membrane (e; arrow). Random fluorescing spots throughout the core of the ganglion suggest that this pattern continues in neural processes as well (a). Double labelling with DAPI, specifically binding to DNA, shows a notably denser chromatin in glial cells than neurons (b) and as arrows in the image (a) show the big motorneurons, sometimes, are not cut through their nuclei. ne; neuronal cell body, cg; core of the ganglion, pe; perineurium, gl; glial cells, tr; trachea.
obvious change in the Hsp70 expression pattern throughout the metathoracic ganglion (Figure 14). Additional quantitative analyses performed by measuring the fluorescence intensity in different areas of the tissue (perineurium, trachea, neuron cytoplasm, neuron nuclei, glial cells and core of the ganglion) confirmed this lack of a significant difference between CON and HS tissues stained with Hsp70 antibody (unpaired t-test; Figure 15).
Figure 14. Horizontal sections of MTG were immunostained using polyclonal anti-Hsp70 (against recombinant human Hsp72) and FITC-conjugated polyclonal goat anti-rabbit. Images from CON and HS groups were taken using the same exposure time and studied in the same intensity range. Pictures shown here represent one out of the four replicates. Heat shock does not affect Hsp70 expression throughout the metathoracic ganglion. ne; neuronal cell body, cg; core of the ganglion, pe; perineurium, gl; glial cells, tr; trachea.
Figure 15. Fluorescence intensities in neuron cytoplasm, perineurium, core of the ganglion, glial cells and trachea were normalized to the neuron nucleus intensity and compared between CON and HS tissues. No significant change was observed in these values according to heat shock (unpaired t-test). The data from both CON and HS groups show that the intensity in perineurium is significantly higher than the other parts (asterisk). Also glial cells and trachea displayed a significantly stronger signal than the core of the ganglion (neural processes) and neuron cytoplasm (daggers) (P<0.05: one-way ANOVA followed by Holm-Sidak method).
Chapter 4

Discussion

Previous experiments have shown that exposure to extreme but sub-lethal temperatures can protect neural operation during subsequent extreme temperatures. In locusts, this thermoprotection is well characterized via electrophysiological studies on both ventilatory and flight motor circuits as well as the DCMD (a visual interneuron) activity. The protective effect of heat shock is found to be correlated with a sustained $K^+$ homeostasis in the neurons which is otherwise disturbed by increasing the temperature. However, the exact mechanisms underlying this response are not understood. It has been speculated that Hsp increase, especially that of Hsp70, is responsible for these events, since their protective effects in tissues during high temperatures are well established in most systems. However, the results of my 2-D gel experiments provided no evidence to support a robust change in the protein profile of MTG following heat shock. The localization pattern of Hsp70 in the MTG was also found unchanged. Finally, I found no evidence for any robust change in the Hsp70 level in the MTG of isolated locusts following heat shock.

4.1 2-D gel electrophoresis did not provide evidence of a robust change in the protein profile of MTG following heat shock.

4.1.1 Protein expression in metathoracic ganglion is not affected by the current heat shock treatment.

The first interpretation of the current results is that protein expression in tissue does not change due to the heat shock treatment. Instead, heat shock may confer its thermoprotective effect
on the function of nervous system through some pathways which work independently from protein synthesis.

Neural circuit failure at elevated temperatures is correlated with a reduction in action potential duration and amplitude. This response could be attributed to an increase in potassium current across the cell membrane which exceeds Na\(^+\) influx and results in an incomplete action potential at high temperatures. Heat shock can compensate this effect partly through suppression of K\(^+\) efflux and keeping it in balance with the Na\(^+\) current (Robertson, 2004a). Although this response is not completely characterized at the molecular level, up-regulation of Na\(^+\)/K\(^+\) ATPase, which could accelerate the [K\(^+\)]\(_o\) clearance and/or inhibition of K\(^+\) channels which prevents K\(^+\) diffusion into the extracellular space are two potential mechanisms.

The activity of the Na\(^+\)/K\(^+\) pump could be regulated through various pathways. A long-term effect may happen via synthesis of new pumps (transcriptional regulation) or degradation of the existing ones. The short-term response, on the other hand, happens via changing kinetics of the existing pumps or translocation of Na\(^+\)/K\(^+\) ATPase from intracellular pools into the plasma membrane (Therien et al., 2000).

