PROTEIN CHAPERONES AND WINTER COLD HARDINESS IN INSECTS: HEAT SHOCK PROTEINS AND GLUCOSE REGULATED PROTEINS IN FREEZE-TOLERANT AND FREEZE-AVOIDING SPECIES

By

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ABSTRACT

Two species of insects with different overwintering strategies were chosen as model organisms to assess the role of chaperone proteins in insect cold hardiness. *Epiblema scudderiana*, a freeze-avoiding gall moth, and *Eurosta solidaginis*, a freeze-tolerant gall fly, were used to compare and contrast the two overwintering strategies; both species overwinter in the diapause stage as last instar larvae inside galls on the stems of goldenrod plants. The research reported in this thesis analyzed changes in molecular chaperone levels over the winter season in insects sampled from outdoors as well as in larvae given laboratory exposures to subzero temperatures and hypoxia. Multiple proteins in the heat shock (Hsp10, Hsp40, Hsp60, Hsp70, Hsp110) and glucose-regulated (Grp75, Grp78, Grp94, Grp170) families of chaperones were assessed as well as other chaperones (TCP-1, αA- and αB-crystallins). The heat shock transcription factor (HSF-1) was also analyzed. Despite an overall suppression of transcription and translation during winter diapause, selected chaperone proteins were differentially expressed in the larvae in response to low temperatures, freeze/thaw, or hypoxia exposures. Most of the chaperones were up-regulated directly or indirectly by subzero temperature and/or anoxia exposures to enhance their actions in the stabilization, repair or elimination of misfolded or denatured proteins. These chaperones function in cytoplasmic, endoplasmic reticulum and mitochondrial compartments, indicating that multiple endogenous pathways are engaged in maintaining and/or restoring cellular homeostasis in the larvae in response to these insults. Elevated HSF-1 levels indicated that Hsp enhancement in the larvae was due to up-regulation of the genes involved.

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List of Abbreviations

AA	amino acid
ADP	adenosine diphosphate
ATF	activating transcription factor
ATP	adenosine triphosphate
cDNA	complementary deoxyribonucleic acid
Cpn	chaperonin
CREB	cAMP response element binding protein
DEPC	diethylpyrocarbonate
DnaJ	*Escherichia coli* Hsp40 protein
DnaK	*Escherichia coli* Hsp70 homologue
DTT	dithiolthreitol
ECL	enhanced chemiluminescence
ETC	electron transport chain
EDTA	ethylenediamine tetraacetic acid
eIF2α	eukaryotic initiation factor 2 α subunit
ER	endoplasmic reticulum
ERAD	ER associated degradation
ERSE	ER stress-response element
GRP	glucose regulated protein
HSF	heat shock factor
HSE	heat shock element
HSP	heat shock protein

IRE1	Inositol requiring kinase 1
kDa	kilodalton
MAPK	mitogen-activated protein kinase
mRNA	messenger RNA
OGP	oxygen regulated protein
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PERK	PKR-like endoplasmic reticulum kinase
PVDF	polyvinylidine fluoride
RACE	rapid amplification of cDNA ends
ROS	reactive oxygen species
rRNA	ribosomal RNA
RT	room temperature (~21°C)
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
Tb	body temperature
TCP	Tailless complex polypeptide
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
UCP	uncoupling protein
UPR	unfolded protein response
UPRE	unfolded protein response element

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CHAPTER 1

Introduction

1.1 Dealing with the cold winter

At high latitudes or altitudes, ectothermic animals have developed, over evolutionary time, strategies to cope with the cold temperatures that they must endure. Locally, over a typical Ottawa winter, temperatures stay below 0°C for many weeks at a time and temperatures of -30°C or lower can occur for a few days each winter. There are multiple strategies that animals can use to deal with winter. Some species have chosen to avoid the cold altogether. Monarch butterflies and many species of birds migrate south for hundreds of kilometres to escape the cold Northern winter. Other species remain in the general area, but select hibernacula that protect them from the extreme cold. Thus, animals that winter under water (e.g. aquatic larvae and amphibians) or burrow underground can gain enough insulation so that they need only endure temperatures in the 0 to 5°C range. More fascinating, are those cold-hardy species that have evolved to stand and face the cold with little or no thermal buffering. These organisms have developed behavioural, physiological, and biochemical adaptations that allow survival at temperatures below the normal freezing point of their body fluids. Two fundamental strategies of overwintering are generally recognized: freeze tolerance and freeze avoidance (Storey and Storey, 1988).

1.1.1 Freeze tolerance

Freeze tolerance is used by a wide range of animals to survive the winter cold, and is very common in the insect world (Storey and Storey, 1992; Duman et al., 1991). Freezing, however, is not a process which is left to chance, for highly supercooled tissue is likely to suffer ice nucleation in both extracellular and intracellular loci (Mazur, 1984)

and intracellular ice formation is invariably lethal in Nature. Instead, freeze tolerant animals use a variety of adaptations to initiate, control, and limit ice formation to extracellular spaces only.

To do this, freeze tolerant animals use ice nucleators to trigger extracellular ice formation at relatively high subzero temperatures where the rate of ice formation can also be relatively slow. Sometimes nucleators are non-specific (e.g. bacteria on the skin) but often insect synthesize specific ice nucleator proteins (INPs). These typically initiate ice formation at temperatures above -10°C, even in the presence of high concentrations of colligative solutes (Duman et al., 1991). As the growing extracellular ice crystals are made up of pure water, the remaining extracellular liquid surrounding them become more concentrated, which draws water out of cells and, as a consequence, the freezing point of the cytoplasm decreases by colligative action. In essence, a freeze tolerant animal system then consists of frozen extracellular spaces surrounding unfrozen, shrunken cells.

Water loss to the extracellular ice can damage cells for beyond a critical minimal volume, compression stress on membrane bilayer structure becomes too great and the membrane collapses irreversibly into a gel state. To limit the amount of cell shrinkage, animals synthesize large amounts of low molecular weight colligative cryoprotectants (e.g. glycerol, glucose) that limit the amount water that can freeze by elevating both intracellular and extracellular solute concentrations. In addition, other cryoprotectants are produced (e.g. trehalose, proline) that act as membrane stabilizers to further reduce membrane damage by stabilizing the bilayer structure of membranes.

1.1.2 Freeze avoidance

Freeze avoiding animals use a different strategy to survive the cold of winter by preventing their bodies from freezing by deep supercooling – remaining liquid below the equilibrium freezing point of body fluids (Storey and Storey, 1988). This form of cold hardiness is commonly used by small invertebrates including many kinds of insects and other arthropods (e.g. spiders, tick, etc.) (Storey and Storey, 1989). Perfectly pure water, if cooled slowly, can remain liquid down to–40°C when spontaneous freezing occurs at the homogeneous nucleation temperature. However, this is a metastable state, and as soon as nucleator (a structure that resembles an ice crystal or surface which promotes the rearrangement of water molecules into an ice-like configuration) is added to the supercooled liquid, spontaneous ice formation will occur at any temperature below 0°C. Because of such heterogeneous nucleation, water in nature typically freezes at 0°C. However, many animals exploit the supercooling strategy to endure subzero temperatures without freezing by using strategies that stabilize the supercooled state. The strategy is effective but also risky for if they come in contact with nucleators, lethal freezing can occur instantaneously.

Several strategies can help freeze avoiding insects to avoid spontaneous nucleation of internal body fluids and/or prevent inoculation due to contact with external ice. These include: (A) choosing a hibernaculum which limits physical exposure to nucleators (e.g. environmental ice) and/or forming a protective barrier such as a silk cocoon to prevent contact with external ice (Bale, 1987), (B) eliminating internal nucleators from the body, notably by emptying the contents of the gut prior to cold exposure, (C) the synthesis of thermal hysteresis (or antifreeze) proteins, (D) production of high concentrations of low molecular weight carbohydrates that depress the freezing

and supercooling points of body fluids by colligative action, and (E) controlled dehydration of the body which effectively increases the osmotic concentration of remaining body fluids (Storey and Storey, 1989; Zachariassen, 1985).

The freezing point of water is a colligative property and is altered by the concentration of osmotically active molecules in solution. The addition of osmotically active solutes decreases both the freezing point (FP) and the supercooling point (SCP: the temperature below the FP at which spontaneous ice nucleation occurs). However, high concentrations of solutes must be added for significant depression of the FP and the SCP (Hochachka and Somero, 1984). Therefore, for molecules to be useful as colligative cryoprotectants they must be of low molecular weight, highly water soluble, and nontoxic to cells. The most common cryoprotectants in nature are the polyhydroxy alcohols and some sugars (Hochachka and Somero, 1984) and, of these, glycerol is by far the most widely used. Other commonly found colligative cryoprotectants in insects are sorbitol, mannitol, inositol, ribitol, erythritol, and ethylene glycol (Gehrken, 1984; Storey and Storey, 1989); the latter is the polyol that we use in car radiators, and possesses some well known toxic effects on higher animals. The haemolymph sugars, trehalose and to a lesser extent glucose and fructose, may also be elevated in winter, normally with a concurrent increase in polyols (Duman et al., 1991). Multicomponent systems consisting of a number of polyols and/or sugars are also known in some insect species. Colligative antifreezes are usually synthesized from the breakdown of glycogen stores in insect fat body (Storey and Storey, 1988).

Thermal hysteresis proteins (THPs; also known as antifreeze proteins), which decrease the FP of water in a non-colligative manner, have been identified in over 26

species of insects (Duman et al., 1991) These proteins depress the freezing point, but not the melting point, of water and hence produce a thermal hysteresis between FP and MP. These proteins can prevent the freezing of an aqueous solution in the presence of a seed ice crystal by as much as 90°C below the freezing point of the solution. THPs are believed to function by adsorption onto the small seed ice crystals through hydrogen-bonding, inhibiting further growth (Raymond et al., 1989). Via the use of THPs in combination with high polyol concentrations, many temperate zone insects can supercool to -40°C whereas polar species may supercool as low as -70°C.

Many ectothermic species have evolved special characteristics to make them cold hardy. The group of animals with the largest number of species in this category are the insects, including both freeze tolerant and freeze avoiding species. For the present work presented in this thesis, two species of insects with different overwintering strategies have been chosen as model organisms. *Epiblema scudderiana*, a freeze-avoiding gall moth, and *Eurosta solidaginis*, a freeze-tolerant gall fly, were used to compare and contrast the two types of overwintering strategies employed by insects at a molecular and biochemical level. Both species overwinter as last instar larvae inside galls on the stems of goldenrod plants, genus *Solidago.* The galls can frequently project above the snowline and consequently the insects inside them are subjected to temperatures as low as –30°C in the Ottawa area (Rickards *et al.*, 1987).

1.2 The damage caused by low temperatures

There are two types of injuries that can be caused by low temperatures in

nonhardy cells: chilling injury and freezing injury. The deleterious effects of chilling injury arise from the altered physical properties of molecules and rate processes (Q_{10} effect) at low temperatures. This includes improper orientation, mobility and fluidity of membrane lipids and membrane-bound proteins (Franks, 1985; Hochachka and Somero, 1984), which affects diffusion and transport across membranes as well as other metabolic processes involving membrane biochemistry. Low temperatures also influence various classes of weak bonds to alter individual protein structures as well as interactions between proteins in large cascades or enzymatic pathways (Hazel, 1984; Locker and Hernendez, 2004). Low temperatures also disrupt cellular ATP generation and the balance between net ATP production and consumption. Because of ATP limitation in the cold (also perhaps due to direct temperature effects), critical cellular processes can be disrupted. These problems involving energetics are the basis of many hypothermia injuries in nonhardy species including man. These same problems of disrupted cellular energetics also arise as a result of other stresses including oxygen limitation (hypoxia or anoxia). Therefore, the summed result of subzero temperature exposure on non cold-hardy cells is an overall disruption of many metabolic processes, in particular cellular energetics, which can become lethal if exposure is for an extended period.

The main injuries during freezing are the potential for physical damage from the growth of ice crystals within confined spaces (e.g. the lumen of capillaries) and the resulting freeze concentration of remaining body fluids when free water is incorporated into ice. Freeze concentration is frequently lethal in freeze-intolerant species because it results in osmotic shock to cells, severe dehydration so that cell volume shrinks below a critical limit, and elevated solute levels that can affect membranes or protein structure

and function (Franks, 1985; Mazur, 1984). In addition, extracellular ice formation cuts off oxygen supply to cells so that cellular energetics are disrupted. Intracellular ice formation appears to be lethal to all organisms – although a couple of reports dispute this – whereas with adaptation, large numbers of organisms can endure ice formation in extracellular spaces.

1.3 Survival during the winter

Cells respond to various environmental or physiological stresses with a transient arrest of the cell cycle that is accompanied by widespread changes in macromolecular synthesis, degradation, trafficking, overall cellular metabolism and signal transduction pathways to cope with the stressful conditions until more favorable conditions are encountered (Feige et al. 1996). Under these conditions, a variety of molecular and biochemical adaptations are activated, overall metabolic rate is strongly suppressed, and some specific genes/proteins are induced. Much of the work on cell stress responses has been done on insects.

1.3.1 Hypometabolism

Insects typically respond to the unfavourable growth conditions of winter (cold temperature, lack of food) with metabolic rate depression. Many species enter a programmed state of developmental arrest called diapause that may last several months, whereas others experience quiescence. Given limited endogenous fuel reserves (particularly for species that will not eat again in their life cycle, such as the two species studied here), insects must rebalance ATP-utilizing and ATP-producing reactions to

ensure energy conservation for long-term survival. Oxygen consumption, largely used as an indicator of overall metabolic rate, typically falls to less than 5% of the normal rate during diapause (Danks, 1987; Storey and Storey, 1987).

1.3.2 Stress proteins conferring cold-hardiness

A common response to stresses of many kinds, is the shut-down of most protein synthesis and the activation instead of the production of a suite of proteins that are commonly called shock proteins, most of which function as chaperones. It is well established that diverse stresses including heat, cold, hemodynamics, mutant proteins, UV radiation, heavy metals, and oxidative stress can produce multiple injuries to cells that can ultimately affect protein structure/function and one consequence is the destabilization of protein conformation, leading to unfolding and aggregation of existing proteins or the malfolding of nascent proteins during their synthesis (Goldberg 2003). The job of protein chaperones is to correct these problems.

What is a protein chaperone? A protein chaperone is a protein that binds to and stabilizes an otherwise unstable conformer of another protein and, by controlled binding and release of the substrate protein, facilitates its correct fate *in vivo*, be it folding, oligomeric assembly, transport to a particular subcellular compartment, or controlled switching between active/inactive conformations (Hendrick and Hartl 1993). Many types of chaperones are constitutive in cells and guide the normal production and subcellular localization of proteins. However, under environmental and chemical stresses, cells can experience a huge increase in unfolded and malfolded cells. In these cases, cells typically respond to such stresses with a rapid shutdown of the synthesis of most proteins and with

a dramatic transient increase in the synthesis of several families of small proteins. Studies have identified these stress-induced proteins as heat shock proteins (Hsps), glucose regulated proteins (Grps) and transcription factors (TFs), which are mainly located in the cytoplasm/mitochondria, endoplasmic reticulum (ER), and nuclei of cells, respectively. Hsps and Grps are protein chaperones whereas TFs are the regulators of the transcription of these protein chaperones.

1.3.2.1 Heat shock proteins and heat shock factor

Heat shock proteins (Hsps), also called stress proteins, are so-called because they were first discovered in salivary glands and other tissues of *Drosophila melanogaster* that were given transient sublethal heat shock (Tb elevated by ~5°C). Hsps are a group of proteins that are present in all cells in all life forms. They are induced when a cell undergoes various types of environmental stress like heat, cold and oxygen deprivation. To date, in eukaryotic cells, six families of Hsps have been identified based on their molecular weight including HSP110, HSP90, HSP70, HSP60, HSP40, and the small Hsps (Katschinski, 2004). HSP families have both constitutive and stress-inducible members whose primary functions are to interact with naive and denatured proteins to prevent the aggregation of unfolded proteins, facilitate the folding of naive proteins and the re-folding of misfolded proteins, and aid intracellular protein trafficking (Gething *et al.*, 1992; Becker and Craig, 1994). These functions make contributions to the maintenance of cellular homeostasis and promote cell survival in response to stressful conditions.

The transcription of *hsp* genes is regulated by heat shock factors (HSFs), which

are constitutively synthesized cellular transcription factors. In human tissues, three HSFs have been discovered (HSF1, HSF2, HSF4) but only HSF1 is involved primarily in the stress response. The HSF is negatively regulated since it can bind to DNA only under stress conditions (Chang *et al.,* 2001).

1.3.2.2 Glucose regulated proteins (Grps)

Another set of stress proteins were first identified in 1977 as responses to glucose starvation of cells and are called glucose regulated proteins (GRPs) (Lee, 1992; Little *et al.,* 1992; Lee, 2001). Accumulation of misfolded protein causes ER stress and leads to activation of a complex signal transduction cascade known as the unfolded protein response (UPR) (Lee, 1992; Harding, 2002). One result of the UPR is expression of Grps. Like HSPs, Grps also are molecular chaperones. They play important roles in protein folding and secretion, and can confer protection against cell death by regulating signal transduction and protein translation under a variety of stress conditions (Gazit et al., 1995; Lee, 1987; Yoshida et al., 1998).

1.3.2.3 Other molecular chaperones

Other proteins including TCP-1 and the crystallin family also operate as protein chaperones, showing binding, holding and folding capacity towards proteins but, unlike Hsps and Grps, they actions are not yet clearly understood. These molecular chaperones were not considered to be stress proteins until recently. TCP-1 is essential for the correct folding of cytoskeletal proteins including actin and tubulin (Spiess *et al.*, 2004).

Crystallins cannot directly renature denatured proteins, but, they can help other proteins chaperones to finish this job (Narberhaus et al., 2002).

All molecular chaperones, alone or in cooperation with "partner" chaperones (e.g. other ER chaperones and mitochondrial chaperones), actively participate in the rearrangement of cellular processes including cytoprotection, correct folding and assembly of proteins, transport of proteins to specific intracellular locations, and protein degradation. These cytoprotective stress proteins decrease or neutralize the deleterious effects of various stresses by preventing inappropriate intra- and intermolecular interactions of polypeptide chains that can lead to protein denaturation. Accumulating evidence has indicated that the main functions of molecular chaperones in cells are to maintain polypeptide chains in an appropriate conformation suitable for translocation across organelle membranes, actively trigger stress-regulated mechanisms and other cytoprotective stress proteins; and to assist in degrading toxic metabolites by promoting ubiquitination and proteasome lysis (Chiang *et al.,* 1989; Knowleton, 1995).

1.4 Purpose and Hypothesis

Stress response mechanisms such as the production of protein chaperones (Hsps and Grps), as well as HSF and other TF mediated regulation of these chaperones has been extensively explored in mammalian systems and in various other animals. However, relatively little is known to date about whether these stress response mechanisms contribute to animal response to environmental cold temperatures and whether they are part of the natural cold-hardening that prepares ectothermic animals for winter survival.

The responses of these proteins in freeze tolerant and freeze avoiding insects are unknown. To our knowledge, most studies regarding HSP and GRP induction have generally been confined to model organisms grown under standard laboratory conditions and, therefore, have failed to examine the real-life situations regarding thermal stress in natural populations. This present research aimed to provide an understanding of the potential role of these mechanisms in the winter survival of freeze tolerant (*E. solidaginis*) and freeze avoiding (*E. scudderiana*) goldenrod gall insects by identifying and analyzing the expression of various molecular chaperones under both outdoor natural field conditions and indoor laboratory stress conditions.

The research is based on the proposal that stressors that cause protein unfolding, misfolding or aggregation will trigger a stress response that leads to the induction of gene transcription for chaperone proteins with the capacity to stabilize and re-fold proteins, thereby re-establishing the balance between protein synthesis, assembly and degradation. For cold-hardy insects, adaptations and modification of molecular and biochemical machinery that support winter survival should predictably include changes to the protein make-up of cells to deal with a number of stresses and problems. These could include protein changes that allow cells to maintain coordinated functions under low temperature or freezing/thawing exposures, or that may deal with potential anoxia/hypoxia stresses (e.g. freezing cuts of oxygen supplies to tissues). Furthermore, because protein synthesis is strongly suppressed during diapause for 3-4 months in each species, mechanisms may be needed to help stabilize cellular proteins over the long term. These actions may be jobs for Hsps and Grps in these two insects. In this thesis I have tested two hypotheses:

1. Low temperature or freeze/thaw induces stress proteins (Hsps and Grps) and their expression is regulated by specific TFs.

2. The roles and expression patterns of Hsps and Grps differ in freeze-avoiding (*E. scudderiana*) and freeze-tolerant (*E. solidaginis*) species.

In Chapter 3, the effects of low temperature and anoxia on Hsp protein expression is examined in both *E. solidaginis* and *E. scudderiana* larvae. To identify the natural roles of Hsps in these two insects, both indoor laboratory and outdoor field experiments were used. These included: (a) winter profiles of outdoor larvae sampled at multiple times from September to April, (b) laboratory exposure of larvae to subzero temperatures (including those that cause freezing in *E. solidaginis*), and (c) anoxia exposure. Expression profiles of HSF were also quantified in these two insects to help understand the regulation of Hsp expression.

In Chapter 4, the responses of Grps are quantified in both *E. solidaginis* and *E. scudderiana* under the same experimental conditions as in Chapter 3. The possible roles of other molecular chaperones (TCP-1, crystallins) in winter cold hardiness are also evaluated in both species.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animal treatments

Galls containing last instar larvae of *Eurosta solidaginis* or *Epiblema scudderiana* were collected in early September from the stems of goldenrod plants in fields around the Ottawa area. Galls were divided randomly into two main experimental groups: (a) animals that were left outdoors over the winter and (b) animals that were brought into the laboratory for indoor experiments.

For outdoor experiments, *E. solidaginis* and *E. scudderiana* galls were stored in cloth bags hung on a fence where they experienced variable, diurnal ambient winter temperatures. Galls were sampled over the 1999-2000 winter in the second week of each month from September to April. (For outdoor winter temperature profiles in Ottawa for that winter, see http://www.climate.weatheroffice.ec.gc.ca/climateData/monthlydata_e.html?) At each sampling time, galls were brought into the laboratory at ~9:00 a.m. and briefly stored in an incubator set to the current outdoor temperature. Galls were opened as soon as possible and larvae were flash-frozen in liquid nitrogen and then stored at –80°C.

Other September-collected galls were brought into the laboratory and acclimated at a constant 15°C in large incubators (constant darkness) for a period of two weeks. After this time, some galls were opened and insects were sampled as above. The remaining galls were divided into two experimental groups and given either cold stress and anoxic stress. Animals destined to part of the cold stress group were left in their galls until immediately before they were flash-frozen in liquid nitrogen. Insects to be part of a anoxia timecourse were removed from their galls and stored in petri dishes containing a

piece of moist filter-paper in the bottom to prevent desiccation.

Two different protocols for cold-exposure were employed in different species. For *E. scudderiana* this was a three-staged decease in temperature – from 15°C down to 4°C and then maintained for 7 days followed by a second acute decrease from 4°C to -4°C for 7 days and finally an acute decrease from -4°C to -20°C for 7 more days. Insects were removed and flash-frozen after 1 and 7 days exposure to each temperature. For *E. solidaginis,* the exposure periods were only 24 hours at each temperature with sampling at the end of the 24 hours. They started at 15°C (control) and then temperature was acutely lowered to +3°C for 24 hours, then down to –16°C for another 24 hours (a freezing stress for this species), and then back to +3°C for a final 24 hours of thawed recovery. Both species were also subjected to quick drops from 15°C immediately down to -4°C and animals were flash frozen after 1 and 4 hours at subzero temperature. RNA isolated from these two stress points was eventually pooled to make up 1 experimental sample.

Both *E. solidaginis* and *E. scudderiana* were also given low oxygen exposure. To do this, insects that were acclimated to 15°C were removed from their galls and placed on petri dishes. The petri dishes were then sealed in a plastic container with two valves on the top, one to introduce nitrogen gas and one to vent the gas. The container was flushed with 100% nitrogen gas for 20 minutes and then both valves were sealed and the container was replaced at the appropriate incubation temperature. Two anoxia exposure timecourses were generated, for *E. solidaginis,* the larvae acclimated to 15°C for two

weeks, and for *E. scudderiana,* the larvae acclimated to 4°C (also for 2 weeks). Animals were sampled at set time intervals by rapidly opening the container, removing the insects and dropping them into liquid nitrogen.

2.2 Sample preparation

2.2.1 Isolation of total protein

Whole frozen larvae were weighed and then quickly homogenized in a buffer that was designed to inhibit the activities of endogenous protein phosphatases and kinases: 20 mM HEPES, 400 mM NaCl, 20% v:v glycerol, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM NaF, 10 mM β-glycerophosphate, and 1 mM Na_3VO_4. The buffer also contained the appropriate protease inhibitors to protect the sample from degradation: 1 μM each of phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, and benzamidine. The homogenate was centrifuged at 10,000 x g at 4°C for 10 minutes and the supernatant was transferred to a fresh tube and stored in a freezer at -80°C.

2.2.2 Isolation of nuclear extracts

Samples of frozen larvae (0.1 g) were weighed and added to 500 μl of homogenization buffer (1:1 w:v) containing 10 mM HEPES, pH 7.9, 10 mM KCl, 10 mM EDTA, 10 mM dithiothreitol (DTT), and 10 μl of protease inhibitor cocktail (1 mM PMSF, 0.015% w/v aprotimin, 10 μM leupeptin, 1 μM pepstatin, 1 mM NaF). Samples were disrupted with 20 piston strokes of a teflon-glass homogenizer and then centrifuged at 10,000 x g for 10 min at 4°C. The supernatant representing the cytoplasmic extract was

removed into a sterile Eppendorf tube and frozen at -80°C. The pellet was resuspended in extraction buffer containing 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% v/v glycerol, 0.1 mM DTT and 1.5 µl of protease inhibitor cocktail. This resuspension was held on ice and shaken for 1 h followed by centrifuging at 10,000 x g for 10 min at 4°C. The second supernatant was saved as the nuclear extract and stored at -80°C.

2.2.3 Measurement of protein concentration

Total protein concentrations in each sample were determined by the Coomassie blue dye-binding method (Bradford, 1976) using the Bio-Rad prepared reagent with bovine serum albumin as the standard. The reagent was diluted 5-fold with distilled water and protein extracts of tissues were diluted 1:20 or 1:40 (v:v). For assay, 10 µl aliquots of diluted protein samples were added to 190 µl of dye reagent in microplate wells followed by mixing and 10 min incubation at room temperature (RT). Absorbance at 595 nm was measured and protein concentration was determined from a pre-programmed standard curve using BioLinx 2.0 software.

2.3 Western blotting

Western blot analysis is a technique that allows the study of antigen-antibody interactions. Proteins are separated by electrophoresis on SDS-polyacrylamide gels and transferred onto nitrocellulose or PVDF membranes (Banins, 2002). The membrane is blocked with nonfat milk, then probed first with antibody specific to the protein of interest and then a second antibody, an anti-immunoglobulin linked to an enzyme

(horseradish peroxidase; HRP). Detection is performed using ECL reagents.

2.3.1 SDS-polyacrylamide gel electrophoresis

Protein samples were mixed 1:1 (v:v) with 2x SDS-PAGE sample buffer containing 100 mM Tris-HCl (pH 6.8), 4% SDS (w/v), 20% glycerol (v/v), 5% β-mercaptoethanol (v/v) and 0.2% bromophenol blue (w/v) and boiled for 5 min. On any given gel, the same amount of protein was loaded into each well. Generally, aliquots containing 10-20 μg protein were loaded into each well of 6% to 15% acrylamide gels (acrylamide:bis-acrylamide 29.2:0.8; w/w) using the discontinuous buffer system of Laemmli (1970). The acrylamide percentage varied depending on the estimated subunit molecular weight of the target protein to be analyzed. Electrophoresis was carried out on a Bio-Rad mini-gel apparatus at 180 V for 40-50 min at RT with 1x running buffer containing 3.03 g Tris base, 14.4 g glycine and 1 g SDS per liter, pH ~8.3. This procedure is also called one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) to distinguish it from two-dimensional gel electrophoresis (2D-PAGE).

2.3.2 Two dimensional gel electrophoresis

First dimension isoelectrofocusing gels were prepared in capillary gels; gel composition was 9.2 M urea, 4% acrylamide, 1.6% pH 5-8 ampholytes, 0.4% pH 3.5-10 ampholytes, 0.01% ammonium persulfate and 0.1% TEMED. The gels were pre-electrophoresed at 200 V for 10 min, 300 V for 15 min and 400 V for 15 min with 100 mM NaOH as the upper chamber solution and 10 mM H_3PO_4 as the lower chamber

solution using the Modular Mini-Protean II System (Bio-Rad). Protein samples were mixed 1:1 v:v with first dimension sample buffer containing 9.5 M urea, 5% v/v 2-mercaptoethanol, 1.6% pH 4-6 ampholytes and 0.4% pH 3.5-10 ampholytes. After incubation for 10-15 min at 21°C, aliquots of 15-20 µg protein were loaded into the capillary sample reservoirs and overlaid with first dimension overlay buffer (9 M urea, 0.8% pH 5-8 ampholytes, 0.2% pH 3.5-10 ampholytes, 0.005% w:v bromophenol blue). Gels were run at 500 v for 10 min and then 750 v for 3 h or 300 v overnight.

After isoelectrofocusing, gels were removed from the capillary tubes and incubated in SDS equilibration buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% w:v SDS, 5% 2-mercaptoethanol, 10% v/v glycerol and 0.00125% w:v bromophenol blue) for 10 min at 21°C. Capillary gels was then positioned onto a 10% SDS-PAGE gel and overlaid with overlay buffer containing 1% v/v agarose, 62.5 mM Tris-HCl, pH 6.8, 2.3% w/v SDS, 5% v/v 2-mercaptoethanol, 10% v/v glycerol and 0.00125% w/v bromophenol blue. SDS-PAGE was run at 180V for 30-45 min at 21°C in 1x running buffer (as for 1D gels).

2.3.3 Protein transfer

After electrophoresis, 1-D or 2-D PAGE were immersed in transfer buffer (25 mM Tris pH 8.5, 192 mM glycine and 20% v/v methanol) 10 minutes and then assembled with a polyvinylidene difluoride (PVDF) membrane (Immobilon-P Transfer Membrane, Millipore Corp. Bedford, MA) in a transfer stack. The electrotransfer was carried out in the blotting apparatus at 180 mA for 1.5 h at 4° C within transfer buffer.

2.3.4 Gel stain

To confirm that all the proteins transferred off to PVDF, we can strain the gel with either silver or Coomassie blue dye. For Coomassie staining, gels were stained for 1 h in 0.25% w/v Coomassie brilliant blue R, 50% v/v methanol, and 10% v/v acetic acid. The gels were destained in 25% v/v methanol, and 10% v/v acetic acid until background levels were acceptable. Silver staining was performed as described by Blum *et al.* (1987). First, gels were fixed by incubating in 25% v/v methanol and 10% v/v acetic acid for 30 min. The gels were washed for 3 x 5 min in distilled water and incubated in fresh sodium thiosulphate solution (0.2 g/L) for 2 min. The gels were then rinsed with distilled water (3 x 30 sec) and incubated in silver nitrate (0.1g/mL) for 20 min. Then the gels were washed with distilled water (3 x 1 min) followed by incubation in developer solution: 12 g sodium carbonate and 0.1 ml 37% v/v formaldehyde in 100 ml total volume. Color development was stopped by a 5 min incubation in 5% v/v acetic acid and the gels were stored in distilled water.

2.3.5 Antibody incubations

After the transfer of protein onto the PVDF membranes, the membranes were blocked with 2.5% non-fat milk in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 10~60 min at RT. The membrane was then rinsed with TBST and incubated with primary antibody on a shaking platform for either for 2~3 h at RT or overnight at 4 °C. Blots were then washed twice with TBST for 10 min and incubated

with secondary antibody at the appropriate dilution in TBST according to manufacturer's instructions for 1~2 h at RT. Blots were then washed 2 times for 20 min with TBST. Additional or longer washes were used to reduce background in some cases.

2.3.6 Stripping and reprobing

As appropriate, a single blot on PVDF membranes was stripped and re-probed with different antibodies up to three times. For stripping, membranes were placed in Falcon tubes with stripping buffer (62.5 mM Tris-HCl, pH 6.7, 2% w:v SDS, 100 mM 2-mercaptoethanol) and rotated at 50°C for 30 minutes. Membranes were then washed with distilled water, then TBST, and then re-probed with another antibody as described above beginning with blocking the membrane with nonfat milk.

2.3.7 Detection and quantification

Blots were developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer's protocol. Immunoblotted bands were visualized under a ChemiGenius (Syngene, MD, USA). After all immunoblotting was complete, the PVDF membranes were stained for protein using Coomassie blue (see 2.3.3) and bands were also visualized also by ChemiGenius.

Intensities of immunoblot bands and selected Coomassie-stained bands were quantified using the GeneTools program (Syngene, MD, USA). On each blot, Coomassie-stained bands that did not change in intensity between control and experimental states were chosen and quantified. Immunoblot band intensities in each lane

were then normalized against the corresponding intensity of the Coomassie blue stained bands in that lane to correct for any minor variations in sample loading.

2.3.8 Statistical analysis

Statistical significance of the differences between normalized experimental band intensities (n=4) and normalized control values (n=4) was evaluated using the Student's t-test. The probability level of significance was fixed at $P<0.05$.

2.4 RT-PCR

2.4.1 RNA isolation and purity assessment

Gloves were worn at all times while handling RNA. To avoid RNAase contamination, all solutions and materials used during RNA handling were treated overnight with 1% (v/v) diethylpyrocarbonate (DEPC) at RT and later autoclaved.

Frozen larvae were homogenized 1:4 w/v in Trizol™ reagent (Gibco-BRL, Bethesda, MD) followed by a 5 min incubation at room temperature. Next, chloroform (0.2ml/ml of Trizol) was added and samples were shaken for 15 sec and incubated at room temperature for an additional 2-3 min. Samples were centrifuged at 12,000 x g for 15 min at 4°C. This step separated the sample into three layers: a bottom layer containing protein, an interface containing DNA and an upper aqueous layer containing RNA. The upper aqueous layer was transferred to an RNase-free 1.5 mL Eppendorf tube where it was combined with an equal volume of isopropanol and incubated for 10 min at room temperature to allow RNA precipitation. Subsequent centrifugation at 12,000 x g for 10

min at 4°C pelleted the RNA. The supernatant was removed and the pellet was washed with 1 mL of 70% ethanol. A final spin at 7500 x g for 5 min recovered the washed RNA. The ethanol was discarded and the RNA was re-suspended in DEPC-treated water and stored at –80°C until further use.

To determine the quantity and quality of the isolated RNA, samples were assayed spectrophotometrically at 260nm and 280nm. At 260nm, an optical density of 1.0 is equivalent to an RNA concentration of 40 μg/mL. The ratio of A_{260}/A_{280} was measured to determine RNA quality. A value of 1.6 or higher indicated that the RNA samples were relatively pure, with few contaminating proteins or phenols.

Denaturing agarose gels were also used to assess the quality (size) of the total RNA (Sambrook *et al.*, 1989). A 1.2% w:v gel was prepared by melting 2.4 g of solid agarose (electrophoresis grade, GIBCO BRL) in 169 ml DEPC-treated water, followed by cooling to 60°C before the addition of 20 ml 10× MOPS buffer (0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0) and 11 ml of 37% v/v formaldehyde. The mixture was then poured into a 20 x 35 cm gel mold to set. To prepare RNA samples, 15 or 20 μg of total RNA from each sample was made up to 10-20 μL with sterile DEPC-treated water, heated at 65°C for 3-5 min, and then chilled on ice. To this was added 19 μL of a stock mixture (made of 12.5 μl formamide, 2.5 μL 10× MOPS buffer and 4 μL 37% v:v formaldehyde) and 2.5 μL of 10× RNA loading buffer (0.9 mL of 50% v:v glycerol, 2 μL of 0.5 M EDTA, pH 8.0, 50 μL of 0.25% bromophenol blue, 50 μL of 0.25% w:v xylene cyanol FF). After a quick spin, the RNA sample was loaded onto the agarose gel that had

been pre-run at 90 V (4.5 V/cm) with 1× MOPS as the electrophoresis buffer for 10-15 min. A 7 μg aliquot of RNA ladder (1 μg/μl, 0.24-9.5 kb, GIBCO BRL) was applied as a size marker in one lane. The gel was run at 70 V for 1 h until the blue dye migrated 5 cm away from the gel wells. The gel was photographed under a UV light transilluminator or using the SynGene. High quality of RNA was assessed by the presence of sharp and distinct 28S and 18S rRNA bands.

2.4.2 First strand synthesis

For cDNA first strand synthesis, 15 μg of total RNA in 10 μl of DEPC-treated water was placed in a 0.5ml tube to which 1 μl 200 ng/μl of Oligo dT primer (5'-TTTTTTTTTTTTTTTTTTTV-3'; V=A or G or C) (Sigma Genosys) was added, and the tube was put in a 65°C water bath for 5 min. Then the following components were added: 2 μl DTT, 4 μl of 5X sample buffer, 1 μl of 10 μM dNTPs, and 1 μl reverse transcriptase (Superscript, GIBCO). The mixture was incubated at 40°C for 40 min to reverse transcript the mRNA to cDNA. Then a series of cDNA dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were made and stored at 4°C.

2.4.3 Gene specific primer design and synthesis

Degenerate primers were designed using DNAMAN 4.11 (Lynnon Biosoft) and Primer Designer software (Scientific and Educational Software). The sequence for the gene of interest was retrieved from NCBI Genbank for several species and then aligned and compared using DNAMAN. Highly conserved regions were identified and these were then used to design primers for the gene of interest using Primer Designer based on

the variables including primer length, melting temperature, G/C content and 3'-end sequence. The designed primers were then synthesized by Sigma Genosys and were aliquoted into 300 pmol/µl stocks in DEPC-treated water and stored at -20°C.

2.4.4 mRNA hybrid by RT-PCR

Reverse transcription and polymerase chain reaction amplification (RT-PCR) was used to amplify cDNA and quantify the relative levels of selected mRNA species in tissue extracts of control versus experimental larvae. A 5 µl aliquot of first strand cDNA product was mixed with 45 µl DEPC-treated water to produce a 10^{-1} dilution; this procedure was then repeated to produce serial dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. The PCR reaction mixture contained 15.5 µl distilled water, 2.5 µl 10X PCR buffer, 1.25 µl 50 mM $MgCl_2$, 0.5 µl 10 mM dNTP mix, 0.125 µl Taq DNA polymerase (5 U/µl, Gibco-BRL) and 1.25 µl primer mixture (1:10 dilution of 300 pmol stock). Each PCR reaction contains 5 µl of serial diluted cDNA and 21.125 µl PCR reaction mixture. After mixing well and a quick spin, PCR reactions were performed in the thermocycler with the following steps: (a) 95°C pre-heat for 3 min, (b) 37 cycles of running: 95°C for 30 sec (denaturation), 55°C for 30 sec (annealing) and 72°C for 30 sec (elongation), (c) final elongation at 72°C for 7 min, and (d) hold at 22°C. The total running time was 136 min.

PCR products were mixed with DNA loading buffer containing 40% w:v sucrose, 0.25% w:v bromophenol blue and 0.25% w:v xylene cyanol FF. Samples were loaded onto 1% agarose TAE gels (1% w/v agarose, 40 mM Tris-acetate, 1 mM EDTA, pH 8.3, 0.0005% v:v EtBr) with 1% TAE running buffer (40 mM Tris-acetate, 1 mM EDTA,

pH8.3) and separated by electrophoresis at 130 V for the 22-well gel tray or at 90 V for the 16-well gel tray. DNA was visualized using the UV transilluminator function of the ChemiGenius and band intensities were analyzed with the GeneTools program.

CHAPTER 3

Heat Shock Proteins and Heat Shock Factor

3.1 Introduction

A common feature of the response to environmental stresses is the induction of a group of proteins which were first termed heat shock proteins (HSPs) due to their initial discovery in cells exposed to hyperthermia (elevated temperatures). Although the precise functions of most HSPs are not yet fully understood, it is clear that they play essential roles in protecting cells against the damaging effects of stressful conditions. HSPs act as molecular chaperones, alone or in cooperation with "partner" chaperones (e.g. other ER chaperones and mitochondrial chaperones), and they actively participate in the rearrangement of cellular processes including cytoprotection, correct folding and assembly of proteins, transport of proteins to specific intracellular locations, and protein degradation. Multiple families of these proteins has been identified based on their molecular weights including HSP110, HSP90, HSP70, HSP60, HSP40, HSP27 and HSP10 (Katschinski, 2004).

The mechanism of HSP induction in eukaryotes is relatively well understood (see Figure. 3.1) and involves binding of an activated heart shock factor (HSF) to a responsive heat shock element (HSE) in the genome, which initiates the transcription of heat shock genes and subsequently the translation of the proteins (Sorger, 1991; Morimoto *et al.*, 1997; Chang *et al.,* 2001.) Multiple HSFs (HSF-1, HSF-2, HSP-3, HSF-4) exist in higher eukaryotes whereas yeast and *Drosophila* have only a single HSF-1 involved primarily in the stress response (Wu *et al,* 1995; Santoro *et al.* 2000*).*

In eukaryotes, HSP proteins are found in the cytoplasm, nucleus and mitochondria. In addition to heat shock, several other stresses can trigger their synthesis including hypoxia, ischemia, inflammation, reactive oxygen species and exposure to

toxins including heavy metals and endotoxins. The physiological roles and the protective potential of HSPs under pathophysiological circumstances have been identified clearly in animal models. However, relatively little is known to date about the possible roles of HSPs in organismal responses to cold. Previous studies in our lab have shown that HSPs are induced and play a role in supporting natural freeze tolerance in organs of the wood frog, *Rana sylvatica*, during freezing (Li, 2004) and in ground squirrel tissues at the near 0°C body temperatures encountered during hibernation (Yan, 2005). Therefore, I hypothesized that HSPs would be up-regulated and play a role in the subzero survival of goldenrod gall fly larvae, *E. solidaginis* and *E. scudderiana*, over the winter months.

