**MOLECULAR AND BIOCHEMICAL ADAPTATIONS
CONFERRING COLD-HARDINESS IN TWO GALL INSECTS**

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of the requirements for the degree of

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ABSTRACT

Terrestrial insects have evolved molecular and biochemical adaptations that have allowed them to exploit virtually all climates on earth including adaptations that allow survival at subzero temperatures during the winter months by insects living in temperate and polar climates. Two strategies of insect cold-tolerance have emerged: freeze-tolerance and freeze-avoidance. Freeze-tolerant insects can endure extracellular ice formation, whereas freeze-avoiding species strongly depress the supercooling point of their body fluids to remain liquid over the winter. The research reported in this thesis analyzed molecular strategies of cold tolerance, hypometabolism and hypoxia tolerance in both kinds of insects using as models the freeze-avoiding gall moth, *Epiblema scudderiana*, and the freeze-tolerant gall fly, *Eurosta solidaginis*. Activities of ion motive ATPases [Na^+K^+ ATPase, sarco(endo)plasmic Ca^{2+} ATPase] were strongly reduced over the winter months, contributing to energy-saving hypometabolism, and *in vitro* studies indicated that the control mechanism involved was reversible protein phosphorylation. Differential gene expression in response to cold, subzero or hypoxia exposures was evaluated using three different methods (cDNA library construction and screening, nylon macroarrays, DNA microarrays) to identify key protein products that could contribute to cold and hypoxic tolerance. These studies identified a wide variety of genes as cold responsive that have never previously been associated with cold tolerance. Enzyme activities, transcript levels, and DNA content of mitochondrial genes were evaluated under cold and hypoxia stresses and indicated a role for mitochondria in cold and hypoxia tolerance that is more dynamic than previously understood and that differs significantly between freeze avoiding and freeze tolerant species. Other studies indicated an important

role for signal transduction via the mitogen-activated protein kinase (MAPK) superfamily in cold and hypoxia tolerance, particularly the role of the p38 MAPKs, which showed clear differences between the freeze-tolerant and freeze-avoiding species. Finally, examination of the nuclear factor (NF)- κ B signaling pathway indicated that the suppression of the inflammatory response appears to be significant in the winter survival of both species and may be a crucial difference between hypoxia-tolerance and hypoxia-intolerance.

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LIST OF ABBREVIATIONS

AA	amino acid
% <i>a</i>	percent glycogen phosphorylase in the active <i>a</i> form
AMP, ADP, ATP	adenosine mono- di- or triphosphate
BHBDH	β -hydroxybutyrate dehydrogenase
cDNA	complementary DNA
COX	cytochrome c oxidase
COX 1	gene encoding cytochrome c oxidase subunit I
CS	citrate synthase
Cy3/Cy5	cyanine dye 3 or 5
cAMP	3', 5'-cyclic adenosine monophosphate
cGMP	3', 5'-cyclic guanosine monophosphate
DEPC	diethylpyrocarbonate
DOC	deoxycholate
DTT	dithiothreitol
EDTA	ethylenediamine tetra-acetic acid
EGTA	ethyleneglycol bis(β -aminoethyl ether) N, N'-tetraacetic acid
Elf2 α	eukaryotic initiation factor 2 α subunit
ERK	extracellular regulated kinase
EtBr	ethidium bromide
FP	freezing point
HSP	heat shock protein

IEF	isoelectric focusing
I κ B	inhibitor of NF- κ B
kb	kilobase
kDa	kilodalton
k _m	Michaelis-menton
MAPK	mitogen activated protein kinase
mRNA	messenger RNA
NF- κ B	nuclear factor kappa B
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethysulfonyl fluoride
Q ₁₀	factor by which a 10°C temperature change reduces the rate of a chemical reaction
RHCC	Reduced horse cytochrome c
rRNA	ribosomal RNA
RT	reverse transcription
SDS	sodium dodecyl sulfate
SERCA	sarco(endoplasmic reticulum Ca ²⁺ ATPase
SSC	saline sodium citrate
Tris	tris(hydroxymethyl)aminomethane
V _{max}	maximal enzyme velocity

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Chapter 1

General Introduction: Insect cold-tolerance

The cold winter temperatures of temperate and polar climates pose a severe challenge to cold-blooded animals inhabiting these areas. Common strategies to elude injury from subzero temperature exposure include migration to more suitable climates or avoidance of cold temperatures by spending the winter in thermally buffered hibernacula. Some species have adopted other overwintering strategies that allow them to deal with harsh winter conditions head-on. These species have evolved specific physiological and biochemical characteristics that confer cold-hardiness.

Subzero temperatures are dangerous to ectothermic animals due to the increased potential for the formation of ice within body tissues. For non-tolerant species, it is not just the risk of intracellular ice but of any ice forming within the body because the resulting ice expansion in fluid spaces such as capillaries, ventricles of the brain, the lumen of the gut, the abdominal cavity or bladder can cause major physical damage to the animal. In addition, extracellular ice formation places both severe osmotic and anoxic stresses on cells. Intracellular ice formation appears to be lethal to all organisms – although a couple of reports dispute this – whereas with adaptation, a variety of organisms can endure ice formation in extracellular spaces. Two strategies have been adopted by cold-hardy ectotherms that need to survive weeks or months of subzero exposure during the winter. These are freeze-tolerance and freeze-avoidance. Freeze-tolerant organisms can endure the formation of ice in extracellular spaces while using biochemical adaptations that prevent intracellular ice growth. Freeze-avoiding species resist any ice formation within their bodies by depressing the supercooling point of their body fluids to temperatures well below those they would encounter in their natural environment. These two

strategies are discussed in detail in multiple review articles (Aarset, 1982; Asahina and Tanno, 1969; Baust, 1973, 1981; Murphy, 1983; Zachariassen, 1985; Storey and Storey, 1988; Duman, 2001; Zachariassen and Kritiansen, 2000).

Low temperatures can cause two types of injuries 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 (Bock and Frieden, 1978; Hazel, 1984; Hatley and Franks, 1986; Locker and Hernandez, 2004). Low temperatures also change the dielectric permittivity and ionic characteristics of cellular solutions. For example, the intracellular pH increases as the temperature decreases. However, all animal systems use intracellular histidine buffers that are based on the buffering of imidazolium groups on histidine. The change in pH with temperature of imidazole exactly matches the change in pH of water with temperature and this has the effect of maintaining a constant degree of ionization on proteins as temperature changes. This buffering system is known as alpha-stat which occurs even in nonhardy organisms so its not too likely that pH change caused by temperature would be a big factor in cold injury although it could contribute, especially when cell temperatures exceed the range that is normally experienced by the species.

Low temperature can also disrupt cellular ATP generation and the balance

between net ATP production and ATP consumption. Because of ATP limitation in the cold (also perhaps due to direct temperature effects) critical cellular processes can be disrupted. For example, an imbalance between the activities of ATP-dependent ion pumps and ATP-independent ion channels results when ATP is limiting and leads to membrane depolarization in non-tolerant cells. These problems involving energetics are the basis of many hypothermia injuries. These same problems also arise as a result of hypoxia as well due to the limited ATP production capacity of anaerobic processes. Therefore, the summed result of low temperature on non cold-hardy cells is an overall disruption of metabolic processes, in particular cellular energetics, which can become lethal if exposure is for an extended period.

The main threats of injury 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 as 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). Even freeze tolerant animals are subject to these same injuries if freezing is at temperatures far below the normal range to which the organism is adapted. Thus, freeze tolerant animals employ colligative cryoprotectants and other adaptations that are just sufficient to allow their cells to remain above the critical minimum cell volume at the lower end of the natural temperature range

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. Freeze tolerant insects are found most commonly in the orders Coleoptera, Diptera, Hymenoptera, and Lepidoptera (Baust, 1981; Baust and Ring, 1982; Block, 1982; Danks, 1978, Hansen, 1980; Lee *et al.*, 1986; Kostal *et al.*, 2004; Sinclair *et al.* 2004). Survival freezing at -30°C is common and there are even some Arctic species known to survive freezing at temperatures down to -70°C (Miller, 1982; Ring, 1981, 1982).

Two species of insects with different overwintering strategies have been chosen as model organisms for the work presented in this thesis. *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). There are many factors necessary to confer either freeze-tolerance or freeze-avoidance and several are common to both strategies.

Freeze-avoiding species resist the formation of ice at temperatures below 0°C by depressing the supercooling point of their body fluids (Storey and Storey, 1988; Zachariassen, 1985). Under carefully controlled laboratory conditions, minute droplets of ultrapure water can be supercooled to -40°C (the homogeneous nucleation point) but this does not normally occur because heterogeneous nucleation of water (stimulated by solutes, particles, surfaces, disturbances, etc.) can be triggered at any temperature below 0°C . Insects and other invertebrates that use freeze-avoidance use a variety of

mechanisms to push the supercooling point of their body fluids downwards as far as possible but they always run the risk of spontaneous freezing should winter temperatures drop below their supercooling point or should they come in contact with powerful nucleators (e.g. external ice crystals touching their body surface). Therefore, it is necessary that they take extensive measures to stabilize the supercooled state. The process of preparing to supercool (or to freeze in freeze-tolerant species) is called cold-hardening. During the cold-hardening process, freeze-avoiding insects take several steps to reduce the probability that they will freeze at subzero temperatures. These steps include: 1) finding hibernacula which will protect the insects from nucleators such as contact with environmental ice, and/or forming a protective barrier against such contact (e.g. a silky cocoon in the case of *E. scudderiana*), 2) elimination of potential ice nucleators from within the body; this includes clearing all gut contents as well as seasonal elimination of some protein types from the haemolymph, 3) the synthesis of thermal hysteresis proteins (antifreeze proteins), 4) reduction of the amount of free water available for freezing in the body by reducing total body water content over the winter, and 5) the accumulation of high concentrations of cryoprotectants, usually polyhydric alcohols, that lower the supercooling point of body fluids in a colligative manner. For example, the supercooling point of *E. scudderiana* drops from -15°C to -38°C as it cold-hardens over the autumn months (Rickards *et al.*, 1987).

The use of thermal hysteresis proteins, also known as antifreeze proteins, seems to be a universal characteristic of insects and other animals (e.g. marine fish living in polar waters) that have perfected the freeze avoiding strategy. These proteins have an affinity for ice (Davies *et al.*, 2002). Most proteins are excluded from growing ice crystals and

are thus pushed ahead of an expanding ice head. Antifreeze proteins are unique because they will bind to the ice head and prevent the further growth of ice beyond a microscopic size (Raymond and DeVries, 1977). This effectively lowers the freezing point in a non-colligative and non-equilibrium fashion. However, the presence of these proteins does not affect the melting point and therefore solutions containing these proteins exhibit a thermal hysteresis (temperature difference) between the melting and freezing points. The presence of thermal hysteresis proteins alone can often lower the supercooling point of insect hemolymph to values of -10 to -15°C and they are often the only adaptation needed by insects that spend the winter under the snow. However, other mechanisms that help to push the supercooling point even lower are needed by species that winter above the snowpack (such as *E. scudderiana*) or in very cold polar or alpine environments.

Lowering the total amount of bulk water in the body also aids freeze avoidance and freeze-avoiding insects show substantial dehydration as they cold harden. For example, the larvae of *E. scudderiana* lose up to 25% of their free body water over the autumn months (Rickards *et al.*, 1987). This effectively increases solute concentrations in the body and contributes to lower freezing and supercooling points of body fluids.

Because the freezing point of water is a colligative property, it is controlled by the concentration of osmotically active solutes in solution. As a result, an increase in solute concentration leads to a depression in both the freezing and supercooling points. The addition of one mole of solute lowers the freezing point of a solution by -1.86°C (1L solution) and can lower the supercooling point by up to three times that value (Hochachka and Somero, 1984). Insects make effective use of osmotically active solutes for cold hardening. Most insect cryoprotectants are low molecular weight polyhydric

alcohols, glycerol being the most common (Zachariassen, 1991). These types of cryoprotectants are advantageous because they can be easily manufactured from stored glycogen via existing metabolic pathways (Storey and Storey, 1983), they can easily pass through membranes, they have high solubility, low reactivity, and are non-toxic so they can be accumulated in high concentrations without negative effects on cell functions. The colligative depression of freezing and supercooling points by polyols is an invaluable component of freeze-avoidance. In *E. scudderiana* glycerol concentrations can rise to over 2 M (Rickards et al., 1987) and levels as high as 5 M have been measured in the wasp, *Bracon cephi* (Lee, 1991). High concentrations of polyols, combined with thermal hysteresis proteins, allow many freeze avoiding species to supercool to -40°C or even lower.

As opposed to freeze-avoiding species, freeze tolerant insects allow the formation of ice in their extracellular body fluids. Most species, including the *E. solidaginis* larvae studied here, can endure the conversion of about 65% of total body water into ice. Rapid uncontrolled ice growth can be lethal even in freeze tolerant species. Therefore, freeze-tolerant species take measures to promote the slow and controlled growth of extracellular ice by initiating ice formation at relatively high subzero temperatures. In fact, many freeze tolerant species actually elevate their supercooling points as they cold-harden, even in the presence of high concentrations of polyol cryoprotectants (Duman *et al.*, 1981). Control over ice formation is achieved by using ice-nucleating agents to initiate ice formation within extracellular fluid compartments. Sometimes these agents are nonspecific; for example, bacteria in the gut or on the skin surface act as the nucleating agents for some species. Other species add specific ice nucleating proteins to their

hemolymph and these trigger ice formation at very precise subzero temperatures. Ice nucleating proteins are believed to mimic the structure of embryonic ice crystals and provide a template for new ice crystal growth.

Having permitted ice formation in extracellular fluid spaces, the cells of freeze-tolerant species are then susceptible to the consequence of locking a high percentage of total body water into extracellular ice. The concentration of solutes in remaining extracellular fluids rises dramatically and this places a strong osmotic stress on cells that causes an outflow of water, a rise in intracellular ion and solute concentrations, and a major decrease in cell volume. These changes result in a colligative depression of the freezing and supercooling points of intracellular fluids but excessive cell shrinkage below a critical minimum cell volume can irreparably damage cell membranes. Excessive cell shrinking and intracellular freezing are resisted by the production and accumulation of high concentrations of polyhydric alcohols, the same strategy employed by freeze-avoiding insects. In *E. solidaginis*, for example, two polyols are produced, glycerol and sorbitol. Glycerol accumulates over the autumn months in response to cool temperatures below 15°C and is maintained at maximal concentrations throughout the winter. Sorbitol is produced only in response to temperatures below 5°C and concentrations fluctuate with rising and falling temperatures over the winter (Storey and Storey, 1988).

Freeze tolerant insects also take steps to stabilize their membranes against the compression stresses imposed during cell shrinkage. The disaccharide, trehalose, and the amino acid, proline, are frequently used to stabilize lipid bilayers. Both accumulate in *E. solidaginis* over the winter months.

Freeze-tolerant insects also employ most of the other adaptations used by freeze

avoiding insects to enhance their cold hardiness. They reduce the amount of body water available to freeze. This occurs, not by whole body dehydration as in freeze avoiding insects, but by altering the cellular composition of macromolecules and subcellular components in a way that increases the amount of bound, unfreezable water, thereby decreasing the amount of bulk, freezable water. In *E. solidaginis*, the amount of bound water increased approximately three-fold during cold-hardening (Storey *et al.*, 1981). High concentrations of polyols are also used to strongly reduce the supercooling point of intracellular water and to retard water loss into extracellular ice crystals (Lee, 1981; Storey and Storey, 1988). Many freeze tolerant insects also have thermal hysteresis proteins in addition to ice nucleating proteins. This seeming contradiction was explained when it was demonstrated that thermal hysteresis proteins inhibit recrystallization, the process by which small ice crystals reform into larger and larger crystals. Inhibition of recrystallization is important to prevent crystals from growing to sizes that could do physical damage to delicate tissues.

In climates where much of the year is unsuitable for ongoing development due to reasons such as shortage of food supplies, extreme low or extreme high temperatures or shortage of water, many insects have developed the ability to enter a state of arrested development called diapause. Diapause is characterized by strong metabolic rate depression and entry into a torpid state and diapause in cold hardy insects has many similarities to winter hibernation in mammals. Diapause is associated with a stockpiling of energy reserves before feeding ends, a regulated decrease in metabolic rate, and an interruption of development (Denlinger, 2000). There are two types of diapausing species, obligatory and facultative. Some species enter obligatory diapause, a state that is

genetically programmed to occur at a specific stage of development regardless of environmental cues. However, the majority of insects that use diapause enter facultative diapause, a state that is triggered by environmental cues such as shortened photoperiod and cooler temperatures. A main advantage of diapause is the reduction of metabolic rate that allows the insect to conserve fuel reserves and energy and to survive for extended periods of time until more favourable conditions return. In most species that show a wintering diapause, the rate of oxygen consumption in diapause falls to 5% or less the normal rate at the same body temperature (Danks, 1987; Storey and Storey, 1987). The two species studied here both appear to have winter diapause. For *E. solidaginis* this has been well documented (Irwin *et al.*, 2001). Gall fly larvae in the Ottawa area seem to require a 3-4 month period of cold exposure, with diapause lasting until about early February. If removed from cold storage earlier the insects will not pupate but just die (J. Storey, personal observations). 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.

To date there has been much information published about the physiological and metabolic processes that occur during the overwintering of freeze-avoiding and freeze-tolerant insects, including *E. scudderiana* and *E. solidaginis* (Storey and Storey, 1981, 1983, 1991, 1992; Holden and Storey, 1993, 1994; Churchill and Storey, 1989a-d). However, at the time that my thesis research was started, there was very little information about the biochemical control mechanisms involved in regulating cold hardiness and/or diapause in cold hardy insects or the gene expression responses that support survival at subzero temperature. The research undertaken in the present thesis aimed to identify and

analyze various molecular and biochemical mechanisms employed by these insect species to support cold hardiness and to regulate reversible entry into the diapause state.

Specifically my hypotheses were:

1. Changes in gene expression support insect survival at subzero temperatures.
2. The hypometabolic diapause state in cold hardy insects is aided by changes in mitochondrial metabolism, specifically DNA abundance and transcription patterns.
3. The hypometabolic diapause state is aided by suppression of the activities of ATP-expensive ion motive ATPases, specifically $\text{Na}^+\text{K}^+\text{ATPase}$ and $\text{Ca}^{2+}\text{ATPase}$, the probable mechanism being a covalent one to make stable, yet reversible, changes to enzyme activities.
4. Metabolic and gene expression changes during cold hardening are regulated by changes in the activities and expression of selected protein kinases, protein phosphatases and transcription factors.

To address the first hypothesis changes in gene expression patterns were examined in both *E. scudderiana* and *E. solidaginis* in response to low temperature and/or freezing exposures. Three separate approaches were employed to assess gene expression changes in the two species: cDNA library screening, cDNA macroarrays, and cDNA microarrays. The goal of these experiments was to identify genes or families of genes whose protein products could contribute to cold hardiness, freezing survival or diapause regulation. Although various protein adaptations may be put in place during the autumn months in advance of cold exposure, other gene/protein responses may be up-regulated as direct responses to subzero exposure and the present experiments were designed to highlight those acute changes. Furthermore, it is now known that despite the

reduced energy available in the winter and the overall reduction in energy consuming processes, insects do continue to alter their protein make-up during diapause, either in response to acute stress or as part of “diapause development”, the slow program of metabolic change that ultimately results in diapause termination (Xu and Denlinger, 2004; Gotto and Denlinger, 2002; Bilgen *et al.*, 2001; Rinehart *et al.*, 2000; Yocum *et al.*, 1998). The gene screening experiments would also highlight such gene expression changes that are associated with diapause development in the insects. Finally, the comparison of two species, one freeze avoiding and one freeze tolerant, provides the opportunity to search for both common and disparate responses to low temperature, in particular with an opportunity to highlight gene changes that may serve freezing survival.

Mitochondria are the powerhouse of the cell and entry into a prolonged state of diapause with low metabolic rate might be aided by alterations to mitochondrial abundance or performance. Indeed, previous studies on a high Arctic caterpillar that spends up to 11 months of the year in diapause indicated that a large percentage of mitochondria were degraded during the non-active part of the year (Kukal *et al.*, 1989). Studies in our lab showed that the activities of a number of mitochondrial enzymes involved in energy metabolism decreased over the winter months in both species (Joanisse and Storey, 1994), but the mechanism(s) of these changes are unknown. To address my second hypothesis, studies were designed to analyze changes in mitochondrial DNA abundance and transcription patterns using molecular techniques such as PCR and RT-PCR. Studies analyzed mitochondrial responses to cold and freezing as well as to reduced oxygen availability, hypoxia/anoxia being one of the consequences of freezing for *E. solidaginis*.

When an organism makes a shift to a hypometabolic state, it is important that the cell maintain homeostasis. All cells have the ability to increase or decrease energy output based on demands; however, in a hypometabolic state such as diapause the cells must scale down ATP turnover dramatically but still maintain the balance between ATP consuming and ATP generating processes. One of the reasons why intolerant cells die in energy compromised states is because they are not able to rebalance ATP turnover in a way that maintains vital life processes without exhausting limited fuel supplies. Ion motive ATPases are major users of cellular energy and energy limitation to these ion pumps leads to rapid metabolic damage and loss of membrane potential difference when pump activity cannot keep pace with the activity of oppositely directed ion channels. Therefore, I hypothesized that both *E. scudderiana* and *E. solidaginis* would show active regulation to suppress the activities of ion motive ATPases both as part of diapause and in response to environmental stresses including low temperature and the hypoxia/anoxia associated with freezing in *E. solidaginis*. To address this third hypothesis, studies focused on two of the major ion motive ATPases, Na⁺K⁺-ATPase of the plasma membrane and Ca²⁺-ATPase of the sarco(endo)plasmic reticulum to analyze changes in enzyme activities in response to temperature, freezing and low oxygen stresses and to investigate the molecular mechanisms, such as reversible protein phosphorylation, involved in enzyme regulation.

Much work has been devoted to investigating how organisms can switch between states of high metabolic output down to a hypometabolic state. One mechanism of protein/enzyme regulation that is commonly used is reversible protein phosphorylation. The activities of many proteins can be activated or repressed by the addition or removal

of covalently bound phosphate groups via the actions of protein kinases or protein phosphatases. Modification of enzyme activities by this mechanism can often result in on-off control of enzyme function and reversible phosphorylation also frequently regulates the ability of transcription factors to bind to DNA and stimulate gene expression. Control by reversible phosphorylation of proteins is a much more energetically favourable way to regulate existing proteins/enzymes, particularly in response to transient stress, rather than to use coarse controls such as protein synthesis/degradation. Hence, I hypothesized that protein kinases and phosphatases would play significant roles in controlling both metabolic and gene expression changes in response to low temperature, freezing and low oxygen stresses in cold-hardy insects. To address this, I monitored changes in the activity states of multiple protein kinases, and transcription factors in the two insect species under different experimental states. Protein kinases and phosphatases typically act in response to extracellular signals (often as part of a long signal transduction cascade) or to internal cues such as cellular energy charge. My studies focused particularly on the MAPK and NF- κ B signal transduction pathway because they have been linked previously to stress survival.

In summary, then, the goals of this project were to shed light on the molecular and biochemical processes involved in insect cold tolerance. The information reported here has enhanced our understanding of this topic but has also opened new doors to even more questions that will need to be addressed in future projects.

Chapter 2

Regulation of Na⁺K⁺-ATPase and SERCA

INTRODUCTION

The harsh winter temperatures found in the northern terrestrial latitudes provide a severe environmental challenge that must be overcome by indigenous animals. Not only are environmental temperatures very low (often below 0°C), but these conditions also limit the availability of food resources for many months of the year and, therefore, are not conducive to active development of most species. Many species of insects have adopted a strategy of winter diapause – a state of arrested development with low metabolic rate similar to that of hibernation or estivation. Prior to diapause, insects build up sufficient fuel reserves to last the winter, and then metabolic rate is reduced, and development ceases as diapause is entered. *E. solidaginis* larvae enter diapause in mid-October and remain quiescent until early February. Normal development does not resume even if the larvae are exposed to warm temperature before at least mid-December (Irwin *et al.*, 2001). To date, there have been no specific experiments to test for diapause in *E. scudderiana* but, typically, overwintering larval/pupal lepidopterans are in diapause and *E. scudderiana* larvae that our lab has tested in the past would not pupate if taken out of cold storage in November or December (Storey and Storey, 1988).

Careful control of energy reserves is a necessity for all overwintering species. The greatest energy-consuming process in most cells is the active transport of ions across membranes (Claussen, 1986). All plasma membranes have an electrical potential difference across them with the cytoplasm negative relative to the outside of the cell. This is conducive to the entry of positively charged ions into cells and opposes the entry of negative ones. The transmembrane concentrations of many solutes in living cells are far from equilibrium. To maintain these gradients across membranes, energy input is

needed, usually supplied by the hydrolysis of ATP and the action of enzymes that act as transmembrane ion pumps. If ion pumps fail, the potential difference across the membrane is dissipated and this leads to a variety of degenerative events that can result in cell death. Because membrane pumps consume a large fraction of total cellular energy and are crucial for maintaining proper cellular homeostasis, it stands to reason that the activity of these enzymes would be suppressed in order to achieve a stable state of reduced energy expenditure in any hypometabolic state including diapause. I hypothesized, therefore, that the activities of membrane ion pumps would be strongly suppressed in overwintering larvae of *E. solidaginis* and *E. scudderiana*. Possible mechanisms involved could include changes in the total protein content of these enzymes, reversible control of pump activity via protein phosphorylation or dephosphorylation, and low temperature effects on enzyme activity or kinetic properties.

The above hypothesis was tested by studying two groups of the P-type ion-motive ATPases. The name "P-type" is derived from the fact that all members of this family have a phosphorylated intermediate as part of their catalytic cycle. These pumps undergo large-scale conformation changes upon substrate binding that leads to the movement of ions from one side of the membrane to the other. P-type ion pumps hydrolyse ATP to move ions across a membrane and against a concentration gradient. They have been found in bacteria, plants and animals and there are five branches of the family determined by the set of ions that they translocate: H^+ , Na^+/K^+ , H^+/K^+ , Ca^{2+} , and heavy metals (Axelsen and Palmgren, 1998). All P-type ATPases have four protein domains that are well conserved across phylogeny as well as ten transmembrane α -helices that make up

the ion translocation site and three cytoplasmic domains (N, P, and A) that complete the ATP hydrolysis. The 2 types studied here are the Na^+/K^+ and Ca^{2+} -ATPases.

Na^+/K^+ -ATPase, the largest member of the P-type family of ATPases, is a ubiquitous component of the plasma membrane that uses the energy of 1 molecule of ATP to drive the influx of 2 molecules of K^+ and the concomitant export of 3 molecules of Na^+ . This pump is the major contributor to the differential concentrations of Na^+ and K^+ inside and outside of cells: K^+ concentration inside a cell is 10-20 times higher than outside the cell and, conversely, intracellular Na^+ is 10-20 times lower than its extracellular concentration. Depending on the type of tissue, Na^+/K^+ -ATPases consume between 5-40% of a cell's energy (Claussen, 1986). Na^+/K^+ -ATPase has many important roles including contributing to the regulation of cell volume, maintaining resting membrane potential in nerve and muscle cells, and providing ion gradients that facilitate the movement of many other solutes. In many species including mammals, the active form of Na^+/K^+ -ATPase is a heterodimer consisting of α and β subunits. Several different genes have been found to code for each subunit resulting in many different isoforms that are expressed in a tissue specific manner (Jorgensen *et al.*, 2003). Recent evidence from insect studies indicates that the alpha and beta subunits may be functional on their own (Baxter *et al.*, 2003). To date, 14 different isoforms of P-type ATPase have been identified in the fruit fly, *Drosophila melanogaster* (P-type ATPase database; <http://biobase.dk/~axe/Patbase.html>).

Ca^{2+} -ATPases are responsible for maintaining Ca^{2+} homeostasis within the cell. They can be divided into two main groups depending on where they are located; plasma membrane Ca^{2+} -ATPases (PMCAs) or sarco/endoplasmic reticulum Ca^{2+} -ATPases

(SERCAs) (Pedersen and Carafoli, 1987). These two subtypes differ slightly in their mechanistic details and stoichiometry. PMCAs pump 1 molecule of Ca^{2+} across the membrane for every ATP hydrolysed whereas SERCAs sequester 2 molecules of Ca^{2+} into the sarcoplasmic reticulum for every ATP hydrolysed. The gradients generated by these pumps are used for a variety of signalling cascades. There are three forms of SERCA coded by closely related genes (Magyar and Varadi, 1990). SERCA1 is the fast-twitch skeletal muscle form, SERCA2 is the slow twitch muscle and cardiac form, and SERCA3 is a broadly expressed form found in muscle and non-muscle tissues. All three forms are inhibited by thapsigargin, a chemical extracted from the root of the plant, *Thapsia garganica*. Thapsigargin binds to SERCA (but not PMCA) in the third transmembrane domain and locks the enzyme into an irreversible inactive state (Sagara *et al.*, 1992).

Phospholamban (PLN), a 52-amino acid integral membrane protein is known to be the primary regulator of SERCA in cardiac sarcoplasmic reticulum. It interacts with cardiac SERCA at specific cytoplasmic domains (Toyofuku *et al.*, 1994a, b). The dephosphorylated form of phospholamban inhibits Ca^{2+} -ATPase by lowering the enzyme's sensitivity towards free Ca^{2+} (MacLennan *et al.*, 1992; Voss *et al.*, 1994; Autry and Jones, 1997). Phosphorylation of phospholamban at Ser¹⁶ or Thr¹⁷ results in an increase in SERCA activity by lowering the affinity of PLN for SERCA.

