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Heat shock proteins in Mojave Desert dragonflies

Carol Juanita Promisel

HEAT SHOCK PROTEINS IN MOJAVE DESERT DRAGONFLIES

A Thesis
Presented to the
Faculty of
California State University,
San Bernardino

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
in
Biology

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ABSTRACT

The synthesis of hsp70 at normal physiological and elevated temperatures was compared between two groups of *Belonia saturata* from distinct habitats. *Belonia saturata* from desert communities are able to withstand 4-9 C higher thoracic temperatures than those from cooler coastal areas. *Belonia saturata* were captured from their natural environments in order to compare the relative level of hsp70 present at each dragonfly's normal temperature as well as the results after heat-shock of the desert insects.

Based upon prior research such as that conducted by Ulmasov *et al.* (1992), it was expected that desert insects would have significantly higher levels of hsp70 present at normal temperatures than semi-desert samples. According to Ulmasov's general rule, there is a direct correlation between the environment and the amount of hsp70 proteins in the cells at normal temperature (Ulmasov *et al.*, 1992).

The relative quantity of heat shock proteins produced in the two distinct climates was compared on a molecular level utilizing a monoclonal antibody, biotinylated labeling and densitometer analysis. The results showed no significant difference between the two groups at normal temperatures. This refutes Ulmasov's rule perhaps because Ulmasov's research is limited by the fact that he used many different species of laboratory-raised lizards.

The relative amount of hsp70 was highest in the heat-shocked desert flies. However, this difference was not significant, perhaps because the heat shock used was too intense for optimum hsp production. Further research using a variety of incubation temperatures and time lengths would be necessary to find an optimum hsp70 heat shock for *Belonia saturata* and to confirm the role of hsp70 in the development of thermotolerance.

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INTRODUCTION

Temperature plays a crucial role in the constant interaction between organisms and their environment. It affects development and rates of physiological functions as well as survival of organisms. For over one hundred years scientists have studied the relationship between genes and the thermal environment. As early as 1854, George Dorfmeister demonstrated that extreme heat or cold could change the pattern on a butterfly's wings. Later researchers primarily utilized *Drosophila* in their studies due to its short life cycle and small genome. Goldschmidt showed in the 1930's that many different developmental defects could be induced in *Drosophila* by heating during the pupal stage. The inducible defects resembled mutant defects but they were not inherited. Goldschmidt coined the term "phenocopy" to describe environmentally induced developmental defects due to their resemblance to mutant phenotypes (Petersen and Mitchell, 1991).

Developmental Defects. Since Goldschmidt's pioneering work, geneticists working with *Drosophila* have routinely utilized heat stress to induce developmental defects. In addition, heat-induced defects have been studied in many other organisms including mammals. Exposure of pregnant guinea pigs, hamsters, mice or rats to temperatures above 42 C have induced a variety of developmental defects depending on the stage of development at the time of heating. In 1967, Edwards outlined time periods for heat induction of microcephaly and several limb malformations in guinea pigs. In 1978, Cockroft and New showed similar defects were induced in rat embryos at temperatures above 41 C. In the 1980's a series of researchers demonstrated

that severe cases of cleft palate, cleft lip, and encephaly resulted when pregnant mice were exposed to heat stress of 43 C for short periods (see Petersen, 1990 for review).

Retrospective studies in humans have linked anencephaly, mental deficiency, facial dysmorphogenesis, and altered muscle tone to maternal exposure to heat through prolonged high fever or excessive sauna or hot tub use (Pleet *et al.*, 1981; Fisher and Smith, 1981; Miller *et al.*, 1978). Other studies contradict these findings and it is difficult to determine the actual contribution of heat to the incidence of human birth defects (Petersen, 1990). Whether or not heat causes these defects in humans, it seems certain from studies on insects and many mammals that heat affects development. Heat shock exhibits many of the characteristics found in classical chemical teratogens; it is stage specific, induces similar defects, and interacts with the genetic background in a way similar to other teratogens (Petersen, 1990).

Normal Growth Temperature. Animal life is mainly limited to a narrow temperature range, from a few degrees below 0 C to approximately 50 C (Burdon, 1986). Temperatures as little as 4-8 C above ambient can be lethal to cells. At the molecular level, heat stress can alter the structure of the cytoskeleton and the functioning of the cell membrane. It can slow or stop cell metabolism and adversely effect the synthesis of essential proteins and nucleic acids (Burdon 1988). Somero and Low (1976) found that temperature had a profound affect on enzymic activities in a variety of organisms including chickens, rabbits, lobsters and tuna. They demonstrated that changes in temperature greatly alter rates of catalysis and can also

affect the weak bonds or secondary interactions that stabilize enzyme-ligand complexes and the higher orders of protein structure.

Heat Shock Response. A variety of organisms as diverse as yeast and mammals respond to heat stress in a similar way. When eukaryotic cells are exposed to temperatures 5-10 C above their optimum for growth, they respond by producing a family of heat-shock proteins (hsps) (Velazquez and Lindquist, 1984). At the same time, there is at least partial suppression of the synthesis of normal proteins (Velazquez, *et al.*,1983).