The regulatory effect of hormones on the Na\(^+\)/K\(^+\) ATPase for example could happen both acutely and chronically. The acute effects are best characterized for the peptide hormones which exert their effects through cAMP-, cGMP- or IP\(_3\)- mediated pathways, upon their attachment to the membrane receptors. The kinetic behavior of Na\(^+\)/K\(^+\) ATPase could be regulated by protein kinases or phosphatases whose activation is normally a downstream event in these pathways. Whether Na\(^+\)/K\(^+\) ATPase is activated or inhibited by its phosphorylation or dephosphorylation depends on the tissue type or the enzyme isoform (reviewed by Therien et al., 2000).
A similar mechanism could be induced by heat shock preconditioning to confer its thermoprotective effect on the stability of ion gradient across neural membranes. The correlation between heat shock and protein phosphorylation, in general, has been examined in human fibrosarcoma cell lines. In this study, 90 proteins underwent a significant change in their phosphorylation status following heat shock (Kim et al., 2002).

Although the overall activity of Na\(^+\)/K\(^+\) ATPase was not significantly different in homogenates prepared from CON and HS ganglia, it could be assumed that the differential activity may not appear until the K\(^+\) concentration is increased in extracellular space as a consequence of hyperthermia. Therefore, application of some alternative assays seems necessary for confirming the obtained results (Rodgers et al., 2007). It could also be speculated that the overall pump activity is unchanged in the ganglion but there are some minor changes in particular neurons which are either caused by an increase in the number of pumps or activity of the existing ones.

Moreover, involvement of K\(^+\) channels in the heat shock response has been evidenced by their pharmacological blockade using TEA. This treatment mimicked thermoprotective effects of heat shock on neural activity and suggested that the K\(^+\) conductance could be decreased following heat shock in the ion channels (Wu et al., 2001). The gating properties of these channels are modulated via different mechanisms; the attachment of a neuromodulator, for example, will alter the ion conductance of the channel via a second messenger pathway. Activation of adrenergic receptors in cardiac muscle induces phosphorylation of L-type Ca\(^{++}\) channels by a cAMP-dependent serine-threonine kinase and enhances the overall Ca\(^{++}\) current through the cell membrane. Similarly, tyrosine phosphorylation has been observed to affect physiological properties of ion channels including the K\(^+\) conducting ones (Davis et al., 2001). A similar
pathway might be triggered by a physiological stress to modulate the activation voltage of ion channels and alter the membrane conductivity.

While Na\(^+/K^+\) pumps or channels can be up-regulated independently from protein synthesis according to any of the explained mechanisms, their number can also increase in the plasma membrane without any net increase in the cell. In other words, the population of these proteins can increase in the cell surface by their translocation from intracellular pools. In skeletal muscle, for example, recruitment of Na\(^+/K^+\) ATPase from its sub-cellular stores has been observed as an acute response to remove excess potassium ions from the extracellular space following a meal or an exercise (Benziane et al., 2008). If this event is also happening following heat shock, the overall capacity of the neurolemma to handle accumulated K\(^+\) in the extracellular space could increase without any change in the overall number of the pumps or even their intrinsic activity.

In addition to the neuron-based mechanisms which were discussed above, the role of glial cells in buffering the extracellular K\(^+\) concentration should not be underestimated. In fact, the extracellular build-up of K\(^+\) is associated with a decrease in its electrical gradient across the glial membranes and leads to the inward current of K\(^+\) ions through the membrane of glial cells down their concentration gradient (Kandel et al., 1991).

Therefore, although it was postulated that a proteomic change is responsible for the physiological thermotolerance observed in the nervous system following heat shock, there are still other potential pathways for driving these effects independently from protein synthesis. The results obtained for the effect of octopamine on the nervous system, however, are in contrast with this statement (Armstrong et al., 2006). Injection of ACTD or CHX (transcription or translation blockers, respectively) to the MTG abolishes the thermoprotective effect of octopamine. There is a possibility though that ACTD or CHX interfere with the octopamine effect through disrupting
normal protein synthesis in the cells rather than inhibiting production of specific proteins induced by octopamine. Still, some may argue that octopamine-induced thermoprotection does not appear in less than one hour which indicates involvement of a transcription/translation pathway. However, other signal transduction pathways in the cell may also display their maximum effect over a similar time if they involve a long signaling cascade or if they function in an accumulative manner (Lodish et al., 2007). Meanwhile, assuming that the octopamine effect is dependent on protein synthesis, there is still not enough evidence to show how much of the heat shock effect in the nervous system is caused by the increased level of octopamine in the hemolymph.