Studies presented in this chapter examine the protein levels, activities and regulation of Na^+K^+ -ATPase and SERCA in both cold-hardy insect species. Strong suppression of the activities and changes in the kinetic properties of both enzymes were found over the winter months and regulatory mechanisms including temperature effects

on enzyme function, reversible protein phosphorylation, and phospholamban inhibition of SERCA were identified.

MATERIALS AND METHODS

Treatment of Animals.

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. Some insects were used for a winter timecourse (September to April) and, for this, galls were stored outdoors in cloth bags hung on a fence where they experienced ambient winter temperatures. They were sampled in the second week of each month. For this, galls were brought into the lab 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 .

For laboratory experiments, other September-collected galls were brought into the lab and acclimated to 15°C in large incubators for a period of two weeks. After this time, some galls were opened and insects were sampled as above. Remaining insects in their galls were then exposed to lower temperatures for various incubation periods. After the appropriate incubation period, galls were opened and the insects were removed and frozen.

Other insects were 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 container was flushed with 100% nitrogen gas for 2

minutes and then both valves were sealed and the container was replaced at the appropriate incubation temperature. Two anoxia exposure timecourses were generated, one with larvae acclimated to 15°C for two weeks, and the other with 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. The containers were then refushed with nitrogen gas.

Colorimetric assay for ion motive ATPases using malachite green-molybdate

Na⁺K⁺-ATPase and Ca²⁺ATPase were assayed using a stop-time procedure. Assays were started at time zero with the addition of ATP substrate and stopped at a predetermined time by the addition of the malachite green-molybdate acidic colorimetric reagent, which detects inorganic phosphate released from ATP hydrolysis and forms a coloured complex that can be measured at 595 nm. Initial trials optimized the assay with respect to amount of enzyme extract used and ATP substrate concentration, confirmed a linear rate of reaction over the assay time used, and also determined that the malachite green color development (which is pH dependent) was not affected by adding different amounts of assay mixture to the colorimetric reagent.

The malachite green-molybdate colorimetric assay for phosphate detection was conducted essentially as described by Ekman and Jager (1993). All glassware was washed with 6 M HCl to remove dirt, bound protein and phosphate. To prepare the malachite green reagent, one volume of 10% (w/v) (NH₄)₆Mo₇O₂₄·4H₂O in 4 M HCl was mixed with 3 volumes of 0.2% (w/v) malachite green in 4 M HCl. After stirring for 30 min at room temperature, the solution was gravity filtered using filter paper (Whatman)

and funnel that were prewashed with 6 M HCl. The first 25% of the filtrate was discarded since it was diluted with HCl. The reagent was kept in a dark glass bottle at room temperature for up to 1 month.

Phosphate standards were generated by preparing a stock solution of 10 mM potassium phosphate that was diluted 100-fold to give a final concentration 0.1 mM orthophosphate. Standards ranging in concentration from 1 to 12 nmol inorganic phosphate (Pi) in 1 ml final volume were used to generate the standard curve. Distilled water was used as a blank. The equation of the straight-line relationship between [Pi] standard and $A_{595\text{nm}}$ was used to determine the Pi concentrations in experimental samples.

Assaying Na^+/K^+ -ATPase

Frozen larvae were quickly weighed and homogenized 1:4 w/v in homogenization buffer (25 mM imidazole, pH 7.0, 250 mM sucrose, 10 mM β -mercaptoethanol, and 0.2% w/v deoxycholate). To prevent proteolysis, several protease inhibitors (PMSF, leupeptin, aprotinin) and were added at the time of homogenization. The homogenate was immediately centrifuged at 10,000 x g for 10 min at 4°C. A 300 μL aliquot of the supernatant was removed and centrifuged through a 3 ml column of Sephadex G-50 equilibrated with homogenization buffer to remove low molecular weight metabolites and ions including endogenous Pi. Desalted samples were kept on ice until assay.

Na^+/K^+ -ATPase assays were conducted with paired reaction tubes, one containing ouabain with no KCl, whereas the other contained KCl but no ouabain. Ouabain specifically inhibits Na^+/K^+ -ATPases whereas KCl is one of the substrates. Buffer A contained 100 mM NaCl, 20 mM KCl, 5 mM MgCl_2 , 20 mM imidazole, pH 7.0 and

buffer B contained 120 mM NaCl, 5 mM MgCl₂, 20 mM imidazole, pH 7.0, and 1 mM ouabain. The reaction mixture consisted of 825 µl assay buffer (A or B), 75 µL enzyme extract, and 100 µL of 30 mM ATP (3 mM final concentration). Two microliters of thapsigargin (1 mg/mL), an inhibitor of SERCA, was added to reduce background in the assay. The reaction was started by adding ATP, and standard assays were allowed to run for 15 min at 21°C and then stopped by removing a 50 µL aliquot and adding it to 750 µL of ddH₂O and 200 µl malachite green dye in the wells of a microplate. Samples were incubated at room temperature for 10 minutes for color development and then absorbance at 595 nm was measured using a Dynateck MR5000 microplate reader. Inorganic phosphate released from ATP hydrolysis was quantified by comparison with the standard curve. Na⁺K⁺-ATPase activity was expressed as the difference between the enzyme activity obtained in the presence of KCl (in buffer A) versus in the presence of ouabain (in buffer B). All samples were measured in triplicate and averaged. Activity was expressed as nmol phosphate released/min/mg soluble protein in the desalted extract. Soluble protein content in the extracts was quantified by the Coomassie blue dye-binding method using the Bio-Rad prepared reagent.

Phosphorylation and dephosphorylation of Na⁺K⁺-ATPase

Control (15°C acclimated for 2 weeks) *E. solidaginis* larvae were homogenized 1:2 w/v in the same homogenization buffer as above. Homogenates were immediately centrifuged at 10,000 x g for 10 min at 4°C. A 300 µL aliquot of the supernatant was removed and centrifuged through a 3 ml column of Sephadex G-50 equilibrated with incubation buffer (50 mM imidazole, pH 7.0, 25% v/v glycerol, 0.2% w/v deoxycholate,

10 mM β -mercaptoethanol, and 0.1 mM EDTA). A 40 μ L aliquot of desalted homogenate was added to 80 μ L incubation buffer and the appropriate effectors were added to give a final volume of 150 μ L. The final mixture was incubated at 21°C for 3 hours to allow the protein kinases or phosphatases to alter Na^+K^+ -ATPase activity. The exact incubation conditions are described in the figure legends.

Assaying Ca^{2+} -ATPase

Tissues from either control or stressed insects were homogenized in 25 mM imidazole, pH 7.0, 2 mM EDTA, 20 mM β -glycerophosphate, 250 mM sucrose, 10 mM β -mercaptoethanol, 10% (v:v) glycerol, and 1mM PMSF using a Polytron PT10 homogenizer. Homogenates were centrifuged at 5000 x g for 10 minutes before a 300 μ L aliquot of supernatant was desalted through a 3 mL Sephadex G-50 column equilibrated with homogenization buffer.

Ca^{2+} -ATPase activity was also measured in stop-time assays that quantified inorganic phosphate production by the malachite green-molybdate dye method. Reactions were initiated by adding 50 μ L of desalted enzyme extract to each of 2 separate 1.5 mL Eppendorf tubes containing 950 μ L of reaction buffer. The first tube contained 50 mM imidazole, pH 7.0, 100 mM NaCl, 20 mM KCl, 5 mM MgCl_2 , 5 mM ATP, 1 mM calcium ionophore A23187, and 0.125 mM CaCl_2 . This measured total Ca^{2+} -ATPase (PMCA + SERCA, as well as non-specific ATPases). The second tube was identical except that it contained 2 μ L (1 mg/mL) of thapsigargin, a SERCA-specific inhibitor. Standard assays were incubated at 21°C for 20 min before a 20 μ L aliquot was removed and added to 780 μ L water and 200 μ L malachite green dye. Color development proceeded for 10 minutes

and was measured at 595 nm on a Dynateck MR5000 microplate reader and compared to a standard curve generated from known concentrations of inorganic phosphate. All samples were measured in triplicate and averaged. SERCA activity was defined as the difference in activity between the uninhibited and thapsigargin-inhibited reactions. SERCA activity was expressed as nmol phosphate released/min/mg protein.

Phosphorylation and dephosphorylation of Ca^{2+} -ATPase

Endogenous kinases and phosphatases were stimulated to test their effect on SERCA activity. Frozen insects were homogenized 1:4 w:v in homogenization buffer containing 25 mM imidazole, 250 mM sucrose, 10% w:v glycerol, 10 mM β -mercaptoethanol, and 1 mM PMSF. The homogenate was desalted by low speed centrifugation through a Sephadex G-50 column equilibrated homogenization buffer. A 100 μL aliquot of enzyme extract was then combined with 50 μL of the appropriate incubation buffer that contained the same reagents as the homogenization buffer plus the following additions. The control incubation buffer contained 10 mM MgCl_2 and 10 mM ATP. The kinase stimulation buffer contained 5 mM MgCl_2 , 5 mM ATP, 20 mM β -glycerophosphate and either 3 mM cAMP (final concentration 1 mM, to stimulate protein kinase A), 3 mM cGMP (final concentration 1 mM to stimulate protein kinase G), or 3.9 mM CaCl_2 and 21 $\mu\text{g/mL}$ phorbol 12-myristate 13-acetate (final concentration 1.3 mM CaCl_2 and 7 $\mu\text{g/mL}$ phorbol 12-myristate 13-acetate to stimulate protein kinase C). Phosphatases were stimulated with buffer containing 10 mM MgCl_2 and 3.0 mM CaCl_2 . Also, an additional incubation with added calf intestine alkaline phosphatase (CIAP) was

used to further dephosphorylate the enzyme. All samples were incubated at 30°C for 1, 3, or 5 h (as per fig legends) before assay for Ca^{2+} -ATPase activity.

Western blotting

Whole larvae were homogenized in a buffer that was designed to inhibit any endogenous protein phosphatases and kinases present: 20mM HEPES, 400mM NaCl, 20% v:v glycerol, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM NaF, 10 mM β -glycerophosphate, and 1 mM NaVO_3). The buffer also contained the appropriate protease inhibitors to protect the sample from degradation (PMSF, leupeptin, aprotinin, and benzamidine, 1 μM each). The homogenate was centrifuged at 10,000 x g for 10 minutes and the supernatant was transferred to a fresh tube. Total protein concentration of each sample was determined using the Coomassie blue dye-binding method with the Bio-Rad prepared reagent and then a standard amount (10 or 20 μg) of protein was loaded into each well of an SDS-polyacrylamide gel. The gels varied between 8-15% acrylamide: bisacrylamide, depending on the size of the protein being separated. The running buffer was diluted from a 10X stock solution, which contained 30.3 g Tris-base (pH 8.3), 144 g glycine and 10 g SDS per liter. Gels were run at 200 volts for 1 h and then proteins were transferred to a PVDF membrane (Immobilon-P Transfer Membrane, Millipore Corp., Bedford, MA, U.S.A.) with a wet transfer procedure using a current of 300 mA for 3 h. The transfer buffer contained 25 mM Tris-base (pH > 8.3), 192 mM glycine, and 20% v/v methanol, chilled to 4°C. Membranes were blocked with 5% non-fat dried milk in TBST (10 mM Tris-base, pH 7.0, 150 mM NaCl, 0.05% Tween 20) for at least one hour at 4°C to prevent non-specific binding. The membranes were then incubated with the primary

antibody at a concentration determined by the supplier (usually 1:1000 to 1:5000 v:v) overnight at 4°C. The unbound primary antibody was removed with several five-minute washes with TBST. This was followed by the addition of the secondary antibody raised against the primary antibody. The proteins were visualized using the enhanced chemiluminescence assay (ECL; NEN Life Science Products, Inc., Boston, MA, U.S.A) and band densities were quantified using the Syngene (Bio-Rad) with GeneTools software (v3.00.02).

RESULTS

Na⁺K⁺-ATPase activity in E. solidaginis

Figure 1A shows the pattern of Na⁺K⁺-ATPase activity in *E. solidaginis* larvae over the winter. Activity was high in the fall (October) but was strongly reduced during the coldest winter months (an 85% decrease from October to November). Activity began to rise again in February and by April about 3-fold higher than the October value. To investigate the biochemical mechanisms controlling Na⁺K⁺-ATPase activity and potentially identify those involved in the winter suppression of enzyme activity, a variety of additional studies were undertaken.

Low temperature exposure and/or tissue freezing could stimulate changes in Na⁺K⁺-ATPase activity in this freeze tolerant species. To test this, 15°C-acclimated larvae were shifted to 3°C for 24 h and then frozen at -16°C for a further 24 h (Figure 2.2A). Cold exposure at 3°C did not affect the maximal activity of Na⁺K⁺-ATPase in *E. solidaginis* but freezing did with activity nearly 2-fold higher in frozen larvae than in controls. Activity remained high after 24 h thawed at 3°C. Anoxia-induced suppression of Na⁺K⁺-ATPase activity is one of the major energy-saving mechanisms of metabolic rate depression in anoxia-tolerant vertebrates (Hochachka et al., 1996). To determine if Na⁺K⁺-ATPase activity was similarly responsive to hypoxia in insects, *E. solidaginis* larvae were exposed to a nitrogen gas atmosphere for 24 h at 15°C (Figure 2.2A) followed by 24 h aerobic recovery. However, neither hypoxic exposure nor subsequent recovery significantly altered enzyme activity.

Na⁺K⁺-ATPase in other systems is known to be subject to control by reversible protein phosphorylation and Figure 2.3A shows the results of *in vitro* incubation studies

that examined the effects of stimulating protein kinase or protein phosphatase activities on Na^+K^+ -ATPase activity. Incubations of the enzyme with Mg.ATP and specific protein kinase second messengers strongly decreased Na^+K^+ -ATPase activity. Stimulation of cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG) or Ca^{2+} /phospholipid-dependent protein kinase (PKC) all resulted in a large decrease in Na^+K^+ activity to values just 4 to 8 % of the control values. By contrast, incubations with exogenous calf intestine protein phosphatase (CIAP) had no effect on Na^+K^+ -ATPase activity, as it was the same level as the control sample.

Na^+K^+ -ATPase activity in *E. scudderiana*

The winter profile of Na^+K^+ -ATPase activity was also assessed in freeze-avoiding *E. scudderiana* larvae (Figure 2.4A). Maximum activity of Na^+K^+ -ATPase was highest in larvae sampled from outdoor populations in October and, like *E. solidaginis*, activity decreased significantly over the winter months. Activities in larvae sampled between November and February were ~25% of the October mean value. Na^+K^+ -ATPase activity began to increase again in the spring.

Ca^{2+} -ATPase activity in *E. solidaginis*

Figure 1B shows the winter profile of SERCA activity in the freeze tolerant *E. solidaginis* larvae. Similar to the results for Na^+K^+ -ATPase, enzyme activity was highest in the fall, fell to low values in midwinter, and then rose again in the spring. The observed activity in February was only about 20% of the September value. The effects of low temperature and hypoxia exposures on the maximum activities of SERCA in *E.*

solidaginis are shown in Figure 2.2B. Low temperature (3°C) exposure reduced SERCA activity by about one-third and activity stayed low in frozen (-16°C) larvae and after 24 h thawed at 3°C. Hypoxia exposure showed a trend toward reduced SERCA activity in the larvae but the effect was not statistically significant.

As can be seen from Figure 2.3B, the activity of SERCA in *E. solidaginis* also responded to *in vitro* treatments that stimulated the actions of protein kinases. Incubations with Mg.ATP plus protein kinase second messengers (cAMP, cGMP, or CaCl_2 + PMA) all resulted in decreased in SERCA activity; stimulation of PKA reduced activity by about 25% whereas PKG and PKC action lowered activity by about 50%. However, incubation with CIAP did not significantly affect SERCA activity.

Some kinetic properties of *E. solidaginis* were analyzed, comparing the enzyme from 15°C acclimated (control) and -16°C (frozen) larvae. The sensitivity of SERCA to changing concentrations of ATP and Ca^{2+} is shown in Figure 2.5. Velocity *versus* substrate concentrations were hyperbolic relationships in all cases. K_m values were determined for each substrate with no significant differences found for the enzyme from control versus frozen larvae. The mean $K_m(\text{ATP})$ was 0.501 ± 0.016 mM for control larvae and 0.448 ± 0.009 mM for frozen larvae ($n = 4$ in both cases). The $K_m(\text{Ca}^{2+})$ in control larvae was 0.306 ± 0.014 mM versus 0.273 ± 0.014 mM in frozen larvae.

The effect of assay temperature on *E. solidaginis* SERCA is shown as an Arrhenius plot in Figure 2.6A. Activity was measured at six temperatures (0, 5, 10, 15, 20, and 30°C) and the plot of $\log v$ versus $1/K$ yielded a straight line over the full range. Using the equation of the line the activation energy was calculated to be 15.74 ± 0.55 kcal/mol (65.87 ± 2.28 kJ/mol)($n=5$).

Changes in enzyme activity can result from changes in the amount of enzyme protein present and so, to determine if this was a factor in SERCA regulation in *E. solidaginis*, Western blotting was used with primary polyclonal antibodies raised against rabbit SERCA (Developmental Studies Hybridoma Bank, University of Iowa). The antibody recognized a 50 kDa fragment resulting from trypsin-digestion of the full length 110 kDa SERCA protein. Figure 2.7A shows representative Western blots and histograms of mean band densities for multiple trials detecting SERCA protein levels in larvae treated to acute low temperature and freezing exposures as well as anoxic stress and recovery. The results showed no significant difference in the amount of SERCA protein in the larvae under any of these conditions. Figure 2.7B analyzes SERCA protein content in the larvae between September and April. Again, no significant change in SERCA protein was found.

Ca²⁺-ATPase activity in *E. scudderiana*

Figure 2.4B shows the activity profile of SERCA in the freeze-avoiding *E. scudderiana* larvae throughout the winter. Enzyme activity was very high in September and fell to a low point by December (just 36% of the September value). In late winter and spring, SERCA activity rose again to a level that was about 58% of the September value. Figure 2.8A shows the activities of SERCA in *E. scudderiana* larvae exposed to low temperatures or hypoxia. A temperature decrease from 15°C to 4°C (for 7 days) resulted in a significant decrease in SERCA activity in the larvae and the subsequent transfer to -20°C for 7 days depressed activity even more; activities were reduced to 80 % and 35%

of the control values, respectively. Hypoxia exposure and recovery had no significant effect on SERCA activity as compared with the normoxic control.

The effects of *in vitro* incubations that stimulated endogenous kinase and phosphatase activities on SERCA activity is shown in Figure 2.8B. Activity was measured after 1, 3 or 5 h of incubation. Under control conditions activity declined over time probably due to instability of the desalted enzyme or possibly due to the presence of added Mg.ATP that could stimulate a low rate of endogenous protein kinase activity. When PKA was stimulated by the addition of cAMP, SERCA activity was strongly reduced (by about 50%) compared with the corresponding control activities at each time point. Similarly, stimulation of PKC by the addition of CaCl_2 and PMA significantly reduced the activity of SERCA at 1 and 5 h compared with the corresponding controls. Stimulation of PKG with cGMP also reduced SERCA activity but due to high variability in the data sets, the effect was significantly different from the corresponding control only after 5 h of incubation. By contrast, conditions that promoted endogenous phosphatase activity while repressing endogenous kinases had no significant effect on SERCA activity. Interestingly, addition of exogenous CIAP strongly reduced SERCA activity even more effectively than did stimulation of the endogenous kinases.

The sensitivity of *E. scudderiana* SERCA to changing concentrations of ATP and Ca^{2+} can be seen in Figure 2.9 comparing 15°C acclimated control larvae with larvae exposed to subzero temperature (1 week at 4° followed by 1 week at -20°C). In each case, the relationship between velocity and substrate concentration was hyperbolic. Calculated $K_m(\text{ATP})$ values were 0.658 ± 0.016 mM for controls and 2.085 ± 0.300 mM for cold-exposed larvae ($n = 4$ for both), a significant 3.2-fold increase in $K_m(\text{ATP})$ of

SERCA in the cold larvae ($P < 0.05$). Hence, the sensitivity of *E. scudderiana* SERCA to ATP was greatly reduced in cold-exposed larvae and this may contribute to the reduced activities of the enzyme observed in the colder winter months. However, the $K_m(\text{Ca}^{2+})$ changed only slightly between the two groups; the control value was 0.044 ± 0.002 mM and 0.030 ± 0.003 mM in cold-exposed larvae ($n = 4$ for both).

The effect of temperature on *E. scudderiana* SERCA was measured at eight different temperatures ($-4, 0, 5, 10, 15, 20, 25$, and 30°C) and plotted as an Arrhenius plot (Figure 2.6B). There was a clear break in the line at the 10°C . The calculated activation energy for temperatures $>10^\circ\text{C}$ was 10.64 ± 0.81 Kcal/mol (44.36 ± 3.43 KJ/mol). At temperatures below 10°C , the activation energy increased sharply to 28.38 ± 1.54 Kcal/mol (120.32 ± 5.57 KJ/mol).

Figure 2.10 shows Western blot analysis of SERCA protein levels in *E. scudderiana* under low temperature and anoxia exposures as well as throughout the winter. Figure 2.10A shows that the amount of SERCA protein did not change with low temperature exposures: 1 day at 4°C , 1 or 7 days at -4°C , or 1 week at -20°C . Similarly, anoxia exposure did not alter protein levels. However, Figure 2.10B shows that amount of SERCA protein in *E. solidaginis* larvae steadily increased from autumn fall to spring, despite the fact that activity of the enzyme was suppressed until late spring.

Figure 2.11 shows the protein bands and accompanying histogram of phospholamban levels over the winter in *E. scudderiana*. The commercial polyclonal antibody raised against the phosphorylated form of mouse cardiac phospholamban cross-reacted with a protein in *E. scudderiana* of the proper size of ~ 25 kDa. The total amount of phosphorylated protein decreased by about 80% in January and February as compared

to levels in October. This is consistent with the observed decrease in SERCA activity measured in *E. scudderiana*. The antibody to phospho-phospholamban did not cross-react with any proteins from *E. solidaginis*.

Figure 2.1. Maximal activities of Na^+K^+ -ATPase (A) and Ca^{2+} ATPase (B) in *Eurosta solidaginis* larvae over the winter months. Values are means $\pm$ S.E.M., $n = 5$ (Na^+K^+ -ATPase) or $n=4$ (Ca^{2+} ATPase) determinations on separate homogenates, each prepared from a pool of 3 insects. *- Significantly different from earliest autumn value as determined by the Student's t-test, $P<0.05$.

A. Na^+/K^+ -ATPase **B. Ca^{2+} -ATPase**

Figure 2.2. Maximum activities of Na^+K^+ -ATPase (A) and Ca^{2+} -ATPase (B) in *Eurosta solidaginis* larvae exposed to different experimental conditions. Controls were autumn-collected insects that were acclimated to 15°C for 2 weeks before sampling. Three groups of insects were then given low temperature exposure. Temperature was dropped to +3°C for 24 hours (after which the 3°C group was sampled) and then further decreased to -16°C for 24 hours (-16°C group). The third group was returned from -16°C to +3°C and sampled after 24 hours thawed (thaw group). Other groups were given hypoxia exposure under nitrogen gas at 15°C for 24 hours (hypoxic group) and then restored to normoxia for 24 hours (recovered group). Data are means $\pm$ S.E.M., n = 8 (for Na^+K^+ -ATPase) or n = 4 (for Ca^{2+} -ATPase) determinations on separate homogenates, each made from 3 larvae. *- Significantly different from the control value using the Student's t-test, $P < 0.05$.

A. Na^+/K^+ -ATPase **B. Ca^{2+} -ATPase**

Figure 2.3. Effect of stimulating endogenous protein kinases or the addition of exogenous calf-intestinal alkaline phosphatase (CIAP) on *Eurosta solidaginis* Na⁺K⁺-ATPase (A) and Ca²⁺-ATPase (B). Extracts of insects acclimated to 15°C were used. For Na⁺K⁺-ATPase control incubations contained 10 mM ATP, 30 mM NaF, and 15 mM MgCl₂. Kinases activities were stimulated by the further addition of second messengers: (a) 0.3 mM cAMP to stimulate PKA, (b) 0.3 mM cGMP to stimulate PKG, or (c) 1.3 mM CaCl₂ + 7 µg/mL phorbol 12-myristate 13-acetate to stimulate PKC. Phosphatase effects on the enzyme were tested by incubating with 27 units of CIAP in the presence of 15 mM MgCl₂ + 1.3 mM CaCl₂ (without ATP or NaF). Samples were incubated at 30°C for 3 hours before assay. For SERCA, incubations contained 10 mM ATP and 15 mM MgCl₂ (control) with the addition of second messengers: 1.0 mM cAMP, 1.0 mM cGMP or 1.3 mM CaCl₂ + 7 µg/mL phorbol 12-myristate 13-acetate. CIAP incubation was as above. Samples were incubated at 30°C for 1 hour before assay. Values are means ± S.E.M., n = 3 (Na⁺K⁺-ATPase) or n=6 (Ca²⁺-ATPase) determinations on separate homogenates, each containing 3 insects.. *-Significantly different from control value as determined by the Student's t-test, P<0.05.

A. Na⁺/K⁺-ATPase **B. Ca²⁺-ATPase**

Figure 2.4. Maximal activities of Na^+K^+ -ATPase (A) and Ca^{2+} ATPase (B) in *Epiblema scudderiana* larvae over the winter months. Values are means $\pm$ S.E.M., n = 5 (Na^+K^+ -ATPase) or n=6 (Ca^{2+} -ATPase) determinations on separate homogenates, each containing 3 insects. *- Significantly different from earliest autumn value using Student's t-test, $P < 0.05$.

A. Na^+K^+ -ATPase **B. Ca^{2+} -ATPase**

Figure 2.5. Representative velocity versus substrate concentration curves for *Eurosta solidaginis* SERCA showing dependence on ATP concentration (A) and Ca^{2+} concentration (B) for the enzyme isolated from control (●) and frozen (■) larvae. Control insects were maintained at +15°C for 2 weeks and frozen insects were cooled to +4°C for 1 week before being frozen at -20°C for another week. Assays were run at pH 7.2 with fixed co-substrate concentrations: for ATP curves the $[\text{Ca}^{2+}]$ was 0.6 and for Ca^{2+} curves, the $[\text{ATP}]$ was 3 mM.

A. *Eurosta solidaginis* SERCA : ATP dependence

B. *Eurosta solidaginis* SERCA : Ca^{2+} dependence

Figure 2.6. Arrhenius plot of the effect of temperature on SERCA from control *Eurosta solidaginis* (A) and control *Epiblema scudderiana* (B). Reaction mixtures were pre-incubated for 5 minutes to allow temperature equilibration before initiating the reaction by the addition of ATP. Optimal assay conditions were used and assays were run for 20 minutes. Temperature is shown in Kelvin. Data are means $\pm$ S.E.M., for n = 4 determinations for *Eurosta*, n=5 for *Epiblema*.

A. *Eurosta solidaginis* Ca^{2+} -ATPase

B. *Epiblema scudderiana* Ca^{2+} -ATPase

Figure 2.7. Western blots showing the SERCA protein content in *Eurosta solidaginis*.

(A) Representative blot of SERCA levels under six experimental conditions and histogram showing densitometric analysis of SERCA levels in control (+15°C), cold (+3°C, 24 h), frozen (-16°C, 24 h), thawed (+3°C, 24 h), 24 h hypoxic exposure, and 24 h normoxic recovery from hypoxia. (B) Representative blot and histogram showing densitometric analysis of SERCA protein levels in *E. solidaginis* from September to April. Equal amounts of protein (10 µg) were loaded in each well and blots were probed using polyclonal antibodies raised rabbits against mouse SERCA and recognizing a single band at ~50 kDa.

A. *Eurosta solidaginis* SERCA: protein levels under different conditions

B. *Eurosta solidaginis* SERCA: protein levels under over the winter

Figure 2.8. Ca^{2+} -ATPase in *E. scudderiana*.

(A) Effect of different experimental conditions on SERCA activity in the larvae. Control insects were maintained at 15°C for 14 days. Subsequent low temperature exposure was +4°C for 7 days (4°C group) followed by -20°C for 7 days. The hypoxic time-course required a separate control due to different collection times. Other groups were given hypoxia exposure under nitrogen gas at 15°C for 24 hours and then restored to normoxia for 24 hours (recovered group). Values are mean $\pm$ S.E.M, n = 4 determinations on separate homogenates, each combining 3 larvae. * - Significantly different from the corresponding control value, $P < 0.05$.

(B) Effect of stimulating the activities of endogenous protein kinases and protein phosphatases as well as treatment with exogenous calf-intestinal alkaline phosphatase (CIAP) on SERCA activity *in vitro*. Samples were incubated at 30°C for 1, 3, or 5 h before assay. Control and protein kinase incubations contained 10 mM ATP and 15 mM MgCl_2 (control) with the further addition of second messengers: 1.0 mM cAMP to stimulate PKA, 1.0 mM cGMP for PKG, or 1.3 mM CaCl_2 + 7 $\mu\text{g/mL}$ phorbol 12-myristate 13-acetate for PKC. Endogenous protein phosphatases were stimulated by incubating with 2 mM EDTA (to inhibit kinases) + 3.9 mM CaCl_2 in the absence of ATP (did contain 15 mM MgCl_2) and NaF. In the final condition, extracts were incubated with exogenous CIAP (27 U, Invitrogen Life Technologies). Values are means $\pm$ S.E.M for n = 5 determinations on separate extracts, each containing 3 insects. * - Significantly different from corresponding control value using the Student's t-test, $P < 0.05$.