A simple elevation of temperature alters the entire nature of *Drosophila* gene expression in a rapid and highly orchestrated manner (Lindquist, 1980). In *Drosophila* cells, transcription of heat shock genes is induced when the normal growing temperature of 25 C is raised to temperatures between 29 C and 39 C. At 37 C, hsp transcription is maximal and the transcription of previously active genes is repressed. Heat shock also affects translational activity. Although hsp mRNA's are rapidly translated at 37 C, preexisting mRNA's are translationally inactive. These messages are not degraded but rather are stored intact and translated during recovery from heat shock (Yost and Lindquist, 1988).

It should be noted that in addition to heat, a variety of other stressor agents induce the heat-shock protein response. Treatments such as exposure to drugs (Buzin and Bournais-Vardiabasis, 1984), amino acid analogs (Welch and Feramisco, 1982) and metals such as arsenate, cadmium and mercury (Bournais-Vardiabasis *et al.*, 1990) give rise to the hsp response . Likewise, serum withdrawal has been

found to induce the response in cultured cells (Chiang *et al.*, 1989). The biological significance of these and many other inducing agents is uncertain but there is strong evidence for some biological importance of induction by heat (Velazquez and Lindquist, 1984).

Thermotolerance Induction. The theory that hsps protect cells from the damaging effects of heat is supported by the strong correlation between their induction and the induction of thermotolerance (the ability to withstand higher temperatures without damage). Experiments on cultured cells and a wide variety of organisms have shown similar results. A group of cells or organisms dies rapidly when heat-shocked directly from their normal temperature to a much higher temperature. In contrast, when a group is first given a mild heat shock to induce hsp production, it has a higher survivorship when subsequently exposed to extreme temperatures (Nagao *et al.* 1990). In her work on *Drosophila*, Lindquist (1980) found that a gradual increase in temperature greatly extends the temperature range of a heat shock response by allowing cells to produce substantial quantities of heat-shock mRNAs and proteins before they are subjected to the stress of extreme temperatures. A pretreatment of 34 C results in increased survival and protection from phenocopy in larvae later exposed to 42 C (Lindquist, 1980).

Research on brine shrimp demonstrated that either acclimation to increasing temperatures or a brief heat shock gives rise to thermotolerance. The brine shrimp *Artemia* have an optimal growth temperature of 28 C. McLennan and Miller (1990) exposed some newly hatched shrimp to a 60 minute heat shock at 40 C while others were

acclimated at 37 C for five hours. When both groups were subjected to a 30 minute heat shock at 42 C, they were completely protected. Without either pretreatment, temperatures of 42 C caused 100% lethality.

In addition to providing protection from elevated temperatures, Chen *et al.* (1990) demonstrated a connection between pretreatment and the survival of cold shock in the flesh fly, *Sarcophaga crassipalpis*. When flies were taken from their optimal temperature of 25 C, a 10 minute exposure to 0 C was enough to prevent injury to a later cold shock at -10 C. A 30 minute pretreatment of 40 C was required to confer protection against a heat shock of 45 C. It is interesting to note that the high temperature protected the flies from a mild cold shock while the low temperature pretreatment did not protect them from a future heat shock (Chen *et al.* 1990, 1991).

A variety of organisms from *E.coli* to mammals show similar results (Johnston and Kucey, 1988; Nguyen *et al.* 1989). It seems that the heat shock proteins are induced by non-lethal stresses which subsequently protect the organism from lethal stress (Lindquist and Craig 1988). The mechanism(s) of this protection is not well-understood as heat shock alters many complex cellular characteristics such as protein synthesis, nucleolar morphology, intermediate filament network distribution, and RNA splicing (Nguyen *et al.* 1989). It is thought that heat shock proteins may protect cells either through the prevention of thermal denaturation of "normal" proteins or through the rapid removal of "abnormal" proteins and aggregates that might form (Nguyen *et al.* 1989). Whatever the mechanism, strong support for the essential role of heat shock proteins in the development of thermotolerance

is provided by mammalian research. Cultured mammalian cells in which certain heat shock proteins are not made or are inactivated by antibody binding are not capable of developing thermotolerance (Petersen, 1990).

Low Molecular Weight hsp. Identification of the heat shock proteins (hsp) involved in thermotolerance were first reported in *Drosophila* by Tissieres and his colleagues in 1974. Since that time, several hsps have been reported in a wide range of organisms and every species studied to date has been found to express hsp (with the exception of one species of Hydra). The exact number of different types of hsp varies in different organisms and cell types but are commonly categorized as either low or high molecular weight heat shock proteins. The small hsps are a diverse group. Different organisms have different numbers of low molecular weight (LMW) hsps ranging from one in *S. cerevisiae* to as many as 30 in some plant species. The molecular weights range from 16 kd in the nematode *C. elegans* to 40 kd in the protozoan *S. mansoni* (See Lindquist and Craig, 1988 for review). In contrast to animal systems where the most abundant hsp family is HSP70, the LMW hsps are the most abundant proteins induced by heat shock in most plant species (Nagao *et al.* 1990).