3.2 Materials and methods

3.2.1 Antibodies

All HSPs and HSF-1 antibodies were purchased from Stressgen Biotechnologies Corp. Secondary antibodies were purchased from Cell Signaling Technology Inc. The dilutions of stock antibodies used in the studies are listed in Table 3.1.

3.2.2 Western blotting

To separate, identify, and detect the expression levels of HSP proteins and HSF-1 in *E. solidaginis* and *E. scudderiana* larvae, Western blotting was employed (see Chapter 2).Antibody were tested for specificity by the use of antigene peptides and/or 2D gels (the data were not shown). The gels and the conditions used are summarized in Table 3.1.

3.2.3 Statistical analyses

Band intensities were quantified and plotted using the GeneTools and Excel programs (see Chapter 2). Statistical significance of differences between normalized experimental band intensities (n=4) and normalized control values (n=4) was evaluated using the Student's t-test.

3.3 Results

Mammalian antibodies raised against HSP110, HSP70, HSP60, HSP40, HSP10 as well as HSF-1 were used to evaluate the responses of heat shock proteins in cold-hardy insects under three types of experimental conditions: (a) winter profiles of outdoor larvae sampled at multiple times from September to April, (b) laboratory acclimation of larvae to low temperatures, and (c) anoxia exposure. Except for HSP10 in *E. solidaginis* which was not detectable using the rabbit anti-HSP10 antibody, all other HSPs as well as HSF-1 were detected in both species with the mammalian antibodies used (Table 3.1). The HSP70 antibody used in all of these studies detected the inducible HSP70 which is also known as HSP72. Initial trials were also done with a mammalian antibody for the constitutive HSP70 protein (also called HSc70 or HSP73) but, even after a variety of manipulations of experimental conditions, no bands were detected in either insect species when using this antibody.

3.3.1 HSP 110 in *E. solidaginis*

The commercial polyclonal antibody raised against HSP110 from rabbit cross-reacted with a protein in *E. solidaginis* extracts of the proper size of ~110 kDa. Figure

3.2A shows representative Western blots of HSP110 expression under the different experimental conditions. Figure 3.2B shows the mean levels of HSP110 (± SEM, n=4) in larvae sampled from outdoors over a winter season (from September to April). HSP110 protein expression showed a parabolic trend over time. Compared with the level in September, the relative expression levels of HSP 110 increased in October (1.60 fold higher than the September value, $P<0.05$) and December (1.79 fold, $P<0.05$), stayed high in February (1.69 fold, $P<0.05$) and March (1.40 fold), and finally decreased in April to 0.92 fold (compared with September).

Figure 3.2C shows HSP110 responses in larvae exposed to the following low temperature regimen in the laboratory: (a) controls were larvae acclimated at 15°C, (b) larvae were then shifted from 15 down to 3°C and cold-exposed for 24 hours, (c) larvae were next shifted to –16°C and frozen for 24 hours, and (d) larvae were transferred back to +3°C and were sampled after 24 hours thawed. The relative expression levels of HSP 110 were unchanged when larvae were cold exposed at 3°C or frozen at -16°C, as compared with controls, but HSP110 levels increased significantly ($P<0.05$) by 1.46-fold when the larvae were thawed at 3 °C.

Figure 3.2D shows the effect of anoxia exposure for 4 or 24 hours (at 15°C) on HSP110 expression in *E. solidaginis*. Compared with controls, anoxia exposure led to reduced expression of HSP110; mean levels of the protein were 82% of the control value (but not significantly different) after 4 hours of anoxia and decreased further to 58% of control (significantly different, $P<0.05$) after 24 hours of anoxia.

3.3.2 HSP 70 in *E. solidaginis*

The rabbit anti-HSP70 polyclonal antibody strongly recognized an ~70 kDa band

on blots of *E. solidaginis* proteins. Figure 3.3A shows representative Western blots for inducible HSP70 expression in *E. solidaginis* under three experimental regimes. Figure 3.3B shows the relative expression levels of HSP70 over the course of the winter. Like HSP110, the protein expression pattern showed significant increases in HSP70 content over the midwinter months; levels were 1.20, 1.42, and 1.33 fold higher than control values in December, February, and March, respectively ($P<0.05$). HSP70 content decreased again to control levels in April.

Figure 3.3C shows the changes in HSP70 expression with cooling, freezing and thawing. Compared with controls (15°C, 1 wk), HSP70 levels showed an increasing trend after 3°C exposure for 24 hours, and were significantly elevated by 1.43 fold ($P<0.05$) after freezing at –16°C for 24 hours. Thawing resulted in dramatic decrease in HSP70, falling to 56% of the value in frozen larvae and to 80% of control values ($P<0.05$) after 24 hours back at 3°C.

Figure 3.3D shows the effect of anoxia exposure on HSP70 expression levels in *E. solidaginis*. Compared with controls, the expression of HSP70 was increased after anoxia exposure, the mean levels of the protein were 1.12 fold higher than the control value (but not significantly different) after 4 hours of anoxia and increased further to 1.50 fold to control (significantly different, $P<0.05$) after 24 hours of anoxia.

3.3.3 HSP 60 in *E. solidaginis*

The mouse anti-HSP60 monoclonal antibody recognized a ~60 kD protein on *E. solidaginis* Western blots. Figure 3.4A shows representative Western blots for HSP60 expression under the three experimental regimes. Figure 3.4B shows the relative

expression levels of HSP60 in the larvae over the winter months from September to April. The expression levels of HSP60 changed oppositely compared with the other HSPs (HSP110, HSP70, HSP40, HSP10). The pattern was a concave curve, with a downward trend being indicated first in October and significantly reduced levels of HSP60 in December, February and March; levels were 48, 42 and 51% of September values, respectively ($P<0.05$). HSP60 levels rebounded again between March and April back to control levels.

Figure 3.4C shows the relative expression level of HSP60 in *E. solidaginis* over a cycle of cooling, freezing and thawing. Comparing with control levels, HSP60 levels were unchanged by the treatments.

Figure 3.4D shows that HSP60 expression levels decreased in *E. solidaginis* under anoxic conditions. Compared with controls, a downward trend was noted after 4 hours of anoxia and levels were significantly reduced to 62% of the control value ($P<0.05$) after 24 hours of anoxia.

3.3.4 HSP 40 in *E. solidaginis*

The rabbit anti-HSP40 polyclonal antibody recognized an ~40 kDa protein in *E. solidaginis* extracts. Figure 3.5A shows representative Western blots for HSP40 expression under the various experimental conditions. HSP40 levels increased in the larvae during the winter (Figure 3.5B) with significantly higher levels of HSP40 found in October (1.56 fold, $P<0.05$) and December (1.62 fold, $P<0.05$). Levels were still elevated in February and March (but not significantly different due to high variation), and fell back to control values April.

Figure 3.5C shows the expression of HSP40 over the freeze/thaw exposure. Compared with controls (15°C, 1 wk), HSP40 levels were unchanged by 24 hours of cold exposure at 3°C. However, levels increased significantly (1.50 fold, $P<0.05$) during freezing (–16°C, 24 hours) and then remained high (1.61 fold, $P<0.05$) after thawing at +3°C for 24 hours.

Figure 3.5D shows the changes in HSP40 expression levels in *E. solidaginis* under anoxic conditions. Expression was not significantly different from the control value after either 4 or 24 hours of anoxia exposure.

3.3.5 HSF-1 in *E. solidaginis*

The commercial polyclonal antibody raised against HSF-1 from rabbit cross-reacted with two protein bands in *E. solidaginis*, one at ~85 kDa and one at ~95 kDa. These correspond to the known molecular masses of the inactive and activated forms, respectively, of HSF-1 in other systems. Figure 3.6A shows representative Western blots for HSF-1 expression in the larvae under the different experimental regimes. In almost all cases, the blots showed bands for both the inactive and activated proteins and experimental conditions could alter both the overall expression levels of the two forms and the relative amount of active HSF-1 compared with the inactive form.

Figure 3.6 B shows the relative expression levels of HSF-1 (both inactive and activated forms) in *E. solidaginis* over the winter months. Note that the expression levels of both activated and inactive forms are graphed relative to the September levels of inactive 85 kDa HSF-1. In September and October the amount of activated HSF-1 was significantly higher (by 1.56-2.00 fold) ($P<0.05$) than the corresponding amount of

inactive HSF-1 in the same larvae. However, over the remainder of the time course, the levels of active and inactive HSF-1 were similar at each sampling point. The amount of inactive HSF-1 protein in the larvae showed a concave expression pattern over the winter-time course. The amount inactive HSF-1 began a downward trend in October and was significantly reduced in December and February to 65% and 63% of September values ($P<0.05$), before rising again in March and April (to 84-85% of September values). The expression pattern of activated HSF-1 was similar with minimum levels in December and February that were about one-third of the September levels (significantly different, $P<0.05$). Levels rebounded somewhat (0.51-0.61%) to in March and April but were still significantly lower than September ($P<0.05$).

Figure 3.6C shows the responses of inactive and activated HSF-1 protein expression to freeze/thaw. Cold exposure at 3°C had no effect on HSF-1 expression but freezing at -16°C resulted in a strong increase in the amount of active HSF-1. Levels rose by ~1.85 fold higher ($P<0.05$) compared with the corresponding amount of inactive protein at the same temperature (as opposed to an approximate 1:1 ratio of activated vs. inactive at the other sampling points). The amount of active HSF-1 at -16°C was also 1.46 fold ($P<0.05$) higher than the amount in control larvae (15°C, 1 wk). After thawing (3°C for 24 hours) the amounts of both activated and inactive forms were significantly reduced to 63% ($P<0.05$) and 76% of the corresponding 15°C control values. Activated HSF-1 protein expression also decreased by ~50% as compared with the level in frozen larvae ($P<0.05$).

Figure 3.6 D shows HSF-1 expression in response to anoxic conditions. Levels of active HSF-1 were significantly higher than the corresponding inactive form under all

conditions. Compared with aerobic control larvae, the expression of inactive HSF-1 increased significantly under anoxia, rising to 2.15 and 2.80 fold higher than controls after 4 or 24 hours of anoxia ($P<0.05$). An even greater increase was seen for active HSF-1 expression which increased by 3.26 fold after 4 hours and by 10.25 fold after 24 hours ($P<0.05$).

3.3.6 HSP 110 in *E. scudderiana*

Comparable sets of studies evaluated HSP expression in the freeze-avoiding insect *E. scudderiana* and, as for the freeze tolerant larvae of *E. solidaginis*, these included a profile of protein levels over the winter months as well as a time course of anoxia exposure (1, 4 and 24 h at 4°C). A low temperature acclimation experiment followed a different course and involved acclimation of controls at 15°C, followed by 1 week at 4°C, then 1 day and 1 week at -4°C, and finally 1 week at -20°C. Note that the subzero exposures do not cause freezing to these larvae that use the supercooling strategy of winter survival.

The rabbit anti-HSP110 polyclonal antibody recognized an ~110 kDa protein on blots of *E. scudderiana* extracts. Figure 3.7A shows representative Western blots for the different experimental treatments. Figure 3.7B shows the relative expression of HSP110 in *E. scudderiana* larvae over the winter months. Levels increased over the autumn and were 2.13 fold higher in December than in September animals ($P< 0.05$). HSP110 levels remained high through April (1.90 fold, $P< 0.05$) compared to September.

Figure 3.7C shows that HSP110 expression responded to cold exposure. Chilling at 4°C did not affect HSP110 levels but subzero temperatures stimulated an increase

resulting in 1.4-fold higher levels of HSP110 after 1 week at -4°C and 1.56-fold higher levels after 1 week at -20°C.

Figure 3.7D shows the effect of anoxia on the expression level of HSP110 in *E. scudderiana* larvae. It was clear that anoxia decreased the expression of HSP110; a decreasing trend was seen after 1 and 4 h of anoxia exposure and by 24 h anoxic levels were significantly reduced to just 48% of the control value ($P<0.05$).

3.3.7 HSP 70 in *E. scudderiana*

The rabbit anti-HSP70 antibody recognized an ~70 kDa fragment in *E. scudderiana* extracts. Figure 3.8A shows representative Western blots for inducible HSP70 under the three experimental procedures. Histograms showing mean HSP70 expression in larvae over the winter-time course are shown in Figure 3.8B. Expression was lowest in September but had increased strongly by 2.15 fold in October ($P<0.05$). Levels remained high in December and January, 2.20 and 1.92 fold higher than September values ($P<0.05$), and then began to decline in March (1.60 fold; $P<0.05$). April values were not significantly different than September levels.

Figure 3.8C shows that HSP70 expression levels increased in response to subzero temperature exposure. Levels were unchanged by 4°C exposure or by 24 h at -4°C but after 1 week at -4°C, levels were 1.50 fold higher than controls ($P<0.05$). HSP70 rose even higher to 2.22 fold higher than control values after 1 week at -20°C. A significant difference was also found between 1week at -4°C and -20°C ($P<0.01$).

Figure 3.8D analyzes the effects of anoxia on the expression level of HSP70 in *E. scudderiana* larvae. No significant effects of anoxia exposure were found.

3.3.8 HSP 60 in *E. scudderiana*

The mouse anti-HSP60 antibody recognized an ~60 kDa protein in *E. scudderiana* extracts. Figure 3.9A shows representative Western blots for HSP60 expression under the three experimental procedures. Contrary to the results for the freeze tolerant *E. solidaginis* larvae, Figure 3.9B shows that expression levels of the mitochondrial HSP60 rose dramatically over the winter months. Compared with September values, the levels of HSP60 were 2.06, 2.58, 1.88, and 2.03 fold higher in October, December, January and March (all significantly different, $P<0.05$). In April, however, HSP60 levels had returned to control values.

Figure 3.9C shows the relative expression levels of HSP60 in *E. scudderiana* larvae during laboratory cold exposures. Although not significantly different from 15°C control values, levels of HSP60 appeared to be generally reduced in larvae exposed to 4 or -4°C (73-80% of control values) and HSP60 levels were significantly lower (62% of control) after 1 week -20°C ($P<0.05$).

Figure 3.9D shows the expression levels of HSP60 in the larvae under anoxic conditions. No change in HSP60 expression level was seen after 1 or 4 hour anoxia exposures, but levels had decreased significantly to 75% of control values after 24 hours of anoxia exposure ($P<0.05$).

3.3.9 HSP 40 in *E. scudderiana*

The rabbit anti-HSP40 antibody recognized an ~40 kDa protein in *E. scudderiana* extracts. Figure 3.10A shows representative Western blots for HSP40 expression levels

under the three experimental procedures. Figure 3.10B shows that HSP40 expression was elevated over the midwinter months. The level of HSP40 increased gradually, being 1.6 fold higher in October than in September (not significantly different) and increased further to 2.16 fold higher in December ($P<0.05$) and 2.67 fold higher in January ($P<0.05$). Levels had decreased somewhat by March (1.80 fold higher than September, $P<0.05$) and were 1.63 fold higher than September values in April (but not significantly different).

Figure 3.10C shows the effect of low temperature exposures on HSP40 in *E. scudderiana*. HSP40 levels were not affected by any of the laboratory low temperature acclimations. Figure 3.10D analyzes the effect of anoxia exposure on the expression level of HSP40 in *E. scudderiana* larvae. A decreasing trend was noted but anoxia exposure for as long as 24 hours had no statistically significant effect on HSP40 levels.

3.3.10 HSP 10 in *E. scudderiana*

The HSP10 antibody recognized a 10 kDa protein in *E. scudderiana* extracts. Figure 3.11A shows representative Western blots for HSP10 under the three experimental conditions used. HSP10 expression levels were largely unchanged over the winter months (Figure 3.11B). Only in December was a significant change seen; levels were 1.40 fold higher than in September ($P<0.05$).

Figure 3.11C shows HSP10 expression levels in *E. scudderiana* over the course of laboratory cold exposures to 4, -4 and -20°C. Levels of HSP10 in the larvae were not significantly changed by cold exposure. Figure 3.11 D shows the effects of anoxia exposure on expression levels of HSP10 in *E. scudderiana* larvae. Short term anoxia had

no effect but HSP10 levels rose by 1.44 fold after 24 hours of anoxia exposure ($P<0.05$).

3.3.11 HSF-1 in *E. scudderiana*

The polyclonal antibody raised against HSF-1 from rabbit cross-reacted with proteins of ~85 kDa and ~95 kDa on SDS-PAGE immunoblots of *E. scudderiana* extracts, corresponding to the molecular masses of the inactive and activated forms of HSF-1, respectively. Figure 3.12A shows representative Western blots for the three experimental treatment groups. Almost all of the blots showed bands for both inactive and activated forms.

Figure 3.12B shows the changes in HSF-1 expression levels (both inactive and activated forms) in the larvae over the winter months. All data are expressed relative to the level of inactive 85 kDa HSF-1 in September larvae. Overall, the level of activated HSF-1 was always higher than the amount of inactive HSF-1 (maximally 3-fold higher in October) and these amounts were significantly different in all months except April. Levels of inactive HSF-1 rose during the winter reaching 2.07 and 2.38 fold higher than September values in December and January ($P<0.05$). Levels were reduced in March but dramatically increased again in April (3.02 fold over September values, $P<0.05$). Activated HSF-1 protein expression showed a similar pattern. The levels increased strongly in October (by 2.58 fold higher than September values, $P<0.05$) and remained high in December and January (3.22 and 2.67 fold, $P<0.05$). The content of activated HSF-1 dropped March but increased again in April (2.64 fold over September values, $P<0.05$).

Figure 3.12C shows HSF-1 expression levels in *E. scudderiana* during laboratory

cold exposures. The amount of inactive HSF-1 did not change under any condition. However, levels of activated HSF-1 were significantly reduced after 4°C exposure for 1 week (to 69% of the control value, $P<0.05$). Levels of activated HSF-1 increased again during -4°C exposure and were strongly increased by 1.78 fold ($P<0.05$) at the coldest temperature (-20°C for 1 week).

Figure 3.12 D shows the effects of anoxia exposure on HSF-1 expression levels in *E. scudderiana.* Levels of activated HSF-1 were all much lower than the amounts of the inactive form under all experimental conditions (both control and anoxia). Compared with aerobic controls, the expression of inactive HSF-1 decreased significantly after 4 h or 24 h of anoxia; levels fell to 76% and 55% of control values ($P<0.05$). However, opposite results were found for activated HSF-1 expression; levels gradually increased after 4 and 24 hours of anoxia to 1.32 and 1.86 fold higher than controls ($P<0.05$).

3.4 Discussion

Hsp110 was one of the earliest HSPs described in mammalian cells and has been examined in numerous studies (Subjeck *et al.*,1982; Landry *et al.*,1982; Welch *et al.*, 1983). Many studies have shown that HSP110 and its family members have plentiful expression levels in different cell lines and tissues and play significant roles in both stress responses and in the normal functioning of non-stressed cells (Hatayama *et al.*, 1998; Kaneko *et al.*, 1997; Kojima *et al*, 1996; Muller *et al.*, 1996). The role of the HSP110 family in cellular physiology is not well understood. Oh *et al.* (1997) have shown that HSP110 confers thermal tolerance when over-expressed in cells and that it can prevent aggregation of denatured proteins in vitro. Similar results have been obtained by Brodsky

(1999), who reported that yeast HSP110 prevents thermal aggregation of luciferase and holds it in a folding competent state. Recent biochemical studies suggest that HSP110 chaperones may be involved in the regulation of HSP70 ATP hydrolysis and that HSP70 may have some stimulatory activity on HSP110 ATPase as well (Yamagishi *et al.* 2004; Steel *et al.* 2004). These studies indicate that HSP110 works in concert with other chaperones (i.e. HSP70) and has a very important role in protein refolding.

In our studies, HSP110 was responsive in both *E. solidaginis* and *E. scudderiana* to all experimental conditions tested (outdoor winter timecourse, laboratory experimental cold exposure, and anoxia stress). This shows that HSP110 is responsive to environmental stresses that are naturally encountered by both species and suggests that HSP110 has a role to play in the adaptive response to cold and anoxic stresses. HSP110 levels were elevated in both insects over midwinter months, particularly in December, suggesting that HSP110 has a natural role to play in the winter survival of both insect species at cold temperatures. In the indoor cold stress experiments, similar results were found for *E. scudderiana*; HSP110 levels rose significantly with both -4 and -20°C exposure. However, in *E. solidaginis,* HSP110 levels were not affected by experimental freezing (-16°C, 24 h) but increased during the thawing period (24 h back at 3°C after -16°C exposure). This different response between the two species to subzero temperature exposure may be due to their freeze tolerant versus freeze avoiding strategies of cold hardiness. *E. scudderiana* supercools and remains liquid down to about -40°C. As such, protein synthesis can still occur at -4 or -20°C and so the insects can enhance their HSPs levels as the weather grows colder outdoors. However, *E. solidaginis* larvae freeze below about -8°C and freezing induces anoxia and places tissues under an energy-

stressed state. ATP-expensive processes such as protein synthesis are typically suppressed in the frozen state but when the animals thaw and oxygen supply is restored, the insects can rapidly initiate protein synthesis responses to the freezing stress. Under normal circumstances outdoors, *E. solidaginis* larvae would freeze and thaw many times over the winter. It is possible that overnight freezing exposures in the early autumn are responsible for triggering the up-regulation of heat shock proteins (note that active HSF-1 is strongly increased by -16°C exposure; Figure 3.6C) but that the major synthesis might occur when larvae thaw again during the day. In both species HSP110 levels decreased under anoxic conditions. This may suggest that HSP110 is not needed to aid cell survival under anoxic conditions or it is possible that the protein is normally degraded quite quickly in cells but cannot be replaced easily under anoxic conditions. It is interesting that activated HSF-1 responded very strongly to anoxia (Figure 3.6D), as it also did to freezing so it is possible that HSP110 levels might rise quickly during the recovery after anoxia, as they did during recovery after freezing.

HSP70, also known as inducible HSP70 or Hsp72, is a member of the HSP70 family which contains a number of highly-related protein isoforms ranging in size from 66 kDa to 78 kDa. These proteins are the best-studied of all HSPs and they include both cognate members that are found within major intracellular compartments and highly inducible isoforms which appear to be predominantly cytoplasmic or nuclear in distribution. Under unstressed conditions Hsp70 proteins can stabilize unfolded nascent precursor peptides (Beckmann *et al.*, 1990; Hartl and Martin, 1992). Hsp70 has also been found to associate with cytoskeletal proteins (Tsang, 1993) and under stress conditions, Hsp70 translocates into the nucleus, particularly to the nucleolus (Welch *et al*, 1984). It is

well-known that inducible HSP70 is synthesized under multiple stress conditions in cells. Expression of HSP70 can significantly reduce nuclear protein aggregation and accelerate refolding of luciferase after heat shock (Nollen *et al.*, 1999; Stege *et al.*, 1994). HSP70 contains a peptide-binding site and an enzymatic catalytic site. The assisted protein folding activity, or chaperone activity, of HSP70 protein is dependent on a close interaction between the substrate binding domain and the ATPase domain. The binding of ATP triggers a critical conformational change leading to the release of the bound substrate protein (Fink, 1999).

It is believed that HSP70, as a protein chaperone in mammals, is in very low levels in unstressed conditions but is highly induced by various stresses (Snoeckx *et al.*, 2001). However, the situation in the two insect species studied here was different with HSP70 protein levels expressed strongly in all unstressed controls. This high expression of HSP70 in these two insect larvae suggests that HSP70 may also play an important role in the larval stage of these two insects and may have constitutive expression.

HSP70 levels were elevated in both *E. solidaginis* and *E. scudderiana* over the winter months outdoors, which suggests that HSP 70 has an important role in adaptation to environmental cold stress. Analysis of HSP70 protein expression under laboratory cold exposures showed that freezing of *E. solidaginis* for 24 hours at –16°C or supercooling of *E. scudderiana* at -4°C or -20°C also elevated HSP70 protein. This indicated that protein expression was directly responsive to cold temperatures. The present data agree with other reports (Goto *et al.*, 1998; Li *et al.*, 1999; Martinez *et al.*, 2001; Sonna *et al.*, 2002; Sejerkilde *et al.* 2003), that showed that low temperatures can induce an increase in HSP70 in some species. This is further supported by the fact that HSP70 levels decreased

in *E. solidaginis* when the larvae were thawed/warmed (returned from -16°C to +3°C for 24h). Since HSP70 is the most plentiful HSP in normal cells, this observation may have important implications for HSP function at low temperatures in these two insects.

Several authors have reported that HSP70 protein or mRNA is overexpressed under anoxia stress (Dwyer *et al.*, 1989; Iwaki *et al.*, 1993; Drummoun *et al.*, 1987; Ma *et al.*, 1997; Scott *et al.*, 2003; Prentice *et al.*, 2004). In those studies, most animals responded rapidly to anoxia and up-regulated HSP70 protein or mRNA expression both during and/or after anoxia (recovery). Our data show that in *E. solidaginis* the levels of HSP70 increased significantly after 24 h under anoxic conditions. This also correlated with the increased levels of both inactive and activated HSF-1 during anoxic exposure, particularly after 24 h anoxic stress (see Figure 3.6D). Our results indicate that HSP70 may play a protective role against degradation of proteins caused by anoxia in *E. solidaginis*. Interesting, we found that HSP 70 levels were stable in *E. scudderiana* under anoxic conditions. The difference between these results and those of other studies could be due to species differences (e.g. Dwyer and Iwaki used rat cells, Drummoun and Ma studied *Drosophila*, and Scott and Prentice investigated turtles) as well as time course differences; furthermore, we did not analyze the response by HSP70 during aerobic recovery after anoxia in *E. scudderiana*.

HSP40 is another protein chaperone that is inducible by various environmental stresses (Ohtsuka *et al.* 1990; Kaneko *et al.* 1995). Studies have revealed that HSP40 is localized mainly in the cytoplasm under normal growth temperatures and translocates into the nuclei and nucleoli upon heat shock (Hattori *et al.* 1993; Ohtsuka *et al.*1993; Yamane *et al.* 1995). This is very similar to the pattern of response by HSP70 and

suggested that these two proteins act together in protecting cell functions from heat shock. Indeed, HSP40 is now known to work with HSP70 to repair denatured proteins (Suzuki *et al.* 1999; Ohtsuka and Hata 2000). HSP40 stimulates the low basal ATP hydrolysis activity of its partner HSP70, and in doing so, enhances HSP70 substrate binding activity and hence their chaperone activity (Cheetham and Caplan 1998; Yamagishi *et al*, 2000; Yaohui *et al.*, 1999; Suh *et al.*, 1999). Meanwhile, HSP40 preferentially binds hydrophobic polypeptides (Rudiger *et al.* 2001) and can prevent the aggregation of some unfolded proteins (Langer *et al.* 1992; Cyr *et al.* 1995). In addition, Hsp40 promotes the multimerization of Hsc70 (King *et al.*, 1999).

The present data show that HSP40 levels are elevated in both insect species over the winter months in the field. The pattern of HSP40 expression also follows that of HSP70 quite well, suggesting that the two may be coordinately up-regulated to deal with winter conditions. In *E. solidaginis*, both HSP40 and HSP70 were also elevated during -16°C exposure of the larvae in the laboratory. However, in *E. scudderiana*, HSP40 was unchanged with cold exposure whereas HSP70 increased. Neither species showed a change in HSP40 content under anoxia stress and this correlated with a similar lack of change in HSP70 in *E. scudderiana* but HSP70 rose during anoxia in the freeze tolerant *E. solidaginis*.

HSP60, also called chaperonin (Cpn 60), is a ring-shaped oligomeric protein complex which binds nonnative proteins within a large cavity. In eukaryotes HSP60 is constitutively expressed and highly abundant; it is synthesized in the cytoplasm and then transported into the mitochondria. Its functions are dependent on HSP10 (also called Cpn 10), which binds to HSP60 and regulates its substrate binding and ATPase activity

(Agsteribbe *et al.*, 1993; Cheng *et al.,* 1989). HSP60 is associated with the mitochondrial matrix and participates in the folding and assembly of proteins that are transported into the mitochondria (Cheng *et al.,* 1989; Itoh. *et al.*, 1995; Brosnan *et al.,* 1996; Xanthoudakis *et al.*, 1999). A major role of HSP10 is to assist the HSP60-mediated folding of newly imported precursor proteins inside the mitochondrial matrix (Hohfel *et al.*, 1994). HSP60 alone or with HSP10 was reported to protect substrate proteins from thermally-induced aggregation (Martin *et al.*, 1992). It has been shown that the role of HSP10 in the folding cycle is to regulate the rate of ATP hydrolysis by HSP60 (Dubaquie *et al.*, 1997). However, Burston (1995) reported that even in the absence of HSP10 only HSP60 at a time hydrolyzes ATP. Recently, it was shown that HSP60 and its co-chaperone HSP10 may also function in the regulation of procaspase-3 activation in mitochondria and hence participate in apoptotic signaling pathways (Samali *et al.*, 1999).

Both HSP60 and HSP10 were found in the freeze-avoiding *E. scudderiana* larvae under all the stress conditions. The data show that the HSP60 response to "cold" stress had different patterns in the field and in the laboratory. HSP60 expression levels were significantly increased over the winter months in the outdoor population but protein levels decreased somewhat under indoor laboratory cold acclimation conditions, particularly in supercooled larvae at -20°C (1 week). Similar results were seen for HSP10 in *E. scudderiana.* Levels had increased by 1.4 fold in December in outdoor animals but were unaffected by laboratory cold acclimation. These data for both HSP60 and HSP10 in *E. scudderiana* larvae suggest that levels of these mitochondrial chaperon proteins are probably not responding to a low temperature signal when they accumulate over the winter. Other possible triggers could include internal hormonal signals or environmental

signals such as photoperiod. The inducibility of HSP60 expression during the winter months was higher than that of HSP10; HSP60 levels rose by 2.5 fold whereas HSP10 rose only 1.4 fold. This difference probably reflects the known 2:1 stoichiometry arrangement of the HSP60/HSP10 chaperonin complex *in vivo* (Xu and Sibler 1998). The coordinated increase in both of these proteins also suggests the involvement of HSP10 in the HSP60-dependent stabililization of proteins over the winter months. Interestingly, HSP10 was up-regulated after 24 h anoxia exposure in *E. scudderiana* whereas the opposite was true of HSP 60. These results imply that HSP10 may not always function with HS60 and it alone may have an enhanced role to play in the protection of mitochondrial proteins under anoxic conditions.

In *E. solidaginis,* only HSP60 was detected, and it showed a very different expression pattern over the outdoor winter course compared with all the other HSPs. HSP60 levels was strongly reduced during midwinter in outdoor larvae, falling by February to just 40% of September values. HSP60 was also suppressed under anoxic conditions such as would occur naturally when the larvae freeze. The present results agree in part with those of Martinez (2001) who reported that HSP60 decreased after cold treatment in two *Trichinella* species. Singh (2000) reported that in three insects, the synthesis of HSP64, a member of the HSP60 family, is different from other HSPs after heat shock and Subhash (2002) found in *D. melanogaster* larvae that HSP 64 polypeptides synthesized in response to heat shock are degraded rapidly. These studies imply that HSP60 maybe regulated differently in insects and have a special role related to the specific cellular physiological requirement in *D. melanogaster* larvae. The above implication supports our results that in these two insects HSP 60 expression is different

from other HSPs after the various stresses and this difference implies the special role of HSP60 in these two larvae. Previous studies in our lab (Joanisse and Storey, 1995) showed that the activities of multiple mitochondrial enzymes decreased over the winter months in *E. solidaginis*. The parallel between the drop in HSP60 levels over the winter and the drop in the activities of multiple mitochondrial enzymes suggests that HSP60 levels are falling along with the overall abundance of mitochondria in the larvae while they are in diapause (torpor).

We failed to detect expression of HSP 10 in *E. solidaginis*. Several possible reasons might be suggested. These include (a) the commercial antibodies used were unable to bind to the *E. solidaginis* protein; indeed, both the antibody listed in Table 1 which worked for *E. scudderiana* and a second source of HSP10 (SPA-781) did not work, (b) HSP60 and HSP10 do not always act as a single functional unit, since only newly imported proteins are severely affected by inactivation of HSP10 (Hohfel *et al.*, 1994); cellular conditions in the autumn-collected, nonfeeding *E. solidaginis* larvae may have required little or no HSP10, and (c) the greatly reduced expression level of HSP60 in *E. solidaginis* may suppress the synthesis of its cochaperone, HSP10.

Hence, from the above results we know that both *E. solidaginis* and *E. scudderiana* express some HSPs in response to environmental stresses (both cold and anoxia). But how are HSPs regulated at the transcriptional level?

Stressors that cause protein unfolding, misfolding or aggregation trigger a stress response that leads to the induction of transcription of genes encoding HSPs which then act to stabilize and re-fold proteins, thereby re-establishing the balance between protein

synthesis, assembly and degradation (Pirkkala *et al.* 2001). It is believed that activation of HSF via trimerization, phosphorylation and nuclear localization is a key step in HSP expression (Sorger, 1991; Xiao *et al.*, 1988). There are at least four HSFs in mammalian cells but only HSF-1 has been found in *D. melanogaster* and appears to be the primary mediator of the heat shock response system (Wu *et al.*, 1995; Santoro *et al.*, 2000). HSF-1 is constitutively present in a non-activated, monomeric state and, in response to stress, becomes activated to form a trimer that is capable of high affinity binding to a specific DNA response element, the heat shock element (HSE, 5 bp DNA consensus nGAAn) in the promoter regions of heat shock genes (Klemenz *et al.*, 1991; Zhong *et al,* 1998). The number and arrangement of the HSEs affects the expression of HSPs (Wu *et al.*, 1995). Activation of HSF-1 also leads to exposure of a transcriptional activation domain leading to increased transcription of heat shock genes, which results in accumulation of HSPs (Wu *et al.* 1995; Morano *et al.* 1999). The transcriptional activation domains contained in all HSFs are regulated by protein phosphorylation (Cotto *et al.* 1996; Xia and Eoellmy 1997; Fritsch and Wu, 1999) whereas interactions with molecular chaperones lead to transcriptional repression (Shi *et al.* 1998; Zou *et al.* 1998).

Both inactive (unphosphorylated) and activated (phosphorylated) forms of HSF-1 were detected in *E. solidaginis* and *E. scudderiana* under unstressed control conditions. Since denatured or nonnative proteins are the proximate activation trigger for HSF (Ananthan *et al.* 1986; Pirkkala *et al.* 2001; Voellmy 2004), it was unexpected that activated HSF-1 would be present in unstressed larvae. Our data agree with some studies from mammalian and insect cells where activations of HSFs have been found under non-stressed conditions (Hubel *et al.* 1995; Clos *et al.*1993). This is also in agreement with

Jedlicka (1997) who reported that fruit fly HSF is required during oogenesis and early larval development. The expression of activated HSF-1 in these two insect larvae under nonstressed conditions can also partly explain the high expression levels of inducible HSP70 in these larvae, which is unlike the situation that is typically reported (Snoeckx *et al.*, 2001).

The current data demonstrate that in *E. scudderiana* under natural winter conditions outdoors the relative levels of activated HSF-1 are much higher than the inactive form. This implies that winter creates stress conditions that require elevated levels of HSPs over the winter months. Furthermore, the protein levels of both inactive and activated HSF-1 were strongly increased during the winter months (except in March), and this indicates that HSF-1 protein is inducible under natural cold temperatures and implies that genes under HSF-1 control would be up-regulated during the winter. This conclusion is also supported by the results of the laboratory cold stress experiments with the amount of active HSF-1 rising significantly in larvae acclimated at -20°C for 1 week. However, low temperature is probably not the only influence on HSF-1 activation in nature for two reasons: (a) HSF-1 is strongly activated in October in outdoor larvae which is long before they would encounter low subzero temperatures (such as -20°C) naturally, and (b) chilling of larvae at 4°C for 1week actually reduced the amount of activated HSF-1 (Figure 3.12C). Further studies will be needed to determine what other influences can trigger HSF-1 activation in the *E. scudderiana* larvae.

The expression of HSF-1 in *E. scudderiana* under anoxic conditions in the laboratory is quite different and interesting. The amount of activated HSF-1 increased during the anoxic period, suggesting that anoxia can trigger the activation of HSF-1.

Oppositely, the amount of inactive HSF-1 decreased during the anoxia, implying that the inactive HSF-1 is converted to the activated form during anoxia; hence, total HSF-1 levels appear to remain stable (Figure 3.12D). Comparing the activation ability of cold and anoxic stresses we see that the amount of activated HSF-1 is lower than inactive HSF-1 under anoxia whereas activated HSF-1 is higher than inactive HSF-1 under cold exposures (both field and laboratory experiments). This suggests that HSF-1 is less sensitive to anoxia stress in *E. scudderiana* than it is to cold stress. This lower expression of activated HSF-1 under anoxia also agrees with the decreased or stable expression profiles of HSP110, HSP70, HSP60 and HSP40 under anoxic exposure in *E. scudderiana*. Hence, we can conclude that this insect is not very sensitive to anoxia.

HSF-1 expression in *E. solidaginis* was very different from that in *E. scudderiana.* In the winter months, both inactive and activated HSF-1 showed a concave expression pattern with highest levels in September and October and minimum levels in February. Furthermore, the content of activated versus inactive HSF-1 was much higher during September and October. These data indicate that HSF-1 action in stimulating gene expression is high during the autumn months. This may be due to a higher turnover of HSPs in the larvae at higher temperatures requiring HSP synthesis and/or it may set the stage for the build-up of selected HSPs as outdoor temperatures cool over the autumn and into early winter (ie. HSP110, HSP70, HSP40 were all elevated in December). However, by midwinter HSF-1 levels were reduced (both inactive and activated) in outdoor larvae which may suggest that new HSP synthesis is low when environmental temperatures drop below 0°C (December, February). Over these midwinter months, the insects are in diapause and in the dormant state there may be relatively little new protein synthesis,

particularly when the larvae are frozen and energy-restricted. However, in the laboratory cold exposure experiments, different results were seen. Transfer of larvae from 3°C to -16°C resulted in a strong increase in the amount of activated HSF-1 when larvae were frozen for 24 h. This shows that HSF-1 activation is cold and/or freeze responsive and that this shock response is activated by an abrupt decrease in temperature. Hence, the high amounts of activated HSF-1 seen in September and October larvae might be due cold temperature shocks as a result of nightly decreases in environmental temperature. Both inactive and activated HSF-1 decreased when the larvae were thawed (transfer from -16°C to +3°C for 24 h) which indicates that the cold-activation of HSF-1 at -16°C is reversible by warming/thawing. This is consistent with the lower levels of activated HSF-1 in March and April and suggests that HSP synthesis is turned down as environmental temperatures warm up in the spring (note that HSP110, HSP70 and HSP40 levels fell in April). However, HSP60 responded oppositely and rose sharply in April which suggests that HSF-1 may not be regulating the HSP60 response. Under anoxic conditions, both inactive and activated HSF-1 were significantly increased in *E. solidaginis*, particularly after 24 h of anoxia exposure where the inactive form increased by 3-fold and the activated form by 10-fold. This suggests that HSF-1 is both inducible and high activated by anoxia in *E. solidaginis*. Hence, the strong activation of HSF-1 seen when larvae were exposed to -16°C might be triggered both by cold and by the anoxia/ischemia associated with freezing.

A comparison of the HSF-1 expression patterns with the patterns of expression of the individual HSPs in each species allows us to assess the possible role of HSF-1 in the regulation of each type of HSP. In *E. scudderiana*, the expression of the following HSPs

were positively correlated with the expression of activated HSF-1: all HSPs over the winter timecourse, HSP 70 and 110 in laboratory cold acclimation, and HSP 10 in anoxia. In *E. solidaginis*, the expression profiles of HSP 70 and 40 during freeze/thaw and HSP 70 in anoxia were also correlated with levels of activated HSF-1. The above data suggest that in these two insect species, constitutive and inducible activation of HSF-1 correlates with elevated levels of some HSPs and, hence, indicates that HSF-1 regulates the production of these HSPs under environmental stresses.

However, the expression of some HSPs did not correlate with HSF-1 expression; e.g. in *E. scudderiana* this included the levels of HSP 110 in anoxia and HSP 60 in cold and anoxia, whereas in *E. solidaginis* this included HSP 110 in thawing and anoxia and HSP 60 in cold and anoxia. How can the transcription of heat shock genes be regulated by the same HSF-1, but the HSPs themselves be expressed differentially? First of all, HSF activation of gene transcription is only one aspect of the regulation of HSP expression. Other regulatory mechanisms play important roles including promoter sequences as well as regulation of HSP message degradation and translation (Kregel 2002). Secondly, recent studies (Xiao *et al.* 1999; Trinklein *et al.* 2004) have shown that HSF-1 also regulates some inducible genes with likely HSE sequences. Thirdly, even the regulation of HSF-1 activation itself is very complicated (Voellmy 2004). Studies suggest the some stresses that induced trimerizaton and acquisition of high affinity DNA binding (after phosphorylation) by HSF-1 appear to be separable from the stresses that induced activation of transcription of heat shock genes (Jurivich *et al.* 1992; Lee *et al.* 1995; Cotto *et al.* 1996). In their studies, they found stresses that could induce trimerizaton and high affinity DNA binding, but did not result in increased transcription of heat shock

genes. In addition, some studies (Bruce *et al.* 1993; Hensold *et al.* 1990) showed that transcriptional activation of the HSP70 gene was independent of protein synthesis (ie. HSP70 mRNA could increase in response to activation of HSF after stress without increasing expression of HSP70 protein). Moreover, other studies (Shi *et al.* 1998; Zou *et al.* 1998; Bonner *et al.* 2000) have suggested that some HSPs (particularly HSP70) can physically interact with HSF. Hence, the cellular level of HSPs, directly or indirectly, feedback regulates HSF activity. However, other reports do not agree with this suggestion. Hjorth-Sorensen *et al.* (2001) believe that the activation of HSFs is not influenced by the level of expression of HSPs. Furthermore, recent studies show that except for the above aspects, the interactions with regulatory cofactors and/or other transcriptional factors carefully control the regulation of HSF (Connell *et al.* 2001; Boellmann *et al.* 2004).