A. *Epiblema scudderiana* SERCA: activities under different conditions

B. *Epiblema scudderiana* SERCA: in vitro incubations

Figure 2.9. Representative velocity versus substrate concentration curves for *Epiblema scudderiana* SERCA showing dependence on ATP concentration (A) and Ca^{2+} concentration (B) for the enzyme isolated from control (●) and cold-exposed (■) larvae. Control insects were maintained at +15°C for 2 weeks and cold-exposed insects were cooled to -20°C for one week. Assays were run at pH 7.2 with fixed co-substrate concentrations: for ATP curves, the $[\text{Ca}^{2+}]$ was 0.6 mM and for Ca^{2+} curves, the $[\text{ATP}]$ was 3 mM.

A. *Epiblema scudderiana* SERCA : ATP dependence

B. *Epiblema scudderiana* SERCA : Ca^{2+} dependence

Figure 2.10. Western blots showing SERCA protein content in *Epiblema scudderiana*.

(A) Representative Western blot showing SERCA levels under six conditions and histogram showing densitometric analysis of SERCA protein levels in control (+15°C), cold-exposed (+4°C, 24 h; -4°C, 24 h; -4°C, 1 wk.; -20°C, 1 wk.) or anoxic (24 h at +15°C) insects. (B) Representative Western blot and histogram showing densitometric analysis of SERCA protein levels from September to April. Total protein extracts were separated by electrophoresis (10 µg per lane) and then probed using polyclonal antibodies against the SERCA from mouse raised in rabbits; the antibody cross-reacted with a single band at ~50 kDa. Data in histograms are mean $\pm$ S.E.M., n = 3. *- Significantly different from the September value using the Student's t-test, P<0.05.

A. *Epiblema scudderiana* SERCA: protein levels under different conditions

B. *Epiblema scudderiana* SERCA: protein levels over the winter months

Figure 2.11. Western blots showing changes in the levels of phospho-phospholamban in *E. scudderiana* over the winter months. Each lane was loaded with 20 µg of total protein and probed with a polyclonal antibody raised against the phosphorylated form of mouse cardiac phospholamban. The antibody bound to a protein about 25 kDa in size. Only the bottom band was used for quantification. Histogram shows means ± SEM for n = 3 blots using protein extracts from different animals. *- Significantly different from October value, P<0.05.

Table 2.1. Summary of activities of sarcoplasmic reticulum Ca^{2+} -ATPase from *E. scudderiana* and *E. solidaginis*. *-Significantly different from control value.

	<i>Epiblema</i>		<i>Eurosta</i>	
	Control	Cold-exposed	Control	Cold-exposed
	(+15°C)	(-20°C)	(+15°C)	(-20°C)
Maximal Activity (nmol/min/mg protein)	59.40 ± 3.09	21.49 ± 2.42	21.29 ± 1.34	5.79 ± 0.35
K _m (ATP) (mM)	0.66 ± 0.02	2.09 ± 0.30*	0.50 ± 0.02	0.45 ± 0.01
K _m (Ca^{2+}) (mM)	0.044 ± 0.002	0.030 ± 0.003*	0.306 ± 0.014	0.273 ± 0.014

DISCUSSION

Despite the fact that both *E. scudderiana* and *E. solidaginis* overwinter in galls on the same plants in the same environment, they have adopted different strategies to combat the challenges of a harsh northern winter. Gall moth caterpillars, *E. scudderiana*, employ freeze-avoidance, whereas gall fly larvae, *E. solidaginis*, use freeze-tolerance. Each strategy has its pros and cons. Supercooling requires less extensive adaptations but is a risky strategy because if the supercooled state is broken, lethal freezing occurs instantly. Freeze tolerance requires animals to be well equipped to deal with multiple freeze-thaw cycles and all of the associated stresses that this involves. These include hypoxia during freezing and reperfusion-like events when oxygen is reintroduced upon thawing as well as huge changes in cell volume, ionic strength and osmolality due to the freezing and thawing of as much as 65% of total body water in extracellular spaces.

Although their cold-hardiness strategies differ, the two species seem to use a common strategy of diapause for fuel/energy conservation over the winter. Winter diapause has been experimentally demonstrated in *E. solidaginis* (Irwin *et al.*, 2001) and observational evidence indicates that it also occurs in *E. scudderiana*. Hence, both species require 3-4 months of cold exposure before they can resume development when returned to room temperature; if rewarmed earlier they will not pupate but die (J. Storey, personal communication). An important element of diapause is metabolic rate depression, the coordinated suppression of the rates of ATP-utilizing and ATP-producing reactions in cells so that net ATP turnover is reduced to a low value (Storey and Storey, 2004). Previous studies in our lab have shown that the activities of various mitochondrial enzymes are reduced in both insect species over the midwinter months, suggesting a

reduced capacity for ATP output from aerobic metabolism (Joanisse and Storey, 1994). Activities of citrate synthase, NAD-isocitrate dehydrogenase and glutamate dehydrogenase were lowest between November and March, which correlates well with the patterns of Na^+K^+ -ATPase and Ca^{2+} -ATPase seen in Figures 1 and 4.

Na^+K^+ -ATPase is a P-type ion pump found in virtually all multicellular organisms. It is composed of two subunits, the ~100 kDa catalytic alpha subunit and the smaller glycoprotein beta subunit. The alpha subunit contains the binding sites for ATP, Na^+ , K^+ , and the cardiac glycoside, ouabain. The β -subunit functions as an adhesion molecule (Gloor *et al.*, 1990). The activity of Na^+K^+ -ATPase was measured by carrying-out two simultaneous reactions; one activated by the presence of K^+ ions and the other inhibited with ouabain.

Many insect species have evolved different methods to maintain proper ion balance in a tissue-specific manner. Na^+K^+ -ATPase is the most common method of regulating insect haemolymph and transmembrane K^+ and Na^+ gradients; however, there are many other variations (Fitzgerald *et al.*, 1996). The tissue distribution of other transporters such as the H^+/K^+ -ATPase as well as different anion or cation-dependent transporters vary greatly among species (Lebovitz *et al.*, 1989; Emery, 1995).

Hormones have been shown to play a role in the regulation of Na^+K^+ -ATPase in insects and other organisms but it often remains unclear as to whether the hormones are affecting the total amount of Na^+K^+ -ATPase units or altering the activity of the existing units. Infusion of juvenile hormone (JH) III and methoprene altered Na^+K^+ -ATPase activity in *Musca domestica* and *Locusta migratoria* (Davey *et al.*, 1993; Gong *et al.*, 1994). In *Rhodnius prolixus*, JH I initiated the PKC-dependent phosphorylation of

Na^+K^+ -ATPase (Savala and Davey, 1993). Methoprene, JH and methyl farnesoate all increased Na^+K^+ -ATPase activity in the crustacean, *Artemia salina* (Ahl and Brown, 1991). In addition, JH is involved in triggering the synthesis of antifreeze proteins and polyols in some cold hardy species and is also involved in the maintenance of diapause in various species (Denlinger, 2002). In species where diapause and cold-hardiness are linked, both events are often linked with JH concentration. The reappearance of ecdysone terminates diapause and cold hardiness. Hence, it is possible that JH may be the triggering factor that sets off the signal transduction pathways that lead to coordinated suppression of multiple cell functions during the winter diapause including the suppression of the ion motive ATPases.

This effect of -16°C exposure in increasing *E. solidaginis* Na^+K^+ -ATPase activity is difficult to explain. It suggests that in the early autumn larvae respond differently to an acute low temperature or freezing exposure by increasing Na^+K^+ -ATPase activity and/or protein content as opposed to the different long term response of Na^+K^+ -ATPase suppression that is seen when seasonal patterns are followed (Figure 2.1A and 2.2A). For larvae given acute subzero exposure when perhaps not yet fully cold-hardened (e.g. larvae acclimated to 15°C would not have high cryoprotectant levels), it may be necessary to have elevated Na^+K^+ pumping capacity to help deal with osmotic/ionic imbalances over the freeze/thaw cycle as cell volume shrinks and swells. Most of the upset during freeze/thaw is water movement between the extracellular and intracellular spaces and maybe there is a need for ion pumps to be involved as well.

The overwinter profile of Na^+K^+ -ATPase enzyme activity clearly demonstrated a reduction in activity in the cold winter months and a large increase in activity in April.

These changes could result from changes in the total amount of Na^+K^+ -ATPase protein in membranes over the winter months or from reversible changes in the activities of existing protein. The latter would allow the insect to adjust Na^+K^+ -ATPase activities when needed without the need for new protein synthesis. The results from *in vitro* incubation studies that stimulated the activities of endogenous kinases with the appropriate second messengers indicated that *E. solidaginis* can regulate the activity of its Na^+K^+ -ATPase via reversible protein phosphorylation. The data suggest that all three protein kinases tested (PKA, PKC, and PKG) may be involved with the suppression of Na^+K^+ ion pump activity in times of energy stress. The activity of Na^+K^+ -ATPase in *E. scudderiana* was also significantly depressed over the winter and returned to higher levels in the spring. Active regulation of Na^+K^+ -ATPase is an important part of each of the overwintering strategies adopted by these two insect species. Many efforts were made to assess total Na^+K^+ -ATPase protein content in these two insect species by Western blotting but none of the commercial antibodies tested cross-reacted with a protein of the appropriate size.

SERCA is a Ca^{2+} -ATPase located in the sacro- or endoplasmic reticulum and is responsible for sequestering Ca^{2+} ions following a signal transduction event or muscle contraction that triggered Ca^{2+} release into the cytoplasm. As with Na^+K^+ -ATPase, SERCA activity is actively regulated in both *E. scudderiana* and *E. solidaginis*. Both species reduced SERCA activity in response to acute cold-shock as well as over the winter months. In both species *in vitro* stimulation of the three major protein kinases resulted in a significant reduction of SERCA activity.

The midwinter suppression of Ca^{2+} -ATPase activities could result from reduced levels of the enzyme protein in cells over the winter months. However, analysis of

SERCA protein levels showed unchanged SERCA protein content in *E. solidaginis* between September and April (Figure 2.7) and rising levels of SERCA protein in *E. scudderiana* over the colder months (Figure 2.10). Hence, it appears that enzyme activity is independent of protein amount and this suggests control of activity by post-translational or other mechanisms.

One of the widely used mechanisms for changing the activity states of enzymes is reversible protein phosphorylation via the actions of protein kinases and protein phosphatases. Reversible phosphorylation provides virtual on-off control of the activity of many enzymes (e.g. glycogen phosphorylase) and interconverts other enzymes between states of high and low activities (e.g. pyruvate kinase). Indeed, this mechanism is a key element of metabolic rate depression in all hypometabolic systems that have been examined to date (Storey and Storey, 2004). The present data suggest that the mechanism is also important for the regulation of Na^+K^+ -ATPase and Ca^{2+} -ATPase in cold-hardy insects. Na^+K^+ -ATPase activity in *E. solidaginis* (control 0.78 ± 0.03 nmol/min/mg) was very strongly reduced by more than 90% when the enzyme was incubated with Mg.ATP plus the second messengers that activate PKA (0.03 ± 0.01 nmol/min/mg), PKG (0.06 ± 0.03 nmol/min/mg.) or PKC (0.02 ± 0.02 nmol/min/mg.) (Figure 3A). Similarly, SERCA activity was suppressed in both species in incubations that stimulated protein kinase activities (Figure 3B, 8B). However, treatment with protein phosphatase did not alter either Na^+K^+ -ATPase and Ca^{2+} -ATPase activity in incubations with extracts from 15°C-acclimated *E. solidaginis*. This suggests that the ion motive ATPases in warm-acclimated larvae are in their low phosphate (or dephosphorylated) forms and that phosphorylation suppresses their activity. Furthermore, the results of these incubation

experiments suggest that the stable suppression of ion motive ATPase activities over the winter months (without a change in protein content) could be due to phosphorylation of the enzymes. The results for *E. scudderiana* SERCA also generally agree with this interpretation; stimulation of endogenous protein kinases suppressed activity whereas stimulation of endogenous phosphatases had no effect. However, treatment of *E. scudderiana* SERCA with calf intestine alkaline phosphatase strongly reduced enzyme activity (Figure 2.8B), which suggests additional factors in the control of the enzyme in this species.

The responses by Na^+K^+ -ATPase and Ca^{2+} -ATPase to stimulation of all three protein kinase activities suggests that these important ion motive ATPases can be regulated in response to multiple signals in insect cells, mediated by at least three different signal transduction pathways. These results are in agreement with those found for the regulation of ground squirrel Na^+K^+ -ATPase (MacDonald and Storey, 1999). All three second messengers strongly suppressed Na^+K^+ -ATPase activity. The suppression was reversed by the activation of endogenous phosphatases. In another example, dephosphorylation by protein tyrosine phosphatase-1B increased the activity of Na^+K^+ -ATPase in the human eye lens by 50% (Bozulic *et al.*, 2004). The protein kinase(s) responsible for the natural suppression of ion motive ATPases over the winter months in the two insects remains to be determined.

Low temperature suppressed SERCA activity in both species (Fig. 2.2 and 2.8) but did not alter total SERCA protein content (Figure 2.7 and 2.10). This argues again for a probable covalent modification such as reversible phosphorylation because a stable reduction in enzyme activity was found when activities of both control and cold-exposed

larvae were measured at a constant temperature. The freeze tolerant and freeze-avoiding species showed slightly different patterns of regulation. In *E. solidaginis*, SERCA activity was reduced by 3°C exposure and did not decrease further with freezing at –16°C. In *E. scudderiana*, SERCA activity decreased somewhat at 4°C and was strongly suppressed by –20°C exposure. The differences here could result from the different incubation periods (*E. solidaginis* was exposed at low temperature for 1 day only whereas *E. scudderiana* was exposed for 7 days at each temperature). However, these differences could also be part of the different overwintering strategies of the two species. Because *E. scudderiana* remains in the liquid state the entire winter, a more controlled regulation of enzyme activity may be required. This could suggest that low temperature facilitates the suppression of SERCA activity, clearly by a stable mechanism since the extracts were run through a Sephadex column to remove all small molecules prior to measuring activity and the assays were run at a constant high temperature. This is strong evidence for a covalent modification of the enzyme by a mechanism such as reversible phosphorylation. There is precedence already for such forms of regulation in these cold-hardy insects. The activation of glycogen phosphorylase (GP) by cold in both *E. scudderiana* and *E. solidaginis* is a prime example. The low temperature mediated increase in the percentage of GP in the active a form is due to differential effects of low temperature on the activity of GP kinase *versus* GP phosphatase (PP-1). The kinase showed a normal decrease in activity with temperature whereas the phosphatase was very strongly inhibited at low temperature (Hayakawa, 1985; Hayakawa and Chino, 1982 and 1983). Hence, the net effect of low temperature exposure was a rise in %GPa at low temperature, starting at

about 5°C. The end result was the activation of glycogenolysis and the production of the polyol cryoprotectants.

Previous experiments with *E. scudderiana* have shown that low temperature metabolism does result in an energy stress (Churchill and Storey, 1989b). After 36 h at -4°C, ATP levels had dropped by 50%, AMP levels increased 300%, the total pool of adenylates was reduced, and the total energy charge had dropped from 0.94 to 0.83. However, after 10 days at -4°C, the energy charge had returned to control levels. In another study both *E. scudderiana* and *E. solidaginis* (acclimated to 3°C for several months) were subjected to alternating cycles of 24 h at -16°C followed by 24 h at 3°C (Churchill and Storey, 1989a). Once again, cold-exposure placed an energy stress on the *E. scudderiana* larvae. ATP concentration decreased and ADP and AMP levels increased with each cold-exposure; however, the energy charge quickly returned to control levels once the larvae were returned to 3°C. Repeated cycles of cold-exposure had a more profound effect on *E. solidaginis*. At -16°C, oxygen becomes limited in *E. solidaginis* due to the formation of extracellular ice. The 24 h freeze-thaw cycles were not long enough to cause a build-up of the by-products of anaerobic metabolism (lactate, alanine, etc.) but they were sufficient to have a cumulative detrimental effect on the energy charge of the larvae. While frozen, the ATP levels fell to 56% of those at 3°C. ATP levels returned to control values at 3°C for the first 2 cycles only; in subsequent cycles, the return to 3°C did not help to increase ATP levels. This was initially compensated for in the insects by decreasing the total adenylate pool (ADP and AMP) to keep the energy charge high but by the 6th cycle this process was no longer possible and energy charge fell to 0.70-0.75. The fact that the strain on energy charge takes a long time to be

realized means that the regulation of Na^+K^+ -ATPase and SERCA in both *E. scudderiana* and *E. solidaginis* is not dependent solely on energy charge or adenylate levels but substrate availability may be a contributing factor.

The data in Table 2.1 showed evidence of cold-induced changes in enzyme affinity for ATP or Ca^{2+} for *E. scudderiana* SERCA which could be the result of reversible phosphorylation of the enzyme but reduced ATP substrate availability could also have an influence on enzyme activity *in vivo*. However, the ATP and Ca^{2+} K_m data for *E. solidaginis* SERCA would seem to indicate that the effect of phosphorylation on the enzyme from this species is not to change substrate affinities but just the $V_{\max}$ (and/or possibly properties that were not measured).

E. scudderiana SERCA showed a distinct increase in $K_m(\text{ATP})$ in the enzyme isolated from cold exposed insects that suggests that one effect of probable phosphorylation at low temperature is on the substrate affinity for ATP. Interestingly, and opposite to *E. solidaginis*, long-term cold exposure of *E. scudderiana* (up to 77 days at -20°C) had virtually no effect on larval energy reserves (ATP and arginine phosphate) after an initial “shock” period of several days (Churchill and Storey, 1989). Hence, the unfrozen *E. scudderiana* larvae remain aerobic and maintain their energy charge so if they need to suppress the rates of ATP-utilizing processes, they cannot do so with reduced ATP substrate availability but they can complete this task with a stable modification of the enzyme protein that reduces affinity of the enzyme for ATP.

The Arrhenius plots showing the effect of temperature on SERCA activity showed different patterns for the enzyme from the two insect species. The plot for *E. solidaginis* SERCA was a straight line over the entire temperature range tested but the

plot for *E. scudderiana* SERCA had a clear break at $\sim 10^{\circ}\text{C}$. A break in an Arrhenius plot indicates a distinct conformational change in the protein that alters activity. A sharp break in the Arrhenius plot below $\sim 10^{\circ}\text{C}$ has been documented for a variety of enzymes from *E. scudderiana* including PKA, protein phosphatase 1, glycogen phosphorylase, glycogen synthase, hexokinase and phosphofructokinase (Pfister and Storey 2002a,b; Muise and Storey, 2001). In all cases, activation energies and Q_{10} values increased substantially over the lower temperature range compared with the higher range and this could be one factor that contributes to metabolic suppression at low environmental temperatures. Thus, this serves as another way to suppress activity at low temperature even though the ATP substrate availability does not change.

Using cDNA array technology (see Chapter 3), phospholamban was identified as one of the genes that was up-regulated in *E. scudderiana* in response to low temperature shock. Phospholamban is a primary regulator of SERCA in cardiac sarcoplasmic reticulum and has previously been identified only in cardiac tissue (Zhoa *et al.*, 2004). The identification in *E. scudderiana* of both phospholamban mRNA transcripts and a protein that cross-reacts with antibodies raised against phosphorylated phospholamban provides strong evidence for the presence of an insect homologue of phospholamban and a role for this protein in the regulation of insect SERCA. Western blots with a phospho-specific antibody towards phosphorylated phospholamban showed a pattern of seasonal change that was highly consistent with the pattern of SERCA activity changes measured over the winter in both insects. Western blots revealed that the amount of the phosphorylated form of phospholamban decreased over the winter but increased again in the spring. It is the dephosphorylated form of phospholamban that represses the activity

of SERCA and, hence, the results imply that the level of dephosphorylated phospholamban must rise over the winter months. Hence, phospholamban inhibition of SERCA may be a key mechanism for the reversible suppression of SERCA activity over the winter. This may be particularly important in *E. scudderiana* because the amount of SERCA protein in the larvae increased strongly over the winter months (to about 8-fold higher in April than in September) (Figure 2.10B) and yet measurable SERCA activity in this species was much lower in the winter than in September (Figure 2.4B).

Suppression of the activities of ion motive ATPases (typically via reversible protein phosphorylation) is a documented response to anoxia exposure by anoxia tolerant vertebrates (Hochachka *et al.*, 1996). However, anoxia exposure had no effect on either Na^+K^+ -ATPase or Ca^{2+} -ATPase activities in these insect species, although both species can survive prolonged periods of anoxia (Storey and Storey, 1988). For the freeze tolerant *E. solidaginis*, this suggests that low oxygen and ischemia during freezing are probably not the signals responsible for the suppressed activities of these enzymes over the midwinter months (Figure 2.1) or the reduced Ca^{2+} -ATPase activity during experimental freezing (Figure 2.2B).

At higher temperatures, a significant decrease of ion pumping ATPase activity would be detrimental towards cell viability. In overwintering insects, a decrease in activity of these enzymes is necessary in order to prevent the exhaustion of limited energy resources during the long winter diapause. If Na^+K^+ -ATPase activity failed during environmental stress, the result could be cell swelling, loss of membrane potential and eventually cell death. The same fate would be inevitable with a loss in Ca^{2+} -ATPase activity. Cell signals could not be terminated and, therefore, new signals could not be

propagated. Maintaining P-type ATPase function at low temperature is a key adaptation of all cold-hardy species.

It is fascinating that both of these insect species are able to maintain necessary cellular functions throughout prolonged periods of cold-exposure. Proper regulation of energy-intensive processes, such as active transport of ions, in times of energy stress is crucial to survival. A coordinated suppression of energy consuming processes is required to maintain a suitable energy charge.

Chapter 3

Analysis of gene expression in cold-tolerance

INTRODUCTION

Temporal and spatial regulation of gene expression profiles is an invaluable component of the development and survival of all organisms. In overwintering insects there must be changes in gene expression to support the development of cold hardiness. Even insects that are in a full state of diapause continue to accumulate diapause-specific proteins and show a slow program of “diapause development” that ultimately ends diapause and allows development to resume (Denlinger, 2002). Gene expression changes that support cold hardiness in insects may be triggered in two ways: (1) seasonal and/or environmental cues such as changes in photoperiod and/or thermoperiod may initiate hormone responses and regulate a coordinated suite of gene expression changes, and (2) direct responses to acute temperature changes. Many examples of the first mechanism are known, such as the accumulation of antifreeze proteins as well as the necessary enzymes to produce the polyol cryoprotectants used by many cold-hardy insects. The shorter daylengths and cooler temperatures seen in late summer in temperate climates forewarn the insects of the impending winter and allow them to be prepared well before cold temperatures arrive. The first example of acute cold-induced gene expression in insects was the synthesis of small heat shock proteins in *Sarcophaga crassipalpis* (Denlinger *et al.*, 1991). Since then, several other genes have been shown to be induced by low temperature exposure in insects (Yocum *et al.*, 1998; Tammarielo and Denlinger, 1998; Rinehart and Denlinger, 2000). Our early gene expression studies also identified *EsMlp* (Genbank accession number AF206698) as a cold-induced gene product in *E. scudderiana* (Bilgen *et al.*, 2001). This protein belongs to a family LIM proteins whose consensus sequence includes a pair of zinc fingers and the presence of a glycine-rich

domain further classified *EsMlp* as belonging to the CRP subgroup of the LIM family. LIM proteins are involved in myogenesis during both embryogenesis and metamorphosis. The mRNA transcripts for this gene doubled in concentration within 24 hours when larvae were transferred from 4°C to -20°C. Western blots confirmed that the gene product (EsMlp) was also elevated over the winter and decreased in the spring.

I hypothesized that cold exposure would up-regulate the expression of multiple genes in both freeze tolerant and freeze avoiding insect species. Some of these might be common responses to cold by both species whereas others might address specific issues of the freeze tolerance *versus* freeze avoiding strategy. For example, freezing imposes an ischemic and hypoxic state on tissues since oxygen (and nutrients) cannot diffuse easily through ice to reach cells. Hence, some of the gene expression responses to low temperature/freezing in *E. solidaginis* may support hypoxia survival. The goals of the studies presented in this chapter were to identify the genes that are up-regulated in *E. solidaginis* and *E. scudderiana* in response to cold exposure and/or hypoxia. Three different techniques were used to search for cold-induced or hypoxia-induced gene products in these cold-hardy insects: cDNA library screening, cDNA arrays, and DNA microarrays.

A cDNA library (see Figure 1) is a representative collection of the mRNAs found in a tissue (or organism). The mRNAs are retrieved, converted into cDNA and then inserted into a vector, usually phage DNA. The phage is then used to infect a bacterial lawn and as the phage multiplies in number, so does the copy number of the inserted cDNA. Eventually the phage will burst the host bacterial cell leaving behind a “plaque” containing multiple copies of the cDNA message. The cDNA is then transferred to a

neutral or positively charged membrane (nylon) and covalently cross-linked to prevent desorption. The library is constructed using mRNA isolated from the stressed condition (ie. cold or hypoxia) and then differentially screened with labelled probe from both the control and stressed conditions. The idea is to look for plaques that either appear solely or are strongly increased in amount in the stressed condition. Once putatively up-regulated clones are identified from differential screening, the original plaque is recovered from the master plate and used to recover the phage containing the cDNA insert. A second or third round of screening may be completed to reduce the number of false positives. Clones that are identified as putatively up-regulated from the screening must be then confirmed by using either Northern blots or RT-PCR. If the clone is proven to be up-regulated, the gene that it encodes is then sequenced and studied in more detail. Advantages of cDNA screening as a technology include a low incidence of “false-positives” and, very importantly, the potential to identify novel genes that may be unique to the animal or tissue under investigation. Disadvantages of the technique include the fact that cDNA library screening is a long arduous procedure, it can be very costly, and it is not very efficient for finding differential expression by mRNA transcripts that are present in low copy number.

A newer, more sensitive and more efficient method of gene screening is the use of cDNA arrays and this chapter describes the results from screening with two kinds of arrays. Commercial cDNA nylon arrays are constructed with hundreds or up to a few thousand spots containing cDNAs for known gene sequences. The cDNA fragments are usually between 200 and 600 bases in length and are spotted on the nylon array by a robot. The long length of the cDNA allows for high hybridization efficiency. The cost of

cDNA arrays has become more affordable in recent years and they are easy to use. In only 2-3 days, a researcher can simultaneously screen the expression pattern of a few thousand genes. Also, nylon arrays are big enough so that no special visualization techniques are necessary. Many different arrays are now available that have families or classes of genes organized in functional groups which provides the researcher with a way to assess the responses to stress by major functional units of cells. However, limitations to the use of cDNA array technology include the fact that only identified sequences are present (ie. novel genes will not be detected) and that arrays are available only for a limited number of model species such as human, mouse, rat, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Arabidopsis thaliana*, *Escherichia coli*, and *Saccharomyces cerevisiae*. When working with a different model animal there are two options: (1) take on the expensive and time-consuming endeavour of creating a species-specific array, or (2) undertake heterologous probing using labelled probe made from the animal of interest but hybridized with a commercial array for one of the model systems. In studies presented in this chapter, a Clontech Rat 1.2 II nylon array containing 1188 individual cDNAs was screened using probe created from warm- versus cold-exposed *E. scudderiana*. Two separate regimes of cold-shock were examined: a 15°C down to 4°C shift and a 4°C down to -5°C shift. Despite the evolutionary distance between the freeze-avoiding insect and the rat, the percent cross-reactivity for this analysis was impressive and the data gained proved to be valuable for downstream analysis.

The limited number of cDNAs present on a nylon array has now been remedied by the next generation of array: DNA microarrays that can contain 20-30 thousand individually arrayed cDNAs. The wealth of information coming out of DNA microarray

experiments is growing by leaps and bounds. A simple search in PUBMED demonstrates the huge number of research articles that have been published over the last few years using microarray technology for many different applications. DNA microarrays are the most common form of microarray (for review see Eddy and Storey, 2002; Lobenhofer et al., 2001; Burgess, 2001; Blohm and Guisseppi-Elie, 2001) but there are also protein microarrays (Templin *et al.*, 2004) and even carbohydrate microarrays (Wang, 2003).

There are two main ways of producing modern DNA arrays. The first and most common method spots PCR amplified cDNAs onto the solid support (nylon, glass, or plastic). Spotting cDNA onto glass slides was first developed at Stanford University in the mid-1990's (Schena *et al.*, 1995). The other method, created and distributed by Affymetrix (Pease et al., 1994), involves the light-directed synthesis of oligonucleotides on the solid-support using photolithographic protecting groups. This method is usually only used for creating oligonucleotides of up to 100 nucleotides with longer oligonucleotides best prepared by PCR amplification followed by deposition onto the support (Gabig and Wegrzyn, 2001). The use of DNA microarrays allows the researcher to undertake the simultaneous profiling of many thousands of genes in a period of less than a week. However, to complete the analysis, a large financial investment must be made. The control and stress probes are labelled with different fluorescent tags (usually Cy3 and Cy5), then mixed and allowed to competitively hybridize with the microarray. The chip is then scanned by a machine that excites each fluorescent label independently and measures the intensity of the control and stress probe bound to each spot. One of many different software programs is then used evaluate the results and calculate ratios of fluorescence intensities that indicate which genes are up- or down-regulated or

unaffected. Any data retrieved from array analysis must be further validated with Northern Blots or RT-PCR. Results from our lab and others have shown that genes identified as up-regulated on DNA arrays are generally confirmed as up-regulated by downstream quantitative methods (Bartosiewicz et al., 2000). In fact, the fold change suggested by the microarray is often an underestimate compared with the fold change determined by Northern blot (Taniguchi et al., 2001).

