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The expression of stress proteins in response to temperature extremes in insects

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The Ohio State University, 1992
THE EXPRESSION OF STRESS PROTEINS IN RESPONSE TO TEMPERATURE EXTREMES IN INSECTS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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1992

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To my Lord
Jesus Christ
Who alone gives hope, and meaning to life
Soli Deo gloria!
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# TABLE OF CONTENTS

**DEDICATION**

**ACKNOWLEDGMENTS**

**VITA**

**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>II. Prolonged Thermotolerance in the flesh fly, <em>Sarcophaga crassipalpis</em>. Does Not Require Continuous Expression or Persistence of the 72 kDa Heat Shock Protein</td>
<td>11</td>
</tr>
<tr>
<td>Abstract</td>
<td>11</td>
</tr>
<tr>
<td>Introduction</td>
<td>12</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>13</td>
</tr>
<tr>
<td>Results</td>
<td>16</td>
</tr>
<tr>
<td>Discussion</td>
<td>18</td>
</tr>
<tr>
<td>III. Induction and Decay of Thermosensitivity in the Flesh Fly, <em>Sarcophaga crassipalpis</em></td>
<td>30</td>
</tr>
<tr>
<td>Abstract</td>
<td>30</td>
</tr>
<tr>
<td>Introduction</td>
<td>31</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>32</td>
</tr>
<tr>
<td>Results</td>
<td>34</td>
</tr>
<tr>
<td>Discussion</td>
<td>37</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

FIGURES

1. Red eye pharate adults of S. crassipalpis were exposed to 35 (open squares), 40 (open circles) or 45°C (solid circles) for varying durations. After 24 h at 25°C pharate adults were exposed to 45°C for 90 min. Survival was based on the percent of flies to survive to adult emergence (mean ± S.E.); three replicates of 15 flies each for each time point................................................................. 20

2. Red eye pharate adults of S. crassipalpis were pretreated at 40°C for 2 h (A) or 45°C for 30 min (B). After 24 h at 25°C pharate adults were exposed to 45°C for varying durations. Survival was based on the percent of pretreated (solid circles) and non-pretreated (open circles) flies to survive to adult emergence (mean ± S.E.); three replicates of 15 flies each for each time point................................................................. 22

3. Red eye pharate adults of S. crassipalpis were pretreated at 40°C for 2 h and then exposed to 45°C for 90 min after 0, 4, 8, 12, 24, 48 or 72 h at 25°C to determine the onset and duration of thermotolerance. Survival was based on the percent of pretreated (solid circles) and non-pretreated (open circles) flies to survive to adult emergence (mean ± S.E.): three replicates of 15 flies each for each time point................................................................. 24

4. Red eye pharate adults of S. crassipalpis were pretreated at 40°C for 2 h, and after 0 (A), 4 (B), 24 (C), 48 (D), or 72 (E) h at 25°C 5 brains were dissected and labeled for 1 h at 25°C with 10 μCi of 35S-methionine. Controls were held continuously at 25°C (F) or
heat shocked at 43°C for 1 h and then immediately labeled 1 additional h at 43°C (G). Samples were separated on a 10% SDS-PAGE gel. The 72 kDa heat shock protein is indicated.

5. Brains from 5 red eye pharate adults of S. crassipalpis were cultured at 40°C (A, B) or 43°C (C) for 1 h then labeled with 10 μCi 35S-methionine for one additional hour at the same temperature. Protein expression was either stopped immediately by boiling (A, C) or brains were washed with Grace's medium and cultured for 24 h at 25°C (B). Samples were separated on a 10% SDS-PAGE gel.

6. Pharate adults (red-eye stage) of S. crassipalpis reared at 25°C were exposed to 45°C for 0 (open circle), 30 (solid circles) or 60 min (squares). After 24 h at 25°C pharate adults were exposed to 45°C for varying durations. Survival was based on the percent of flies that survived until adult emergence (mean ± S.E., three replicates of 15 flies each for each time point).

7. Pharate adults (red-eye stage) of S. crassipalpis were pretreated at 45°C for 1 h. After 24 (A), 48 (B) or 72 (C) h at 25°C pharate adults were exposed to 45°C for varying durations. Survival was based on the percent of pretreated (open circles) and non-pretreated (solid circles) flies to survive until adult emergence (mean ± S.E., three replicates of 15 flies each for each time point).

8. Pharate adults (red-eye stage) of S. crassipalpis were pretreated at 45°C for 1 h. After 24 h at 25°C pharate adults were exposed to 30 (A) or 35°C (B) for varying durations. Survival was based on the percent of pretreated (open circles) and non-pretreated (solid circles) flies to survive until adult emergence (mean ± S.E., three replicates of 15 flies each for each time point).

9. Pharate adults (red-eye stage) of S. crassipalpis were exposed to 40°C for 4 h then immediately exposed to 45°C for 1 h (open circles), 45°C for 1 h only (squares), or remained at 25°C (solid circles). After 24 h at 25°C pharate adults were exposed to 35°C
or 45°C for varying durations. Survival was based on the percent of flies that survived until adult emergence (mean ± S.E., three replicates of 15 flies each for each time point).

10. Pharate adults (red-eye stage) of S. crassipalpis were held at 25°C (A, C) or pretreated at 45°C for 1 h (B, D). After 24 h at 25°C brains were cultured at 25°C for 1 h with 10 μCi 35S-methionine (A, B) or at 43°C for 1 h then labeled for 1 additional h at 43°C (C, D). Samples were separated on a 10% SDS-PAGE gel. The 72 kDa heat shock protein is indicated.

11. Heat shock protein expression in diapausing pharate first instar larvae of L. dispar. Pharate larvae dissected from their chorions were exposed to the indicated temperatures (°C) for 1 h and labeled for an additional hour at the same temperature. Proteins were separated on a 10% SDS-PAGE gel. Molecular weights of heat shock proteins are expressed in kilodaltons.

12. Heat shock protein expression in diapausing pharate larvae of L. dispers during recovery from a 2 h exposure to 40°C. Pharate larvae were dissected from their chorions at 0(A), 2(B), 4(C) or 6(D) h after heat shock and labeled for 1 h at 25°C. (E) 25°C control, (F) heat shock control, labeled at 40°C. Proteins were separated on a 10% SDS-PAGE gel. Molecular weights of heat shock proteins are expressed in kilodaltons.

13. Heat shock protein expression in diapausing pharate first instar larvae of L. dispers during recovery at 25°C from cold shock. Following a 2 h exposure at either -10 (A, B, C, D) or -20°C (E, F, G, H) pharate larvae were dissected from their chorions at 0 (A, E), 2 (B, F), 4 (C, G) or 6 (D, H) h after cold exposure and labeled for 1 h at 25°C. (I) 25°C nonshocked control, (J) 40°C heat shock control. Proteins were separated on a 10% SDS-PAGE gel. Molecular weights of heat shock proteins are expressed in kilodaltons.
14. Heat shock protein expression in diapausing L. dispar pharate larvae during recovery at 4°C from a cold shock at -20°C for 24 h. Following a 24 h exposure pharate larvae were dissected from their chorions at 0(A), 24(B), 48(C), 72(D) or 96(E) h after cold shock and labeled for 24 h at 4°C. (F) heat shock control 2 h at 40°C, (G) 4°C nonshocked control, (H) 25°C nonshocked control. Proteins were separated on a 10% SDS-PAGE gel. Molecular weights of heat shock proteins are expressed in kilodaltons.

15. Western transfer of heat shock proteins in diapausing pharate first instar larvae of L. dispar. (A) heat shock for 2 h at 40°C, (B) cold shock for 2 h at -20°C, (C) 4°C nonshocked control. Nitrocellulose filter was screened with the monoclonal antibody C92F3A-5 which is specific for the inducible form of the human heat shock 70 kDa proteins. Arrows indicate lightly bound heat shock proteins in lane A.

16. Western transfer of heat shock proteins in diapausing pupae of S. crassipalpis. Heat shocked nondiapause (A); diapause (B); nonshocked nondiapause (C). Proteins were separated on a 10% SDS-PAGE gel. Nitrocellulose filter screened with a polyclonal antibody against the human 70 kDa heat shock protein family.

17. Protein expression in diapausing and nondiapause S. crassipalpis pupae. Nonshocked nondiapause (A); diapausing (B, E, F, G; heat shocked nondiapause 2 h at 43°C (C, D). Diapausing pupae were approximately 1 (E), 25 (F) or 55(G) days into diapause. Proteins were separated on a 10% SDS-PAGE gel. Molecular weight expressed in kilodaltons.
CHAPTER I

Introduction

Exposure to supraoptimal temperatures can increase (make thermotolerant) or decrease (thermosensitize) an organism's survival to a second thermal challenge. Most mammalian cell lines examined to date develop thermotolerance when exposed to supraoptimal temperatures below 42.5-43°C but are rendered thermosensitive when exposed to temperatures above this range (Jung and Kolling, 1980; Jung, 1982; Nielsen et al., 1982; Dikomey et al., 1984). Chinese hamster ovary cells (CHO) exposed to 40°C (1-16 hours) develop thermotolerance against 43°C, whereas the same CHO cells pretreated at 43°C (15-90 minutes) become thermosensitive to the normally nonlethal temperature of 40°C (Jung, 1982). Malignant mouse lung cells (L1A2) exposed to 42 or 45°C experience a marked increase in sensitivity to thermal stress (Lindegaard and Nielsen, 1990). Exposure to a supraoptimal temperature can also protect an organism against suboptimal temperatures (Burton et al., 1988; Chen et al., 1991).

Although the precise mechanism(s) of protection against nonoptimal temperatures is (are) not known, in response to nonoptimal temperatures organisms adjust their physiological properties and processes via hormonal
changes (Ingram et al., 1980), activation of a preexisting enzyme (Chen and Denlinger, 1990), increased synthesis of enzymes (Lover et al., 1985), changes in membrane components (Cress and Gerner, 1980), increased or decreased rate of synthesis of certain proteins (Lindquist, 1986; Kroeker and Walker, 1991), and expression of preexisting mRNA (Reiter and Penman, 1983).

The best documented case of proteins protecting against environmental stress is the SOS response in *Escherichia coli*. Agents (e.g., ultraviolet light) that damage *E. coli* DNA induce the proteins of the SOS response, which repair the damage (reviewed Walker, 1984).