3.5 Conclusions

The results presented above suggest that inducible HSPs are important for fitness under environmental conditions in both *E. solidaginis* and *E. scudderiana* larvae. As part of the protein quality control system, HSPs play a major role in the struggle to maintain functional cellular machinery upon exposure to environmental stresses and in survival during the extreme cold of winter. Our results also indicate that constitutive expression and rapid induction of some HSPs is accomplished through mechanisms of transcriptional activation and preferential translation; and HSF-1 is involved in regulation of the inducible synthesis of HSPs during development and adaptation to environmental stress (cold and anoxia) in these two cold hardy larvae.

Table 3.1 Antibodies and experimental conditions used for both *E. solidaginis* and *E. scudderiana*.

Antibodies	Sources	Acrylamide Gel used	Blocking time with 2.5% milk (min)	Primary antibodies dilution factor (V:V)	Primary antibodies incubation time (4°C)	Secondary antibodies used and dilution factor
HSP 110 SPA-1103	Stressgen	8%	20	1:1,000	Over night	Anti-rabbit, 1:1,500
HSP70 SPA-812	Stressgen	10%	45	1:150,000	4h	Anti-rabbit, 1:2,000
HSP 60 SPA-805	Stressgen	10%	30	1:1,000	Over night	Anti-mouse, 1:2,000
HSP 40 SPA-400	Stressgen	12%	20	1:10,000	Over night	Anti-rabbit, 1:2,000
HSP 10 SPA-110	Stressgen	15%	10	1:2,000	2 days	Anti-rabbit, 1:1,500
HSF 1 SPA-901	Stressgen	10%	15	1:10,000	Over night	Anti-rabbit, 1:2,000

Fig. 3.1 Regulation of transcription of *hsp* genes by HSF1.

Under normal conditions, HSF1 exists primarily as a latent monomer in the cytoplasm. Upon exposure to cytotoxic conditions such as heat shock, oxidative and others stresses, phosphorylated HSF1 trimerizes and migrates to the nucleus. In the trimeric state, HSF1 binds to the HSE, forming a complex that has the potential to activate the transcription of *hsp* genes. Figure retrieved from: www.umich.edu/~protein/ hsff/HSF1fig.JPG

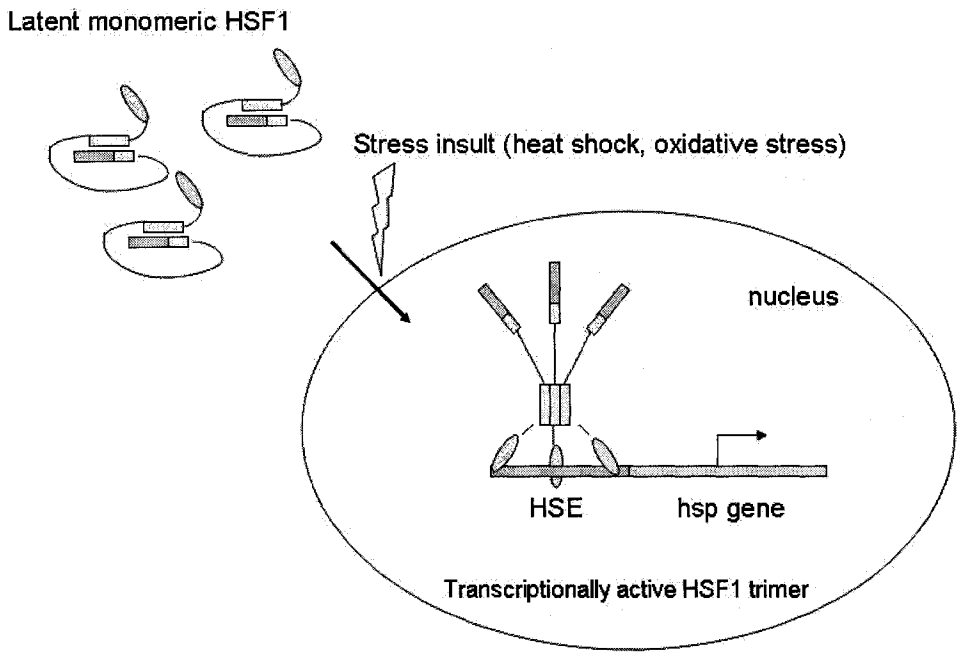


Fig. 3.2 HSP110 protein expression in *E. solidaginis* larvae.

A. Representative Western blots showing total HSP110 levels over the winter, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; Ch - Chilled; Fr - Frozen; Th - Thawed; An – Anoxia.

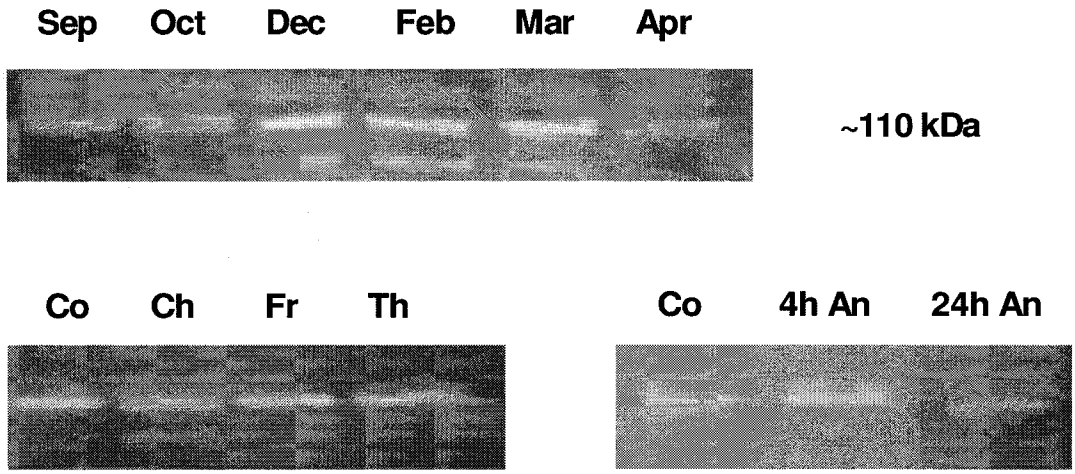
B. Histogram showing relative expression (densitometric analysis) of HSP110 protein in outdoor larvae over the winter months.

C. Histogram showing relative expression of HSP110 in laboratory low temperature experiments: control (+15°C acclimated), chilled (+3°C, 24 h), frozen (-16°C, 24 h), and thawed (+3°C, 24 h).

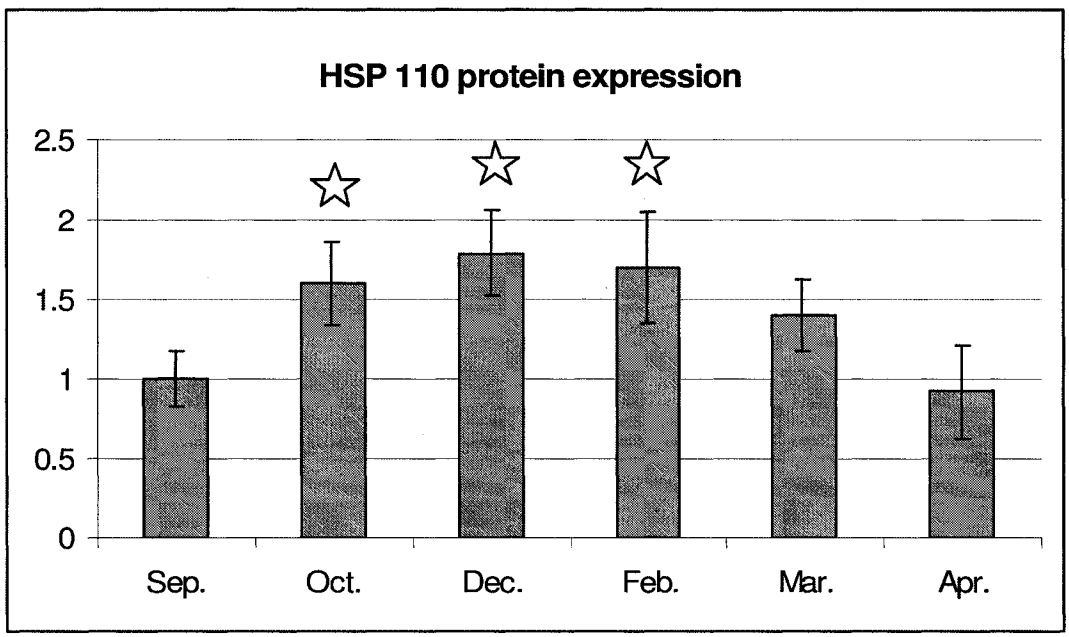
D. Histogram showing relative expression of HSP110 in larvae under aerobic control (+15°C) versus anoxia exposure (4 h or 24 h at +15°C) conditions.

Note: All data are means ± S.E.M. (n=4). ☆ - Significantly different from the corresponding September or control value as determined by the Student's t-test, $P<0.05$.

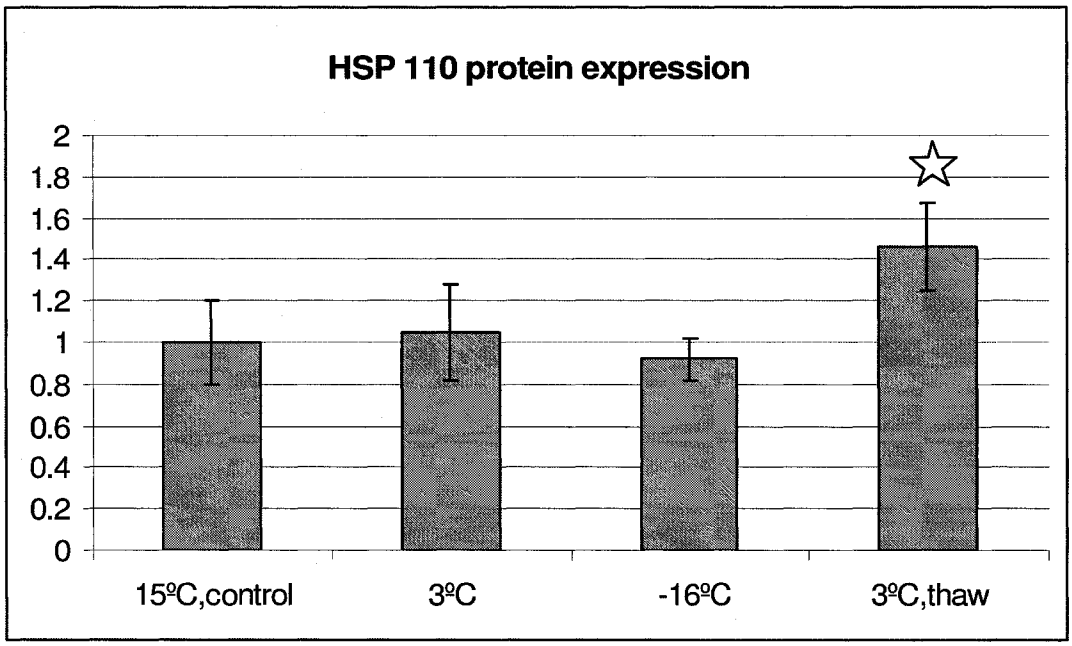
A.



B.



C.



D.

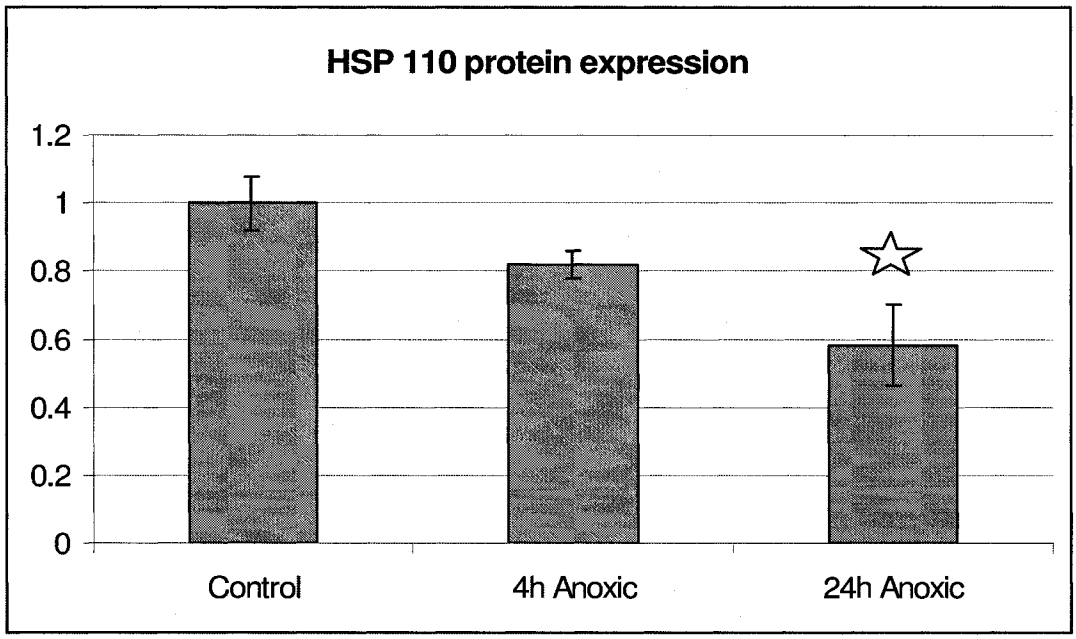


Fig. 3.3 HSP70 protein expression in *E. solidaginis* larvae.

A. Representative Western blots showing total HSP70 levels over the winter, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; Ch - Chilled; Fr - Frozen; Th - Thawed; An – Anoxia.

B. Histogram showing relative expression (densitometric analysis) of HSP70 protein levels over the winter months outdoors.

C. Histogram showing relative expression of HSP70 in control (+15°C), chilled (+3°C, 24 h), frozen (-16°C, 24 h), and thawed (+3°C, 24 h) larvae in indoor laboratory experiments.

D. Histogram showing relative expression of HSP70 under control (+15°C) and anoxic (4 h or 24 h at +15°C) conditions.

Note: All data are means ± S.E.M. (n=4). ☆– Significantly different from the corresponding September or control value as determined by the Student's t-test, $P<0.05$. ♡ –Thawed value is significantly different from the frozen value, $P<0.05$.

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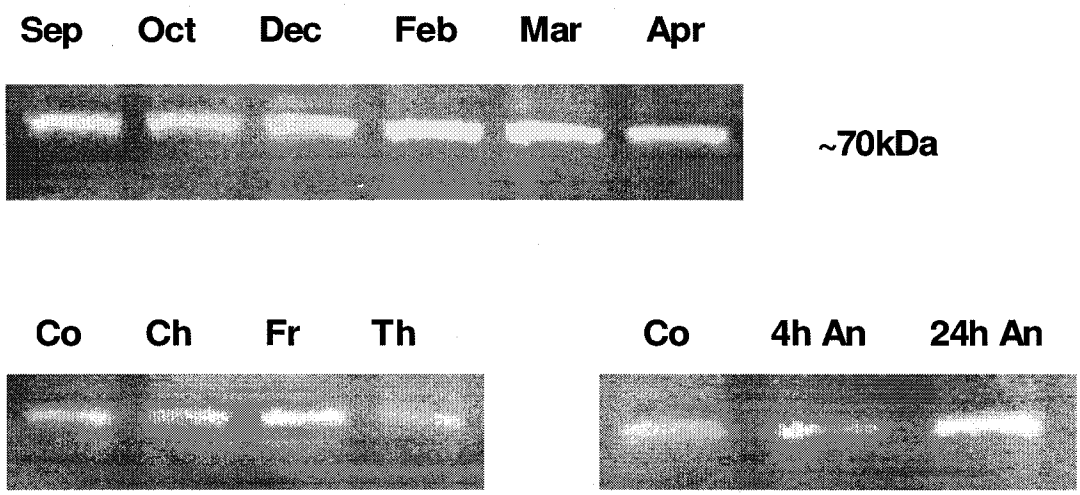

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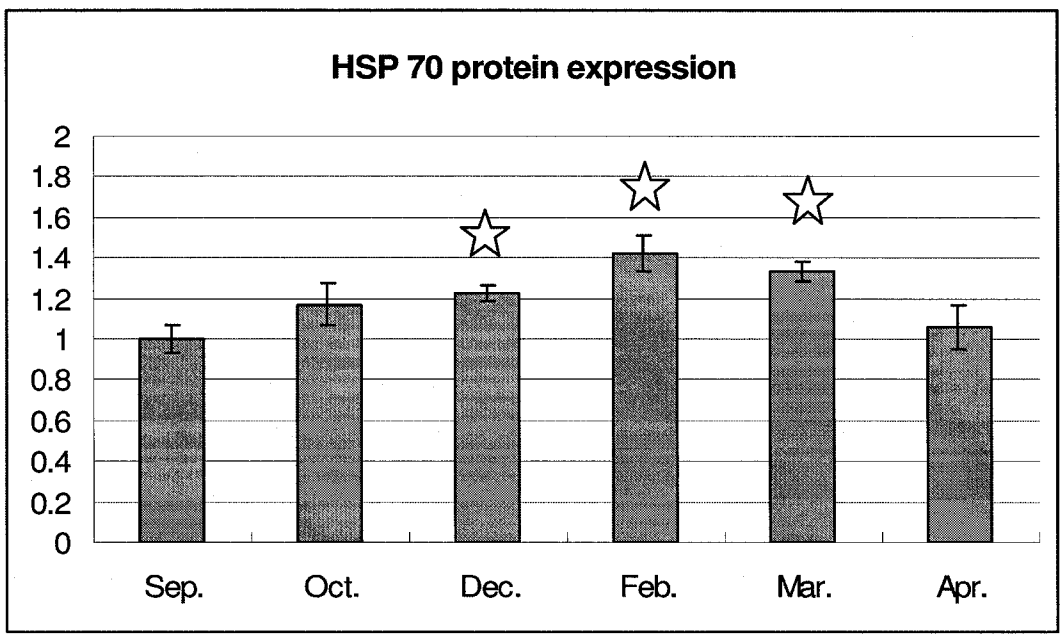

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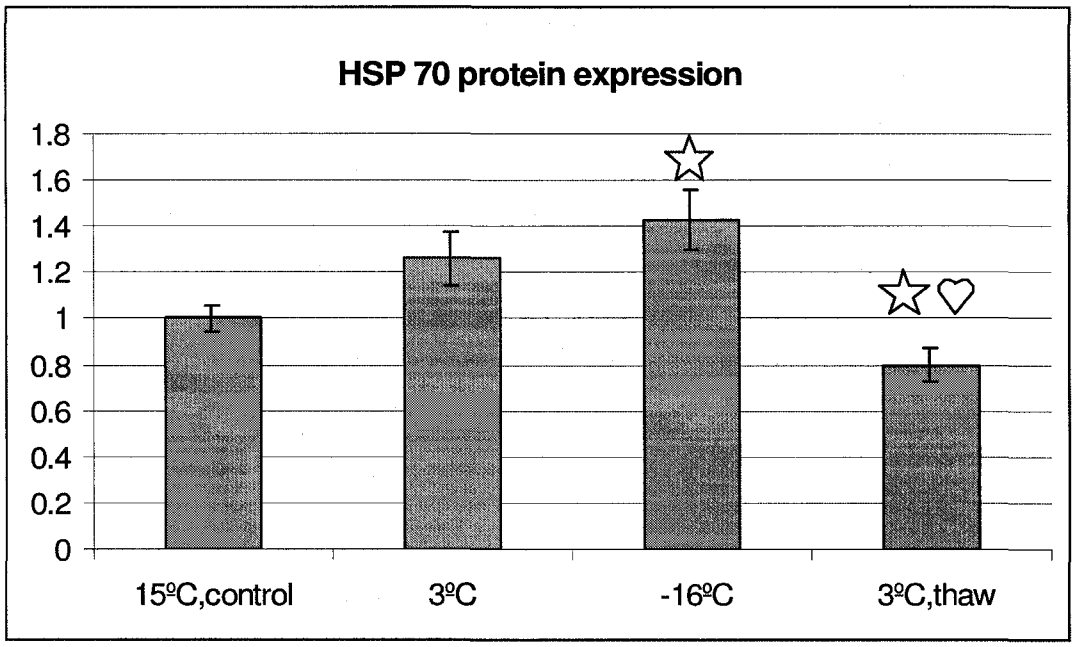

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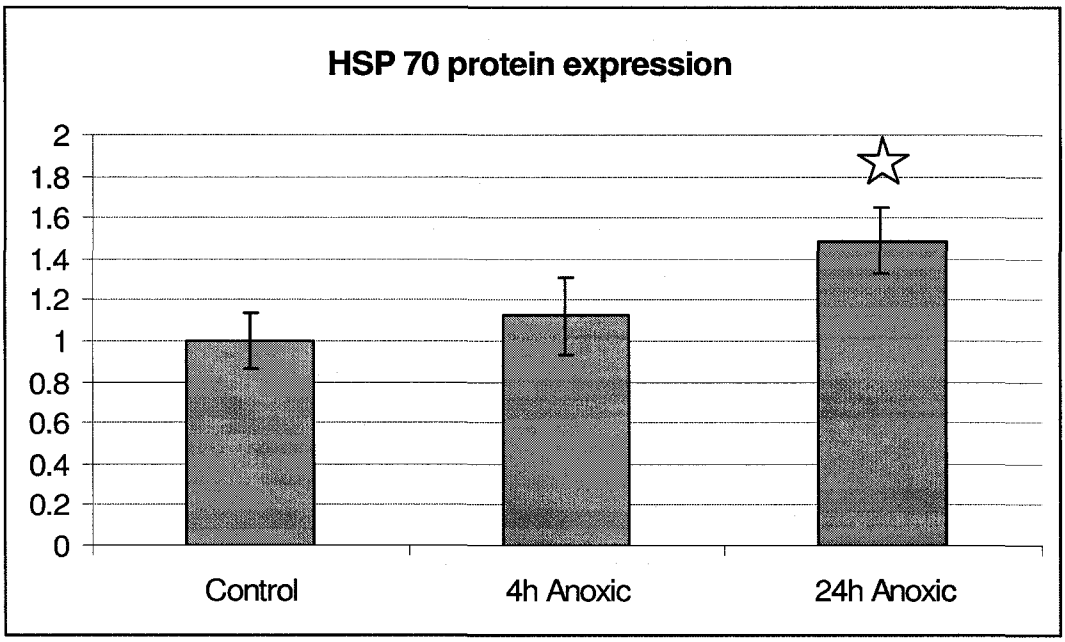

Fig. 3.4 HSP60 protein expression in *E. solidaginis* larvae.

A. Representative Western blots showing total HSP60 levels over the winter, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; Ch - Chilled; Fr - Frozen; Th - Thawed; An – Anoxia.

B. Histogram showing relative expression (densitometric analysis) of HSP60 protein over the winter months outdoors.

C. Histogram showing relative expression of HSP60 in control (+15°C), chilled (+3°C, 24 h), frozen (-16°C, 24 h), and thawed (+3°C, 24 h) larvae in indoor laboratory experiments.

D. Histogram showing relative expression of HSP60 under aerobic control (+15°C) versus anoxic (4 h and 24 h at +15°C) conditions.

Note: All data are means ± S.E.M. (n=4). ☆– Significantly different from the corresponding September or control value as determined by the Student's t-test, $P<0.05$.

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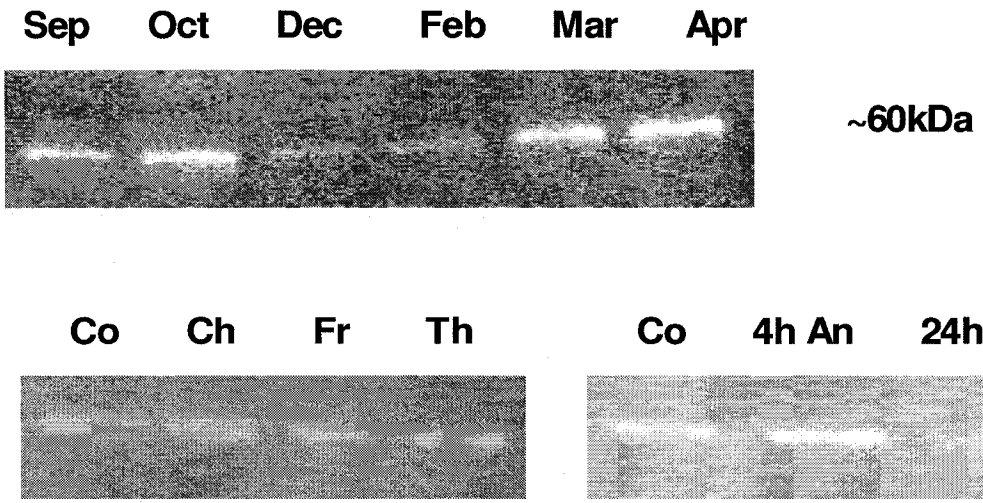

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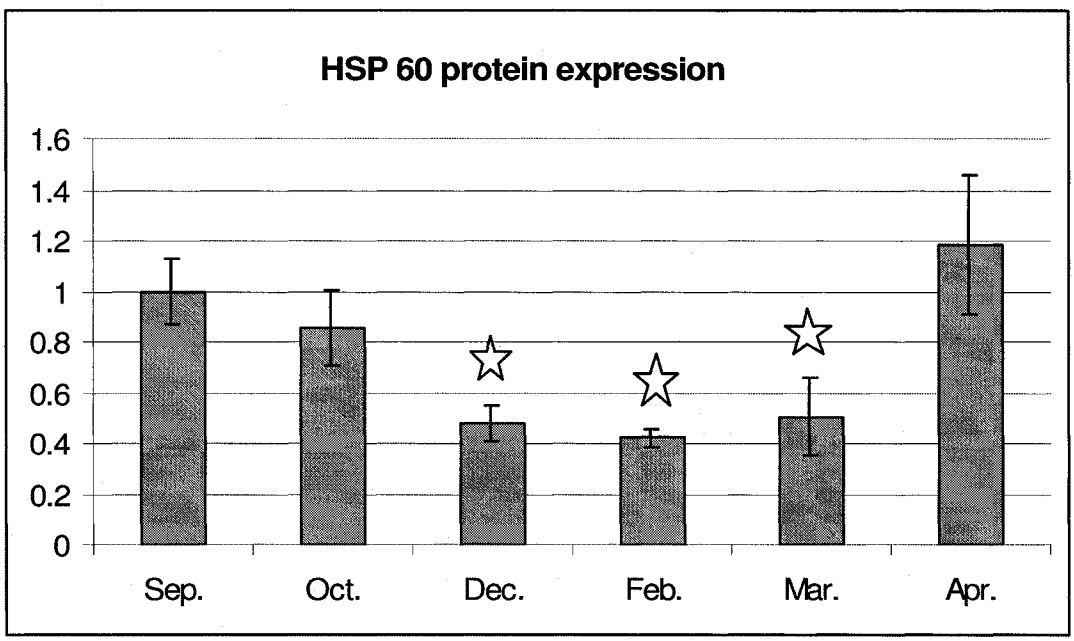

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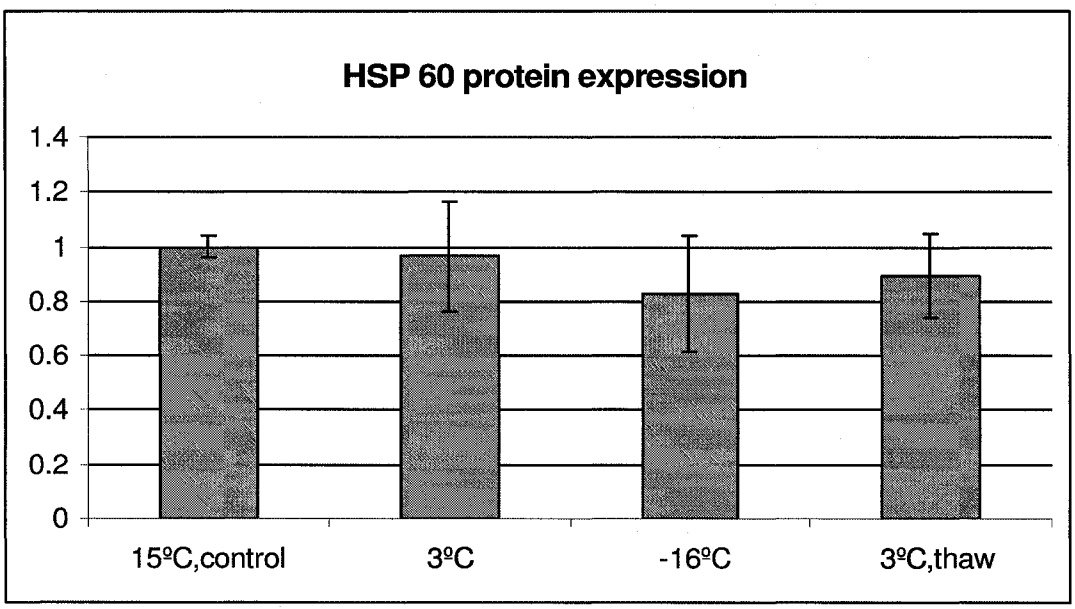

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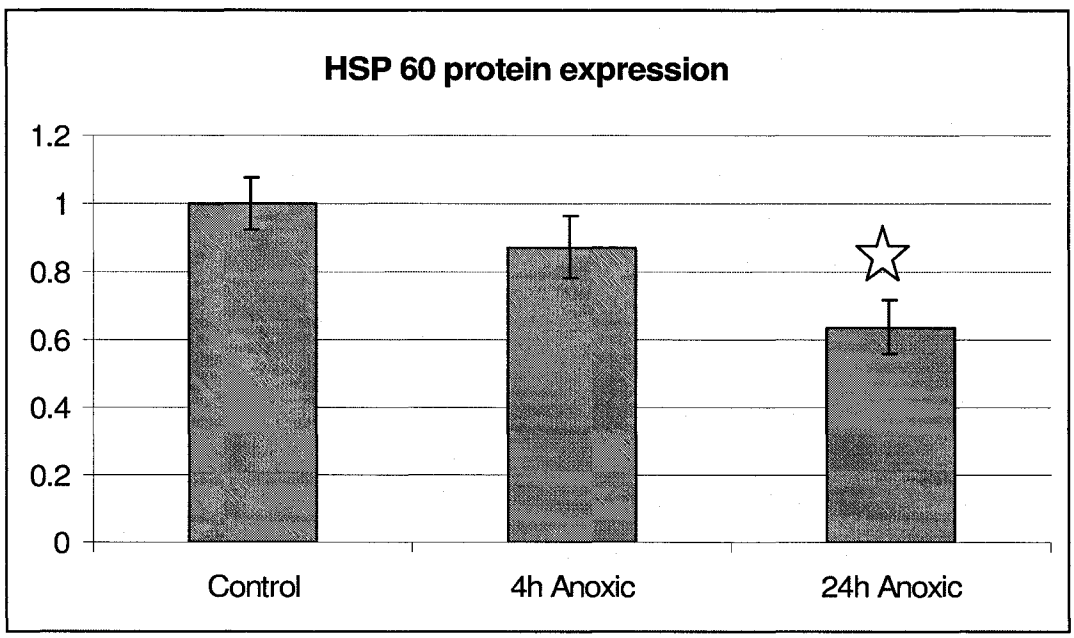


Fig. 3.5 HSP40 protein expression in *E. solidaginis* larvae.

A. Representative Western blots showing total HSP40 levels over the winter, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; Ch - Chilled; Fr - Frozen; Th - Thawed; An – Anoxia.

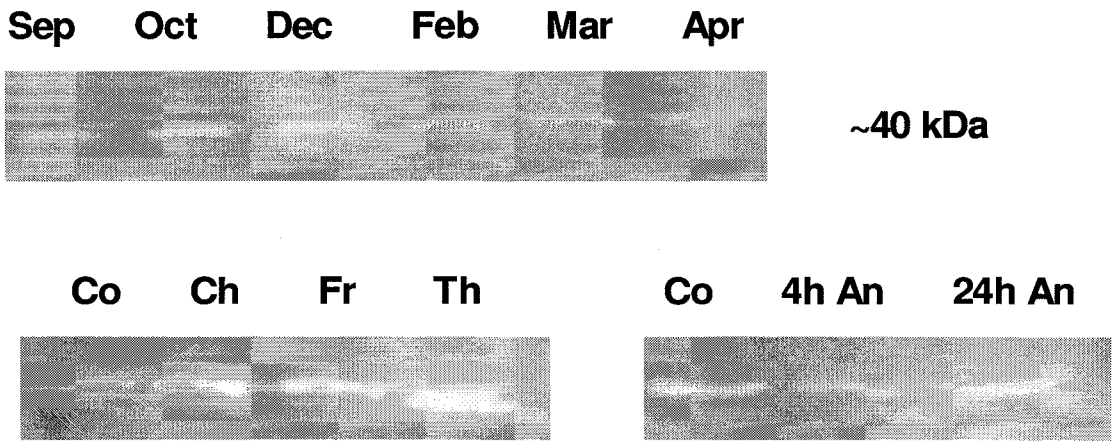
B. Histogram showing relative expression (densitometric analysis) of HSP70 protein over the winter months outdoors.

C. Histogram showing relative expression of HSP40 in control (+15°C), chilled (+3°C, 24 h), frozen (-16°C, 24 h), and thawed (+3°C, 24 h) larvae in indoor laboratory experiments.

D. Histogram showing relative expression of HSP40 in aerobic control (+15°C) versus anoxic (4 h and 24 h +15°C) larvae.

Note: All data are means ± S.E.M. (n=4). ☆– Significantly different from the corresponding September or control value as determined by the Student's t-test, $P<0.05$.

A.



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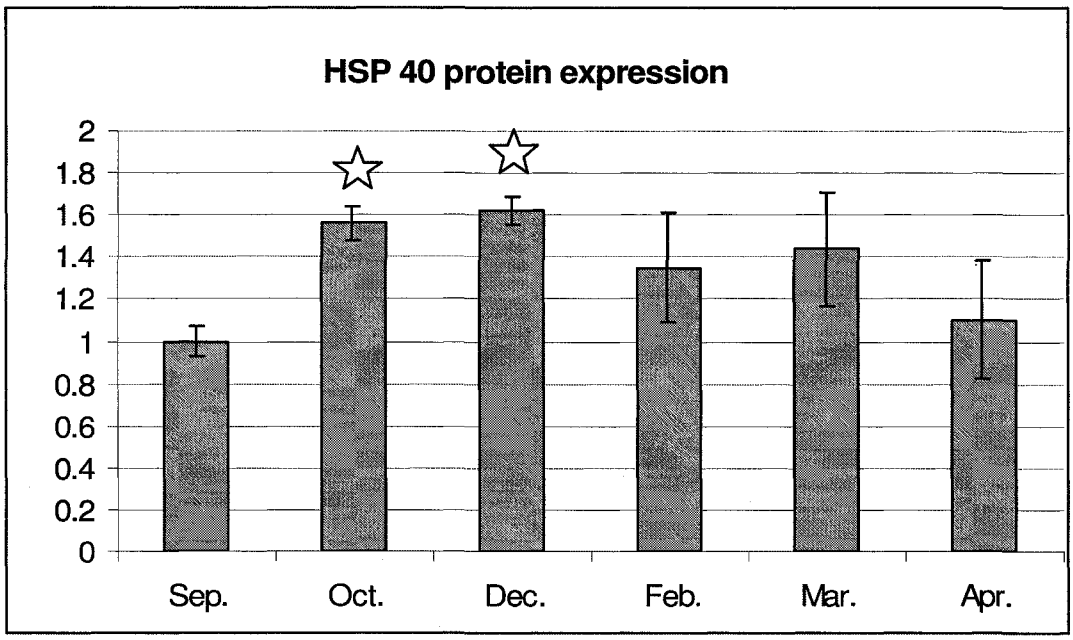

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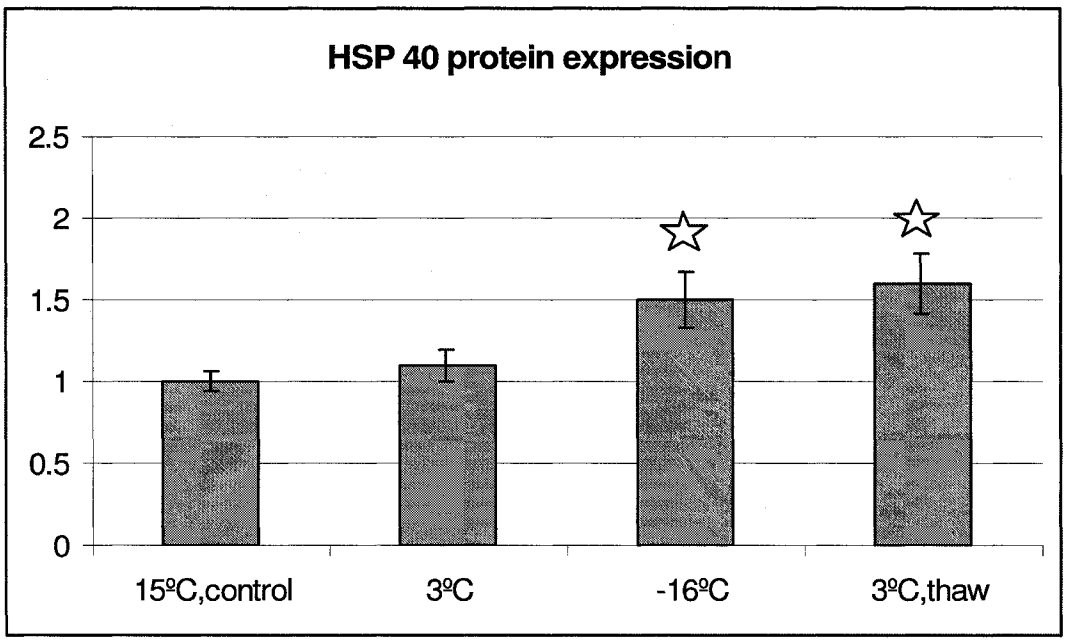

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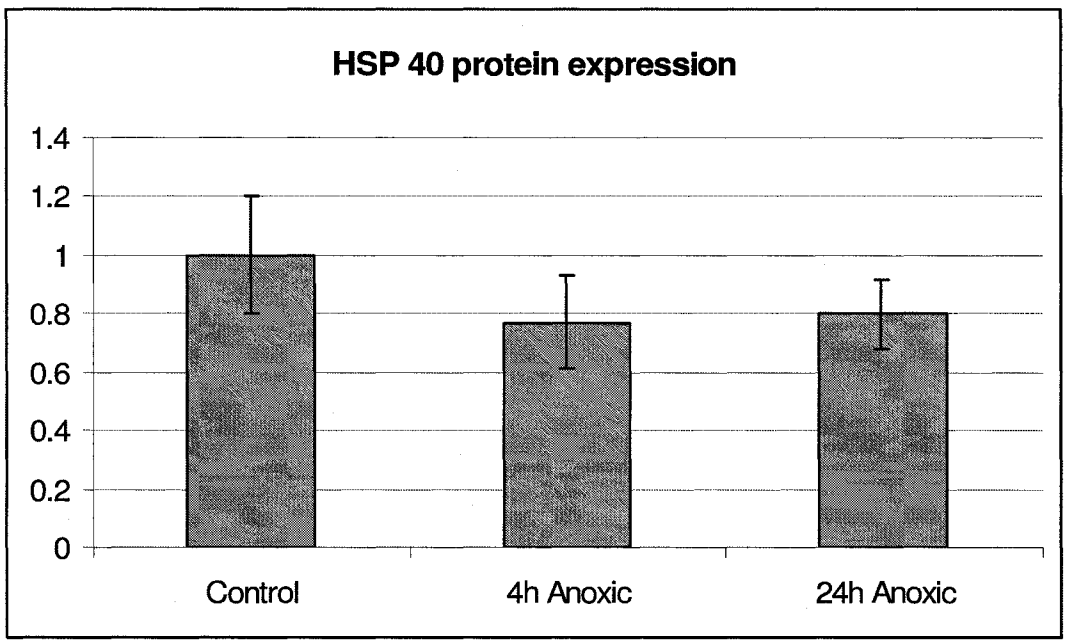


Fig. 3.6 HSF-1 protein expression in *E. solidaginis* larvae.

A. Representative Western blots showing total HSF-1 levels over the winter, both inactive (85 kDa) and activated (95 kDa) forms, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; Ch - Chilled; Fr - Frozen; Th - Thawed; An – Anoxia.

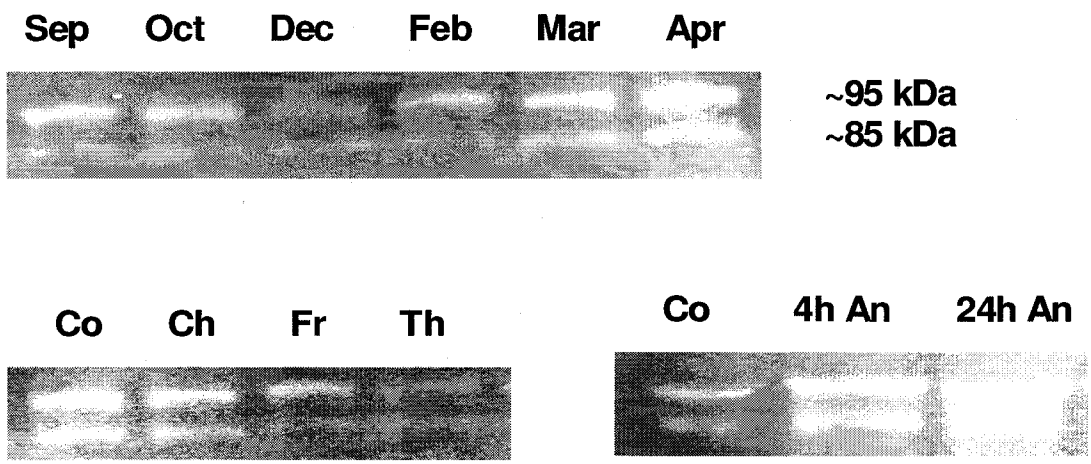
B. Histogram showing relative expression (densitometric analysis) of both inactive and activated HSF-1 protein over the winter months outdoors.