The LMW hsps have been classified by size into three Families: 15 to 18 kd, 21 to 24 kd and 26 to 28 kd (Nagao *et al.* 1990). The LMW hsps show much greater homology within organisms than between organisms. For example, members of a LMW hsp subgroup in soybeans have 90% amino-acid identity with each other but only 20% amino-acid identity with the proteins of *D. melanogaster, C.elegans* and *X. laevis*. None of the well-characterized hsps of *E.*

coli are significantly homologous to those of higher cells. It seems that the genes are subject to relatively frequent coversion events or they have been deleted and expanded frequently during evolution (Lindquist and Craig, 1988). Despite the divergence in sequence, the LMW hsps are conserved in their structural properties. They tend to form highly polymeric structures called heat-shock granules and they are phosphorylated under similar conditions. In addition, the LMW hsp particles isolated from Drosophila, sea urchin and tomato cells have all been found to contain RNA. It is thought that the hsp may serve to preserve translationally inactive messenger RNA although this finding has been disputed (Lindquist and Craig, 1988). Currently, the functions of LMW hsps are not well understood. They are induced both by heat shock and by normal developmental cues. They are remarkably diverse and seem to compare more in structure than in amino-acid sequence. While some evidence suggests they are important in thermotolerance, genetic evidence in yeasts disputes this connection (Lindquist and Craig, 1988).

High Molecular Weight hsp. High molecular weight (HMW) heat shock proteins include three major families: HSP110, HSP90 and HSP70. Although most eukaryotes in response to high temperatures produce proteins of greater than 100kd, the HSP110 family has predominantly been studied in mammals (Burdon, 1986). The function of these proteins may be revealed by their location in cells. The 110kd protein is mainly found in the nucleus and both control and heat-shocked cells exhibit a concentration of HSP110 in the nucleolus. The protein separates from the nucleolar body and forms a nucleolar cap when cultures growing at normal

temperatures become confluent, are incubated without serum, or are treated with actinomycin. Brief heat shock does not cause the hsp110 to separate but longer heat shock causes the protein to form a ring-like structure at the nucleolar periphery (Lindquist and Craig, 1988). Immunoelectron microscopy indicates that hsp110 associates with the fibrillar component of nucleoli where nucleolar chromatin (rRNA) is located. It is speculated that hsp110 is induced to protect ribosome production, a process which is very sensitive to heat (Lindquist and Craig, 1988).

A second well-studied set of HMW heat shock proteins belongs to the HSP90 family. This family includes proteins of varying size such as *Drosophila* HSP83, yeast HSP90, and chicken HSP89 determined by their mobilities on polyacrylamide gels. Despite the variance in size, HSP90 represents a highly conserved family of HSPs. Members of this family have been cloned and sequenced from evolutionarily diverse organisms including *Drosophila*, yeast, chickens, mammals, trypanosomes, plants and bacteria. Proteins from even the most distantly related eukaryotes have 50% amino acid sequence identity and they all have greater than 40% identity with the corresponding *E.coli* protein (Nagao, *et al.*, 1990).

Proteins of the HSP90 family are typically present at normal temperatures but are elevated five- to ten-fold by heat shock (Nagao, *et al.*, 1990). According to McLennan and Miller (1990), hsp90 is a component of most steroid receptors and may be involved in the transport or regulation of some protein kinases. To assess the role of 90kd heat shock protein in the stress response, Bansal *et al.* (1991) used an anti-sense RNA approach to reduce the levels of the protein in cultured mammalian (mouse) cells.

They discovered that cells exhibit reduced growth at mildly elevated temperatures (39 C for 4 days) and reduced survival at highly elevated temperatures (42 C for two hours) (Bansal *et al.*, 1991).

Research on crustaceans has revealed temperature- and time-dependent increases in hsp89. A 40 C heat shock enhanced the expression of hsp89 after only five minutes while lower temperatures required a longer heat shock. At 40 C, maximal induction of hsp89 occurred after 15 minutes. Exposure to 40 C for periods as long as 60 minutes resulted in complete repression of non-hsp protein synthesis (McLennan and Miller, 1990).

HSP70. The most abundant hsp in many organisms has a molecular weight between 68K and 74K. Commonly referred to as HSP70, this protein family was named for the major *Drosophila melanogaster* hsp for which the initial characterization was done (Moran *et al.* 1982). HSP70 has been highly conserved throughout evolution. For example, the hsp70 proteins of *Drosophila* and yeast have a 72% amino acid identity. The protein sequence derived from the human HSP70 gene is 73% homologous to *Drosophila* hsp70 and that from maize is 68% homologous *to Drosophila* (Burdon, 1986). Moran *et al.* (1982) report 85% gene homology between mouse and *Drosophila*. The similarity is great enough that the cloned gene for *Drosophila* hsp70 cross-hybridizes with DNA sequences from humans, mice, chickens, lizards, yeast and corn (Velazquez *et al.*, 1983).