Two types of DNA microarrays were used to explore gene expression patterns in *E. solidaginis* and *E. scudderiana*: the *Drosophila* 7K and human 19K DNA arrays. A 1.5-fold cut-off was used to identify genes that were putatively up-regulated in response to temperature shifts or the availability of oxygen. These experiments produced a huge amount of data that has to be organized to group the genes in terms of function, family, common pathways, or any other way that provides information about the mechanisms that underlie the stress being studied. This provides enough clues for new experiments to last a very long time.

MATERIALS AND METHODS

Treatment of Animals

Two experimental groups of *E. solidaginis* and *E. scudderiana* were generated by manipulating their incubation conditions: cold-stress and hypoxic 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 hypoxia 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 each species, a slow-staged decrease in temperature and a quick drop from control down to subzero temperatures. The control group for low temperature experiments were insects acclimated to 15°C for 14 days. For both species the staged low temperature exposure involved a drop from 15°C down to 4°C that was maintained for 7 days followed by a decrease to -4°C for 7 days and finally a decrease to -20°C for 7 more days. Insects were removed and flash-frozen after 1, 3, and 7 days acclimation to each temperature for use here or in other experiments. Another shorter timecourse was also created for *E. solidaginis*. For these animals, the acclimation periods were only 24 hours long. They started at 15°C (control) and then temperature was lowered to +3°C for 24 hours, then down to -16°C for another 24 hours, and then recovered at +3°C for a final 24 hours. 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 this subzero temperature. RNA isolated from these two stress points was eventually pooled to make up 1 experimental sample. For hypoxia exposures, insects were treated as described in Chapter 2. Two groups of hypoxic animals were generated: (1) those acclimated to 15°C, and (2) those acclimated to 4°C. Presumably, the animals at 15°C (with higher metabolic rates) would experience a greater (or more rapidly developed) stress from hypoxia than animals exposed to hypoxia at 4°C.

Isolation of Total RNA.

All chemicals were of molecular biology grade or equivalent unless otherwise specified. Gloves were worn at all times while handling RNA. Deionized-distilled water was treated with 0.05% v/v DEPC at room temperature overnight and then autoclaved.

Non-autoclavable stock solutions, such as those containing SDS, were prepared with sterile DEPC-treated water. Since Tris buffers can inactivate DEPC, all Tris-containing stock solutions were made with DEPC-treated water that was previously autoclaved.

Frozen larvae were homogenized 1:4 w/v in TrizolTM 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 -70°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 90 V for 4-5 h, then removed, rinsed in DEPC-treated water, stained with EtBr (0.5 µg/ml in ddH₂O) for 20 min, destained in ddH₂O for 15-30 min, and then photographed on a UV light transilluminator (Foto/UV 26, Fotodyne Inc.) at 300 nm using a DS-34 camera with Polaroid instant pack films (Type 667). The bands of RNA ladder as well as ribosomal RNA in the total RNA sample could also be visualized by staining blots with methylene blue (see below under Northern blotting). Blots were treated in 5% v:v acetic acid for 10 min, stained in 0.05% w/v methylene blue (in 0.5 M sodium acetate, pH 5.2) for a few minutes and then destained with ddH₂O until the clearest band resolution was obtained (Herrin *et al.*, 1988; Sambrook *et al.*, 1989).

Construction and Differential Screening of the cDNA library

A cDNA library was constructed using the Uni-Zap-cDNA synthesis kit (Stratagene, La Jolla, CA) with mRNA from *E. solidaginis* larvae that was isolated from four cold-exposed treatment groups; an equal amount of mRNA from each treatment group (4°C 1 week, -20°C for 8 h, 24 h, and 1 week) was pooled. To screen the library, NZY agar plates were prepared to contain between 10-20,000 plaques. To do this, XL1-Blue cells were cultured overnight at 37°C in LB broth. The cells were pelleted and resuspended in 10 mM MgSO₄. A 100 µL aliquot of titered library stock was added to 2 volumes of XL1-Blue cells and incubated at room temperature for 20 min before being added to 50 ml of molten NZY agar and poured on a large (24 cm x 24 cm) plate. The plates were incubated at 37°C for 6-10 h, or until the plaques reached a diameter of 2 mm. The phage DNA was transferred to Hybond-N 0.45µm nylon membranes (Amersham, Cleveland, Ohio). The membranes were then soaked in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 2 min, neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0) for 5 min, and rinse solution (0.2 M Tris-HCl, pH 7.5, 2 x SSC) for 30 sec. (SSC is prepared as a 20 x stock solution containing 3 M NaCl and 0.3 M Na₃citrate.2 H₂O) The membranes were dried on Whatman 3M filter paper prior to cross-linking the DNA using a UVP Ultraviolet Cross-linker Model CL-1000 (VWR Canlab, London, ON) set for 120,000 µjoules/cm² (254 nm). Membranes were then placed between two pieces of Whatman 3M filter paper and desiccated in a Bio-Rad 583 gel dryer for 90 min at 80°C.

Construction of probe was carried out using first-strand synthesis. Briefly, total RNA was incubated with an oligo-dT primer and reverse transcriptase in the presence of

radiolabelled deoxycytosine (alpha ^{32}P -dCTP). Probes were prepared from both control (autumn-collected larvae acclimated to 15°C for 14 days) and cold-exposed (equal amounts of RNA pooled from the 4 treatment groups) animals. The plaque lifts were probed first using radioactive cDNA probe synthesized from total RNA isolated from control *E. solidaginis*. All membranes were prehybridized in 25% v/v formamide, 0.25 M $\text{Na}_2\text{P04}$, pH 7.2, 0.25 M NaCl, 1 mM EDTA, 7% w/v SDS, and 10% w/v PEG for at least 30 min at 50°C in a Labline Model 308 hybridization oven (VWR Canlab, London, ON). The radioactive probe was placed in boiling water for 5 min to remove any secondary structure and cold-snapped on ice before being added to the membranes. At this point the temperature of the hybridization oven was reduced to 42°C.

Following 24 h hybridization, the membranes were washed in 2 x SSC, 0.1% w:v SDS until the radioactivity was reduced to approximately 1500 cpm (as measured with a Geiger counter). Membranes were then placed on aluminum foil and covered with plastic wrap and plaques were visualized by exposure to Kodak XAR5 X-ray film at -70°C for 1-2 days depending on the amount of radioactivity on the membranes. The film was then developed manually using Kodak chemicals (Rochester, NY).

After autoradiography, the membranes were stripped in a high stringency wash buffer (0.2 x SSC, 0.2% w/v SDS) until the radioactivity was reduced to background levels (<100 cpm). Membranes were then re-probed using radioactive cDNA probe prepared from the cold-exposed animals. Comparison of pairs of autoradiograms identified clones that were either up-regulated or unique to the cold-exposed *Eurosta* larvae. Plaques representing potentially up-regulated clones were removed from the NZY plate with a sterile Pasteur pipette. The core of NZY agar containing the appropriate

plaque was placed in a sterile 1.5 mL centrifuge tube along with 500 µl of SM buffer and 100 µl chloroform. The tubes were then vortexed and allowed to sit at room temperature for a minimum of 30 min. Next, the tubes were centrifuged at 5000 x g for 15 min at 4°C and the supernatant that contained the phage was removed and transferred to a new tube and stored at 4°C until use. All potentially up-regulated clones were then individually plated onto small (10 cm) NZY plates, each containing 50-100 plaques. These smaller plates were subjected to a second round of screening. All clones surviving this stage were considered as strong candidates for cold-induced genes and were further tested by Northern Blot analysis.

In vivo Excision of Clones

This protocol was adapted from the manufacturer's instructions (Stratagene). Putatively up-regulated clones identified from the secondary screening were selected and single plaque isolation, phage release and titering were performed as described previously. The phage (Uni-ZAP XR vectors) were excised in *E. coli* strains using a helper phage (ExAssist, Stratagene) as follows. In a 1.5 mL tube, 0.1 ml of the XL1-blue MRF' cells ($OD_{600} = 1$ in 10 mM $MgSO_4$), 0.1 mL isolated phage suspension ($>5 \times 10^5$ pfu/ml) and 0.5 µl ExAssist helper phage ($>1 \times 10^6$ pfu/ml) were added, and the mixture was incubated at 37°C for 15 min. Then 1 ml of LB broth was added and incubated at 37°C for 3 h with gentle shaking. The mixture was then heated at 70°C for 15 min and centrifuged at 1,000 g for 5 min. The supernatant containing single-strand Bluescript phagemids was stored at 4°C for a few months or kept in aliquots with DMSO (0.7% v:v) for long storage at -80°C. SOLR (another *E. coli* strain) cells were grown (with a single

colony from fresh streaking plate or glycerol stock) in LB broth at 30°C overnight and then kept at room temperature until use. An aliquot of 200 µl SOLR cells ($OD_{600} = 1$ in LB broth) was mixed with 100 µl of the above phagemid suspension, incubated at 37°C for 15 min, and then 100 µl of the cell mixture (or a 10-fold dilution in LB broth) was plated on LB-ampicillin agar plates (50 µg/mL) and incubated overnight at 37°C. The colonies appearing on the plate contained the Bluescript double-stranded phagemid (pBluescript plasmid) with the cloned DNA insert, which can propagate in the SOLR cells and can be used directly for double strand DNA sequencing due to a choice of several primer sites (T3 and T7 primer, etc.). The SOLR colony was then streaked on a new LB-ampicillin agar plate, and a single colony was picked and inoculated into 10 ml of LB broth with ampicillin (25 µg/mL) overnight at 30°C. Sterile glycerol-LB broth solution (4.5 ml, 1:1 mix glycerol:LB broth) was added to the bacterial culture and then it was stored in aliquots at -70°C.

DNA Agarose Gel Electrophoresis

The purified Bluescript plasmid DNA was treated with restriction enzymes (*EcoR* I for ZAP cDNA library clones; *EcoR* I and *Xho* I for the Uni-ZAP cDNA library clones) and then analyzed on an agarose gel to estimate the size of the insert and the purity of the mini-prep. *EcoR* I, *Xho* I and related enzyme buffers were obtained from NEB. For analyzing insect cDNA clones, a sample of DNA (2-10 µg) was adjusted to 6 µl with sterile ddH₂O, then 2 µL of 10 × enzyme reaction buffer, 1 µL of 10 U/µL *EcoR* I and 1 µl of 10 U/µL *Xho* I were added. The mixture was incubated at 37°C for 1-2 h, heated at 70°C for 3-4 min and chilled on ice. A 2 µL aliquot of 6× DNA loading buffer [60 mM

NaCl, 6 mM EDTA, 60 mM Tris-HCl (pH. 8.0), 15% w/v Ficoll-400 (Sigma), 0.25% w/v bromophenol blue and 0.25% w/v xylene cyanol FF] was then added and the sample was loaded on an agarose gel. A 1 kb DNA ladder (GIBCO BRL) was also usually loaded. The gel (0.8-1.2% w:v agarose) was prepared with 1× TAE buffer [made from 50× TAE stock: 242 g Tris base, 20 mL of 0.5 M EDTA (pH 8.0), 57.1 mL glacial acetic acid per liter] and electrophoresis was carried out with a medium-size agarose gel apparatus (10 × 20 cm) (Bio-Rad) at 80 V for ~1.5 h, followed by ethidium bromide staining.

Generating ³²-P labeled probe by random primers

The synthesis of ³²P-labeled DNA probe was performed with the random priming method by a protocol adapted from Sambrook *et al.* (1989). Approximately 50-250 ng of insert DNA was adjusted to 10 µl with sterile ddH₂O, heated in boiling water for 3-4 min and chilled on ice. Then 1 µl of 1 mM dNTPs (without dCTP), 2 µl of 10× Reaction Buffer (NEB) (100 mM Tris-HCl, 50 mM MgCl₂, 75 mM DTT, pH 7.5), 1.5 µl of DNA random primer d(N)₆ (1 µg/µl, NEB), 0.5 µl of Large Fragment of DNA Polymerase I (Klenow fragment, exonuclease minus, 5 U/µl, NEB), 5 µl of [α-³²P]dCTP (3,000 Ci/mmol, Amersham) were added and the mixture was incubated at 37°C for 30 min. The reaction was stopped by adding 1 µL of 0.5 M EDTA (pH 8.0) and then the radiolabelled mixture was passed through a Sephadex G-50 column. TE buffer was added to the probe sample to a 0.5 ml final volume and radioactivity was measured with a liquid scintillation counter set on the ³²P-channel.

Northern Blot Analysis

Northern blots were used to confirm that putatively up-regulated genes showed increased levels of gene transcripts in the stressed insects. Samples of total RNA (15 or 20 µg each) or mRNA (1 µg) from tissues of control or stressed animals were loaded on a formaldehyde agarose gel and run as described above for agarose denaturing gel electrophoresis. After electrophoresis, the RNA agarose gel was rinsed with ddH₂O and soaked in 10× SSC and then RNA was transferred onto a nylon membrane (pore size 0.2 µm, Schleicher & Schull, Keene, NH; or Hybond-N, Amersham) by capillary action with the same buffer (10× SSC) (Sambrook *et al.*, 1989). Total RNA was fixed to the membrane by the protocol described above and the membrane was hybridized with specific cDNA probe derived from the cloned insert DNA. As described above, the insert was isolated by restriction enzyme digestion of the plasmid DNA and the restriction fragments were separated by agarose gel electrophoresis. The insert DNA was then purified from the gel with GeneClean III kit (BIO 101) and used as a template to create a radioactive probe using the random primer method (see above).

The procedure for hybridization of Northern blots was very similar to that for screening a cDNA library although fewer counts of radiolabelled probe were used (1-2 × 10⁶ cpm/per ml of hybridization solution) and higher stringency washing at 50-65°C was usually required. Each blot could be reused up to 5 times, and probe used for a previous hybridization was stripped off the membrane by treating the blot in a boiling solution of 0.1× SSC and 0.2% w:v SDS for 1-2 min.

Autoradiography and X-ray film development were carried out as previously described. Band intensities on autoradiograms were measured by densitometry to

quantify peak area (cm²) with the use of computer imaging software. A Scan Jet3C scanner (Hewlett-Packard, Palo Alto, CA) was used along with Deskscan II v2.2 software (Hewlett-Packard) to render an autoradiogram into a form recognizable by Imagequant software v3.22 (Innovative Optical Systems Research), which was then used to quantify relative band intensity.

cDNA Microarrays

Microarrays were purchased from the Canadian Drosophila Microarray Centre, University of Toronto, Toronto, Ontario. A complete list of all the genes found on the arrays and their corresponding positions on the chips is available at:

<http://www.flyarrays.com/downloads.html>.

First strand cDNA synthesis

Construction of first strand cDNA was performed using 40 µg of total RNA. This was combined with 2 µl of oligo(dT)₂₅ (Sigma Genosys, Oakville, Ontario) primer (200 ng/µl) and heated to 68°C for 5 minutes, then chilled on ice. This was followed by the addition of 8.0 µl 5X first strand buffer (Invitrogen Life Technologies), 4.0 µl 0.1M DTT, 3.0 µl 20 mM dNTP (without C, Amersham Pharmacia Biotech), 1.0 µl 2 mM dCTP (Amersham Pharmacia Biotech), 1.0 µl of the appropriate fluorescent dye (cyanine 3 or cyanine 5, Amersham Pharmacia Biotech; reciprocal labelling was performed with each sample), and finally 1.0 µl Superscript II reverse transcriptase (Invitrogen Life Technologies). The final mixture was incubated at 42°C for 120 minutes. The reaction was stopped and the RNA was degraded by the addition of 4 µl 50 mM EDTA and 2 µl

10 N NaOH with heating to 65°C for 10 minutes. The samples were neutralized by adding 4 µl 5 M acetic acid (or more until neutral as determined by pH paper). At this point, cDNA probes made from control and experimental samples (one labelled with cy3 and one with cy5) were combined in 1 tube and precipitated by the addition of 100 µl isopropanol and incubated at -20°C for 30 minutes. The probe was pelleted by centrifugation at 12,000 x g at 4°C for 15 minutes. The pellet was then washed with 300 µl cold 70% ethanol, centrifuged again at 12,000 x g at 4°C for 5 minutes, and the ethanol removed by aspiration. The pellet was dried at room temperature for 5 minutes and then resuspended in 5 µl of TE buffer (10 mM TRIS-HCl, pH 7.5, and 1 mM EDTA).

cDNA Microarray Hybridization

The glass chips containing the spotted cDNAs were pre-warmed to 37°C in a hybridization chamber that contained 100 µl DIG Easy Hyb (Roche) in a humidifying well at the bottom of the chamber. All 5 µl of the fluorescent probe was combined with 75 µl of DIG Easy Hyb solution, heated to 68°C for 5 minutes and then chilled on ice for 2 minutes. The 80 µl of probe/hyb mixture was added to the spot-side of a microarray. This was followed by the addition of a plain glass slide to the top of the microarray forcing the probe to spread over the entire surface of the chip. The glass slides were placed back inside the humidifying chamber and incubated in the dark at 30°C overnight (16 hours).

Microarray washing

The sandwiched glass slides were separated under a solution of 2X SSC (3 M NaCl, 0.3 M Na₃·citrate·2H₂O). Once separated, the microarrays were washed 3 times for 5 minutes at room temperature in 2X SSC containing 0.05% w:v SDS with gentle shaking. To remove any residual SDS, the slides were rinsed in a solution of 1X SSC and then dipped in isopropanol before being spun dry in a swing bucket rotor for 5 minutes at 500 rpm in the dark. All slides were stored in the dark until they were scanned.

Microarray scanning and data analysis

Fluorescence intensity readings of each individual spot was obtained by scanning the chips in the laboratory of Dr. R. Walker or Dr. J. Nash, National Research Council (NRC), Ottawa. The information generated from the scanner (Vertek) was exported in tiff file format. Two black and white tiff images were generated for each chip scanned, one representing Cy3 intensity and the other representing Cy5 intensity. The program Arraypro (in the lab of Dr. J. Nash) was then used to analyze the data generated by the scanner in order to compare the relative intensity of the probe signal in control versus stress of every spot on the chip.

RESULTS

cDNA Screening and Northern Blot Analysis

A cDNA library was constructed from *E. solidaginis* larvae using the Uni-ZAP cDNA synthesis kit (Stratagene, La Jolla, CA). The library was constructed by pooling 1

μg of mRNA from each of the cold-exposed groups of larvae giving a total of 4 μg of RNA (chilled at 4°C, and frozen at -20°C for 8 h, 24 h, and 1 week). Using multiple NZY plates (24 x 24 cm) and the subsequent plaque lifts made from them, over a million plaques from the *E. solidaginis* library were differentially screened. Many rounds of primary screening were performed in order to identify 4 putatively up-regulated clones. These were then subjected to secondary screening and then underwent *in vivo* excision. The new plasmids were amplified overnight in SOLR cells and then isolated and subjected to restriction endonucleases to recover the clone insert from the plasmid. Each recovered insert was then used as a template to create a radioactive probe using the random primer method. Four different inserts were recovered: *eur-2* (558 bp), *eur-3* (742 bp), *eur-4* (1187 bp), and *eur-5* (560 bp). Subsequent sequencing by Canadian Molecular Research Services (CMRS), Ottawa, Canada revealed that the inserts present in *eur-2* and *eur-5* were clones of the same mRNA transcript and that *eur-3* and *eur-4* were also clones of the same transcript. The longest clone of each gene (*eur-4* and *eur-5*) was then chosen for further analysis.

The insert DNA from *eur-4* and *eur-5* was used to make radiolabelled probes for Northern blotting. Analysis confirmed the cold-induced up-regulation of each gene. Figure 3.2A shows the pattern of *eur-4* mRNA expression in response to experimental cold exposures in the lab. Compared with 15°C-acclimated controls, progressive cold exposure at 4°C (1 week) followed by freezing -20°C (8 h, 24 h or 1 week) showed a trend towards increased *eur-4* transcript levels although even the peak elevation (1.49-fold higher than control after 1 week at -20°C) was not significantly different from the control value. *Eur-4* message levels were also evaluated over a winter timecourse (Figure

3.2B) and a significant elevation of transcripts was seen in midwinter (January, February). Maximum transcript levels were reached in February with a mean value that was 1.66-fold higher than the level in October. However, by April, *eur-4* mRNA transcript levels had returned to a value not significantly different than in October.

Comparable analysis of *eur-5* gene expression is shown in Figure 3.3. Exposure to 4°C did not alter transcript levels but levels rose significantly by 1.47-fold after 24 h at -20°C and remained at 1.34-fold higher than controls after 1 week at low temperature (Figure 3.3A). As also occurred for *eur-4*, transcript levels of *eur-5* rose during the midwinter months with mean values in January and February that were 1.69- and 1.62-fold higher than controls, respectively. By April, *eur-5* RNA levels had returned to the same levels as in October.

EUR-4 AND EUR-5 Sequence Analysis

Nucleotide and putative amino acid sequences of *eur-4* and *eur-5* are shown in Figures 3.4 and 3.5, respectively. Any new nucleotide sequence retrieved from a library screen must first be examined to determine if it contains the trademark characteristics of an actual transcribed gene. This includes analysis of each of the three positive reading frames to identify at least one open reading frame of sufficient length, hopefully containing both start and stop codons (although clones retrieved from a cDNA library quite often lack the full 5' end of the sequence). Other features that identify a true protein-coding sequence include the presence of a Kozak sequence (ribosomal binding site to increase efficiency of translation), a polyadenylation signal, poly-A tail and a 3' untranslated region. Both *eur-4* and *eur-5* contained all of the above trademarks of a

transcribed gene. Each clone contained only one possible open reading frame with both start and stop codons. *Eur-4* coded for 133 amino acids and *eur-5* coded for 123 amino acids in their potential primary protein structure (Figure 3.4A and 3.5A).

Both sequences were submitted to BLAST for a nucleotide sequence comparison (<http://www.ncbi.nlm.nih.gov/BLAST>) to seek the possible identities of the genes. The BLASTn (nucleotide) search did not produce any significant homology matches for either *eur-4* or *eur-5*. Other search engines and databases were then used that can identify domains or motifs in the sequence that may give clues as to the function of the putative protein. Although some of these programs appear to be redundant, they all use different algorithms so it was useful to try them all. The programs tested included the following.:

1. SOSUI (<http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html>): Uses physicochemical (e.g. hydrophobicity) properties of an input amino acid sequence to predict secondary structure.
2. PROSITE (<http://expasy.ch/prosite/>): Uses amino acid primary sequence to compare against a database of conserved protein motifs.
3. PredictProtein (<http://www.embl-heidelberg.de/predictprotein/predictprotein.html>): Another tool that uses primary structure and attempts to predict protein structure using similar sequences as a model.
4. Protein sequence analysis (PSA) (<http://bmerc-www.bu.edu/psa/request.html>): Uses primary structure to predict secondary and tertiary structures.
5. PSORT (<http://psort.nibb.ac.jp/>): Searches the amino acid sequence for potential “transport” or “localization” signals that are responsible for determining the final

cellular destination for a mature protein.

6. SignalIP. (<http://www.cbs.dtu.dk/services/SignalIP/>): Identifies potential signal peptides and their potential cleavage sites.

The putative amino acid sequences of *eur-4* and *eur-5* did show some functional domains and motifs that were similar to other known proteins. The closest domain match to *eur-4* was a zinc metalloprotease from *Streptococcus pneumoniae*. The amino acid identity was only 30% and is not above the threshold to be considered a significant match (Figure 3.4B). This putative match suggests that the Eur-4 protein may be a metalloproteinase or a zinc-binding protein but more information is required to make a positive identification. PROSITE identified other markers in the putative Eur-4 protein that may be instructive about protein function including an alanine-rich region between AA 34-79, four possible Casein kinase II phosphorylation sites (AA 60-63, 64-67, 68-71, and 112-115), and two potential PKC phosphorylation sites (AA 64-66, 131-133).

The putative protein encoded by *eur-5* showed a stronger match to other known proteins, all belonging to the eukaryotic aspartyl protease family. Aspartyl (aspartic acid) proteases include pepsins, cathepsins, and renins. The closest match for the putative Eur-5 protein was a 43% amino acid identity with cow pepsinogen A (Figure 3.5B). The gall fly protein also showed 39% identity with a lysosomal aspartic protease precursor from the yellow fever mosquito (*Aedes aegypti*). The typical active site of aspartyl proteases contain two 12 amino acid consensus sequences; however, *eur-5* only contained 1 such consensus sequences. Structural analysis via PROSITE revealed five potential N-myristolation sites (AA 3-8, 8-13, 47-52, 104-109) and three potential Casein kinase II phosphorylation sites (AA 52-55, 56-59, 108-111).

NYLON DNA ARRAY

Clontech Rat 1.2 II nylon arrays were used to monitor gene expression changes in the freeze-avoiding species, *E. scudderiana*. Two regimes of cold temperature stress were assayed: insect switched from the control situation (+15°C, 2 weeks) to be chilled at +4°C for 24 h and insects switched after 1 week at +4°C to a subzero temperature (-5°C, 24 h). The mRNA from each larval group was isolated and used to create cDNA by reverse transcription. Radioactive ³²P was incorporated into the cDNA and then the ³²P-labeled cDNA was used to probe the nylon membranes. Following autoradiography, the membranes were stripped and re-probed with another sample. The level of cross-reactivity for these heterologous probing experiments was approximately 80%. Both temperature shifts resulted in the up-regulation of genes. The exposed films were scanned and the pixel density of each spot was measured using Imagequant (molecular Dynamics) software. A 1.5-fold cut-off was used to determine up-regulation. In the shift from 15°C to 4°C a total of 9 genes were identified as putatively up-regulated (Figure 3.6) whereas the second shift from 4°C down to -5°C resulted in the putative up-regulation of 33 genes (Figure 3.7). It appears that shift to subzero temperature is a much more stressful for *E. scudderiana*. The complete lists of genes that were identified as up-regulated by the nylon DNA array screening are given in Tables 3.1 and 3.2.

DNA MICROARRAYS

Two types of DNA microarrays were used to study gene expression in the two cold-hardy insects: *Drosophila* 7K arrays (7,222 genes) from the Canadian *Drosophila*

microarray center and the human 19K (19,000 genes) arrays from the Ontario Cancer Institute. Obviously, both were cases of heterologous probing. However, *Drosophila* genes would be expected to have a closer evolutionary relationship to the two insect species; indeed, *Drosophila* and *Eurosta* are both flies belonging to the Acalyptratae subgroup of the Muscomorpha infraorder of the Order Diptera. The first step in each case was to optimize all procedural conditions, such as hybridization and washing, in order to achieve optimal hybridization between the array and the gall insect cDNA. Dipping the slides in isopropanol following mild washing was an effective way of cleaning the slides without being too stringent. Under optimal conditions, I was able to achieve 89% cross-hybridization between *E. scudderiana* cDNA and the *Drosophila* array and slightly higher, 91% cross-hybridization for *E. solidaginis* cDNA. This means that ~6,400 genes could be assessed on the arrays. In each species of cold-hardy insect, the results showed that the expression pattern for ~90% of genes was unchanged during cold (4°C) or subzero (-16°C for *E. scudderiana*, -20°C for *E. solidaginis*) exposure, whereas 4.7% of cross-reacting genes (~340 genes) were putatively up-regulated in the experimental condition by 1.5-fold or more and a similar number were down-regulated by the same amount (Storey and McMullen, 2004). All experimental conditions that were assessed by microarray screening in *E. scudderiana* and *E. solidaginis* are summarized in Tables 3.3 and 3.4. The results of one example microarray screening experiment can be seen in appendix A1.

All data from heterologous probing of cDNA arrays must be verified by other quantitative methods such as Northern blotting or RT-PCR. At this point, many of the genes identified as putatively upregulated by DNA arrays have been confirmed to be

differentially expressed at the gene and/or protein level during temperature stress. Many others are the focus of ongoing studies in our laboratory.

Figure 3.1. Schematic depiction of cDNA library screening. Clones are bound to duplicate membranes, which are then screened with a radioactive probe for control and stressed experimental samples. Clones that are differentially expressed between the two states can then be isolated and identified.

Figure 3.2. Northern blot analysis of *eur-4*. (A) Relative mRNA abundance of *eur-4* during acute cold/freezing stress in *E. solidaginis*. (B) Relative expression of *eur-4* transcripts in *E. solidaginis* over the winter. Values are means $\pm$ S.E.M. for $n = 3$ independent determinations. *- Significantly different from October value using Student's t-test, $P < 0.05$.

A.

B.

Figure 3.3. Northern blot analysis showing the relative mRNA expression of *eur-5* in *E. solidaginis* larvae during acute temperature stress (panel A) and over the winter (panel B). Values are $\pm$ S.E.M. for $n = 3$ determinations. *- Significantly different from control value (October in panel B), using Student's t-test, $P < 0.05$.

A.

B.

Figure 3.4. (A) Nucleotide (upper) and deduced amino acid (lower) sequences from the potential open reading frame of the cDNA clone *eur-4* from *E. solidaginis*. Nucleotides and amino acids (in bold) are numbered on the left and start and stop codons are in bold. (B) Amino acid sequence alignment between *eur-4* and the closest potential homologue as identified by PSI-Blast, zinc metalloprotease from *Streptococcus pneumoniae* (accession number NP_358175).

A.