4.1.2 Selectivity of 2-D gel electrophoresis should be considered while studying subtle changes in the protein profile.

Similar to many other quantitative techniques in biology, results of the 2-D gel experiments should be interpreted in the context of its efficiency and performance under various conditions.

The expression range of proteins in very complex samples such as the total protein extracted from tissues could be as broad as 1-10^5. In other words, the most abundant protein could be 10^5 times more copious than the least abundant protein in the sample. However, the 2-D gel technique can cover only a range of 1-10^4 in the most favorable conditions (Rabilloud, 2002). It means that information about scarce proteins in the sample is lost in favor of the most plentiful ones or vice versa, simply through changing the protein load on the gel or application of different protein enrichment techniques. A similar scenario exists for the pH range of the proteins in a sample, their size and their solubility properties; in other words, a protocol which is optimized for a subset of proteins may not be the best for another group. In this study, the lysis buffer chosen for the total protein extractions was designed to extract as many proteins from the tissue as possible. A high concentration of urea in combination with thiourea normally tends to extract hydrophobic membrane proteins. The applied buffer also contained three different detergents including ASB-
14 and Triton X-100 which have been successfully used to extract membrane proteins from bacteria (Lilley et al., 2002). However, it should be remembered that there is not any single buffer able to meet the criteria for extracting every protein with the same efficiency. Therefore, application of several lysis buffers with various compositions of chaotropes and detergents is always recommended in 2-D gel electrophoresis (Gorg et al., 2004). The sequential extraction of proteins by different lysis buffers which was applied in my experiments, to some extent, addressed this issue. One advantage of this approach is that only one sample is examined with different buffers. The other advantage is that instead of including every compound in a single buffer, which could interfere with the IEF step, they are added to the separate buffers. The major disadvantage associated with this approach is caused by its long process of sample preparation (repeated protein solubilization and precipitation). Thus the resulting gels are less reproducible than the ones made from the total protein extracts. As a supplementary experiment, therefore, 2-D gel electrophoresis could be repeated with different lysis buffers applied to separate replicate samples.

Extremely acidic (< 3) or alkaline (> 10) proteins cannot be separated even with broad pH range classical IPG strips (3-10) (Celis et al., 1999). In conventional 2-D gel electrophoresis, ultraacidic proteins which are mainly created by post-translational modifications migrate out of the IPG strip into the electrode wicks. As a solution, anodic acidic gels have been recently developed and successfully used for quantitative analyses of these proteins (Hempel et al., 2008). On the other hand, specific narrow IPGs 10-12 or 9-12 have been designed to resolve strongly basic proteins such as ribosomal and nuclear proteins (Boguth et al., 2000). In my experiments IPG strips with a pH range of 5-8 were used based on preliminary experiments showing that the proteins in the sample mainly reside in this range. However, the possibility of some proteins in the sample being out of this range cannot be totally eliminated.
In a similar manner, extremely small proteins (<10 kD) appear on 2-D gels with a relatively low spot resolution and extremely big proteins (>100 kD) are often underrepresented due to their incomplete absorption into the IPG strips. Active rehydration of IPG strips has improved the situation for the latter; however, this still remains a challenge in 2-D gel electrophoresis (Garfin, 2003). These proteins, however, have a better chance to be separated on a 1-D SDS gel, due to their lower diversity in biological samples. Small proteins or polypeptides, on the other hand, could be separated by other techniques such as HPLC which are also compatible with the later mass-spectrometry techniques (Issaq, 2001). As a matter of fact, the majority of proteins (~85%) in the Entrez Protein (NCBI protein database) reside between 10 and 100 kD which means they could be successfully detected and studied by 2-D gel electrophoresis.

