TARGETED OVER-EXPRESSION OF HSP22 AND THE MAINTENANCE OF LOCOMOTOR ACTIVITY OF THIRD INSTAR LARVAE OF *DROSOPHILA MELANOGASTER* AT HIGH TEMPERATURES

by

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Abstract

Hsp22 has been implicated in stress tolerance and longevity in various organisms though its role in *Drosophila melanogaster* larval thermal tolerance has not yet been investigated. I undertook this project to determine if over-expression of hsp22 in either muscle or motor neurons could alter locomotor ability at high temperature in third instar larvae of *D. melanogaster*. A combination of the UAS-gal4 and tet-On promoter systems was used to over-express transgenic hsp22 in the larvae. A β-galactosidase assay was used to determine the level of gene expression following administration of different amounts of tetracycline. A concentration of 100 μg/ml of tetracycline was found to elicit appreciably higher expression of the reporter gene than 0 and 0.1 μg/ml of tetracycline. Locomotor ability of larvae was assessed at a temperature of approximately 40^0C by measuring the time to movement failure (TMF). Larvae that were fed 100 μg/ml of tetracycline showed a significant decline in the TMF, which could be attributed to the presence of tetracycline at a concentration of 100 μg/ml. Possible reasons behind the lack of a noticeable effect of hsp22 over-expression on the TMF are discussed. The detrimental effect of tetracycline could be attributed to the decline in mitochondrial translation or a decline in the population of endogenous bacteria, which are known to exert positive effects on the development and function of *Drosophila* larvae.

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24B	A driver that expresses gal4 protein in the muscle
Akt	A serine/threonine kinase involved in apoptosis. Also called protein kinase B (PKB)
CA3	*Cornu ammonis* 3, a part of the hippocampus
Ca^{2+}	Calcium ions
CPG	Central pattern generator
CPRG	Chlorophenol red-β-d-galactopyranoside
D42	A driver that expresses gal4 protein in the motor neurons
EJP	Excitatory Junction Potential
Flp	A recombinase enzyme that binds FRT region of DNA
FRT	Flp recombinase target
Gal4	A yeast transcription factor
H11	Another name for hsp22
HspB8	Another name for human hsp22
HSE	Heat shock elements
HSF	Heat shock factor
Hsps	Heat shock proteins
Hsps	Heat shock genes
K^{+}	Potassium ions
kDa	Kilo Dalton
LacZ	A reporter gene that encodes β-galactosidase enzyme
MDa	Mega Dalton

mM	Milli molar
MPP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
NMJ	Neuromuscular junction
O26	A driver that expresses gal4 protein in the motor neurons
p38	A mitogen-activated protein kinase (MAP) involved in apoptosis.
ROS	Reactive oxygen species
Shsps	Small heat shock proteins
rtTA	Reverse tetracycline-inducible transactivator
Tet	Tetracycline
TetO	Tet-Operator region of the DNA
TMF	Time to movement failure
tTA	Tetracycline-inducible transactivator
UAS	Upstream activating sequence
w1118outX	*Drosophila melanogaster* flies that contain a mutation in the gene for eye colour, making them white.

Chapter 1

Introduction and Literature Review

1.1 Stress tolerance and heat shock proteins:

Organisms face various stresses in their lifetime, ranging from endogenous stressors such as reactive oxygen species generated by the body, to exogenous ones such as heat, irradiation and toxic chemicals. Each stress is capable of harming the vital functions of these organisms. Neural circuits are particularly vulnerable as their activity can be disrupted by stresses that are not potent enough to destroy cells or tissues. Several of these neural circuits form the basis for important behavious such as predator detection and avoidance, movement to less stressful microenvironments and control of ventilatory activity (Robertson, 2004). Their disruption would prove perilous to the organism even when the stress itself is not potent enough to kill it. Furthermore, poikilotherms are particularly vulnerable to such temperature-mediated disruption of neural activity because their body temperature mimics that of the environment. Therefore, an adaptive mechanism against such stresses would prove extremely useful.

The consistent protective effects of a sub-lethal heat shock from subsequent, more severe ones were the first indications of the existence of such adaptive mechanisms. These protective adaptations form part of the organism's heat shock response. During the course of this response, organisms express specific proteins in increasing amounts, called heat shock proteins (hsps), which were first discovered more than four decades ago as uncharacteristic puffs observed in the polytene chromosomes of the salivary glands of *Drosophila* larvae that had been accidentally exposed to high temperatures (Ritossa, 1962). This altered pattern of chromosomal swelling was

later found to indicate increased transcription of *hsp* genes in response to stress (Tissieres *et al.,* 1974).

Expression of hsp following a stressful event is achieved by transcription as well as translation. For transcription of *hsps* to occur, their promotor regions need to be bound by the Heat Shock Factor (HSF), a transcription factor that is constitutively expressed in monomeric form and diffused across the nucleus. In order to be active, it needs to convert to a trimeric state, which occurs following exposure to a stress. Preferential translation of hsp-mRNA occurs over normal mRNA because it contains no introns and untranslated sequences, which promote translation (reviewed in Michaud *et al.,* 1997).

These proteins are divided into distinct families based on their amino acid sequences, molecular weights and functions, namely hsp100, hsp90, hsp70, hsp60, hsp40 and small hsps (shsps), many of which are up-regulated during stress (Parsell and Lindquist, 1993). The up-regulation of hsps following exposure to sub-lethal, conditioning stresses is correlated with a longer time to failure and shorter period of recovery in neuronal circuits that are exposed to potentially debilitating stresses (Robertson, 2004). Hsp up-regulation might protect neuronal transmission by protecting important proteins from losing their native conformations, stabilizing cytoskeletal structures and modulators of apoptosis (Arya *et al.*, 2007). Neuronal conduction requires myriad proteins, enzymes and ion-channels, which are susceptible to denaturation during stress. Similarly cytoskeletal structures are important for several activities such as internal vesicle transport and exocytosis to release neurotransmitter molecules.

An increase in the duration of action potentials during stress to sustain flight rhythms in heat-shocked locusts and similar protective effects induced by use of K^+ channel blocking agents link modulation of K^+ channel activity to synaptic thermoprotection by hsps (Wu *et al.,* 2001). Furthermore, an increase in extracellular K^+ concentration is correlated with failure of ventilation

central pattern generator circuits in *Locusta migratoria* during heat stress. Moreover, the decline in concentration of extracellular K^+ is correlated with recovery following stress termination (Robertson, 2004). These observations indicate a possibility of modulation of ion channel activity by hsps to protect neuronal transmission during stress.

Regulation of apoptosis is another potential mechanism by which hsps might protect neuronal circuits. For instance, hsp27, a member of the shsp family, interacts with Akt and p38, which are involved in apoptosis. Moreover, it maintains Akt in its phosphorylated state and its over-expression in cells is accompanied by a decline in cell death associated with withdrawal of NGF (nerve growth factor, whose withdrawal is normally associated with cell death by apoptosis) (Mearow *et al.,* 2002).

Hsps also interact with the cytoskeleton. Use of cytoskeleton-protecting agents such as concanavalin A revealed protective effects on ventilator pattern generation in *Locusta migratoria* and neuromuscular transmission in the extensor tibiae during stress which were similar to those induced by prior heat shocks or over-expression of hsps while cytoskeleton-destabilizing agents such as colchicines, had the converse effect (Klose *et al.*, 2004; Garlick and Robertson, 2007). Furthermore, while hsps such as hsp70 are known to protect microtubules and intermediate filaments from stress-induced disassembly, shsps such as hsp27 and 20 increase the survival of cells during stress by increasing the stability of actin filaments (Mounier and Arrigo, 2002). This is hypothesized to occur by the binding of phosphorylated forms of the shsps to the actin filaments, thereby preventing their disassembly.

Synaptic transmission at the neuromuscular junction (NMJ) of *D. melanogaster* larvae is protected from high temperature stress by increasing the bouton size and cumulative area of boutons to release increased amounts of neurotransmitter into the synaptic cleft. The presence of increased amounts of hsp70 in motor neurons is correlated with this process (Xiao *et al.,* 2007).

Increased release of neurotransmitter could also be achieved through changes in cytosolic Ca^{2+} levels. Increase in extracellular Ca^{2+} levels mimic the protective effects of a prior heat shock on transmission at the NMJ of *D. melanogaster* (Barclay and Robertson, 2003). Hsp22, a shsp found in the mitochondria and known to affect lifespan and stress tolerance in adult *D. melanogaster*, could be involved in protection of neuronal transmission by pre-synaptic modulation of cytosolic Ca^{2+} levels as it is localized in the mitochondria, which store intracellular Ca^{2+}. Release of Ca^{2+} into the cytosol facilitates exocytosis of neurotransmitter vesicles and an increase in this process could protect neuronal conduction during high temperatures. I therefore, chose to analyze the effect of up-regulation of this protein in larval locomotor ability during high temperature stress. Any indications of a protective effect could be investigated further by analyzing the activity of the larval NMJ.

1.2 Small Heat Shock Proteins (shsps):

Small hsps are a family of proteins that are more diverse than members of the other hsp families, although they do share certain common characteristics listed below:

1. a conserved α-crystallin domain of approximately 90 residues,
2. a monomeric molecular mass between 12-43 kDa,
3. formation of large oligomers of up to 0.8 MDa size,
4. a dynamic quaternary structure with rapid sub-unit exchange even at room temperature and
5. stress induction (reviewed in Haslbeck *et al.*, 2005, Nakamoto and Vigh, 2007).

Though the individual amino acids in the α-crystallin domain are variable, in most shsps, it is organized into a β-sheet that is bound by the N-terminal on one side and the C-terminal on the other. This domain is needed for dimerization, though higher-order assembly requires all three regions to be present. Of the three regions, the N-terminal is the most variable between members (Nakamoto and Vigh, 2007).

Oligomerization is an important characteristic of most shsps and believed to be vital for substrate binding and their role as chaperones, though exceptions have been found such as human hsp22 and hsp20, which exist as monomers and dimers respectively. Organisms have been found to exhibit variation in the nature and size of the oligomers that they form. For instance, members from *Mycobacterium tuberculosis* (hsp16.3), *Methanococcus jannaschii* (hsp16.5) and *Triticum aestivum* (hsp16.9) form oligomers of defined size whereas mammalian shsps such as hsp25 and α-crystallin exhibit greater variation (Nakamoto and Vigh, 2007). Moreover, oligomers of shsps are dynamic, with smaller-order subunits being continuously exchanged between molecules. This exchange increases with temperature and is believed to be important in preventing aggregation of various proteins following denaturation (Haslbeck *et al.,*2005).

Oligomer disassembly increases the hydrophobic surfaces exposed for interaction with and binding to substrates such as misfolded proteins (Haslbeck *et al*., 2005). The interaction between the hydrophobic surfaces of the unfolding substrate and the newly exposed sites on the chaperone are considered vital for chaperone activity. For instance, in α-crystallin, a vertebrate eye-lens shsp, the hydrophobic N-terminal phenylalanine-rich region and the C-terminal are suggested to be necessary for chaperone activity (Reddy *et al.*, 2006). However, hydrophobicity could be one of many other factors that determine chaperone activity of shsps. These proteins lack a conserved hydrophobic residue(s). Instead, the hydrophobic surfaces that appear on attaining the final conformation are considered important. In addition, in proteins such as α-crystallin, the heat

shock domain, which contains hydrophobic surfaces, fails to show any chaperone activity when excised from the rest of the protein. These examples undermine the initial assumption of hydrophobic interactions being the sole determinant of chaperone activity among shsps. Other factors that could affect chaperone activity are oligomeric size/state, subunit exchange, quaternary structure and stability of the protein and the ionic interactions between the chaperone and substrate, particularly between residues at the C-terminal (Reddy *et al.*, 2006).