Also well-known are proteins that are closely associated with environmental stresses, but whose presumed protective functions are not yet certain. The best known examples of such proteins are the heat shock proteins, a group of highly conserved proteins (yeast to human) induced by a variety of environmental and physiological stresses (reviewed Lindquist, 1986), including exposure to low or high temperatures (Joplin et al., 1990). Though heat shock proteins are the most thoroughly studied stress-associated proteins, other proteins are also induced by stress. Under desiccating conditions *Tenebrio molitor* fat body secretes a 28 kDa protein (Kroeker and Walker, 1991). Upon a sudden increase in temperature 50 proteins are induced from preexisting mRNA in human HeLa cells (Reiter and Penman, 1983). The main focus of this dissertation will be the possible protective role of proteins against both low and high temperature extremes.
Protein involvement in survival at low temperature

In a great variety of organisms, exposure to low temperatures induces proteins that are associated with increased capacity of the organism to survive or recover from low temperature. Cold temperature acclimation of spinach (*Spinacia oleracea*) and winter rye seedlings (*Secala cereale*) induces a unique set of proteins: in spinach there is a positive correlation between the presence of the cold acclimation proteins and freeze tolerance (Uemura and Yoshida, 1984; Guy and Haskell, 1988). Shifting *Escherichia coli* cultures from 37 to 10°C increases the rate of synthesis of 13 proteins. These proteins have been tentatively identified as enzymes involved in transcription, translation and mRNA degradation (Jones et al., 1987). Exposing pharate adults of the flesh fly *Sarcophaga crassipalpis* to temperatures of 0 to -18°C induces a 72 kDa protein which is immunologically related to the heat shock 70 protein family of *Drosophila melanogaster* (Joplin et al., 1990). *D. melanogaster* larvae accumulate heat shock mRNA following a 13 hour exposure to 0°C. Exposure for longer than 13 hours decreases both the accumulation of heat shock mRNA and larval survival (Petersen et al., 1990).

Inducing the heat shock proteins by a mild heat shock prior to exposing larvae of *D. melanogaster* to 0°C greatly increases their survival (Burton et al., 1988), though survival at 0°C may be due to an inducible factor(s) other than the heat shock proteins. Culturing the conidiospores of *Neurospora crassa* with cycloheximide did not alter the protection against
freezing generated by a mild heat shock (Guy et al., 1986). The most significant evidence for the protective function of heat shock proteins against low temperatures comes from mutants in yeast. Saccharomyces cerevisiae possessing the double mutation YG101\, YG103', which is missing two cognate genes of the heat shock 70 family, have a significantly reduced growth rate at lower temperatures. The double mutant grows 1.9 times slower at 23°C compared to the wild type, and its optimum growth temperature is shifted from 30 to 37°C (Craig and Jacobsen, 1985). These results indicate that the heat shock proteins (both cognate and inducible) are somehow involved in controlling the range of temperature at which yeast can survive.

Protein involvement in survival at high temperature

Long term protection against lethal temperatures induced by a brief exposure to a supraoptimal temperature is well documented (Urano et al., 1980; Li et al., 1983; Landry et al., 1982; Li and Werb, 1983). Treating the Morris hepatoma 7777 cell line to 43°C for 30 minutes induces heat shock proteins up to 8 hours after the treatment at which time thermotolerance is maximized. As the induced heat shock proteins degrade over the next 60 to 80 hours there is a corresponding decrease in thermotolerance (Landry et al., 1982). A positive correlation between the synthesis and decay of heat shock proteins and the onset and decay of thermotolerance has been reported in Chinese hamster ovary fibroblasts (HA-1) (Li and Werb, 1982). Agents other
than supraoptimal temperatures which induce heat shock proteins induce thermotolerance as well. Sodium arsenite and ethanol induce both heat shock proteins and thermotolerance in HA-1 cells (Li, 1983). The rate of synthesis of heat shock proteins decreases significantly at about 5 hours post ethanol treatment but thermotolerance persists for over 24 hours. This led Li (1983) to suggest that it is not the synthesis but the level of heat shock proteins that is critical in thermotolerance. The strongest evidence for the involvement of heat shock 70 proteins in protection against high temperatures comes from experiments which either inhibited the expression or lowered the titer of the 70 kDa heat shock proteins. Mammalian fibroblast cells injected with heat shock 70 monoclonal antibodies were killed by a 30 minute exposure to 45°C, though cells injected with control antibodies survived this treatment (Riabowol et al., 1988). Introduction and amplification (15 X 10^3 to 25 X 10^3) of a heat shock 70 gene fragment containing only the binding site of the heat shock transcription factor into CHO cells greatly reduced the ability of those cells to express the 72 kDa heat shock proteins in response to a thermal challenge. These modified cells were shown to be more thermosensitive than the controls (Johnson and Kucey, 1988).

Though there is a very strong correlation between the presence of heat shock proteins and thermotolerance in many studies, other reports are calling into question the necessity of heat shock proteins in thermotolerance. Culturing Saccharomyces cerevisiae with amino acid analogs (which should result in synthesis of nonfunctional proteins) or with cycloheximide (protein
synthesis inhibitor) did not block the induction of thermotolerance by exposure to supraoptimal temperatures (Hall, 1983). From these results Hall (1983) proposed that thermotolerance must be based upon a preexisting cellular agent, which is either activated or deactivated by the shift to elevated temperatures. *S. cerevisiae* mutants lacking either the inducible heat shock 70 or 82 genes or the heat shock cognate 82 gene showed reduced growth at elevated temperatures (37–37.5°C), yet these deletion mutants were able to develop thermotolerance (Craig and Jacobsen, 1984; Borkovich et al., 1989). Craig and Jacobsen (1984) proposed that the heat shock 70 protein is needed for sustained growth at elevated temperatures (i.e. 37°C), but some other proteins are involved in protection against short exposure to severe conditions (i.e. 52°C). *Escherichia coli* with an adenylate cyclase deletion mutant (cya) are not able to synthesize heat shock proteins but are capable of expressing thermotolerance. The *cya* mutant becomes more thermotolerant than wild types when exposed to supraoptimal temperatures (Delaney, 1990). *E. coli* containing a htpR gene (F33.4 32) fused to an isopropyl-thio-β-D-galactoside (IPTG) inducible promoter express heat shock proteins but not thermotolerance in the presence of IPTG (VanBogelen et al., 1987).

Agents which induce heat shock proteins (e.g. ethanol, copper sulfate, hydrogen peroxide, or temperature shifts) also induce increased titers of the disaccharide trehalose in yeast (Attfield, 1987). Trehalose titers correlate well with the development of thermotolerance in *S. cerevisiae* (Hottiger et al., 1987, 1989). Culturing *S. cerevisiae* with canavanine induces heat shock
proteins but does not elevate trehalose titers and thermotolerance does not develop (Hottiger et al., 1989). As with heat shock proteins the role of trehalose in thermotolerance has been questioned. *S. cerevisiae* strain Klg 102 cannot produce trehalose but still develops thermotolerance (Panek et al., 1990).

Lipids may also play a part in thermotolerance. Exposure to supraoptimal temperatures that induce thermotolerance in C3H mammary adenocarcinoma tumors alters the incorporation of [³H]-acetate in the cellular membrane lipids, significantly decreasing the [³H]-cholesterol/[³H]-lecithin ratio (Harms-Ringdahl, 1989). As with heat shock proteins and trehalose, the role of lipids in thermotolerance has been questioned. The development of thermotolerance in mouse fibroblast LM cells did not alter the cholesterol, phospholipid or protein levels in the plasma membrane, endoplasmic reticulum, or mitochondrial, lysosomal, or nuclear membranes (Konings and Ruifrok, 1985). Thermotolerance in the Chinese hamster ovarian fibroblast appear to be independent of changes in membrane lipid composition (Gonzaley-Mendey, 1982).

Functions of the heat shock proteins

The role of the 70 kDa family of heat shock proteins in the nonstressed cell is largely that of protein folding and translocation across membranes (reviewed by Gething and Sumbrook, 1992). Ribosomal fractions from HeLa cells contain the 72 and 73 kDa heat shock protein
bound to the unreleased nascent protein (Beckman et al., 1990). *S. cerevisiae* mutants lacking a subset of the 70 kDa heat shock protein (Ssa1p, Ssa2p and Ssa4p) contain in the cytosol proteins destined for the endoplasmic reticulum (prepro-α-factor and preprocarboxypeptidase Y) and for the mitochondria (F1 β) in the unprocessed precursor forms (Deshaiyes et al., 1988). Addition of the missing 70 kDa heat shock proteins to isolated yeast microsomes stimulates the uptake of prepro-α-factors, thus clearly showing the 70 kDa heat shock protein’s involvement in membrane translocation of proteins (Chirico et al., 1988). Protein folding in mitochondria of *N. crassa* requires the 60 kDa heat shock protein and ATP. In ATP-depleted mitochondria, 60 kDa heat shock protein can be isolated bound to nonfolded mitochondrial proteins (Ostermann et al., 1989).

Besides their involvement with protein transportation and folding, heat shock proteins are also associated with mature proteins. Human 73 kDa heat shock protein in the presence of ATP and MgCl2 significantly increases protein degradation in cell-free lysosomal proteolytic assays (Chiang et al., 1989). *E. coli* DnaK (70 kDa heat shock protein family) protein dissolves RNA polymerase aggregates formed by high temperature and reactivates newly dissolved RNA polymerase (Skowyra et al., 1990).

Members of the 70 kDa heat shock protein family can be both induced by denatured proteins (Ananthan et al., 1986) and bind to denatured proteins (Skowyra et al., 1990). It is believed that when an organism is stressed the 70 kDa heat shock protein family binds to denatured proteins either to reanimate the denatured proteins (Skowyra et al., 1990) or to
target them for lysosomal degradation (Chiang et al., 1989). Key to both functions is the ability of the 70 kDa family of heat shock proteins to modify protein folding, an ability clearly demonstrated in nonstressed organisms. Whereas the 70 kDa heat shock protein family binds a wide variety of proteins the 90 kDa heat shock protein family binds to a limited range of proteins (i.e. steroid receptors, protein kinases, tubulin and actin). The main function of the 90 kDa heat shock protein family appears to be to maintain the steroid receptors and protein kinases in an inactive or unassembled state (reviewed Gething and Sambrook, 1992). This contrasts with the activation (foiling and membrane translocation) of proteins by the 70 kDa heat shock protein family. The role of the 90 kDa family of heat shock proteins during stress is not known. The small heat shock proteins (16-40 kDa) are induced by stress and expressed during certain developmental stages. Their function(s), as Lindquist and Craig (1988) stated, is "a complete mystery".