The visualization method used in 2-D gel electrophoresis is another determining factor for its accuracy and reliability. An ideal stain is the one having a high sensitivity, which means being capable to detect very low-abundance proteins in the sample. It should also have a broad linear dynamic range which is the range of protein quantities providing a linear intensity of staining. The other factor which should be considered is the compatibility with post-2D gel analyses such as mass spectrometry. The silver staining which has been used in this study is one of the most sensitive approaches, being able to detect proteins as low as ~0.1 ng/spot compared to the Coomassie Brilliant Blue (CBB) with a detection limit of ~200 ng/spot. Although the dynamic range of silver staining (~10 X) is slightly smaller than CBB, the former was preferred in these experiments due to its power to detect very low-abundance proteins and, as a result, the relatively small amount of protein needed to be loaded on the IEF strips (Gorg et al., 2004). This could be particularly advantageous regarding the small size of the metathoracic ganglion in locusts.
4.1.3 The constitutive level of heat shock-related proteins in *L. migratoria* might be too high to allow identification of any further changes in the protein expression.

*Locusta migratoria* is well adapted to the semiarid regions of equatorial Africa where the average day temperature is around 32°C (Uvarov, 1966). Radiant heat from the sun or metabolic heat produced during flight could frequently push the internal body temperature to above 40°C (Chapman, 1976). In this animal, the constitutive amount of heat shock-related proteins might be relatively high in which case any subtle change in the protein level due to the current protocol would be buried in a high background of the existing proteins. In fact, there is evidence that the level of Hsps is constitutively up-regulated in the tissues of animals inhabiting areas with extreme temperatures. In other words, a big reservoir of Hsps in their tissues helps them to be constantly prepared for the harsh conditions which may suddenly happen in their environment. Therefore, these thermoadapted species do not display a drastic increase of the Hsps in their tissues following hyperthermia or other environmental stresses and their general adaptions to these stresses could be greatly modified (Evgen'ev *et al.*, 2007; Zatsepina *et al.*, 2000). The effect of thermal stress on the locomotor behavior of larvae has been compared between two ecologically distinct *Drosophila* species: *D. melanogaster* and *D. arizonae*. The latter which is a desert species was found more thermoresistant compared to the first; however, the level of Hsp70 which was constitutively high in the tissues of *D. arizonae* did not change appreciably following heat shock (Newman *et al.*, 2005). Furthermore, other studies show that over-expression of Hsp70 is detrimental especially during the course of embryogenesis, therefore animals which are constantly exposed to high temperatures in their environment apply mechanisms to maintain the Hsp70 level low in their tissues (Shilova *et al.*, 2006).

In locusts, applying ELISA to measure the Hsp70 change in the fat bodies and ganglia of heat shocked animals yielded a modest increase (less than 2-fold; Qin *et al.*, 2003). Similar results
have been obtained by measuring the Hsp70 transcript in locust fat bodies, muscle and ganglion (Shoemaker et al., unpublished data). The results of 2-D gels in my study are further evidence for not having a robust change following heat shock in the protein profile of the tissues, including heat shock and non-heat shock proteins.

However, probing newly synthesized proteins in the cultured fat bodies and ganglion of locusts has shown a significant increase in the synthesis of some proteins with the same size as the Hsps (Whyard et al., 1986; Qin et al., 2003). If the observed discrepancy is not due to the difference between in vivo and in vitro responses then it could be attributed to the high constitutive level of Hsps in the locust tissues.