C. Histogram showing relative expression of both inactive and activated HSF-1 in control (+15°C), chilled (+3°C, 24 h), frozen (-16°C, 24 h), and thawed (+3°C, 24 h) larvae in indoor laboratory experiments.

D. Histogram showing relative expression of both inactive and activate HSF-1 in aerobic control (+15°C) and anoxic (4 h and 24 h +15°C) larvae.

Note: All data are means ± S.E.M. (n=4). ☆ – Mean value of inactive HSF-1 is significantly different from the corresponding September or control value, $P<0.05$; △ – Mean value of activated HSF-1 is significantly different from the corresponding September or control value, $P<0.05$; ◇ – Mean value of activated HSF-1 is significantly different from the corresponding inactive HSF-1, $P<0.05$; ♡ – activated HSF-1 thawed value is significantly different from the activated HSF-1 frozen value, $P<0.05$.

A.



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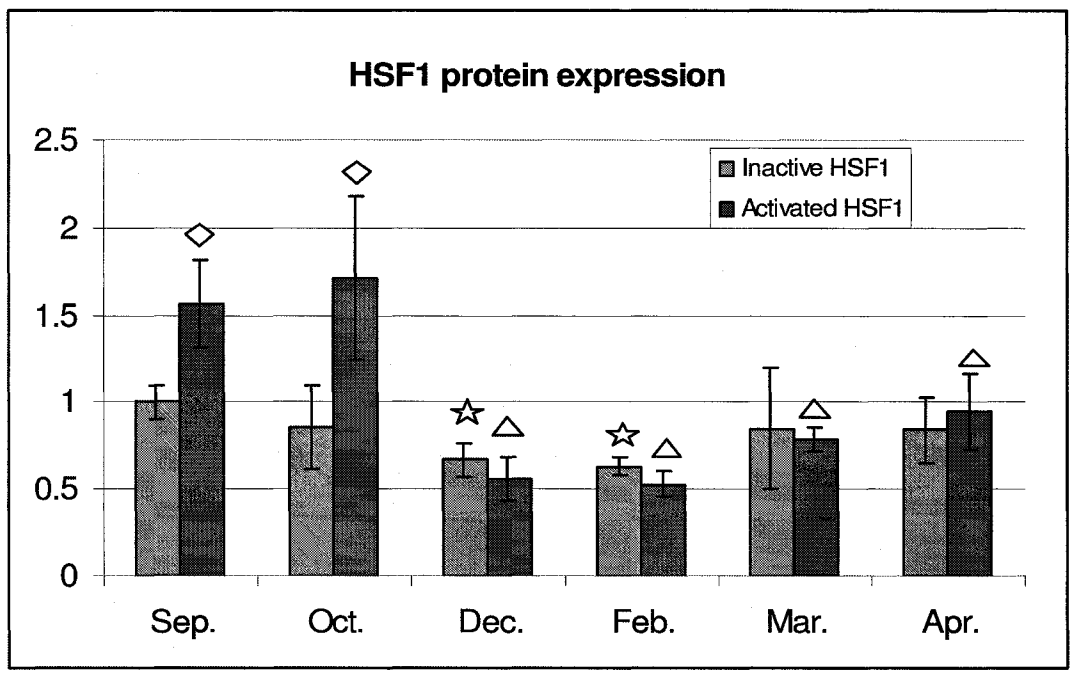

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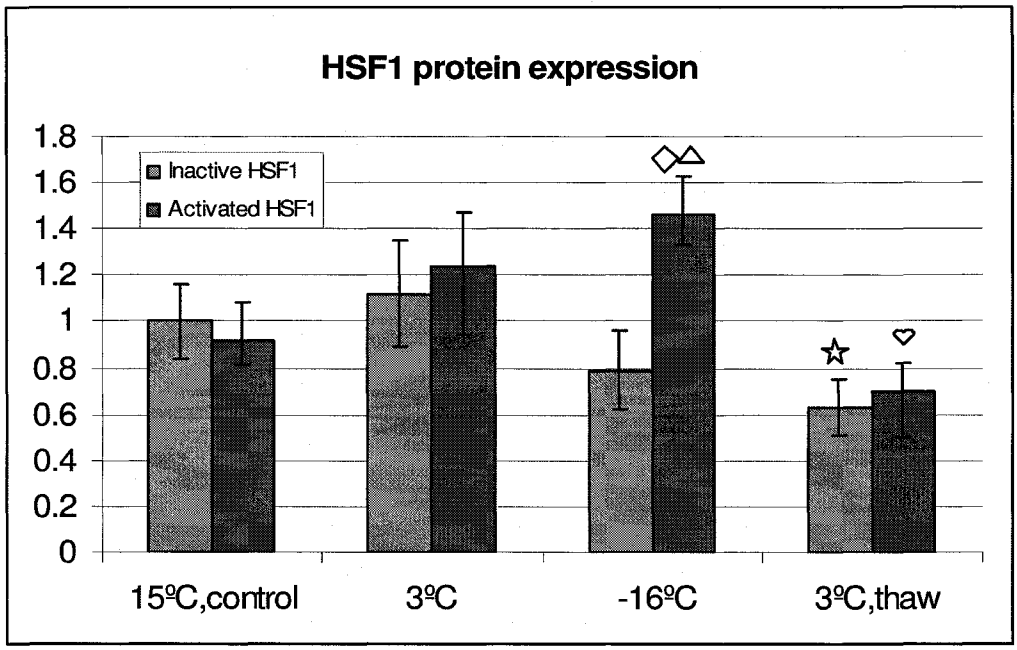

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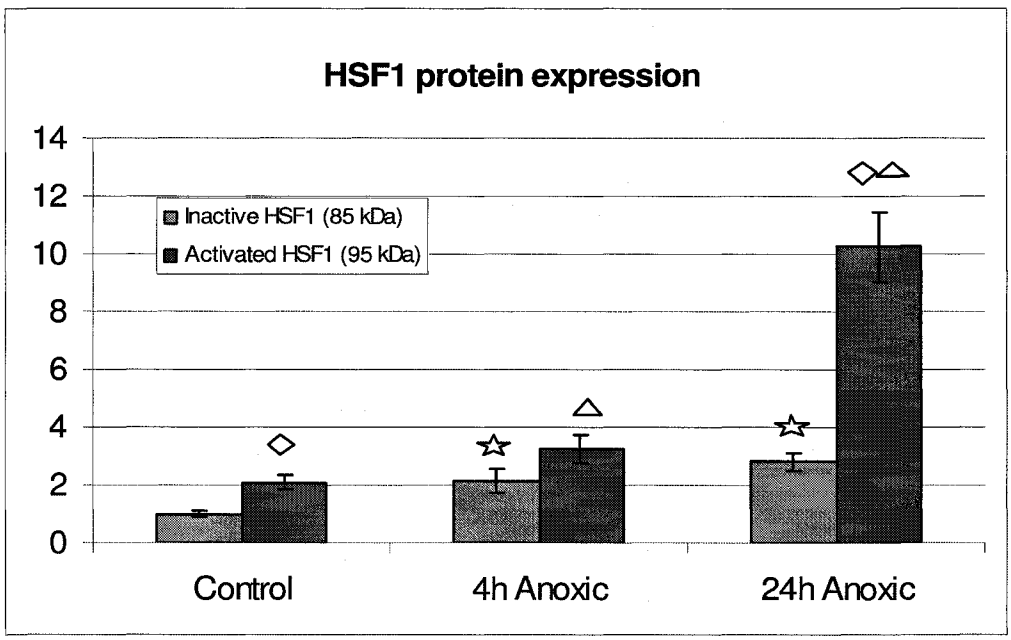


Fig. 3.7 HSP110 protein expression in *E. scudderiana* larvae.

A. Representative Western blots showing total HSP110 levels over the winter months, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; An – Anoxia.

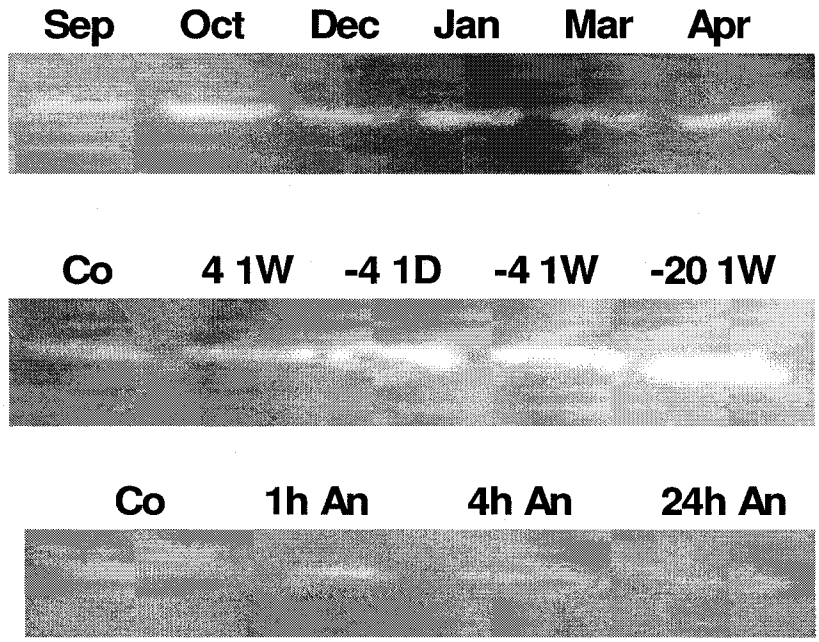
B. Histogram showing relative expression (densitometric analysis) of HSP110 protein over the winter months outdoors.

C. Histogram showing relative expression of HSP110 in indoor laboratory experiments: 15°C, control; 4°C, 1 week; -4°C, 24 h; -4°C, 1 week; and -20°C, 1 week.

D. Histogram showing the relative expression of HSP110 in control (+4°C, 2 weeks) versus anoxic (1 h, 4 h, or 24 h at 4°C) larvae.

Note: All data are means ± S.E.M. (n=4). ☆– Mean value is significantly different from the corresponding September or control value as determined by the Student's t-test, $P<0.05$.

A.



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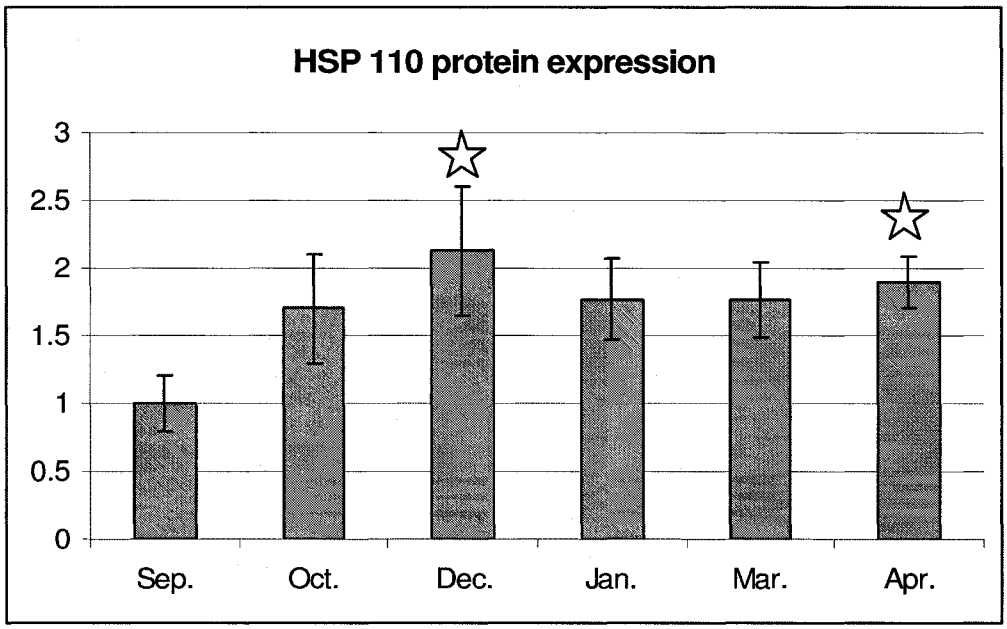

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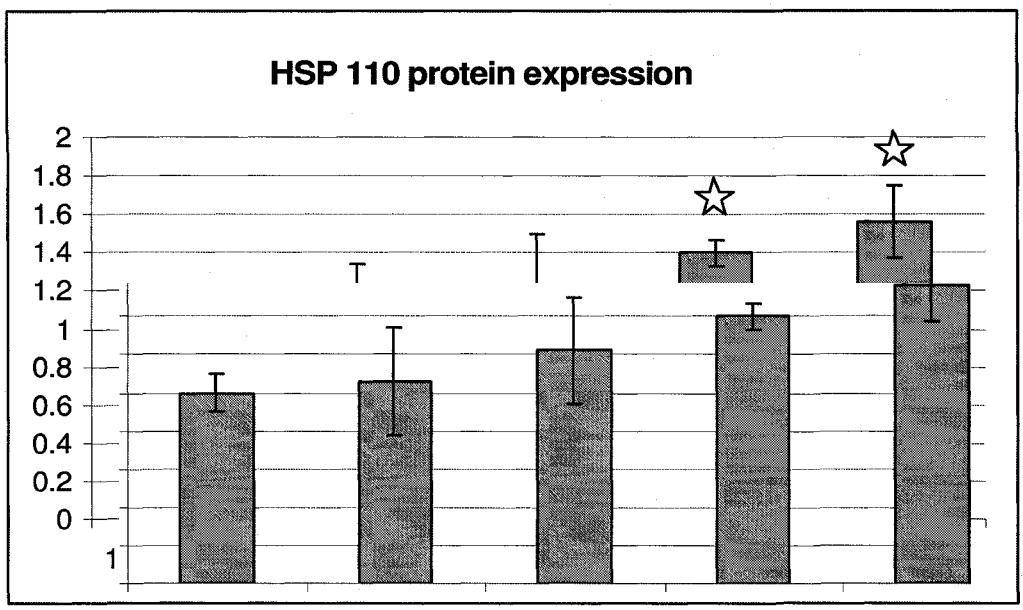

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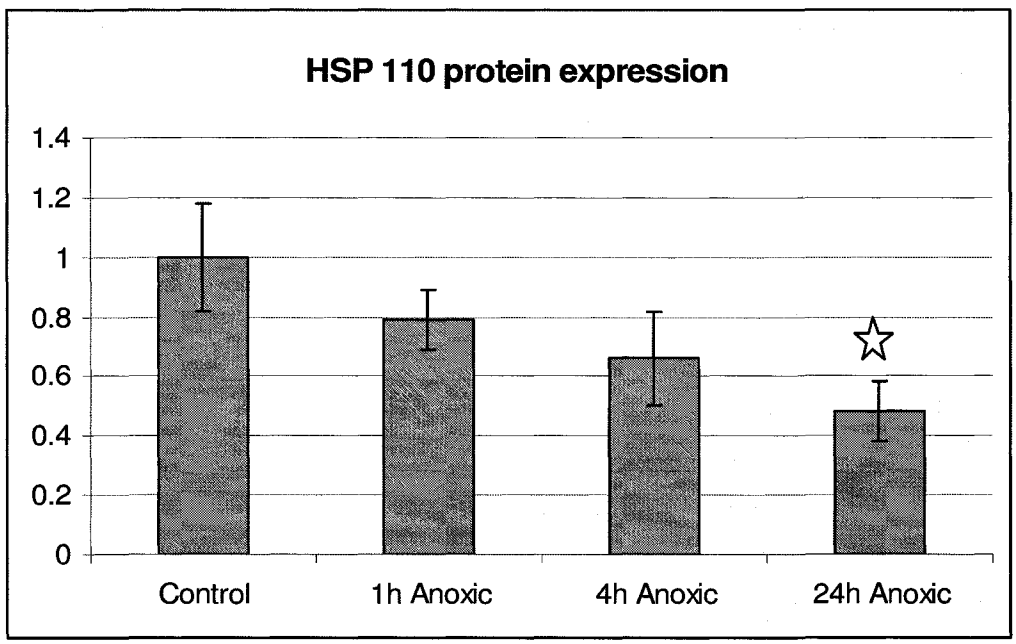


Fig. 3.8 HSP70 protein expression in *E. scudderiana* larvae.

A. Representative Western blots showing total HSP70 levels over the winter months, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; An – Anoxia.

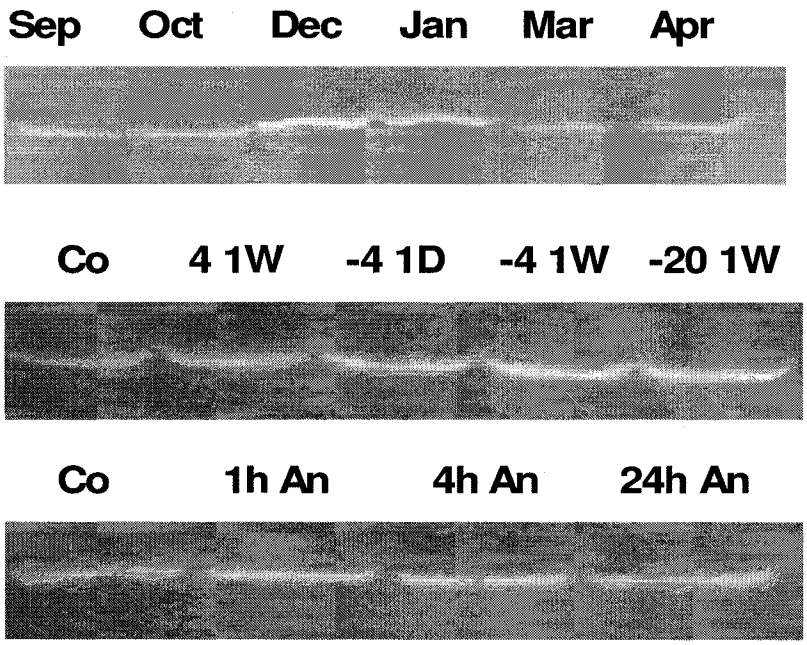
B. Histogram showing relative expression (densitometric analysis) of HSP70 protein over the winter months outdoors.

C. Histogram showing relative expression of HSP70 in the indoor laboratory experiments: 15°C, control; 4°C, 1 week; -4°C, 24 h; -4°C, 1 week; and -20°C, 1 week.

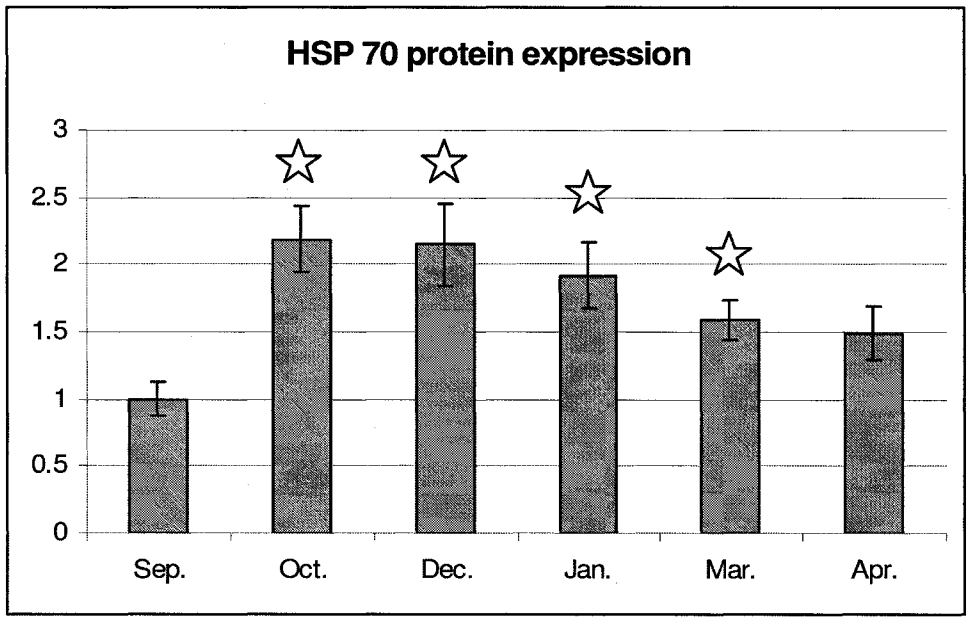
D. Histogram showing relative expression of HSP70 in aerobic control (+4°C, 2 weeks) versus anoxic (1 h, 4 h, or 24 h at 4°C) larvae.

Note: All data are means $\pm$ S.E.M. (n=4). ☆– Mean value is significantly different from the corresponding September or control value as determined by the Student's t-test, $P<0.05$.

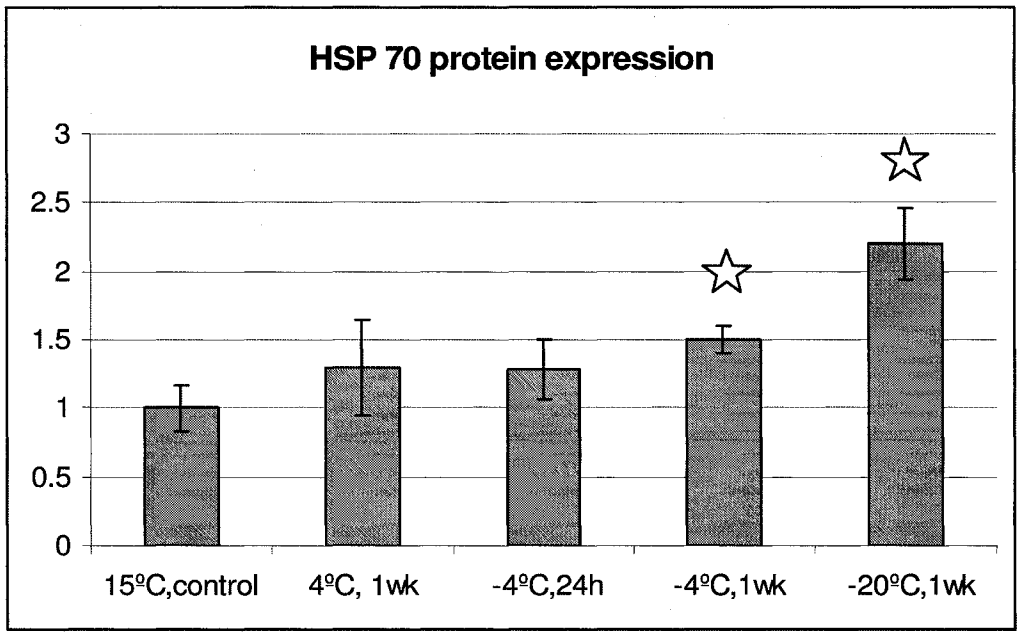
A.



B.



C.



D.

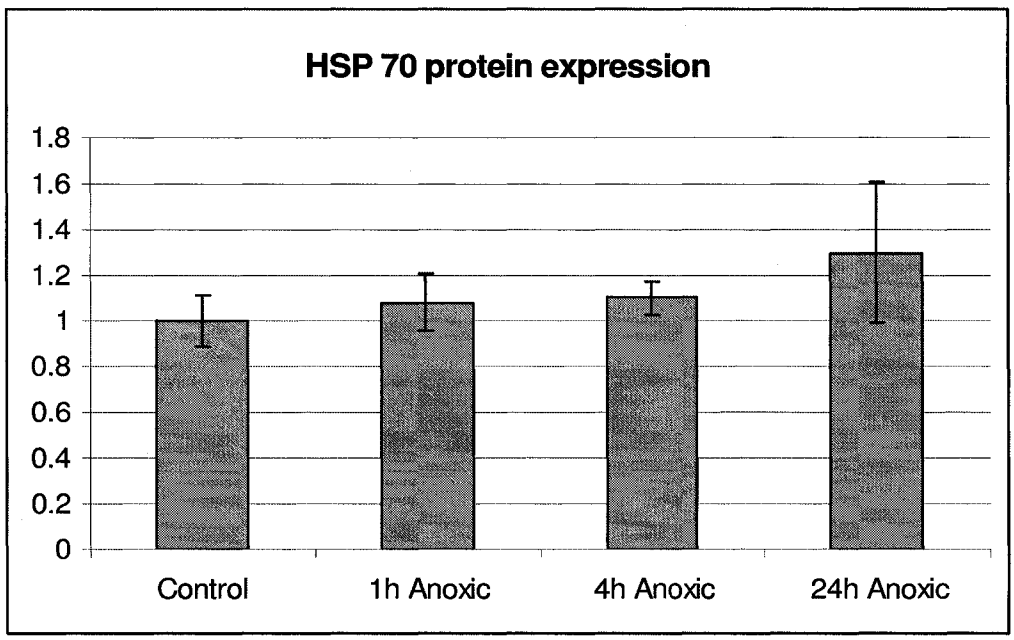


Fig. 3.9 HSP60 protein expression in *E. scudderiana* larvae.

A. Representative Western blots showing total HSP60 levels over the winter, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; An – Anoxia.

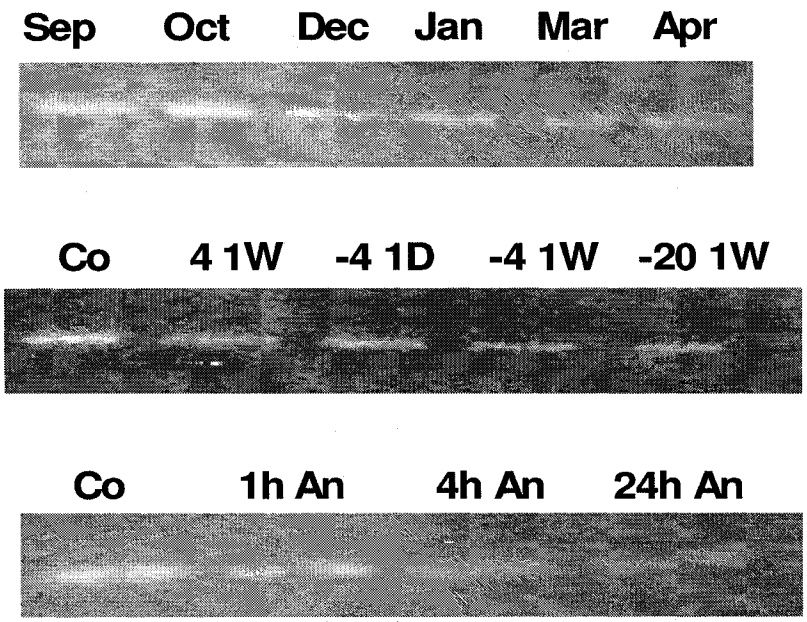
B. Histogram showing relative expression (densitometric analysis) of HSP60 protein over the winter months outdoors.

C. Histogram showing relative expression of HSP60 in indoor laboratory experiments: 15°C, control; 4°C, 1 week; -4°C, 24 h; -4°C, 1 week; and -20°C, 1 week.

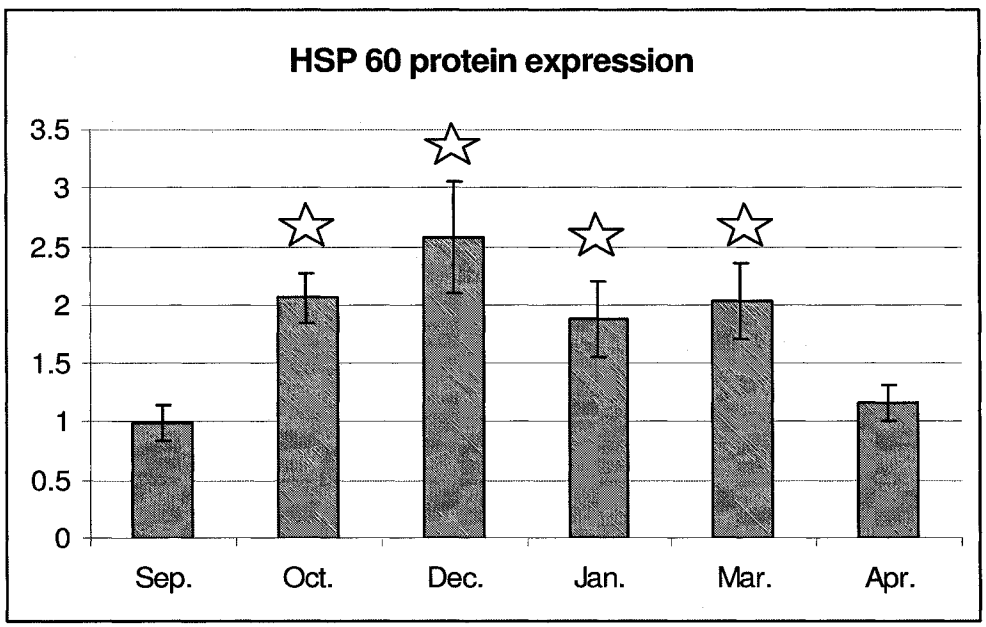
D. Histogram showing relative expression of HSP60 in aerobic control (+4°C, 2 weeks) versus anoxic (1 h, 4 h, or 24 h at 4°C) conditions.

Note: All data are means ± S.E.M. (n=4). ☆– Mean value is significantly different from the corresponding September or control value as determined by the Student's t-test, $P<0.05$.

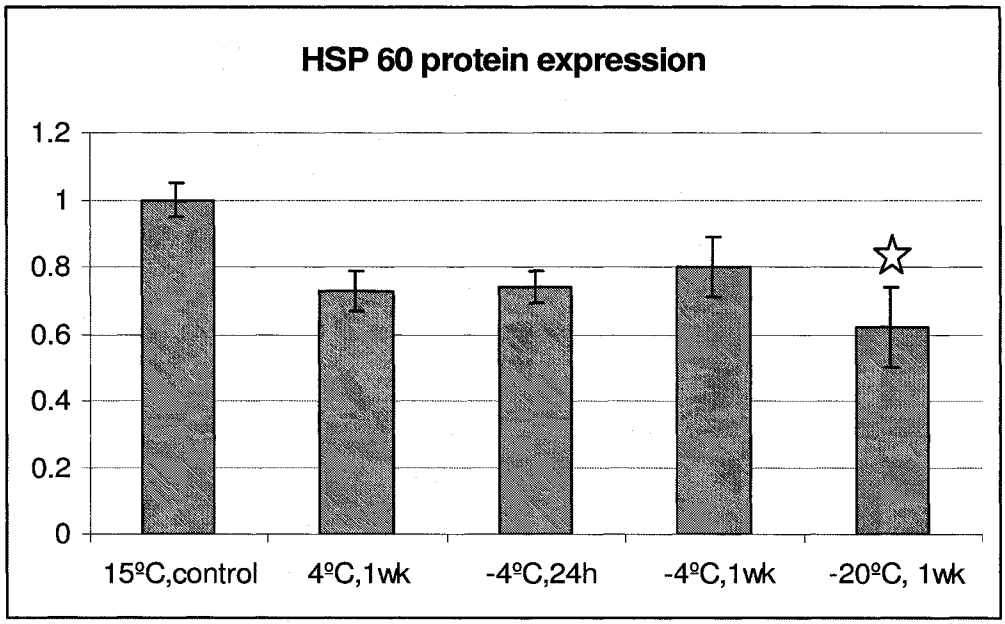
A.



B.



C.



D.

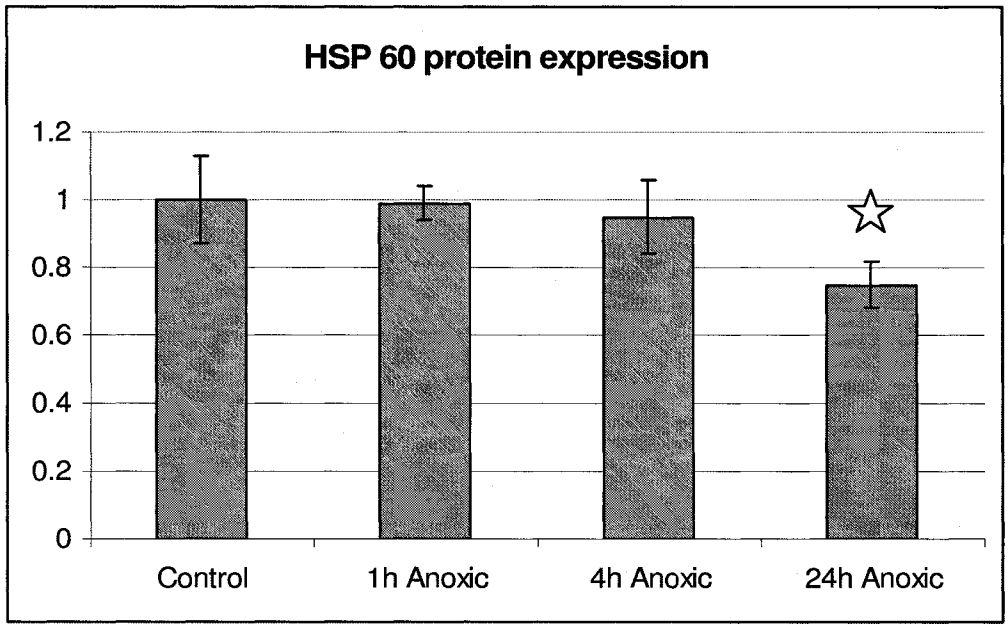


Fig. 3.10 HSP40 protein expression in *E. scudderiana* larvae.

A. Representative Western blots showing total HSP40 levels over the winter, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; An – Anoxia.

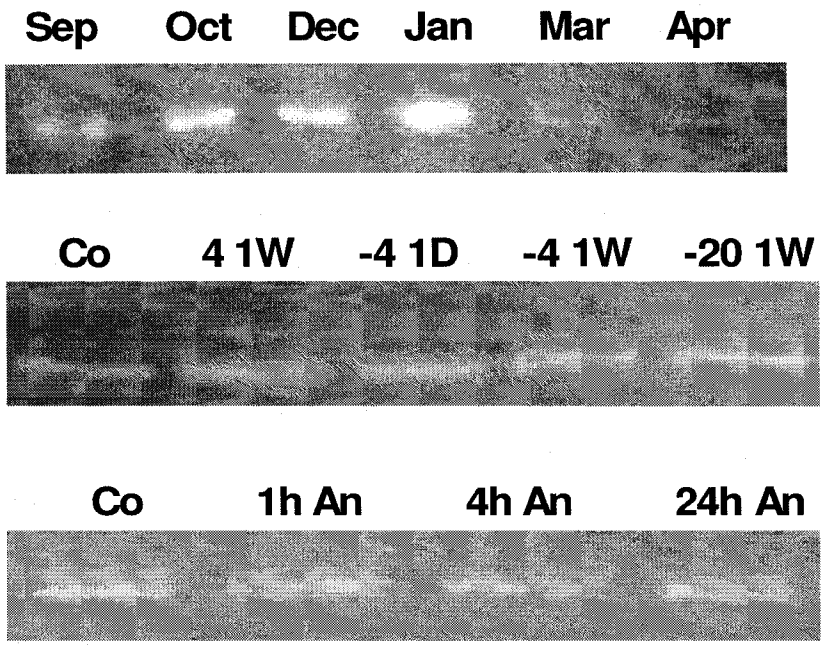
B. Histogram showing relative expression (densitometric analysis) of HSP40 protein levels over the winter months outdoors.

C. Histogram showing relative expression of HSP40 value in the indoor laboratory experiments: 15°C, control; 4°C, 1 week; -4°C, 24h; -4°C, 1 week; and -20°C, 1 week.

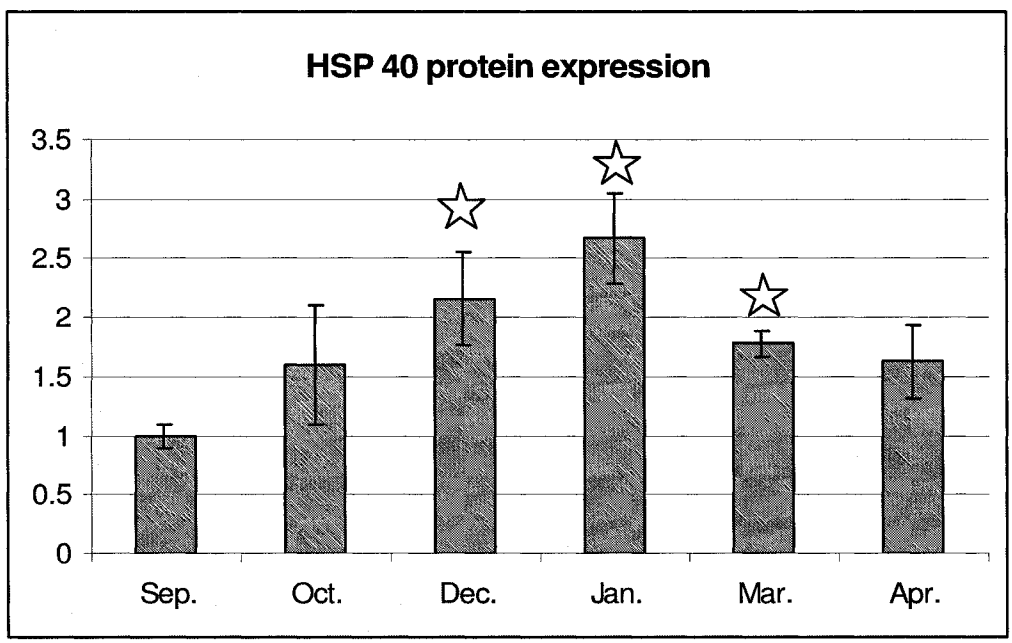
D. Histogram showing relative expression of HSP40 in aerobic control (+4°C, 2 weeks) versus anoxic (1 h, 4 h, or 24 h at 4°C) larvae.

Note: All data are means $\pm$ S.E.M. (n=4). ☆– Mean value is significantly different from the corresponding September value as determined by the Student's t-test, $P<0.05$.

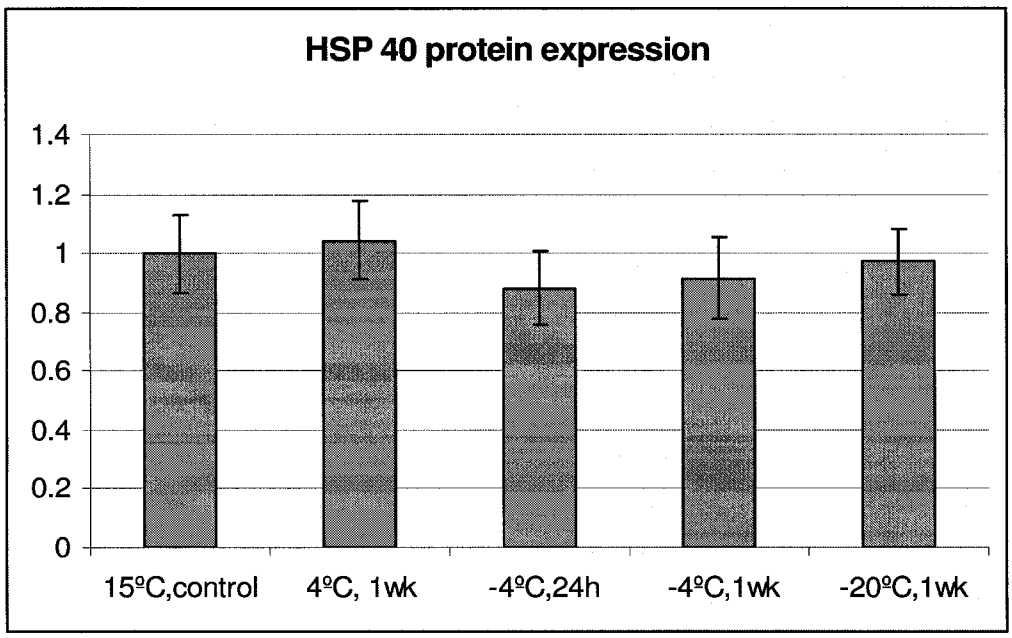
A.



B.



C.



D.

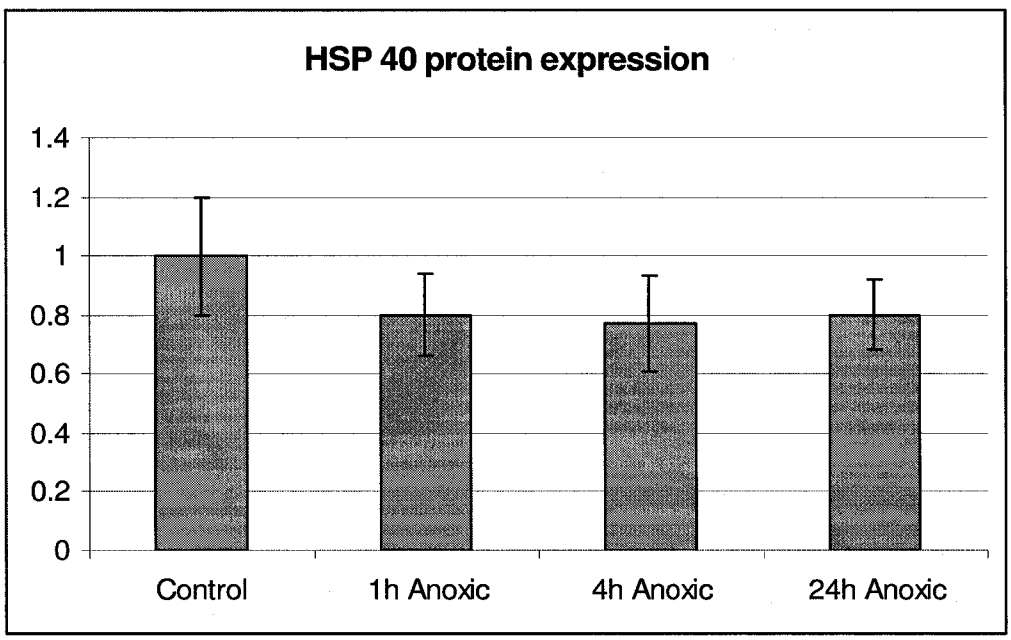


Fig. 3.11 HSP10 protein expression in *E. scudderiana* larvae.