Dissertation goals

Are heat shock proteins ecologically significant? Or, to ask the question another way, do heat shock proteins enable organisms to survive seasonal temperature extremes? Previous studies of the protective role of heat shock proteins focused on the short term titer changes related to increased survival of normally lethal stresses. There are no studies in the literature in which a model system has been developed to address the question of the ecological significance of heat shock proteins.
It is the goal of this dissertation to lay the foundation for exploration of the possible ecological significance of heat shock proteins in insects. To accomplish this my dissertation is divided into two sections. The first section deals with the possible role of the heat shock proteins in protecting *S. crassipalpis* from supraoptimal temperature. To achieve this the following questions were investigated. Can *S. crassipalpis* develop thermotolerance? Is there a correlation between the onset and decay of thermotolerance and the titer of the heat shock proteins? Can *S. crassipalpis* develop thermosensitivity, and if so, can thermosensitized flies express heat shock proteins? The second section investigates the possible protective role of heat shock proteins in overwintering diapausing insects, and asked the following questions. Can diapausing insects express heat shock proteins at low temperatures after experiencing a cold shock? Does the expression of heat shock proteins differ between diapausing and nondiapausing insects? Are there unique heat shock proteins in diapausing as compared to nondiapausing individuals as a normal component of the diapause syndrome?
CHAPTER II

Prolonged Thermotolerance in the Flesh Fly, *Sarcophaga crassipalpis*, Does Not Require Continuous Expression or Persistence of the 72 kDa Heat Shock Protein

**Abstract** A brief exposure to a supraoptimal temperature (35-45°C) induced thermotolerance in pharate adults of the flesh fly *Sarcophaga crassipalpis*. While a 90 min exposure to 45°C was normally lethal to flies reared at 25°C, a brief pretreatment at high temperature generated protection from heat shock injury. The induction of thermotolerance was dependent upon both the temperature and duration of the pretreatment, and in this study maximum thermotolerance was induced by a 2 hour exposure to 40°C. Thermotolerance induced by a 2 hour exposure to 40°C decayed slowly over a 72 hour period. This prolonged thermotolerance appears to be independent of the continuous synthesis and persistence of heat shock proteins.
INTRODUCTION

Acquired thermotolerance due to exposure to a supraoptimal temperature has been clearly demonstrated in flies (Milkman, 1962; Mitchell et al., 1979; Chen et al., 1990; Chen et al., 1991; Carretero et al., 1991), yeast (McAlister and Finkelstein, 1980), mice (Li et al., 1983) and mammalian cell cultures (Li and Werb, 1982; Landry et al., 1982). In mice and mammalian cell cultures, thermotolerance may take days to decay. Little is known about the potential for such prolonged (days) thermotolerance in insects, although thermotolerance against phenocopy defects lasting over 25 h has been observed in Drosophila melanogaster (Milkman, 1963), and thermotolerance lasting more than 72 h has recently been reported in Chironomus thummi (Carretero et al., 1991).

Increased thermotolerance has been correlated with the expression of a group of stress proteins known as heat shock proteins. In Sarcophaga crassipalpis exposure to a temperature range of 40 to 43°C inhibits normal protein synthesis and induces expression of heat shock proteins (90, 72 or 65 kDa, and a cluster of small proteins in the 20-30 kDa range), (Joplin and Denlinger, 1990). The function of the heat shock proteins is not yet clear, but they are believed to protect cells against several types of stress (reviews by Lindquist, 1986; Lindquist and Craig, 1988). In certain organisms, the synthesis and persistence of the higher molecular weight (68-107 kDa) heat shock proteins correlate well with the development and decay of thermotolerance (Li and Werb, 1982, Landry et al., 1982, Li, 1983). Stresses
other than heat that induce the heat shock proteins also increase thermotolerance, e.g., ethanol (Li, 1983; Plesset et al., 1982), hypoxia (Li and Werb, 1982) and heavy metals (Li, 1983). Though the treatments that induce thermotolerance are normally short (< 3 h), the thermotolerance may persist for days (Urano et al., 1980; Li et al., 1983; Landry et al., 1982). There is a strong association of heat shock proteins and thermotolerance, but a growing body of literature questions the necessity of heat shock proteins in the development of thermotolerance (Hall, 1983; Ramsay, 1988; Bols et al., 1990; DeLaney, 1990).

This study asks two basic questions. 1.) Can S. crassipalpis pharate adults develop extended thermotolerance as seen in mice and mammalian cell cultures? 2.) Is synthesis and persistence of the 72 kDa heat shock protein, the most highly expressed heat shock protein in S. crassipalpis (Joplin and Denlinger, 1990), required for thermotolerance?

**MATERIALS AND METHODS**

Flies

*Sarcophaga crassipalpis* was reared as described (Denlinger, 1972) using an LD 15: 9 (light: dark) cycle at 25°C.
Temperature Treatment

Three replicates of 15 red eye pharate adults were placed into thin-walled pyrex test tubes (13 mm X 100 mm) and capped with a cotton plug. Temperature precision was achieved in a Lauda model RM20 glycerol bath. After high temperature exposure pharate adults were placed in petri dishes and stored at LD 15:9 and 25°C.

Protein labeling

Pharate adult brains were dissected under saline (Ephrussi and Beadle, 1936). Five brains were cultured together in 1.5 ml vials containing 20 μl of methionine-free Grace’s culture medium (Grace, 1962) with 1 μl [10 μCi (370 kBq)/μl] methionine (Tran-35S label™; ICN). After 1 h Grace’s medium was removed, 20 μl of sample buffer (0.06% Tris, 10% glycerol, 2% SDS, 10% β-mercaptoethanol, bromophenol blue) was added, subsequently the sample was boiled for 5 min and stored at -70°C.

In examining the duration of expression of the heat shock proteins after a 2 h exposure to 40°C red eye pharate adults were placed at 25°C for 0, 2, 4, 8, 12, 24 or 48 h. Brains were then dissected and cultured as above. In the pulse chase experiment brains were exposed to 40°C for 1 h then labeled with 35S-methionine for an additional hour at 40°C. After labelling brains were washed 3X with 100 μl Grace’s medium with methionine and
then cultured 24 h at 25°C in 100 μl Grace's medium with methionine. All gels represent duplicated samples.

Protein electrophoresis and detection

The amount of 35S incorporation for each sample was determined by trichloroacetic acid precipitation (Mans and Novelli, 1961). Polyacrylamide gel electrophoresis (PAGE) was run using equal counts per sample on a discontinuous 10% gel (Laemmli, 1970). Gels were run at 10 mA, fixed and stained using a modified Coomassie blue method (Zehr et al., 1989), destained with acetic acid: methanol:water (0.18:1:1) and saturated with 1 M sodium salicylate (Chamberlain, 1979). Gels were dried onto Whatman filter paper and exposed to Kodak X-Omat X-ray film at -70°C.

Statistics

All data were normalized by arcsin transformation (Sokal and Rohlf, 1981). Data represented in figures as curves were analyzed by ANOVA without replication to verify there was no significant variance within groups. If variance within groups was insignificant, then the possible differences between individual curves was determined by ANOVA with replication for equal sample size (Sokal and Rohlf, 1981). All other data were analyzed by the Tukey-Kramer T-test (Sokal and Rohlf, 1981).
RESULTS

Optimizing thermotolerance

To optimize long term protection of pharate adults to lethal hyperthermic stress (90 min at 45°C), pupae were first exposed to 35, 40, or 45°C for various durations and then 24 h later exposed to 45°C (Fig. 1). Pharate adults exposed to 35°C for 30 to 120 min gave no clear dose response and were afforded very little long term protection to lethal exposure to 45°C; the best protection generated at 35°C (yielding 28% adult emergence) was obtained with a 60 min exposure. Pretreating pharate adults at 40°C for various durations gave a clear dose response with maximum protection (83% emergence) occurring with a 2 h pretreatment. Pretreatment at 45°C gave maximum protection after only 30 min, but this level (46% emergence) was only about half the level of protection generated by a 2 h pretreatment at 40°C. Pretreatment longer than 30 min at 45°C was detrimental to survival at 45°C.

Pharate adults were also pretreated at 40°C for 2 h (Fig 2A) or 45°C for 30 min (Fig 2B), then 24 h later exposed to 45°C for 30 to 150 min. Both pretreatments gave statistically significant (ANOVA; \( P<0.001 \)) long term protection as compared to controls that were not pretreated. The greatest protection was generated by a 2 h pretreatment at 40°C, e.g. following a 90 min exposure to 45°C, 71% of the flies pretreated at 40°C emerged but only 40% of the flies pretreated at 45°C emerged.
Kinetics of thermotolerance

What are the kinetics of the protection, that is when does thermotolerance start and how long does it last? Pharate adults were pretreated 2 h at 40°C and after 0, 4, 8, 12, 24, 48 or 72 h at 25°C were exposed to 45°C for 90 min (Fig. 3). Protection started immediately after the pretreatment (93% emergence), decreased to 50% emergence by 48 h, and disappeared 72 h after pretreatment.

Heat shock protein expression

Are heat shock proteins involved in the long term protection? While a set of heat shock proteins, including the highly expressed 72 kDa heat shock protein, was synthesized at 43°C (Fig. 4G), synthesis of heat shock proteins stopped when flies were returned from 40°C to 25°C. In brains labeled immediately after a 2 h exposure to 40°C heat shock protein synthesis ceased and normal protein synthesis resumed within the first hour after transferring the brains to 25°C. No heat shock protein synthesis was observed in any samples (0 to 72 h) after removal from 40°C (Fig. 4).

Can the persistence of heat shock proteins that have been synthesized during pretreatment account for the observed thermotolerance? A pulse chase experiment showed that the 72 kDa heat shock protein is being
synthesized during the 2 h exposure to 40°C but is not present 24 h after removing the brains from 40°C (Fig. 5).