Aside from being adapted to a harsh environment, the high population density similar to the condition of our gregarious locust colony could also be stressful to the animals and increase the level of Hsps in tissues. This is based on the fact that animals, in such a condition, have to compete for the limited resources, including food, in their habitat. Fifth instar nymphs of gregarious locusts have been compared with their solitarious counterparts in terms of Hsp expression in different body regions. According to this study, the amount of Hsp70 cDNA was about 2.5 times greater in the thorax of gregarious nymphs compared to solitarious ones (Wang et al., 2007). Therefore, it was presumed that the animals being isolated for a substantial period of their life (except for the embryonic stage) would display a more significant increase of Hsps in their tissues following stress than the gregarious ones, mainly because they have a lower constitutive level of Hsps. Hsp70 extracted from tissues (MTG and muscle) of both gregarious and isolated locusts was immunoblotted and compared between CON and experimental animals. In addition to heat shock, anoxia was examined as another environmental stress which has a convergent thermoprotective effect on the nervous system (Wu et al., 2002). These studies yielded a negative result for both treatments and in both tissues. There was never a consistent
change in the Hsp70 level and the observed changes were always below 2-fold. The difference between my results and the results obtained by Wang et al. (2007) might be caused by the life stage of the animals used in the two studies; in the previous work, 5th instar hoppers were examined, whereas the animals used in my study were all three-week old adults. It was also evidenced by Wang et al. (2007) that Hsp70 expression is significantly different during various developmental stages, although adults were not examined. Also, a change in the cDNA level cannot be always interpreted as an equivalent change in protein level since the final amount of protein is determined by its translation and its rate of proteolytic turnover as well (Gebauer et al., 2004). Another discrepancy might have come from the difference between extreme solitarious and isolated animals. Although the rearing procedure shifted animals’ morphometrics significantly toward solitarious, other characteristics of phase change including some of the molecular ones may happen over generations (Pener, 1991). Finally, although the MTG and muscle 112 (both studied in my experiments) are located in thorax, there are still other tissues including hymolymph, gut, integument and many more muscles which contribute in the protein profile obtained for the thorax studied by Wang et al. (2007). If none of above, it could be concluded that heat shock elicits the same proteomic response in the MTG of both gregarious and isolated (and solitarious) locusts.

4.2 The Hsp70 expression pattern throughout the metathoracic ganglion is not changed by heat shock.

According to the immunohistochemistry experiments in this study, the distribution pattern of Hsp70 in the horizontal sections of metathoracic ganglion was not affected by heat shock treatment. These observations reject the possibility of local over-expression of Hsp70 that had
been assumed undetectable in the total protein extracted from tissue. The IHC experiments also provided some information about the distribution of constitutive Hsp70 in the metathoracic ganglion of locusts. According to these data, in both heat shocked and non heat shocked tissues, the perineurium contains the biggest amount of Hsp70. This tissue is an active barrier between hemolymph and nervous system. In other words, nutrients and excretions are actively transferred into and out of the ganglion through perineurium (Wigglesworth, 1959). Its role in K⁺ homeostasis in the ganglion is also well established (Schofield et al., 1985). Since some housekeeping activities, such as folding newly-synthesized proteins, are also reported for Hsps, their over-expression in the tissues with a high metabolic activity such as perineurium is not unexpected (Bukau et al., 2006). According to the current data, Hsp70 also has a higher expression in glial cytoplasm compared to the neuron cytoplasm; this pattern resembles the differential expression of Hsp70 in the glial cells and neurons of the rat brain following hyperthermia (McCabe et al., 1993; Pavlik et al., 2003). Moreover, in vitro studies evidence that both Hsc70 and Hsp70 are secreted by glial cells and taken up by neurons (Guzhova et al., 2001).

My experiments also show that, in neurons, Hsp70 is more accumulated in the cell membrane. Localization of both Hsc70 and Hsp70 (the latter following heat shock) in the membrane-associated lipid rafts isolated from rat brain is similar to the current observation (Chen et al., 2005).

The strong signal coming from tracheae or tracheoles, on the other hand, should be interpreted cautiously. Such a signal could not be detected with the same intensity in the slides not receiving the first antibody; however, some level of contrast was always observed between trachea and the rest of tissue while the signals within a lower intensity range were studied. The addition of Hsp70 antibody to the tissue, of course, intensifies the signal and makes it more localized within the tracheal cells. An intensive literature search was performed to compare these
observations with results of similar studies. No evidence was obtained for a previous Hsp70 localization in the nervous system of insects; however over-expression of Hsp70 in the endothelium of vessels inside the mammalian brain is reported following the heat shock (Li et al., 1992). Although these two tissues are not considered equal, still the respiratory role of blood vessels in the mammalian brain is carried out by the tracheal system in the insects.

4.3 Future directions

Based on the results obtained in this study, further experiments could be suggested for future work.