A. Representative Western blots showing total HSP10 levels over the winter, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; An – Anoxia.

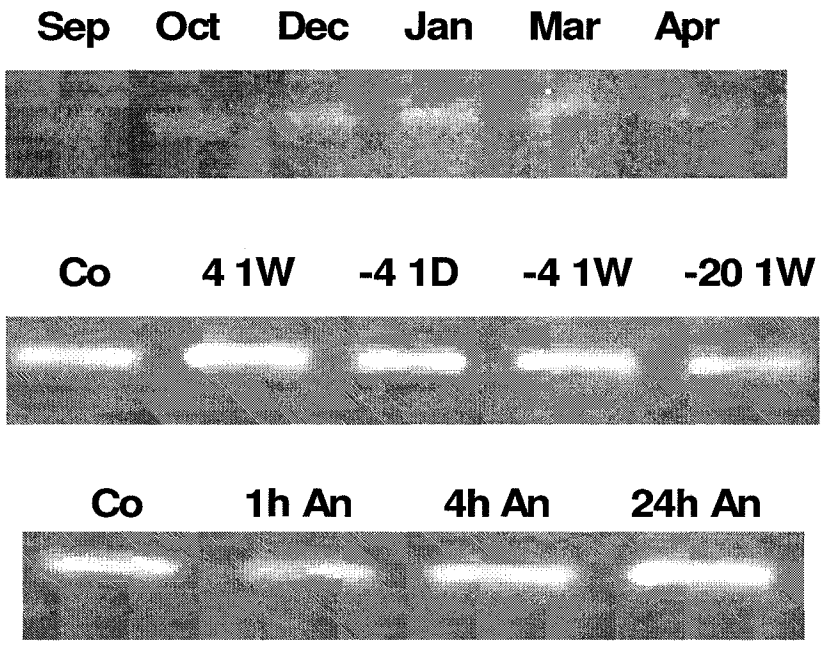
B. Histogram showing relative expression (densitometric analysis) of HSP10 protein levels over the winter months outdoors.

C. Histogram showing relative expression of HSP10 in indoor laboratory experiments: 15°C, control; 4°C, 1 week; -4°C, 24 h; -4°C, 1 week; and -20°C, 1 week.

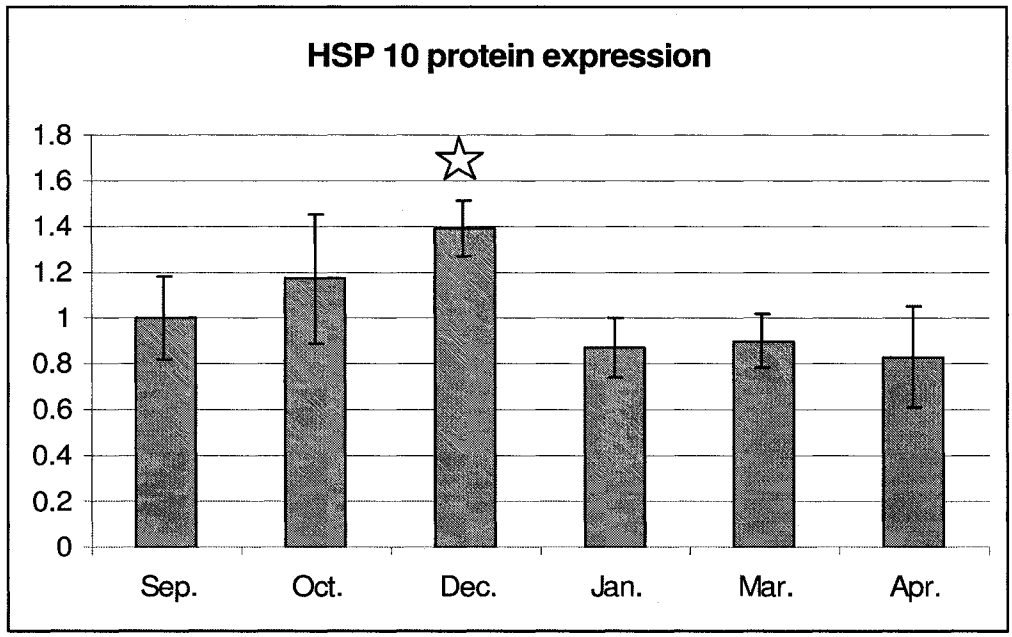
D. Histogram showing relative expression of HSP10 in aerobic control (+4°C, 2 weeks) versus anoxic (1 h, 4 h, or 24 h at 4°C) larvae.

Note: All data are means $\pm$ S.E.M. (n=4). ☆– Mean value is significantly different from the corresponding September or control value as determined by the Student's t-test, $P<0.05$.

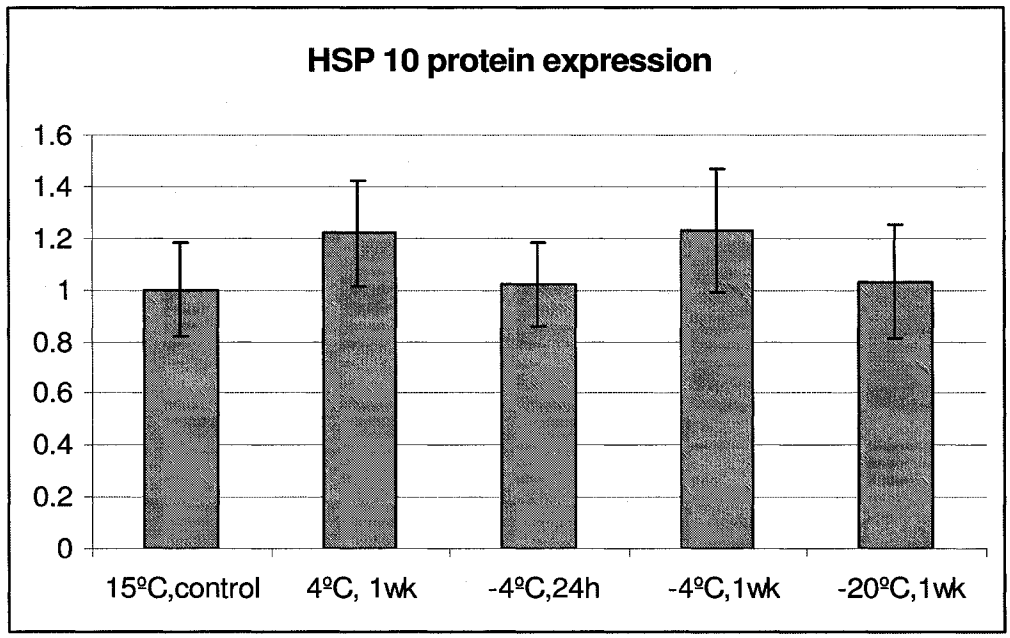
A.



B.



C.



D.

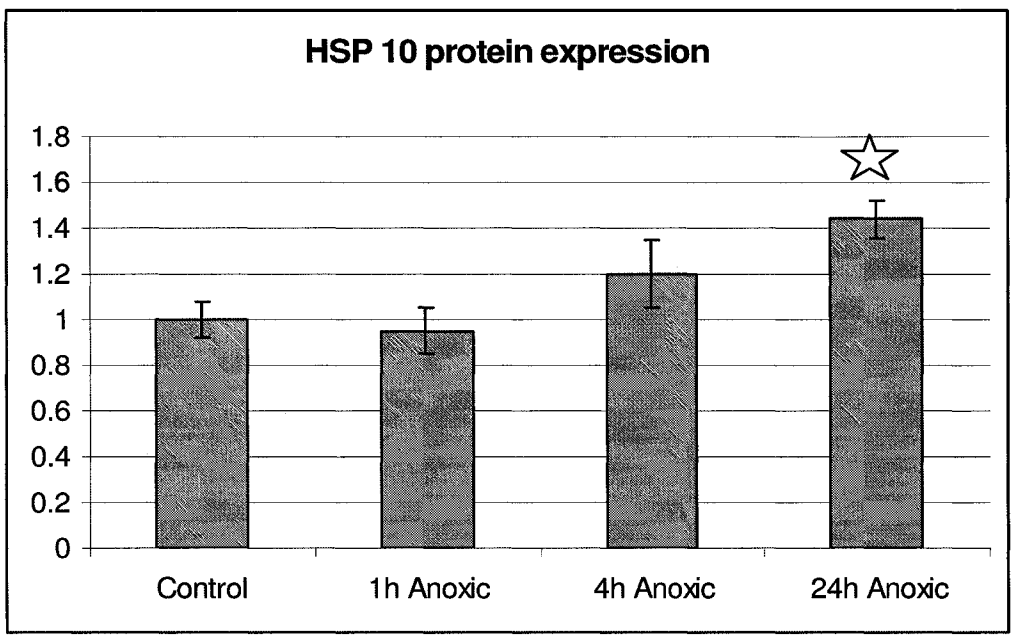


Fig. 3.12 HSF-1 protein expression in *E. scudderiana* larvae.

A. Representative Western blots showing total HSF-1 levels, both inactive (85 kDa) and activated (95 kDa) forms, over the winter months, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; An – Anoxia.

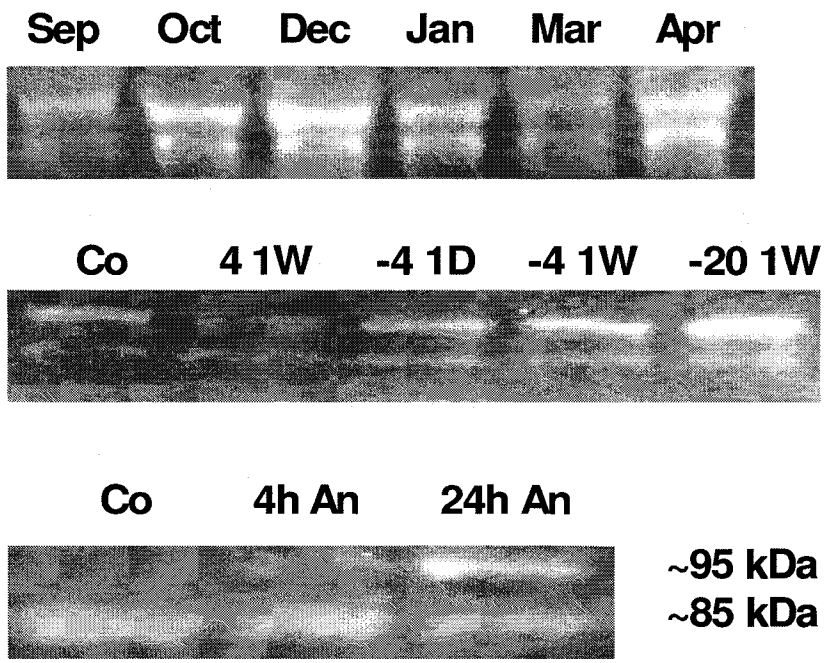
B. Histogram showing relative expression (densitometric analysis) of both inactive and activate HSF-1 protein levels over the winter months outdoors.

C. Histogram showing relative expression of both inactive and activate HSF-1 in indoor laboratory experiments: 15°C, control; 4°C, 1 week; -4°C, 24h; -4°C, 1 week; and -20°C, 1 week.

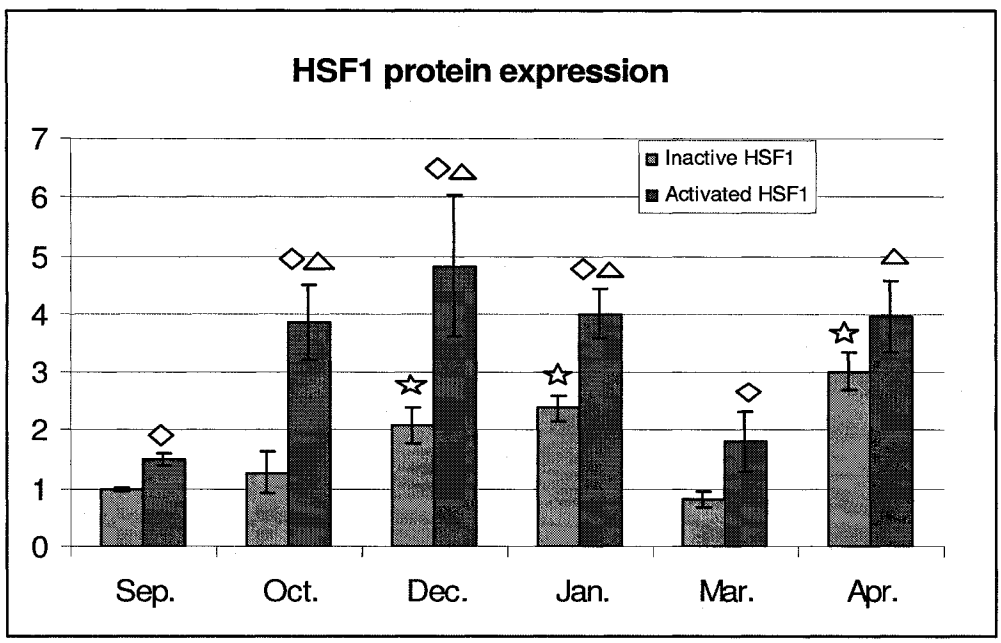
D. Histogram showing relative expression of both inactive and activate HSF-1 in aerobic control (+4°C) versus anoxic (4 h or 24 h) larvae.

Note: All data are means ± S.E.M. (n=4). ☆ – Mean value of inactive HSF-1 is significantly different from the corresponding September or control value, $P<0.05$; △ – Mean value of activated HSF-1 is significantly different from the September or the control value, $P<0.05$; ◇– Mean value of activated HSF-1 is significantly different from the corresponding inactive HSF-1, $P<0.05$.

A.



B.



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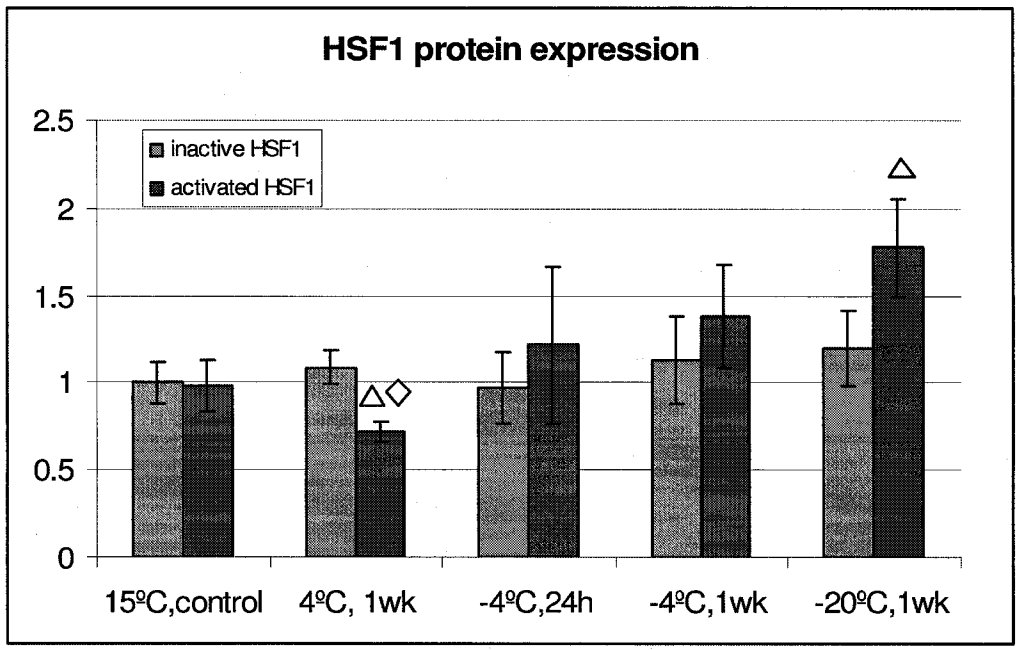
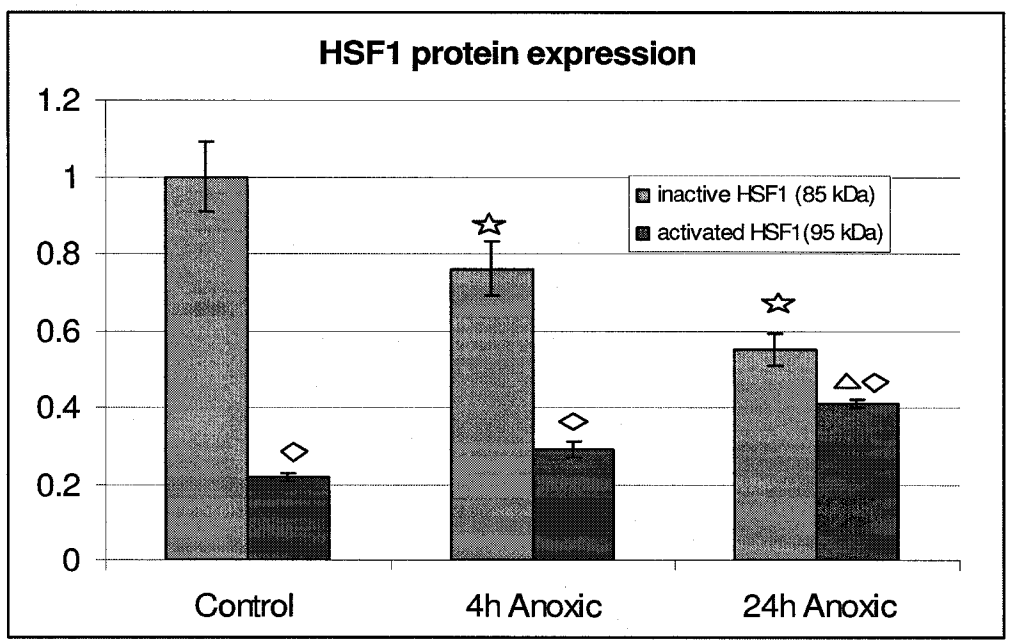


D.



CHAPTER 4

Glucose regulated proteins and other molecular chaperones

4.1 Introduction

Apart from HSPs, other groups of proteins also respond to environmental stresses. The glucose regulated proteins (GRPs) are one of these groups. The term encompasses a variety of endoplasmic reticulum (ER) chaperones that can be induced in cell culture under conditions of glucose starvation (Lee, 1992; Little et al., 1992). GRPs were first discovered in 1977 by Ira Pastan through the observation that two proteins of molecular sizes 78 and 94 kDa were strongly induced in chicken embryo fibroblasts cultured in a glucose free medium (Shiu et al., 1977). These proteins were subsequently identified as Grp 78 and Grp 94. Hypoxia exposure as well as other perturbations of ER function, such as agents that affect calcium stores or inhibit glycosylation, also trigger GRP synthesis. These stresses result in the accumulation of misfolded proteins in the ER, and the up-regulation GRP genes has been become known as a marker for the unfolded protein response (UPR). The principal GRPs now known in mammals are Grp 57, Grp75, Grp78, Grp94, and Grp170. These proteins have been shown to bind to newly synthesized, unfolded, and/or incompletely glycosylated proteins in the lumen of the ER, the mitochondria, and other compartments of cells.

The ER is an important site of biosynthesis for sterols, lipids, membrane-bound and secreted proteins, and glycoproteins. Approximately one-third of all cellular proteins are synthesized on the membranes of the rough ER (Matlack et al., 1999). Disruption of ER stability can directly stimulate apoptosis or cell death. This response is induced by "ER stresses" including accumulation of unfolded or misfolded proteins, depletion of

oxygen, glucose and ATP limitation (Lee, 2001). In mammals, the ER responds to the accumulation of unfolding proteins by triggering the production of ER-specific chaperones that include GRPs and other glycoproteins. The best-studied ER-resident chaperones are Grp78 and Grp94. Grp78 is evolutionarily conserved from yeast to humans, and Grp 94 is the most abundant glycoprotein in the ER. They play important roles in protein folding and secretion, and can confer protection against cell death by regulating signal transduction and protein translation under a variety of stress conditions (Gazit et al., 1995; Lee, 1987; Yoshida et al., 1998). These proteins have also proven to be markers for diseases that result from misfolding of secretory and transmembrane proteins (Hamman et al., 1998).

In our lab, Li (2004) working on wood frogs and Yan (2005) and Hapstaou (2006) working on ground squirrels have identified GRPs that are induced and play a role in supporting natural freeze tolerance and mammalian hibernation. So, I wondered whether GRPs would also be a part of the natural response to winter subzero temperatures by cold-hardy goldenrod gall insects, the freeze tolerant gall fly larvae, *E. solidaginis* and freeze avoiding gall moth larvae, *E. scudderiana*. In their role as chaperones these proteins could act to minimize the accumulation of unfolded/misfolded proteins under the cold and/or hypoxia stresses and aid the correct synthesis of specific freeze-induced proteins. Furthermore, to more fully understand the importance of chaperone-mediated protein folding to cold survival by these two insects, the responses by other two molecular chaperones, TCP-1 and α-cystallins, are also studied in this chapter.

4.2 Materials and methods

4.2.1 Antibodies

Grp 78, Grp 57 and TCP-1 antibodies were purchased from Stressgen Biotechnologies Corp. Grp 94, Grp 75, αA-crystallin, and αB-crystallin were purchased from Santa Cruz. Biotechnology, Inc. Anti-rabbit and anti-rat secondary antibodies were purchased from Cell Signaling Technology, Inc. The dilutions of stock antibodies used in the studies are listed in Table 4.1.

4.2.2 Western blotting

To separate, identify, and detect the expression levels of protein chaperones in *E. solidaginis* and *E. scudderiana* larvae, Western blotting was employed as described in see Chapter 2. Antibody were tested for specificity by the use of antigene peptides and/or 2D gels (the data were not shown). The gels and the conditions used are summarized in Table 4.1.

4.2.3 Statistical analyses

Selected band intensities were quantified and plotted using the GeneTools (see Chapter 2) and Excel programs. The final results are shown as the relative expression levels of different chaperones in each group under each experimental condition. Statistical testing compared normalized experimental band intensities (n=3-5) and normalized control values (n=3-5) using the Student's t-test with significant differences

accepted when $P<0.05$.

4.3 Results

Mammalian antibodies raised against Grp 170, Grp 94 Grp 78, Grp 75, Grp 57, and other protein chaperones (TCP-1, αA-crystallin, and αB-crystallin) were used to evaluate the chaperone responses in cold-hardy insects under three types of experimental conditions: (a) winter profiles of outdoor larvae sampled at multiple times from September to April, (b) laboratory acclimation of larvae to low temperatures, and (c) anoxia exposure. Except for Grp 57 which was not detectable using the rabbit anti-Grp 57 antibody, all other GRPs as well as TCP-1 and crystallins were detected in at least one of the insect species with mammalian antibodies under the conditions used (Table 4.1). The Grp 78, Grp 75, TCP-1, αA-crystallin and αB-crystallin antibodies worked with *E. solidaginis* extracts whereas Grp 170, Grp 94, Grp 78 and TCP-1 worked with *E. scudderiana* larval extracts.

4.3.1 Grp 78 in *E. solidaginis*

The rabbit anti-Grp 78 polyclonal antibody recognized an ~78 kDa band on blots of *E. solidaginis* proteins. Figure 4.1A shows representative Western blots for inducible Grp 78 expression in the freeze tolerant species under the experimental regimes. Figure 4.1B shows the relative expression levels of Grp 78 (± SEM, n=3) over the course of the winter in larvae sampled from outdoors between September and April. The protein

expression pattern showed a significant increase in Grp 78 content over the midwinter months; levels were 1.82, 1.93, 1.81, and 1.67 fold higher than September values in October, December, February, and March, respectively ($P<0.05$). Grp 78 content decreased again in April.

Figure 4.1C shows Grp 78 levels in larvae that were exposed to the following low temperature and freezing regimen: (a) controls were larvae acclimated at 15°C, (b) larvae were then shifted acutely from 15° down to 3°C and cold-exposed for 24 hours, (c) larvae were then shifted acutely from 3°C down to –16°C and were frozen for 24 hours, and (d) larvae were then transferred back to +3°C and were sampled after 24 hours thawed. Compared with control larvae, Grp 78 levels were stable after 3°C exposure for 24 hours, and after freezing at –16°C for 24 hours. However, thawing resulted in a dramatic decrease in Grp 78 content, falling to 47% ($P<0.05$) of the control value and to 49% ($P<0.05$) of the value in frozen larvae.

Figure 4.1D shows the changes in Grp 78 expression levels in *E. solidaginis* under anoxic conditions. No change in Grp 78 expression level was seen after 4 hours of anoxia exposure, but levels increased significantly by 1.43-fold after 24 hours of anoxia exposure ($P<0.05$).

4.3.2 Grp 75 in *E. solidaginis*

The goat anti-Grp 75 polyclonal antibody recognized an ~75 kDa protein in *E. solidaginis* extracts. Figure 4.2 A shows representative Western blots for inducible Grp 75

under the three experimental procedures. Histograms showing mean Grp 75 (± SEM, n=3) expression in larvae over the winter-time course are shown in Figure 4.2 B. The level of Grp 75 increased gradually, being 1.31 fold higher in October than in September (not significantly different) and increased further to 1.79 fold higher in December ($P<0.05$). Levels had decreased somewhat by February (1.32 fold higher than September, but not significantly different) and returned to September values in March and April.

Figure 4.2 C shows the response of Grp 75 to subzero exposure. Compared with controls (15°C, 1 wk), Grp 75 levels were unchanged by 24 hours of cold exposure at 3°C. However, levels increased significantly (1.78 fold, $P<0.05$) during freezing (–16°C, 24 hours) and then decreased again when larvae were thawed. Figure 4.2 D analyzes the effects of anoxia exposure (1, 4 or 24 hours) on the expression level of Grp 75 in *E. solidaginis* larvae. No significant effects of anoxia exposure were found.

4.3.3 TCP-1 in E. *solidaginis*

The rat anti-TCP-1 monoclonal antibody recognized an ~60 kDa protein on blots of *E. solidaginis* extracts. Figure 4.3 A shows representative Western blots for inducible TCP -1 under the three experimental procedures. Changes in TCP-1 levels over the winter months are shown in Figure 4.3 B. Compared with September values, TCP-1 protein levels were significantly increased in October and December (by 1.41 and 1.40 fold; $P<0.05$), but then fell again to control (September) levels over the rest of the winter.

Figure 4.3 C shows TCP-1 expression in the larvae over the cycle of cooling,

freezing and thawing. TCP-1 levels were unchanged under chilled and frozen treatments, but increased strongly during thawing (1.54 fold higher than controls and 2.02 fold higher than freezing). Figure 4.3 D shows the effects of anoxia exposure on TCP-1 levels. Expression was not significantly different from the control value after either 4 or 24 hours of anoxia.

4.3.4 αA-crystallin in E. *solidaginis*

The commercial polyclonal antibody raised against αA-crystallin from goat cross-reacted with a protein in *E. solidaginis* extracts of the proper size of ~20 kDa. Figure 4.4 A shows representative Western blots of αA-crystallin expression under the different experimental conditions. Figure 4.4 B shows the mean levels of αA-crystallin ($\pm$ SEM, n=4) in larvae sampled from outdoors over a winter season. αA-crystallin protein levels were unchanged during the winter months except for a rise in March to a level that was 1.71 fold higher ($P<0.05$) compared with the September value.

Figure 4.4 C shows αA-crystallin expression levels in *E. solidaginis* over a cycle of cooling, freezing and thawing. Protein levels were unchanged when larvae were cold exposed at 3°C or frozen at -16°C, as compared with controls, but αA-crystallin levels increased significantly ($P<0.05$) by 1.63-fold when the larvae were thawed at 3°C. Figure 4.4 D shows the effects of anoxia exposure on levels of αA-crystallin in the larvae. Short term anoxia had no effect but levels rose by 2.0 fold after 24 hours of anoxia exposure ($P<0.05$).

4.3.5 αB-crystallin in E. *solidaginis*

The commercial polyclonal antibody raised against αB-crystallin from goat cross-reacted with a protein in *E. solidaginis* extracts of the proper size of ~20 kDa. Figure 4.5 A shows representative Western blots of αB-crystallin expression under the different experimental conditions. Figure 4.5 B shows the mean levels of αB-crystallin (± SEM, n=4) in larvae sampled from outdoors over a winter season. Only in March was a significant change seen; levels were 1.84 fold higher than in September ($P<0.05$).

Figure 4.5 C shows αB-crystallin expression levels in the larvae over a cycle of cooling, freezing and thawing. Levels of αB-crystallin were unchanged when larvae were cold exposed at 3°C or frozen at -16°C, but protein content increased significantly ($P<0.05$) by 1.63-fold when the larvae were thawed at 3°C. Figure 4.5 D shows the effects of anoxia exposure on the expression levels of αB-crystallin in the larvae. Short term anoxia had no effect but αB-crystallin levels rose by 1.89 fold after 24 hours of anoxia exposure ($P<0.05$).

4.3.6 Grp 170 in *E. scudderiana*

Comparable sets of studies evaluated chaperone protein expression levels in the freeze-avoiding insect *E. scudderiana* and, as for the freeze tolerant *E. solidaginis*, these studies included a profile of protein levels over the winter months as well as a time course of anoxia exposure (1, 4 and 24 h at 4°C). The low temperature acclimation

experiment with this species followed a different course involving acclimation of controls at 15°C, followed by 1 week at 4°C, then 1 day and 1 week at -4°C, and finally 1 week at -20°C. Note that neither subzero exposure causes freezing of this species.

The rabbit anti-Grp 170 antibody recognized an ~170 kDa protein in *E. scudderiana* extracts. Figure 4.6 A shows representative Western blots for Grp 170 expression levels under the three experimental procedures. Figure 4.6 B shows that Grp 170 expression ($\pm$ SEM, n=4) was elevated over the midwinter months. The level of Grp 170 increased gradually, being 1.35 fold higher in October than in September (not significantly different) and increased further to 2.82 fold higher in December, 2.38 fold higher in January and 3.08 fold higher in March (all $p<0.05$). Levels then declined sharply in April, falling to 78% of the September value.

Figure 4.6 C shows Grp 170 expression levels in the larvae during laboratory cold exposures. Chilling at 4°C did not affect the protein but subzero temperatures stimulated an increase resulting in a 1.40-fold increase in Grp 170 after 1 week at -4°C and a 1.80 fold rise after 1 week -20 °C ($P<0.05$). Figure 4.6 D analyzes the effects of anoxia exposure (1, 4 or 24 hours) on the expression level of Grp 170 in *E. scudderiana* larvae. Grp 170 did not change after 1 hour anoxia exposure, but levels increased strongly after 4 and 24 hours of anoxia (1.72 and 1.83 fold higher than controls, respectively, $P<0.05$).

4.3.7 Grp 94 in *E. scudderiana*

The goat anti-Grp 94 antibody recognized an ~94 kDa band on *E. scudderiana*

blots. Figure 4.7 A shows representative Western blots for inducible Grp 94 under the three experimental procedures. Histograms showing mean Grp 94 (± SEM, n=3) expression in larvae over the winter-time course are shown in Figure 4.7 B. Expression was unchanged in the autumn (October) compared with September levels but the protein was elevated during the mid-winter months (December, January, March) by 1.37, 1.66 and 1.81 fold as compared with September values ($P<0.05$). Grp 94 levels fell in April to a level not significantly different from the September value.

Figure 4.7 C shows the effect of low temperature exposures on Grp 94 in *E. scudderiana*. A increasing trend was noted but cold exposure for as long as 1week at -20°C had no statistically significant effect on Grp 94 levels. Figure 4.7 D analyzes the effects of anoxia on Grp 94 levels. No significant effects of anoxia were found.

4.3.8 Grp 78 in *E. scudderiana*

The rabbit anti-Grp 78 polyclonal antibody recognized an ~78 kDa fragment in *E. scudderiana* extracts. Figure 4.8 A shows representative Western blots for inducible Grp 78 under the three experimental procedures. Grp 78 expression levels were largely unchanged over the winter months (Figure 4.8 B). Only in December was a significant change seen with protein content rising to 1.65 fold higher than in September ($P<0.05$).

Figure 4.8 C shows that Grp 78 expression levels increased strongly in response to cold temperature exposure. Grp 78 levels had increased significantly by 1.96 fold after 1 week at 4°C and rose to even higher values, 2.20-2.46 fold higher than controls, after 1

day or 1 week at -4°C, or 1 week at -20°C (all $P<0.05$). Figure 4.8 D shows the effect of anoxia exposure on Grp 78 expression in *E. scudderiana* larvae. Compared with controls, a short anoxia exposure led to reduced expression of Grp 78; mean levels of the protein were 59% of the control value ($P<0.05$) after 1 hour of anoxia. However, with longer anoxia exposure, Grp 78 levels returned to control value.

4.3.9 TCP-1 in E. *scudderiana*

The rat anti-TCP-1 monoclonal antibody recognized an ~60 kDa protein band on *E. scudderiana* blots. Figure 4.9 A shows representative Western blots for TCP -1 under the three experimental procedures. Figure 4.9 B shows the mean changes (± SEM, n=3) in TCP-1 protein expression in *E. scudderiana* over the winter months. TCP-1 expression was unchanged over the autumn (October) but increased during the midwinter months (December and January) to levels 1.66 and 1.40 fold higher than September values ($P<0.05$). Protein levels declined again in March and April.

Figure 4.9 C shows TCP-1 expression in the larvae in response to low temperature exposures. TCP-1 levels were unchanged by any of the laboratory low temperature acclimations. Figure 4.9 D shows the effects of anoxia exposure on TCP-1 in *E. scudderiana* larvae under anoxic conditions. Expression was not significantly different from the control value after 1, 4 or 24 hours of anoxia exposure.

4.4 Discussion

The ER is a perinuclear, cytoplasmic compartment where proteins and lipids are synthesized. When cells are subjected to physiological stress conditions that affect the ER, they respond by activating a defense mechanism referred to as the UPR, a mechanism evolutionarily conserved from yeast to human (Patil et al., 2001; Schroder et al.,2005). A major cellular target of the UPR is the resident ER chaperone proteins (Rutkowski and Kaufman, 2005). These facilitate proper protein folding, maintain proteins in a folded state, and prevent protein folding intermediates from aggregating. Grp 78 is the best characterized of these chaperones. It was first discovered as a 78 kDa protein whose synthesis was enhanced in cultured cells grown in medium that was deprived of glucose (Shiu et al., 1977). Subsequently, Grp 78 was determined to be an ER resident protein whose synthesis is stimulated by a variety of environmental and physiological stress conditions that perturb ER function and homeostasis (Lee, 1992; Little et al., 1992). Grp 78 is a member of the HSP 70 family of molecular chaperones that are involved in the folding and assembly of newly synthesized proteins (Kozutsumi et al., 1988) and is also commonly referred to as BiP, the immunoglobulin heavy chain-binding protein. BiP was originally found to bind to the immunoglobulin heavy chains of pre-B cells. Grp 78 is essential for the proper glycosylation, folding and assembly of many membrane bound and secreted proteins (Lee, 2001) and is now known to be critical for maintenance of cell homeostasis and the prevention of apoptosis (Yang, et al., 2000). Furthermore, Grp 78 accounts for most of the calcium buffering capacity of the ER (Fasolato et al. 1998). Grp 78 is ubiquitously expressed at a basal level but overproduced in cells of ischemic tissues,

as well as in cultured cells exposed to hypoxia or glucose starvation (Lee, 1992; Little et al., 1992; Lee, 2001). Grp 78 levels have been shown to be a reliable biomarker for the onset of ER stress and the UPR (Hong et al., 2005).

In our studies, Grp 78 was detected in both *E. solidaginis* and *E. scudderiana* under all experimental conditions tested (outdoor winter timecourse, laboratory cold exposures, and anoxia stress). This strongly suggests that Grp 78 has a role to play in the adaptive response to cold and anoxic stresses since it responded to environmental stresses that are naturally encountered by both species.

Grp 78 levels were elevated in both insect species over the midwinter months, particularly in December, suggesting that Grp 78 has a natural role to play in the winter survival of both insect species at cold temperatures. In the indoor cold stress experiments, similar results were found for *E. scudderiana*; Grp 78 levels rose significantly with all cold exposures (including 1 week at 4, -4, or -20°C). However, in *E. solidaginis,* Grp 78 levels were not affected by experimental freezing (-16°C, 24 h) although Grp78 levels decreased during thawing (24 h back at 3°C after -16°C exposure). This different response between the two species to subzero temperature exposure may be related to their freeze tolerant versus freeze avoiding strategies of cold hardiness. *E. scudderiana* supercools and remains liquid down to about -40°C. As such, protein synthesis can still occur at 4, -4 or even -20°C and so the larvae can enhance their GRPs levels as needed when temperatures fall. However, *E. solidaginis* larvae freeze below about -8°C and freezing places cells under an energy-stressed state. ATP-expensive processes such as

protein synthesis are typically suppressed in the frozen state. Hence, if Grp 78 is needed for winter survival (as Fig. 4.1 B suggests), then a trigger other than subzero temperature exposure would be needed in order to ensure that Grp 78 accumulates before the larvae freeze and protein synthesis becomes restricted. Interestingly, Grp 78 levels were decreased when *E. solidaginis* larvae thawed. A possible reason for this could be a down regulation of ER-resident stress proteins in response to oxidative damage (Paschen et al., 2001). When the larvae thaw and oxygen supply is restored, the insects will undergo oxidative stress, and oxidation blocks the ER resident stress response. Our data agree with Paschen (2001) and Dey (2006) who reported that oxidative stress triggered a significant decrease in Grp 78 expression in cells. On the other hand, the data showing that Grp 78 protein levels were significantly increased after 24 h anoxia exposure *E. solidaginis* could suggest that falling oxygen levels is the trigger for Grp 78 production in this freeze tolerant species. Hence, the natural trigger for Grp 78 synthesis in *E. solidaginis* may be oxygen limitation. Interestingly, however, anoxia did not affect Grp 78 expression in *E. scudderiana* after either 4 or 24 h of exposure.

Grp 94 (also known as gp96 and Erp 99) is a member of the HSP 90 family of proteins and is another stress-inducible protein that is one of the most abundant and well-characterized ER molecular chaperones. Grp 94 has been studied extensively because it is involved in antigen processing and has a potential use in immune therapy (Argon and Simen, 1999; Nicchitta et al., 2004; Gidalevitz et al., 2004). However, the

physiological roles of Grp 94 are far less understood. As a molecular chaperone, Grp 94 can bind to malfolded proteins and unassembled complexes (Kozutsumi et al., 1988) that accumulate under stress conditions and are posttranscriptionally modified into biologically inactive forms after removing the stress (Argon and Simen, 1999). The promoter of the *grp94* gene is well-conserved and can interact with several transcription factors to induce transcription (Little et al., 1994; Yoshida et al., 2001; Parker et al., 2001). Paris et al. (2005) found that there are three hypoxia response elements (HRE) in the human Grp 94 promoter and Grp 94 was up-regulated in endothelial cells in response to hypoxia. Jeon et al. (2004) suggested that Grp 94 may stabilize the astroglial cytoskeleton and participate in astroglial antioxidant mechanisms.

In the present studies, Grp 94 was detected only in *E. scudderiana,* and its levels increased significantly over the midwinter months in larvae sampled from outdoors. This indicates that the protein has a role to play in the winter survival of the species, perhaps in long term protein stabilization. However, lab experiments indicated that neither low temperature nor anoxia exposures triggered Grp 94 expression. Hence, winter accumulation of this protein might not be directly triggered by environmental factors (temperature, oxygen) but instead might be an innate response. Both *E. scudderiana* and *E. solidaginis* have a one-year life cycle that follows a strong genetic program. Once the final instar larva reaches full size in the autumn, it enters a state of arrested development called diapause that lasts for 3-4 months (Irwin et al., 2001). Diapause is characterized by strong metabolic rate depression that suppresses all metabolic functions, including

protein synthesis and protein degradation (Denlinger, 2002); hence, protein turnover is greatly reduced. Enhanced expression of chaperone proteins could be an important part of survival during diapause, providing a way to stabilize and protect cellular proteins over the long term. Indeed, production of various HSPs is a feature of diapause in other insect species (Denlinger, 2002).

The present data also show that both Grp 94 and Grp 78 are increased in *E. scudderiana* over the winter months outdoors (particularly in December), suggesting the two may be coordinately up-regulated to deal with winter conditions. Indeed, similar to the present results, Liu et al. (1991) indicated Grp 94 and Grp 78 are transcriptionally correlated due to similar control elements.

Grp170, which is also known as oxygen-regulated protein (Orp150), is another ER-resident chaperone that is induced by hypoxia/ischemia stress. Ozawa (1999) suggested that Orp 150 has an important cytoprotective role in hypoxia-induced cellular perturbation. Yoshitane et al. (1998) found that Grp170 was up-regulated in human breast tumors compared with normal breast tissue. In addition to a protective role for cells under stress conditions, Grp170 also contributes to the import of proteins and peptides and is known to be an efficient ATP-binding protein (Dierks et al., 1996). Grp170 is also involved with peptide transport into the ER via the transporter associated with antigen processing, suggesting that Grp170 may be involved in the antigen presentation pathway (Spee et al., 1999).

Grp 170 was not detected on blots of *E. solidaginis* extracts, but was found in *E. scudderiana*. Grp 170 levels were elevated throughout the winter from December to March, which suggests that Grp 170 has an important role to play in winter survival. Grp 170 levels also rose after laboratory cold exposure at -20°C for 1 week, which suggests that Grp 170 protein may be directly responsive to cold temperatures. Furthermore, Grp 170 also increased after 4 and 24 hours of anoxia exposure, consistent with the known response of the protein to low oxygen in other systems.