In *Drosophila* the HSP70 genes are present in several copies. The organization of the members of the HSP70 family in *Drosophila* and related insects is unusual in that it is an example of dispersed multicopy genes (Moran *et*

al., 1982). The complexity and number of genes in the HSP70 family vary among species. It is difficult to make generalizations about the HSPs because of the variety as well as the fact that there also exist a closely related set of proteins known as heat shock cognates which are not heat inducible. Depending on the cell or tissue type, they exhibit constitutive expression or developmentally regulated expression (Nagao *et al.*, 1990).

Early research did not differentiate between the HSP genes and the cognate genes and so certain articles contradict each other when discussing whether HSP70 genes are transcribed constituitively. Velazquez *et al.* (1983) settled the debate by demonstrating that *Drosophila* cells growing at normal temperature do not contain significant quantities of hsp70 (1/1000th of the level present after heat shock). Velazquez hypothesized that conflicting results were due to stressful culture conditions or an inclusion of the cognates.

Unlike other HSP genes, the HSP70 family does not appear to play a role in early development. *Drosophila* and *Sacchararomyces* demonstrate a strikingly similar pattern of expression: LMW HSPs and HSP90 proteins are expressed as a result of early developmental cues but hsp70 is not. In *Sacchararomyces,* hsp70 is not even inducible with heat shock (Kurtz *et al.*, 1986). During the *Drosophila* life cycle, there is only one stage in which animals are unable to acquire thermotolerance: the 0-3 hour preblastoderm embryo. This is also the only stage in which synthesis of hsp70 is not observed (Velazquez and Lindquist, 1984). This suggests a direct correlation between hsp70 and the induction of thermotolerance.

In *Drosophila* larvae, hsp70 concentrates strongly in

the nucleus during periods of stress. With treatment of heat or anoxia, hsp70 is transported to the nucleus very rapidly. Within 30 minutes of heat shock, mRNAs for hsp70 are made, transported to the cytoplasm, translated into protein and that protein is transported to the nucleus. Some of the protein, however, is retained in the cytoplasm even during periods of extreme stress. During recovery the hsp70 moves back to the cytoplasm at a much slower rate than its entry into the nucleus. It has been suggested that this return is necessary for the cell to return to normal patterns of translation after heat shock (Velazquez and Lindquist, 1984).

Hsp70 is an RNA-binding protein whose synthesis is required for the recovery of normal protein synthesis and the repression of further hsp synthesis. It is the only hsp whose synthesis bears a specific quantitative relationship to both the reactivation of pre-existing synthesis and the repression of hsp synthesis. It has been suggested that hsp70 promotes both aspects of recovery through direct interaction with messenger RNA (Velazquez and Lindquist, 1984).

HSP70 in Belonia saturata. Much research has supported the significance of hsp70 in the stress response of a variety of organisms. This level of conservation combined with the fact that HSP70 encoded abundant 70K hsp in previous research, makes hsp70 an ideal protein for study. This project focuses on the hsp70 response in the dragonfly *Belonia saturata*.

Dragonflies from desert communities are able to withstand higher body temperatures than those from cooler coastal areas of the same species. Despite air temperature

changes within an area, the body temperature remains relatively stable. In general, the thoracic temperatures of the desert dragonflies are 4-9 C higher than their counterparts from a cooler habitat (Polcyn, 1988). *Belonia saturata* from these two environments were analyzed in order to compare the level of hsp70 expression in adults. By capturing samples in their natural environment it was possible to test the relative level of hsp70 present at each dragonfly's normal temperature as well as the results after heat-shock of the desert insects.

Similar research was recently conducted on lizards. Ulmasov *et al.* (1992) studied the synthesis of hsps at normal and elevated temperatures in nine different lizard species. They concluded by stating a general rule which they felt was applicable to most organisms. The rule postulates a "direct correlation between the characteristic temperature of the ecological niche of a given species and the amount of hsp70-like proteins in the cells at normal temperature" (Ulmasov *et al.*, 1992). Following this "rule", the desert *Belonia saturata* should have significantly higher levels of hsp70 than the semi-desert dragonflies.

MATERIALS AND METHODS

Collection of Specimens. *Belonia saturata* used in this study were collected at two distinct sites. The Santa Ana River site is a semi-desert area in San Bernardino, California. At this site, the air temperature ranged from 17 C to 34 C. The average air temperature was 26 C at the time of capture . The Soda Springs Desert Studies Center at Zzyzx, California is in the Mojave Desert and had temperatures ranging from 28 C to 37 C. The average air

temperature was 34 C at the time of capture.

The *Belonia saturata* were all captured during normal flight activity. Within 10 minutes of capture, some individuals were subjected to heat-shock. Half of these were incubated for 90 minutes at 45 C in the dark at 100% relative humidity. The others were incubated for 60 minutes at 45 C then at room temperature for 60 minutes. After incubation, the thorax was cut open and flight muscle tissue was extracted from each dragonfly. The tissue was homogenized with 0.1% phenylmethylsulfonyl fluoride (PMSF) protease inhibitor in 95% ethanol. The insects which did not undergo incubation had flight muscle extracted and homogenized the same way but within 10 minutes of capture. After homogenization, all samples were stored in the freezer.