1 GGCACGAGGTTGCTTCTACCATTCGGACGTGCTTGTCTTTTGCTATTTTGTCCGTCAAGT
 61 AATCTTTTTTAATACAAAAAGAAAAAGTTGGTAAAGTTTTCAAATTATTTTTCATCGCGC
 121 ATTTATTTAGCATAATAATTACTTGCTAAATTCTTGTGTTTCATACATAAAACAACAAAT
 181 TAGCGCTTTTAATATTCATTCTGCACGTAAAGAAGAAAAAATACAATACAGACAAAATG
 1 M
 241 GCCGATGTTGTTGAAAAGAACGATACTCCAATTGTCGAGAAAGTCGCTGCTGAAGAAGTA
 2 A D V V E K N D T P I V E K V A A E E V
 301 GAAGCAAAAAAAGATAAAAAAGAGGTTGTTGATGATGCAGCACCAGCCGCTGAAAACGGT
 22 E A K K D K K E V V D D A A P A A E N G
 361 AAGGCTGACAGTGCAACCGCCGCCAAAGAAAATGGTGATGCTGTAGCAGCTGAATCCACA
 42 K A D S A T A A K E N G D A V A A E S T
 421 ACTGAAACACCAAAAGAAACATCTGCCGAGGCTGAAGCTGCTGCTCCCGCCGCTGAGGAA
 62 T E T P K E T S A E A E A A A P A A E E
 481 AATGGCAAAGATGAAGAGGCGGAGAGCGAAGCAACTGACTCAGCACCAACAGAGGCTGTT
 82 N G K D E E A E S E A T D S A P T E A V
 541 AAGCGAAAAGTCGGCGAAGTTGAATCTAAATCTAAAACAGAAGAATCCACTGCTGCTGTA
 102 K R K V G E V E S K S K T E E S T A A V
 601 GCAACGCCAGAGAAAAAAGCTAAGCAGACGAGCCGGTAAAGGAAGACAACCAAAATGGTT
 122 A T P E K K A K Q T S R *
 661 CAGAGGCTTCAGAAGTTGCGGCTTAACCTAGATCTTAATATTACATTTAAACTATTCAAA
 721 ACATATTACAGTAACAAAAAAGAAAAAAGTCTCGAKAKCTCTCTTTTTTTTCTCTCT
 781 CTCTMDCGCTCAGCACACATTCTCGCSAGATAATAAWATCTATCAYATATATATAGTTAT
 841 ACAGTGTGTGCTCATTTCATGTATGTCTGTTTGTATWTCCSYTTAAGGGATGCTAWAGA
 901 TTTTYYTCTTTATTTTGGAGGTTTCTTGAADATATTAWTTTGWATATATATATTAWYTTG
 961 CACATKCGCACATCYCTCATTATGGGRTTATGTATATCCCATATATAGTACWYTTGCTTA
 1021 WAATCTTCMTTAAATTAAGWCMACACTATWGCARGCRTCACCCACVWGDGYCAAGAGA
 1081 CTGAAWGATTAGTMGACCTGGCTGCATDGGTCGRAGCGTGTGWGGGCGCGTCCACGWGCS
 1141 MGTTGTGCKCTCACGCCWGRCCCGSMGMTCGSGDTATAGCTATAAAG

B.

<i>S.pneumoniae</i>	196	VVETEEAPKEEAPKTEESPKEEPKSEVKPTDDTLPKVEEGKEDSAEPAPV
<i>E.solidaginis</i>	4	VVEKNDTPIVEKVAAEEVEAKKDKKEVV..DDAAPAAENGKADSATAAKE
<i>S.pneumoniae</i>	256	EEVGGEVESKPEEKVAVKPESQPSDKPAEESKVEQAGEPVAPRKDEQAPV
<i>E.solidaginis</i>	62	NGDAVAAESTTETPKETSAAEAAAPAAEENGKDEEAESEATDS...APT

Figure 3.5. (A) Nucleotide (upper) and deduced amino acid (lower) sequences of the cDNA encoding the putative aspartyl protease from *E. solidaginis*. The start and stop codons are in bold and the polyadenylation signal is underlined. (B) Cladogram showing the percent amino acid identity between *E. solidaginis* aspartyl protease and the amino acid sequences of cow (*Bos taurus*, accession number NP_001001600) and chicken (*Gallus gallus*, accession number NP_990209) pepsinogen, and yellow fever mosquito lysosomal aspartic protease precursor (*Aedes aegypti*, accession number Q03168) protein.

A.

1 CGGCACGAGATATTATCAAGGTGATCTAACCTATGTACCAATATCTCAACCAGGTTATTG

61 GCAATTTAGC**ATGG**GATGGTGCTACCATTGGTGGTCAACCTATGTGCGGCAGTGGTTGTCA
1 M D G A T I G G Q P M C G S G C Q

121 AGCTATCGCTGATACTGGCACCTCCCTAATTGTTGCGCCATACTACGCCTACATAACTTA
18 A I A D T G T S L I V A P Y Y A Y I T Y

181 CATGAATGTGGTTGATCCCGATCAGGATGGTGGTTCGATTGCTCACTCATTGACTCATT
38 M N V V D P D Q D G V V D C S L I D S L

241 ACCCGATATGGAATTCATTATTGGTGGCAAGACTTTCTCTGTACCAGCTTCGCAATATAT
58 P D M E F I I G G K T F S V P A S Q Y I

301 TCTTAACGAGGATGGTGAATGCTCACCTGCTGTCAGCTATATGGGTAGTGATTTCTGGAT
78 L N E D G E C S P A V S Y M G S D F W I

361 TTTGGGTGATATCTTCATTGGCATGTACTACACTGAATTCGATATGGGTAATAATCGTAT
98 L G D I F I G M Y Y T E F D M G N N R I

421 TGGTTTTGCTCCAGTCGCT**TAAG**TAGCTTTTGTTATTAATTATGAAGCAGATGGAAACTA
118 G F A P V A *

481 GATGAGCTTTATTTAGTATTTAAGTTCGATTTTGTAAAAAATAAAAAAAATAAGTTATTTG
541 TTTTAAATGAAAAAAAAAAAAAAAAAAAA

B.

Figure 3.6. Nylon cDNA arrays hybridized with radioactive-cDNA derived from *E. scudderiana*. Each Clontech 1.2 II nylon microarray contained 1188 cDNAs that were hybridized with ^{32}P -labeled cDNA. (A) Control (+15°C, 2 weeks) versus (B) cold shock (+4°C, 24 hours).

+15°C, 2 wks.

+4°C, 24 h

Figure 3.7. Nylon cDNA arrays hybridized with radioactive cDNA derived from *E. scudderiana*. Each Clontech 1.2 II nylon microarray contained 1188 cDNAs that were hybridized with ^{32}P -labeled cDNA. (A) Control (+4°C, 2 weeks) versus (B) cold shock (-5°C, 24 hours). Examples of four cold-induced genes are circled for easier identification.

+4°C, 2 wks.

-5°C, 24 h

Table 3.1 Putative up-regulation of genes in *E. scudderiana* larvae stimulated by cold exposure at 4°C for 24 h as determined with the Clontech Rat Array series 1. Controls were larvae acclimated to 15°C for 2 weeks.

Gene	Clontech ID
Hypoxia Inducible factor 1 α	A10A
krox 20	A05D
Outer mitochondrial membrane receptor rTOM20	B12A
RT6.2	C09E
Defensin RatNP-3 precursor	D13I
2'5' oligoadenylate synthetase-2	D05K
Histone H10	D06M
Membrane and microfilament associated protein p58	E01L
Nuclear receptor (RNR-1)	F01F

Table 3.2. Putative up-regulation of genes in *E. scudderiana* stimulated by subzero exposure at -5°C for 24 h as determined with the Clontech Rat Array series 1.2. Controls were larvae held at +4°C for 1 week.

Gene	Clontech ID
Hairless protein	A09C
HNF-3/forkhead homolog-1 (HFH-1)	A04D
NF1-X1, partial cds	A10D
ALP mRNA for brevican	A12E
transcription factor USF-1	A05L
IgE Fc receptor beta subunit	B01A
C7-1 protein (C7-1)	B08B
120Kd lysosomal membrane glycoprotein	B12D
Golgi SNARE GS15	B14E
retinal dehydrogenase type III	C14B
serine pyruvate aminotransferase	C11D
RT6.2	C09E
VD3 1a hydroxylase	C12H
isopentenyl diphosphate: dimethylallyl diphosphate isomerase	C14H
endoplasmic reticulum alpha-mannosidase	C04I
putative pheromone receptor (Go-VN2)	D08E
defensin RatNP-3 precursor	D13I
ribosomal protein S10	D03K
ribosomal protein S14	D03M
FceRI gamma chain interacting protein SH2-B (SH2-B)	D14L
testis specific histone H1t and histone H4t	D06N
lymphotoxin (TNF-beta) gene, tumor necrosis factor (TNF-alpha) gene	D13N
SNF-1 related kinase	E03B
activin receptor-like kinase 7 (ALK7)	E14H
hepsin	E11K
E10L PC12 cells, carboxypepsidase Z	E10L
pituitary adenylate cyclase activating polypeptide precursor protein	E09N
NORBIN	F06B
bithoraxoid-like protein	F06D
factor X	F12F
stannin	F06H
CLIP-115 protein	F02I
phospholamban	F08I

Table 3.3. List of *E. scudderiana* experimental conditions analyzed on *Drosophila* and human microarrays.

CONTROL	STRESS
15°C, 14 days	+4°C, 24 hours
15°C, 14 days	+4°C, 1 week
15°C, 14 days	-4°C, 24 hours (staged decrease)
15°C, 14 days	-4°C, 1 week (staged decrease)
15°C, 14 days	-20°C (pooled 1 and 7 days)
15°C, 14 days	-4°C, (pooled 1 and 4 hours)
15°C, 14 days	24 hours hypoxic (15°C)
4°C, 7 days	24 hours hypoxic (4°C)

Table 3.4. List of *E. solidaginis* experimental conditions analyzed on *Drosophila* and human microarrays.

CONTROL	STRESS
15°C, 14 days	+3°C, 24 hours
15°C, 14 days	-16°C, 24 hours
15°C, 14 days	Thaw (+3°C for 24 h after -16°C, 24 hours)
15°C, 14 days	24 hours hypoxic
15°C, 14 days	24 h recovery (from 24 h hypoxia)
15°C, 14 days	-4°C (pooled 1 and 4 h) (direct from +15°C)
4°C, 7 days	24 hours hypoxic (4°C)

DISCUSSION

Three different techniques were employed in a concerted effort to answer the question: which genes are activated in cold-hardy insects in response to temperature or hypoxic stress to aid in the survival of these natural environmental challenges. The aim was to identify gene expression patterns that could be classified into different categories such as (a) genes unique to the overwintering survival of *E. solidaginis* or *E. scudderiana*, (b) expression patterns that distinguish freeze-tolerant versus freeze-avoiding strategies, and (c) global expression patterns that have evolved in many cold-hardy species, not just insects.

Construction and screening of cDNA libraries is a time-intensive process but it has many advantages. The fact that this technique supports the discovery of novel genes that are unique to an animal/tissue or stress model may be its greatest asset. For example, cDNA library screening in our lab has found three novel freeze-responsive proteins that support freezing survival in the wood frog, *Rana sylvatica* (Storey, 2004). However, due to the limited sensitivity of this approach, cDNA library screening is not well-suited to be the main gene-discovery technique of a research laboratory. Given the advances in microarray technology in recent years, it would be a mistake to not take advantage of this breakthrough in screening power. DNA microarrays allow the researcher to simultaneously screen for changes in expression of hundreds or thousands of genes. A key advantage of array screening is the ability to survey the responses of functionally related groups of genes (e.g. enzymes of a pathway or of a signal transduction cascade, transporter families, etc.) to look for coordinated responses by selected cell functions to the imposed stress. Data from DNA microarray screening is typically highly reliable but

must still be validated on a gene by gene basis, particularly when heterologous screening has been used. This can be done effectively by RT-PCR, first using a consensus sequence designed from multiple species to create a primer to amplify the species-specific product and then using species-specific labelled probe to amplify and quantify message levels in control versus experimental states and to retrieve a full gene sequence from a cDNA library screen.

Over a million plaques were screened from the *E. solidaginis* cDNA library in order to identify two up-regulated clones, *eur-4* and *eur-5*. Northern blotting verified that mRNA levels of these genes were indeed elevated in response to cold or subzero temperature exposure and/or over the winter months. Indeed, in the laboratory low temperature exposures, the highest transcript levels for *eur-4* and *eur-5* were in the larvae at -20°C, a temperature at which *E. solidaginis* are frozen (they freeze below about -10°C). This correlates well with the observation that transcript levels were also highest in January/February, which are the months when the larvae would experience the longest and deepest freezing exposures in nature. Hence, the data suggest that the protein products of the two genes probably contribute to freezing survival.

Sequence analysis revealed that both *eur-4* and *eur-5* had the necessary primary structural characteristics to be an actual transcribed gene. The putative proteins encoded by these clones most closely resembled members of the protease family. *Eur-4* showed a weak domain homology to a zinc metalloprotease from the bacterium, *Streptococcus pneumoniae* but more characterization of this gene/protein will be required to confirm its true function in the insect. *Eur-5* seems to belong to the family of aspartyl proteases. The aspartyl proteases, also called acid proteases, are known to exist in fungi, plants, viruses,

some retroviruses, and vertebrates (Foltmann, 1981). Eukaryotic aspartyl proteases are monomeric proteins that contain two domains, each with a catalytic aspartyl residue in the center of the active site. Members of the vertebrate family include cathepsins, pepsins, chymosin, and rennin. Viral and retroviral aspartyl proteases are often heterodimers comprised of 2 chains, each 90-125 amino acids in length and with a single active site per chain (Davies, 1990). The protein encoded by *eur-5* also seems to contain only 1 active site and is much smaller than traditional eukaryotic aspartyl proteases. It is not very likely that the *E. solidaginis* aspartyl protease homologue (*eur-5*) has a format similar to the viral aspartyl proteases, therefore the clone *eur-5* probably represents a truncated portion or the actual gene sequence. Also, the Northern blots showed that *eur-5* probe bound to a mRNA band of ~1.5 Kb whereas the size of the *eur-5* retrieved from the cDNA library was 0.56 Kb. Several attempts were made to retrieve a larger *eur-5* clone from the library but all clones examined turned to be the same size or smaller than the original *eur-5* used to “bait” homologous clones.

The cDNA macroarray is a useful tool for profiling hundreds or a few thousand genes in a single experiments. Many macroarrays are now commercially available with various classes of genes on them; e.g. apoptosis arrays, cancer arrays, signalling pathway array, etc. The data presented in this chapter was derived from screening a Clontech 1.2 II Rat array with probe created from *E. scudderiana*. Even though this was a case of heterologous probing, we achieved an impressive level of cross-hybridization. The data (see Tables 3.1 and 3.2) suggested that exposure to subzero (-5°C) temperatures elicited a more pronounced change in the gene expression profile than did exposure to cold (+4°C) temperatures. Some of the genes that were putatively upregulated are discussed below,

beginning with some that were putatively up-regulated in response to the initial 15°C to 4°C shift in temperature (Table 3.1). For example, Krox 20 is a transcription factor that has been linked previously to cell stress. A major function of Krox-20 is to suppress the c-Jun NH(2)-terminal protein kinase (JNK)-c-Jun pathway, activation of which is required for both proliferation and apoptosis (Parkinson *et al.*, 2004). Therefore, activation of Krox 20 may be involved in a suppression of metabolic processes at low temperature or may serve to inhibit apoptosis that might otherwise be triggered by a sharp decrease in temperature.

Tom20 is a major receptor of the mitochondrial preprotein translocation system and is anchored to the outer membrane by an N-terminal transmembrane domain (Kanaji *et al.*, 2000). Most mitochondrial proteins are produced in the cytosol and contain an N-terminal pre-sequence that directs the peptide to the mitochondria. The function of Tom20 is to transport the pre-proteins across the outer mitochondrial membrane. The mechanisms of mitochondrial outer membrane transporters are still poorly understood. As will be discussed in Chapter 5, regulation of mitochondrial activity seems to be an important part of overwintering in both insect species.

The switch from 15°C to 4°C also triggered putative up-regulation of the hypoxia-inducible factor-1 α (HIF-1 α) subunit. Much research in recent years has centered on gene expression responses to low oxygen and the role of the HIF-1 transcription factor that stimulates and coordinates the up-regulation of multiple genes in response to hypoxia signals (Haddad, 2000). Among the genes regulated by HIF-1 are those involved in anaerobic glucose metabolism, erythropoiesis, and vascular growth (Bunn and Poyton, 1996; Gleadle and Ratcliffe, 1998). HIF-1 is a heterodimer formed from 2 basic-helix-

loop-helix-PAS (bHLH-PAS) proteins, HIF-1 α and HIF-1 β . Under oxygenated conditions, HIF-1 α has a short half-life *in vivo* and is rapidly inactivated by oxygen-dependent targeting of the protein for degradation. However, when oxygen is low this process is impeded, and HIF-1 α can bind with HIF-1 β (also called the aryl hydrocarbon receptor nuclear translocator, ARNT), which is constitutively expressed. The transcription factor is translocated to the nucleus to activate gene expression via binding to a conserved DNA sequence, the hypoxia response element (HRE), in target genes (Ratcliffe *et al.*, 1996 and Bunn *et al.*, 1996).

A similar system of oxygen-dependent signalling has been identified in insects in studies with *Drosophila melanogaster* (Nambu *et al.*, 1996; Nagao *et al.*, 1996). A protein called Similar (Sima) is now known to be functionally and structurally analogous to HIF-1 α whereas Tango (Tgo) is the equivalent of HIF-1 β . Sima protein accumulates in *Drosophila* cells following hypoxia and Hep3B cells transfected with Gal4-Sima fusions showed inducible activity following exposure to stimuli that activate HIF-1 including hypoxia, cobaltous ions and desferrioxamine (Bacon *et al.*, 1998). Although Sima is considerably larger than HIF-1 α (Sima contains 1505 amino acids compared with 826 in HIF-1 α), the two proteins show substantial sequence identity especially within the bHLH and PAS domains that are responsible for dimerization and DNA binding (Ratcliffe *et al.*, 1998). Hence, the heterologous screening used in the nylon macroarray studies undoubtedly represent cross-hybridization between HIF-1 α cDNA on the array and Sima cDNA in *Epiblema*. Indeed, this has been followed up in our lab and hypoxia and cold-responsive up-regulation of Sima has been confirmed in collaborative studies by myself and P. Morin (data not reported in this thesis). The insect protein also shows

conservation of two proline residues (Pro747 and Pro850) that appear to constitute the oxygen-dependent degradation domain so the mechanism of targeting Sima for degradation appears to be the same as in HIF-1 α (Lavista-Llanos *et al.*, 2002). The main difference between the two proteins is a long (~500 amino acid) C-terminal domain in Sima that is not present in HIF-1 α (Bacon *et al.*, 1998). The hypoxia inducible factor 1 α (HIF-1 α) is an important transcription factor that triggers the up-regulation of a suite of genes in response to hypoxia signals (Zhu *et al.*, 2002). The putative up-regulation of *sima* that is indicated by the array screening results suggests that various genes that are regulated by *sima* are probable targets of up-regulation in response to cold and/or hypoxia stresses in insects.

Array screening to reveal genes that were putatively upregulated in response to subzero exposure (-5°C for 24 h as compared with 4°C controls) revealed other interesting genes whose protein products may contribute to cold hardiness (Table 3.2). One of these was USF-1, which forms a homodimer or a heterodimer with USF-2 and binds to the E-box motif of selected enhancer regions (Sirito *et al.*, 1992). USF (upstream stimulatory factor) belongs to the bHLH superfamily of transcription factors and regulates the tissue-specific expression of a number of genes linked to C/EBP (Timchenko *et al.*, 1995), pyruvate kinase (Vallet *et al.*, 1998), and fatty acid synthase (Wang and Sul, 1995). More recently, activation of USF has been linked to the in vitro repression of the pro-inflammatory cytokine, interleukin-1 β (Gupta and Kone, 2002). This topic will be discussed further in Chapter 5.

Two ribosomal proteins, S10 and S14, were also putatively upregulated in *E. scudderiana* in response to subzero temperature exposure. Ribosomal proteins are known

to play a major role in ribosome function and stability. Eukaryotic ribosomes are made up of four types of ribosomal RNAs and over 80 ribosomal proteins. Gene expression and protein synthesis of ribosomal proteins is tightly regulated and is responsive to changes in physiological condition, indicating that regulation of these proteins may be important to stress survival. Ribosomal protein L26 is upregulated in the marine snail, *Littorina littorea*, during anoxia and has been proposed to play a role in increasing structural stability of the ribosome in stress (Larade and Storey, 2001). Recently, the function of ribosomal protein S14 has been suggested to be 3' end processing of 20S pre-rRNA (Jakovljevic *et al.*, 2004). As of yet, the function of S10 is unclear. Work in this lab has found that many ribosomal proteins are putatively upregulated, as determined by microarray analysis, in other animal systems responding to environmental stress. More work is needed to understand the complex role of the ribosomes in survival during stress.

Phospholamban was also upregulated by subzero temperature exposure in *E. scudderiana*. This protein is a regulator of SERCA and Western blotting studies reported in Chapter 2 suggested that increased levels of the phosphorylated form of phospholamban could contribute to the suppression of SERCA activity during cold exposure. The present data indicating that phospholamban transcript levels are upregulated during cold exposure suggests that an additional mode of SERCA control could come from increased phospholamban gene expression producing increased total phospholamban protein levels under cold and/or midwinter conditions. In fact, phospholamban was also putatively upregulated in the skeletal muscle of the little brown bats during hibernation as determined by screening with the human 19K cDNA array (S. Eddy, Ph.D. thesis, Carleton University, 2004).

Advances in DNA chip technology, miniaturization, and data analysis software have made microarrays an incredibly powerful research tool. Even labs that cannot afford to produce their own arrays or buy the equipment necessary to scan and analyze DNA chips will soon have access to facilities that will run and quantify samples at an affordable cost. Microarrays are now being employed for numerous different applications. Some examples include using microarrays to monitor gene changes in prosthetic arterial grafting to promote smooth muscle cell proliferation and matrix production (Willis *et al.*, 2004), finding potential therapeutic drug targets (Butte, 2002), signaling pathways in plants (Hugouvieux *et al.*, 2001), and following the progression of various diseases (Onda *et al.*, 2004). Each analysis of a control *versus* stress experiment (Table 3.3 and 3.4) yields an enormous amount of data. As mentioned early, each stress situation that was screened for the two insect species resulted in ~5% of gene transcripts being assessed as putatively upregulated by 1.5-fold or more, ~5% of genes that were putatively downregulated, and ~90% of the genes that were unchanged by stress. At approximately 85% cross-reactivity of probe signal, this resulted in a list of about 300 genes that were putatively up- or down-regulated as determined from screening the *Drosophila* 7K arrays and more than 500 genes whose expression either increased or decreased were revealed from screenings using the human 19K arrays for each experiment. All samples were run in reciprocal fashion and the gene lists were compared for genes that were upregulated in both cases to reduce the number of false positives. The next step was to analyse all the gene lists and identify common patterns in gene expression profiles.

One pattern of interest, was the strong upregulation of plasma membrane

transporters in *E. scudderiana* as a result of exposure to -20°C . Within this group was included:

- (1) Concentrative nucleoside Na^+ transporter 2 (CNT2): CNTs cotransport sodium and nucleosides unidirectionally against the nucleoside concentration gradient (Cabrita *et al.*, 2002) and function in both salvage pathways for nucleic acid synthesis and in the transport of nucleosides that have important roles in signaling (e.g. adenosine plays a key role in metabolic rate depression; Storey, 2004).
- (2) Na (K) Cl cotransporter: These electroneutral cotransporters are nearly ubiquitous and have an important role in sodium chloride absorption/secretion and in cell volume regulation during hypertonic and other volume challenges (Kaplan *et al.*, 1996).
- (3) Na, Pi cotransporter: Inorganic phosphate has critical roles, both in intermediary metabolism and in the structure of macromolecules. However, due to the negative electrochemical potential across the cell membrane, inorganic phosphate cannot enter cells by diffusion and, hence, is imported by Na^+ -dependent Pi cotransporters that have important roles in regulating phosphate availability (Werner and Kinne, 2001).
- (4) Na-Ca exchanger (NCX): These exchangers are found in the plasma membrane of most cells and are integral to Ca^{2+} homeostasis (Omelchenko *et al.*, 2003). In heart, for example, NCX provides the principle mechanism for Ca^{2+} extrusion after contraction. NCX may also function in Ca^{2+} influx, particularly under pathophysiological conditions. Isoform 1 is found in a macromolecular complex with protein kinase A, its anchoring protein AKAP, protein kinase C and protein

phosphatases 1 and 2A and beta-adrenergic mediated phosphorylation of NCX increases exchanger current.

- (5) Dicarboxylate transporter (cation-independent): This sodium-independent transporter in *Drosophila* was named INDY (I'm not dead yet) because mutation of the gene to create a disfunctional protein resulted in a 2-fold extension of the average adult lifespan (Inoue *et al.*, 2002). INDY transports a variety of tricarboxylic acid cycle intermediates, including citrate and succinate, and it is hypothesized that disfunction of INDY reduces substrate availability for energy metabolism, resulting in life extension effects similar to those achieved by caloric restriction.
- (6) Monocarboxylate transporter (MCT): Monocarboxylic acids such as lactate and pyruvate play central roles in metabolism and are often transported between tissues that produce versus catabolize these compounds. Their transport across the plasma membrane is via proton-linked MCTs that occur in especially high levels in white muscles and are also up-regulated by increased work load in heart (Halestrap and Price, 1999).

Coordinated up-regulation of a variety of plasma membrane transporters in response to acute subzero exposure is an intriguing response that has not previously been reported as a part of cold acclimation. Up-regulation may be needed to enhance the overall levels of transporters in the cold or perhaps to alter the relative levels of different isoforms of each transporter. Further studies will verify and explore changes in both mRNA and protein levels of each of these transporters and aim to discover how they contribute to re-establishing homeostasis at low temperature.

Results from *E. solidaginis* array screenings were largely similar to those from *E. scudderiana*. Once again, solute transporters were putatively upregulated during cold and freezing stress as well as in response to hypoxia. Another class of interest noted from *E. solidaginis* array screenings were mitochondrial transporters, both those located on the inner and outer mitochondrial membranes. Most mitochondrial proteins are transported into the mitochondria after their synthesis in the cytosol; however, some proteins are encoded by the mitochondrial genome. As a result, the production of nuclear-encoded and mitochondrial-encoded proteins must be coordinated so that an imbalance of the two types of protein does not occur. Regulation of this balance is now believed to respond to changing environmental conditions (Devaux et al., 2002; Zhao et al., 2002; Liu et al., 2003). Exactly how and why these mitochondrial transporters are regulated needs to be elucidated.

Several chaperone proteins were also putatively upregulated but one that showed up on just about every list of upregulated genes in *E. solidaginis* in response to cold stress was Hsp70. This protein is now known to regulate apoptotic cell death both directly by interfering with the function of several proteins that induce apoptotic cell death as well as indirectly by increasing levels of the anti-death protein bcl-2 (Giffard and Yenari, 2004). Hsp70 also serves to deliver protein to the mitochondrial import receptors on the outer mitochondrial membrane (Kanaji et al., 2004).

Several ribosomal proteins, including L10, L31, S3, S10, and S14 were revealed as putatively upregulated in *E. solidaginis* in response to subzero temperature exposure (-16°C). These proteins will be discussed briefly in Chapter 5. Multiple protein kinases and protein phosphatases were also putatively upregulated in the cold. As already

discussed, reversible phosphorylation is an efficient and common mechanism for controlling the activity of many proteins and pathways. Reversible phosphorylation has emerged as a conserved mechanism employed by virtually all species as a means of coordinating entry into or arousal from a hypometabolic state. Each of the protein kinases revealed by array screening will need to be examined further.

A final gene that deserves comment is the FOXO transcription factor that was also identified as putatively upregulated during subzero exposure from microarray screening of *E. solidaginis*. FOXO is involved in regulating the cell cycle and apoptosis and inhibits NF- κ B (Giannakou and Partridge, 2004). A member of the FOXO forkhead type transcription factors, it is markedly upregulated in skeletal muscle in energy-deprived states (Kamei et al., 2004). Intense research is now being focused on this family of transcription factors as it has been linked to hibernation and hypoxia survival.

The information presented above represents just a small percentage of the leads that have been generated from cDNA microarray analysis of the responses to cold, freezing and hypoxia by freeze tolerant and freeze avoiding insects. It will require several years to validate and follow up the information gathered from these sets of experiments. The value of this technology is only now starting to be realized and has become a dominant force in the field of gene expression studies.

Chapter 4

Overwinter fate of the mitochondrion

INTRODUCTION

Mitochondria are membrane-enclosed organelles that contains their own genetic material and the reaction pathways for much of aerobic metabolism including the β -oxidation of lipids, the tricarboxylic acid cycle, the electron transport chain and oxidative phosphorylation. As such, the mitochondria are the main site of cellular ATP production in aerobic cells. Because energy balance is crucial to cold-tolerance and metabolic regulation and because suppression of the rate of ATP production is a key part of metabolic rate depression in diapause, it seemed logical to investigate the properties of the mitochondria during hypometabolism and in response to cold and low oxygen stresses in the cold-tolerant insects, *E. scudderiana* and *E. solidaginis*.