Considering all the up regulated ER-resident GRPs (Grp 170, Grp 94, and Grp 78) in *E. scudderiana* during the winter months, the present results indicate that GRP induction is a protective response mechanism by which the cells of *E. scudderiana* larvae adapt to survive winter conditions that would otherwise be injurious or lethal.

Grp75 (also known as Mortalin-2/mthsp70/PBP74), a member of the Hsp70 family of proteins, has been localized to mitochondria, ER, plasma membrane (Shin et al., 2003), and cytoplasmic vesicles (Domanico et al., 1993; Singh et al., 1997) but mitochondria seem to be the dominant location (Ran et al., 2000). Mizzen et al. (1991) showed that Grp75 interacts with and facilitates the folding and assembly of proteins as they enter the mitochondria. The protein is also required for degradation of misfolded peptides in mitochondria (Liu et al., 2001). Evidence suggests that Grp 75 cooperates with Hsp 60 and Hsp 10 chaperones for folding of imported proteins into functionally competent forms in the mitochondria. (Cechetto *et al.*, 2000; Strub *et al.*, 2001). Recent

studies have shown that Grp 75 also binds other proteins such Grp 94 and p53 (Takano et al., 2001; Wadhwa et al., 2002). The level of Grp 75 protein can be enhanced by exposure to low levels of ionizing radiation, glucose deprivation (Merrick et al., 1997), and calcium ionophore (Massa et al., 1995). In humans, Grp75 is up-regulated in tumours and in transformed and tumour cell lines (Kaul et al., 1998; Taknao et al., 1997; Bini et al., 1997). Taken together, these studies have shown that overexpression of Grp 75 confers proliferation-growth advantage to cells in vitro and in vivo (Wadhwa et al., 2002).

The present study of cold-hardy insects found that Grp 75 was only detectable in *E. solidaginis*. Levels were significantly elevated in the larvae in December and the protein also increased significantly in response to experimental freezing (-16°C, 24 h). This suggests that Grp 75 has a natural role to play in the winter survival of these freeze tolerant larvae. Furthermore, the strong response of Grp 75 to freezing at -16°C suggests that the protein may have a particular function when freezing occurs. Such an action could include facilitating the correct folding/assembly of specific freeze responsive proteins or a more general role in the protection/preservation of proteins (perhaps especially mitochondrial proteins) under the cellular conditions of the frozen state. In particular, ice formation in extracellular compartments causes cellular dehydration and a major reduction in cell and organelle volumes that places compression stress on membranes. This could have a serious effect on the import and assembly of mitochondrial proteins and, therefore, might require enhanced levels of Grp 75 chaperone to guide this process. Interestingly, although Grp 75 is known to cooperate with Hsp 60

and Hsp 10 in other systems, the studies reported in Chapter 3 showed that levels of the mitochondria-resident HSP 60 were strongly reduced in *E. solidaginis* larvae over the winter months. Hence, Grp 75 may assume a greater relative importance in the folding/assembly mitochondrial proteins during the winter months.

Interesting, the data in Figure 4.2 C show that Grp 75 levels were reduced again when the larvae were thawed (24 h back at 3°C after -16°C exposure). This finding is similar to the pattern of Grp 78 expression in *E. solidaginis*. It is possible that both proteins might be degraded under oxidative stress occurring after thawing. Alternately, since anoxia did not affect Grp 75 (Fig. 4.2 D), it is possible that Grp 75 simply responds directly response to either cold temperatures or freezing and that when these conditions are reversed, the levels of Grp 75 are reduced.

Tailless complex polypeptide 1 α (TCP-1 α) is a 60 kDa subunit of a cytosolic hetero-oligomer chaperone that is known to be involved in the folding of action and tubulin(LIorca, *et al.*, 1999: Liorca, *et al.*, 2001; Martin-Benito *et al.*, 2002). Recent studies show that TCP-1 (also called Tric/CCT) also binds to numerous non-cytoskeletal substrate proteins (Dunn *et al.*, 2001; Spiess *et al.*, 2004). Like another chaperonin, HSP 60, TCP-1 can assemble into the shape of a toroid, usually composed of two rings placed back to back (Carrascosa et al., 2001). TCP-1 is also functionally similar to HSP 60, assisting in the folding of proteins upon ATP hydrolysis (Gomez-Puertas, *et al.*, 2004). However, TCP-1 can function alone whereas HSP 60 functions with its co-chaperone,

HSP 10. TCP-1 transiently interacts with ~10% of newly synthesized proteins and the overall expression of TCP-1 in mammalian cells is primarily dependent on cell growth (Yokota et al., 1999). TCP-1 has not been considered a stress inducible protein as a result of the limited information on it (Ursic *et al.*, 1992; Soares *et al.*, 1994). However, some studies have shown that heat and chemical stress can induce the TCP-1 expression (Schena, *et al.*, 1996; Yokota, *et al.*, 2000) and recent studies have shown that cold stress also induces TCP-1 expression (Somer *et al.*, 2002; Kayukawa, *et al.*, 2005). The eukaryotic chaperonin TCP-1 ring complex is indispensable for cell survival because the folding of an essential subset of cytoskeletal proteins (e.g. actin, tubulin) requires TCP-1, and this function cannot be substituted by other chaperones (Spiess *et al.*, 2004). This specificity indicates that TCP-1 has evolved structural and mechanistic features that distinguish it from other chaperones.

TCP-1 expression levels increased significantly in both *E. solidaginis* and *E. scudderiana* over the winter months in the outdoor population, particularly in December, which suggests that TCP-1 has a role to play under natural cold stress. Laboratory subzero temperature exposures (24 h at –16°C for *E. solidaginis* or 1 week at –4°C or -20 °C for *E. scudderiana*) did not directly affect the expression of TCP-1 in either species, nor did anoxia exposure, which may argue that these environmental stresses do not directly trigger expression of this chaperone. However, TCP-1 levels did increase strongly when *E. solidaginis* larvae were thawed and this fits well with a recent study of CCT in yeast. Somer et al. (2002) found that CCT is a cold shock protein in

Saccharomyces cerevisiae; Northern blot analysis showed that mRNA transcript levels rose when yeast were given cold exposure at 4°C but Western blots showed that levels of CCT subunits did not increase until yeast were returned to a higher temperature (10°C). A similar pattern may occur in *E. solidaginis*; subzero freezing exposures may trigger mRNA transcription with translation and protein accumulation occurring only after thawing. Hence, day/night cycles of temperature change could then account for TCP-1 accumulation in both species over the midwinter months as well as for TCP-1 elevation when *E. solidaginis* larvae were transferred back from -16°C (frozen) to 3°C (thawed). Since actin and tubulin are the major substrates for TCP-1, and cold injuries can cause depolymerization of these cytoskeletal proteins, Somer et al. (2002) hypothesized that upregulation of TCP-1 is related to the reorganization of actin and tubulin monomers during the recovery from cold stress.

Crystallins were originally found in the lens of the vertebrate eye, where they maintain the transparency and refractive index of the lens (Jaenicke et al., 2001). Crystallins are divided into α, β and γ families, and the α-crystallin group consists of three gene products, αA, αB, and αC-crystallin, which are members of the small HSPs (Klemenz et al., 1991; Narberhaus et al., 2002). Although the α-crystallins are major components of the lens, they are now known to occur in a variety of other tissues (Iwaki et al., 1989; Longoni et al., 1990; Kato et al., 1991; Srinivasan et al., 1992). α-Crystallins and β-crystallins both act as molecular chaperones to suppress thermal, oxidative and

UV-induced aggregation of other crystallins and proteins in the lens (Horwitz et al., 1992; Wang et al., 1995; Borkman et al., 1996). As chaperones, α-crystallins can hold denatured proteins in large soluble aggregates but unlike other molecular chaperones, α-crystallins do not renature these proteins (Narberhaus et al., 2002). Studies show that α-crystallins can stabilize denatured proteins and transport them to another chaperone, which is not likely to be present in the vicinity of the damaged protein (Wang and Spector, 2000). Hence, α-crystallins act co-operatively with other chaperones to renature stressed proteins.

In *E. solidaginis,* both αA-crystallin and αB-crystallin were responsive to all experimental conditions tested; levels rose in late winter in the outdoor timecourse, and in response to laboratory freeze/thaw and anoxia exposures. Furthermore, the expression profiles of αA- and αB-crystallins were virtually identical, both qualitatively and quantitatively, suggesting that they are co-regulated in *E. solidaginis*. However, the winter expression profile of both crystallins (significantly higher levels in March) was different from that of the other chaperones which rose in early and mid-winter. This suggests that the chaperone function of α-crystallins is probably not in cold stabilization of proteins but, instead, becomes important when environmental temperatures are rising in the spring. This is also supported by the elevated α-crystallin levels that were seen in thawed larvae after -16°C freezing exposure, again linking α-crystallins not with cold stress but with rewarming.

Depolymerization of actin and tubulin at low temperature has been recognized

as an important component of cold injury in a number of systems (Upadhya and Strasberg, 1999; Egierszdorff and Kacperska, 2001). Wang and Spector (1998) reported that α-crystallins can stabilize polymerized actin and prevent cytochalasin-induced depolymerization. Bluhm *et al.* (1998) showed that overexpression of αB-crystallin enhanced microtubule integrity under conditions of simulated ischemia in rat neonatal cardiac. Enhanced expression of TCP-1 is also known to be a cytoprotective response to denatured actin and tubulin caused by cold stress and, indeed, TCP-1, αA-crystallin and αB-crystallin were all elevated when *E. solidaginis* were thawed after freezing exposure. Taken together, this suggests that upregulation of α-crystallins and TCP-1 may act cooperatively to stabilize actin and tubulin, key protein of the cytoskeleton, over the wide range of environmental temperatures encountered over the winter by cold-hardy insect larvae. The late winter (March) increase in crystallins in *E. solidaginis* may also be related to entry of the larvae into a post-diapause state where development can begin again. Enhanced levels of cystallins at this time could support renewed synthesis of cytoskeletal proteins as the animals transition from the larval to the pupal stage.

4.5 Conclusion

The data in the present chapter indicates that GRPs and other chaperones have an important role in winter survival for both *E. solidaginis* and *E. scudderiana* larvae. Thus, in all cases, Grp expression was maximal in midwinter. Our results also indicate that the constitutive expression and rapid induction of some other molecular chaperones (ie.

TCP-1, crystallins) is associated with maintaining functional cellular machinery upon exposure to environmental stresses (cold, freezing, anoxia) in these two cold hardy insect larvae.

Table 4.1 Antibodies and experimental conditions used for both *E. solidaginis* and *E. scudderiana*.

Antibodies	Sources	Acrylamide Gel used	Blocking time with 2.5% milk (min)	Primary antibodies dilution factor (V:V)	Primary antibodies incubation time (4°C)	Secondary antibodies used and dilution factor
Anti-rabbit Grp 170	Gift form Japan	6%	60	1:1,000	Over night	Anti-rabbit, 1:1,500
Grp 94 Sc-1794	Santa Cruz	10%	20	1:500	Over night	Anti-goat, 1:2,000
Grp 78 SPA-805	Stressgen	10%	30	1:1,000	Over night	Anti-rabbit, 1:2,000
Grp 75 sc-1058	Santa Cruz	10%	20	1:500	Over night	Anti-goat, 1:2,000
TCP-1 CTA-191	Stressgen	10%	30	1:750	2 days	Anti-rat, 1:1,500
αA-crystallin sc-22390	Santa Cruz	15%	20	1:500	Over night	Anti-goat, 1:2,000
αB-crystallin sc-22391	Santa Cruz	15%	20	1:500	Over night	Anti-goat, 1:2,000

Fig. 4.1 Grp 78 protein expression in *E. solidaginis* larvae.

A. Representative Western blots showing total Grp 78 levels over the winter, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; Ch - Chilled; Fr - Frozen; Th - Thawed; An – Anoxia.

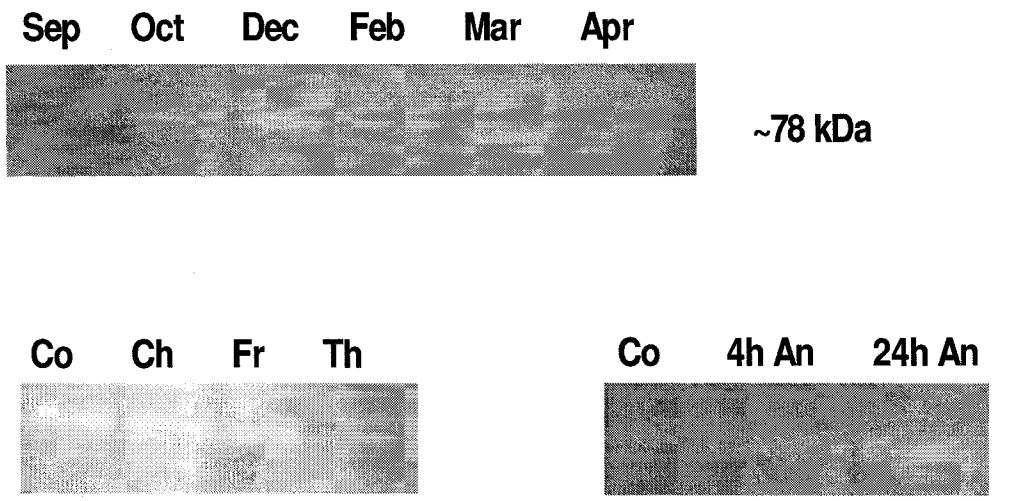
B. Histogram showing relative expression (densitometric analysis) of Grp 78 protein in outdoor larvae over the winter months.

C. Histogram showing relative expression of Grp 78 in laboratory low temperature experiments: control (+15°C acclimated), chilled (+3°C, 24 h), frozen (-16°C, 24 h), and thawed (+3°C, 24 h).

D. Histogram showing relative expression of Grp 78 in larvae under aerobic control (+15°C) versus anoxia exposure (4 h or 24 h at +15°C) conditions.

Note: All data are means ± S.E.M. (n=3). ☆- Significantly different from the corresponding September or control value as determined by the Student's t-test, $P<0.05$. ♡ –Thawed value is significantly different from the frozen value, $P<0.05$.

A.



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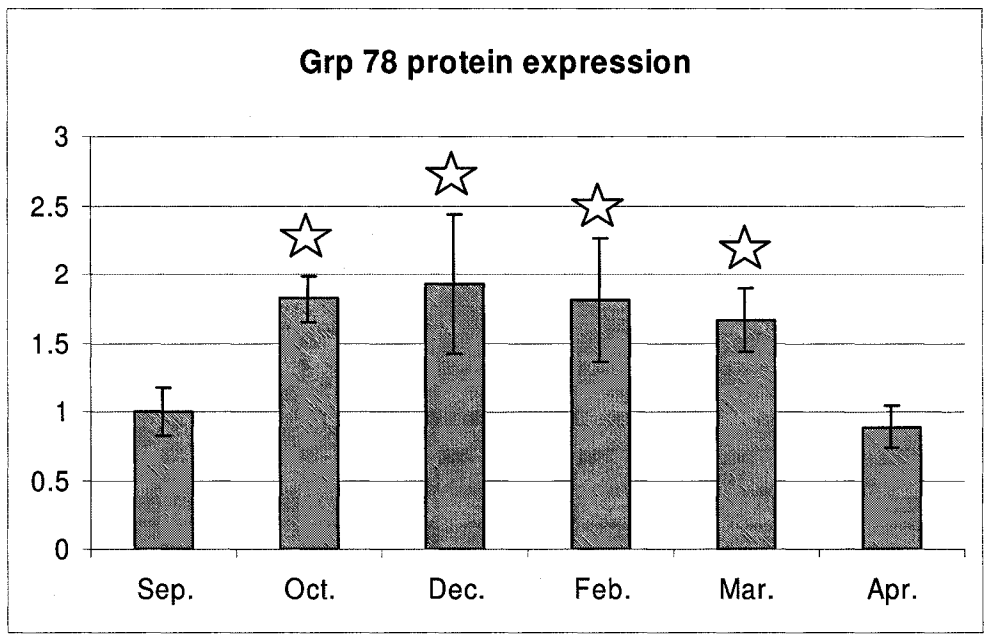

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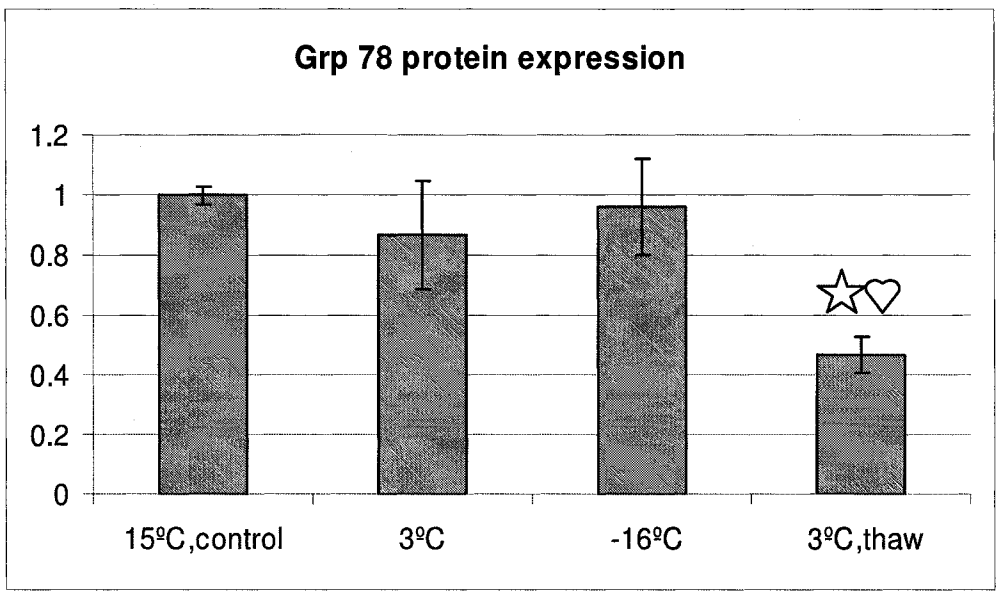

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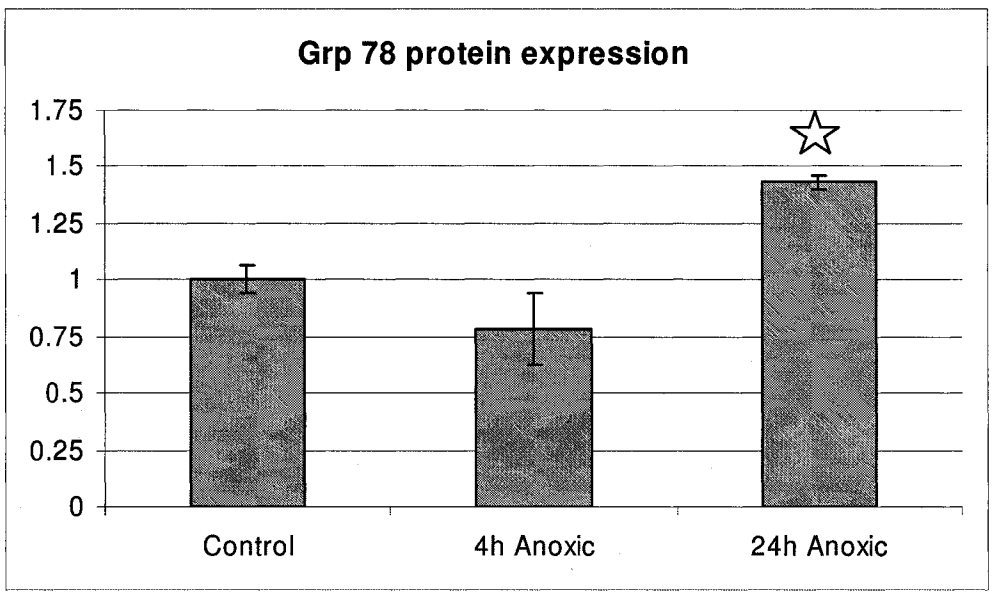


Fig. 4.2 Grp 75 protein expression in *E. solidaginis* larvae.

A. Representative Western blots showing total Grp 75 levels over the winter, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; Ch - Chilled; Fr - Frozen; Th - Thawed; An – Anoxia.

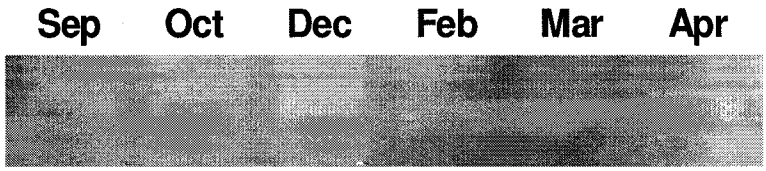
B. Histogram showing relative expression (densitometric analysis) of Grp 75 protein levels over the winter months outdoors.

C. Histogram showing relative expression of Grp 75 in control (+15°C), chilled (+3°C, 24 h), frozen (-16°C, 24 h), and thawed (+3°C, 24 h) larvae in indoor laboratory experiments.

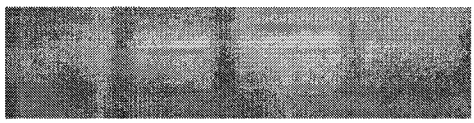
D. Histogram showing relative expression of Grp 75 under control (+15°C) and anoxic (4 h or 24 h at +15°C) conditions.

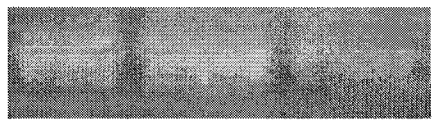
Note: All data are means ± S.E.M. (n=3). ☆– Significantly different from the corresponding September or control value as determined by the Student's t-test, $P<0.05$. ♡ –Thawed value is significantly different from the frozen value, $P<0.05$.

A.



~75kDa





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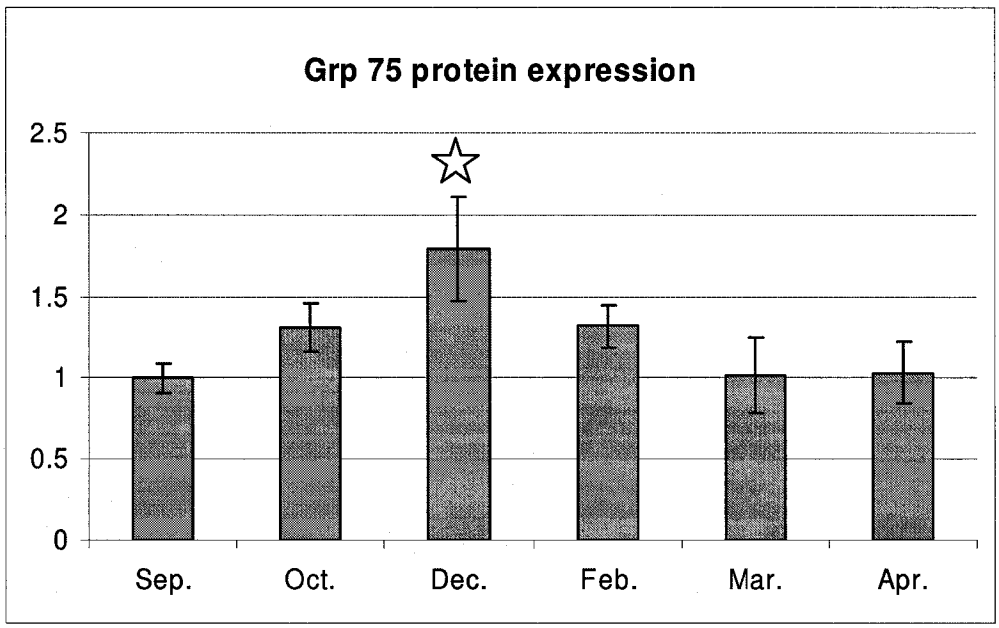

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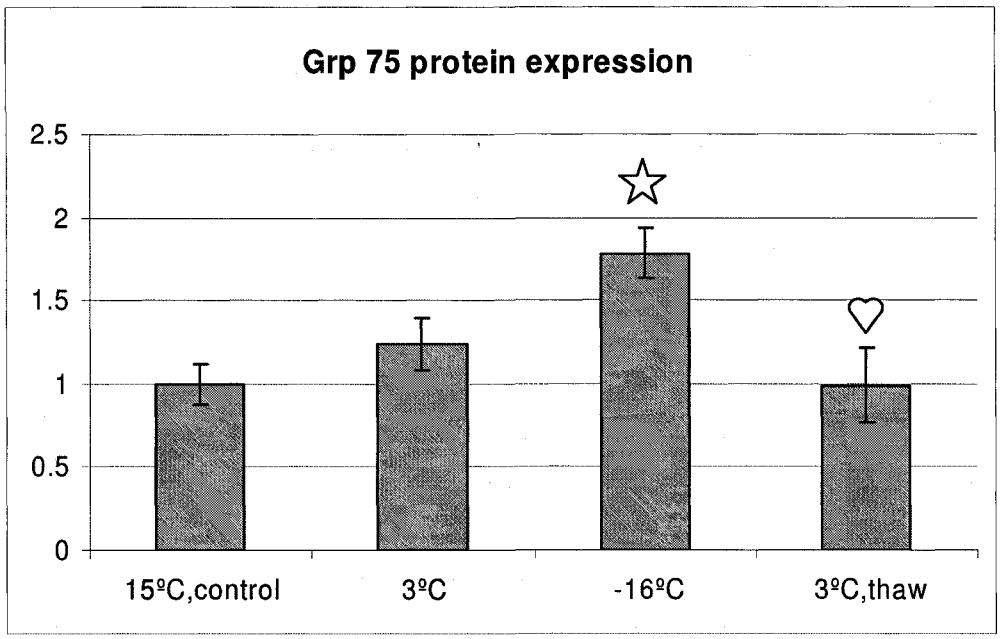

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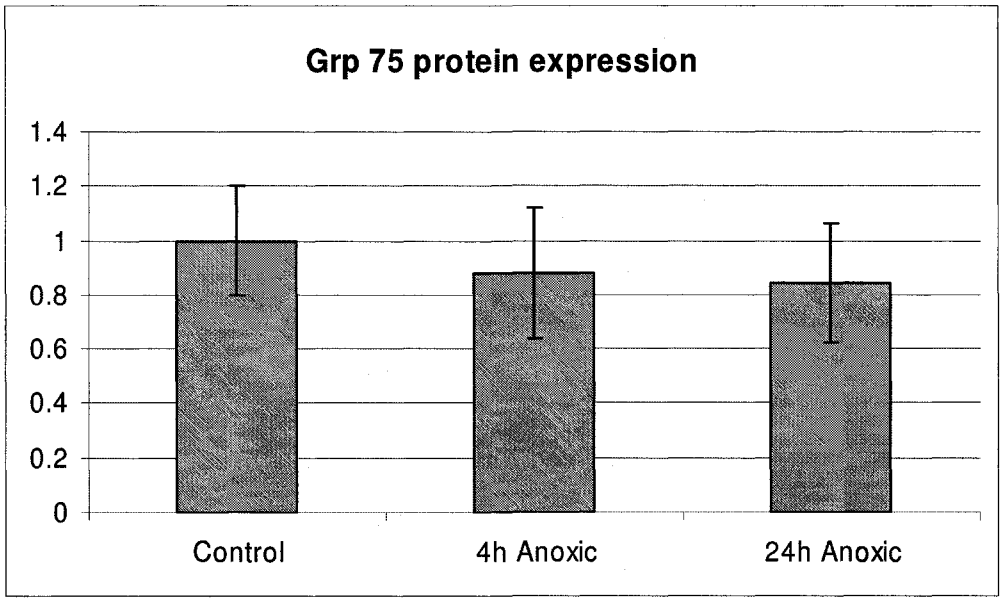


Fig. 4.3 TCP-1 protein expression in *E. solidaginis* larvae.

A. Representative Western blots showing total TCP-1 levels over the winter, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; Ch - Chilled; Fr - Frozen; Th - Thawed; An – Anoxia.

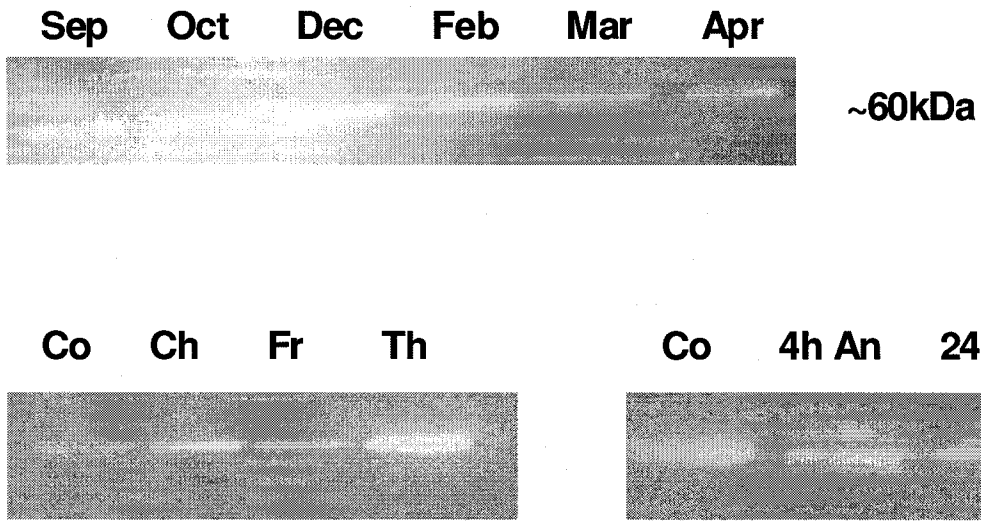
B. Histogram showing relative expression (densitometric analysis) of TCP-1 protein over the winter months outdoors.

C. Histogram showing relative expression of TCP-1 in control (+15°C), chilled (+3°C, 24 h), frozen (-16°C, 24 h), and thawed (+3°C, 24 h) larvae in indoor laboratory experiments.

D. Histogram showing relative expression of TCP-1 under aerobic control (+15°C) versus anoxic (4 h and 24 h at +15°C) conditions.

Note: All data are means ± S.E.M. (n=4). ☆– Significantly different from the corresponding September or control value as determined by the Student's t-test, $P<0.05$. ♡ –Thawed value is significantly different from the frozen value, $P<0.05$.

A.



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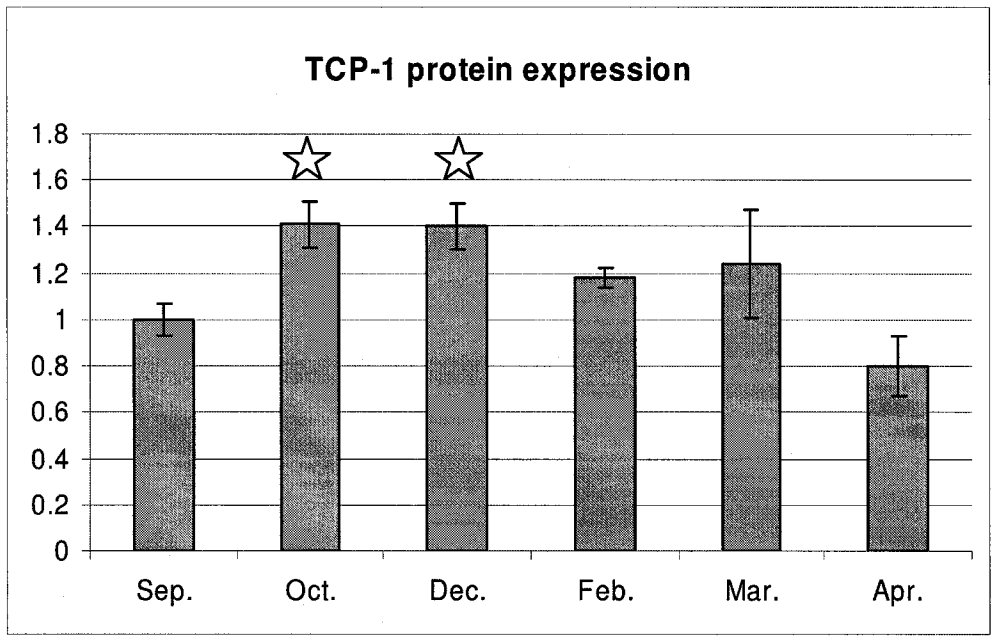

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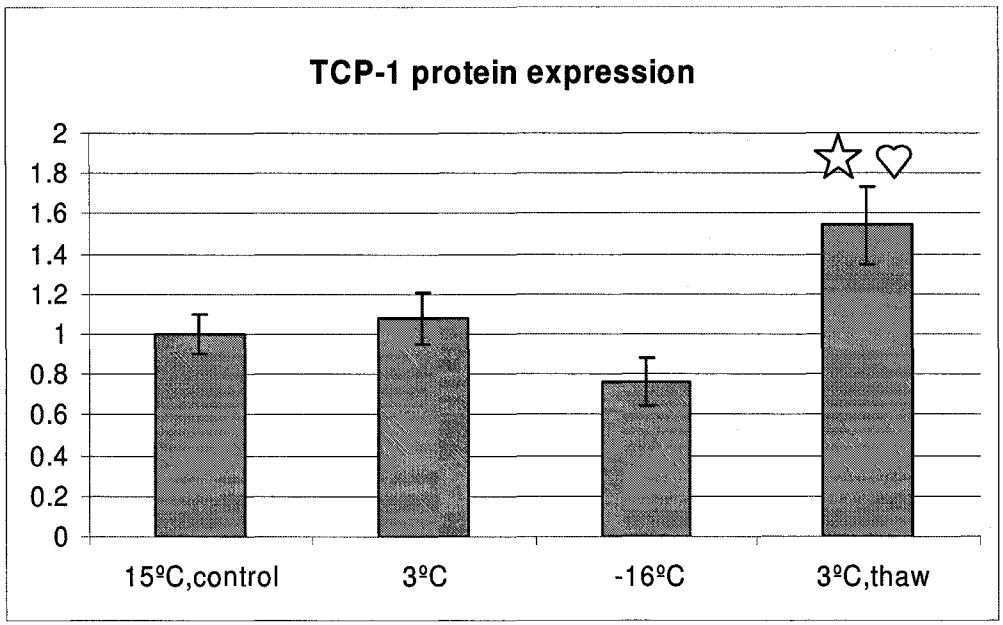

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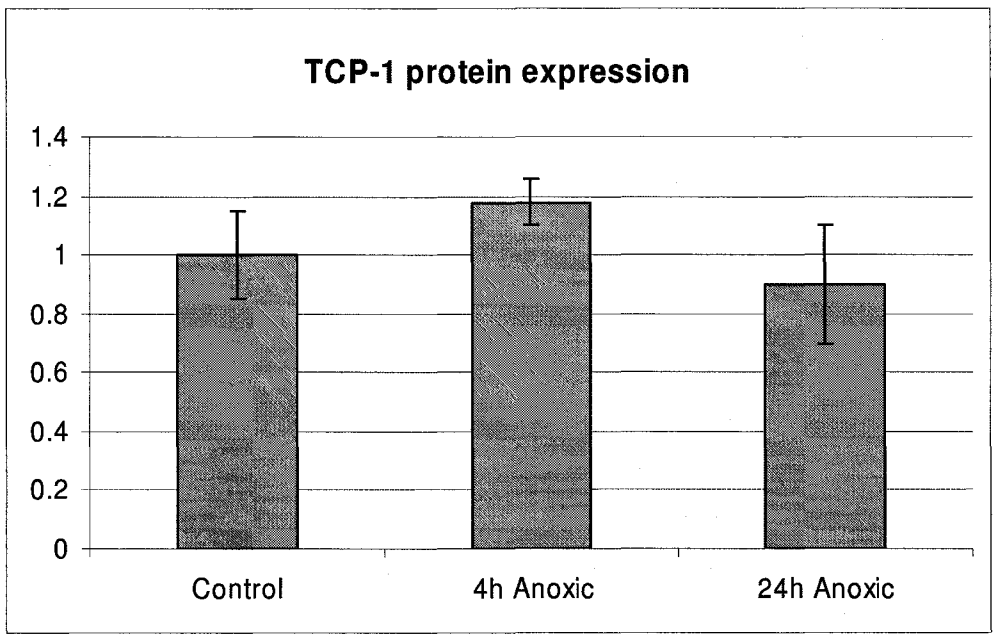


Fig. 4.4 αA-Crystallins protein expression in *E. solidaginis* larvae.

A. Representative Western blots showing total αA-Crystallins levels over the winter, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; Ch - Chilled; Fr - Frozen; Th - Thawed; An – Anoxia.

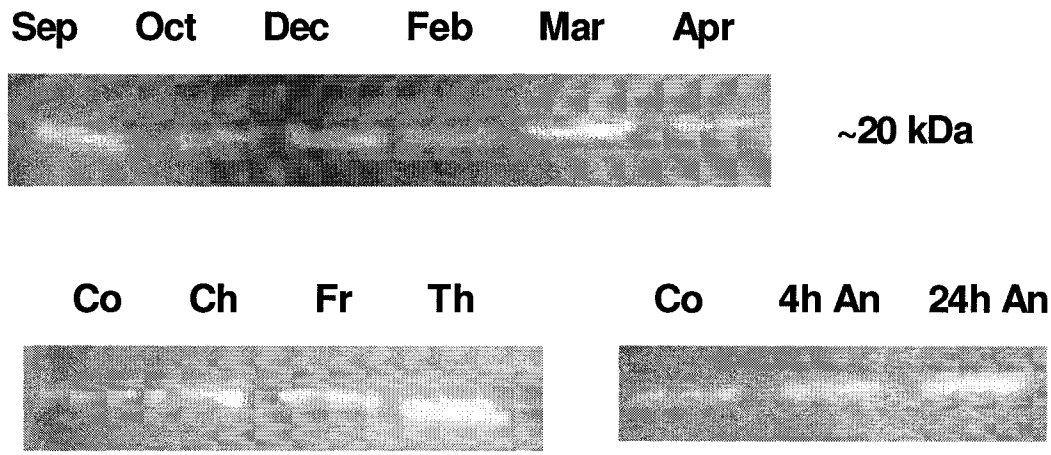
B. Histogram showing relative expression (densitometric analysis) of αA-Crystallins protein over the winter months outdoors.

C. Histogram showing relative expression of αA-Crystallins in control (+15°C), chilled (+3°C, 24 h), frozen (-16°C, 24 h), and thawed (+3°C, 24 h) larvae in indoor laboratory experiments.

D. Histogram showing relative expression of αA-Crystallins in aerobic control (+15°C) versus anoxic (4 h and 24 h +15°C) larvae.

Note: All data are means ± S.E.M. (n=4). ☆– Significantly different from the corresponding September or control value as determined by the Student's t-test, $P<0.05$.

A.



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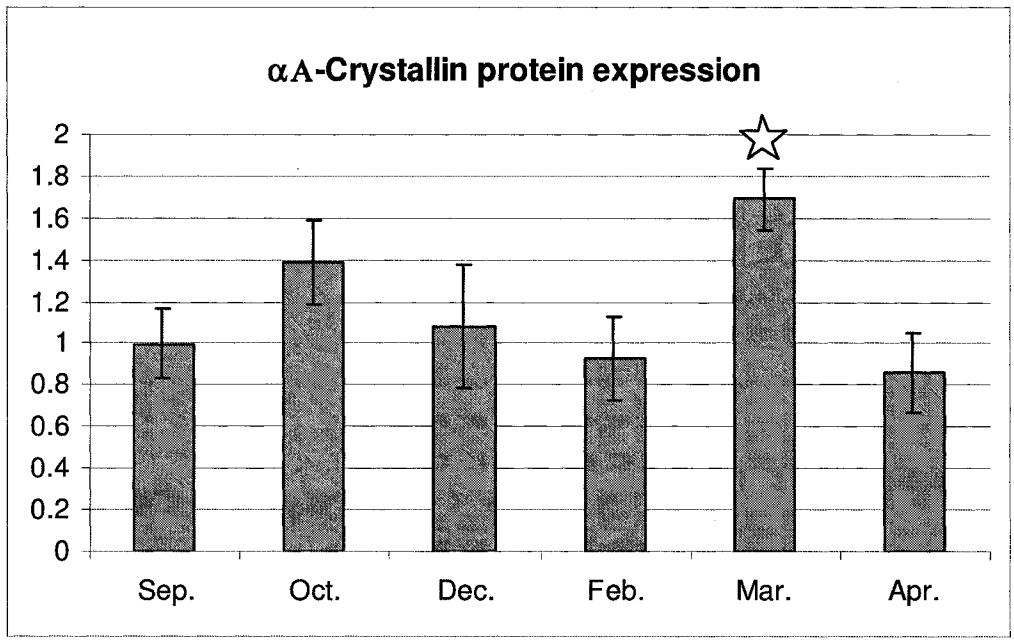

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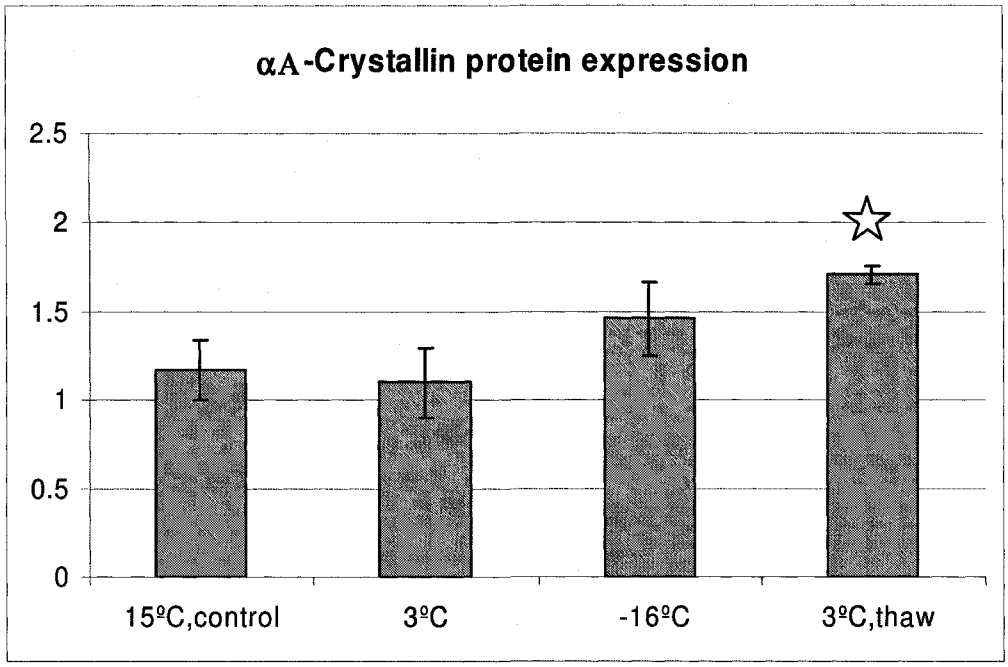

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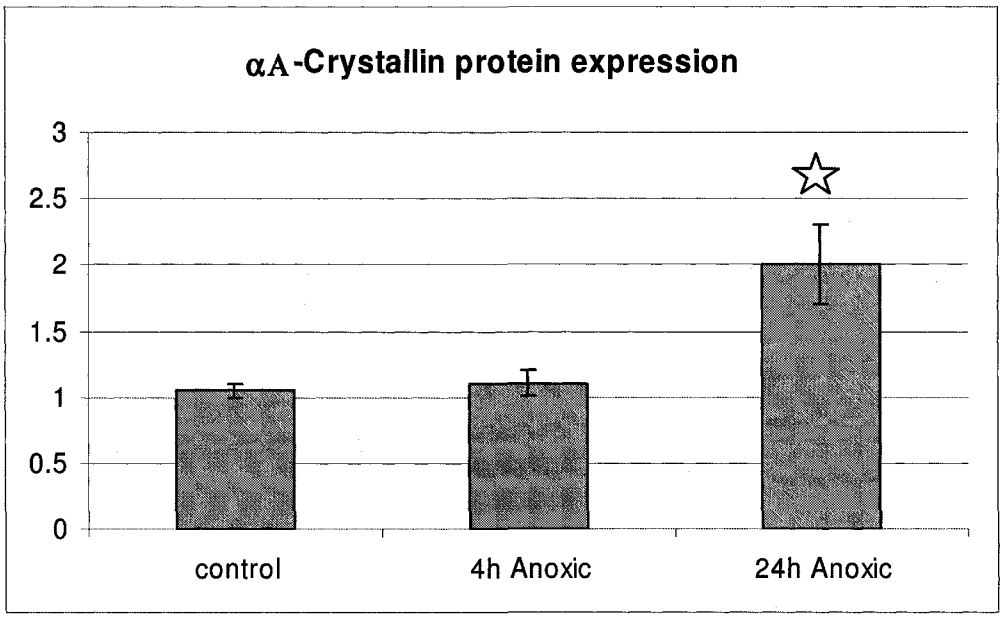


Fig. 4.5 αB -Crystallins protein expression in *E. solidaginis* larvae.