Under normal conditions, continued subunit exchange prepares shsps for activities such as regulation of the cytoskeleton and protection from apoptosis. Moreover, it prepares the cell for a rapid response upon stress exposure. Besides temperature, other factors that influence this dynamic assembly/disassembly are tissue age, pH, ionic strength, phosophorylation and protein-concentration (Sun and MacRae, 2005). Subunit exchange can occur between oligomers of the same hsps or different ones.

Once the stress is terminated, shsps need the help of ATP-dependent chaperones such as hsp70 to release and refold proteins. (Sun and MacRae, 2005). Furthermore, a single shsp complex can bind several non-native polypeptide chains (Haslbeck *et al.*, 2005). This could protect several proteins from stress-mediated damage simultaneously, in an otherwise crowded cell.

Drosophila melanogaster has four main shsps, namely hsp22, hsp23, hsp26 and hsp27. These are localized in different compartments of cells, with hsp22 found in the mitochondria, hsp27 in the nucleus and hsp23 and 26 in the cytosol (Morrow *et al.,* 2006). In spite of similarities in their sequence, different locations in the cell are indicative of different functions during the normal course of events. Chaperone activity is common to all the above, though its efficiency differs between them. Hsp22 and hsp27 were most efficient in preventing heat-induced aggregation of proteins such as citrate synthase and luciferase (Morrow *et al.,* 2006). This ability also varies with

the nature of the substrate as well. Moreover, hsp26 and 27 have revealed thermo-protective and lifespan-enhancing roles in various organisms including *D. melanogaster*.

Many members of the shsp family have been implicated in stress tolerance. For instance, over-expression of hsp27 in transgenic mice lowers the severity of kainite-induced seizures and the subsequent mortality and decline in neuronal cell death in the CA3 region of the hippocampus (Franklin *et al.,* 2005). Similarly, over-expression of hsp26 or hsp27 increased the lifespan and stress tolerance of young adult *D. melanogaster* (Haslbeck *et al.,* 1999 and Wang *et al.*, 2004). Resistance to heat and paraquat was increased following over-expression of either of the shsps. Paraquat is a herbicide that bears striking similarity to a dopaminergic neurotoxin called 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPP) and is often used to mimic oxidative stressors in experiments as it generates reactive oxygen species that are harmful to the organism. The extent of resilience to paraquat indicates the ability to cope with oxidative stress. In addition, up-regulation of human hsp27 in Chinese hamster cells was found to confer resistance to heat and toxic chemicals such as cisplatin and anti-cancer agents such as doxyrubicin (Fortin *et al.*, 2000). Hsp16.2, another member of the family, enhanced the survival of *E. coli* following exposure to heat stress and increased resistance to ectopiside-induced apoptosis. Ectopiside causes the release of cytochrome c from the mitochondria, triggering apoptosis and also prevents the activation of caspase-3, another component of the apoptosis machinery of the cell (Bellyei *et al*, 2007). However, the mechanisms underlying these protective effects of different shsps still need to be determined to get a complete picture of their activity.

1.3 Heat Shock Protein 22 (hsp22):

Hsp22 was first isolated from the genome of *D. melanogaster* in 1980 by Craig and McCarthy whereas its mammalian equivalent was first isolated in 2001 (Benndorf *et al.*, 2001). The protein itself is referred to by several names such as hsp22, hspB8, H11 kinase and E21G1, depending on the research team. For instance, Bendorf *et al.* referred to it as hspB8 as it was the eighth human shsp to be discovered (2001). Similarly, following the discovery of its serine/threonine kinase activity, it was given the name H11 (Hase *et al.*, 2005). In general, its been seen that human or mammalian hsp22 is referred to as hspB8, while hsp22 from other organisms such as *D. melanogaster* is referred to as simply hsp22.

It contains all three regions that characterize hsps namely the C-terminal, which extends from 1- 85 residues, the α-crystallin domain, which extends from 86-176 residues, and the variable N-terminal (Hu *et al,* 2007). Whereas the α-crystallin domain helps form dimers, higher-order assembly needs the presence of the variable N- and C-terminals. Moreover, the α-crystallin domain of hsp22 is unique as it does not display the usual β-sheet conformation. Instead, it exists as a randomly coiled structure (Hu *et al.*, 2007).

The gene for this protein is 1.1 kb long in *D. melanogaster* and has three heat shock elements (HSEs) up-stream of the transcribed region. Two of these are near the TATA box whereas the third lies further upstream along the genome. It localizes in the mitochondrial matrix in *Drosophila* and mammalian cells (Morrow *et al.*, 2006 and Hu *et al.*, 2007). However, the former exist as oligomers whereas the latter as a monomer. In fact mammalian hsp22 is unique in this respect. It is the only shsp that is capable of chaperone activity while existing as a monomer (Kim *et al.*, 2004 and Chowdhary *et al.*, 2004).

Among the known functions of hsp22 are chaperone activity, autokinase activity and apoptotic activity (Hu *et al.*, 2007). Chaperone activity of hsp22 is similar to that of other shsps in its mechanism. Moreover, hsp22 along with hsp27 is among the more efficient shsp-chaperones in organisms such as *D. melanogaster* (Morrow *et al.*, 2006). Its chaperone activity is believed to be vital to its protective activity during stressful conditions and aging. Induction of *hsp22* following exposure to an endogenous or exogenous stress is triggered by the presence of denatured proteins, which cause the activation of heat shock transcription factors (HSFs), by trimerization. An activated HSF moves into the nucleus and binds heat shock elements (HSEs) in the promoter regions of *hsps* genes and facilitate their transcription (Lindquist, 1986).

The hsp22 protein in *D. melanogaster* could affect stress tolerance and longevity. Its noticeable up-regulation with age lends credibility to the theory that it might play a part in countering age-accumulated stresses (King and Tower, 1999). Moreover, its presence in the mitochondria could be indicative of a role in countering the ROS generated there or of controlling the Ca^{2+} dynamics of the cell to regulate neuronal conduction. However, research on this protein and its role in stress tolerance is scant, particularly in *D. melanogaster*. Moreover, its up-regulation in *D. melanogaster* adults is correlated with a decreased lifespan and stress tolerance (Bhole *et al.*, 2004). It therefore seemed prudent to analyze this protein to illuminate its role in stress tolerance. I undertook this study to determine the effect of increased expression of hsp22 on thermal tolerance in the larval system.

1.4 *Drosophila melanogaster* as a model organism:

Drosophila melanogaster is a frequently used model system. Its larval and adult stages provide useful models to study complex behaviours such as locomotion and synaptic transmission. The

low cost associated with its rearing is an asset along with its short generation time. Its life cycle comprises four stages: egg, larva, pupa and adult. The time spent in each stage and the generation time vary with temperature. At room temperature (25^0C), the egg stage lasts a day, the larval stages take up six days while pupation lasts for about five days while the adults last for 25 days (Ashburner, 1989).

The availability of a variety of genetic tools such as a battery of mutants, genetic manipulation systems and balancer chromosomes is another vital reason behind the popularity of *D. melanogaster* as a model. Gene expression systems can be used to manipulate spatial and temporal expression of genes such as in the gal4-UAS system, Flp/FRT-system and the tet-On promoter system. Besides, *D. melanogaster* shares genes and fundamental aspects of basic cell biology and higher order events such as organ construction with higher eukaryotes. In fact, its neuromuscular system has been used as a model to study synaptic transmission. Its easy accessibility and ease of manipulation make it a popular model (Keshishian *et al.*, 1996). Not surprisingly, its been as a model for human neurodegenerative diseases such as spinal muscular atrophy, Alzheimer's and Parkinson's diseases (Cauchi and Heuvel, 2006).

1.5 Gene expression system used:

To achieve spatial and temporal specificity of gene expression, a combination of the UAS-gal4 and Tet-ON system was used. While the UAS-gal4 system allows genes to be expressed in specific tissues (Fischer *et al.,* 1988), the Tet-On system can be used to turn genes on during specific stages of the organism's lifecycle (Bieschke *et al.*, 1998).

Gal4 is a yeast transcription factor that can activate transcription of genes by binding the Upstream Activating Sequences (UAS) (Fischer *et al.*, 1988). Tissue-specific promoters joined to the *gal4* gene regulate its site of induction and the gene of interest (attached to the UAS-sequence). Thus, the UAS-gal4 system allows one to express the gene of interest in specific tissues. It is one of several conditional gene expression systems that are available. However, lack of temporal control is considered a major drawback of this system. Most *gal4*- drivers induce the gene-of-interest right from the embryonic stages, often resulting in premature lethality and severe developmental abnormalities (Bello *et al.*, 1998).

Tetracycline-inducible systems provide temporal control by using a relatively benign molecule to regulate transcription. They rely on the ability of the tet-repressor protein found in *E. coli* or its derivatives to bind to tet-operator sequences and promote transcription. In the tet-Off system, the tet-repressor protein is bound to the activation domain of the Herpes Simplex Virus and the conjoined product is referred to as the tetracycline transactivator protein (tTA) (Bieschke *et al.,* 1998). In the absence of an antibiotic, transcription occurs and the product of interest is produced. However, this process is stalled in the presence of an antibiotic. Another similar system is the tet-On system, which employs a mutated form of the tTA protein, which can bind to tet-operator sequences only in the presence of tetracycline and its analogs. This allows the researcher to activate a gene of interest in the presence of the antibiotic (Bello *et al.*, 1998 and Stebbins *et al.*, 2001).

I used a combination of the gal4-UAS and tet-On promoter systems. At least one copy of each of the following DNA constructs needs to be present in the test organism:

1. Tissue-specific driver-Gal4 construct. e.g. 24B-gal4 construct would be needed to drive a gene in the muscle (Brand and Perrimon, 1993) and D42-gal4 to drive it in the motor neurons (Parkes *et al.,* 1998)

2. UAS-rtTA

3. TetO-gene of interest (e.g. TetO-lacZ for the initial β-galactosidase assay and TetO-hsp22 for the subsequent locomotor analyses)

The tissue-specific driver allows the generation of gal4-protein in specific tissues, where it binds to the UAS sequence and leads to generation of the rtTA protein. rtTA can bind to the tet-operator region only in the presence of tetracycline or its analog. Thus, this system provides spatial and temporal control of gene expression to better understand its function *in vivo*.

1.6 Rationale behind the project:

I chose to analyze the effect of increased expression of hsp22 in the maintenance of locomotion at high temperatures. I chose hsp22 as it is localized in the mitochondria, which contain Ca^{2+} in noticeable amounts and could be involved in modulating Ca^{2+} dynamics in neurons. Altering level of extracellular Ca^{2+} in the NMJ of *D. melanogaster* larvae has been found to affect synaptic transmission. Increasing extracellular Ca^{2+} mimics the protective effects of a prior heat shock, i.e. an increase in EJP amplitude and failure temperature and quicker recovery (Barclay and Robertson, 2003). Moreover, recent research has linked it to stress resistance and longevity, as outlined below:

1. *Drosophila melanogaster* expresses the hsp22 along with hsp70 in increased amounts with age (King and Tower, 1999). These proteins could help the organism counter age-associated physiological decline that occurs due to an increase in the intensity of certain stresses with age and an accompanying decline in the organism's ability to cope with them. Furthermore, hsp70 has been established as an important protector of neural circuit function during stresses such as

hyperthermia by protecting pre-synaptic activity at the level of the neuromuscular junction (Xiao *et al.*, 2007). Therefore, it is feasible that a hsp that is up-regulated along with hsp70 would also play a similar protective role.

2. Hsp22 works as a chaperone, with temperature-dependent activity (Morrow *et al.,* 2006). Its chaperone function might be involved in protecting different cellular (mitochondrial) proteins from temperature-induced disassembly and denaturation and allow them to function at higher temperatures.