2-D gel experiments could be repeated while using fluorescent dyes such as SYPRO Ruby which have a broader linear dynamic range (~\(10^3\) X) than silver staining and their detection limit (~ 1-2 ng/spot) is also comparable (Gorg et al., 2004). Moreover, proteins could be extracted from the tissue using a variety of different lysis buffers since each of these buffers may result in a slightly different protein profile of the tissue (Lilley et al., 2002). As an approach to decrease horizontal streaks on the gels which have been prepared from total protein extracts, samples could be subjected to enzymatic deglycosylation. This method will help to eliminate possible effects of saccharide groups on the isoelectric focusing of proteins (Kleinert et al., 2007). Other protein precipitation methods such as chloroform-methanol extraction could be also applied to the samples in order to remove possible non-protein molecules (Stadler et al., 2002). Furthermore, samples could be pre-fractionated by other methods such as chromatography or solution phase IEF before their isoelectric focusing (Ahmed et al., 2005; Smejkal et al., 2005). Similar to the sequential extraction of proteins already examined, the two latter methods will help to decrease the complexity of the sample. Application of alternative procedures, however, will increase the chance of proteins possibly lost in one method being detected by the other.
As mentioned earlier in this document, post-translational modifications, particularly protein phosphorylation, have a well established role in the regulation of proteins including ion pumps and channels. Hence, the phosphorylation pattern of the proteins in CON and HS samples could be compared after targeting phosphate groups on the 2-D gels with one of the available methods for staining phosphoproteins such as ones using antibodies or fluorescent phosphoprotein gel stains (Yan et al., 1998; Patton, 2004). The addition of phosphate groups to the proteins drives their isoelectric focusing point more acidic. Therefore, different phosphorylated isoforms of the protein appear as individual spots on the gel. An example of this spot multiplicity which came under attention during the current study was for ATP-synthase β. Different isoforms of this protein with similar sizes and different isoelectric points might be differentially phosphorylated states of the protein. A tentative phosphorylation prediction in NetPhos 2.0 Server for fruit fly sequence of ATP-synthase β resulted in eleven serine, seven threonine and one tyrosine residues with a greater than 50% chance of phosphorylation. Among these sites, five serines and three threonines are more than 90% likely to be phosphorylated (Blom et al., 1999). Other studies also show that the addition of phosphate groups to the ATP-synthase subunits has an important role in its assembly and function (Del Riego et al., 2006). Results of western blots do not show any evidence of overall increase in the amount of this protein; however, the phosphorylation pattern of proteins still might be different for CON and HS samples as a response to energy crises during stress. If such heat shock-associated PTMs happen in the epitope recognized by the antibody, it might have even interfered with the protein-antibody interaction and, therefore, affected quantitative analyses by eliminating the modified portion of the protein (Maya et al., 2000). Heat shock might have also changed phosphorylation pattern in some other proteins whose involvement in the response may have not been even documented before, or the ones which are suspected to be involved in heat shock response such as Na⁺/K⁺ ATPase. In order to monitor the
first group, the whole protein content of the sample could be separated by 2-D gels and examined using an appropriate method which targets phosphoproteins, whereas for the second group, the protein of interest could be purified first and its phosphorylation state could be studied subsequently.

In order to investigate whether the lack of a significant change in Hsp level is due to a high basal level of heat shock-related proteins in locust tissues, the constitutive level of these proteins, e.g. Hsp70, could be measured in the metathoracic ganglion of locusts. This amount could be then compared with the amount of protein in the tissues from a few other animals such as D. melanogaster for whom a robust heat shock response has been documented (Lindquist et al., 1988). Moreover, in a similar approach as of the one taken for isolated locusts, Hsp70 levels could be measured in the MTG of solitary animals following heat shock. Proteins extracted from MTG of the latter group could be even used in the 2-D gel experiments in order to investigate phase specific protein changes associated with stress in L. migratoria. Possible difference in the heat shock response of isolated animals and extremely solitary ones will be reflected in such experiments.