Before use, the frozen samples were thawed, centrifuged and the pellets were resuspended in 1000uL Tris buffered saline (TBS) with 0.5% sodium dodecyl sulfate (SDS) detergent (appendix) and homogenized. The samples were then centrifuged at 2,000 rpm for four minutes at room temperature, the supernatant was collected in microcentrifuge tubes for hsp analysis; the pellets were placed in the -20 C freezer for future use.

Anti-hsp Antibody. The anti-hsp monoclonal antibody was obtained from Dr. Susan Lindquist at the University of Chicago. It is a rat antibody from a rat spleen/mouse myeloma fusion, thereby requiring an anti-rat secondary antibody. The antibody recognizes members of the HSP70 family from virtually every eukaryote tested at the University of Chicago including insects, yeasts, trypanosomes, mice and soybeans. Recently, this antibody

has also become available commercially through Affinity BioReagents (Lindquist 1992).

Dot Blot of hsp70. An immunostaining protocol was utilized to determine the relative amount of hsp70 in each sample. Nitrocellulose paper was incubated in a dish of TBS buffer. The wet nitrocellulose paper was placed into a Bio Rad Bio-Dot™ apparatus and twelve different samples were tested on each blot. (One sample, chosen at random, was run on every blot in order to provide a standard for comparison.) Each blot contained eight different serial dilutions of each of the twelve samples to ensure a successful concentration. To prepare the serial dilutions, 100uL of TBS buffer was placed into every well of a 96-well microtiter plate using a multichannel pipet. 100uL of sample was added to the first well in a column. After mixing, 100uL was transferred to the second well in that column. This was repeated for the remaining six wells in each column so that each would contain half the concentration of sample present in the previous well with concentrations ranging from 1:2 to 1:256. 100uL of the TBS/sample mixture was discarded from the last well in each column to control the amount of mixture in every well at 100uL. These dilutions were then transferred to the blot apparatus in sequence (Figure 1).

The dot blot apparatus was connected to a vacuum pump and the wells evacuated until the blot was dry (approximately 30 minutes). The nitrocellulose paper filter was removed and all non-specific binding was blocked by incubating in BLOTTO (appendix) for 30 minutes at room temperature. Next, the filter was placed in a dish containing 5uL of rat anti-hsp antibody mixed with 25mL

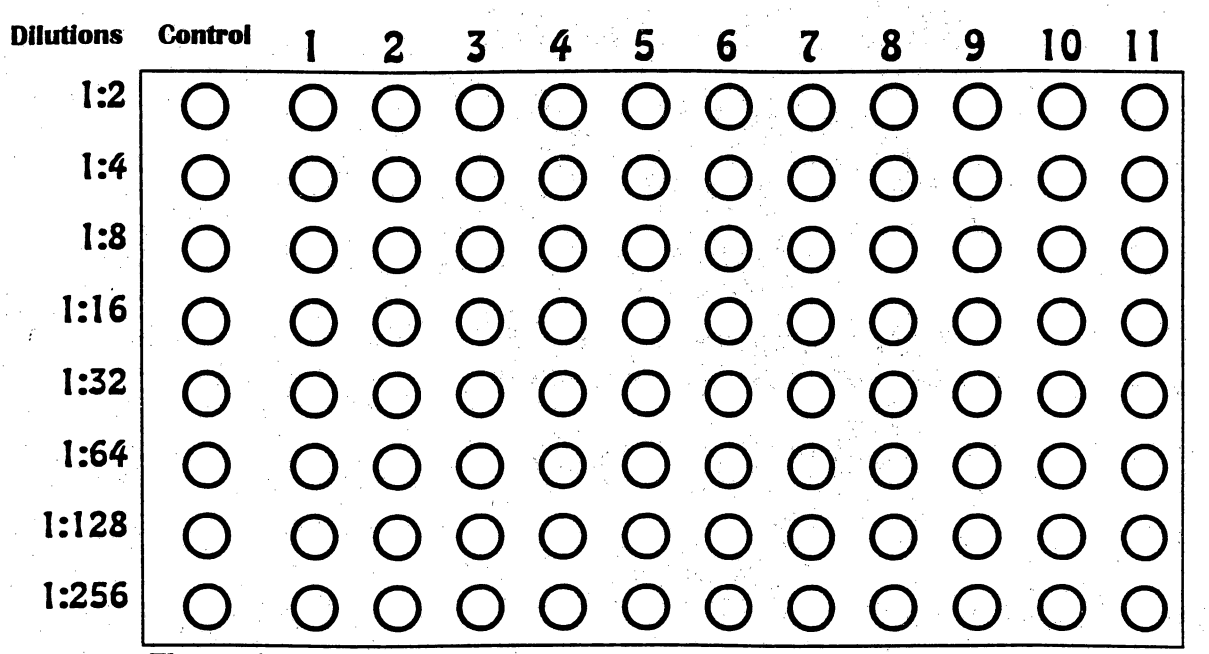

Figure 1.