3. Moreover, other members of the shsp family such as hsp20, hsp26 and hsp27 have revealed thermoprotective effects.

The current study was designed along the lines of previous ones conducted in the Robertson lab, to analyze the effect of targeted over-expression of transgenic hsp22 on locomotion, at high temperatures. A combination of the gal4-UAS and Tet-On promoter systems was used to up-regulate hsp22 in the muscles and motor neurons. Based on the information known so far, I hypothesized that hsp22 over-expression in either tissue could affect locomotor activity at elevated temperatures. The presence of the protein in the mitochondrion and its increase in aging organisms lend credibility to the idea that hsp22 could be playing a protective function in organisms, particularly by helping it recover from ROS-induced protein damage. No studies have analyzed the effects of over-expression of this protein in *D. melanogaster* larvae though it is found to affect longevity and stress tolerance in adults (Bhole *et al.,* 2004).

1.7 Project plan:

The project consisted of three parts that were performed sequentially, as listed below:

Part 1 involved a β-galactosidase analysis of transgenic larvae that were capable of over-expressing the bacterial β-galactosidase enzyme in the muscle and motor neurons.

Part 2 comprised locomotor analyses of larvae that were capable of over-expressing transgenic hsp22 in the muscle.

Part 3 involved locomotor analyses of larvae capable of over-expressing transgenic hsp22 in the motor neurons.

I used a conditional gene expression system that combines the gal4-UAS and tet-On promotor systems. It requires tetracycline or its analog to be present in appropriate amounts in the medium to initiate transcription of the gene of interest in specific tissues. Thus, before using such a system, it is vital to determine an appropriate concentration of the antibiotic being used for maximal expression. In order to do so, I decided to run a β-galactosidase assay on larvae (of the appropriate genotype) that had been raised on one of three different concentrations of tetracycline- no tetracycline, 0.1 μg/ml and 100 μg/ml of tetracycline. These treatments were referred to as 0-tet, 0.1-tet and 100-tet respectively. Thus, the initial part of the project was run to determine answers to the following questions:

1. What is the level of expression of the protein among larvae that differed in the position of the transgenic inserts in the genome? A difference in the position of an insert can lead to differences in level of protein expressed and consequently in the extent of its activity.

2. What was the difference in the level of protein expression achieved by the different concentrations of tetracycline in the medium?

Based on the results of the assay, specific stocks and concentrations of tetracycline were chosen for the locomotor analyses.

Locomotor analyses were done using third instar larvae and gauged using a variable called Time to Movement Failure (TMF), which measures the duration for which the test animal exhibits movement (twitching, writhing, forward and backward movement) while subjected to a stress (which in this case was a temperature of 40^0C). A significant increase in the average TMF of larvae expressing hsp22 in specific tissues would indicate a protective role for the protein.

Chapter 2
Materials and Methods

2.1 Transgenic flies:

Dr. J. Tower from the University of Southern California provided the stocks of flies homozygous for the tetO-hsp22. These stocks differed in the position of the insert in the genome and in the subsequent level of gene expression in response to antibiotics. USC22S(3)22A showed a higher level of expression than USC22S(2)23 (Bhole *et al.*, 2004) . The stock containing 24B-gal4 construct and the one containing the D42-gal4 construct that were used in my study were originally generated by Brand and Perrimon (1993).

Four UAS-rtTA lines, which differed in the position of the insert in the genome, were obtained from Dr. Jerry Yin from the University of Wisconsin. The UAS-rtTA construct is located on chromosome 2 for all stocks. The scheme outlined below was used to generate UAS-rtTA; 24B-gal4 and UAS-rtTA; D42-gal4 lines:

Cross 1	Parents:	UAS-rtTA	X	w^{+}; T(2;3)ap^{Xa}, ap^{Xa}/CyO; TM3 Sb (Flybase Stock# 2475)
	F1:	UAS-rtTA/+; TM3/+ (Selfing)		
	F2:	UAS-rtTA; TM3/+		
Cross 2	Parents:	Driver-gal4	X	w^{+};T(2;3)ap^{Xa}, ap^{Xa}/CyO; TM3 Sb (Flybase Stock# 2475)
	F1:	CyO/+; Driver-gal4/+ (selfing)		
	F2:	CyO/+; Driver-gal4		

Cross 3	Parents:	UAS-rtTA; TM3/+	X	CyO/+;	Driver-gal4
	F1:	UAS-rtTA/+; Driver-gal4/+ (selfing)			
	F2:	UAS-rtTA; Driver-gal4 (selfing)			
	F3:	UAS-rtTA; Driver-gal4 (stock generated)			

(Note: 'X' refers to the fact that the stock on the left of the 'X' was mated with that listed on its right while 'F1' refers to the progeny obtained by crossing the 'parents' listed immediately above it)

Four UAS-rtTA; 24B-gal4 and three UAS-rtTA; D42-gal4 stocks were generated using this crossing scheme, which differed in the position of the UAS-rtTA construct in the genome. The generation of UAS-rtTA; 24B-gal4 and UAS-rtTA; D42-gal4 was done by Mr. Luc Poirier.

2.2 Generating the test animals:

I used a combination of the tet-On system and the gal4-UAS system to generate test animals that could express the protein of interest (β-galactosidase enzyme for the CPRG assay and hsp22 for the locomotion experiments) in the muscle and motor neurons. In order to do so, I crossed flies from two different stocks:

1. Homozygous for the tetO-gene of interest construct

2. Homozygous for the tissue-specific driver-gal4 and UAS-rtTA constructs.

To generate the appropriate controls, I crossed flies that contained the tetO-gene of interest construct with w1118outX stocks (Appendix 1).

2.3 Maintaining the test animals:

Flies were reared on standard fly food prepared in the Seroude Lab by Mrs. R. Kristensen and Mrs. F. Seroude. The following recipe was used which is adapted from Ashburner (1989) and makes approximately 10L of food. First, 6.1L water was heated in a cook pot and 0.6L molasses was added to it, after which 70.7g agar was slowly added and the mixture was boiled for 10 minutes. In addition, 614.8g of cornmeal and 253.3g of yeast were first blended and the mixture was then added to 2.5L of water and worked by hand till a smooth mix was obtained. This mix was added to the cook pot and boiled for 10 minutes to kill off the yeast. After the heat is turned off, tegosept (13.8g methyl-p-hydrobenzoic acid in 138.3ml 95% ethanol) and 49.2ml propionic acid were added to the food and it was poured in containers.

The test animals and controls of each genotype (generated by the crosses mentioned earlier) were raised in one of three treatments, namely 0-tet, 0.1-tet or 100-tet. They differed in the amount of tetracycline present in the food. While 0-tet treatment comprised standard fly food, 0.1-tet and 100-tet contained fly food supplemented with 0.1 μg/ml and 100 μg/ml of tetracycline, respectively.

In order to add tetracycline to the food, a stock solution of 100mg/ml was first prepared using tetracycline and distilled water. Tetracycline was obtained from a stock of tetracycline hydrochloride (Bioshop Canada Inc.). This was then diluted serially to obtain a 0.1mg/ml stock solution. Both 100 mg/ml and 0.1mg/ml stock solutions were then diluted by a 1000-fold using flyfood (liquefied and held at a temperature at approximately 55^0C). The food was immediately dispensed into appropriate containers and stored in a dark, cold environment until use to prevent it from getting spoilt. Tetracycline-containing food older than 3 days was not used for the experiments.

2.4 Protocol for the beta-galactosidase assay:

In order to determine the level of marker expression in the third instar *D. melanogaster* larvae in response to the different experimental conditions (tetracycline concentration and genotype), a β-galactosidase assay was performed twice using standard protocol (Seroude *et al,* 2002). The first assay analyzed the levels of enzyme following treatment with 0, 0.1 μg/ml or 100 μg/ml of tetracycline. The subsequent assay looked at enzyme levels following administration of 0, 100 and 250 μg/ml of tetracycline.

During the assay, each larva that was to be tested was transferred to a 1.7 ml Eppendorf tube (Axygen Scientific Inc., CA, USA). A hundred microlitres of the assay buffer (50 mM potassium phosphate, 1 mM magnesium chloride, pH= 7.2), containing a mixture of protease inhibitors was added to each sample (1 Complete Cocktail Tablet of protease inhibitor for 20 ml buffer) (Boehringer-Manheim, catalogue# 1697498). The extract was homogenized using a homogenizer and centrifuged for a minute at a speed of 12492 x *g* following which 10 μL of the supernatant was put in a well of a 96 well micro-plate. Finally, 100 μL of CPRG substrate (Boehringer-Manheim, catalogue# 45601222) was added to each well of the plate, which was then read for an hour at the rate of one reading per minute in a spectrophotometer set at 562 nm. The spectrophotometer ascertains the amount of the product formed per unit time by the breakdown of the CPRG substrate by the β-galactosidase enzyme. It is measured as milli-optical density per minute per larvae (ΔmOD/min/larvae). Three to four larvae were tested from each group individually and the average of their absorbance was plotted along with the standard deviation values using Microsoft Excel. Each reading obtained for the CPRG assay was normalized against the total protein content of the original sample.

2.5 Protocol for determining the protein content of the samples:

To measure the total protein content of the samples, the protocol listed in section 2.3 of the Bio-Rad Protein Assay booklet, which accompanies its products (used for such assays and mentioned later in this section) was used. A stock solution of 1 mg/ml albumin (Bioshop Canada, Inc., catalogue# ALB003) was prepared and subsequently diluted with the distilled water to get 100, 200, 300, 400 and 500μg/ml standards. In addition, a working dye reagent solution was prepared by adding four parts of distilled water to one part of Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc., Catalogue# 500-0006). Ten microlitres of each of the standards and the samples to be tested were put in individual wells of a 96-well microplate. Two hundred microlitres of working dye reagent was added to each well and the plate was read using a spectrophotometer at 595 nm. The five standards were used to generate a standard curve by plotting absorbance against concentration. This curve was used to extrapolate the protein content of the samples from their absorbances.

2.6 Details of the locomotor assay:

Locomotion was measured as the time to locomotion failure (measured in minutes). In order to do so, the third instar larvae that were raised on either standard food or food containing the appropriate amount of tetracycline were placed in an aluminum dish containing a thin layer of 1% non-nutritive agar solution. The dish had been divided into seven compartments using circular metal rings of 9 mm diameter. Of these, three compartments were randomly chosen for each trial and larvae of each treatment of a genotype were placed there for testing. All three treatments of a genotype (0-tet, 0.1-tet and 100-tet) were tested together. Once the larvae were placed in the dish,

it was put in a water bath set a room temperature and the locomotor activity recorded for 5-minutes using a Logitech webcam at a speed of 1 frame/sec. Thereafter, the dish was put in a water bath set at 44.5^0C (which would enable the temperature of the agar to reach 40^0C) and locomotor activity was recorded until all the test animals came to a standstill. Cessation of any twitching, writhing and backward and forward movements was taken to mean locomotion failure. The video recordings were used to determine the time to movement failure for each larva. The average values obtained for each treatment were plotted along with their corresponding standard error values using Microsoft Excel.

2.7 Statistical Analyses:

Microsoft Excel was used for graphing purposes whereas JMP 5.1 (SAS Institute) was used to perform the statistical analyses. A one-way ANOVA was used to analyze differences between treatments in a group. Tukey's HSD was used as a post-hoc test to determine the specific treatments that were different from each other, when a significant difference was revealed by ANOVA. Significance was assessed at $\alpha = 0.05$.