**DISCUSSION**

Pretreating *D. melanogaster* (Milkman, 1963) and *C. thummi* (Carretero et al., 1991) at supraoptimal temperatures induces thermotolerance that persists for more than 25 and 72 h, respectfully. Exposing *S. crassipalpis* to a temperature within a 35 to 45°C range provided protection against 90 min at 45°C for more than 24 h. Optimum protection was provided by a 2 h exposure to 40°C, and this pretreatment provided protection beyond 48 h. These results would appear to conflict with a report indicating loss of thermotolerance within 4 h after pretreatment in *S. crassipalpis* (Chen et al., 1991), but this disparity is likely due to differences in the severity of the final high temperature shocks employed. In this study a 90 min shock at 45°C was the maximum used, while Chen et al. (1991) exposed flies to a 2 h shock at 45°C.

Whether heat shock proteins are essential for thermotolerance may be dependent upon the organism being examined. Exposing the Morris hepatoma 7777 cell line to 43°C for 30 min induces heat shock proteins up to 8 h after treatment, at which time thermotolerance is maximized. As the induced heat shock proteins degrade over the next 60 to 80 h there is a corresponding decrease in thermotolerance (Landry et al., 1982). The presence of heat shock proteins also correlates well with thermotolerance in
the midge, *C. thummi* (Carretero et al., 1991). In contrast, the role of heat shock proteins in thermotolerance of yeast *Saccharomyces cerevisiae* is questionable. Neither culturing *S. cerevisiae* with amino acid analogs (which should result in synthesis of nonfunctional proteins) nor with cycloheximide (which blocks protein synthesis) prevents induction of thermotolerance by exposure to supraoptimal temperature (Hall, 1983). Thermotolerance in *S. crassipalpis* appears not to be dependent upon the synthesis or persistence of heat shock proteins. Synthesis of heat shock protein stops and normal proteins synthesis resumes within the first hour after removal from a 2 h exposure to 40°C. The pulse chase experiment clearly shows that heat shock proteins are being synthesized during the 2 h at 40°C and that those proteins synthesized during that exposure are degraded within 24 h after removal from 40°C. Yet, thermotolerance persists beyond 48 h. A role for heat shock proteins in establishing thermotolerance in *S. crassipalpis* remains a distinct possibility, but neither the continuing synthesis nor persistence of heat shock proteins appears critical in maintaining thermotolerance.

In conclusion we report in this article 1.) The development of long term (> 48 h) thermotolerance after a brief exposure to supraoptimal temperature in *S. crassipalpis*. 2.) Expression of thermotolerance is dose dependent on both the duration of the exposure and the temperature of the pretreatment. 3.) The expression of thermotolerance in *S. crassipalpis* does not appear to be dependent upon the synthesis or the persistence of the 72 kDa heat shock protein.
Figure 1. Red eye pharate adults of *S. crassinalpis* were exposed to 35 (open squares), 40 (open circles) or 45°C (solid circles) for varying durations. After 24 h at 25°C pharate adults were exposed to 45°C for 90 min. Survival was based on the percent of flies to survive to adult emergence (mean ± S.E.); three replicates of 15 flies each for each time point.
Figure 2. Red eye pharate adults of *S. crassipalpis* were pretreated at 40°C for 2 h (A) or 45°C for 30 min (B). After 24 h at 25°C pharate adults were exposed to 45°C for varying durations. Survival was based on the percent of pretreated (solid circles) and non-pretreated (open circles) flies to survive to adult emergence (mean ± S.E.); three replicates of 15 flies each for each time point.
FIGURE 2

% Adult Emergence (mean ± S.E.)

Time at 45° C (min.)
Figure 3. Red eye pharate adults of *S. crassipalpis* were pretreated at 40°C for 2 h and then exposed to 45°C for 90 min after 0, 4, 8, 12, 24, 48 or 72 h at 25°C to determine the onset and duration of thermotolerance. Survival was based on the percent of pretreated (solid circles) and non-pretreated (open circles) flies to survive to adult emergence (mean ± S.E.): three replicates of 15 flies each for each time point.
FIGURE 3

% Adult Emergence (mean ± S.E.)

Time after Pretreatment (h)

0 20 40 60 80
Figure 4. Red eye pharate adults of *S. crassipalpis* were pretreated at 40°C for 2 h, and after 0 (A), 4 (B), 24 (C), 48 (D), or 72 (E) h at 25°C 5 brains were dissected and labeled for 1 h at 25°C with 10 μCi of $^{35}$S-methionine. Controls were held continuously at 25°C (F) or heat shocked at 43°C for 1 h and then immediately labeled 1 additional h at 43°C (G). Samples were separated on a 10% SDS-PAGE gel. The 72 kDa heat shock protein is indicated.
Figure 5. Brains from 5 red eye pharate adults of S. crassipalpis were cultured at 40°C (A, B) or 43°C (C) for 1 h then labeled with 10 μCi $^{35}$S-methionine for one additional hour at the same temperature. Protein expression was either stopped immediately by boiling (A, C) or brains were washed with Grace's medium and cultured for 24 h at 25°C (B). Samples were separated on a 10% SDS-PAGE gel.
CHAPTER III

Induction and Decay of Thermosensitivity in the Flesh Fly, 
Sarcophaga crassipalpis

Abstract  When pharate adults of the flesh fly Sarcophaga crassipalpis are exposed to 40°C for 4 h they become more tolerant of high temperatures that are normally lethal (thermotolerance). In contrast, a 1 h exposure to 45°C decreases tolerance to a subsequent high temperature challenge (thermosensitivity). While control flies experience little mortality when held at 35°C for 24 - 48 h the thermosensitized flies die when exposed to 35°C. Sensitivity to a second thermal challenge slowly decays over a 72 h period. The acquisition of thermotolerance prevents the development of thermosensitivity. Brains from thermosensitized flies cultured at 43°C express the 72 kDa heat shock protein and normal protein synthesis is inhibited. This implies that development of thermosensitivity is not associated with a loss in the capacity to express the 72 kDa heat shock protein.
INTRODUCTION

The ability of an organism to survive a stress depends in part upon the stress history of that individual organism. Exposure to a supraoptimal temperature can increase (make thermotolerant) or decrease (thermosensitize) an organism's ability to survive a future temperature stress. Development of thermotolerance or thermosensitivity depends upon the temperature to which the organism is exposed. Most mammalian cell lines develop thermotolerance when treated at temperatures below 42-43°C, but become thermosensitive when treated at temperatures above this range (Nielsen et al., 1982; Jung and Kolling, 1980; Jung 1982; Dikomey et al., 1984). Chinese hamster ovary cells exposed to 40°C (1 to 16 h) develop thermotolerance to 43°C, whereas a 15 to 90 min exposure to 43°C induces thermosensitivity to 40°C (Jung and Kolling, 1980; Jung, 1982).

In response to thermal challenge (as well as other stresses) a group of stress proteins commonly known as heat shock proteins are induced. These stress proteins are believed to protect the organism against future stress(es) (reviewed by Lindquist, 1986). There is a positive correlation between the expression of the heat shock 70 family of proteins and thermotolerance in the midge Chironomus thummi (Carretero et al., 1991) and certain mammalian cell cultures (Li and Werb, 1982; Landry et al., 1982). The strongest evidence for the involvement of the heat shock 70 family in thermotolerance comes from experiments which reduced the level of
expression or titers of 70 kDa heat shock proteins thereby rendering the test organism thermosensitive to normally nonlethal conditions (Riabowol et al., 1988; Johnson and Kucey, 1988). However, a number of studies question the involvement of the heat shock proteins in thermotolerance. In the flesh fly Sarcophaga crassipalpis (Yocum and Denlinger, 1992), Escherichia coli (Ramsay, 1988; DeLaney, 1990), and Saccharomyces cerevisiae (Hall, 1983) thermotolerance is not always correlated with the expression of the heat shock 70 kDa family of proteins.

Though thermotolerance has been demonstrated in insects (e.g., Milkman, 1962; Carretero et al., 1991; Chen et al., 1990; Chen et al., 1991; Yocum and Denlinger, 1992), thermosensitivity has yet to be examined in insects. This study seeks to answer two basic questions: 1.) Is the flesh fly S. crassipalpis capable of developing thermosensitivity and, if so, is thermosensitivity in the flesh fly similar to that observed in mammalian cell lines? 2.) Can thermosensitized flies express heat shock proteins?

**MATERIALS AND METHODS**

**Flies**

*S. crassipalpis* was reared in accordance with Denlinger (1972). All life stages were maintained under a long day regime of 15:9 LD and 25°C.
Temperature treatment

As determined by preliminary experiments pharate adults (red eye pigmentation was visible) were thermosensitized by pretreating at 45°C for 60 min. Three replicates of 15 pharate adults were placed in thin-wall pyrex test tubes with a cotton plug inserted in the end. High temperature exposures were attained using a Lauda model RM20 glycerol bath. After both the pretreatment and final treatment the pharate adults were returned to 15:9 LD and 25°C. Only those adults flies that emerged completely from the puparium were counted as surviving the treatment.

Statistics

Data was arcsin transformed in accordance with Sokal and Rohlf (1981) for percentage data. Curves were first analyzed by ANOVA without replication to verify that there was no significance in variance within groups. Curves passing this test were then tested against other curves using ANOVA with replication for equal sample sizes (Sokal and Rohlf, 1981). When calculating ANOVA with equal sample sizes, time points not present in both curves were not used.
Protein labeling and electrophoresis

Pharate adults were either pretreated 1 h at 45°C or maintained at 25°C; 24 h after the pretreatment brains from both groups were dissected under insect saline (Ephrussi and Beadle 1936). Brains from flies that were not heat shocked were cultured 1 h at 25°C in 20 μl of methionine-free Grace's medium (Grace 1962) with 1 μl [10 μCi (370 kBq)/μl] methionine (Tran-35S label; ICN). Brains for heat shock samples were cultured at 43°C for 1 h then labelled for an additional 1 h at 43°C. Labelling was stopped by removing the culture medium and boiling the samples 5 min in 20 μl of SDS sample buffer (0.06% Tris, 10% glycerol, 2% SDS, 10% β-mercaptoethanol, bromophenol blue). Samples were stored at -70°C. Electrophoresis was carried out as described previously (Yocum and Denlinger 1992), using 10% polyacrylamide gels and equal counts per sample as determined by trichloroacetic acid precipitation.