BioRad Bio-Dot™ apparatus set up. Each blot contained eight different serial dilutions of each of the twelve samples. A randomly chosen sample was placed in the first column of every blot to serve as a control.

BLOTTO and incubated for 1 hour at 37 C with rocking.

The excess anti-hsp antibody was removed, and non-specific binding inhibited, by rinsing the nitrocellulose paper three times with BLOTTO, 10 minutes each at room temperature with rocking. Next, 5uL of biotinylated goat anti-ratIgG antibody (Pierce) was mixed with 25mL BLOTTO and the nitrocellulose paper incubated in this mixture for three hours at room temperature with rocking. The blot was then rinsed with TBS, three times for 10 minutes each time.

Immunostaining. The presence of hsp was determined with the biotin alkaline phosphatase (biotin-AP) staining method (Becker *et al.* 1990). The nitrocellulose paper was first placed in a solution containing 30uL streptavidin (Pierce) mixed with 15mL of Buffer 1 (appendix) for 10 minutes with rocking. It was rinsed three times with Buffer 1 for three minutes each time. Then the nitrocellulose paper was placed in a mixture of 15uL biotin-AP (Vector Laboratories) and 15mL Buffer 1 for 10 minutes with rocking. It was rinsed three times with Buffer 1 as above and then it was rinsed with Buffer 3 (appendix) two times for three minutes each time.

The dye solution was prepared immediately prior to use by adding 132uL nitroblue tetrazolium (NBT) to 30mL Buffer 3 and mixing gently by inverting. Next, 100uL bromo--4-chloro-3-indolyl phosphate (BCIP) was added and mixed gently. The nitrocellulose paper was placed inside a sealable plastic bag with this dye mixture. It was incubated in the dark at room temperature until color developed (approximately two hours). Once a visible color developed, the paper was soaked in stop buffer (appendix),

dried and stored in the dark.

Quantification of hsp70. A Bio-Rad 620 video densitometer was used to determine the relative quantity of hsp70 in each sample by reflectance densitometry. Each blot was placed into the densitometer and the optical density (OD) value was read directly for each dot and background filter. The sample OD number was obtained by subtracting the background OD from the sample OD. The sample OD number was then divided by the control OD to arrive at a relative OD (sample/control).

Quantification of Total Protein. Because the samples varied in the amount of tissue extracted, it was necessary to calculate the total protein in each sample. The Lowry assay was used for protein determination (Becker *et al.* 1990). A series of bovine serum albumin (BSA) standard dilutions were prepared and 250uL of each dilution was transferred to a tube containing 750uL distilled water. 250uL of TBS with 0.5% SDS was mixed in a tube with 750uL distilled water to produce a "blank". After 1.0mL Lowry reagent solution was mixed into each tube, they were left at room temperature for 20 minutes. With rapid and immediate mixing, 0.5mL Folin and Cocalteu's Phenol Reagent Working solution was added to each tube. Tubes were allowed to stand at room temperature for 30 minutes while the color developed. Next the solutions were transferred to cuvettes and a Milton Roy Spectronic 3000 spectrophotometer was used to measure absorbance at 750nm. All readings were completed within 30 minutes to ensure accuracy. A calibration curve was created by plotting absorbance versus concentration for the standards. The

Lowry assay was repeated for all the *Belonia saturata* samples and the quantity of protein was determined by locating the sample absorbance on the calibration curve. Finally, the relative OD from above (sample OD/ control OD) was divided by the total protein amount for each sample. The resulting numbers represented the relative hsp70 OD per mg total protein.

Comparison between OD and Air Temperature. The dragonflies were collected at a variety of air temperatures, as previously stated. It was also mentioned that earlier research did not detect a correlation between air temperature and thoracic body temperature (Polcyn, 1988). In order to support these findings, and eliminate the possibility that higher capture temperatures could induce greater hsp70 production within each habitat, the relative hsp70 OD was compared to air temperature. The analyses were carried out using a Microsoft Excel simple linear regression analysis.

RESULTS

The relative amount of hsp70 was highest in the heat-shocked desert flies, as expected. They had a mean of 22.1 relative hsp70 OD per mg protein. The amount present in the desert and semi-desert *Belonia saturata* was 18.61 and 20.44 respectively (Figure 2). Although the graph displays a visible difference between these numbers, there was no significant difference as determined by the T-test. Between the desert heat-shocked and the desert control group, $t=0.32$, $n_1=18$, $n_2=6$, $p>0.05$. In the comparison between desert and semi-desert heat-shocked, $t=0.26$, $n_1=18$, $n_2=11$, $p>0.05$.