Chapter 3

Results

3.1 Results of the β-galactosidase assays:

An initial β-galactosidase analysis was performed because I had more than one kind of UAS-rtTA; driver-gal4 stocks available and wanted to pick out one that could be crossed with tetO-hsp22 construct-containing stocks to generate progeny, which showed the maximum and most consistent expression of the protein in response to tetracycline. This assay also allowed me to compare the level of protein expression in response to the different concentrations of tetracycline (Fig. 1 and 2).

Larvae obtained by crossing flies containing a tetO-LacZ construct with w1118outX flies and raised on one of the three treatments, were used as a negative control. Since they lack the tissue-specific-driver-gal4 and UAS-rtTA constructs, any expression of lacZ gene seen in these larvae would be attributed to the inherent leakiness of the tetO-LacZ strains. A noticeable leakiness in the tetO-LacZ stock used was observed in my experiments with the level of expression in the three treatments being nearly same as for the positive control.

The larval groups called M75,24B, M78,24B, F83,24B and F84,24B differ in the position of the UAS-rtTA construct in the chromosome. Each is capable of expressing the β-galactosidase enzyme in the muscle tissue. Similarly, the groups M78,D42, F83,D42 and F84,D42 are capable of expressing the enzyme in motor neurons though each differs in the position of the UAS-rtTA insert in the genome. All test groups show similar enzymatic activity in response to each of the three treatments. In each case, the 100-tet treatment elicited noticeably higher expression of β-

galactosidase whereas the other two treatments did not show any consistent difference in β-galactosidase expression (Fig. 1).

Another similar assay using 250 μg/ml of tet instead of 0.1 μg/ml of tet was performed to determine if any higher level of expression could be achieved. However, there was no difference in the amount of enzyme expression between larvae that were fed 100 and 250 μg/ml of tetracycline (Fig. 2). I concluded that 100-tet treatment elicited the maximum possible protein expression in the test larvae using this protocol.

I therefore, chose 0-tet, 0.1-tet and 100-tet treatments for the subsequent experiments. The 0.1-tet concentration was chosen because such smaller concentrations have been consistently found to help prolong the lifespan of adult *D. melanogaster* in the Seroude lab and I was interested in seeing if it would have a similar protective effect in my test assays. Moreover, based on these analyses, I chose the stocks labeled M78,24B and M78,D42 for the locomotion experiments as they displayed the most consistent expression in response to each treatment in both assays.

3.2 Locomotion analysis of larvae over-expressing hsp22 in muscle and motor neurons:

An initial analysis was done to determine the effect of tetracycline on the locomotor ability of third instar larvae at high temperature. To do so, larvae containing only the tetO-hsp22 DNA construct that differed in the amount of tetracycline that they were raised on, were tested (Fig. 3). The three treatments 0-tet, 0.1-tet and 100-tet referred to a concentration of 0, 0.1μg/ml and 100μg/ml of tetracycline respectively. The 100-tet larvae were found to have a significantly lowered TMF at high temperature as compared to the other test groups. Based on the results of

the β-galactosidase assay, the 100-tet treatment was found to induce considerably higher expression in the larvae. As these larvae were not capable of expressing the transgenic hsp22, the decline in locomotor ability can be attributed to the tetracycline present in the medium. The experiment was done using two different fly stocks that differed in the position of the tetO-hsp22 construct in the genome. Whereas the stock labeled "USC22S(3)22A" contained the tetO-hsp22 construct in the third chromosome, the stock labeled "USC22S(2)23" contained the tetO-hsp22 construct in the second chromosome. These differences in the position of the insert are known to have effects on the level of expression of the transgene, with USC22S(3)22A showing higher expression, as noted in an earlier study (Bhole *et al.*, 2004). Each control group was tested twice and the results of each experiment are shown in Fig. 3 (i.e. Day 1 for results of the first experiment and Day 2 for those of the repeat experiment). Clearly, a concentration of 100μg/ml of tetracycline significantly decreased the average TMF of the larvae while the 0-tet and 0.1-tet groups revealed similar results ($p < 0.0001$, one-way ANOVA with Tukey's HSD). This decline was noted in both USC22S(3)22A and USC22S(2)23 groups on both days. Thus, it wase concluded that a concentration of 100 μg/ml of tetracycline significantly lowers the TMF of the third instar larvae.

A similar decline was noted for larvae capable of over-expressing the transgene in motor neurons and muscles (Fig. 4 and 5). From results of the β-galactosidase assay, it can be said that larvae fed 100 μg/ml of tetracycline (100-tet) were capable of expressing a transgene whereas the other two treatments did not show any appreciable expression. "USC22S(3)22A" and "USC22S(2)23" are test groups that differ in the position of the tetO-hsp22 construct that they contain. The remaining two constructs (i.e. the tissue-specific driver-gal4 and UAS-rtTA construct) are identical in both groups. For larvae containing the USC22S(3)22A construct, the average TMF for the 0-tet and 0.1-tet was found to be significantly higher than that for the 100-tet treatment ($p < 0.0001$, one-way ANOVA with Tukey's HSD). Similarly, for larvae containing the

USC22S(2)23 construct, average TMF was significantly higher for the 0-tet and 0.1-tet treatments than 100-tet ($p < 0.0001$, one-way ANOVA with Tukey's HSD). However, since this decline mirrored the decline noted in the control groups in Fig. 3, it was attributed to the 100 μg/ml of tetracycline present in the medium rather than the over-expression of transgenic hsp22.

Figure 1. Induction of the lacZ reporter gene in response to different concentrations of tetracycline. Columns represent mean±SD ($3<n<4$) and positive control refers to larvae that contain lacZ attached to a constitutively active promoter called armadillo or arm. Also, the negative controls refer to larvae obtained by crossing w1118outX flies with ones containing the tetO-lacZ construct alone. The current figure shows the effect of 0, 0.1 and 100μg/ml of tetracycline.

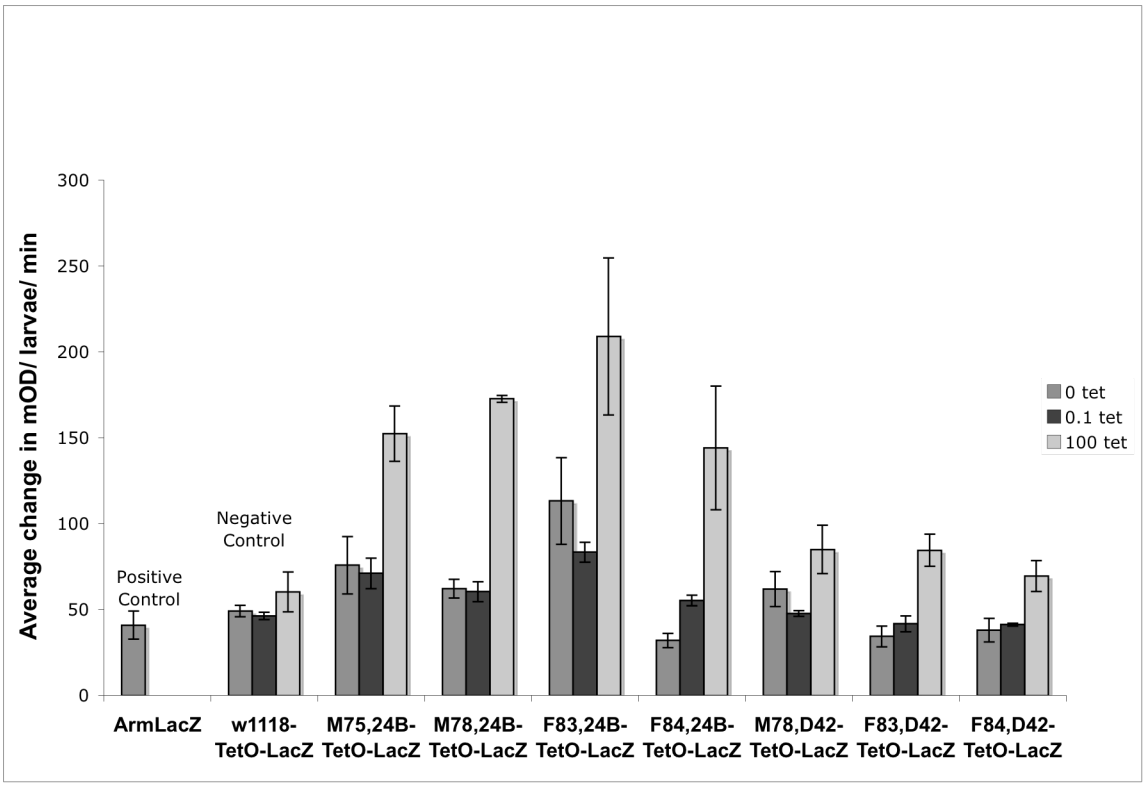

Average change in mOD/ larvae/ min
300
250
200
150
100
50
0
Positive Control
Negative Control
0 tet
0.1 tet
100 tet
ArmLacZ
w1118-TetO-LacZ
M75,24B-TetO-LacZ
M78,24B-TetO-LacZ
F83,24B-TetO-LacZ
F84,24B-TetO-LacZ
M78,D42-TetO-LacZ
F83,D42-TetO-LacZ
F84,D42-TetO-LacZ

Figure 2. Induction of lacZ in response to 0, 100 and 250μg/ml of tetracycline. Columns represent mean±SD (3<n<4) and positive control refers to larvae that contain lacZ attached to a constitutively active promoter called armadillo or arm. Also, the negative controls refer to larvae obtained by crossing w1118outX flies with ones containing the tetO-lacZ construct alone.

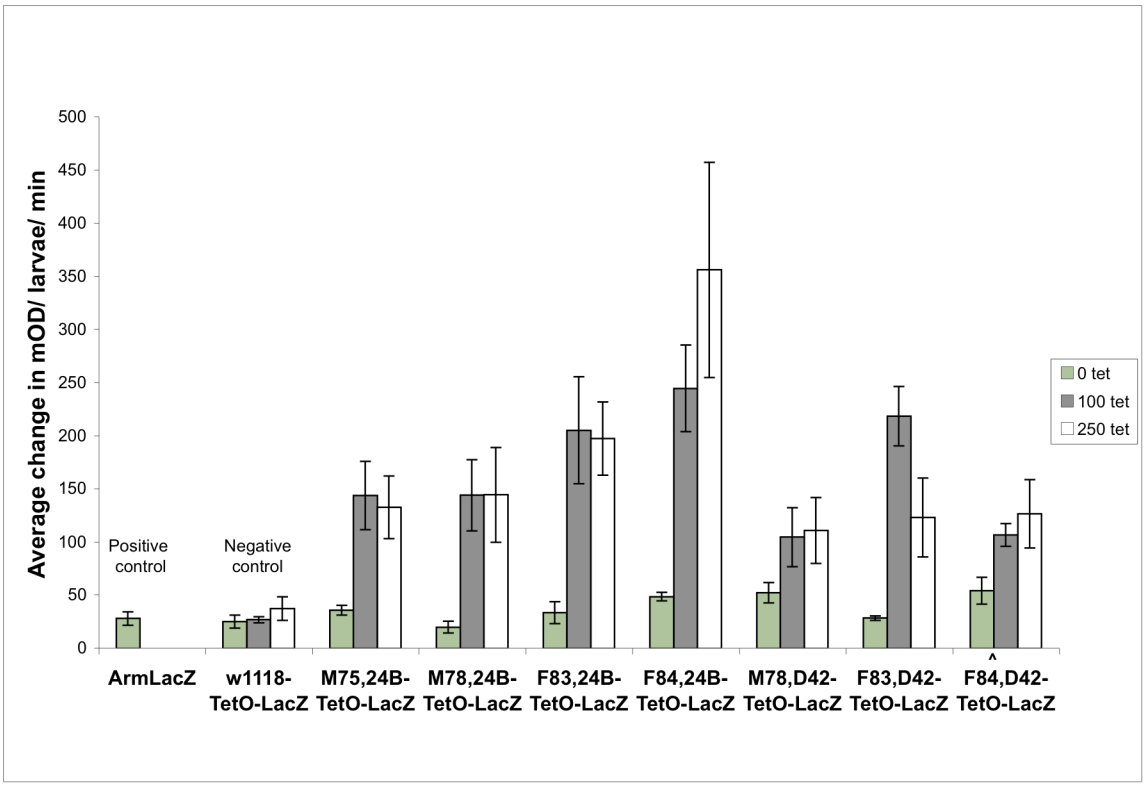

Average change in mOD/ larvae/ min
500
450
400
350
300
250
200
150
100
50
0
Positive control
Negative control
0 tet
100 tet
250 tet
ArmLacZ
w1118-TetO-LacZ
M75,24B-TetO-LacZ
M78,24B-TetO-LacZ
F83,24B-TetO-LacZ
F84,24B-TetO-LacZ
M78,D42-TetO-LacZ
F83,D42-TetO-LacZ
F84,D42-TetO-LacZ