The complete nucleotide sequence of the mitochondrial genome is known for several species including human (Anderson *et al.*, 1981, 1982), mouse (Bibb *et al.*, 1981), cow, *Xenopus laevis* (Roe *et al.*, 1985), *C. elegans* (Okimoto *et al.*, 1992), several fish (Tzeng *et al.*, 1992; Chang *et al.*, 1994), and at least five species of insects: *Drosophila yakuba* (Clary and Wolstenholme, 1985), *Apis mellifera* (Crozier and Crozier, 1993), *Anopheles quadrimaculatus* (Mitchell *et al.*, 1993), *Anopheles gambiae* (Beard *et al.*, 1993), and *Drosophila melanogaster* (Lewis and Kaguni, 1995). The mitochondrial DNA from all metazoans examined to date codes for either twelve or thirteen proteins that are subunits of the electron transport chain protein complexes or the F_1F_0 ATPase as well as for twenty-two tRNAs and two mitochondrial ribosomal RNAs (rRNA) that are needed for protein synthesis within the mitochondria (Wolstenholme, 1992). The gene arrangements can differ from species to species with the degree of variation being higher in invertebrates than in vertebrate species (Desjardins and Morais, 1990). Within insects,

gene arrangements are largely constant except for the position of some tRNAs (Clary and Wolstenholme, 1985; Garesse, 1988; Crozier *et al.*, 1988; Mitchell *et al.*, 1993; Beard *et al.*, 1993).

The mitochondrial genome is transcribed in two directions (Figure 4.1). The fact that different genes are coded by opposing DNA strands allows for a smaller piece of DNA to code for more genes. One direction is the heavy chain and the other direction is the light chain. All the genes of the heavy chain are transcribed together (as are all the genes of the light chain) as one large polycistronic RNA from promoters in the control region of the mitochondrial genome. The polycistronic RNA is then processed to produce the individual mature mRNAs (Lehninger *et al.*, 1993; Alberts *et al.*, 1994; Lewin, 2000).

Previous studies have suggested that insect cold hardiness and/or diapause is associated with reduced numbers of mitochondria. For example, the contents of cytochromes *b* and *a+a₃* were significantly reduced in *Hyalophora cecropia* during pupal diapause (Shapiro and Williams, 1957). In a more extreme scenario, Kukal *et al.* (1989) reported that the numbers of mitochondria in the fat body and brain of the high Arctic caterpillar *G. groenlandica* decreased about 10,000-fold in animals acclimated to -15°C versus controls at 15°C.

Previous work in our lab suggested that a similar situation might occur in our two model insects, *E. solidaginis* and *E. scudderiana*. Both showed a greater than 50% reduction in the activities of three mitochondrial enzymes (citrate synthase, glutamate dehydrogenase, NAD-isocitrate dehydrogenase) over the winter months which could suggest reduced mitochondrial numbers in the overwintering larvae (Joanisse and Storey,

1994). However, earlier studies showed that *E. solidaginis* mitochondria do remain capable of carrying out the oxidation of specific substrates throughout the winter (Ballantyne and Storey, 1985).

Because mitochondria are important regulators of cellular energy charge, I hypothesized that active regulation of maximal mitochondrial output would be a crucial component of winter survival in *E. scudderiana* and *E. solidaginis*. The effects of cold exposure and hypoxia on mitochondria were evaluated by measuring: (1) the maximal activity of a key enzyme located in the mitochondria, cytochrome c oxidase (COX) (the whole COX enzyme is not mitochondrially encoded. Only 3 of its 13 subunits are, the other 10 are nuclear-encoded, see figure 4.1), (2) the level of transcription of one heavy and one light-chain coded gene (COX subunit 1 and 12S rRNA, respectively), and (3) the ratio of mitochondrial DNA to nuclear DNA.

MATERIAL AND METHODS

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

cDNA was amplified from total RNA isolated from either species (see Chapter 3 for Trizol isolation of RNA). A volume containing 5 µg of total RNA was diluted to 10 µL with DEPC-treated water and then combined with 1 µL of RNase inhibitor (RNA guardTM, Amersham) and 1 µL of 0.2 µg/µL oligo(dT)₁₈ primer (Sigma Genosys). The samples were heated to 68°C for 5 min and then chilled on ice for another 2 min. The following ingredients were then added: 4 µL of 5X first-strand buffer (supplied with Superscript IITM), 2 µL 0.1 M DTT, 1 µL of 10 mM dNTP mix, and 1 µL of Superscript IITM (200U/µL). The mixture was incubated at 42°C for 45 min. The reverse transcriptase

was inactivated by incubating the mixture at 94°C for 3 minutes. The cDNA was stored at 4°C for up to 2 weeks.

The initial PCR reactions were performed on serial dilutions of the cDNA in order to determine the optimal cDNA concentration for use. PCR reactions were conducted with 5 µL of diluted cDNA, 15 µL sterile water, 2.5 µL 10 x PCR buffer (provided with Taq), 1.25 µL 50 mM MgCl₂ (Invitrogen), 0.5 µL dNTPs (Invitrogen), 1.25 µL of forward and reverse primers (30 µM each), and 0.125 µL Taq polymerase (Invitrogen). Most PCR amplifications followed a program similar to the following: denature at 94°C for 30 sec, anneal for 1 min at a predetermined temperature, and extend for 1 min at 72°C. These steps were repeated for 35 cycles and followed by a final 7 min extension at 72°C. The optimal annealing temperature was determined from initial gradient PCR runs. PCR products were sequenced by Canadian Molecular Research Services Inc., Ottawa, Ontario.

After the correct sequence was obtained, RT-PCR was used to quantify the relative expression of the gene under investigation. First strand cDNA was normalized to confirm that there were equal amounts of starting material in control and stress samples. This was accomplished by creating serial dilutions and performing PCR on these dilutions using primers for a constitutively expressed gene, α -tubulin. The PCR amplified DNA was then subjected to agarose gel electrophoresis and visualized by ethidium bromide staining to confirm that the expression level of α -tubulin was the same in control and stressed samples. Band intensities were quantified using the Syngene with GeneTools software (Synoptic, Ltd.). If some samples contained too little or too much starting material, they were concentrated or diluted until all samples were normalized.

Following normalization, PCR amplification was performed on the serial dilutions using primers specific for the genes of interest. The sequence of the primers used for α -tubulin, 12S mitochondrial ribosomal RNA, and cytochrome c oxidase subunit I were:

α -tubulin forward: 5'-GGTCACATTTTCACCATCTG-3'

α -tubulin reverse: 5'-AAGGAAGATGCTGCCAATAA-3'

12S rRNA forward: 5'-TACTATGTTACGACTTAT-3'

12S rRNA reverse: 5'-AAACTAGGATTAGATACCC-3'

COX 1 forward: 5'-TGATCAAATTTATAAT-3'

COX 1 reverse: 5'-GGTAAAATTAAAATATAAACTTC-3'.

Mitochondrial DNA analysis

The relative amount of mitochondrial DNA present in a sample was determined by using PCR to compare the copy number of the mitochondrial specific DNA encoding cytochrome c oxidase subunit 1 (COX 1) to that of the nuclear specific DNA encoding α -tubulin. Genomic DNA was isolated from *E. scudderiana* using the DNeasy Tissue kit from Qiagen (cat. no. 69504) as per the manufacturer's instructions. Briefly, insect tissue was frozen in liquid nitrogen and pulverized with a mortar and pestle. Ten milligrams of tissue was then combined with 180 μ L of tissue lysis buffer (provided with the kit) and 20 μ L of proteinase K. The tissue was digested at 55°C overnight. RNA was degraded in the samples by incubating with 4 μ L RNase A (100mg/mL, Sigma Aldrich Chemical Co.). Samples were then vortexed for 10 sec, neutralized with 200 μ L neutralization buffer and then incubated at 70°C for 10 min. At this point, the DNA was precipitated by the addition of 200 μ L of 96% ethanol and vortexed for another 10 sec. Samples were

then loaded onto small silica gel columns that bind DNA and centrifuged at $6,000 \times g$ for 1 minute. The columns were washed twice with 500 μL of wash buffer (with centrifugation after each wash followed by a final centrifugation at $20,000 \times g$ for 3 min to remove as much liquid as possible from the column). The DNA bound on the column was eluted with 200 μL DEPC-water at pH 7.5 and then serially diluted in fractions ranging from 10^0 down to 10^{-6} . PCR was performed on the samples as described earlier and PCR products were separated on agarose gels and visualized with ethidium bromide. The band intensity of the COX 1 DNA was normalized against the corresponding band intensity of α -tubulin in the same sample.

DnaseI treatment of total RNA

Because isolation of RNA can result in the carry-over of DNA contamination, total RNA samples were treated with DNase I. PCR primers designed for RT-PCR have the proper orientation and directionality to bind and amplify genomic DNA if it is contained within an RNA sample. To degrade DNA, 5 μg aliquots of sample RNA were combined with DEPC-water to a final volume of 10 μL and then samples were incubated at 95°C for 2 min and then placed on ice to degrade secondary structure. One μL of DNaseI (2 U/ μL) was then mixed into the each sample, followed by incubation at 37°C for 25 min. The DNase I was inactivated with 5 μL of DNase inactivating reagent (Ambion, TX) and then samples were centrifuged at $10,000 \times g$ for 2 min to pellet the inactivating reagent. The DNA-free RNA was then transferred to a fresh tube and stored at -80°C until use.

Cytochrome c oxidase (COX) assay

The substrate for the COX assay is reduced horse heart type III cytochrome c (RHCC) (Sigma) which must be fully reduced before use. To do this, 10 μ L of ascorbic acid (reducing agent), pH 6-7, was added to 300 μ L of cytochrome (40 mg/mL) and the solution was mixed until the color changed from dark maroon to light red, indicating complete reduction of the RHCC. The ascorbic acid was then removed by low speed (benchtop) centrifugation of the reduced RHCC solution through a 3 mL column of Sephadex G-50 (Sigma) previously equilibrated with 25 mM Na_2HPO_4 , pH 7.0. The buffer-exchanged RHCC solution was then placed on ice for immediate use or stored at 4°C for up to 1 week.

The multi-subunit nature of COX renders it sensitive to shear and other forms of harsh mechanical disruption (e.g. sonication) and, therefore, a dounce homogenizer was used for tissue disruption. Furthermore, homogenization in the presence of a detergent (0.2 % deoxycholate [DOC]) releases submitochondrial particles (SMP), which contain the active enzyme. Samples of frozen larvae were crushed under liquid nitrogen and washed with 100 mM Na_2HPO_4 , pH 7.0, and 0.2 % DOC (1ml per 100 mg tissue). The washed tissue was resuspended in 100 mM Na_2HPO_4 , pH 7.0, 0.2 % DOC (1 ml per 100 mg tissue) and 1 crystal of PMSF and then disrupted in a pre-chilled dounce homogenizer using a firm constant pressure until the homogenate was free of large pieces. The crude homogenate was transferred to a fresh 1.5 mL centrifuge tube and incubated on ice for 30 min prior to centrifugation at 10,000 x g for 10 min. The supernatant was then transferred to another fresh tube and stored on ice. Protein concentration was determined by the BioRad colorimetric assay as described earlier.

A Cary 3 UV/VIS spectrophotometer was used to obtain absorption spectra for reduced and oxidized cytochrome c. The reduced form (RHCC) showed two distinct peaks, one of which (550 nm) was used to monitor the rate of oxidation of RHCC by the COX activity in the clarified tissue extracts. Assay blanks contained 25 mM Na₂HPO₄ pH 7.0 and 0.2 % DOC. A time-zero reading was obtained by combining 800 µL of 25 mM Na₂HPO₄ pH 7.0, 0.2 % DOC and 50 µl of the RHCC solution in a 1mL glass cuvette and inverting twice. Fifty µL of enzyme extract was then added and the decrease in absorbance was monitored at 0.2 sec intervals as RHCC was oxidized by COX. The change in absorbance over time was used to calculate the activity of COX, which was expressed as nmol RHCC oxidized/min/mg protein.

RESULTS

The maximal activity of COX was assayed during acute stress and throughout the winter in both *E. scudderiana* and *E. solidaginis* larvae. The substrate used for the assays was horse heart type III cytochrome c. The reduced form of the substrate absorbs maximally at 550nm (Figure 4.2A), whereas the oxidized form does not absorb at this wavelength. Before starting the assay, a wave-scan from 450-600nm was performed on the substrate to be sure that it was in the fully reduced state. Assay conditions were also optimized for time and enzyme amount to be sure that a linear reaction rate was maintained for a sufficient time to accurately measure activity (Figure 4.2B).

The maximal activity of COX in *E. scudderiana* was affected by both low temperature exposure and lack of oxygen (Figure 4.3A). Mean activity in control larvae acclimated at 15°C was 46.7 ± 3 nmol/min/mg protein. The activity dropped as the

larvae were exposed to low temperatures. After 1 week at 4°C, COX activity had fallen to 33.3 ± 1.9 nmol/min/mg protein and after a further temperature decrease to -4°C for 1 week activity was just 13.8 ± 0.7 nmol/min/mg protein or 30% of the control value. Activity in larvae shifted to -20°C for 1 week was 27.2 ± 0.8 nmol/min/mg protein. Similarly, 24 hours of hypoxia exposure at 15°C reduced COX activity significantly to 25.2 ± 1.4 nmol/min/mg protein.

Seasonal changes in COX activity in *E. scudderiana* are shown in Figure 4.3B. Activity was high in September at 41.7 ± 8.4 nmol/min/mg of protein but was sharply reduced by December to just 13.6 ± 0.65 nmol/min/mg protein or 33% of the September value. Activities remained low at both the February and April sampling times.

Changes in COX activity in *E. solidaginis* in response to cold exposure and hypoxia stress are shown in Figure 4.4A. The mean activity in control larvae at 15°C was 47.1 ± 0.3 nmol/min/mg protein. Activity was reduced by cold exposure, dropping to 72% of the control value (33.9 ± 3.25 nmol/min/mg protein) in larvae transferred to +3°C for 24 h. Activity was similar after freezing exposure at -16°C for 24 h (39.3 ± 5.0 nmol/min/mg protein) but rose substantially when larvae were thawed (+3°C, 24 h) to a value 29% higher than the control and 54% higher than the value in -16°C exposed larvae. Unlike the situation in *E. scudderiana*, however, 24 h of hypoxia exposure had no effect on COX activity in *E. solidaginis* nor did activity change during recovery after hypoxia.

The seasonal pattern of COX activity in *E. solidaginis* was similar to that recorded in *E. scudderiana* (Figure 4.4B). Highest activity was in September larvae and activity had decreased by 52% to 22.7 ± 6.2 nmol/min/mg protein in December. In

February, COX activity was just 31% of the control value and it remained low in April (14.4 ± 3.2 and 18.4 ± 3.1 nmol/min/mg protein, respectively).

Transcriptional activity of the mitochondrial genome was assessed using RT-PCR. Transcript levels of COX 1 and 12S rRNA were determined, representing genes on the heavy and light chains of the mitochondrial genome, respectively, to determine whether transcriptional activity of the two chains might vary. Figure 4.5 shows the winter profile of the relative transcript levels of COX 1 and 12S rRNA in *E. scudderiana*, normalized against α -tubulin transcript levels in the same samples. Tubulin is a constitutively-expressed nuclear gene that is commonly used to standardize the expression levels of other genes. RT-PCR produced a single band for each PCR product. The COX 1 PCR product was ~600 bp in length whereas the 12S fragment was ~500 bp. The data in Figure 4.5A show that the level of transcription of each of these two genes did not vary significantly from September values throughout the winter.

Genomic DNA was also isolated and PCR was used to amplify the mitochondrial DNA for COX 1 and the nuclear DNA for α -tubulin from *E. scudderiana* (Figure 4.5B). A decrease in the DNA ratio for COX 1 *versus* α -tubulin could indicate that the total number of mitochondria was reduced in the larvae over the winter. However, the data showed that the DNA ratio of the mitochondrial versus nuclear gene was unchanged throughout the winter. This result indicated that the winter reduction in COX activity seen in *E. scudderiana* was not the result of decreased numbers of mitochondria.

The effects of hypoxia on the rate of transcription of COX 1 and 12S rRNA were measured in both *E. scudderiana* and *E. solidaginis* (Figure 4.6). In both species the mRNA levels of the two genes were reduced under hypoxia with levels lowered to 35-

45% of control values after 24 hours of hypoxia. The ratio of mitochondrial DNA to nuclear DNA was also measured in *E. solidaginis* during hypoxia to see if the reduced transcriptional status was due to reduced mitochondrial DNA. As can be seen in Figure 4.7, the ratio of mitochondrial to nuclear DNA was unaffected in *E. solidaginis* by 24 hours of hypoxia exposure.

Figure 4.1. Schematic diagram of the mitochondria genome from *Drosophila melanogaster*. Picture taken from Lewis *et al.*, 1995.

Figure 4.2. (A) Wavelength scan showing the absorbances of the fully reduced and fully oxidized forms of the cytochrome c oxidase substrate, heart type III cytochrome c. The peak of maximum absorbance for the fully reduced form of the substrate was at 550nm and this wavelength was used in subsequent studies to monitor the enzymatic reaction.

(B) Typical absorption (A_{550}) profile of cytochrome c in the presence of the oxidizing enzyme, COX. The spectrophotometer was zeroed with 25 mM Na_2HPO_4 pH 7.0, 0.2 % DOC.

A.

B.

Figure 4.3. Measured activities of cytochrome c oxidase in clarified supernatants from *E. scudderiana*. (A) Activity of COX after low temperature exposure to 4°C, -4°C or -20°C and after 24 h hypoxia exposure at 15°C, compared with control larvae held at 15°C. Each low temperature exposure was for one week followed by a shift to the next lower temperature (B) COX activities in larvae sampled at different times over the winter. Data are means $\pm$ S.E.M. (n=3). *-Significantly different from control values (+15°C values in panel A or September values in panel B) as determined by the Student's t-test, $P < 0.05$.

A.

B.

Figure 4.4. Measured activities of cytochrome c oxidase in clarified supernatants from *E. solidaginis*. (A) Effects on COX activity of acute temperature change (24 h at 3°C, followed by 24 h at -16°C, followed by 24 h thawed at 3°C) or hypoxia exposure (24 h at 15°C under nitrogen gas) and aerobic recovery (at 15°C), compared with control larvae at 15°C. (B) Changes in COX activity throughout the winter. Data are means $\pm$ S.E.M. (n=3). *-Significantly different from control values (+15°C for panel A and September for panel B) as determined by the Student's t-test, $P < 0.05$.

A.

Figure 4.5. (A) COX 1, 12S rRNA and α -tubulin mRNA transcript levels in *E. scudderiana* over the winter months as determined by RT-PCR. PCR reactions were conducted with four different dilutions of template. Bands densities for COX1 or 12S rRNA were normalized against the corresponding tubulin bands for the same sample and histograms show mean values ($\pm$ S.E.M.) for $n = 3$ independent trials. (B) Genomic DNA was isolated and amplified by PCR and the levels of DNA for the mitochondrial gene (COX 1) and the nuclear gene (α -tubulin) were quantified over the winter months. The 10^{-3} dilution was used to quantify relative band intensities. Histograms show the ratio COX1: α -tubulin, means $\pm$ S.E.M. ($n = 3$).

A.

B.

Figure 4.6. Histograms showing expression levels of two mitochondrially-expressed genes, COX and 12S, in *E. scudderiana* (A) and *E. solidaginis* (B) over a time course of hypoxia exposure at 15°C. The cDNA levels of each gene were normalized against the housekeeping gene, α -tubulin. Data are means $\pm$ S.E.M. (n = 3). * - Significantly different from the control value as determined by the Student's t-test, $P < 0.05$.

A.

B.

Figure 4.7. Relative amounts of COX mitochondrial DNA versus α -tubulin nuclear DNA in *E. solidaginis* larvae given 1, 4 or 24 h of hypoxia exposure. Ethidium bromide stained gels show amplified PCR products from three different dilutions of larval DNA. Histograms show the ratio COX: α -tubulin PCR products, means $\pm$ S.E.M. (n = 3). In this example, the 10⁻³ dilution was used to quantify relative intensities.

DISCUSSION

Both *E. scudderiana* and *E. solidaginis* enter diapause, a state of reduced metabolic demands and output, in the autumn and do not exit until late winter. Inhibition of mitochondrial oxygen consumption and ATP output is clearly one of the defining elements of a hypometabolic state such as diapause but whether this is done by biochemical inhibition of the mitochondrial enzymes/pathways, by reducing the amounts of various enzymes/proteins present in each organelle, or by reducing the overall numbers of mitochondria in cells is largely unknown. In a situation where diapause extends over many months, the last option, degradation of the organelles, may be useful. Indeed, an extraordinary example of such a strategy has been reported in the high Arctic caterpillar, *G. groenlandica*; the number of mitochondria in the brain and fat body of this species was reported to be reduced by 10,000-fold in cold-adapted larvae (Kukal *et al.*, 1989). More recent data on *G. groenlandica* also suggests that mitochondrial degradation occurs in this species although not to the same extent. Levin *et al.* (2003) used a 16S rRNA probe with dot blots to measure the relative amount of mitochondrial DNA in *G. groenlandica* under different conditions. Compared with summer active animals, caterpillars collected from hibernacula or after short or long freezing exposures showed about half the amount of mitochondrial DNA.

The same method was used to analyze mitochondrial DNA content in *E. solidaginis* in summer, autumn and winter and the authors found that frozen, winter-collected larvae had about half of the mitochondrial DNA content as compared with summer active larvae held at 15°C (Levin *et al.*, 2003). This agrees quite well with the data for seasonal changes in COX activity in *E. solidaginis* seen in Figure 4.4B; the

measurable activity of this mitochondrial enzyme was reduced over the winter months to 30-50% of the value seen in September-collected larvae. Similarly, Joannis and Storey (1994) reported that the maximal activities of three other mitochondrial (but entirely nuclear-encoded) enzymes (citrate synthase, glutamate dehydrogenase, NAD-isocitrate dehydrogenase) were also reduced by ~50% in *E. solidaginis* over the midwinter months as were the activities some mitochondrial enzymes involved in fatty acid synthesis (malic enzyme) and fatty acid oxidation (carnitine palmitoyl transferase, 3-hydroxyacyl-CoA dehydrogenase and thiolase)(Joannis and Storey, 1996). Furthermore, Levin *et al.* (2003) showed that metabolic rate of the larvae over the autumn/winter was reduced to 35-40% of the September value (when both were measured at 15°C). Hence, the above evidence suggests that mitochondrial capacity is reduced over the winter months in *E. solidaginis* and that the mechanism of this might be a reduction in the number of mitochondria by about one-half. Furthermore, Levin *et al.* (2003) suggest that this situation can be rapidly reversed since the measurable amount of mitochondrial DNA doubled within 5 h when January-collected larvae were brought into the lab and warmed to 10 or 15°C. This situation could serve a freeze tolerant insect well. While the animal is frozen for many weeks during the winter, metabolism is anaerobic and lactate accumulates as an end products (Storey and Storey, 1985). Lipid fuels cannot be used when oxygen supply is interrupted in frozen animals and, furthermore, mitochondria may be vulnerable to structural or metabolic damage arising due to extreme cell volume reduction and oxygen limitation in the frozen state. Therefore, it may be beneficial for *E. solidaginis* larvae to reduce their number of mitochondria over the winter months.

The situation does not appear to be the same for *E. scudderiana*. The data in

Figure 4.5 shows that the amount of mitochondrial DNA is stable in the larvae over the winter months as are the transcript levels of two mitochondrially-encoded genes, COX 1 and 12 S rRNA. However, COX activities decreased in the winter to just 33-38% of the summer values (Figure 4.3) and citrate synthase, glutamate dehydrogenase, NAD-isocitrate dehydrogenase and malic enzyme activities were all reduced by about 50% (Joanisse and Storey, 1994, 1996). Unlike the situation in the freeze tolerant larvae, however, activities of mitochondrial enzymes involved in fatty acid oxidation (3-hydroxyacyl-CoA dehydrogenase and thiolase) rose two to four-fold over the winter in *E. scudderiana* (Joanisse and Storey, 1996) which suggests that aerobic lipid oxidation supports metabolic needs in the supercooled larvae even at low subzero temperatures. Indeed, lactate does not accumulate in *E. scudderiana* over the winter months (Rickards *et al.*, 1987). In summary, then, the data for *E. scudderiana* are consistent with reorganized metabolic priorities within mitochondria during the winter but not with a net loss of mitochondrial DNA or of transcripts of mitochondrially-encoded genes and, therefore, not with an overall reduction in mitochondrial numbers in the larvae. The winter suppression of COX activity (Figure 4.3) (and activities of other mitochondrial enzymes) would probably arise instead from enzyme regulation, either inhibitory controls on enzyme function or reduced amounts of enzyme protein in mitochondria during the winter.

The activity of β -hydroxybutyrate dehydrogenase increased over the winter months in both species indicating a greater potential for ketone body metabolism in both cold-hardy species (Joanisse and Storey, 1994). This data for *E. solidaginis* could oppose the theory of decreased mitochondrial numbers over the winter in the freeze tolerant

species but can also be interpreted as showing that, although mitochondrial numbers are reduced overall, some readjustments of the metabolic potential of remaining mitochondria can still be made to adjust mitochondrial enzymatic make-up for the particular metabolic demands placed on the organelles over the winter.

The data also offer some insights into the regulation of COX expression in the two insect species. Cytochrome c oxidase is located on the inner membrane of the mitochondrion and is the terminal electron acceptor in the respiratory chain. It is responsible for the reduction of dioxygen to water and is one of the three sites of proton pumping across the inner membrane that creates the electrochemical gradient that drives oxidative phosphorylation. Three of the thirteen subunits of COX are encoded on the mitochondrial genome (COX 1, 2 and 3) (Figure 4.1) and other studies in our lab have found that COX 1 expression is up-regulated in response to entry into hibernation in ground squirrels and anoxia or freezing exposures in turtles (Cai and Storey, 1996; Hittel and Storey, 2002). However, the present study shows that hypoxia exposure significantly reduced COX 1 transcript levels in both insect species (Figure 4.6). This is the opposite response to low oxygen as compared with the results from vertebrate studies but, nonetheless, confirms that COX 1 gene expression is sensitive to changing oxygen levels in both vertebrates and invertebrates. The levels of 12S transcripts were also reduced in both species under hypoxia, generally paralleling the changes in COX 1. This suggests that transcription of both the heavy and the light chains of mitochondrial DNA is reduced in both insects under hypoxia (although net transcript levels can also be altered by changing the rate of their degradation). A general reduction in the rates of nuclear gene transcription is a well-known response during entry into hypometabolic states by stress-

tolerant animals such as hibernating mammals and anoxia tolerant turtles and snails and is an energy-saving mechanism (Storey and Storey, 2004). A similar suppression of mitochondrial gene transcription would also contribute to ATP conservation under hypoxia. However, although mitochondrial gene transcript levels were reduced under hypoxia (probably due to a reduced rate of transcription), mitochondrial DNA content did not change over 24 h of hypoxia exposure in *E. solidaginis* (Figure 4.7). This suggests that there is no mitochondrial degradation under hypoxia. Reduced COX 1 transcript levels under hypoxia in *E. scudderiana* correlated with the reduced maximal activity of COX seen after 24 h of hypoxia exposure (Figure 4.3). Although COX 1 is only one subunit of the protein, these results suggest that one of the mechanisms involved in the suppression of COX activity in response to hypoxia may be reduced synthesis of COX subunits.

Chapter 5
Role of reversible phosphorylation and signaling
cascades during stress

INTRODUCTION

In the majority of cases of natural hypometabolism, metabolic rate is decreased to between 5 and 40% of the normal resting metabolic rate (Guppy and Withers, 1999; Storey and Storey, 2004). However, even though there is a major change in the metabolic state, there is usually little or no change in the internal environment of the cells or tissues so that the animal can return rapidly to the normal metabolic rate when favourable conditions return. Early research on metabolic rate depression was focussed primarily in two areas: (1) quantifying physiological parameters of metabolic rate depression, and (2) characterizing the patterns of fuel use, substrate utilization, and end product accumulation. Recent work has been more directed at the biochemical processes that activate and repress the appropriate enzymatic pathways to obtain the necessary coordinated responses needed to re-establish homeostasis at much reduced net rate of ATP turnover. Many different model systems have been used to evaluate the mechanisms of metabolic suppression in situations including estivation, hibernation, diapause, and anoxia/ischemia tolerance (Storey and Storey, 1990; Guppy *et al.*, 1994; Hand and Hardewig, 1996; Hochachka *et al.*, 1996; Denlinger 2001; Hochachka and Lutz, 2001). Even in very distantly related animals, common mechanisms of biochemical regulation are being uncovered.

It is now known that the priorities for energy expenditure are re-sculpted during metabolic suppression in an effort to conserve limited fuel reserves. As a result, some functions are downregulated or turned off in order to maintain the activity of more crucial processes (Hochachka *et al.*, 1996). However, the capacity to upregulate these pathways is not lost, but rather reversible control mechanisms are put in place to allow rapid

fluctuations between high and low output of a specific pathway or process at minimal energy expenditure. The reversible control mechanism that has emerged as the dominant method for regulating transitions to/from the hypometabolic state is reversible protein phosphorylation. The actions of protein kinases and protein phosphatases are able to markedly alter the activity of their protein/enzyme substrates in response to upstream stimuli in a quick and efficient manner. Phosphorylation of proteins is also an important method for increasing the affinity of two molecules in a signalling cascade. The importance of reversible phosphorylation in the control of the activities of ion pumps in *E. scudderiana* and *E. solidaginis* has already been demonstrated in Chapter 2.