RESULTS

Thermal wounding

Twenty-four hours after a 4 h pretreatment at 40°C, red eye pharate adults of S. crassipalpis were thermotolerant: 84±6 (mean±S. E., 3 replications of 15 flies each) of the pretreated flies survived 90 min at 45°C while none of the controls flies survived. In contrast, pretreating the flies at 45°C for 60
min induced significant thermosensitivity (Fig. 6, ANOVA, P<0.001). The LT_{50} (time of exposure that kills 50\% of the pharate adults) of the thermosensitized pharate adults was approximately 20 min at 45°C, whereas the LT_{50} of the controls at 45°C was approximately 90 min. A 30 min exposure to 45°C failed to induce either thermotolerance or thermosensitivity (Fig 6).

Decay of thermosensitivity

Do thermosensitized pharate adults regain normal tolerance to thermal stress? Pharate adults exposed to 45°C for 1 h were significantly (ANOVA, P<0.001) less able to handle an exposure to 45°C 48 h after the pretreatment than controls (Fig. 7B), but by 72 h after pretreatment thermosensitized pharate adults and controls had identical LT_{50}'s of 35 min at 45°C. As the control pharate adults aged they became more sensitive to 45°C: LT_{50} decreased from >80 min on the day of red-eye pigmentation to 35 min 72 h later (Fig. 7).

Mortality at normally nonlethal temperatures

Are thermosensitized pharate adults sensitive to high temperatures that normally would be nonlethal? Thermosensitized pharate adults were placed at 30 or 35°C 24 h after the pretreatment. Significant (ANOVA, P<0.001) mortality was observed for the thermosensitized pharate adults at
35°C but not at 30°C as compared to control pharate adults exposed to the same temperatures (Fig. 8A, B). 24% of the thermosensitized pharate adults emerged after 48 h at 35°C as compared to 98% of the controls. When exposed to 35°C for 72 h only 4% of the thermosensitized flies emerged but 48% of the control flies emerged (Fig. 8B).

Thermotolerance protects against thermosensitivity

Does the active expression of thermotolerance prevent thermosensitivity? A 4 h exposure to 40°C (thermotolerance) immediately prior to a 1 h exposure to 45°C completely prevented thermosensitivity in red eye pharate adults of S. crassipalpis (ANOVA, P<0.001) (Fig. 9A, B). Thermosensitized pharate adults had LT50's of 16 h and 18 min at 35 and 45°C respectively as compared to greater than 72 h and 100 min at 35 and 45°C respectively for the pharate adults protected by the active expression of thermotolerance (Fig. 9A, B).

Expression of heat shock proteins

Can thermosensitized pharate adults express heat shock proteins in response to a second thermal stress? Brains from thermosensitized and control pharate adults were labelled at 43°C, a temperature known to induce heat shock proteins in these flies (Joplin and Denlinger, 1990). Exposing
thermosensitized brains to 43°C induced expression of the 72 kDa heat shock protein and inhibited normal protein synthesis (Fig. 10C, D).

DISCUSSION

Various cell lines exposed to lethal temperatures have similar survival curves that can be divided into two segments, a shoulder area where there is very little cell mortality with increasing time, followed by a section of exponential death with increasing time (Jung and Kolling, 1980; Jung, 1982; Nielsen et al., 1982; Spiro et al., 1982; Dikomey et al., 1984). Pretreating L1A2 cells at 45°C for 10 min (Nielsen et al., 1982) and CHO cells at 45°C for 5-10 min (Spiro et al., 1982) sensitizes these cell to supraoptimal temperatures (40-43°C) and alters the basic shape of their survival curve. A characteristic of the survival curves for these thermally sensitized cells is the reduction or absence of the shoulder (Jung, 1982; Nielsen et al., 1982; Spiro et al., 1982). The survival curve of S. crassipalpis, when given a single exposure of lethal high temperature, has the same basic shape reported for the cell lines. The shoulder of the S. crassipalpis curve extends for about 60 min at 45°C, followed by a section of rapid death with increasing time at 45°C. As in cells lines the survival curve for thermosensitized S. crassipalpis pharate adults shows the same characteristic lack of a shoulder when exposed to a second thermal stress of 35°C or 45°C. A 30 min exposure to 45°C gives what appears to be a conflicting result. We reported previously (Yocum and Denlinger, 1992) that a 30 min exposure to 45°C induced a
minimal degree of thermotolerance. Here we report that a 30 min exposure to 45°C had no effect on surviving a second exposure to 45°C. This suggests that the transition point between the induction of thermotolerance and thermosensitivity is very close to 45°C. All other treatment regimes reported here are consistent with our previous observations.

Very little is known about the nature of the injury causing thermosensitivity. What is known is that the thermal injury can be prevented by the active expression of thermotolerance and that the damage is reversible with time. A 16 h exposure to 40°C blocks the development of thermosensitivity in CHO and R1H cell lines (Jung, 1982; Dikomey, 1984). Likewise, pretreating S. crassipalpis at 40°C for 4 h to induce thermotolerance prevents the development of thermosensitivity following a 1 h exposure to 45°C. The damage caused by a 1 h exposure to 43°C in CHO cells completely decays after 7 h at 37°C (Jung and Kolling, 1980). Thermosensitivity in S. crassipalpis takes much longer (72 h) to decay. Our results demonstrate that thermosensitized S. crassipalpis pharate adults share the same aspects of thermosensitivity found in mammalian cell lines: loss of the survival curve shoulder, mortality at normally nonlethal temperatures, decay of thermosensitivity and protection against the induction of thermosensitivity by thermotolerance.

Experiments that were successful in reducing the level of expression or titer of 70 kDa heat shock protein in test organisms increased their thermosensitivity. Rat fibroblast cells injected with heat shock 70 monoclonal antibodies were killed by a 30 min exposure to 45°C, though
cells injected with control antibodies survived this same treatment (Riabowol et al., 1988). Introduction and amplification (15 X 10^3 to 25 X 10^3) of a heat shock 70 gene fragment (from *Xenopus laevis*) containing only the binding site of the heat shock transcription factor (HFS) into Chinese hamster ovary cells greatly reduced the ability of those cells to express the 72 kDa heat shock proteins in response to a thermal challenge. These modified cells were more thermosensitive than the controls (Johnson and Kucey, 1988). Brains from thermosensitized flies heat shocked at 43°C expressed the 72 kDa heat shock protein and normal protein synthesis was inhibited. Our results indicate that thermosensitivity in *S. crassipalpis* pharate adults is not caused by the loss in capacity to either express the 72 kDa heat shock protein or inhibit normal protein synthesis at high temperatures.
Figure 6. Pharate adults (red-eye stage) of *S. crassipalpis* reared at 25°C were exposed to 45°C for 0 (open circle), 30 (solid circles) or 60 min (squares). After 24 h at 25°C pharate adults were exposed to 45°C for varying durations. Survival was based on the percent of flies that survived until adult emergence (mean ± S.E., three replicates of 15 flies each for each time point).
Figure 7. Pharate adults (red-eye stage) of *S. crassipalpis* were pretreated at 45°C for 1 h. After 24 (A), 48 (B) or 72 (C) h at 25°C pharate adults were exposed to 45°C for varying durations. Survival was based on the percent of pretreated (open circles) and non-pretreated (solid circles) flies to survive until adult emergence (mean ± S.E., three replicates of 15 flies each for each time point).
FIGURE 7

% Adult Emergence (mean ± S.E.)

Time at 45°C (minutes)
Figure 8. Pharate adults (red-eye stage) of *S. crassipalpis* were pretreated at 45°C for 1 h. After 24 h at 25°C pharate adults were exposed to 30°C (A) or 35°C (B) for varying durations. Survival was based on the percent of pretreated (open circles) and non-pretreated (solid circles) flies to survive until adult emergence (mean ± S.E., three replicates of 15 flies each for each time point).
FIGURE 8
Figure 9. Pharate adults (red-eye stage) of *S. crassipalpis* were exposed to 40°C for 4 h then immediately exposed to 45°C for 1 h (open circles), 45°C for 1 h only (squares), or remained at 25°C (solid circles). After 24 h at 25°C pharate adults were exposed to 35°C (A) or 45°C (B) for varying durations. Survival was based on the percent of flies that survived until adult emergence (mean ± S.E., three replicates of 15 flies each for each time point).
FIGURE 9
Figure 10. Pharate adults (red-eye stage) of S. crassipalpis were held at 25°C (A, C) or pretreated at 45°C for 1 h (B, D). After 24 h at 25°C brains were cultured at 25°C for 1 h with 10 μCi $^{35}$S-methionine (A, B) or at 43°C for 1 h then labeled for 1 additional h at 43°C (C, D). Samples were separated on a 10% SDS-PAGE gel. The 72 kDa heat shock protein is indicated.
CHAPTER IV

Expression of Heat Shock Proteins in Response to Both High and Low Temperature Extremes in Diapausing Pharate Larvae of the Gypsy Moth, *Lymantria dispar*

**Abstract** Diapausing pharate first instar larvae of the gypsy moth, *Lymantria dispar*, respond to high temperature (37-41°C) by suppressing normal protein synthesis and synthesizing a set of 7 heat shock proteins with molecular weights of 90, 75, 73, 60, 42, 29, and 22 kDa. During recovery from heat shock at 25°C, synthesis of the heat shock proteins gradually decreases over a period of 6 hours, while normal protein synthesis is restored. A subset of these same heat shock proteins are also expressed during recovery at 4 or 25°C from brief exposures to low temperature (-10 to -20°C), and their expression is more intense with increased severity of cold exposure. During recovery at 4°C after 24 hours at -20°C, both 90 and 75 kDa heat shock proteins are expressed for more than 96 hours. While normal protein synthesis is suppressed during heat shock and recovery from heat shock, normal protein synthesis coincides with synthesis of the heat shock proteins during recovery from low temperatures, thus implying that
expression of the heat shock proteins is not invariably linked to suppression of normal protein synthesis. Western transfer, using a monoclonal antibody that recognizes the inducible form of the human 70 kDa heat shock protein, demonstrates that immunologically related proteins in gypsy moth are expressed at 4°C and during recovery from cold and heat shock.