Figure 3. Effect of tetracycline on the larval average time to movement failure. These animals contain a tetO-hsp22 construct alone and are not capable of expressing the transgene even in the presence of tetracycline. The data sets shown on the left and right parts of the graph differ in the tetO-hsp22 construct used- the former containing the USC22S(3)22A construct and the latter containing the USC22S(2)23 construct. Each experiment was replicated (Day 1, Day 2). Columns represent mean±SE. 0-tet, 0.1-tet and 100-tet represent treatments of 0, 0.1μg/ml and 100 μg/ml of tetracycline respectively. Columns with an asterisk next to them indicate treatments with a significantly different TMF from the rest ($p < 0.0001$, one-way ANOVA with Tukey's HSD, $16<n<20$).

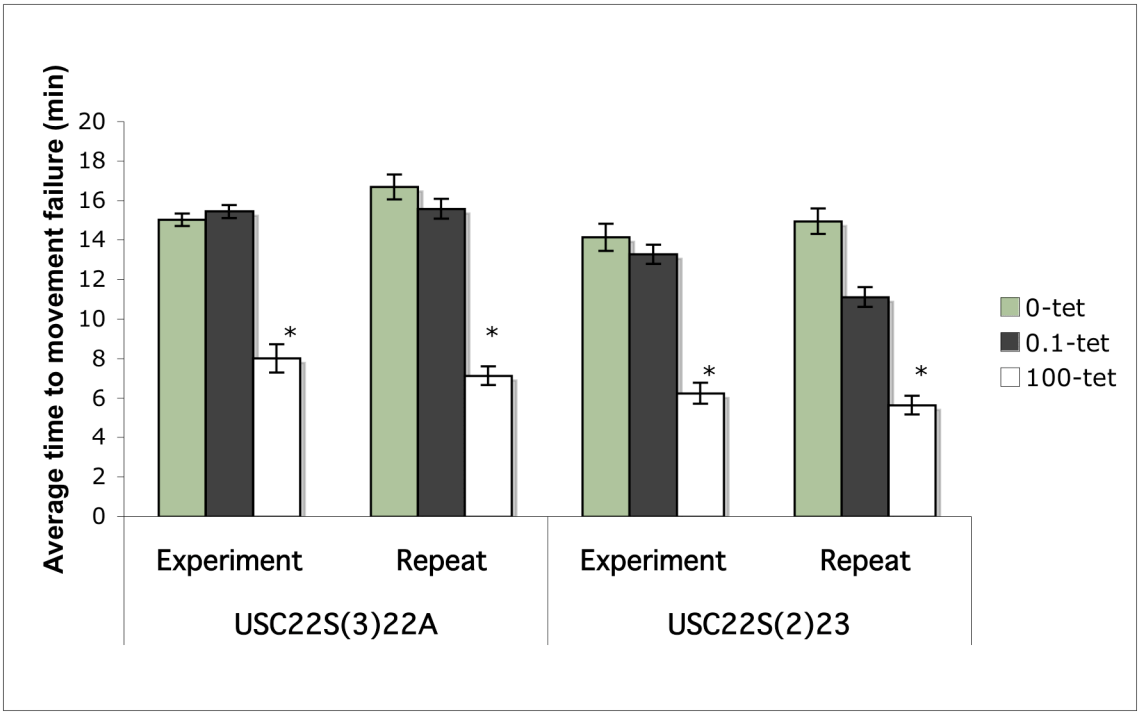

Average time to movement failure (min)
20
18
16
14
12
10
8
6
4
2
0
*
*
*
*
Experiment
Repeat
Experiment
Repeat
USC22S(3)22A
USC22S(2)23
0-tet
0.1-tet
100-tet

Figure 4. Variation in the locomotor ability of larvae capable of over-expressing hsp22 in muscle tissues across the three treatments. The data sets shown on the left and right parts of the graph differ in the tetO-hsp22 construct used- the former containing the USC22S(3)22A construct and the latter containing the USC22S(2)23 construct. Each experiment was replicated (Day 1, Day 2). Columns represent mean±SE. 0-tet, 0.1-tet and 100-tet represent treatments of 0, 0.1μg/ml and 100 μg/ml of tetracycline respectively. Columns with an asterisk next to them indicate treatments with a significantly different TMF from the rest ($p < 0.0001$, one-way ANOVA with Tukey's HSD, $16<n<20$).

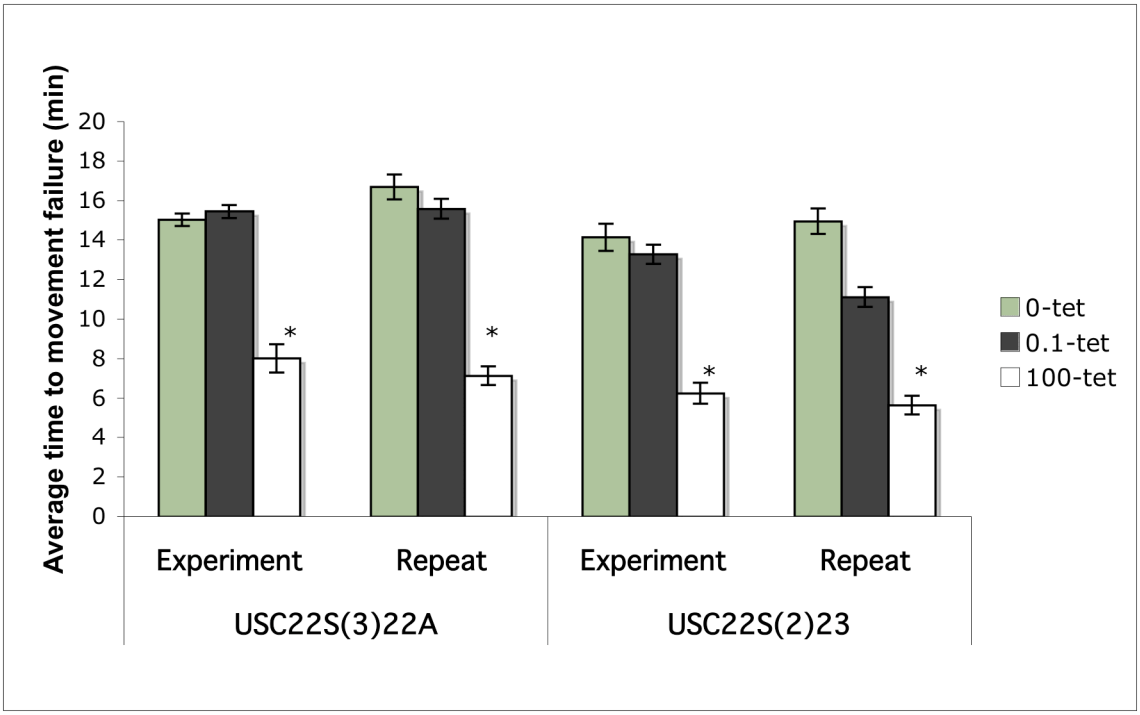
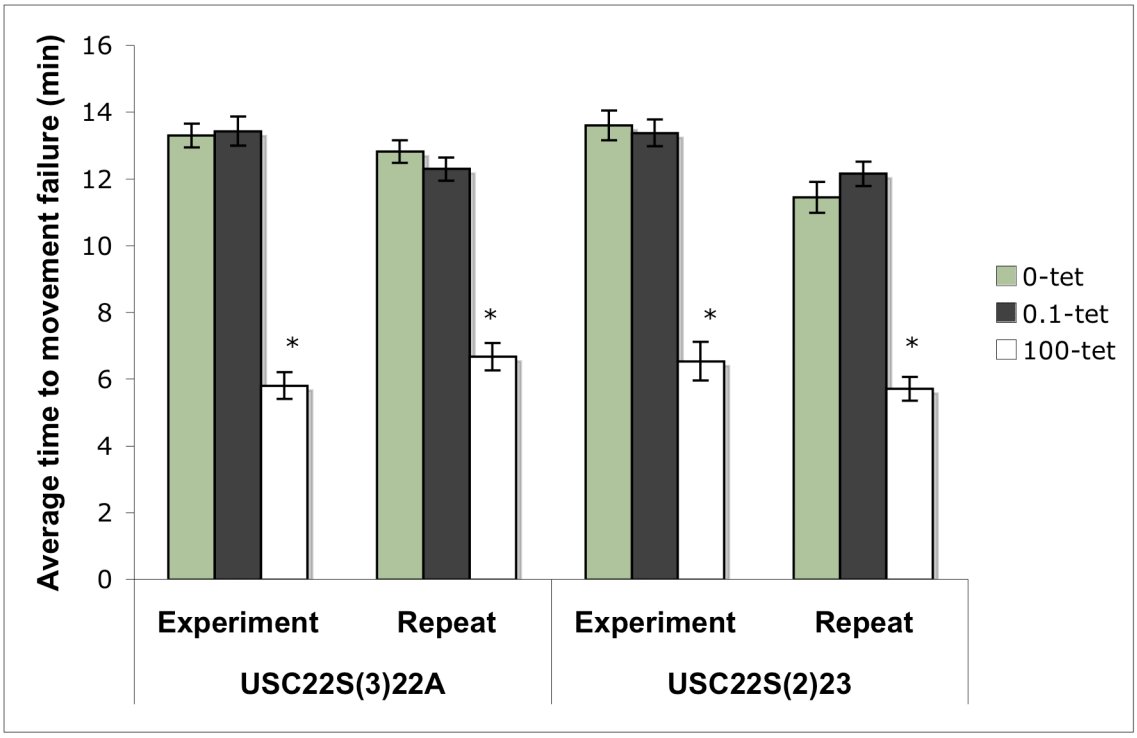

Average time to movement failure (min)
20
18
16
14
12
10
8
6
4
2
0
*
*
*
*
Experiment
Repeat
Experiment
Repeat
USC22S(3)22A
USC22S(2)23
0-tet
0.1-tet
100-tet

Figure 5. Variation in locomotor ability of larvae capable of over-expressing hsp22 in motor neurons. The data sets shown on the left and right parts of the graph differ in the tetO-hsp22 construct used- the former containing the USC22S(3)22A construct and the latter containing the USC22S(2)23 construct. Each experiment was replicated (Day 1, Day 2). Columns represent mean±SE. 0-tet, 0.1-tet and 100-tet represent treatments of 0, 0.1μg/ml and 100 μg/ml of tetracycline respectively. Columns with an asterisk next to them indicate treatments with a significantly different TMF from the rest ($p < 0.0001$, one-way ANOVA with Tukey's HSD, $16 < n < 20$).