A group of kinases that has gained a lot of attention lately is the mitogen-activated protein kinases (MAPK) superfamily (for review see Cowan and Storey, 2003). These kinases are ubiquitous in eukaryotic cells and are involved in regulating cell growth, development, and response to cellular stress. There are three branches of the MAPK superfamily: (1) the extracellular signal-related protein kinases (ERKs), (2) p38 family, and (3) the c-Jun N-terminal kinases or the stress-activated protein kinases (JNKs/SAPKs). Each branch of the MAPK tree operates in the same fashion. In each case, there are three steps involved. MAPKs, which are themselves serine/threonine kinases, are inactive in their dephosphorylated form and are activated by a conformation change when they are phosphorylated (Hoeftlich and Woodgett, 2001). Each MAPK is a substrate for an upstream MAPK kinase (MAPKK), which is itself a substrate for a MAPKK kinase (MAPKKK). The MAPKKK acts in response to other kinases and phosphatases or from signals received from stimulus-activated receptors on the cell surface or through interactions with GTP-binding proteins.

The most prevalent and commonly studied of the ERKs are ERK1 and ERK2. They respond primarily to growth factors and mitogens and initiate transcriptional responses in the nucleus. ERK1 and ERK2 are activated via phosphorylation by MAPK/ERK kinases 1 and 2 (MEK1 and MEK2), respectively. MEK1 and MEK 2 phosphorylate their ERK substrates at a Thr-Glu-Tyr motif (Cobb and Goldsmith, 1995). The MAPKKK of this pathway is c-Raf, which is controlled by growth factor receptors and tyrosine kinases that activate through the adaptor Ras (Moodie and Wolfman, 1994). Once activated, the ERKs translocate into the nucleus and phosphorylate various substrates depending on the initial stimulus. Some known substrates of the ERKs include: p90 RSK S6 (regulation of translation) (Frodin and Gameltoft, 1999), MAPK-activated protein kinase-1, phospholipase A₂, MSK, and several transcription factors such as Elk-1, Ets 1, Sap 1a, m-Myc, STATs, EGF, and estrogen receptors.

The p38 pathway is known to be activated by a number of environmental stresses such as heat, osmotic stress, ionizing radiation, ischemia, cell death and inflammatory cytokines (New and Han, 1998; Ono and Han, 2000). There are at least five isoforms of p38 in mammals (α , β , γ , δ , and p38-2) but the number of insect isoforms is not known. The p38 α protein is the most common and is the isoform that was studied here. The α and β isoforms are ubiquitously expressed whereas γ and δ are tissue-specific (Jiang *et al.*, 1996; Lechner *et al.*, 1996; Jiang *et al.*, 1992). The isoforms also differ in their substrate specificity. All p38 kinases have the Thr-Gly-Tyr (TGY) dual phosphorylation motif that is phosphorylated by the upstream MAPKK (Ono and Han, 2000). The MAPKKs acting on p38 include MKKs 3 and 6, which activate the different isoforms with different efficacy (Chan-Hui and Weaver, 1998). The MKKKs include MEKK,

MLK, and ASK-1. In turn, GTPases such as the Racs, Rhos, and Cdc42s stimulate MKKK activation. In the nucleus, the p38s activate several transcription factors including STAT1, Max/Myc complexes, ATF-2, MEF-2A/C, Elk-1, CREB, and CHOP.

There are three types of JNKs (JNK1, JNK2, and JNK3). These kinases also respond to environmental stresses such as heat shock, osmotic stress, pro-inflammatory cytokines, ischemia, and ionizing radiation (Pombo *et al.*, 1994; Hoeflich and Woodgett, 2001; Irving and Bamford, 2002). The JNKs are phosphorylated by JNKK1 (MEK4) and JNKK2 (MEK7) (Tournier *et al.*, 1999). Once again, the MAPKKKs of the lineage (MEKK 1-4, ASK, and MLK) are activated by GTP-binding proteins belonging to the Rho family (Hirai *et al.*, 1996; Teramoto *et al.*, 1996). Activated JNKs translocate into the nucleus as a dimer, where they are known to activate several transcription factors including AP-1, ATF-2, Elk-1, Myc, Smad3, p53, NFAT-4, DPC4, and MADD (cell death protein) (Atfi *et al.*, 1997; Zhang *et al.*, 1998; Hoeflich and Woodgett, 2001). In contrast to the ERKs and p38s which have substrates both inside and outside the nucleus, the JNKs target only transcription factors. Activation of the JNK pathway in non-tolerant cells (ie. lethal stress) usually results in apoptosis although a few exceptions where JNKs promote survival have been documented (Dougherty *et al.*, 2002).

Members of all three branches of the MAPK superfamily have been identified in *Drosophila melanogaster*; however, the complex roles of these pathways are still poorly understood in insect systems. Analysis of cold or hypoxia responsive gene expression in *E. scudderiana* and *E. solidaginis* using cDNA microarrays (Chapter 3) identified the putative upregulation of several pro-inflammatory genes downstream of the NF- κ B family of transcription factors. Genes that are known to be activated by NF- κ B include:

cytokines such as TNF α , IL-1, IL-6, GM-CSF, chemokines such as IL-8 and MIP-1 α , RANTES, and adhesion molecules such as VCAM-1, ICAM-1 (Ghosh et al., 1998; Li and Verms, 2002; Silverman and Maniatis, 2001; Ghosh and Karin, 2002; Yamamoto and Gaynor, 2001). In mammal, dysregulation of the NF- κ B pathway results in inflammatory states and autoimmune diseases. Similar aberrant conditions have been observed in insects (Silverman and Maniatis, 2001). Other important genes activated by the NF- κ B pathway include the cellular-inhibitor of apoptosis protein (c-IAP 1), c-IAP 2, TNF receptor-associated factor (TRAF) 1, TRAF 2, Bcl-2, Fas, c-myc, and cyclin D1. Therefore, in addition to inflammation activation, the NF- κ B pathway is also involved with cell proliferation and survival (Karin and Lin, 2002). Inhibition of apoptosis under environmental stresses that would otherwise be damaging to non-tolerant species (e.g. cold, hypoxia) might be one of the hallmarks of stress tolerance in organisms that are adapted to extreme environments.

The NF- κ Bs are a family of ubiquitously expressed proteins that typically exists as heterodimers of two proteins, p65 (RelA) and p50. There are several different isoforms but all contain a C-terminal Rel-homology domain (RHD) which is necessary for DNA binding, dimerization, and nuclear translocation. Dimerization is necessary for activity. In unactivated cells, the NF- κ B proteins are sequestered in the cytoplasm by an inhibitor protein called I κ B α . Phosphorylation of I κ B α results in its polyubiquitination and subsequent proteasomal degradation, allowing the released p65-p50 complex to translocate into the nucleus and bind to the promoter region of genes containing the κ B sites recognized by NF- κ B.

Because MAPK and NF- κ B-induced mechanisms of cell survival during stress are highly conserved across a broad phylogenetic range, I hypothesized that they would play key roles in winter survival of cold-hardy insects. Many components of these pathways have been identified in insect systems; however, the role that they play in insect biochemistry is far from understood. The goal of the work presented here was to identify and characterize some of the players in the MAPK and NF- κ B families and their roles in insect cold-hardiness. The work presented in this chapter looks at the p38 and ERK branches of the MAPK superfamily as well as some of the components of the NF- κ B-related pathways.

MATERIALS AND METHODS

2-Dimensional gel analysis.

Samples of frozen larvae were homogenized 1:10 w:v in a buffer that contained 250 mM sucrose, 25 mM HEPES, 25 mM KCl, 1 mM EDTA, 1 mM EGTA, and 0.1% w:v DOC with a cocktail of protease inhibitors added just prior to homogenization. The homogenate was incubated on ice for 20 min, centrifuged at 5000 x g for 5 min, and then the supernatant was removed for immediate use or stored at -20°C . The protein concentration in each sample was quantified using the Coomassie blue dye binding method with the Bio-Rad reagent. Proteins in each sample were separated using 2-D gel electrophoresis with the Bio-Rad Mini-Protean III system. The first dimension IEF gel was run in 0.75 x 100 mm capillary tubes containing 4% w:v acrylamide, 9.2 M urea, 5% Triton X-100, 1.6% v/v broad-range ampholytes, 0.4% narrow range ampholytes, 0.01% APS, and 0.1% TEMED. The pH gradient was established with broad range ampholytes

(pH range 3.0-10.0) and selected narrow range ampholytes (3.5-5.0, 4-6, 5-7, 6-8, or 7-9) were used in different gels to emphasize/stabilize one particular area of the gradient in order to focus on proteins with a specific range of isoelectric points. Before being loaded into capillary tubes, samples were combined with an equal volume of 2X sample buffer (9.5 M urea, 2.0% v:v Triton X-100, 5.0% v:v β -mercaptoethanol, 1.6% v:v narrow range ampholytes, the same as the ampholytes used in the IEF gel being run), 0.4% v:v ampholytes 3.5-10.0, and 0.0025% w/v bromophenol blue. Each capillary tube was loaded with 10 μ g of total protein. Isoelectric focusing was performed for 8 h at 500 v. The upper chamber solution was 100 mM NaOH and the lower chamber contained 10 mM H_3PO_4 .

After isoelectric focusing, the tube gels were excised from the capillary tubes and equilibrated in another buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% w/v SDS, 5% v/v β -mercaptoethanol, 10% w/v glycerol, and 0.05% w/v bromophenol blue). A tube gel was then placed horizontally on top of an SDS-polyacrylamide gel (10-15% w:v acrylamide [depending on the size of the proteins under study], 0.4 M Tris-HCl, pH 8.8, 0.1% w/v SDS, 0.1% w/v APS, 0.04% TEMED) and electrophoresed for 2 h at 100 v. Running buffer was diluted from a 5X stock solution (15.1g Tris-base, 94 g glycine, and 0.5% w/v SDS per liter of ddH₂O). Proteins were visualized by staining the gels 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, 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.

Western blotting

Western blots were prepared as described in Chapter 2. All antibodies were purchased from Cell Signaling (Beverly, MA).

Superarray Mouse NFkB Signaling Pathway Gene Array

Animals: Galls containing larvae of *E. scudderiana* or *E. solidaginis* (larval mass 50-70 mg) were collected in September from the stems of goldenrod plants in fields in the Ottawa area. In the lab insects were removed from their galls, placed in covered petri dishes, and acclimated to 4°C in large incubators. After 14 days, control insects were removed, immediately flash frozen in liquid nitrogen and then stored at -80°C. For hypoxia exposure, petri dishes were sealed in small chambers containing two ports at the top of the chamber, one for the introduction of 100% nitrogen gas and one to vent the chamber. Insects were sampled after hypoxia exposure times of 1, 4, or 24 hours at 4°C and flash frozen.

GEArray Q Series kit, Mouse NF κ B Signaling Pathway: Arrays were purchased from Superarray (Frederick, MD, USA) and contained 96 cDNA fragments of genes related to the NF κ B signal transduction pathway spotted on a 3.8 x 4.8 cm nylon membrane. The identity and corresponding position of all the cDNA fragments can be found at: <http://www.superarray.com/>. All solutions and materials used during RNA handling were treated overnight with 1% v:v diethylpyrocarbonate (DEPC) and later autoclaved. Total RNA was isolated from insects using Trizol reagent (BRL) as described by the manufacturer. RNA concentrations were measured by absorption spectrometry ($\lambda = 260$ nm). Only samples with an $A_{260/280}$ in the range of 1.7-1.9 were used. RNA integrity was further analyzed by electrophoresis of samples on a 1% denaturing formaldehyde gel, staining with ethidium bromide and visual inspection of the bands.

Probe synthesis: Radioactive probe was made from insects from each of the control and experimental (hypoxic) conditions and each was hybridized to a different membrane. Software was used to normalize the signal data between different membranes. Each probe was constructed using 10 μ g of total RNA as template. This was combined with 3 μ l GEAprimer mix (Buffer A, SuperArray) and enough DEPC-treated distilled water to bring the total volume up to 10 μ l. The mixture was heated to 70°C for 3 minutes and then cooled to 42°C for 2 minutes prior to the addition of the labelling mix. One hundred μ l of labelling buffer (Buffer B) was prepared by mixing 50 μ l RT buffer (supplied with kit), 1 μ l 1M dithiothreitol, and 50 μ l dNTP mix (5 mM dATP, 5 mM dGTP, 5 mM dTTP, and 50 μ M dCTP, supplied with the kit). The labelling

master mix was prepared by mixing 4 μ l buffer B, 3 μ l [α - 32 P] dCTP (10 μ Ci/ μ l, Amersham Pharmacia Biotech), 1 μ l RNasin RNase inhibitor (Promega), 1 μ l Superscript II reverse transcriptase (Invitrogen Life Technologies), and 1 μ l RNase-free water. The 10 μ l master mix was pre-warmed to 42°C before being transferred to the total RNA template. Reverse transcription proceeded for 60 minutes at 42°C. The reaction was stopped by the addition of 4 μ l of 0.5M EDTA and 2 μ l of 4M NaOH. The probe was denatured and the RNA degraded by the addition of 10 μ l Denaturation solution (provided with kit) and incubation at 68°C for 20 minutes. The probe was then neutralized by the addition of 20 μ l 2X Neutralization buffer (provided with kit) and incubated at 68°C for another 10 minutes.

Hybridization and Washing: The nylon membranes were soaked in water and then pre-hybridized in 2 ml GEAhyb hybridization solution (provided with kit) at 60°C for 120 minutes. The denatured probe was pre-mixed with 750 μ l GEAhyb solution and added to the hybridization bottle. The probe was allowed to hybridize with the membranes overnight at 42°C while rotating at a speed of 15 revolutions per minute. The membranes were transferred to separate rectangular containers (~ 4.5" x 3.5") and washed on a rocking platform for 10 minutes at room temperature with 2X SSC (diluted from 20 x stock solution, 3 M NaCl, 0.3 M Na₃citrate.2 H₂O) containing 0.5% w:v SDS. This was followed by 3 x 5 minute washes with 1X SSC, 0.5% w:v SDS until the radioactive signal on the blots was reduced to approximately 2000 counts per minute.

Signal Detection and Data Analysis: The washed membranes were placed on aluminum foil, covered with plastic wrap and placed in an autoradiography cassette. The radioactive signal was captured using phospho-imaging screens (Bio-Rad) which were exposed to the radioactive blots at room temperature for 4, 12 or 24 hours. The phospho-screens were scanned using a Bio-Rad phospho-imager equipped with Quantity One 4.1 (Bio-Rad) imaging software. The images from the scanner were exported as tiff files and opened in ImageQuant 3.22 (Molecular Dynamics) for quantification. ImageQuant was used to generate a large grid with 96 sub-grids that measured the pixel density of each cDNA tetra-spot corresponding to the 96 genes on the array. Finally, the data files generated by ImageQuant were imported into GEMArray Analyzer 1.0 (SuperArray). This software was used for normalization and background elimination procedures and matched gene expression data for each spot with the proper gene names.

RESULTS

Two-dimensional gel electrophoresis was used to assess changes in the soluble protein patterns of *E. scudderiana* and *E. solidaginis* larvae. Both season expression of proteins and response to low temperature acclimation was tested with the intention of identifying proteins that were upregulated in association with the acquisition of cold hardiness. Effects of low temperature were evaluated by comparing control larvae at 15°C with larvae sampled after 1 week at 4°C and then a further 1 week at -20°C. Examples of the 2D gels resulting from these experiments are shown in Figures 5.1 (*E. scudderiana*) and 5.2 (*E. solidaginis*). The effects of 24 hours of hypoxia were also analyzed (data not shown). It is clear from these figures that the protein profiles of these

two cold-hardy insects do not change very much in response to changing environmental conditions. Several different conditions were analysed, including using different detergents (anionic, cationic, neutral) and varying the pH range of the IEF gel (pH 4-6, 5-8, 6-9) in an attempt to identify differentially regulated proteins. Many gels were run and analyzed but no instances were found in either species where one or more proteins were identified that were consistently upregulated (or induced) either in response to experimental low temperature exposure or on a seasonal basis. These results indicate that the protein profiles of the relatively abundant proteins of these insects are largely unchanged during cold hardening and suggests that metabolic adjustments for winter cold survival may come mainly from modification of regulatory systems.

The results from the cDNA microarray analysis of gene expression profiles under hypoxia stress in both *E. scudderiana* and *E. solidaginis* (reported in Chapter 2) indicated that components of the NF- κ B signalling pathway are involved in the response to hypoxia in both insects. For this reason, the NF- κ B signalling pathway was studied in more detail. Once again, cDNA arrays were employed to gain information on this pathway. GEMArray Q Series Mouse NF κ B Signaling Pathway Gene Arrays (SuperArray BioScience Corp.) containing 96 genes related to NF κ B mediated signal transduction were probed with cDNA generated from control and hypoxic insects. The level of hybridization was ~ 85% for each species. Two representative arrays are shown in Figure 5.3 comparing of the results for control *E. scudderiana* larvae with larvae exposed to an N₂ gas atmosphere for 1 h. Three of the genes that consistently showed strong up-regulation under hypoxia are circled on the figure. Figure 5.4 summarizes the results for the two species showing the relative intensity of hybridization for 1 h hypoxic larvae

versus controls. Relative transcript levels of the majority of genes were unaffected by hypoxia exposure but a few genes were strongly upregulated in each species. Nine genes in *E. solidaginis* and twelve in *E. scudderiana* showed fold upregulation values ranging from 2.5 to 25 (Table 5.1). Five of these putatively upregulated genes were common to both species and may represent proteins involved in conserved mechanisms of insect hypoxia tolerance. The five genes were interleukin 1 receptor type II, MAP/ERK kinase kinase 1, NF- κ B subunit p100, BALB/cJ small inducible cytokine A2 precursor, and toll-like receptor 8.

Other experiments were conducted to further characterize the NF- κ B pathway. Western blotting was used to assess hypoxia effects on the level of the phosphorylated form of I κ B α , the inhibitor of NF- κ B nuclear translocation. Phosphorylation of I κ B α abolishes its ability to bind to the p65-p50 complex and therefore elevated levels of phospho-I κ B α correlate with an activation of NF- κ B. Figure 5.5 shows the effects of low temperature and hypoxia stresses on the relative amount of phospho-I κ B α in *E. scudderiana*. The data showed no statistically significant decrease in the amount of phospho-I κ B α protein in response to low temperature exposures to 4, -4 or -20°C. However, phospho-I κ B α protein was significantly reduced by ~20% after 4 hours of hypoxia exposure which implies that the amount of active NF- κ B would be reduced in hypoxic insects. Several attempts were made to similarly quantify phospho-I κ B α levels in *E. solidaginis*; however, no antibodies would cross-react in this species.

The p38 signaling pathway has been linked with the NF- κ B pathway and stress survival in many circumstances. The p38 MAPK is essentially inactive in its dephosphorylated form and activated >1000-fold by phosphorylation. Therefore,

antibodies against phospho-p38 were used in Western blotting to quantify the relative levels of the active MAPK in both species and assess the importance of p38 signaling in insect response to low temperature and hypoxia. Figure 5.6A shows that low temperature stimulated an increase in the amount of phospho-p38 in *E. scudderiana* larvae of about 3-fold after 1 week of cold exposure at 4°C. Levels remained high after a further 1 week at -4°C (3.5-fold over control) and were 3.75-fold higher than control values after 1 week at -20°C. However, oxygen limitation had the opposite effect; 24 hours of hypoxia exposure led to a 65% decrease in the amount of phospho-p38 (Figure 5.6B) and phospho-p38 remained suppressed even after 24 hours of aerobic recovery. The responses of p38 to stress in *E. solidaginis* are shown in Figure 5.7. As in *E. scudderiana*, cold exposure of the freeze tolerant insects increased the amount of the p38 phosphoprotein. Levels were 1.93 ± 0.56 , 3.14 ± 1.26 , and 3.97 ± 1.04 fold higher than control values in larvae given cold exposure at +3°C (24 h), frozen at -16°C (24 h), or thawed at +3°C (24 h), respectively. However, in contrast to *E. scudderiana*, hypoxia stress stimulated a rapid and large increase in the amount of phospho-p38 in the freeze tolerant larvae. Levels were increased by 12.0 ± 4.07 , 3.66 ± 0.96 , and 7.21 ± 3.17 -fold over controls after 1, 4 or 24 h of N₂ gas exposure (Figure 5.7B).

Figure 5.8 shows changes in p38 over the winter months for both species. Two antibodies were used for analyses with *E. solidaginis*. Antibodies raised against total p38 α protein showed no significant change in the total amount of this protein over the winter months. However, the relative amount of the active phosphoprotein rose substantially during the midwinter months of December, January and February to 4-5 fold higher than the September values. A similar pattern was seen for phospho-p38 in *E.*

scudderiana with midwinter values being about 2.6-fold higher than in October. In both species, phospho-p38 levels were reduced again in April.

The patterns of ERK1 and ERK responses over the winter months were also examined in both insect species using antibodies raised against the phosphorylated (active) forms of the kinases (Figure 5.9). The activation of ERK1 (p44) via phosphorylation was a strategy employed by both species. In *E. scudderiana*, the level of p44 phosphorylation was increased slightly in October and rose significantly by about 2.5-fold in December. However, the level of phosphorylated ERK1 was near control values in all other months sampled. The amount of phospho-ERK2 in this species was decreased to about 50% of control levels in early fall (October) and remained low for the rest of the timecourse (Figure 5.9A). In *E. solidaginis*, the amount of phosphorylated ERK1 increased by 2.75-fold in December and remained elevated through until February (~2-fold) before returning to control levels in March and April. The level of phosphorylation of ERK2 did not change throughout the winter in this species.

Figure 5.1. Representative two-dimensional gels displaying the protein content of *E. scudderiana* during cold exposure (15°C for 2wks, 4°C for 1wk, and -20°C for 1wk.) and at different times during the winter (September, February, and April). The proteins were visualized with silver stain. The IEF gradient in these gels was from pH 5-8 (left to right) and the second dimension SDS gels (vertical direction; high molecular weight at the top) contained 12% acrylamide.

E. scudderiana, +15°C

E. scudderiana, September

E. scudderiana, +4°C

E. scudderiana, February

E. scudderiana, -20°C

E. scudderiana, April

Figure 5.2. Representative two-dimensional gels displaying the protein content of *E. solidaginis* during cold exposure (15°C for 2wks, 4°C for 1wk, -20°C for 1wk.) and throughout the winter (September, February and April). The proteins were visualized with silver stain. The IEF gradient was from pH 5-8 and the second dimension SDS gels (vertical direction; high molecular weight at the top) contained 12% acrylamide.

E. solidaginis, +15°C

E. solidaginis, September

E. solidaginis, +4°C

E. solidaginis, February

E. solidaginis, -20°C

E. solidaginis, April

Figure 5.3. Representative Superarray blots used to compare the relative expression levels of genes associated with the NF-kB signaling pathway in aerobic control (+4°C) *E. scudderiana* larvae versus larvae exposed to hypoxia for 1 h at 4°C. Blots were probed with ³²P-labelled cDNA. Three upregulated cDNAs are circled for reference. The same procedure was also completed for *E. solidaginis*.

E. scudderiana

Control

1 h hypoxic

Figure 5.4. Bar graph representing the relative expression of 96 genes in the NF- κ B pathway in *E. solidaginis* (top) and *E. scudderiana* as determined from the Superarray mouse NF- κ B cDNA arrays comparing control versus 1 h anoxia exposure. Blots were corrected for background hybridization and normalized to the median pixel density of each blot as well as to three standard genes; β -actin, α -tubulin, and GAPDH.

Figure 5.5. Western blots showing the effects of low temperature or hypoxia exposures on phospho-I κ B α protein content in *E. scudderiana*. (A) Representative blot of phospho-I κ B α levels under four experimental conditions and histogram showing densitometric analysis of phospho-I κ B α levels for control (+15°C), cold (+4°C, 1 wk.), high subzero (-4°C, 1wk), low subzero (-20°C, 1 wk) larvae. (B) Representative blot and histogram showing densitometric analysis of phospho-I κ B α protein levels in *E. scudderiana* over a time course of hypoxia exposure, all at 4°C. Equal amounts of protein (10 μ g) were loaded in each well and blots were probed using polyclonal antibodies raised in rabbits against phospho-I κ B α and recognizing a single band at ~40 kDa. Data in histograms are means $\pm$ S.E.M. (n=3). *-Significantly different from the corresponding control value using the Student's t-test, P<0.05.

A.

B.

Figure 5.6. Western blots showing the content of phosphorylated p38 protein in *E. scudderiana*. (A) Representative blot of phospho-p38 levels under four experimental conditions and histogram showing densitometric analysis of phospho-p38 levels in control (+15°C) larvae and in larvae exposed to cold (+4°C, 1 week), high subzero (-4°C, 1 week), and low subzero (-20°C, 1 week) temperatures. (B) Representative blot and histogram showing densitometric analysis of the effects of 24 hours of hypoxia at 4°C or 24 hours of aerobic recovery after hypoxia exposure on phospho-p38 protein levels in *E. scudderiana*. Equal amounts of protein (10 µg) were loaded into each well and blots were probed using polyclonal antibodies raised against phospho-p38 α what in rabbits and recognizing a single band at ~40 kDa. Data in histograms are means $\pm$ S.E.M. (n=3). *- Significantly different from the corresponding control value using the Student's t-test, P<0.05.

A.

B.

Figure 5.7. Western blots showing the phospho-p38 protein content of *E. solidaginis* larvae exposed to low temperatures or hypoxia. (A) Representative blot of phospho-p38 levels under four experimental conditions and histogram showing densitometric analysis of phospho-p38 levels in control (+15°C), cold (+3°C, 24 h), frozen (-16°C, 24 h), and thawed (+3°C, 24 h) larvae. (B) Representative blot and histogram showing densitometric analysis of phospho-p38 protein levels in *E. solidaginis* over the hypoxia timecourse. Equal amounts of protein (10 µg) were loaded in each well and blots were probed using polyclonal antibodies raised in rabbits and recognizing a single band at ~40 kDa. Data in histograms are means $\pm$ S.E.M. (n=3). *-Significantly different from the corresponding control value using the Student's t-test, $P < 0.05$.

A.

B.

Figure 5.8. (A) Representative western blots showing both total p38 α levels and phospho-p38 content throughout the winter in *E. solidaginis*. (B) Representative western blot showing phospho-p38 content in *E. scudderiana* throughout the winter. Equal amounts of protein (10 μ g) were loaded in each well and the blots were probed with polyclonal antibodies raised in rabbits and recognizing a single band at $\sim$ 40 kDa. Data in histograms are means $\pm$ S.E.M. (n = 3). *-Significantly different from the corresponding control value using the Student's t-test, P<0.05.

A.

B.

Figure 5.9. Relative protein concentrations of phospho-p42 and phospho-p44 over the winter in (A) *E. scudderiana* and (B) *E. solidaginis*. Data are means $\pm$ S.E.M. (n = 3).

* - Significantly different from the September value as determined by the Student's t-test, $P < 0.05$.

A.

B.

Table 5.1. Genes upregulated in *E. solidaginis* (top) and *E. scudderiana* (bottom) in response to 1 h of hypoxia as determined by Superarray mouse NF- κ B cDNA arrays.

E. solidaginis

Gene position	Genebank ID	Name	Fold-upregulated
14	M31585	Mouse intercellular adhesion molecule 1 (ICAM-1)	25.1
27	NM_010555	*-Mus musculus interleukin 1 receptor, type II (Il1r2)	23.8
28	NM_008363	Homo sapiens interleukin-1 receptor-associated kinase	6.0
41	AF117340	*-MAP/ERK kinase kinase 1	17.3
42	AF143094	Mus musculus NF-kappaB inducing kinase (Nik)	8.7
55	AF155373	*-Mus musculus nuclear factor kappa B subunit p100 (Nfkb2)	5.1
56	U36277	Mus musculus I-kappa B alpha chain	6.0
69	NM_011333	*-Mus musculus strain BALB/cJ small inducible cytokine A2 precursor (Scya2) mRNA	5.7
83	AY035890	*-Mus musculus toll-like receptor 8 (Tlr8)	3.4

E. scudderiana

Gene position	Genebank ID	Name	Fold-upregulated
11	L19953	ELK3, ETS-domain protein (SRF accessory protein 2)	2.6
12	NM_010175	Fas-associating protein with death domain (FADD)	2.7
25	M15131	Mouse Interleukin 1-beta (Il-1 mRNA)	2.5
27	NM_010555	*-Mus musculus interleukin 1 receptor, type II (Il1r2)	15.5
37	M16819	Mouse tumour necrosis factor-beta mRNA	2.5
40	U18310	SAPK/Erk kinase 1 MAP kinase kinase 4	2.6
41	AF117340	*-MAP/ERK kinase kinase 1	5.7
55	AF155373	*-Mus musculus nuclear factor kappa B subunit p100 (Nfkb2)	6.5
69	NM_011333	*-Mus musculus strain BALB/cJ small inducible cytokine A2 precursor (Scya2) mRNA	3.4
83	AY035890	*-Mus musculus toll-like receptor 8 (Tlr8)	3.0
87	NM_009399	Mus musculus tumor necrosis factor receptor superfamily, member 11a (Tnfrsf11a)	3.2
88	AF019048	Tumor necrosis factor (ligand) Superfamily member 11	3.7

DISCUSSION

Two-dimensional gel electrophoresis analysis revealed that neither *E. scudderiana* nor *E. solidaginis* make drastic changes to the levels of their major proteins over the winter months or in response to temperature change or hypoxic stress. Indeed, the results from this chapter (and others) are consistent with the idea that cold-hardening does not require major changes in the protein make-up of either species. Rather, the important protein changes seem to be more subtle, less extensive, and less ATP-expensive. Not only does this avoid a need for major protein restructuring at subzero temperatures but also ensures major restructuring is similarly unnecessary when animals are returned to warmer conditions that are favourable for renewed development.