**INTRODUCTION**

Eggs of the gypsy moth, *Lymantria dispar*, are deposited on tree trunks in late summer, and in this exposed site diapausing pharate larvae are subjected to extremes of temperature ranging from the heat of late summer to the severe cold of winter. It is therefore critical for the pharate larvae to have mechanisms for protection against both extremes of this temperature range.

High temperature stress induces a group of proteins commonly referred to as heat shock proteins. These highly conserved proteins are present in all organisms ranging from yeast to human and are believed to confer protection to the stressed organism (review by Lindquist, 1986). Literature on yeast (McAlister and Finkelstein, 1980), insects (Velazguey and Lindquist, 1984; Chen et al., 1990) and mammals (Li et al., 1983) indicates that a mild heat shock will provide protection against subsequent temperatures that would otherwise be lethal. Tolerance to these normally lethal temperatures is positively correlated with the presence of the heat
In addition to providing protection against high temperature, the heat shock proteins may play a role in survival at temperatures below optimum. In the yeast *Saccharomyces cerevisiae*, expression of a 67 kDa protein, a member of the highly conserved 70 kDa heat shock protein family, is increased at lower rearing temperatures (Craig and Jacobsen, 1985). The growth rate of a mutant that lacks the 67 kDa protein is 2.4 times slower at 19°C than the wild type, and its optimum temperature is shifted from 30°C to 37°C. Cold exposure induces heat shock proteins in the fruit fly *Drosophila melanogaster* (Burton et al., 1988), in the flesh fly *Sarcophaga crassipalpis* (Joplin et al., 1990), and in several noninsect species including *Neurospora crassa* (Guy et al., 1986) and *Dictyostelium discoideum* (Maniak and Nellen, 1988).

Though glycerol has been identified in gypsy moth eggs (cited by Madrid and Steward, 1981) and is likely to serve as a cryoprotectant, mechanisms of protection against high temperature stress have not been investigated. Can heat shock proteins be expressed in these diapausing eggs? Flesh fly pupae in diapause are capable of synthesizing heat shock proteins (Joplin, unpublished data), but clearly not all insect developmental stages have that capacity (Eberlein and Mitchell, 1987 and Zimmerman et al., 1983). And, if the gypsy moth at this stage of development can produce heat shock proteins, is synthesis elicited by both high and low temperature?
In this paper we subject gypsy moth eggs (diapausing pharate first instar larvae) to a range of high temperatures, ascertain molecular weights of the heat shock proteins synthesized, and then determine whether these same proteins are synthesized in response to and at low temperature. In addition, Western transfers were used to verify that the major protein induced in gypsy moth by low temperatures is related to a well-characterized 70 kDa heat shock family of proteins.

MATERIALS AND METHODS

Insects

_L. dispar_ eggs (New Jersey strain) were obtained from the United States Department of Agriculture Forest Service in Hamden, Connecticut. Upon receipt the eggs were stored at 4°C. All experiments were conducted on diapausing eggs (pharate first instar larvae) chilled at 4°C for more than 60 days.

Protein labeling

Pharate first instar larvae were dissected from the eggs by mechanically stripping off the chorion under saline (Ephrussi and Beadle, 1936). Four pharate larvae were bisected and cultured together in a 1.5 ml vial containing 10 μl methionine-free culture medium (Grace, 1962).
Cultures were heat shocked (35-45°C) for 1 h in water baths and then labelled for 1 more hour at shock temperatures using 1 μl [10 μCi (370 kBq)] Trans 35S-labelT.M. (1259 Ci/m mole, ICN Radiochemicals). Grace's medium was then removed, the tissue homogenized in 20 μl of sample buffer (0.06% Tris, 10% glycerol, 2% SDS, 10% β-mercaptoethanol, bromophenol blue), boiled for 5 min, and stored at -70°C.

In experiments examining recovery from heat and cold shock, ten eggs were placed in thin walled pyrex test tubes with cotton plugs and exposed either to -20°C in an ethanol bath stored in a freezer or to -10 or 40°C for 2 h in a Lauda circulating bath. Pharate larvae were dissected, cultured, and labelled at 25°C at 0, 2, 4 or 6 h after exposure to cold or heat shock temperatures. To evaluate the expression of heat shock proteins at low temperatures eggs were exposed to -20°C for 24 h then placed at 4°C for 0, 24, 48, 72 or 96 hours. Pharate larvae were then dissected and labelled at 4°C for 24 h.

Protein electrophoresis and detection

The amount of 35S incorporation for each sample was determined by trichloroacetic acid precipitation (Mans and Novelli, 1961). Polyacrylamide gel electrophoresis (PAGE) was run using equal counts per sample on a discontinuous 10% gel (Laemmli, 1970) using a modified stacking gel (Neuhoff, et al., 1986). Gels were run at 15 mA, fixed and stained using a modified Coomassie blue method (Zehr et al., 1989), destained with acetic
acid: methanol:water (0.18:1:1) and saturated with 1M sodium salicylate (Chamberlain, 1979). Gels were dried onto Whatman filter paper and exposed to Kodak X-Omat X-ray film at -70°C.

Western transfer

Larvae were cold shocked at -20°C for 2 hours then placed at 25°C for 3 hours. After a 3 h incubation 5 larvae were homogenized in 20μl of 5% sucrose, and samples of protein were electrophoresed on a 10% nondenaturing gel system (Hoefer Scientific). Proteins were transferred overnight at 25 VDC and 4°C onto a nitrocellulose sheet in transfer buffer (20mM Tris-Cl, 0.18M glycine, 20% methanol, pH 8.0). The filter was washed after transfer with Tris saline solution (10mM Tris-Cl, 150mM NaCl, 0.05% Tween, pH 8.0) containing 5% nonfat dry milk for 30 min. The nonfat dry milk wash was removed and the filter incubated for 30 min in Tris saline with the primary antibody (1:1000 dilution), monoclonal antibody C92F3A-5 which is specific for the inducible form of the human 70 kDa heat shock protein (StressGen). The filter was then washed 3 times with Tris saline and incubated for 30 min with a goat antimouse antibody (1:2000 dilution) conjugated with alkaline phosphatase (Promega). The filter was then washed 3 times with Tris saline. After the final wash the filter was developed 1 h in 10ml alkaline phosphate buffer (100mM Tris-Cl, 100mM NaCl, 5mM MgCl₂, pH 9.5) to which was added 66μl NBT and 33μl BCIP.
RESULTS

Expression of heat shock proteins

The range of temperatures that induce synthesis of heat shock proteins in diapausing pharate larvae of *L. dispar* is shown in Fig. 11. Seven heat shock proteins (90, 75, 73, 60, 42, 29 and 22 kDa) were strongly induced during a 1 h label at 37-41°C following a 1 h incubation at the corresponding temperatures. Synthesis of non-heat shock proteins was progressively suppressed at temperatures of 37°C and above. At 43°C synthesis of the heat shock proteins also declined, and at 45°C all protein synthesis was inhibited (data not shown).

Recovery at 25°C from heat shock

The duration of heat shock protein synthesis was examined after a 2 h exposure to 40°C. After transfer to 25°C, the 90, 75 and 29 kDa proteins continued to be expressed for over 6 h whereas the 73 and 22 kDa proteins were expressed for less than 6 h (Fig. 12). Non-heat shock proteins reappeared within 1-3 h after removal from heat shock. Maximum expression of the heat shock proteins occurred 2 h after removal from 40°C. Expression of the 60 and 42 kDa proteins during recovery from heat shock was difficult to determine because their identity is obscured by the
synthesis of normal proteins with similar molecular weights (see Fig. 12, lane E).

Recovery at 25°C from cold shock

A comparison of heat shock protein expression induced by a 2 h cold shock at -10 or -20°C demonstrated a clear dose-response relationship, with heat shock protein expression increasing with severity of the cold shock (Fig. 13). Exposure to -10°C for 2 h induced primarily the 75 kDa protein whereas a 2 h exposure to -20°C induced the 90, 75 and 29 kDa proteins. Maximum expression of the 90, 75 and 29 kDa heat shock proteins occurred 2 h after removal from -20°C, and expression continued for at least 6 h. As in recovery from heat shock, expression of the 60 and 42 kDa heat shock proteins during recovery from cold shock was obscured by the expression of normal proteins with similar molecular weights. The normal pattern of protein synthesis was restored within the first hour after the cultures were transferred from -20 to 25°C.

Recovery at 4°C from cold shock

Another group of eggs exposed to -20°C for 24 h was returned to 4°C for recovery. Heat shock proteins were expressed at this low temperature, and expression persisted for several days (Fig. 14). As recovery time at 4°C increased (Fig. 14, lanes A though E) the synthesis of 90 and 75 kDa heat
shock proteins increased, reaching a maximum for both proteins 96 h after removal from -20°C. The 29 kDa heat shock protein does not appear to be expressed at 4°C following 24 h exposure to -20°C though it is expressed at 25°C following a lesser cold shock (Fig. 13).

Gypsy moth heat shock protein related to human heat shock protein

When the filter from a nondenaturing gel was screened with the monoclonal antibody C29F3A-5, which binds the inducible form of the human 70 kDa heat shock protein, two doublets were observed in the 4°C control (Fig. 15C) and cold shocked (24 h at -20°C) lane (Fig. 15B), and two single bands were present in the heat shock (2 h at 40°C) lane (Fig. 15A). The two bands in the heat shock lane correspond to the lower bands of each doublet in the control and cold shock lanes. The upper bands in the doublets were less intensely detected than their lower counterparts and were not always present. When samples were run on a denaturing gel just one doublet was observed, indicating that the two doublets found on the nondenaturing gel have the same molecular weight but different charges (data not shown). Results from the Western transfer and pulse labeling suggest that the inducible 70 kDa-related heat shock proteins are also expressed in diapausing L. dispar at 4°C but that both heat and cold shock further increase the expression of this protein.
The basic features of the heat shock response in diapausing pharate larvae of *L. dispar* are similar to those reported for other insects. A limited number of proteins are induced [i.e., 7 in *L. dispar*, 6 in *D. melanogaster* (Lindquist, 1980) and *Locusta migratoria* (Walker et al., 1986)]. There are 3 major heat shock protein families based on sequence homology. Proteins within a family commonly exhibit a limited range of molecular weights. Based upon molecular weights the *L. dispar* heat shock proteins can be assigned to the three major families; the high molecular weight (90-83 kDa) family (*L. dispar* 90 kDa), the 70 kDa heat shock family (*L. dispar* 75, 73 and 60 kDa) and the small heat shock family (*L. dispar* 29 and 22 kDa). Definitive assignment awaits further immunological and sequence studies, but Western transfer results already indicate that at least the 75 kDa *L. dispar* heat shock protein is immunologically related to the human 70 kDa heat shock protein. Western transfer results also indicate that proteins similar to the inducible forms of the heat shock protein are expressed in diapausing *L. dispar* at 4°C, and that heat or cold shock further increases the rate of expression. Verification of this conclusion awaits the cloning of mRNA. In *L. dispar* synthesis of most normal (non-heat shock) proteins is halted during heat shock, as it is in other species (Walker et al., 1986; Fittinghoff and Riddiford, 1988; Joplin and Denlinger, 1990). The temperature at which the heat shock proteins are first expressed varies with the insect tested: around 35°C in *L. dispar* and *D. melanogaster* (Lindquist,
1980), 40°C in *S. crassipalpis* (Joplin and Denlinger, 1990) and 41°C in *S. bullata* (Bultman, 1986). As found in *D. melanogaster* (DiDomenico et al., 1982), normal protein synthesis does not begin immediately when *L. dispar* is removed from heat shock conditions but is delayed 1-3 h.