Average time to movement failure (min)
16
14
12
10
8
6
4
2
0
*
*
*
*
0-tet
0.1-tet
100-tet
Experiment
Repeat
Experiment
Repeat
USC22S(3)22A
USC22S(2)23

Chapter 4

Discussion

Locomotion in larval *Drosophila melanogaster* is not affected by hsp22:

My experiments yielded a negative result with the locomotor ability of third instar larvae of *D. melanogaster* during high temperature being unaffected by genetic manipulation. Several factors could be responsible for these results such as the intruding effects of the use of tetracycline to activate expression of the transgene or information on moderately high temperatures on locomotion, impairment of normal locomotion (i.e. locomotion at room temperature) due to up-regulation of hsp22 and the use of a single variable to gauge locomotion. Each of these will be discussed in greater detail in the subsequent paragraphs.

As mentioned earlier in the text, no work has been done on the effect of hsp22 in larval *D. melanogaster*. Studies on its effect on the adult form have found changes in hsp22 expression to affect lifespan and stress tolerance. Its up-regulation is correlated with decrease in survival and tolerance to stresses such as high temperature and paraquat in adult *D. melanogaster* (Bhole *et al.,* 2004). Furthermore, certain observations about hsp22 seem suggestive of a role in stress tolerance, e.g. its location in a cell and its increased expression in adult *D. melanogaster* with age (King and Tower, 1999). Mitochondria contain stores of Ca^{2+} in a cell. Regulating the release of Ca^{2+} into the cytosol could be an effective means of controlling synaptic transmission as it would regulate the amount of neurotranmitter released into the synapse by exocytosis. Moreover, hsp22 could be employed by the cell to protect its native proteins from the numerous ROS that are generated in the mitochondria during oxidative phosphorylation to generate ATP. Since hsp22

has chaperone activity, it could be employed in protecting the proteins present in the mitochondria from losing their conformation and function.

During the course of my study, I noticed larvae belonging to the 100-tet-group to be visibly smaller in size than the rest irrespective of genotype. This size change could be attributed to the presence of 100 μg/ml of tetracycline in the medium. Tetracycline could have possibly caused a decline in food intake of the larvae or delayed moulting, either of which could be responsible for their reduced size. This is relevant when assessing the effect of hsp22 over-expression because this decline in size is likely to have altered the locomotor ability of the larvae as well and therefore, the larvae being tested might already be at a disadvantage as compared to the other test groups as far as locomotion is concerned. This would in turn, affect the results of my study. For instance, because of a delay in moulting, animals fed 100 μg/ml tetracycline would still in the second instar when the other groups had already progressed to the third instar. In other words, I could have been comparing locomotor activity of third instar control- and 0.1-tet-larvae with that of second instar-100-tet ones. Similarly, a reduced intake of food due to the presence of tetracycline could reduce the size of larvae and therefore, the larvae tested from 100-tet groups would have been smaller than those from the other groups. Therefore, a difference in their locomotor ability from the rest could not be attributed solely to the expression of the transgene. These possibilities could be tested by follow-up experiments. In order to address the possibility of having tested larvae that were reduced in size due to the presence of tetracycline, my study could be repeated using a concentration of tetracycline that was enough to express the transgene but not large enough to affect the size of the larvae. Since 0.1μg/ml of tetracycline did not cause any ill-effects on size in my study while 100μg/ml caused obvious detrimental effects, a number of concentrations between the two could be tested for transgene-expression and effects on size. Similarly, the possibility of tetracycline affecting the interval between moulting at a concentration

of 100 μg/ml could be tested by a study in which the duration of each larval stage was measured for a large number of larvae that are fed either no tetracycline or 100 μg/ml of tetracycline. The stage of larval development can be determined based on the morphological differences in mouth hooks of different instars, as seen under the microscope (Ashburner, 1989). Furthermore, the possibility of tetracycline affecting the food intake of the test animals at a concentration of 100 μg/ml could be tested by measuring the food intake of the animals in each group. A similar study on adult *D. melanogaster* found antibiotic cocktails (500 μg/ml ampicillin, 200 μg/ml rifamycin and 50 μg/ml tetracycline) to not have an effect on food intake, though the results likely differ for larval stages (Brummel *et al.,* 2004).

Another possibility is that the effect of hsp22 up-regulation was too subtle to be detected in an assay such as mine. This difficulty is likely to be accentuated by the pronounced detrimental effect of tetracycline noted in this study. Since tetracycline was administered in appreciably large amounts throughout development and was found to have a pronounced deleterious effect on locomotion, it is likely that any subtle negative or positive effects of hsp22 up-regulation did not show up in my analyses. This problem could be addressed by follow-up studies such as the one mentioned earlier in which a lower concentration of tetracycline than 100 μg/ml would have to be determined that could cause appreciable gene expression following brief exposure while not affecting locomotion or other parameters. This information could then be used in a subsequent experiment to study the effect of up-regulation of the transgene on locomotor ability of larvae at high temperature.

Another possibility that cannot be eliminated is of hsp22 up-regulation affecting normal larval locomotion at room temperature. Over-expression of hsp22 could have adversely affected locomotion at room temperature since *D. melanogaster* do not express this protein during the larval stages (King and Tower, 1999). If so, then the larvae over-expressing the transgene would

have their locomotion impaired even at room temperature. In other words, I might be comparing larvae that differ in their locomotor ability at room temperature. Moreover, in such a case, even if hsp22 did prove helpful at high temperature, this improvement in the locomotor ability might not become apparent unless compared with the results obtained at room temperature for the same larvae. Since I did not measure the locomotor ability of the larvae at room temperature, I cannot eliminate this possibility. During my experiments, I simply put the larvae to be tested at a room temperature water-bath for 5 minutes to help them get accustomed to the locomotion assay before placing them in a hot water bath. A follow-up experiment in which the test variable was measured for the larvae at room temperature and at high temperature could help address this possibility.

The temperature employed in the study is also an important factor in interpreting the results. I worked with a relatively higher temperature (40^0C) than is often used in studies that look at the effects of thermal stress on organisms. Different temperatures indicate different degrees of the same stress. This is likely to affect the outcome of the experiment by altering the nature and extent of proteins up-regulated to cope with the stress. This would in turn, alter the environment in which the target protein functions and hence its resultant effects. A moderately high temperature causes up-regulation of hsp70 family members in *Saccharomyces cervicae* while a much higher temperature is needed to up-regulate these in *D. melanogaster* (Michaud *et al.,* 1997). In studies of the effect of thermal stresses in cells and organisms, it is advisable to use a temperature of no higher than 36^0C because tissue damage occurs at higher temperature. (Karunanithi *et al.,* 1999). However *D. melanogaster* larvae are found to inhabit temperatures between 40^0C-45^0C in necrotic fruit (Feder *et al.*, 1996). A lack of effect of hsp22 noted in my study could imply that this protein does not play a part in tolerance of such high temperatures though it does not eliminate the possibility of its involvement in countering moderately high temperatures. An experiment along the lines of mine though at a lower temperature could be done to explore the possibility of hsp22 being involved in countering moderately high temperatures.

Another important aspect of my study is the fact that I measured locomotion failure without measuring recovery. During the locomotion experiments, the animals were left on the hot surface till locomotor activity failed, by which time they were presumed dead as they did not recover from the stress even when left at room temperature for a considerable period of time. It is useful to measure recovery along side stress tolerance as hsps often play a part in both processes. Hsp22 is believed to bind to misfolded proteins and prevent their aggregation during high temperatures while it works along side ATP-dependent chaperones such as hsp70 to help refold the proteins once the stress has been terminated. To measure both parameters, a similar study could be conducted, albeit at a lower heat-shock temperature such as 37^0C. Moreover, the animals would have to be exposed to such as stress for a relatively shorter period of time. However, comparison of such results with mine would not be entirely feasible as different heat stress temperatures could induce different sets of hsps. An alternative to this study might be one that measures the tolerance and recovery to the same temperature as I used but for five minute-intervals only or intervals that would be adequate to experience the stress but not long enough to kill the animal.

I measured the time to movement failure of the larvae at a temperature of 40^0C. Other variables that have been measured in the past include the velocity of locomotion, the distance travelled over a 5-min interval and frequency of locomotor movements (Xiao *et al.*, 2007). Each of these variables measures a slightly different though related aspect of locomotion. In several studies conducted in the Robertson lab, similar assays have been used to initially determine the effect of altered expression of a gene (s) on locomotion. For instance, the frequency of peristaltic locomotor contractions and locomotion velocity decreased on over-expressing hsp70, which was also accompanied by a decline in synaptic activity at the level of the NMJ (Klose *et al.,* 2005). Similarly, a strong correlation exists between the results obtained by a behavioural locomotor assay (measuring crawling frequency and locomotion failure temperature) and synaptic activity at the NMJ (Newman *et al.*, 2005). In all these cases, each or most of the variables measured were

similarly affected by the experimental treatments. Therefore, though its possible that the variable I tested did not measure a facet of locomotion that was altered by hsp22 over-expression, such a scenario is not very likely.

Finally, over-expression of hsp22 in motor neurons using a D42 gal4-driver could cause over-expression in other parts of the brain as well. A lack of any noticeable effect of the transgene on larval locomotion could be because it causes converse effects on the neuronal activity in the brain and in the motor neurons. Repeating the study using another motor neuronal-driver such as O26, along with D42, could test this possibility.

However, if the follow-up experiments do not support the afore-mentioned possibilities, it could be concluded that hsp22 up-regulation has indeed no effect on larval locomotion during high temperature. A lack of availability of associate hsps in appropriate amounts could provide a possible explanation of this result. Interactions between these hsps could be vital in protecting the organism from stress. Hsp22 has been found to interact with hsp20, hsp27, hspB3 and αB-crystallin in *in vitro* assays (Sun *et al.,* 2004, Fontaine *et al.,* 2005). Mutations in hsp22 lead to abnormal interactions between hsp22 and itself and other shsps such as hsp27 and αB-crystallin. Moreover, such abnormal interactions are also associated with diseases such as distal hereditary motor neuropathy, providing further evidence for their importance to proper physiological functioning and prevention of diseases (Fontaine *et al.,* 2006). The function and mechanism of interaction between these hsps, however, remains to be unravelled.

Furthermore, over-expression of hsp22 alone could create huge reservoirs of hsp22-misfolded protein aggregates as hsp22 binds misfolded proteins to prevent them from getting completely denatured and form aggregations during stress. However, it needs the help of other hsps to refold the proteins following stress termination, such as hsp70 family members (Sun and MacRae, 2005). In order for this to occur, the latter must also be present in equivalent amounts. Therefore,

over-expression of a single shsp, as in the case of my experiments, could fail to confer additional protection because of the absence of other members of the family in adequate amounts.

However, it is important to also note that some other hsps have reported positive effects in similar assays in which only a single hsp was up-regulated and its effect on stress tolerance was noted. For example. Hsp70 has now been established as a thermoprotective hsp that works at the level of the NMJ to protect locomotion during stress (Xiao *et al.,* 2007). Up-regulation of hsp70 in motor neuronal tissue using a gene expression system revealed it to have a thermoprotective role. Similarly, constitutive expression of hsp27 in murine fibroblasts confers resistance to stress (Mehlen *et al.,* 1995). These examples show that interactions between hsps are not vital for the activity of each and every hsp. To determine if they are important, an experiment similar to mine could be carried out though with a prior, sub-lethal heat shock for the test animals. If the animals expressing transgenic hsp22 show an effect of its expression on locomotion at high temperature, it could imply that hsp22 is effective because of the presence of the other hsps that it needs to interact with. To further test this possibility and figure out the specific hsps involved, an experiment involving the simultaneous up-regulation of a few hsps that are known to interact with hsp22 (such as hsp27 and hsp70) could be carried out and its results compared to those obtained from studies such as mine (in which only hsp22 is up-regulated). A significant change in the parameter being tested, if noted only in animals that are simultaneously over-expressing multiple hsps, would imply that those hsps interact with hsp22 during the course of its effect on locomotion.