Data from both sites were pooled for regression

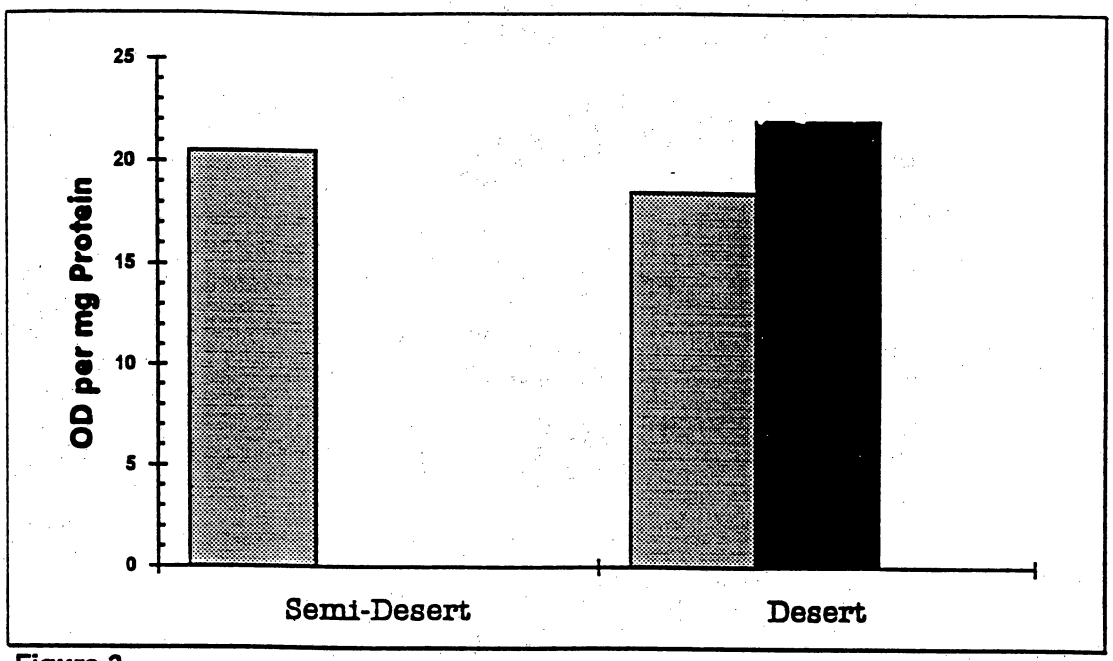

Figure 2.

Amount of heat shock protein from dragonflies during normal activity (gray bars) and following a 60-minute shock (black bar). Desert dragonflies were captured at Soda Springs Desert Studies Center at Zzyzx, California. Semi-desert dragonflies were captured along the Santa Ana River drainage in San Bernardino, California. The amount of heat shock protein is expressed as optical density per milligram of total protein, a relative measure of the staining of dot-blots after treatment with anti-hsp70 monoclonal antibodies and subsequent staining with alkaline phosphatase-mediated chromatagens.

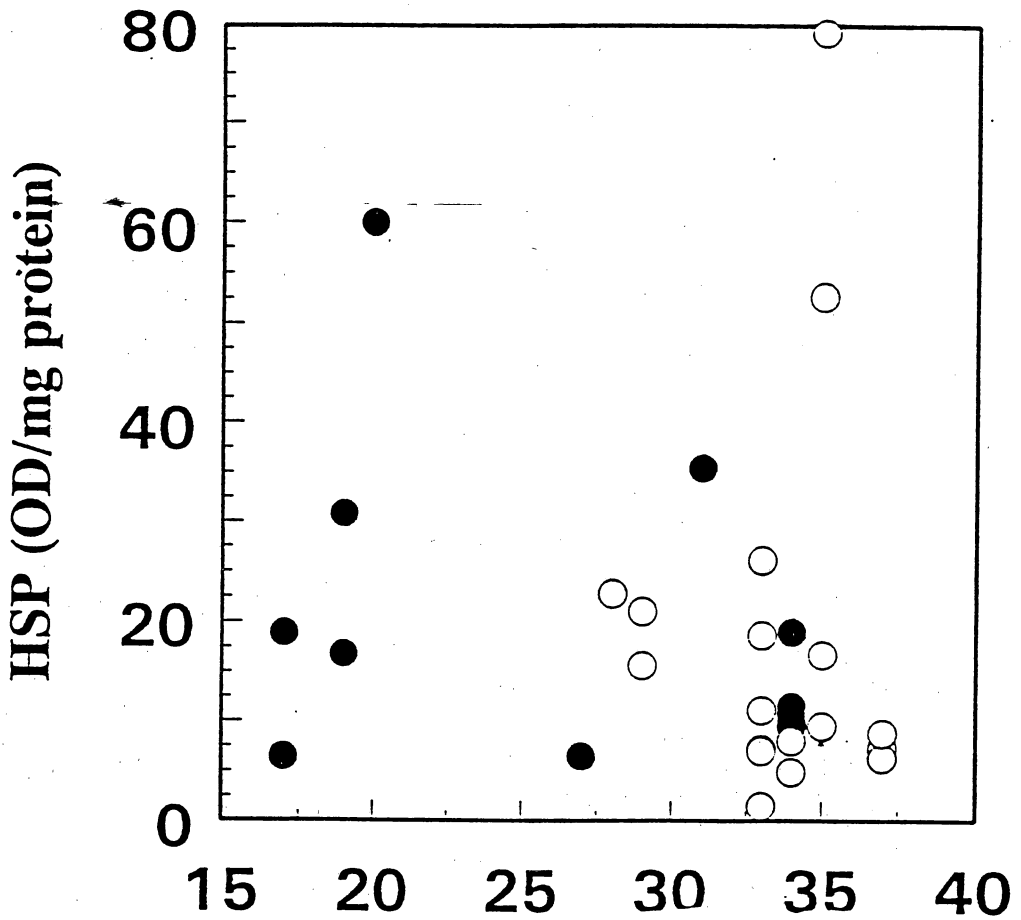

Figure 3.