Diapausing pharate larvae of the gypsy moth are quite tolerant of low temperature: Madrid and Steward (1981) reported only 25% mortality after a 7 day exposure to -20°C. By comparison, the cold exposure necessary to induce the heat shock protein response (2 h at -10°C) is rather mild. The response was dose-dependent at low temperature: 2 h at -10°C apparently induced only the 75 kDa heat shock protein while 2 h at -20°C induced the 90, 75 and 29 kDa heat shock proteins. Dose-dependent induction of heat shock proteins in response to cold shock was also reported for *S. crassipalpis* (Joplin et al., 1990).

Normal protein synthesis is suppressed when heat shock proteins are synthesized in response to high temperature, but following cold shock in *L. dispar* expression of heat shock proteins coincides with synthesis of normal proteins, as it does in *D. melanogaster* (Burton et al., 1988) and *S. crassipalpis* (Joplin et al., 1990). Normal protein synthesis resumes during recovery from heat shock in *Drosophila* cell cultures as levels of the 70 kDa heat shock protein decrease, and a possible regulatory link between the suppression of normal protein synthesis and the synthesis of the 70 kDa heat shock protein has been suggested (DiDomanico et al., 1982; Beckmann et al., 1990). But, our finding and others (Burton et al., 1988; Joplin et al, 1990) showing a maximum expression of heat shock proteins concurrent with normal protein
synthesis during recovery from cold shock indicates that these two events are not necessarily linked. Does the gypsy moth larva see the shift from -20 to 4 to 25°C as a heat shock? This is a possibility, but if so, synthesis of normal proteins presumably would have been suspended as in the heat shock response.

Though many physiological responses are suppressed or eliminated during diapause, diapausing insects clearly maintain the ability to respond to certain forms of environmental stress. Basal metabolism can be temporarily elevated in response to wound healing (Harvey and Williams, 1961; Denlinger et al., 1972), low humidity may be countered by uptake of atmospheric water vapor (Yoder and Denlinger, 1990), introduction of foreign bacteria can stimulate synthesis of new proteins (Boman et al., 1981), and thermal stress can elicit the synthesis of heat shock proteins in diapausing flesh fly pupae (Joplin, unpublished data) and in diapausing first instar pharate larvae of the gypsy moth. Diapausing insects are not environmentally cloistered but are subjected to numerous environmental stresses. The rapid heat shock protein response seen in diapausing L. dispar indicates that these diapausing insects maintain the physiological mechanisms needed to avert injury incurred by sudden thermal stress. And, the fact that our results show expression of heat shock proteins at 4°C implies a possible role for these proteins in providing protection to the diapausing stage during winter conditions.
Figure 11. Heat shock protein expression in diapausing pharate first instar larvae of *L. dispar*. Pharate larvae dissected from their chorions were exposed to the indicated temperatures (°C) for 1 h and labeled for an additional hour at the same temperature. Proteins were separated on a 10% SDS-PAGE gel. Molecular weights of heat shock proteins are expressed in kilodaltons.
Figure 12. Heat shock protein expression in diapausing pharate larvae of L. dispar during recovery from a 2 h exposure to 40°C. Pharate larvae were dissected from their chorions at 0(A), 2(B), 4(C) or 6(D) h after heat shock and labeled for 1 h at 25°C. (E) 25°C control, (F) heat shock control, labeled at 40°C. Proteins were separated on a 10% SDS-PAGE gel. Molecular weights of heat shock proteins are expressed in kilodaltons.
Figure 13. Heat shock protein expression in diapausing pharate first instar larvae of *L. dispar* during recovery at 25°C from cold shock. Following a 2 h exposure at either -10 (A, B, C, D) or -20°C (E, F, G, H) pharate larvae were dissected from their chorions at 0 (A, E), 2 (B, F), 4 (C, G) or 6 (D, H) h after cold exposure and labeled for 1 h at 25°C. (I) 25°C nonshocked control, (J) 40°C heat shock control. Proteins were separated on a 10% SDS-PAGE gel. Molecular weights of heat shock proteins are expressed in kilodaltons.
FIGURE 13
Figure 14. Heat shock protein expression in diapausing *L. dispar* pharate larvae during recovery at 4°C from a cold shock at -20°C for 24 h. Following a 24 h exposure pharate larvae were dissected from their chorions at 0(A), 24(B), 48(C), 72(D) or 96(E) h after cold shock and labeled for 24 h at 4°C. (F) heat shock control 2 h at 40°C, (G) 4°C nonshocked control, (H) 25°C nonshocked control. Proteins were separated on a 10% SDS-PAGE gel. Molecular weights of heat shock proteins are expressed in kilodaltons.
FIGURE 14
Figure 15. Western transfer of heat shock proteins in diapausing pharate first instar larvae of *L. dispar*. (A) heat shock for 2 h at 40°C, (B) cold shock for 2 h at -20°C, (C) 4°C nonshock control. Nitrocellulose filter was screened with the monoclonal antibody C92F3A-5 which is specific for the inducible form of the human heat shock 70 kDa proteins. Arrows indicate lightly bound heat shock proteins in lane A.
CHAPTER V

71 kDa Diapause-specific heat shock protein in the flesh fly.

Sarcophaga crassipalpis

Abstract Using autoradiography we find evidence that a 73 kDa protein is expressed in brains of diapausing and heat shocked nondiapausing pupae but not in nondiapausing pupae that are not heat shocked. Of the three time points tested during diapause (1, 25 or 55 days after the onset of diapause), expression of this 73 kDa putative heat shock protein is greatest at 25 days. With the use of western transfer and a polyclonal antibody to the human inducible 70 heat shock protein we also provide evidence for a 71 kDa diapause-specific heat shock protein. The 71 kDa protein is not detected on autoradiography, thus implying that the 71 kDa diapause specific heat shock protein is either synthesized before diapause begins or is synthesized at a very low level during diapause.

INTRODUCTION

Heat shock proteins are a group of highly conserved proteins that are induced by various stresses, and are believed to protect against future
stress(es) (reviewed Lindquist, 1986; Lindquist and Craig, 1988). Thus, it is logical to investigate the relationship of the heat shock proteins to diapause, a physiological strategy used by many insects to survive the stress of winter. When diapausing Lymantria dispar pharate larvae (Yocum et al., 1991) are cold shocked they express heat shock proteins at low temperatures likely to be experienced by a diapasting insect during winter. Diapausing pharate larvae of L. dispar express the 75 and 90 kDa heat shock proteins at 4°C for more than 96 h following a 24 h exposure to -20°C (Yocum et al., 1991). Cold shock also induces heat shock proteins in nondiapausing Drosophila melanogaster larvae (Burton et al., 1988) and Sarcophaga crassipalpis pupae (Joplin et al., 1990). Because a mild heat shock that induces heat shock proteins in D. melanogaster larvae protects these larvae against a normally lethal exposure to 0°C (Burton et al., 1988), it follows that heat shock proteins expressed following a cold shock also may serve to protect against future cold stress(es).

Although best known for their expression during or following a stress event, certain heat shock proteins are also expressed under nonstress conditions. D. melanogaster has two forms of the 70 kDa heat shock protein, one induced by stress (inducible) and one expressed only under normal growing conditions (cognate, constitutively expressed) (Ingolia and Craig, 1982). Four heat shock proteins immunologically related to the human 70 kDa heat shock protein family are expressed in diapausing larvae of L. dispar (Yocum et al., 1991). In D. melanogaster and Saccharomyces the small molecular weight heat shock proteins (22-26 kDa) are expressed at certain
developmental stages as well as during stress. *D. melanogaster* expresses the 22, 23 and 26 kDa heat shock proteins during late larval and early pupal stages (Cheney and Shearn, 1983; Sirotkin and Davidson, 1982). The 26 kDa heat shock protein is expressed during stationary phase growth and sporulation in *Saccharomyces* (Kurtz et al., 1986).

Are members of the 70 kDa heat shock protein family expressed as a normal part of the diapause syndrome in insects? The only published study on the expression of heat shock proteins during diapause was done in *L. dispar*, which has an obligatory diapause (Yocum et al., 1991). Therefore, there is no comparable (nondiapausing) developmental stage to determine if the observed expression of heat shock protein is unique to diapause or is a developmentally specific event as seen in *D. melanogaster* (Chenev and Shearn, 1983; Sirotkin and Davidson, 1982). In this study, we compared the expression of heat shock proteins in comparable developmental stages [nondiapausing (head everted) and diapausing pupae] of the flesh fly *S. crassipalpis* to determine if the 70 kDa family of heat shock proteins is expressed as a normal part of the diapause syndrome.

**MATERIALS AND METHODS**

Flies

*S. crassipalpis* was reared in accordance with Denlinger (1972). Diapausng pupae were obtained by maintaining the adult flies at room temperature at a light:dark cycle of 12:12 LD and rearing the larvae and
pupae at 12:12 and 20°C. Nondiapausing pupae were obtained by rearing all developmental stages at 15:9 LD and 25°C. After head eversion pupae either continue to develop (nondiapause) or enter diapause. Therefore, we compared protein expression in nondiapausing head everted pupae to diapausing pupae.