The results of my immunohistochemistry experiments show that heat shock does not change the localization pattern of Hsp70 in the metathoracic ganglion of L. migratoria. However, a few of the first and last sections (extremely dorsal or extremely ventral ones) were not included in these analyses, since they did not have the optimum quality. Thus the possibility of having different results for those parts of the ganglion could not be ruled out at this point. An alternative technique which can be used to overcome this issue is a whole mount immunostaining while the permeability of ganglion sheath to the antibodies is increased by its incubation in a solution of collagenase and hyaluronidase (Wildman et al., 2002; Schmah et al., 2003). On the other hand, application of an antibody which specifically binds to the inducible form of Hsp70 (Hsp72) will
eliminate the fluorescent signals originated from the Hsc70 (cognate protein) and will, therefore, provide a better chance for monitoring subtle difference in protein localization after heat shock. The currently used antibody has been produced against the human recombinant Hsp72, however it could also detect a strong band on the western blots prepared from the non-heat shocked human cell lysate. Since the predicted size of Hsc70 (73 kD) and Hsp70 (72 kD) are very close, it is possible that both isoforms have been detected by this antibody in the same band. Also, as emphasized earlier, additional controls are needed to support the current IHC data. One approach to further distinguish non-specific staining in the tissue is to pre-absorb the antibody with an excess amount of Hsp70 (used to raise the antiserum); this solution can be then used to immunostain the tissues. A negative result in such an experiment will confirm the specificity of the current signals. Thus, in future, similar IHC experiments could be performed while a few more Hsp70 antibodies are also examined. Alternatively, the experiments with the current antibody could be supplemented with additional negative and positive controls.

4.4 Conclusion

In animals, nervous system function fails at temperatures which are still not high enough to denature the proteins and kill the cells. There is a great body of evidence indicating that a heat shock pre-treatment can protect nervous system function against hyperthermia by raising its failure temperature and increasing its recovery rate after failure (Robertson, 2004a). This thermoprotective effect of heat shock has been demonstrated in ventilatory and flight circuits of Locusta migratoria as well as the visual system. However, the underlying mechanisms of this response have remained unknown. The results of my 2-D gel experiments in this study do not show any robust change in the protein profile of metathoracic ganglion associated with heat shock. This could mean that heat shock protects nervous system through mechanisms that do not
need increased protein expression for their function; e.g. Na\(^+\)/K\(^+\) ATPase or ion channels can be regulated by their phosphorylation or dephosphorylation through some second messenger involved pathways. Alternatively, the constitutive level of heat shock associated proteins in the locust tissues might be so high that it overshadows subtle changes in protein level caused by the stress. This issue was further investigated by measuring Hsp70 change following stress in the tissues from isolated locusts. These animals were assumed to have a lower basal level of Hsps in their tissues since they have not faced a population density pressure during their life (Wang et al., 2007). However, no evidence was found for Hsp70 increase in the tissues of isolated locusts either. The results still could be different for the solitarious locusts (who have been isolated for several generations), since some of the gregarizing factors could have been transmitted to the isolated animals from their gregarious parents (Uvarov, 1966). If this is not the case, it could be concluded that the isolated (and even the solitarious) animals behave in the same way as their gregarious counterparts in terms of the proteomic response to heat shock. Finally, the immunohistochemistry experiments were performed on the MTG using the Hsp70 antibody. These results did not show any redistribution of Hsp70 in the tissue following heat shock. However, this cannot eliminate the possibility that some other proteins might have been translocated in the tissue due to this treatment.

Phosphoproteomic analyses are suggested for future studies to specifically examine phosphoproteins on the 2-D gel. This group of proteins could be first enriched via phosphoprotein affinity chromatography, then immunoblotted with specific antibodies or visualized on the acrylamide gel with phosphoprotein-specific fluorescent dyes. The resulting image, which is in fact a profile of the protein kinase substrates in metathoracic ganglion, can be compared between CON and HS animals. This approach will help to trace possible signal transduction pathways underlying the heat shock response in the nervous system.
References


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Appendix 1

The spot number and resolution were compared while using two different lysis buffers. a. 7M Urea, 2M Thiourea and 4% CHAPS, b. 7M Urea, 2M Thiourea, 1% CHAPS, 1% ASB-14 and 1% Triton X-100. The second lysis buffer (b) significantly increased the number of spots on the gel.
a. the effect of TCA/acetone precipitation on 2-D gels was examined. This method decreased the number of spots on the gel without a significant improvement in the quality. The current gel also showed that proteins in the sample have a pH range between ~4 and ~8. b. There was a bigger concentration of proteins near pH 7 when IPG strips, pH 4-7, were used. This suggested using IPG strips, pH 5-8 in the next experiments.
Appendix 3