Another factor to consider while assessing my results is the developmental stage of the test organisms. As mentioned earlier, previous work on hsp22 and *D. melanogaster* has focused on the adult organism. While adult *D. melanogaster* express none of the shsps (except for hsp22, whose expression increases with age), larvae express high amounts of most shsps except hsp22

(King and Tower, 1999). This is likely to have an effect on the outcome of studies such as mine because of differences in the backgrounds in which the protein of interest is being analyzed. Therefore, it is possible that hsp22 cannot exert any protective effects on the larval system because of the presence of other shsps in high amounts, which might be playing a similar protective role. Therefore, up-regulation of hsp22 could be redundant in such an organism. Moreover, an inability of hsp22 to protect locomotion in the larvae does not imply a similar role in the adult stage of the organism. As mentioned before, noticeable differences exist between these stages and the roles of proteins such as hsp22 therefore, need to be investigated separately in each.

Tetracycline is detrimental to locomotor ability at high concentrations:

Tetracycline showed significant detrimental effects on larval locomotion when administered at a concentration of 100 µg/ml. This could be due to a direct effect of the antibiotic on the physiologically processes of the larvae or due to a decline in the population of endogenous bacteria, which are believed to perform myriad functions in the organism's body. The effect noted of tetracycline on the growth or size of the larvae (and other processes) could be due to tampering with protein synthesis in the mitochondria as explained below.

Tetracycline belongs to the category of antibiotics, which target protein synthesis of bacteria in order to arrest their growth. To do so, they rely on the structural differences between the ribosomes of eukaryotic cells and prokaryotes. They bind the 30S ribosomes of prokaryotes and block the binding of aminoacyl-tRNA, thereby preventing translation and protein synthesis (Connell *et al.*, 2003). However, in spite of the differences in the eukaryotic and prokaryotic

ribosomes, these antibiotics have been found to have ill effects in the host organism along with the target bacteria. A possible reason for this could be the structural similarity between bacterial ribosomes and mitochondrial ones, which can be attributed to their common origins. Mitochondrial ribosomes have originated from the bacterial ancestors and therefore, their proteins and rRNA share sequence similarities. Therefore, mitochondrial protein synthesis could be susceptible to the effect of antibiotics. *In vitro* analysis of mammalian mitochondrial translation has found it to be affected by tetracycline (Zhang *et al.*, 2005). It is therefore likely that the detrimental effect I noticed in my analyses due to the presence of tetracycline is due to its effect on protein synthesis in the mitochondria of the test organism. Inhibition of mitochondrial protein synthesis could cause a general decline in the activity of most tissues of the body. The possibility of my results having been due to an effect of tetracycline and its analogs that target mitochondrial translation could be tested by repeating my experiments with larvae that had been raised on other antibiotics that are also known to target protein synthesis of bacteria and mitochondria such as thiostrepton and doxycycline along with ones that do not affect mitochondrial translation such as tiamulin, fusidic acid and macrolides (Zhang *et al.,* 2005). Furthermore, the concentration of tetracycline I used (100 μg/ml) could be tested for its effect on mitochondrial translation by using an *in vitro* translation system like the one used by Zhang *et al.,* 2005.

Another possible reason behind the apparent detrimental effect of tetracycline could be due to the general physiological inadequacy that is believed to arise from a decline in the population of endogenous bacteria found in most organisms, particularly in the gut. Nearly 500 species of commensal bacteria are found in the gut, with their proportion increasing from the proximal part of the gut to its distal part. These include aerobes, anaerobes and facultative aerobes. Among the functions served by these gut bacteria in mammals are the absorption of carbohydrates, lipids and micronutrients, metabolism of xenobiotics and endogenous toxins, prevention of pathogenic bacteria from colonizing the gut, priming of the immune system and proper post-natal

development of the intestines (Pal and Wu, 2005). In ruminant mammals, the cellulolytic gut bacteria are needed to digest cellulose, which forms an integral part of their diet. Such bacteria include *Ruminococcus* and *Fibrobacter*, In humans, commensals are needed to metabolise bile acids, bilirubin, cholesterol and short-chain fatty acids, besides being important for synthesis of nutrients such as menaquinones (e.g. vitamin K) (Neish, 2002).

Studies on rodents have also pointed at the importance of gut bacteria in normal gut development and activity. Rodents raised under life-long sterile conditions revealed hypoplasia of the intestinal villi, cuboidal morphology of the enterocytes themselves and a lack of development of the lymphoid follicles and physiological presence of lymphocytes and plasma cells in the lamina propria, which is the conjunctive tissue lining the epithelium of the gut. On being injected with the usual gut commensals, these effects were reversed. The mice also developed luminal mucus inspissation due to lack of degradation of the secreted mucus glycoproteins by the intestinal flora (Neish, 2002).

Similar ill effects have also been noted in the innate immune system following depletion of endogenous bacteria. Immunologically naive pigs showed profound improvements in immune structure and recruitment of dendritic cells to lamina propria and of T-cells to the epithelium and lamina propria when infected with *E. coli* (Haverson *et al.,* 2007).

Drosophila melanogaster are raised on fermenting media and oral intake is the major source of these bacteria. Adult *D. melanogaster* that were raised axenically had a shorter lifespan than ones raised on standard fly food. The former were raised on food containing 500μg/ml of ampicillin, 200μg/ml of rifamycin and 50μg/ml of tetracycline while the latter were fed standard fly food (Brummel *et al.,* 2004). The variables measured included the lifespan of the flies and their dietary intake. The bacteria present in the gut of mammals can have effects on appetite-regulators such as gastrin, ghrelin and leptin. Therefore, in this study, dietary intake was measured to test the

possibility of the antibiotics affecting survival by altering the food intake of the flies. Food intake was measured by adding radioactive tracer ^{32}P α-labelled dCTP to the food. The lifespan of flies raised axenically was decreased by approximately 35% as compared to the control though the amount of food intake was the same in both groups. It was concluded that the bacteria present in the food of flies are important to their survival, though their exact roles and the mechanisms employed to achieve them are not clear. Since I used only a single antibiotic in my experiments, its unlikely that all the endogenous commensals were targeted by it. On the other hand, its likely that certain specific species were targeted by it and a decline in the physiological functioning that is noted could be attributed to a lack of these specific species. A similar locomotor analysis on larvae that were raised axenically could be used to test the hypothesis that the detrimental effects seen in larvae that were fed 100 μg/ml in my study were due to a decline in the population of endogenous commensals. A decline in TMF that mirrors the one obtained in my study would provide support for my hypothesis.

Another important fact to note is that I worked with a juvenile stage of the organism and that they were fed tetracycline right from the egg stage. Therefore, it is highly likely that tetracycline interfered with the different developmental processes rendering them less capable of tolerating stress. Testing larvae that were fed the antibiotic only once they reach the third instar stage could test this possibility. However, the relatively short duration of this stage might pose a problem. To compensate for that, it might be necessary to elevate the concentration of the antibiotic to quite high amounts. The final concentration of antibiotic and the duration of administration should be such that they are able to induce a high level of the transgene without affecting the larval development and locomotor ability.

Summary

My experiments revealed no effect of genetic manipulation on the locomotor ability of third instar *D. melanogaster* larvae at high temperature when expressed in the muscle or motor neurons. The average time to movement failure (used as a measure of locomotor ability) did not show any difference between the control animals and ones over-expressing transgenic hsp22. Follow-up studies suggested in the Discussion could test for possible drawbacks in my protocol such as intruding effects of the use of tetracycline at a concentration of 100 μg/ml or up-regulation of transgenic hsp22 on normal larval locomotion, and help provide a more conclusive answer to whether hsp22 up-regulation affects larval locomotion during high temperatures. If however, these studies also fail to show any effects of up-regulation of the protein, a possible reason could be the lack of the presence of other hsps in appropriate amounts. Shsps are known to interact with one another during the course of their activity and this is believed to be very important to their protective roles during stress. Presumably, up-regulation of hsp22 alone was not able to elicit any protective effects as the other shsps that it needs to associate with were not present in adequate amounts. Moreover, over-expression of a single shsp could also have altered the stoichiometry of these interactions, making it impossible for the proteins to perform their function. Tetracycline, the antibiotic used to turn on the gene expression system (a combination of the UAS-gal4 and tet-On promoter systems) had a detrimental effect on locomotion at a concentration of 100 μg/ml. This could occur partly due to a decline in mitochondrial protein synthesis in the presence of tetracycline and in part, due to a severe decline in the population of certain endogenous commensals. *In vitro* mitochondrial translation is known to be susceptible to tetracycline. Moreover, studies on different organisms have also revealed ill-effects of declining numbers of endogenous micro flora. Their protective roles are known to extend to the

development and functioning of the digestive and immune systems of organisms though the exact mechanism of their activity in each case is not known. Future studies need to focus on unraveling the importance and details of interactions between hsp22 and other hsps along with the mechanistic details of the effects of tetracycline on mitochondrial translation *in vivo*. Finally the mechanisms employed by endogenous bacteria to exert their protective effects need to also be studied.

Literature cited

Ashburner, M (1989). *Drosophila:* A laboratory handbook. Cold Spring Harbor Laboratory Press.

Arya,R, Mallik, M and Lakhotia, S (2007). Heat shock genes- integrating cell survival and death. Journal of Bioscience **32:** 595-610.

Barclay, JW and Robertson, RM (2003). Role for calcium in heat shock-mediated synaptic thermoprotection in *Drosophila* larvae. Journal of Neurobiology **56:** 360-371.

Bello, B, Resendez-Perez, D and Gehring, WJ (1998). Spatial and temporal targeting of gene expression in *Drosophila* by means of a tetracycline-dependent transactivator system. Development **125:** 2193-2202.

Bellyei, S, Szigeti, A, Pozsgai, E, Boronkai, A, Gomori, E, Hocsak, E, Farkas, R, Sumegi, B and Gallyas, F Jr. (2007). Preventing apoptotic cell death by a novel small heat shock protein. European Journal of Cell Biology **86:** 161-171.

Bendorf, R, Sun, X, Gilmont, RR, Biederman, KJ, Molloy, MP, Goodmurphy, CW, Cheng, H, Andrews, PC and Welsh, MJ (2001). Hsp22, a new member of the small heat shock protein family, interacts with mimic of phosphorylated hsp27. Journal of Biological Chemistry **276:** 26753-26761.

Bhole, D, Allikan, MJ and Tower, J (2004). Doxycycline-regulated over-expression of hsp22 has negative effects on stress resistance and life span in adult *Drosophila melanogaster*. Mechanisms of Aging and Development **125:** 651-663.

Bieschke, ET, Wheeler, JC and Tower, J (1998). Doxycycline-induced transgene expression during *Drosophila* development and aging. Molecular And General Genetics **258:** 571-579.

Brand, AH and Perrimon, N (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development **118:** 401-415

Brummel, T, Ching, A, Seroude, L, Simon, AF and Benzer, S (2004). *Drosophila* lifespan enhancement by exogenous bacteria. Proceedings Of The National Academy Of Sciences Of The United States Of America **101:** 12974-12979.

Cauchi, RJ and Heuvel, MVD (2006). The fly as a model for neurodegenerative diseases: is it worth the jump? Neuro-degenerative Diseases **3:** 338-356.

Chowdhary, TK, Raman, B, Ramakrishna, T and Rao, CM (2004). Mammalian hsp22 is a heat-inducible small heat shock protein with chaperone-like activity. Journal of Biochemistry **381:** 379-387.