A. Representative Western blots showing total αB -Crystallins levels over the winter, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; Ch - Chilled; Fr - Frozen; Th - Thawed; An – Anoxia.

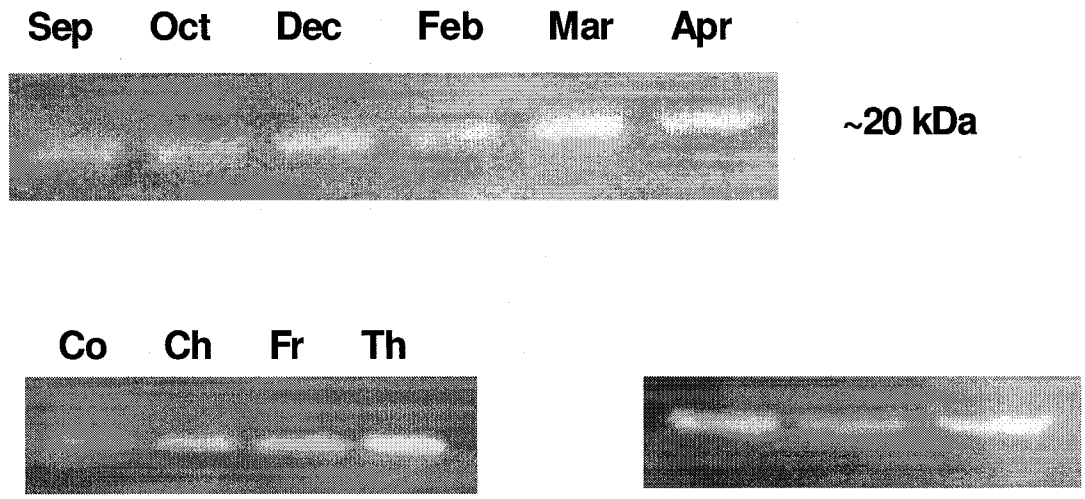
B. Histogram showing relative expression (densitometric analysis) of αB -Crystallins protein over the winter months outdoors.

C. Histogram showing relative expression of αB -Crystallins in control (+15°C), chilled (+3°C, 24 h), frozen (-16°C, 24 h), and thawed (+3°C, 24 h) larvae in indoor laboratory experiments.

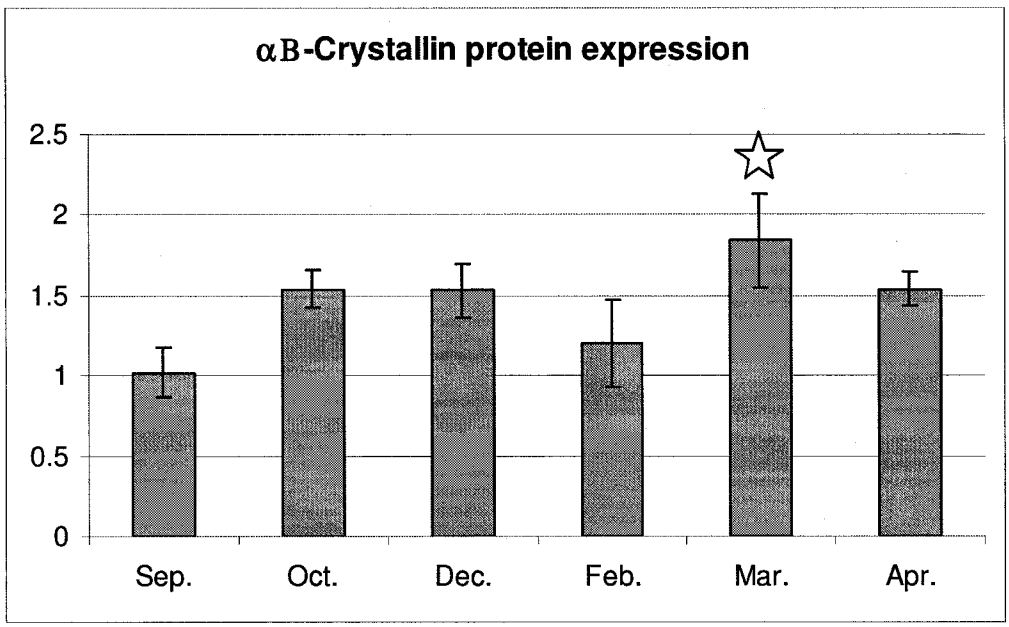
D. Histogram showing relative expression of αB -Crystallins in aerobic control (+15°C) versus anoxic (4 h and 24 h +15°C) larvae.

Note: All data are means ± S.E.M. (n=4). ☆– Significantly different from the corresponding September or control value as determined by the Student's t-test, $P<0.05$.

A.



B.



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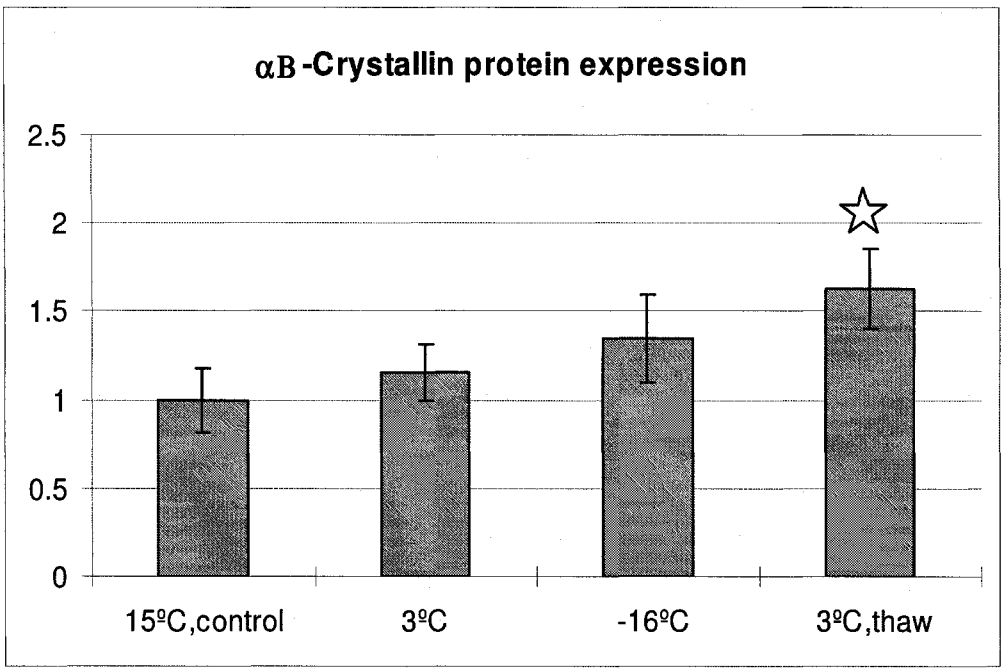

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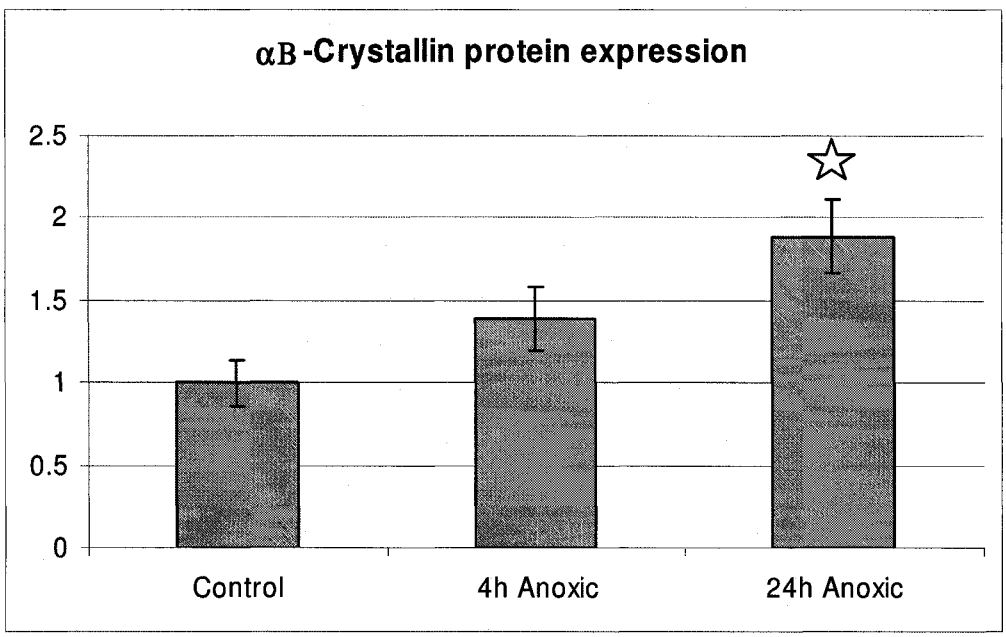


Fig. 4.6 Grp 170 protein expression in *E. scudderiana* larvae.

A. Representative Western blots showing total Grp 170 levels over the winter months, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; An – Anoxia.

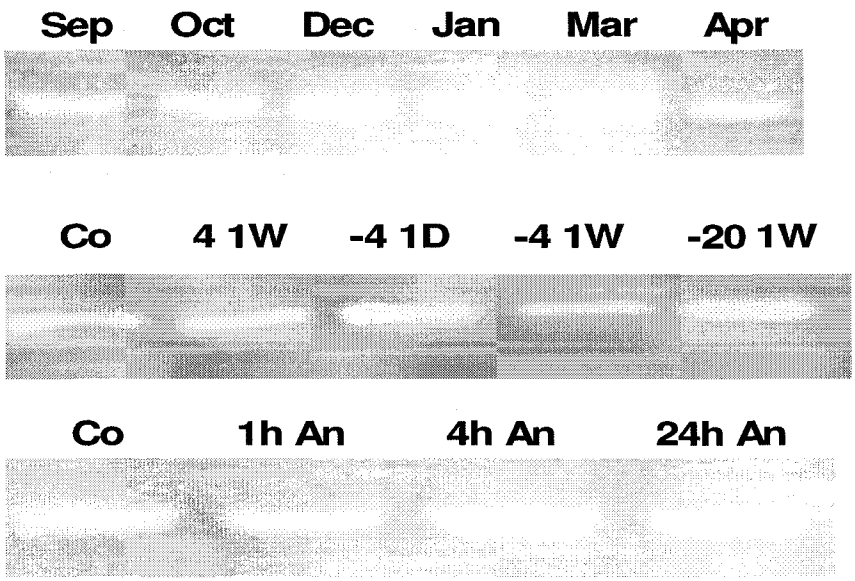
B. Histogram showing relative expression (densitometric analysis) of Grp 170 protein over the winter months outdoors.

C. Histogram showing relative expression of Grp 170 in indoor laboratory experiments: 15°C, control; 4°C, 1 week; -4°C, 24 h; -4°C, 1 week; and -20°C, 1 week.

D. Histogram showing the relative expression of Grp 170 in control (+4°C, 2 weeks) versus anoxic (1 h, 4 h, or 24 h at 4°C) larvae.

Note: All data are means $\pm$ S.E.M. (n=4). ☆– Mean value is significantly different from the corresponding September or control value as determined by the Student's t-test, $P<0.05$.

A.



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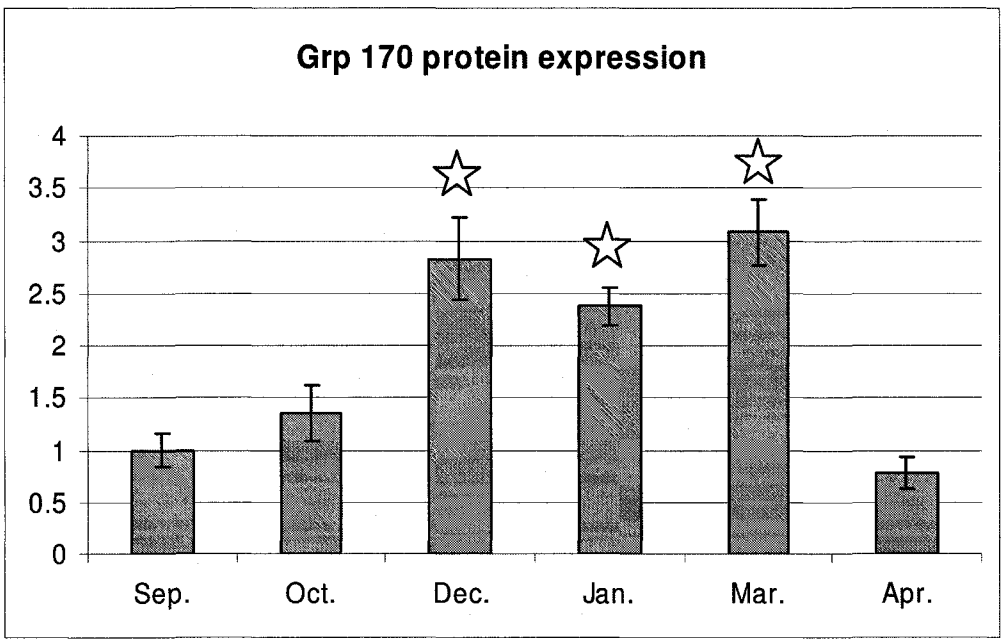

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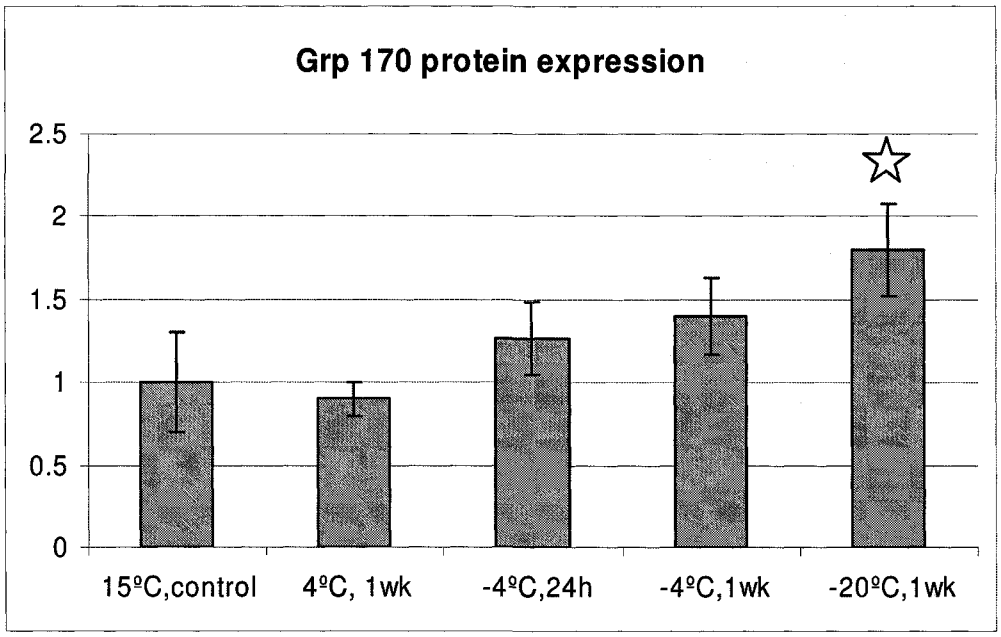

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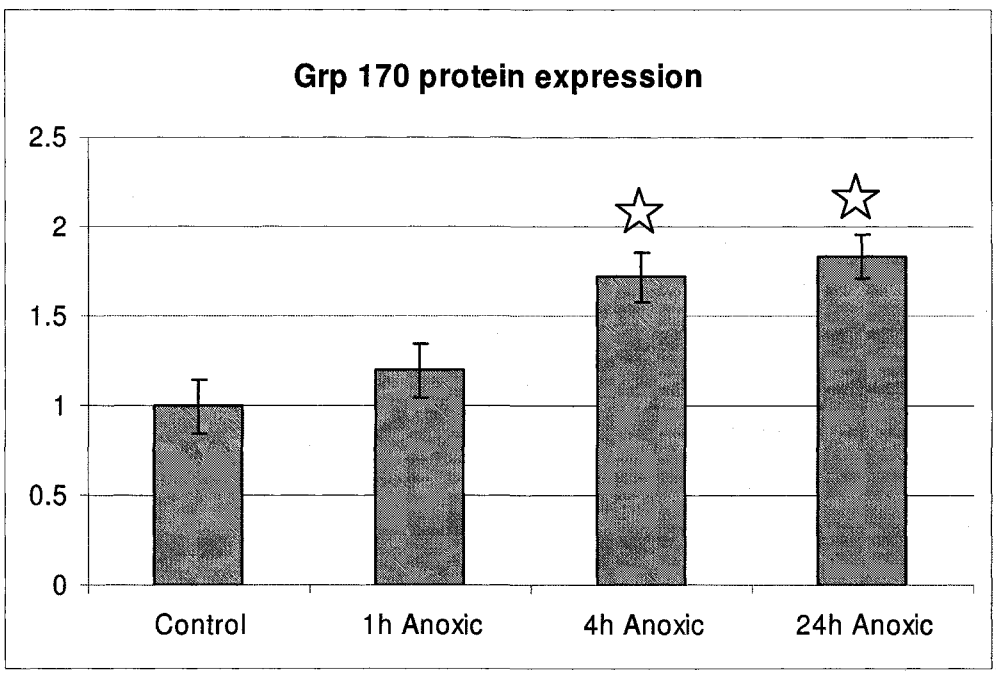


Fig. 4.7 Grp 94 protein expression in *E. scudderiana* larvae.

A. Representative Western blots showing total Grp 94 levels over the winter months, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; An – Anoxia.

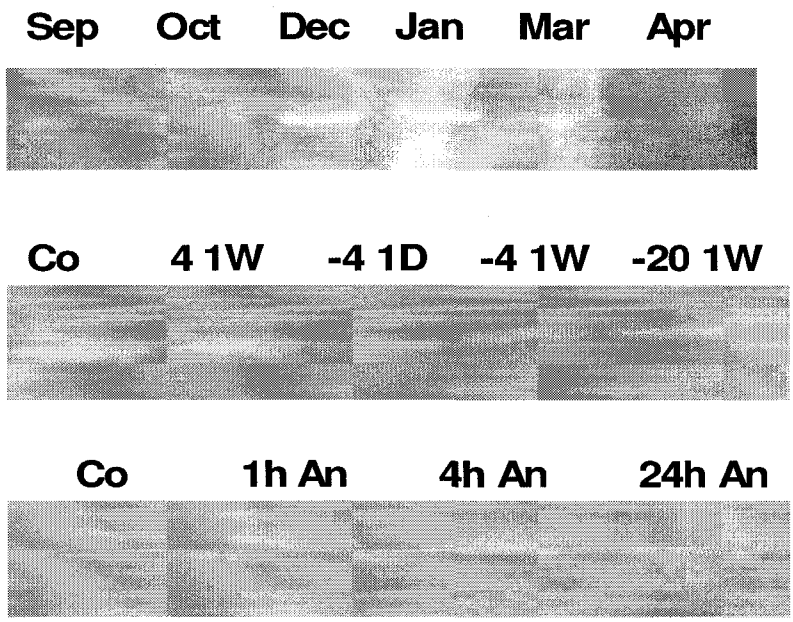
B. Histogram showing relative expression (densitometric analysis) of Grp 94 protein over the winter months outdoors.

C. Histogram showing relative expression of Grp 94 in the indoor laboratory experiments: 15°C, control; 4°C, 1 week; -4°C, 24 h; -4°C, 1 week; and -20°C, 1 week.

D. Histogram showing relative expression of Grp 94 in aerobic control (+4°C, 2 weeks) versus anoxic (1 h, 4 h, or 24 h at 4°C) larvae.

Note: All data are means ± S.E.M. (n=3). ☆– Mean value is significantly different from the corresponding September or control value as determined by the Student's t-test, $P<0.05$.

A.



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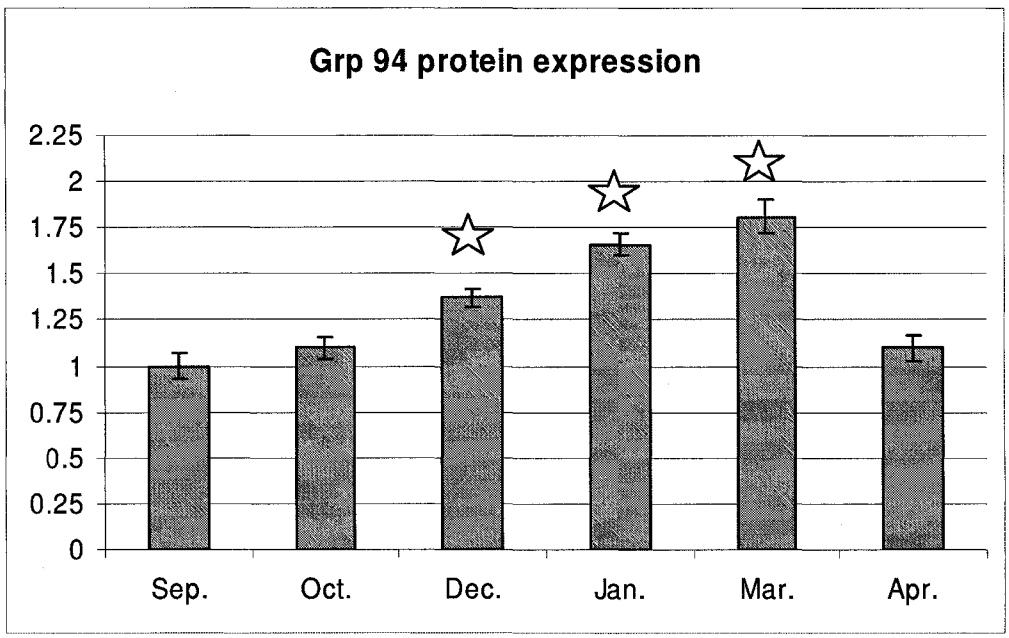
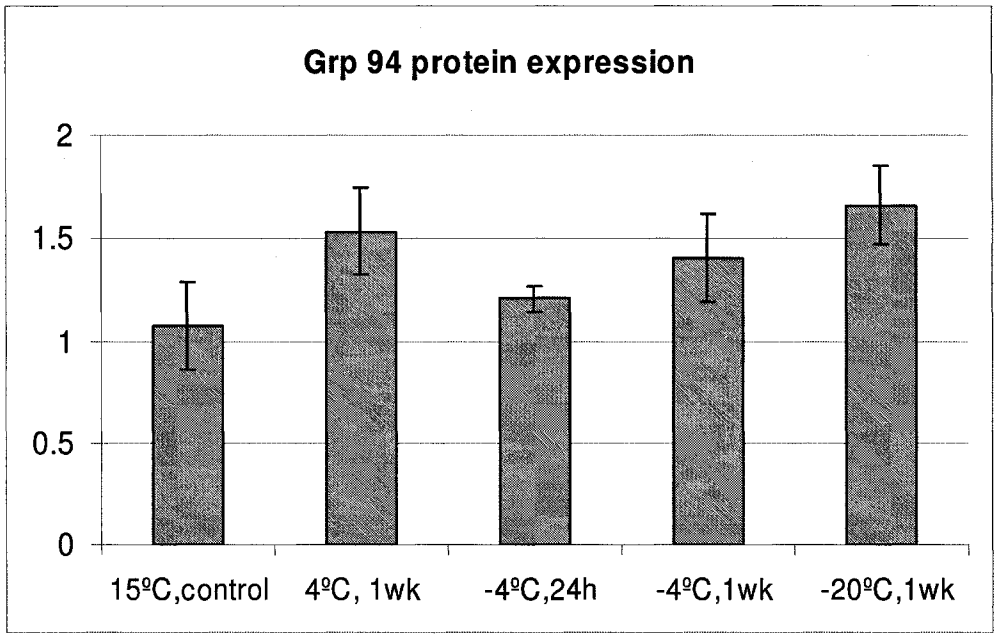


C.



D.

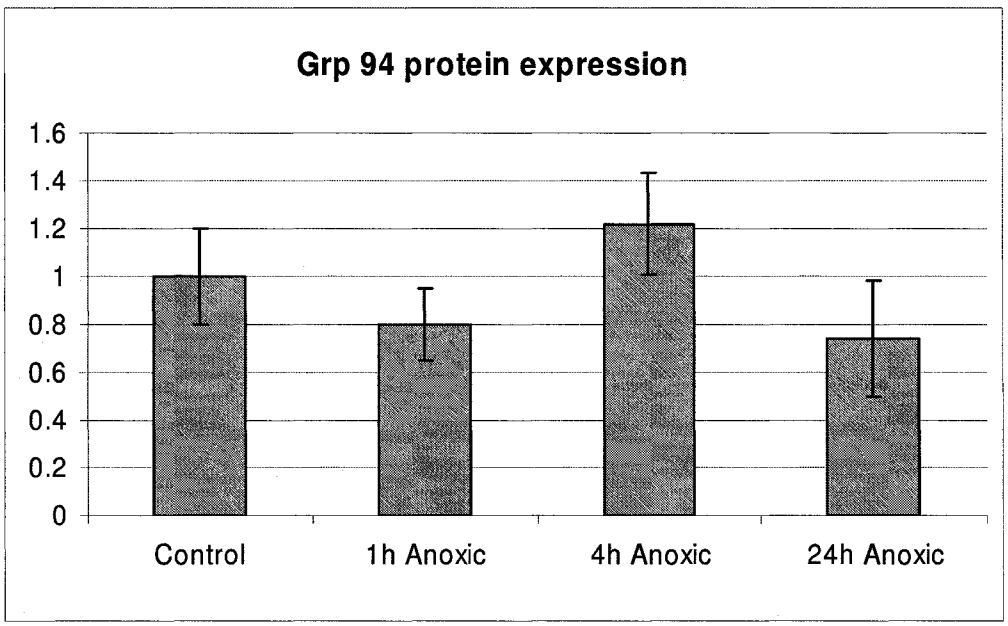


Fig. 4.8 Grp 78 protein expression in *E. scudderiana* larvae.

A. Representative Western blots showing total Grp 78 levels over the winter, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; An – Anoxia.

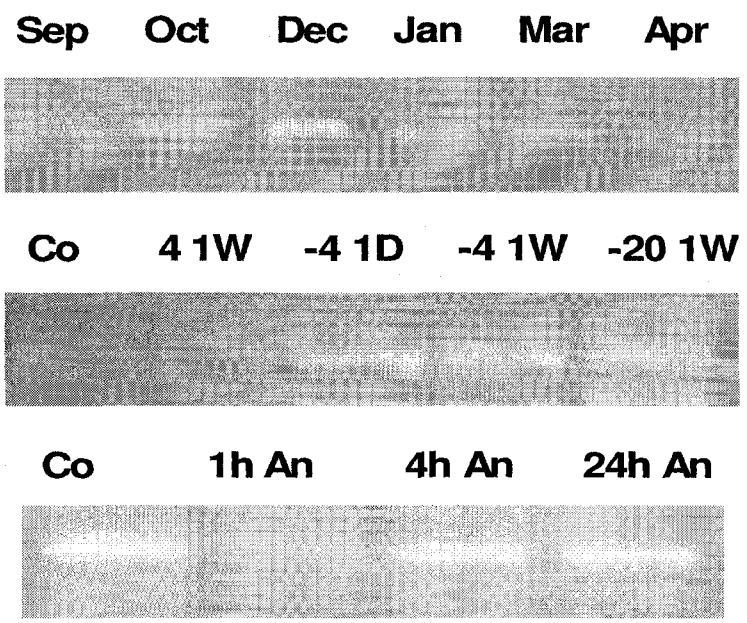
B. Histogram showing relative expression (densitometric analysis) of Grp 78 protein over the winter months outdoors.

C. Histogram showing relative expression of Grp 78 in indoor laboratory experiments: 15°C, control; 4°C, 1 week; -4°C, 24 h; -4°C, 1 week; and -20°C, 1 week.

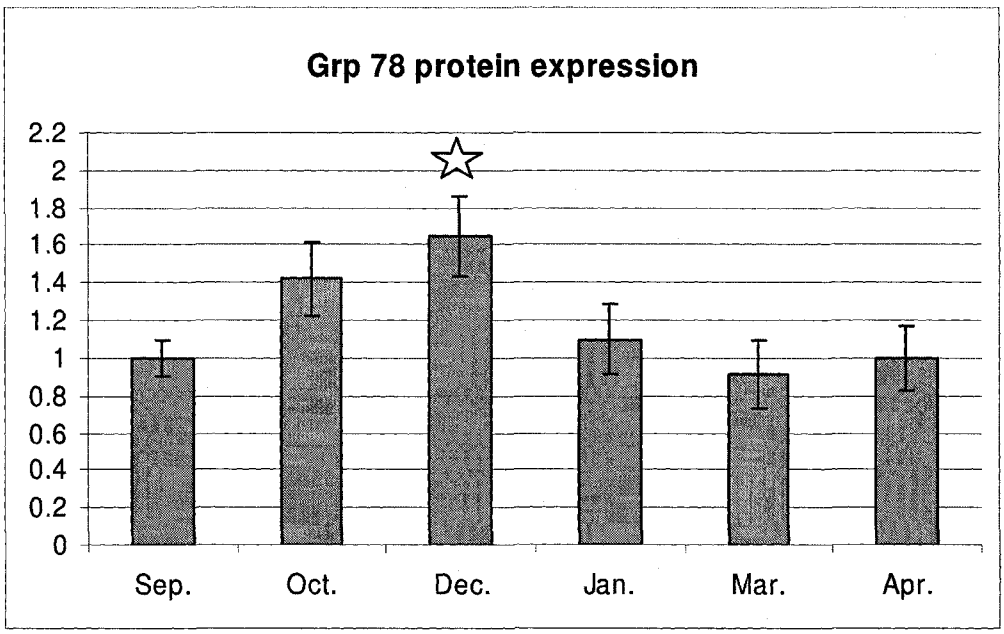
D. Histogram showing relative expression of Grp 78 in aerobic control (+4°C, 2 weeks) versus anoxic (1 h, 4 h, or 24 h at 4°C) conditions.

Note: All data are means $\pm$ S.E.M. (n=3). ☆– Mean value is significantly different from the corresponding September or control value as determined by the Student's t-test, $P<0.05$.

A.



B.



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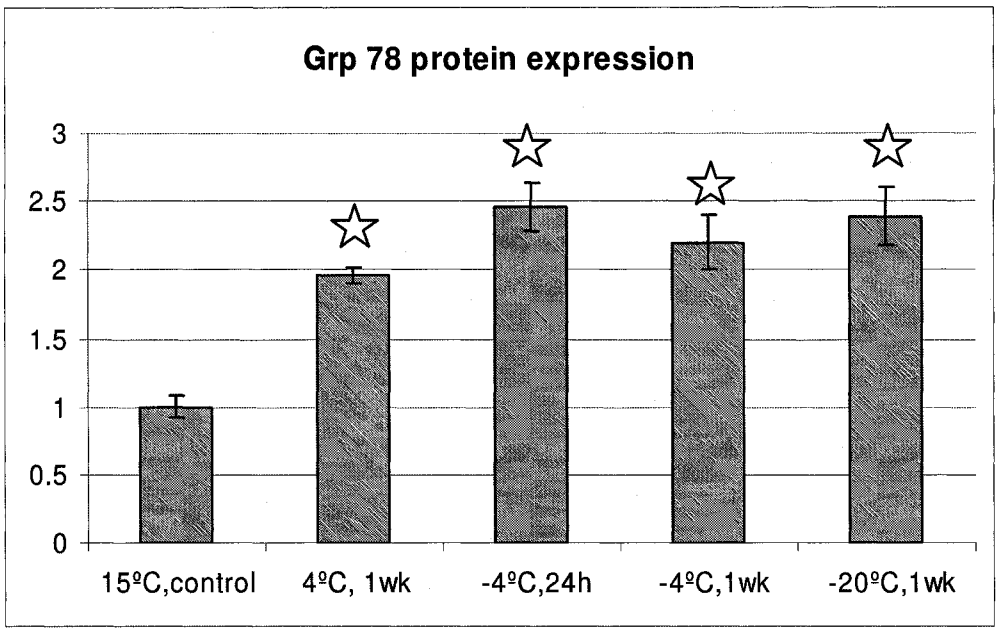


D.

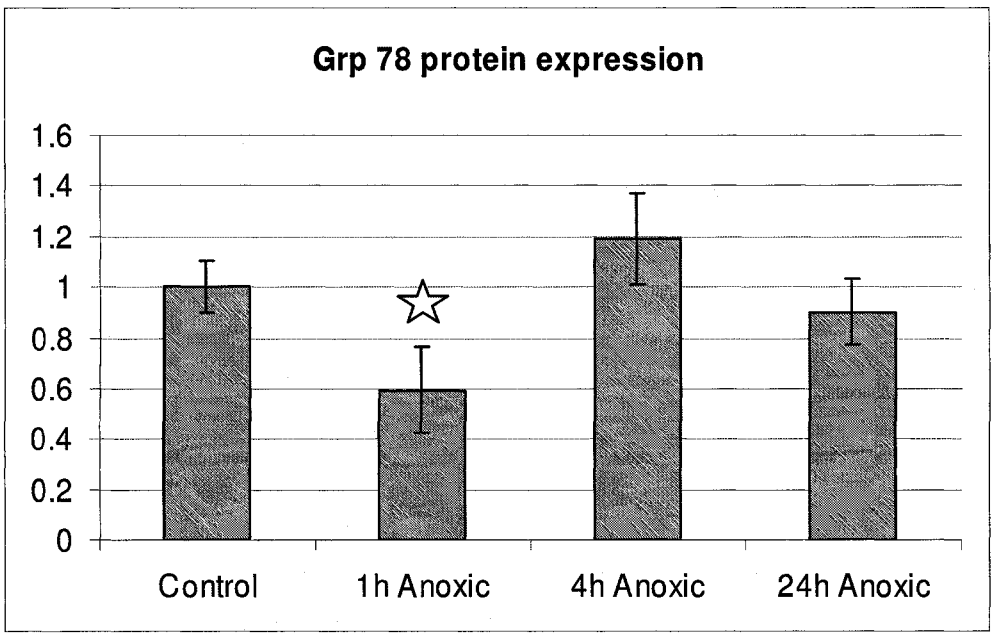


Fig. 4.9 TCP-1 protein expression in *E. scudderiana* larvae.

A. Representative Western blots showing total TCP-1 levels over the winter, during exposures to low temperatures, and over an anoxia timecourse. Abbreviations are: Co - Control; An – Anoxia.

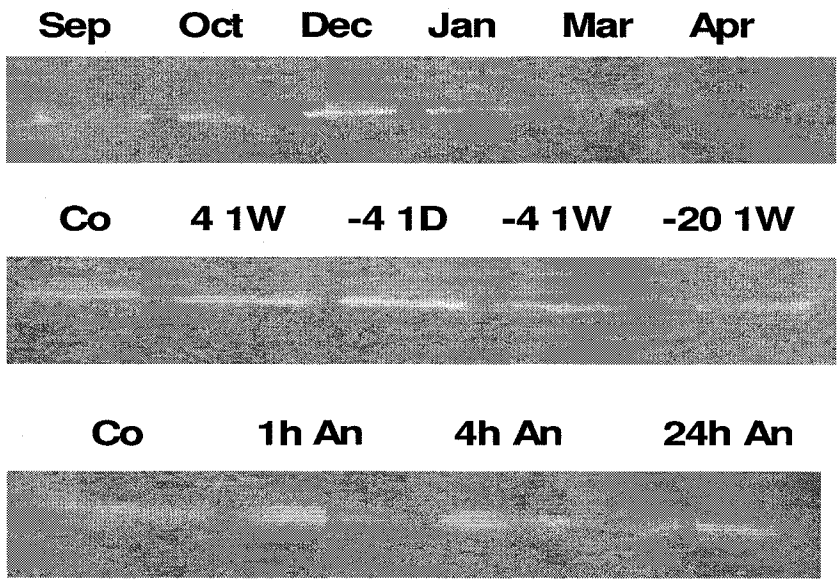
B. Histogram showing relative expression (densitometric analysis) of TCP-1 protein levels over the winter months outdoors.

C. Histogram showing relative expression of TCP-1 value in the indoor laboratory experiments: 15°C, control; 4°C, 1 week; -4°C, 24h; -4°C, 1 week; and -20°C, 1 week.

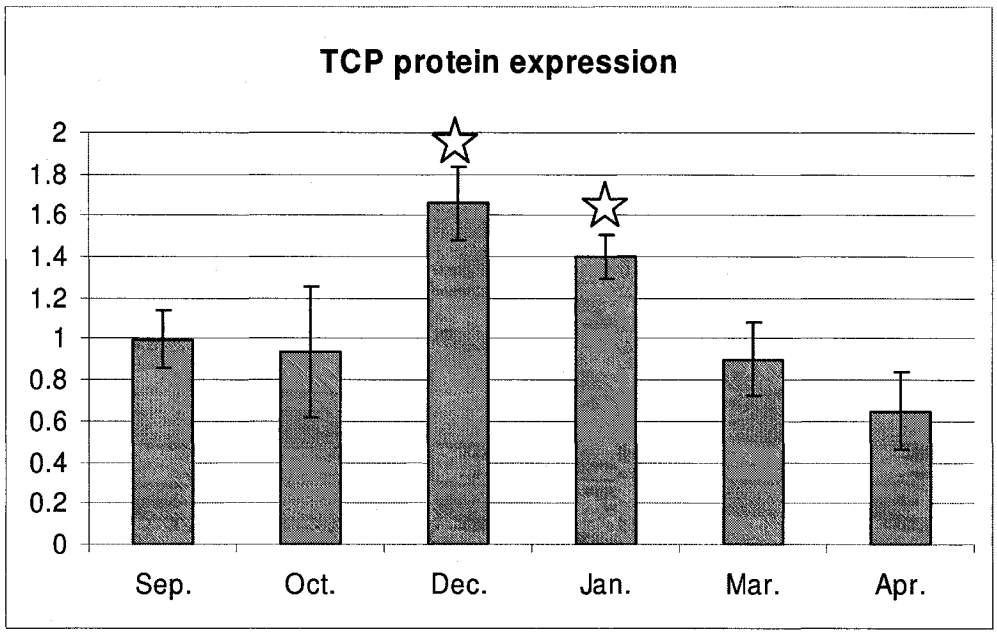
D. Histogram showing relative expression of TCP-1 in aerobic control (+4°C, 2 weeks) versus anoxic (1 h, 4 h, or 24 h at 4°C) larvae.

Note: All data are means ± S.E.M. (n=4). ☆– Mean value is significantly different from the corresponding September value as determined by the Student's t-test, $P<0.05$.

A.



B.