TCA/aceton precipitation procedure;

Ice-chilled acetone (100%) and tricloric acetate (100%) were mixed in 9:1 ratio and added to the protein sample in 4:1 ratio. Proteins were precipitated at -20º C for one hour and samples were centrifuged at 21000 xg for 15 min. Supernatant was discarded and pellet was washed by 1 ml of ice cold acetone. This supernatant was then dissolved in an appropriate amount of rehydration buffer.
The protein map obtained from metathoracic ganglion of *L. migratoria* by 2-D gel electrophoresis. The total protein content of tissue was extracted and separated using IPG Strips (17 cm, pH 5-8) and 12% SDS-PAGE. Two spots with a differential pattern of expression in CON and HS gels are marked on the picture (P=0.032, P=0.028; Mann–Whitney and unpaired t-tests, respectively).
SDS page with 7.5% concentration was used to improve the resolution of spots bigger than ~55 kD by expanding them across the second dimension.
2-D gels prepared from three different sample fractions obtained by sequential extraction of proteins.
Proteins were extracted in three different fractions according to their solubility properties (Bio-Rad sequential extraction kit). The protein extract used to prepare current gels was obtained by the second lysis buffer in the kit containing 8M urea and 4% CHAPS. Five spots had a consistently increased intensity in HS gels compared to the CON ones, although these changes were not statistically significant (unpaired t-test).
MALDI-TOF spectrum resulted from spot 2 and the position of identified peaks in the sequence of ATP-synthase β from *D. melanogaster* (Swiss-Prot AC. Q05825). Similar spectrum was also obtained for spots 1 and 3. Mowse score and sequence coverage for protein identification were 139 and 26%, respectively.
MALDI-TOF spectrum resulted from spot 5 and the position of identified peaks in the sequence of ATP-synthase β from *D. melanogaster* (Swiss-Prot AC. Q05825). Similar spectrum was also obtained for spots 4. Mowse score and sequence coverage for protein identification were 116 and 20%, respectively.
Appendix 10

Using LC/MS/MS analysis, proteins obtained from spot 2 and 5 were both identified as ATP-synthase β. The sequenced peptides from protein 2 are highlighted while underline shows the peptide obtained from spot 5. The sequence shown belongs to *D. melanogaster* (Swiss-Prot AC. Q05825). Obtained fragments from *L. migratoria* had 100% identity, in sequence, with fruit fly’s protein (Mowse scores; 134 and 53, sequence coverages; 7.2%, 2.3% for spots 2 and 5, respectively).
Appendix 11

<table>
<thead>
<tr>
<th>Locust Ganglion (15 µg)</th>
<th>Locust Ganglion (30 µg)</th>
<th>Rat brain (10 µg)</th>
<th>Rat brain (20 µg)</th>
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<tbody>
<tr>
<td></td>
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<td>150 kD</td>
<td>100 kD</td>
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<td>37 kD</td>
<td>25 kD</td>
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</table>

A monoclonal antibody which was produced against the intact rat mitochondrion (Cat. No. ab5432, Abcam) was used to detect the ATP-synthase β in the protein extracts from rat and locust tissues. This antibody did not detect the protein from *L. migratoria* but a clear band (~55 kD) was detected in the positive control prepared from the rat brain by the same procedure.
Arrow shows the spots detected by the polyclonal anti-ATP synthase β (produced against a conserved peptide in the protein sequence across the species) on the 2-D gel and the arrow head shows the migration of ATP-synthase β in the sample which has been directly loaded on the 2-D gel (1-D electrophoresis).
a. Monoclonal antibody against native human Hsp70 (Cat. No. SPA-810, Stressgen) did not detect the protein in three different tissues examined from *L. migratoria* while a strong band (~70 kD) was detected in the hamster and human samples. 
b. Polyclonal anti-Hsp70, produced against recombinant human Hsp70, detected the locust’s protein on the same membrane. Ham; hamster, Loc; locust, Hum cell lys.; human cell lysate, mus; muscle, gang.; ganglion