Connell, SR, Tracz, DM, Nierhaus, KH and Taylor, DE (2003). Ribosomal protection proteins and their mechanisms of tetracycline resistance. Antimicrobial Agents And Chemotherapy **47:** 3675-3681.

Feder, ME, Cartano, NV, Milos, L, Krebs, RA and Lindquist, S (1996). Effect of engineering *hsp70* copy number on hsp70 expression and tolerance of ecologically relevant heat shock in larvae and pupae of *Drosophila melanogaster.* Journal of Experimental Biology **199:** 1837-1844.

Fischer, JA, Giniger, E, Maniatis, T and Ptashne, M (1988). GAL4 activates transcription in *Drosophila*. Nature **332:** 853-856.

Fontaine, JM, Sun, X, Benndorf, R and Welsh, MJ (2005). Interactions of hsp22 (hspB8) with hsp20, αB-crystallin, and hspB3. Biochemical and Biophysical Research Communications **337:** 1006-1011.

Fontaine, JM, Sun, X, Hoppe, AD, Simon, S, Vicart, P, Welsh, MJ and Benndorf, R (2006). Abnormal small heat shock protein interactions involving neuropathy-associated hsp22 (hspB8) mutants. Federation of American Societies for Experimental Biology Journal **20:** 2168-2170.

Fortin, A, Raybaud-Diogene, H, Tetu, B, Huot, J, Landry, J and Deschenes,R (2000). Over-expression of the 27 kDa heat shock protein is associated with thermoresistance and chemoresistance but not with radioresistance. International Journal of Radiation Oncology **46:** 1259-1266.

Franklin, TB, Krueger-Naug, AM, Clarke, DB, Arrigo, AP and Currie, RW (2005). The role of heat shock proteins hsp70 and hsp27 in cellular protection of the central nervous system. International Journal of Hyperthermia **21:** 379-392.

Garlick, KM and Robertson, RM (2007) Cytoskeletal stability and heat shock-mediated thermoprotection of central pattern generation in *Locusta migratoria.* Comparitive Biochemistry and Physiology, Part A **147:** 344-348.

Hase, M, Depre, C, Vatner, SF and Sadoshima, J (2005). H11 has dose-dependent and dual hypertrophic and proapoptotic function in cardiac myocytes. The Biochemical Journal **388:** 475-483.

Haslbeck, M, Walke, S, Stromer, T, Ehrnsperger, M, White, HE, Chen, S, Saibil, HR and Buchner, J (1999). Hsp26: a temperature-regulated chaperone. The European Molecular Biology Organization Journal **18:** 6744-6751.

Haslbeck, M, Franzmann, T, Weinfurtner, D and Buchner, J (2005). Some like it hot: the structure and function of small heat shock proteins. Natural Structural and Molecular Biology **12:** 842-846.

Haverson, K, Rehakova, Z, Sinkora, J, Sver, L and Bailey, M (2007). Immune development in jejunal mucosa after colonization with selected commensal gut bacteria: a study in germ-free pigs. Veterinary Immunnology And Immunopathology (In Press).

Hu, Z, Chen, L, Zhang, J, Li, T, Tang, J, Niangui, T, Xu, N and Wang, X (2007). Structure, function, property and role in neurological diseases and other diseases of the shsp22. Journal of Neuroscience Research **85:** 2071-2079.

Karunanithi, S, Barclay, JW, Robertson, RM, Brown, IR and Atwood, HL (1999). Neuroprotection at *Drosophila* synapses conferred by prior heat shock. Journal of Neuroscience **19:** 4360-4369.

Keshishian, H, Broadie, K, Chiba, A and Bate, M (1996). The *Drosophila* neuromuscular junction: a model system for studying synaptic development and function. Annual Review of Neuroscience **19:** 545-575.

Kim, MV, Seit-Nebi, AS, Marston, SB and Gusev, NB (2004). Some properties of human small heat shock protein hsp22 (H11 or hspB8). Biochemical and Biophysical Research Communications **315:** 796-801.

King, V and Tower, J (1999). Aging-specific expression of *Drosophila* hsp22. Developmental Biology **207:** 107-118.

Klose, MK, Armstrong, G and Robertson, RM (2004). A role for the cytoskeleton in heat shock-mediated thermoprotection of locust neuromuscular junctions. Journal of Neurobiology **60:** 453-462.

Klose, MK, Chu, D, Xiao, C, Seroude, L and Robertson, RM (2005). Heat shock-mediated thermoprotection of larval locomotion compromised by ubiquitous over-expression of hsp70 in *Drosophila melanogaster*. Journal of Neurophysiology **94:** 3563-3572.

Lindquist, S (1986). The heat shock response. Annual Review of Biochemistry **55:** 1151-1191.

Mearow, KM, Dodge, ME, Rahimtula, M and Yegappan, C (2002). Stress-mediated in PC12 cells- the role of small heat shock protein, hsp27, and Akt in protecting cells from heat stress and nerve growth factor withdrawal. Journal of Neurochemistry **83:** 452-462.

Mehlen, P, Preville, X, Chareyron, P, Briolay, J, Klemenz, R and Arrigo, AP (1995). Constitutive expression of human hsp27, *Drosophila* hsp27, or human αB-crystallin confers resistance to TNF- and oxidative stress-induced cytotoxicitiy in stably transfected murine L929 fibroblasts. The American Association of Immunologists **154:** 363-374.

Michaud, S, Marin, R, Westwood, JT and Tanguay, RM (1997). Cell-specific expression and heat-shock induction of hsps during spermatogenesis in *Drosophila melanogaster.* Journal of Cell Science **110:** 1987-1997.

Morrow, G, Heikkila, JJ and Tanguay, RM (2006). Differences in the chaperone-like activities of the four main small heat shcok protesin of *Drosophila melanogaster.* Cell Stress and Chaperones **11:** 51-60.

Mounier, N and Arrigo, AP (2002). Actin cytoskeleton and small heat shock proteins: how do they interact? Cell Stress and Chaperones **7:** 167-176.

Nakamoto, H and Vigh, L (2007). The small heat shock proteins and their clients. Cellular and Molecular Life Sciences **64:** 294-306.

Neish, AS (2002). The gut microflora and intestinal epithelial cells: a continuing dialogue. Microbes And Infection **4:** 309-317.

Newman, AE, Xiao, C and Robertson, RM (2005). Synaptic transmission in a desert-dwelling *Drosophila* species. Journal of Neurobiology **64:** 170-180.

Pal, S and Wu, L (2005). Guts and glory: balancing microbes and the immune response. Developmental Cell **8:** 125-132.

Parkes, TL, Elia, AJ, Dickinson, D, Hilliker, AJ, Phillips, JP and Boulianne, GL (1998). Extension of *Drosophila* lifespan by over-expression of human *SOD1* in motor neurons. Nature Genetics **19:** 171-174.

Parsell, DA and Lindquist, S (1993). The function of heat shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annual Review of Genetics **27:** 437-496.

Reddy, GB, Kumar, A and Kumar, MS (2006). Chaperone-like activity and hydrophobicity of α-crystallin. Life **58:** 632-641.

Ritossa, F (1962). A new puffing pattern induced by heat shock and DNP in *Drosophila.* Experentia **18:** 571-573.

Robertson, RM (2004). Thermal stress and neural function: adaptive mechanisms in insect model systems. Journal of Thermal Biology **29:** 351-358.

Seroude, L, Brummel, T, Kapahi, P and Benzer, S (2002). Spatio-temporal analysis of gene expression during aging in *Drosophila melanogaster*. Aging Cell **1:** 47-56.

Stebbins, MJ, Urlinger, S, Byrne, G, Bello, B, Hillen, W and Yin, JC (2001). Tetracycline-inducible systems for *Drosophila*. Proceedings of The National Academy of Sciences of The United States of America **98:** 10775-10780.

Sun, X, Fontaine, JM, Rest, JS, Shelden, EA, Welsh, MJ and Benndorf, R (2004). Interaction of human hsp22 (HSPB8) with other small heat shock proteins. Journal of Biological Chemistry **279:** 2394-2402.

Sun, Y and MacRae, TH (2005). Small heat shock proteins: molecular structure and chaperone function. Cellular and Molecular Life Sciences **62:** 2460-2476.

Tissieres A, Mitchell, HK and Tracy, UM (1974). Protein expression in salivary glands of *Drosophila melanogaster*: relation tochromosome puffs. Journal of molecular biology **84:** 389-398.

Wang, H, Kazemi-Esfarjani, P and Benzer, S (2004). Multiple-stress analysis for isolation of *Drosophila* longevity genes. Proceedings of the National Academy of Sciences **101:** 12610-12615.

Wu, BS, Walker, VK and Robertson, RM (2001). Heat shock induced thermoprotection of action potentials in the locust flight system. Journal of Neurobiology **49:** 188-189.

Xiao, C, Mileva-SeitZ, V, Seroude, L and Robertson, RM (2007). Targeting hsp70 to motorneurons protects locomotor activity from hyperthermia in *Drosophila*. Developmental Neurobiology **10:** 438-455.

Zhang, L, Ging, NC, Komoda, T, Hanada, T, Suzuki, T and Watanbe, K (2005). Antibiotic susceptibility of mammalian mitochondrial translation. Federation Of European Biochemical Societies Letters **579:** 6423-6427.

Zhao, Y, Sun, H, Lu, J, Li, X, Chen, X, Tao, D, Huang, W and Huang B (2005). Lifespan extension and elevated *hsp* gene expression in *Drosophila* caused by histone deacteylase inhibitors. Journal of Experimental Biology **208:** 697-705.

Appendix I

While using a system that combines the gal4-UAS and tet-On systems, the test animal needs to have at least one copy of each of the following three DNA constructs:

1. Tissue-specific driver-gal4
2. UAS-rtTA
3. TetO-gene of interest

In order to generate the test animals, adult flies homozygous for the tetO-gene of interest construct were crossed with ones homozygous for both tissue-specific gal4 and UAS-rtTA constructs. The former stock of flies is referred to as Stock 1 and the latter as Stock 2. The details of various Stock 1s and Stock 2s that were crossed with each other for different parts of the project are outlined in Table 1a and b.

Table 1. Details of *Drosophila melanogster* stocks that were used to generate the experimental animals. (a) lists the parental stocks to generate larvae for the β-galactosidase assay whereas (b) lists ones used to generate larvae for the locomotion experiments. The different stocks differ in the position of a specific DNA construct in the genome.

(a). For the β-galactosidase assay:

Stock 1:	**Stock 2a**-To over-express gene in muscle(^):
Flies homozygous for the TetO-LacZ construct	1. M75,24B 2. M78,24B 3. F83,24B 4. F84,24B
	Stock 2b- To over-express in motor neurons (#):
	1. M78,D42 2. F83,D42 3. F84,D42
	Stock 2c- Negative control:
	1. W1118outX flies
	(Contain none of the above constructs)

#Homozygous for the D42-gal4 and UAS-rtTA constructs.

^Homozygous for the 24B-gal4 and UAS-rtTA constructs. Each stock differs in the position of the UAS-rtTA insert in the genome

(b). For locomotion experiments with hsp22:

To investigate effect of over-expression of transgene in the muscle:

Stock 1:	**Stock 2:**
Available stocks(+):	
1. USC22S(3)22A	1. M78,24B*
2. USC22S(2)23	

To investigate effects of over-expression of transgene in the motor neurons:

Stock 1:	**Stock 2**:
Available stocks(+):	
1. USC22S(3)22A	1. M78,D42*
2. USC22S(2)23	

+Both are homozygous for the TetO-hsp22 construct and differ in level of expression due to difference in position of insert in genome (Bhole *et al.,* 2004)

*These stocks were chosen based on the results of the β-galactosidase assay