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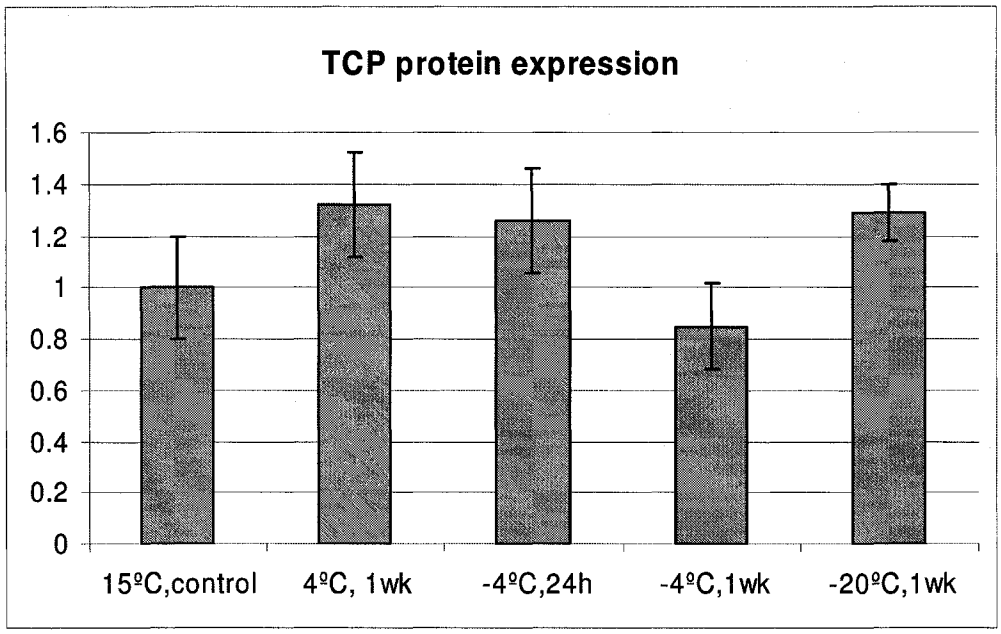

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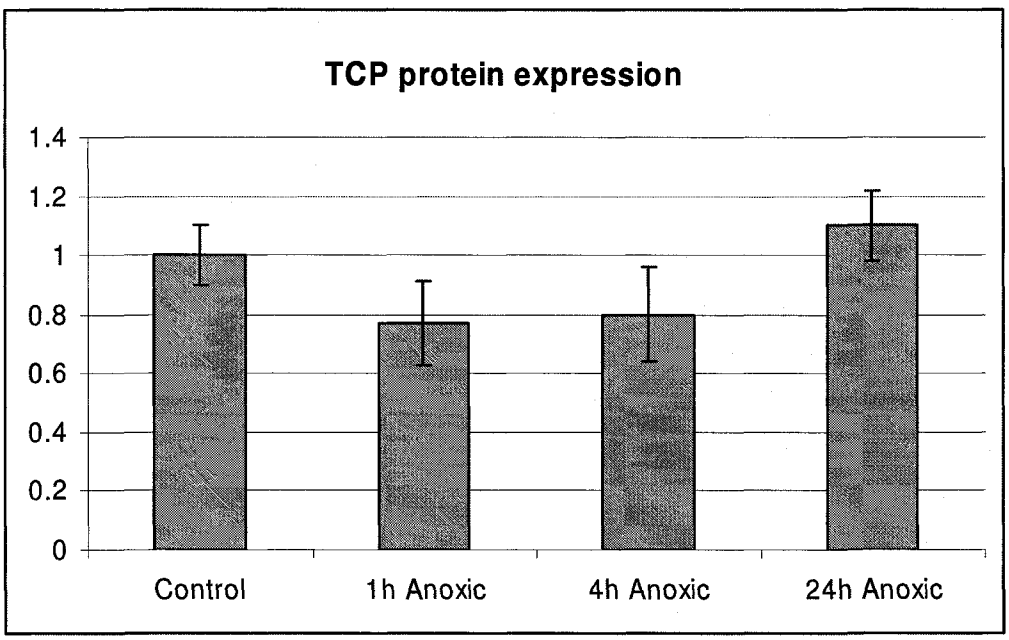


CHAPTER 5

General discussion

5.1 Chaperone-mediated protein folding in the cytosol

The ability of a cell to sense, respond to and circumvent stress is essential for maintaining homeostasis. There are many ways in which stress, either endogenous or exogenous, can be manifested in a cell; these include pathogenic infection, chemical insult, genetic mutation, nutrient deprivation, changes in environmental parameters such as temperature and oxygen content, and even normal differentiation. The process of protein folding is particularly sensitive to such insults (Rutkowski, 2004). Our knowledge about protein folding has increased substantially over the last 30 years. The traditional view that proteins fold spontaneously (Anfinsen, 1973) was revised after it was shown that many proteins in a living cell will not fold correctly without the assistance of molecular chaperones. Chaperones have been defined as "a family of cellular proteins which mediate the correct folding of other polypeptides, and in some cased their assembly into oligomeric structure" (Ellis, 1990). This definition implies that the dominant function of molecular chaperones is to transiently interact with other proteins, thereby preventing the formation of illegitimate interactions that might otherwise lead to deleterious protein aggregation. Chaperones generally seem to bind to exposed hydrophobic surfaces that will ultimately be buried in the folded state. Controlled release of a substrate protein from the chaperone, often driven by ATP hydrolysis, promotes folding into the native state. Repeated cycles of binding and release may be necessary for productive folding.

5.1.1 Protein quality control

One of the most critical events in the biogenesis of a protein is the conversion of its linear amino acid sequence into the properly folded three-dimensional structure. As soon as a nascent polypeptide chain emerges from the ribosome, it is prone to misfolding and subsequent aggregation. Although many proteins may fold spontaneously, the initial folding of a significant portion of cellular proteins requires the assistance of molecular chaperones (Bukau, 2000). It has been estimated that 20 to 30% of all proteins in the prokaryotic cytoplasm transit through the DnaK (HSP 70) or GroEL (HSP 60) chaperone machineries before adopting their final conformation (Ewalt, 1997). Chaperoning occurs both cotranslationally, while the polypeptide is being synthesized, and posttranslationally, after the complete amino acid chain has been released from the ribosome (Bukau, 2000). Cotranslational folding requires HSP 70 and trigger factor, a ribosome-associated protein with peptidylprolyl-*cis-trans* isomerase and chaperone-like activities (Teter, 1999). Both HSP 70 and HSP 60 contribute to posttranslational protein folding. However, despite the actions of chaperones, about 20% of all synthesized polypeptides never reach their final destination because fatal errors have occurred during transcription or translation or because correct folding has not been accomplished (Wickner, 1999). Before such detrimental proteins accumulate, they are removed by ATP-dependent proteases (Gottesman, 1999). Both chaperones and proteases are designed to recognize similar features that are found on unfolded proteins but not on native proteins, namely, surface-exposed hydrophobic patches. The tight combination of chaperone function with

proteases suggests that the initial energy-dependent steps of the two processes are similar. Partial unfolding of substrate proteins is most probably the common prerequisite for both activities (Suzuki, 1997).

Once cellular proteins are correctly folded, various stress conditions still pose a serious threat to their integrity. Temperature variations, ionic changes, antibiotics, solvents, or other chemicals not only interfere with transcription, translation, and protein folding but also often disrupt the faithfully acquired three-dimensional protein structure. The heat shock response elicited by a sudden increase in ambient temperature was the first, and still the most widely used, model system for studying the impact of stress on biological systems. Proteins whose expression is induced by heat shock are generally called heat shock proteins (Hsps). It is not surprising that the classical chaperones and proteases are among them. All players involved in the normal protein quality control system are required to combat the damage inflicted by a thermal insult. In contrast to normal conditions, productive protein folding now occurs predominantly via the chaperone-mediated pathway due to a large number of thermolabile proteins (Mogk, 1999). A large portion of already folded proteins are partially or completely denatured by the thermal insult and reenter the quality control system. When the rate of denaturation outpaces the refolding capacity, increasing amounts of proteolysis-sensitive substrates are generated. The additional demand for folding support during acute stress is met by two strategies: 1) the levels of preexisting quality control proteins are elevated; and 2) additional chaperones that are not expressed or are only weakly expressed under normal

conditions are induced to counteract severe damage. Members of the first class, which are abundant under all metabolic conditions, include DnaK (HSP 70) and GroEL (HSP 60). Some small sized Hsps usually belong to the second class. Since these proteins are barely expressed at nonstress temperatures in most organisms, the induction factors after a temperature upshift can be very high (Richmond, 1999). Proteases are generally less abundant than chaperones regardless of the environmental conditions, indicating that protein refolding is preferred to proteolysis. Transcriptional induction after heat shock of most proteases was measured in the low to intermediate range (Richmond, 1999). Although both chaperones and proteases are upregulated under stress conditions, many damaged proteins seem to escape the quality control system and end up in aggregates.

In short (see figure 5.1), nature has invented an arsenal of powerful strategies to maintain high-quality protein in the cell. Once a nascent polypeptide has folded correctly, it is not left alone. Problems arising within the life span of a protein are solved by chaperones which assist in refolding or by proteases which dispose of proteins that cannot be refolded.

5.1.2 Protein chaperones

Traditionally, Hsps have been grouped into five major families. They were designated Hsp100, Hsp90, Hsp70, Hsp60, and small Hsps according to their molecular masses (Katschinski, 2004). Functional cooperation between different chaperones seems to be a central principle of protein folding in the cell. The two best understood systems

are those of HSP 70 and HSP 60.

5.1.2.1 HSP 70 reaction cycle

The reaction cycle of HSP 70 has been well studied. Many observations indicate that, for maximum functional effect, HSP 70 requires the presence of HSP 40 (Suzuki *et al.* 1999; Ohtsuka and Hata 2000). HSP40 stimulates the ATPase cycle of HSP 70 by increasing the rate of ADP release and HSP 40 increases the rate of hydrolysis of ATP bound to HSP 70 (Cheetham and Caplan 1998; Suh *et al.*, 1999; Yamagishi *et al*, 2000). HSP 70 reaction cycles are involved in a variety of folding processes including folding of newly synthesized polypeptides, refolding of stress- denatured proteins, disaggregation of proteins aggregates, translocation of organellar and secretory proteins across membranes, assembly and disassembly of oligomeric structures, and control over the biological activity and stability of regulatory proteins.

5.1.2.2 HSP 60 reaction cycle

The HSP 60 cycle is by far the best characterized chaperonin reaction cycle. The folding reaction is driven by a cycle of binding and release of the cochaperone HSP10, which alternates with binding and release of the nonnative protein substrate (Fisher, 1994). These cycles are driven by ATP binding and hydrolysis that control the conformation of the chaperonin and its affinity for nucleotides and the cochaperonin HSP 10 (Agsteribbe et al., 1993; Dubaquie et al., 1997). The HSP 60 cycle is involved in many cellular processes, including the de novo folding of newly synthesized polypeptides,

conformational maintenance of pre-existing proteins, secretion, and proteolysis. Although the requirement for HSP 60 for cell growth may result from any of these cellular functions, its role in the de novo folding of proteins may be the most critical, given that some substrates are entirely dependent on folding assistance by HSP 60.

5.1.2.3 Other protein chaperones networks

Whereas HSP 70 and HSP 60 are the main actors on the folding stage, there are many other chaperones adding to the fidelity of protein folding. These chaperones have more specialized functions and extend the limits of HSP 70 and HSP 60 based folding machineries. Hsp110 was one of the earliest HSPs described in mammalian cells (Subjeck et al.,1982). HSP 110 interacts with a large number of chaperones (HSP 70) and cochaperones (HSP40, Hip) in their functional activities (Yamagishi et al. 2000; Steel et al. 2004). Overexpression of HSP 110 in cultured mammalian cells increases thermal tolerance (Oh et al.,1997; Brodsky et al., 1999) and recent studies show that HSP 110 protects cells against protein aggregation when cellular ATP levels decrease markedly under stress conditions (Yamagishi et al. 2003; Yamagishi et al. 2004). TCP-1 and α-crystallin families were not initially considered to be stress proteins because they did not respond to heat shock. However, now they are known to have special protein folding and holding properties. The TCP-1 ring complex has a central cavity that assists in the folding of proteins upon ATP hydrolysis (Gomez-Puertas et al., 2004). Eukaryotic TCP-1 is essential for the correct folding of cytoskeletal proteins, actin and tubulin, its major substrates in vivo (Spiess et al., 2004). α-Crystallins can hold denatured proteins in large

soluble aggregates. However, unlike other molecular chaperones, α-crystallins do not renature these proteins (Narberhaus et al., 2002) and their action is ATP independent. α-Crystallins act co-operatively with other molecular chaperones to renature stressed proteins (Wang and Spector, 2000).

5.1.3 Regulation of Hsps

Heat shock genes are found in all organisms from bacteria to plants and mammals. They are highly conserved and show low between-species variation in the coding regions; for example, the *Drosophila hsp70*-gene and the prokaryotic *Escherichia coli dnaK*-gene show 72% and 50% identity, respectively, with the human *hsp70*-gene (Luc *et al.*, 2001). The transcription of Hsp genes is induced by the interaction of HSF-1 with the HSE in the promoter regions of Hsp genes (Klemenz *et al.*, 1991; Zhong *et al,* 1998). Cell stress causes activation of the inactive cytosolic HSF-1 and phosphorylated HSF-1 trimers then translocate to the nucleus where they induce Hsp gene transcription (see Figure 3.1). Biochemical evidence suggests that the cellular level of HSPs, directly or indirectly, feedback regulates HSF activity (Park and Craig 1989; Nadeau et al. 1993; Shi et al. 1998; Zou et al. 1998; Bonner et al. 2000) although other studies dispute this (Hjorth-Sorensen et al. 2001).

In summary, most of the Hsps function as molecular chaperones involved in the folding, assembly, and/or degradation of proteins and therefore appear to prevent the

accumulation of aggregated, misfolded, or damaged proteins in the affected cells. In the cytosol, heat-shock or other harsh conditions trigger the activation of HSF-1 which subsequently up-regulates the transcription and translation of the Hsp chaperones (e.g. HSP 70). As part of the protein quality control system, HSPs play a major role in the struggle to maintain functional cellular machinery under stress conditions (Figure 5.1).

5.2 Grps involved in ER stress and UPR

Hsps address problems of protein misfolding in the cytosol, mitochondria and nucleus. But malfolded proteins can also accumulate in the ER (a condition referred to as ER stress) and, in this compartment, a different group of ER-resident chaperones is up-regulated by stress, chaperones that are functionally and transcriptionally different from Hsps.

5.2.1 Grp-assisted protein folding in ER

The chaperones in the ER (most are Grps) are structurally related to Hsps but are induced by stresses that disrupt the function of the ER and cause unfolded protein to accumulate in the lumen. The ER is a key compartment in cells that is specialized for protein export and contains many chaperones that are essential for the production of functional proteins for export (Wei, *et al.*, 1996). Protein folding in the ER is more complex than protein folding in the cytosol because proteins are posttranslationally modified. Most secretory proteins and noncytosolic domains of transmembrane proteins are cotranslationally transported in an unfolded state into the lumen of the ER, where

resident enzymatic activities prevent these nascent proteins from aggregating as they fold into their native conformations (Brodsky, 1998). The newly synthesized polypeptides that have not yet folded completely, as well as those proteins that have folded incorrectly (misfolded), are retained in the ER via their interactions with ER-resident chaperones (Ellgaard and Helenius, 2001). Resident protein chaperones such as protein disulfide isomerase (PDI) and *cis-trans* prolyl isomerase catalyze protein-folding reactions and increase the rate by which proteins attain their final correct conformation. Protein chaperones such as Grp78, do not actively catalyze protein folding, but rather maintain proteins in a folding-competent state and prevent protein aggregation (Shen *et al.*, 2004)

5.2.2 ER stress and UPR

A number of biochemical and physiological stimuli can change ER homeostasis, impose stress on the ER, and subsequently lead to accumulation of unfolded or misfolded proteins in the ER lumen (referred to as ER stress). The ER has evolved stress responsive signaling pathways collectively known as the unfolded protein response (UPR) to cope with such ER stress (Shen et al., 2004).

Activation of the UPR is designed to eliminate misfolded proteins in the ER in two ways: 1) by up-regulating the expression of chaperone proteins and degradation factors to refold or eliminate misfolded proteins, and 2) by attenuating translation to reduce the traffic of incoming proteins into the ER (Rutkoeshi and Kaufman, 2004). Genes whose products are involved in metabolism and resistance to oxidative stress are also upregulated to aid the cell in recovering from ER stress. However, under severe

conditions, when the cell is unable to recover from ER stress, apoptosis occurs.

Three ER resident transmembrane proteins have been identified as sensors of ER stress: PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE-1). Binding of molecular chaperones to misfolded proteins is the key step in the UPR. Under normal conditions, the ER chaperone, Grp78, is bound to the ER luminal domain of each sensor. However, when misfolded proteins accumulate in the ER, Grp 78 is removed from these sensors to bind instead to misfolded proteins (Bertolotti *et al.*, 2000). Grp 78 release from PERK and IRE-1 leads to dimerization of their luminal domains, leading to autophosphorylation and activation, whereas Grp 78 release from ATF6 unmasks a Golgi localization signal allowing relocation of ATF6 to the Golgi, where it is cleaved and activated (Shen et al., 2002).

The UPR in eukaryotic cells has evolved to be an integrated signaling network in adaptation to secretory demands (Figure 5.2). ER transmembrane proteins (PERK, IRE1, and ATF6) mediate a three-phase regulation at both transcriptional and translational levels in response to ER stress (Kaufman, 1999). Activation of PERK, as well as Ire1, is controlled by the protein level of Grp78 and under normal conditions PERK and Ire1 both bind Grp78. During ER stress, Grp78 is uncoupled from these kinases to interact with malfolded proteins in the lumen of the ER, permitting PERK and Ire1 activation. One resulting action is the up-regulation and synthesis of more Grp78 to aid protein refolding but ultimately high Grp78 shuts off the stress response due to re-association of Grp78 with the kinases. Other actions of PERK and Ire1 allow for reduction of protein

synthesis workload. An immediate transient attenuation of protein biosynthesis is mediated by PERK phosphorylation of eIF2alpha to reduce the load of new client proteins entering the ER whereas IRE1 and ATF6 mediate the transcriptional activation of genes encoding components that increase ER translocation, protein folding, export, and degradation. Cells that have sustained irreparable levels of damage caused by ER stress are eliminated by programmed cell death (Harding et al., 2002; Kaufman et al., 2002).

In short, to maintain homeostasis, the ER has evolved functions to sense the stress in its lumen and transduce signals to the cytoplasm and the nucleus in an attempt to adapt for survival or, if the stress is too great, induce apoptosis. These responses including transcriptional activation and translational attenuation are collectively termed the UPR and activation of the UPR depends on the dissociation of the proximal signaling molecule Grp 78.

5.2.3 Grp78 and other Grps

Grp 78 is the most studied and best characterized ER chaperone (Kozutsumi et al., 1988; Lee, 2001) and also accounts for most of the calcium buffering capacity of the ER (Fasolato et al. 1998). Grp 78 is critical for maintenance of cell homeostasis and the prevention of apoptosis (Yang, et al., 2000). Grp 78 levels are a reliable biomarker for the onset of ER stress and the UPR (Hong et al., 2005). During conditions of ER stress, the main function of Grp 78 is to bind unfolded proteins, prevent them from aggregating, and aid and monitor their refolding.

Several other ER Grps, including Grp 170, Grp 94 and Grp 58, are known to bind

to some nascent ER proteins (Dierks et al., 1996; Kozutsumi et al., 1988). However, the roles of these chaperones in the UPR and their relationship to Grp 78 have not been clearly elucidated. Grp 75 is not an ER chaperone, but acts in the mitochondria, and is required for the import and folding of mitochondrial proteins (Liu et al., 2001). Little is known about the control of its expression.

In summary, the transcriptional up-regulation of ER chaperones and folding enzymes is the hallmark of the ER stress response. A major role for the increased levels of chaperones during conditions of ER stress is to prevent unfolded and misfolded proteins from aggregating and promote the proper folding and assembly of proteins in the ER. One of the ER chaperones, Grp 78, is central to the cellular mechanisms that sense alterations in the ER environment.

5.3 Stress proteins in cold-hardy insects

This thesis examined stress proteins in the larvae of two species of cold-hardy insects with different overwintering strategies: the freeze tolerant gall fly, *E. solidaginis*, and the freeze-avoiding gall moth, *E. scudderiana*. The goal was to understand the role of protein chaperones in cold hardiness and/or diapause in these larvae by identifying and analyzing the regulation of the various chaperones that are expressed by the two species during both indoor laboratory cold exposures and outdoor natural field conditions.

To survive seasonally recurring, chronic forms of environmental stress, such as

cold or dry seasons, most insects enter the dormant state of diapause (Denlinger 2002). Initiated by environmental cues, diapause is characterized by developmental arrest, strongly suppressed metabolic rate (Tauber et al,. 1986), an increase in resistance to environmental stresses including increased cold hardiness (Adedokun and Denlinger 1984), and the activation of a specific set of genes (Flannigan et al., 1998). Diapause has been well defined in several species, including *E. solidaginis*. This temperate species responds to short daylength and cold temperatures by entering diapause as mature third instar larvae and spends the winter inside galls on the stems of goldenrod plants, genus *Solidago* (Irwin *et al.*, 2001). Less is known about diapause in *E. scudderiana* but observations in the Storey lab indicate that they also will not resume development without a 3-4 month cold exposure; hence, diapause by the mature larvae is indicated.

5.3.1 Regulation and expression Hsps in cold hardy insects

The data presented in my studies show that in *E. solidaginis*, HSP 110, HSP 70, and HSP 40 all rose substantially over the cold months in the field and HSP 70 and HSP 40 also responded to laboratory freezing conditions (-16°C at 24 h). Hence, overexpression of the HSP 70 and its cochaperone HSP 40 appear to have significant roles to play in winter survival in nature. I also showed that levels of activated HSF-1 were increased under frozen conditions, suggesting that HSF-1 activation is cold/freezing responsive and that this shock response is activated by an abrupt decrease in temperature. However, in the field, HSF-1 showed higher levels in the autumn (September, October)

than in the winter (December, February), indicating that HSF-1 action in stimulating gene expression is high before the larvae experience regular freezing bouts outdoors. This may set the stage for the build-up of selected HSPs as outdoor temperatures cool over the autumn and into early winter (ie. HSP110, HSP70, HSP40 were all elevated in December). Over the midwinter months, the insects are in diapause and in the dormant state there may be relatively little new protein synthesis, particularly when the larvae are frozen and energy-restricted. Furthermore, thawing (transfer from -16°C to +3°C for 24 h) decreased both HSP 70 and HSF-1 expression which indicates that the cold-activation of HSF-1 at -16°C is reversible by warming/thawing. This is consistent with the lower levels of activated HSF-1 in March and April and suggests that HSP synthesis is turned down as environmental temperatures warm up in the spring (note that HSP110, HSP70 and HSP40 levels also relative low in March and April). HSP 70 levels also increased significantly under anoxic conditions in *E. solidaginis* and this matches well with the HSF-1 expression pattern under anoxia. Hence, the strong activation of HSF-1 seen when larvae were exposed to -16°C might be triggered both by cold and by the anoxia associated with freezing. Interesting, HSP60 expression responded oppositely to HSP 70, strongly decreasing during the midwinter months and after 24 h anoxic stress. These data suggest that HSP60 (the mitochondrial chaperone) is regulated differently that other chaperones. Indeed, HSP60 patterns mirror the overall decrease in mitochondrial abundance in the larvae while they are in winter diapause.

In *E. scudderiana*, all Hsps (HSP 110, HSP 70, HSP 60, HSP 40, and HSP 10)

were significantly increased during midwinter months in the field, and HSP 110 and HSP 70 were also strongly induced in the indoor larvae that were cold acclimated at -20°C for 1 week. This strongly suggests that up regulated Hsps (particular HSP 110 and HSP 70) aid *E. scudderiana* survival during the winter, perhaps in countering the effects of wide variations in environmental temperature on the conformation and function of cellular proteins. In addition, the Hsp protein data confirms transcriptional data in *E. scudderiana*, which showed that levels of activated HSF-1 increased during the midwinter months and during subzero exposures. Hence, cold stress activates HSF-1 and results in overexpression of Hsps (at least HSP 110 and HSP 70). However, the low temperature may not be the only trigger of activation of HSF-1, since the data showed that 4°C exposure for 1 week actually reduced the amount of activated HSF-1. However, HSP expression in *E. scudderiana* larvae was not sensitive to anoxia stress since levels of HSP110, HSP70, HSP60 and HSP40 decreased or remained stable under anoxic exposure. Interesting, again, we found that HSP 60 expression followed a different pattern (-20°C exposure for 1 week decreased expression), suggesting HSP60 may have specific role in *E. scudderiana* larvae.

For protection against acute forms of environmental stress, insects rely in part on the well-described protective actions of Hsps. These proteins are rapidly upregulated in response to such environmental insults as temperature extremes, anoxia, and various chemical contaminants. Both diapausing and nondiapausing insects have been shown to

increase Hsp production following a cold shock (Denlinger, 2002). Nondiapausing individuals of *S. crassipalpis* (Joplin *et al.*, 1990), *D. melanogaster* (Burton *et al.*, 1988) and *D. triauraria* (Goto et al., 1998) all responded in a similar manner by producing stress proteins in response to low temperatures. Diapausing pharate larvae of *Lymantria dispar* (Yocum *et al.*, 1991) and diapausing adults of *D. triauraria* (Goto *et al.*, 1998) both produced high molecular weight stress proteins after a cold shock. However, the extent to which diapause and the Hsp response are linked is largely unknown.

Our data suggest that Hsps have very important roles in the winter survival of these two diapausing, cold hardy insects. One possible role of the over-expression of chaperone proteins (particularly HSP 70) in these species is to provide a way to maintain the integrity of key metabolic enzymes or structural proteins over the long term during winter diapause. The potential for significant new protein synthesis at subzero temperatures and in the dormant state (diapause) state is low and, therefore, mechanisms that protect existing proteins and renature unfolded, misfolded or aggregated proteins may be very important for long term viability.

5.3.2 The roles of Grps and other chaperones in cold hardy insects

The data showed that in *E. solidaginis* Grp 78 expression levels did not change under laboratory freezing conditions (-16°C at 24 h), indicating that cold/freezing does not directly target expression of Grp 78 in this freeze tolerant species. However, Grp 78 content increased strongly after 24 h anoxia exposure, and elevated levels were also

observed during the winter months; this suggests that Grp 78 may have a very important role to play in the response to freeze-induced hypoxia. Since freezing and hypoxia can induce ER stress, the increase in Grp 78 levels is probably indicative of an activation of the UPR under these conditions. In addition, UPR activation is known to cause attenuation of overall protein biosynthesis. Suppression of protein biosynthesis is an important energy-saving aspect of diapause, and so up-regulation of Grp78 and activation of the UPR may be one mechanism that contributes to the molecular mechanisms of insect diapause.

My studies also found that Grp 75 responds directly to cold temperatures in *E. solidaginis* larvae. This suggests that Grp 75 also has an important role to play in the folding/assembly/protection of mitochondrial proteins during the winter months. The importance of Grp 75 is further highlighted by the fact that other studies have shown that mitochondrial numbers decrease over the winter in this species (Levin *et al.* 2003) as do the activities of multiple mitochondrial enzymes (Joanisse and Storey, 1995) and the other main mitochondrial chaperone, Hsp 60. Hence, the increase in Grp 75 when most other mitochondrial proteins are greatly reduced, indicates an important role for Grp 75.

Most interestingly, the data suggest that upregulation of TCP-1 and α-crystallins may act cooperatively to stabilize actin and tubulin, key proteins of the cytoskeleton, over the wide range of environmental temperatures encountered over the winter by diapausing *E. solidaginis* larvae. It appears that in the late autumn (October) and early winter (December), elevated TCP-1 helps to stabilize newly synthesized cytoskeleton proteins

perhaps as part of a metabolic reorganization as the larvae enter diapause. By contrast, the elevation of α-cystallins in late winter (March) could help thawed larvae to enter post-diapause by holding and transporting denatured proteins (accumulated over the winter) or aiding renewed synthesis of cytoskeletal proteins for the larva to pupa transition by acting as a co-chaperone with another chaperone such as HSP 70.

In *E. scudderiana*, the data showed that all ER-resident GRPs (Grp 170, Grp 94, and Grp 78) were up-regulated during the winter months, however their roles may differ in dealing with cold temperature or diapause. Grp 170, functionally like HSP 110, can refold and reassemble denatured protein caused by cold injury. Grp 94, not directly responsive to cold temperatures, may play a cell protection role in *E. scudderiana* diapause. Grp 78, strongly induced by cold stress, may be responding to activation of the UPR caused by unfolded/misfolded proteins accumulation in ER. Meanwhile, activation of UPR can also support hypometabolism in the diapause stage. So, up-regulation of Grps in *E. scudderiana* could be an important response of this species for dealing with winter survival and diapause development. My results also suggest that TCP-1 may contribute to stabilizing and folding newly synthesized cytoskeleton proteins when *E. scudderiana* enter the diapause stage by folding these important proteins.

Of the chaperone proteins, only Hsp overexpression has been considered to date as a adaptive mechanism of insect cold hardiness and diapause development (Denlinger 2002). My findings not only greatly expand the data for many different kinds of Hsps but

also shows that Grp up-regulation (in particular Grp 78) is also associated with insect cold hardiness and diapause. Possible functions for upregulation of Grp 78 may include both (1) the chaperone function (e.g. transport of peptides into the ER lumen, chaperoning immature secretory proteins, transport of proteins from the ER lumen to the cytoplasm for degradation), and (2) the UPR monitoring and regulating function (activate the UPR under ER stress to help suppress metabolic rate, increase the transcription of chaperone genes, and increase the degradation of malfolded proteins caused by cold shock). My data also indicates that other protein chaperones (TCP-1 and α-crystallins) may play a role in stabilizing actin and tubulin, key proteins of the cytoskeleton, over the wide range of environmental temperatures encountered over the winter.

5.4 Conclusion

In summary, the data in this thesis has shown that low temperature, freeze/thaw, and hypoxia stresses up-regulate a number of chaperone proteins and indicate that multiple endogenous pathways are engaged in maintaining and/or restoring cellular homeostasis in response to these insults. To ensure cell survival, most of the molecular chaperones (Hsps, Grps, TCP-1, α-crystallins) that were assessed in these two cold hardy insect larvae were up-regulated directly or indirectly by subzero temperature and/or anoxia exposures to enhance the protein-folding machinery in order to stabilize, repair or eliminate misfolded or denatured proteins. Furthermore, over-expression of some Hsps and Grps appears to contribute to the molecular and biochemical mechanisms of diapause

in these two insects.

5.5 Remaining questions

The findings of this study have documented possible roles for protein chaperones in the protective response mechanisms used by cells of *E. solidaginis* and *E. scudderiana* to survive winter conditions and sustain viability over long weeks in diapause. But considerable work remains to be done to fully understand all the roles, actions and/or targets of Hsps and Grps.

Induction of multiple of Hsps through the activation of HSF is well characterized and Hsp expression is widely believed to be the fundamental response of cells under various stresses including heat, anoxia, and chemical. However, Hsp induction in response to cold stress still controversial. Various studies have argued that induction of HSPs is not a response to cold but rather to heat, because they found that HSPs did not increase during the period of cold stress itself but during the rewarming afterward (Fujita, 1999; Holland, et al., 1993; Laios, et al., 1997; Liu et al., 1994). However, our results show that some Hsps (in particular HSP 70) respond to cold directly, and this result was also confirmed by HSF induction during cold stress. Future investigations should focus at the mRNA level to determine whether it is cold or warming that activates the Hsp gene expression. It is possible that mRNA transcript levels may be stimulated by cold exposure but that the majority of protein synthesis may be postponed until temperature rises again. Interesting, our data showed that HSP 60 responded to cold/anoxia differently than other Hsps and its

transcription is not regulated by HSF-1 (at least not directly). This indicates a special role of HSP 60 in these two insect species and future studies should be directed at identifying the actual molecular actions of HSP 60 over the winter months.

Although the basic unfolded protein response (UPR) is conserved throughout the eukaryotic kingdom, relative to its yeast counterpart, in higher eukaryotes the response is considerably expanded in scope and consequences. Groundbreaking research over the past few years has led to an exhaustive description of the basic pathways involved in UPR activation, signal transduction and transcriptional activation in mammals (Harding et al., 2002; Kaufman et al., 2002). Much less is known about the finer details of the control and regulation of the UPR in invertebrate species. Our data suggested that Grps (in particular Grp 78) play a very important role in winter survival and/or diapause in insects. However, their functions are not very clear in these two larvae. We do not know if the up-regulation of Grps is a target of the cold stress directly; or is part of diapause. To confirm the Grps do contribute to insect overwintering survival, future studies should aimed on the signal transduction mechanisms. For example, studies should determine whether ATF-6, the main TF that regulates Grps, is also up-regulated in response to cold in the insects. The IRE1/XBP1 pathways should also be examined to see if protein degradation is activated in cold diapausing insects or whether these parts of the UPR are separately regulated under winter conditions. Furthermore, other key downstream genes and their protein products that are part of the UPR (i.e. PERK/eIF2a/ATF4 pathway) could be also investigated to see if they are also altered in cold diapausing insects.

The results in our studies demonstrate that both Hsps and Gps are associated with diapause development in these insect species. However, how these proteins may function in the long-term developmental arrest associated with diapause is unclear. We also do not know if Hsp/Grp transcripts are present throughout diapause or if their expression pattern is limited to specific times. To answer this question, further studies should examine the expression patterns of the mRNA transcripts of chaperone genes both after acute stress and over the winter season.

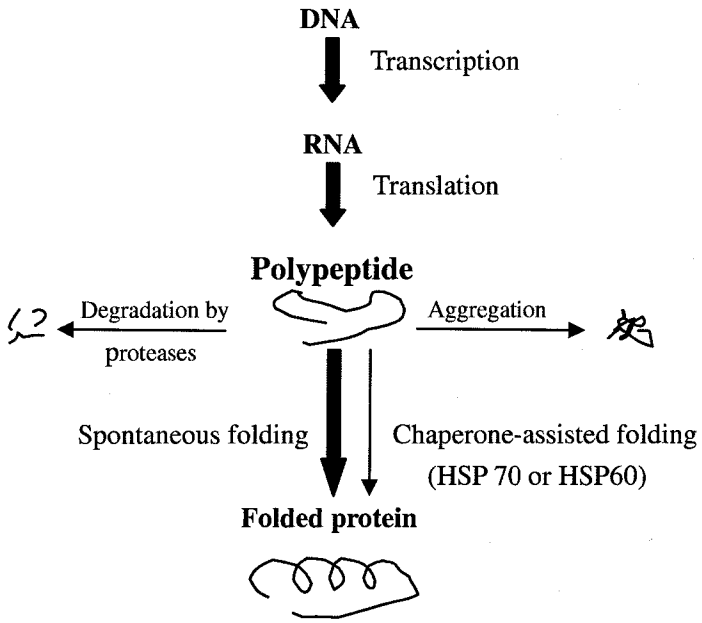
In summary, this thesis provides new insights into the molecular and biochemical adaptations that confer cold- and hypoxia-tolerance in insects. Much work is needed to fully elucidate the complex network of molecular intricacies that mediate stress-tolerance in insects but a good understanding of the role of protein chaperones has been initiated with the work presented here.

Fig. 5.1 Simplified model of protein quality control under normal and stress conditions.

A. Under normal conditions, the rate of transcription and translation is very high. As indicated by the thick arrow, most proteins fold spontaneously only few proteins folding need the assistance of chaperones. Few proteins aggregate and few unfolded proteins are degraded by proteases.

B. After stress, the transcription and translation capacity is reduced. Stress-induced unfolding or misfolding returns previously folded proteins to unfolded stage. The chaperone-dependent quality control system increase action: Some unfolded proteins and misfolded proteins are folded or refolded by HSP 70 and HSP 60 reaction cycle;; some unfolded proteins and misfolded proteins aggregate and few of them disaggregation by HSP 110 and rest of them together with left unfolded and misfolded proteins are removed by proteases.

A.



B.

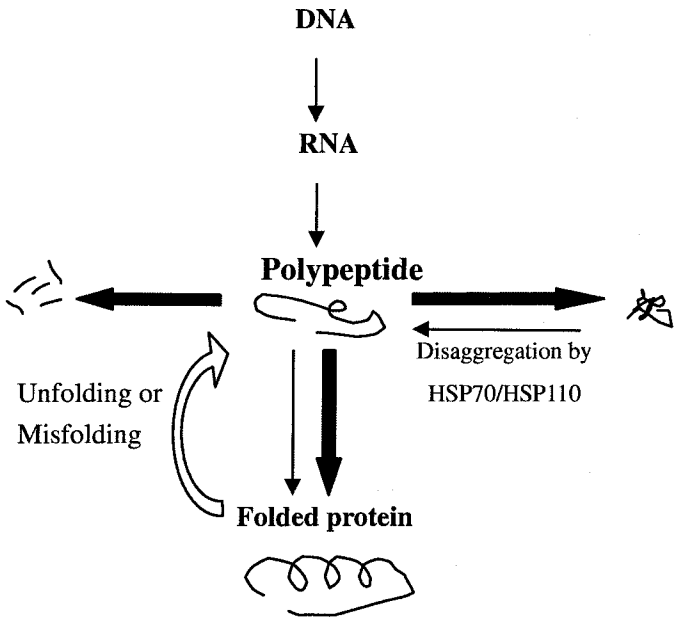
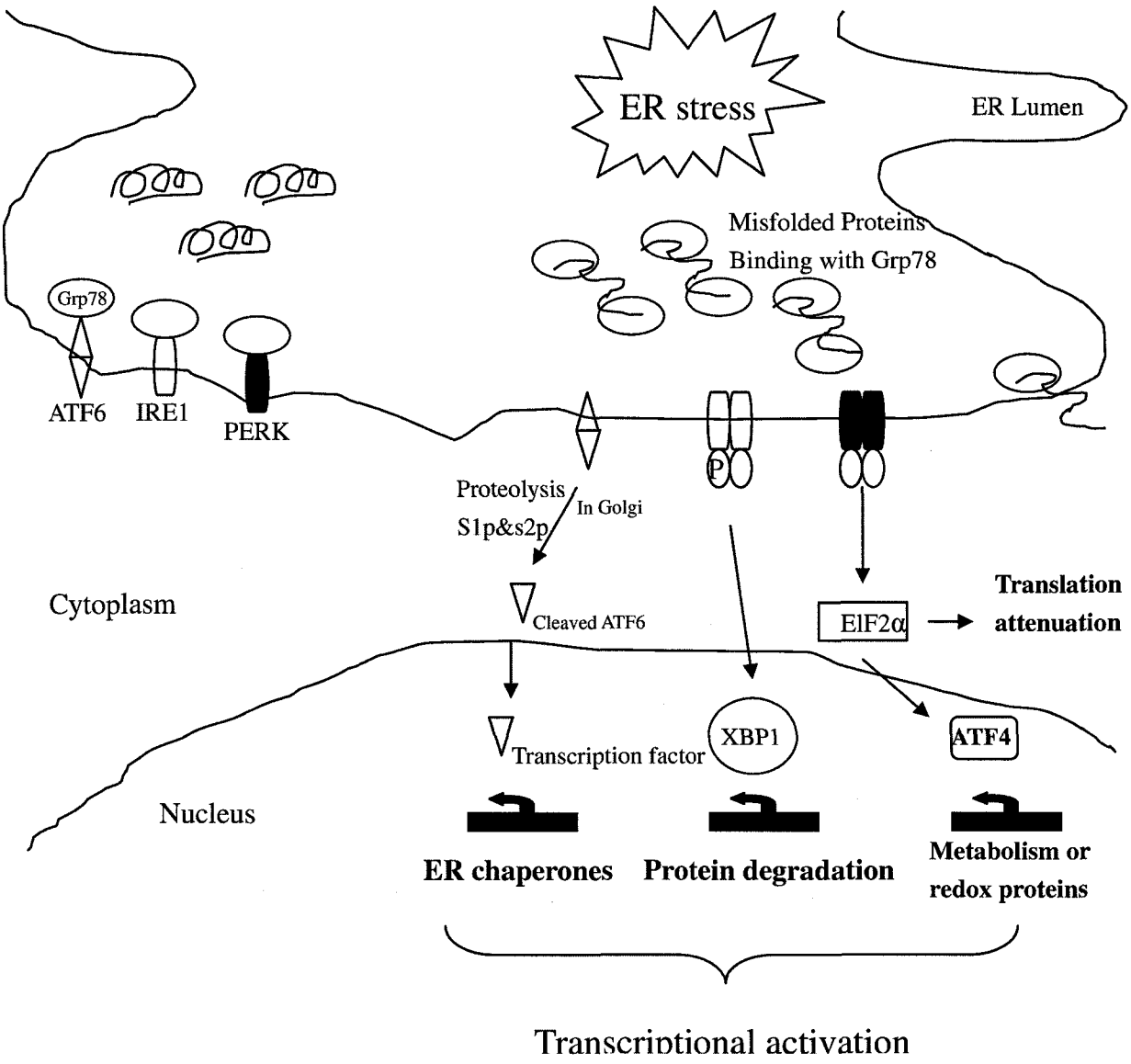


Figure 5.2 Aspects of the unfolded protein response (UPR) and the component of the pathway that regulates them.

Accumulated unfolded proteins titrate the endoplasmic reticulum (ER) chaperone Grp 78 away from association with 3 ER-resident sensors (2 transmembrane kinases (IRE 1 and PERK) and an ER-tethered transcription factor (ATF6)), freeing them for activation and induction of the UPR. A PERK dimerizes and phosphorylates EIF2- α, causing a general inhibition of proteins translation and stimulation of ATF4 synthesis, and ATF4 transcriptionally activates the expression of genes encoding metabolism and redox proteins. IRE1 dimerizes, becomes phosphorylated and then mediates the removal of intron from XBP-1 mRNA. This spliced form of XBP-1 mRNA encodes a TF which transcriptionally activates genes encoding proteins involved in proteins degradation (e.g. degradation factor). ATF6 (90kDa) is cleaved in the Golgi forming an active 50-kDa TF which transcriptionally activates genes encoding ER chaperones.



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