Protein labelling, electrophoresis, and autoradiography

Brains from head everted nondiapausing or diapausing pupae were dissected under insect saline (Ephrussi and Beadle, 1936). Five brains were cultured in a 1.5 ml vial with 20 µl methionine-free Grace’s medium (Grace, 1962). Brains were cultured 1 h at 25°C with 1 µl [10 µCi (370 kBq)] Tran 35S-label™ (1259 Ci/m mole, ICN Radiochemicals). Grace’s medium was removed and brains were boiled 5 min in SDS sample buffer (0.06% Tris, 10% glycerol, 2% SDS, 10% β-mercaptoethanol, bromophenol blue), then stored at -70°C. Heat shocked brains were cultured at 43°C for 1 h then labelled for 1 additional h at 43°C. Electrophoresis and autoradiography were carried out as described previously with equal counts per sample on a 10% polyacrylamide gel (Yocum et al., 1991).

Western transfer

Brains from 5 head everted nondiapausing or diapausing pupae were homogenized in 30µl of 5% sucrose. Heat shock samples of nondiapausing
pupae were first labelled as above before being homogenized. Western transfer and protein blot was carried out as described previously (Yocum et al., 1991), except that the samples were separated on 10% denaturing polyacrylamide gel and the gel was then soaked in TGS buffer (25mM Tris-base, 192mM glycine, 10% SDS) for 2 h at 4°C (Jackson and Thompson, 1984) before the Western transfer. Polyclonal antibody against the inducible human 70 heat shock protein (gift, Dr. R. Tanguay) was used to screen the filter.

RESULTS

The polyclonal antibody against the inducible human 70 heat shock protein binds to a 72 kDa heat shock protein in heat shocked and nonshocked nondiapausing pupae and in nonshocked diapausing pupae (Fig. 1). It has yet to be determined if the 72 kDa heat shock protein detected in the nonshocked nondiapausing pupae and the diapausing pupae is the constitutively expressed or the inducible form of the 72 kDa heat shock protein. Molecular weight of the 72 kDa was determined by autoradiography of the labelled heat shocked samples (data not shown). In addition, a 71 kDa heat shock protein unique to diapausing pupae was detected by this antibody (Fig. 1 B).

A 72 kDa protein is expressed in nondiapausing, diapausing, and heat shocked nondiapausing pupae (Fig. 2). A 73 kDa heat shock protein in heat shocked nondiapausing pupae is detected on the autoradiograph (Fig 2. C)
and a protein with similar molecular weight is also present in the nonshocked diapausing pupae (Fig 2. B). The 73 and 72 kDa heat shock proteins commonly appear on autoradiography as a single band on a 10% polyacrylamide gel (Fig 2. D); an 8% gel would better separate these bands. The expression of the 72 kDa heat shock protein and the 73 kDa protein in diapausing pupae varies over the course of diapause development. Of the three ages tested (1, 25 or 55 days in diapause), expression was greatest 25 days into diapause (Fig. 2. E, F, G). The 71 kDa heat shock protein detected on the Western transfer is not detectable by autoradiography (Fig 2. B).

**DISCUSSION**

Members of the 70 kDa heat shock protein family can be divided into two distinct groups. The constitutively expressed heat shock proteins are only expressed under normal conditions, whereas the inducible forms are expressed under stress (Ingolia and Craig, 1982). Constitutively expressed heat shock proteins are not readily detectable with the pulse-labelling method used in this study (Joplin and Denlinger, 1990). The diapause-specific 71 kDa heat shock protein is likely to be a member of the constitutively expressed heat shock protein family because it is only detectable on the Western blot and not on the autoradiograph. Heat shocked and nonshocked nondiapausing and nonshocked diapausing pupae of *S. crassipalpis* express a 72 kDa protein. Heat shocked nondiapausing and shocked diapausing pupae also expressed a 73 kDa protein. Verification that
the 72 and 73 kDa proteins expressed in the diapausing pupae are indeed heat shock proteins awaits immunoprecipitation or two-dimensional Western blot studies.

Yocum et al. (1991) demonstrated that the human 70 kDa-related heat shock proteins are normally expressed in diapausing *L. dispar* and that cold shock further increases the expression of these proteins. *S. crassipalpis* diapausing pupae (Joplin, personal communication) and nondiapausing *D. melanogaster* larvae also express heat shock proteins following a cold shock (Burton et al., 1988). However, the expression of heat shock proteins after recovery from a cold shock is not universal among diapausing insects. Diapausing larvae of the goldenrod gall fly, *Eurosta solidaginis*, express heat shock proteins in response to heat shock (30-35°C), but not to a cold shock (Joplin and Denlinger, 1992). Both insect species that have been shown to express heat shock proteins in response to a cold shock are freeze intolerant, whereas *E. solidaginis* is freeze tolerant. More species need to be examined to determine if expression of heat shock proteins in response to a cold shock is restricted to freeze intolerant insects.

Heat shock proteins may function in diapausing insects as an overwintering mechanism protecting the insect from low temperatures. Evidence for this function is strong: 1) Diapausing *L. dispar* and *S. crassipalpis* express heat shock proteins in response to low temperature shocks (Yocum et al., 1991; Joplin personal communication). 2) There is a unique diapause-specific heat shock protein (71 kDa) in *S. crassipalpis*. 3) *L. dispar* exercises differential control over which heat shock protein is
expressed following a cold shock. After a cold shock at -20°C and during recovery at 4°C L. dispar expresses only 2 (90 and 75 kDa) of its 7 known heat shock proteins (Yocum et al., 1991). This implies that L. dispar expresses only those heat shock proteins required to survive a particular stress. 4) L. dispar expresses the 90 and 75 kDa proteins at 4°C for more than 96 h after a 24 exposure to -20°C (Yocum et al., 1991). Such a time course of expression would be expected if the heat shock proteins were in fact fortifying the insect against another possible dip to subzero temperatures -- a situation not at all unlikely in the natural habitat of L. dispar. 5) Heat shock proteins have been shown to remain physiologically active at low temperature (10°C) (Ellwood and Craig, 1984). 6) A mild heat shock that induces the heat shock proteins protects D. melanogaster larvae against a normally lethal exposure to 0°C (Burton et al., 1988). The induction by cold shock, expression and physiological activity at low temperatures, and provision of protection against normally lethal low temperatures argue strongly that the heat shock proteins are indeed contributing to overwinter survival. The final determination of this role in diapausing insects awaits studies correlating the expression of heat shock proteins with an increase in cold tolerance.

The possible involvement of heat shock proteins in overwintering insects opens up a new area of research in insect cold tolerance. S. crassipalpis, with its facultative diapause, diapause specific heat shock protein, and variation in expression of possible heat shock proteins (72 and
73 kDa) over the course of diapause development, may serve as an excellent model to investigate the role of these proteins in diapausing insects.
Figure 16. Western transfer of heat shock proteins in diapausing pupae of *S. crassipalpis*. Heat shocked nondiapause (A); diapause (B); nonshocked nondiapause (C). Proteins were separated on a 10% SDS-PAGE gel. Nitrocellulose filter screened with a polyclonal antibody against the human 70 kDa heat shock protein family.
Figure 17. Protein expression in diapausing and nondiapausing *S. crassipalpis* pupae. Nonshocked nondiapausing (A); diapausing (B, E, F, G); heat shocked nondiapausing 2 h at 43°C (C, D). Diapausing pupae were approximately 1 (E), 25 (F) or 55(G) days into diapause. Proteins were separated on a 10% SDS-PAGE gel. Molecular weight expressed in kilodaltons.
The ability of an organism to survive a thermal stress depends upon the stress history of that individual. Red eye pharate adults of the flesh fly Saracophaga crassipalpis exposed to 40°C for 30 - 240 min can survive exposure to subsequent supraoptimal temperatures that are normally lethal (thermotolerance). Thermotolerance induced by a 120 min exposure to 40°C decays slowly over the next 72 h. Thermotolerance appears to be independent of the continuous synthesis or persistence of the heat shock proteins. This result thus challenges the idea that heat shock proteins are the exclusive means for generating thermotolerance.

In contrast to thermotolerance, certain supraoptimal temperature treatments decrease (thermosensitize) rather than increase an organism's ability to survive a future thermal stress. A 60 min exposure to 45°C decreases, not increases, S. crassipalpis tolerance to a second thermal stress. While control flies experience little to no mortality when held at 35°C for 24 or 48 h, the thermosensitized flies suffer significant mortality at this same temperature. Thermosensitivity, like thermotolerance, decays slowly over the 72 h following its induction. Brains from thermosensitized flies expressed the 72 kDa heat shock protein and normal protein expression was inhibited when cultured at 43°C. This implies that development of
thermosensitivity is not associated with the loss of capacity to express the 72 kDa heat shock protein.

Diapausing pharate larvae of the gypsy moth *Lymantria dispar* express 7 heat shock proteins (90, 75, 73, 60, 42, 29 and 22 kDa) when exposed to elevated temperatures (37-41°C). A subset of these stress proteins is also expressed following a cold shock (-10 to -20°C). The 90 and 75 kDa heat shock proteins are expressed for more than 96 h at 4°C following a 24 h exposure to -20°C. These results clearly demonstrate that heat shock proteins are induced by and expressed at temperatures normally experienced by *L. dispar* while overwintering.

In *S. crassipalpis*, I report evidence that a member of the heat shock 70 family of proteins is specific for diapause and is present even under non-stressed conditions. A 73 kDa putative heat shock protein was expressed in non-stressed diapausing and heat shocked nondiapausing pupae. The expression of the 73 kDa protein was not constant throughout diapause: of the ages tested (1, 25 or 55 days after the onset of diapause) maximum expression occurred on day 25. These results demonstrate that expression of heat shock proteins is a normal component of the diapause syndrome.

The induction of heat shock proteins by nonlethal cold shock, expression at low temperatures, and presence of a diapause specific heat shock protein argue forcefully that heat shock proteins are a part of the overwintering mechanism of diapausing insects. My data demonstrate that both *L. dispar* and *S. crassipalpis* would be excellent model systems to
investigate the ecological significance of heat shock proteins in overwintering insects.
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