Regression analysis of OD versus air temperature. Filled circles represent Santa Ana River semi-desert dragonflies. Open circles represent Soda Springs Desert Studies Center samples. The non-linear spread of dots reveals that there is no correlation between hsp OD and air temperature at the time of capture.

analysis. First, a regression of all data revealed no correlation between hsp OD and air temperature. The pooled data yielded y= -0.43x + 32.13, n= 29 and r^2= 0.02 (y= hsp70 OD/mg protein and x= air temperature in degrees Celcius). Next, analyses of individual populations were performed. For the Santa Ana river data, y= -.58x + 35.61, n= 11 and r^2= 0.07. The Zzyzx data yielded y= -0.03x + 19.12, n= 18 and r^2= 0.00 (Figure 3).

DISCUSSION

Ulmasov *et al* (1992) proposed a general rule stating that the level of hsp70 in an organism will show positive correlation with the environmental climate. The results in this study refute Ulmasov's rule which would predict the desert dragonflies would have a significantly higher level of hsp70 than their semi-desert counterparts. Research performed by Ulmasov *et al* and others has shown a positive correlation between habitat and hsp induction (Engen'ev *et al.*, 1987; Ulmasov *et al.*, 1992). The differences cited in the articles, however, could be due to differences in research techniques. For example, Ulmasov *et al* utilized more than one species in their work; their comparisons were not intraspecific. Thus, differences could be due to phylogenetic differences between distantly or more closely related species. The research was also done on laboratory-raised animals. The results attained with such animals may have little, or nothing, to do with the heat shock response occurring in nature. There has not been a single study addressing the relative levels of hsp in free-roaming organisms of the same species which live in different climates. It seems logical that the *Belonia saturata* might not differ in hsp levels despite different habitats purely because they are the same species. If this

is the case, there must be some other physiological or behavioral mechanism which allows the desert dragonflies to withstand higher temperatures.

The regression analysis of OD versus air temperature eliminates the possibility of the results being due to different air temperatures at the time of capture. Because of the range of air temperatures present, there was concern that some of the *Belonia saturata* may have already hit the critical induction state before testing. This possibility was unlikely however, since the air temperature does not normally affect thoracic temperatures within this range (Polcyn, 1988). Regression analysis confirmed that there is essentially no relationship between OD and air temperature as all three analyses had low r^2 values.

It is more difficult to explain why the heat-shocked desert group did not have a significantly higher level of hsp than the normal groups. All the research discussed in the introduction of this paper supports the idea that heat shock induces much greater hsp70 production, yet it was not found in this study. One possible explanation for this unexpected outcome is that the heat shock may have been too intense for optimum hsp70 production. Many of the articles cited have referred to a specific optimal temperature for the induction of HSPs. For *Drosophila*, that temperature is 37 C (Yost and Lindquist, 1988). Perhaps a 60 minute incubation at 45 C created more stress than could be compensated for by the HSP response and resulted in at least partial degradation of the hsp70 present. To test this hypothesis, further work would have to be done. By using a variety of incubation temperatures and time lengths, it would be possible to find an optimum hsp70 heat-shock for *Belonia saturata*.

With the current data, it is impossible to conclude the exact nature of the role hsp70 plays in the development of thermotolerance in *Belonia saturata*. Field work involving organisms in their natural habitat places many limitations on research. However, this type of study provides relevant information on how hsps function in an animal's adaptation to its environment. This study also refutes Ulmasov's general rule by utilizing a more natural, single-species group which was free-roaming instead of laboratory-raised.

APPENDIX

TBS BUFFER

Start with 700 mL distilled water.
Add with stirring:
2.42 grams Tris-base
29.22 grams NaCl
pH to 7.5 with concentrated HCl.
Dilute to 1000 mL with distilled water.

BLOTTO

5.0 grams nonfat dry milk
3.152 grams Tris-HCl
29.22 grams NaCl
100 uL Merthiolate
Dilute to 1000 mL with distilled water.

BUFFER 3

5 mL 1M $MgCl_2$
2 mL 5M NaCl
10 mL Tris-Cl (pH 9.5)
Dilute to 100 mL with distilled water.

BUFFER 1

400 uL 1M $MgCl_2$
4 mL 5M NaCl
20 mL Tris-Cl (pH 7.5)
Dilute to 100 mL with distilled water.

STOP BUFFER

1 mL Tris-Cl (pH 7.5)
0.5 mL EDTA
Dilute to 500 mL with distilled water.

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