Many insects have developed physiological characteristics and biochemical mechanisms that allow them to survive extended periods of environmental stress as part of their natural life cycle (Hoback and Stanley, 2001). Some of the molecular mechanisms supporting this capacity are beginning to be elucidated; however, there are many details yet to be uncovered before anoxia/ischemia tolerance in insects (or any other tolerant species) is fully understood. In an effort to better understand the molecular details of hypoxia tolerance in insects, the effects of 1 h hypoxia exposure on both *E. scudderiana* and *E. solidaginis* were evaluated by screening with nylon arrays containing 96 cDNAs representing genes associated with the NF- κ B signaling pathway. Even though the cDNAs corresponded to sequences from the mouse genome, a high level of cross-reactivity was obtained (~85%). This is yet another example of the validity and usefulness of heterologous probing between distantly related organisms. Of the 96 genes analyzed, the relative expression level of the vast majority did not change with low

oxygen exposure. In *E. solidaginis*, 9 genes were putatively upregulated above the 2.5-fold cut-off whereas 12 genes were putatively upregulated in *E. scudderiana* (Table 5.1). Interestingly, 5 genes were common to both species. These genes were: toll-like receptor 8 (Tlr8), Scya2, nuclear factor kappa B subunit p100, MAP/ERK kinase kinase 1, and interleukin 1 receptor type II (Il1r2). The role of each of these in hypoxia tolerance should be further explored since the present information on each of these proteins suggests that they could perform a potentially important function in hypoxia survival (see below).

In mammals, nuclear factor kappa B subunit p100 is the precursor for one of the less common isoforms of NF- κ B proteins, p52. NF-kappa B-inducing kinase (NIK) induces I kappa B kinase alpha (IKK alpha)-mediated phosphorylation of specific serine residues in the C-terminal domain of p100, leading to recruitment of the SCF (beta-TrCP) ubiquitin ligase (Amir *et al.*, 2004). Subsequent proteolysis releases the active p52. This NF- κ B isoform is missing the C-terminal transcriptional activation domain (TAD) that is usually required for transcription activation (Yamamoto and Gaynor, 2004). However, in some tissues there have been rare examples of p52 induced gene transcription (Ishikawa *et al.*, 1998). Homodimers of p50, another form of the protein that is missing the TAD (DNA binding is normally a function of the p65 subunit), can also bind to κ B sequences in promoter regions and repress transcription of target genes. Homodimers of p50 can compete with traditional NF- κ B isoforms for promoter/enhancer regions. Therefore, cytokine activation of NF- κ B can be inhibited by the replacement of traditional p65/p50 heterodimers with p50 homodimers, thus reducing cytokine-mediated gene expression (Zhong *et al.*, 2002). Homodimers of p52 can also bind κ B domains without activating

transcription. Therefore, it is possible that nuclear factor kappa B subunit p100 is strongly upregulated after 1 hour of hypoxia in both *E. scudderiana* (6.5-fold) and *E. solidaginis* (5.1-fold) in an effort to elevate p52 protein levels and thereby silence the transcription of unnecessary genes in an effort to save energy in the ATP-limited state.

MAP/ERK kinase kinase 1 is the second kinase of the three-tier kinase pathway that ultimately leads to the activation of ERK1, also called p44. ERK1 and ERK2 (also called p42) activation has been shown to have a cytoprotective effect during ischemia and hypoxia in many situations (Gabryel *et al.*, 2004; Henriksson *et al.*, 2004; Kis *et al.*, 2004). Also, ERKs have been demonstrated to be important regulators of insect development, subject to the same temporal and spatial regulation effects observed in mammals (Rintelen *et al.*, 2003). Figure 5.9 shows that the protein levels of the phosphorylated (active) forms of ERK1 do in fact increase in both insect species over the winter. In the coldest winter months, *E. solidaginis* is exposed to multiple hypoxic bouts whenever it freezes. It is known that *E. solidaginis* upregulates the production of SIMA, the insect homologue of HIF-1 α over the winter and in response to cold shock (+3°C, 24 h and -16°C, 24 h) (P. Morin *et al.*, unpublished data). It has also been shown that IL-1 β stimulates the activation of HIF-1 α in an ERK1/2-dependent manner (Qian *et al.*, 2004). Therefore, it is possible that phospho-ERK1 levels are higher over the midwinter months in order to increase the transcription of genes required to endure bouts of oxygen deprivation during freezing. ERK1 was also phosphorylated and thus activated throughout the mid-winter months in *E. scudderiana*, a species that probably does not become hypoxic in the supercooled state. This may suggest that there are additional roles for these kinases in overwintering insects, possibly involvement with cold-hardiness or

with developmental processes in these insects. More work is needed to answer these questions.

Toll-like receptors, including toll-like receptor 8 (Tlr8), belong to the same superfamily as interleukin 1. *Drosophila* Toll is a type I transmembrane protein receptor. The cytoplasmic domain is closely related to the interleukin 1 receptor. This superfamily plays a major role in the activation of the innate immune response triggered by components of both Gram-negative and Gram-negative bacteria (Bartfai *et al.*, 2003). Innate immunity is an ancient host defense system present in all multicellular organisms (Fearon and Locksley, 1996; Hoffmann *et al.*, 1999). Toll was initially uncovered in *Drosophila melanogaster* as a component of dorsoventral patterning in early embryos (Anderson and Nüsslein-Volhard, 1984). In addition, Toll plays a role in the anti-fungal response in *D. melanogaster* (Lemaitre *et al.*, 1996). Moreover, Toll has been implicated in the activation of NF- κ B in humans and mice (Medzhitov *et al.*, 1997; Poltorak *et al.*, 1998). Both the Toll and IL-1 signaling pathways activate the NF- κ B transcription factor through a mechanism conserved between insects and mammals (Belvin and Anderson, 1996). Hence, the putative upregulation of Tlr8 in the two cold-hardy insect species may indicate that changes in NF- κ B signalling in the insects are linked with signals transduced by Toll receptors.

The mRNA levels of Scya2, also known as monocyte chemoattractant protein (MCP-1), have been shown to be responsive to hypoxia under many circumstances. In mammalian studies, macrophages accumulate at highest density in necrotic regions, which are associated with low oxygen tensions (Negus *et al.*, 1998). There are examples of both hypoxia-induced as well as hypoxia-downregulated expression of MCP-1 (Bona

et al., 1999; Ivacko *et al.*, 1997; Safronova *et al.*, 2003). The mechanism by which the hypoxic signal is relayed to MCP-1 is still unknown; however, because regulation occurs in the presence of cyclohexamide, new protein synthesis is not required (Safronova *et al.*, 2003). In addition, there appear to be at least two distinct pathways that are involved with the regulation of MCP-1 as MCP-1 expression reacts differently in IL-1 β -stimulated and unstimulated cells. Recent evidence indicates that MCP-1 activation may be downstream of the HIF-1 α transcription factor in mammals so it is logical that the insect homologue of MCP-1 may be downstream of SIMA (Bourke *et al.*, 2004). Putative upregulation of MCP-1 in response to hypoxia in the two cold hardy insects may, therefore, be one of the results of hypoxia-stimulated SIMA activation.

The fifth gene that was upregulated in each species in response to hypoxia stress showed a very intense response. Interleukin 1 receptor, type 2 (IL1r2) was upregulated by ~24-fold in *E. solidaginis* and 15.5-fold in *E. scudderiana*. Interleukin 1 is a potent pro-inflammatory cytokine that helps regulate components of inflammation, metabolism, and immunology (Dinarello, 1986). There are two forms of IL-1, α and β . IL-1 α can induce gene expression through the transcription factors NF- κ B and AP-1. The genes under IL-1 α regulation are usually related to cell survival and proliferation (Wolf *et al.*, 2001). IL-1 β is known to induce local inflammatory responses and has also been linked to deleterious effects during focal cerebral ischemia in mice and rats (Yang *et al.*, 1998; Betz *et al.*, 1995). In several cases, IL-1 β has been shown to induce the expression of MCP-1 (Bona *et al.*, 1999; Ivacko *et al.*, 1997). Also, increased expression of MCP-1 has been linked with the progression of ischemic brain injury (Ghirnikar *et al.*, 1998; Kennedy *et al.*, 1998). The interleukin receptor type 1 (IL-1r1) binds both IL-1 α and IL-

1 β and initiates signal transduction. The interleukin 1 receptor type 2 (IL1r2) is an inhibitor of IL-1 actions. IL-1r2 binds the ligand but does not initiate the signalling cascade. For this reason, it has been dubbed the decoy receptor. In addition to being a decoy, IL-1r2 removes IL-1 from an area of inflammation by internalizing the cytokine and recycling the receptor back to the surface (Bourke *et al.*, 2003). Therefore, it is a negative regulator of the IL-1 signaling pathway.

Interleukin-1 converting enzyme (ICE) is a cysteine protease that cleaves the inactive pro-IL-1 β to generate the active form of IL-1 β (Dinarello, 1996). ICE is also involved with the apoptotic pathway (Yuan *et al.*, 1993). Xu *et al.* (2001) found that neonatal mice with a homozygous deletion of ICE were protected against brain damage under moderate ischemic insult and that the expression of MCP-1 was attenuated in both IL-1 β -stimulated and unstimulated ICE (-/-) tissue. The resistance to brain injury was believed to result in part by the attenuation of the acute inflammation. Therefore, the putative upregulation of IL-1r2 in response to hypoxia in both *E. scudderiana* and *E. solidaginis* may also be providing cytoprotection by blocking the damaging effects of an acute inflammatory response.

Western blots using an antibody raised against phospho-I κ B α , the inhibitor of NF- κ B, indicated that the level of activation of NF- κ B decreased somewhat as an early response to hypoxia in *E. solidaginis* (Figure 5.5). These results are in agreement with those found recently in human fibroblasts, a hypoxia-intolerant system (Safronova *et al.*, 2003). However, because I κ B is ubiquitinated upon phosphorylation, it may be possible that earlier sampling points would reveal increased concentrations of phospho-I κ B α . Further analysis of this system is required to determine the level of NF- κ B activation in

hypoxia. The inferred decreased activity of NF- κ B with acute temperature stress, as determined by a decreased content of phospho-I κ B protein, may reflect an energy-saving measure employed by the insects.

The difference in the activation pattern of p38 in response to hypoxia in *E. solidaginis* and *E. scudderiana* may reflect different strategies utilized by these insects for dealing with oxygen deprivation. *E. solidaginis* displayed a dramatic 12-fold increase in the amount of phospho-p38 after 1 hour of hypoxia exposure and phospho-p38 remained elevated throughout the hypoxic time course. Conversely, phospho-p38 decreased by 65% after 24 hours of hypoxia exposure in *E. scudderiana*. The tissues of *E. solidaginis* routinely experience hypoxia throughout the winter whenever the animals freeze and may have optimized mechanisms for enduring oxygen shortages that could include p38 mediated responses. The responses of *E. scudderiana* may be those of a much less hypoxia tolerant species. Nonetheless Western blots that quantified phospho-p38 responses to temperature change (Figure 5.6A, 5.7A) indicated that activation of the amount of p38 is important in the response to low temperature exposure by both species of cold-hardy insects.

Chapter 6

Development of semi-quantitative PCR technique

INTRODUCTION

Methods of gene expression analysis have become increasingly important as diagnostic tools, as techniques for biomedical research, and for exploring the wealth of information that has been generated from genome sequencing projects. Furthermore, with the gain in popularity and acceptance of microarray screening, there is an accompanying need for technology to quantify and confirm gene expression levels to validate microarray data. One of the emerging technologies that is employed for this task is quantitative polymerase chain reaction (quantitative PCR or Q-PCR). PCR is typically used for the exponential amplification of a target sequence of interest to generate double stranded DNA products that can then be further analyzed or used for downstream applications. However, because the concentration of a PCR product is directly proportional to the concentration its template (Rasmussen, 2001), the procedure can also be used as a means of estimating the initial levels of template in samples (either cDNA or mRNA). Hence, the procedure could provide an alternative method to the widely used Northern blot technique for quantifying tissue mRNA levels. Initial methods for Q-PCR used the endpoint concentration of the amplicon to estimate the original concentration of the starting target material (Wilhelm *et al.*, 2002). The amount of product produced by the typical PCR trajectory can be described by the equation:

$$P = I * E^n \quad (1)$$

where P is the PCR product concentration, I is initial concentration of template nucleic acid, E is the reaction efficiency and always ranges between 1 and 2, and n is the number of PCR cycles (Tichopad *et al.*, 2003).

However, the problem with using endpoint analysis to calculate initial template

concentration is that the kinetics of amplification can only follow a predictable pattern for a specific period of time known as the log linear phase of amplification. Usually, PCR reactions can be described by three separate phases; the initial exponential phase, followed by the log linear phase, and finally a plateau phase. The log linear phase is the state of constant amplification efficiency. Several factors contribute to the plateau effect including depletion of substrates such as dNTPs or primers, eventual thermal inactivation and/or a limiting concentration of DNA polymerase, enzyme inhibition by increasing concentration of pyrophosphates, increased efficiency of specific product re-annealing (above 10^{-8} M), decreased denaturation efficiency, and increased non-specific binding of the polymerase to the 5' end of the double stranded DNA products (Kainz, 2000; Swillens et al., 2004). Due to these contributors to the plateau effect, endpoint analysis may not be very accurate unless the reaction can always be stopped before reaching the plateau phase. More recent techniques have used the information gathered from the log linear phase to calculate initial target concentrations. Furthermore, the advancement of fluorescent technology has made protocols of PCR-based gene expression simpler and more accurate. These protocols acquire fluorescence data from the growing PCR amplicons during the log linear phase of amplification and use this to quantify the initial template concentration. Because the information accrued is in real time, this technology is called real time-PCR (Deprez *et al.*, 2002). There are two types of fluorescent systems in use. One form uses fluorescently-labeled sequence-specific hybridization probes. These probes are based on fluorescence resonance energy transfer, which takes advantage of changing fluorescence with different physical states (example: bound *versus* free probe) (Clegg, 2000; Selvin, 1995). Common types of probes are Taqman, Molecular

Beacons, LightCycler, and Amplifluor. The other system is a simpler method that uses the DNA binding dye SYBR green I. This dye binds non-specifically to the minor groove of DNA. The DNA-bound form has a fluorescence that is over 100-fold greater than the unbound form and, hence, fluorescence of the system rises strongly as the PCR reaction produces DNA. The advantage of this method is that you do not need to design new primers that are compatible with the gene-specific probes and it is also the less expensive of the two options.

Real-time PCR quantifies an initial template concentration in a PCR reaction based on a property called the amplification cycle threshold (C_t). The C_t is the number of amplification cycles required to produce a predetermined amount of product, or in other words, the point at which the fluorescence signal reaches a threshold value (Wilhelm *et al.*, 2003). The threshold value is an arbitrary noise band that can be determined by the user or automatically by most Q-PCR software. The threshold should ideally be as low as possible without being in the range of background or signal noise. Conversely, if the threshold is set too high, the data will be adversely affected by the plateau effect.

Based on equation (1), the efficiency of the PCR reaction must be calculated in the log linear phase in order to calculate the template concentration. The efficiency of amplification is usually calculated using the C_t values for a dilution series of a known standard sample. Absolute quantification becomes possible if the exact concentration is known for the standard sample. Quantitative PCR is carried out on the dilution series of the standard sample and the C_t value is obtained for each dilution. The obtained C_t values are then plotted against the log of the initial concentration (number of molecules) of each dilution. Ideally the result is a linear graph indicating that the assay has responded

consistently at all concentrations tested. The line of regression through the data points is used to calculate the efficiency of the reaction. This, in turn, is used to calculate the concentration of template in other unknown samples based on their C_t values.

Fluorescence-based real-time PCR is an accurate method of determining gene expression data. However, the method has some drawbacks that include the fact that it can take a long time to optimize all the conditions and that each individual run can be costly due to the price of the reagents, particularly the fluorescent tags. In our lab, we use PCR to validate leads that have been generated from microarray experiments. It would not be feasible to follow up all of our leads from microarray experiments with the time-consuming and costly endeavor of real-time PCR.

The goal of the research described in this chapter was to develop a system that could evaluate our gene expression systems in a more efficient manner. This was done by taking advantage of the system employed by real-time PCR. I used serial dilutions of known concentrations of a standard sample of polyadenylated RNA to create cDNA and then used the cDNA as a template for PCR. The C_t value for each known concentration was obtained and plotted against the log of the known initial concentration of RNA to generate a straight line. This standard straight line was then used in conjunction with the obtained C_t values of other unknown samples to estimate the starting concentration of mRNA in a sample under investigation.

MATERIALS AND METHODS

The kanamycin control RNA was purchased from Promega at a concentration of 1.2625 pMol/ μ L and a 1 μ L of the RNA was used to create cDNA as described in

Chapter 3. Serial dilutions of the cDNA product were then created from 10^{-1} down to 10^{-6} and used as templates for PCR amplification. Primers for the PCR amplification were ordered from Sigma Genosys. The sequence of the upstream primer was 5' - AGCCGCCGTCCCGTCAAGTCAG - 3' and the sequence of the downstream primer was 5' - GCCATTCTCACCGGATTCAGTCGTC - 3'. The PCR reagent concentrations were as described in Chapter 4. A 2x PCR reaction was prepared to give a final volume of 102.5 μ L. The final mixture was divided into 10 tubes each containing a 10 μ L aliquot. The tubes were placed in the thermocycler and the reaction cycle was initiated consisting of 94°C for 3 minutes followed by 40 cycles of 30 seconds at 94°C (denaturation), 1min at 60°C (annealing) and 1 minute at 72°C (extension). PCR was initiated as normal but instead of removing product at the end of the 40 cycles, one tube was removed from the PCR machine at the end of every second 72°C extension step beginning cycle 14 for low dilutions (10^{-1} , 10^{-2}) or up to cycle 26 for higher dilutions (10^{-5} , 10^{-6}). The removed samples were stored on ice until all 10 tubes were removed. Two μ L of DNA loading dye was then added to each sample before they were separated on a 0.8% agarose gel, as described earlier. A 4 μ L aliquot of a low-mass DNA ladder (Invitrogen) was also loaded. The low-mass ladder was chosen because the exact amount of nucleic acid in each band was accurately known. This was useful for quantifying the relative intensity of all bands on a gel. The PCR product was a 323 bp fragment that was visualized with ethidium bromide on the Syngene (Cambridge, UK) imaging system. The band intensities of the products were quantified using the accompanying GeneTools software. In order to normalize all gels to each other the 400 bp band of the low-mass DNA ladder was assigned the arbitrary value of 100. This allowed a correction to be

made between gels for variations arising from staining, exposure times, contrast, background, etc. As a result, a known and precise amount of nucleic acid always had the same relative intensity.

A standard curve was then constructed by plotting the cycle number (x-axis) against relative intensity of each band (y-axis) for each concentration of cDNA used in the PCR reaction. Ideally the regression is a straight line. If the regression line was not straight it was assumed that there was an experimental error and the data point was excluded. The slope of the line generated from the samples taken from each concentration was used to determine the C_t for that concentration of starting material, but excluding any samples that were on the plateau region of the PCR reaction profile. The arbitrary threshold chosen for the C_t was a relative intensity of 100. This value was chosen because it represented an amount of nucleic acid that was easy to detect but far from the plateau range. The calibration curve represented a sum of the data collected from all of the concentrations of cDNA tested. The calibration curve was produced by plotting the logarithm of the template concentration (x-axis) against the observed C_t for the corresponding points. The result was a straight line indicating that our system did in fact respond in a reproducible manner.

RESULTS

Figure 6.1 shows the low-mass DNA ladder that was used to normalize all the gels to each other and give a meaningful value to the relative intensity of the fluorescing PCR bands. The graph of DNA concentration versus relative intensity of the ethidium bromide stained bands demonstrates the linear relationship between these parameters for the 4 μ L aliquot of DNA ladder.

Figure 6.2 shows the PCR product levels for the 10^{-1} through 10^{-4} dilutions of the standard kanamycin RNA sampled after cycles 22 through 40. The PCR product was a single band of 323 bp in size. Serial dilutions of the control RNA were used as templates for reverse transcription and the cDNA products then served as templates for simultaneous PCR reactions. The 2 x PCR (102.5 μ L total volume) reactions were divided into 10 separate PCR tubes that were subjected to increasing cycles of PCR. The PCR products were run out on agarose gels along with 4 μ L of the low-mass DNA ladder. The 400 bp band of the DNA ladder was assigned the arbitrary value of 100. The cycle number was then plotted against relative intensity (Figure 6.2, lower panel) and the slope of the regression line was used to calculate C_t (100). The C_t (100) values were then used to create a calibration curve that related cycle threshold to initial concentration of standard RNA template molecules (Figure 6.3). The equation of the regression line for the standard curve was $y = -3.54x + 47.17$ and the correlation coefficient (R^2) was 0.97. Because the R^2 is over 0.95 the relationship between threshold cycle and copy number of initial target RNA is valid and reproducible in our system. Furthermore, the data shown was collected from amplifications done on three different PCR machines (Amplitrone II, Barnstead Thermolyne Corp.; Eppendorf Mastercycler; BioRad iCycler) which indicates a high degree of reproducibility between machines.

The efficiency of the cDNA synthesis reaction was tested by spiking the kanamycin reaction with contaminating RNA from other sources. Up to 10 μ g of total RNA could be added from *E. scudderiana* to the kanamycin 1st-strand synthesis reaction without any adverse effects (data not shown). This is important because the PCR reactions we conduct are never on a pure sample containing just one species of mRNA.

Figure 6.1. Agarose (0.8%) gel electrophoresis with ethidium bromide staining showing the 323 bp kanamycin band (lane 1) along with three amounts (2 μ L, 4 μ L, and 8 μ L) of the low-mass DNA ladder (lanes 2-4, respectively). Band sizes starting from the top are 2000 bp, 1200 bp, 800 bp, 400 bp, and 200 bp. In the 4 μ L aliquot of ladder the aforementioned bands contained 200 ng, 120 ng, 80 ng, 40 ng, and 20 ng of nucleic acid. The graph shows that the relative intensity of the stained bands increased linearly with increasing DNA concentration.

Figure 6.2. Representative agarose gel (1 x TAE, 0.8% agarose) showing the PCR products of the 10^{-1} through 10^{-4} dilution series of the control RNA template. The kanamycin product was a single band 323 bp in length. Below the gels is a representative graph for the 10^{-6} dilution. The equation of the regression line was used to calculate the C_t (100).

22 Cycle 40 22 Cycle 40

10⁻⁶ dilution

Figure 6.3. Standard curve of the logarithm of the initial template concentration versus the C_t (100) obtained for those concentrations of starting control RNA. This curve represents the data accumulated from 3 separate PCR machines in use in our laboratory (Amplifon, Eppendorf, BioRad). Data points are $\pm$ S.E.M. ($n = 12$).

DISCUSSION

The system employed in our lab is called two-step PCR because the reverse-transcription stage is done prior to and independently of the PCR phase. It is possible to do RT-PCR in one automated step but this does not lend itself well to groups working on several different genes from many different tissue/animal sources due to the increased cost of the one-step procedure. Because we use the two-step procedure, the conditions for each step had to be optimized independently.

Obviously, the first stage of the RT-PCR process is the reverse-transcription step and it must be optimized before moving on to the PCR stage. To do this, it is also useful to do PCR runs on a dilution series of the cDNA construction reaction. The graph of C_t versus log of concentration will identify the linear range of cDNA construction. It is not uncommon for data points to fall outside of the linear region and these conditions must be avoided for accurate quantification. If the RNA in a sample is too concentrated the efficiency of the reverse transcription will be decreased. In addition, there are often inhibitors of the PCR reaction within the cDNA sample that can be diluted out at lower concentrations. Sources of inhibition can include organic solvents and inhibitory proteins carried over from the RNA isolation procedure as well as ethanol from the RNA precipitation or washing steps. Furthermore, if the reverse transcriptase is not properly heat-inactivated it too will inhibit the PCR reactions. Only RNA and reagent concentrations that generate linear standard curves should be used. Any conditions that lead to erroneous slopes and/or efficiencies should be further investigated. Once you know what conditions are ideal and what reagent concentrations may pose problems, you can design more efficient reactions for samples of interest.

With optimal conditions identified, it is possible to generate a standard curve relationship between a known amount of starting material (template RNA) and the minimum number of cycles required to visualize the PCR product by your detection system. I created a standard curve using polyadenylated RNA representing a partial sequence of the kanamycin gene. This RNA was chosen because it represented a sequence that was not present naturally in any of the samples being studied, it contained a poly-A tail so it was amenable to the two-step RT-PCR procedure, and it was commercially available in a precisely known concentration. We used oligo-dT_{18VN} primers in our reverse-transcription reactions. The 18 represents the string of 18 thiamines and the VN represents a random nucleotide (A, C, or G) that helps to anchor the primer at the mRNA/poly-A tail junction and increase the efficiency of the reverse transcription reaction. In fact, the efficiency of this primer method is not significantly different using gene-specific primers to initiate reverse-transcription (Khan *et al.*, 1990; Liang *et al.*, 1993).

A straight-line calibration curve was successfully created with a regression line with a correlation coefficient of 0.97. Typically an R^2 value above 0.95 is acceptable. This high correlation coefficient confirms that our PCR system behaves in a reproducible and predictable manner. We used three different models of PCR machines that employ different heating and cooling procedures and as a result have different ramping speeds (time to reach temperature set points). Despite the differences between PCR machines, there was no significant difference between the PCR reaction efficiencies.

The calibration curve that I developed has been used to estimate initial template concentrations by several different researchers in our lab and on many different tissues.

The results have been compared to previous estimates of relative expression levels generated by comparing the gene in question with the housekeeping gene, α -tubulin. To date, results from both quantification procedure are consistent. In addition, this technique was used in *E. scudderiana* and *E. solidaginis* to confirm that mRNA levels of the “housekeeping” gene, α -tubulin were in fact stable through the stressed conditions and throughout the winter, validating the choice to use α -tubulin as a reference for the other gene expression data presented in this thesis. This was important for the normalization of cDNA array data presented in Chapters 3 and 5. The advantage of using the cycle threshold method is that it requires far fewer reactions and you get quantitative data instead of just relative expression as compared to a housekeeping gene. For sample data using this technique please refer to Appendix I.

Chapter 7

General Discussion

Many organisms share the ability to suppress their metabolism and enter a stable hypometabolic state; this life-saving mechanism is a response to many environmental extremes including high or low temperatures, oxygen limitation, food restriction, and/or water limitation (Storey and Storey, 1990, 2004). The widespread ability of the Class Insecta to exploit the capacity to enter diapause is a major factor contributing to the success of these animals that inhabit virtually all terrestrial and fresh water environments on Earth. Diapause is defined as a state of arrested development accompanied by suppressed metabolism. Two types of diapause exist: (1) obligatory diapause, and (2) facultative diapause. If diapause occurs at a fixed stage regardless of environmental cues, it is obligatory. If diapause induction is stimulated by external cues, it is facultative. Facultative diapause is much more common than obligatory. The ability to enter diapause is usually restricted to a single stage of development (larval instars, pupae, pharate adults, adults) for any given species (Denlinger, 2002).

In order for an insect to enter diapause, it must be able to measure environmental cues and store the information in the brain until to the proper period for diapause arises, coordinate the mechanisms of metabolic suppression and the arrest in development, and eventually reinstate the processes to emerge from diapause and resume development. Most of the past research in this field has focused on understanding the necessary environmental cues (Danks, 1987), the hormone regulation of diapause (Denlinger, 1985, and the so-called “clock mechanism” of diapause (Denlinger *et al.*, 2001). However, very little information has been gathered on the molecular and biochemical adaptations used by insects that allow them to orchestrate the necessary components of metabolic suppression. This thesis has started to examine the underlying molecular responses to the

physiological state of insect diapause. The thesis also addressed the gene expression patterns and biochemical regulatory mechanisms that support cold hardiness in freeze tolerant and freeze avoiding insects looking for common and disparate mechanisms in the two strategies. The studies also addressed the links between cold hardiness adaptations, and the adaptations needed for hypoxia tolerance and diapause that seem to be integral elements of winter survival for these species.

Situations that reduce the potential for ATP production will necessarily affect the rate of ATP-consuming processes. Maintenance of membrane potential difference is a crucial component of cell survival and utilizes a large fraction of total cellular energy. In systems that are stress intolerant a common response to stress is reduced ATP production leading inevitably to an imbalance between the rates of ATP-dependent ion pumps and ATP-independent ion channels that rapidly culminates in a loss of membrane potential difference and with it the ability to conduct many cellular activities (e.g. nerve conductance, voltage-dependent transport of metabolites and ion, etc.) (Hochachka *et al.*, 1996). It is obvious then that regulation of ion pumping capacity would be a crucial component of hypometabolism in stress tolerant cells and, indeed, this has previously been demonstrated for mammalian hibernation and anoxia tolerance in diving turtles.

The data presented here show that regulated suppression of two ATP-dependent ion pumps, Na^+K^+ -ATPase and SERCA, is also a component of cold hardiness and the hypometabolic state of diapause in insects. The activities of both of these enzymes were suppressed by a stable mechanism over the winter months in both *E. scudderiana* and *E. solidaginis* but returned to higher levels in April. Suppression of SERCA activity occurred without a change in total SERCA protein content in *E. solidaginis* and in the

face of rising SERCA protein content in *E. scudderiana* over the winter. Hence, regulation of the winter suppression of enzymatic activity was not due to reduced amounts of these proteins in the larvae but was apparently due to reversible controls on their activities. Activities of both enzymes were reduced when incubated *in vitro* with Mg.ATP plus second messengers of protein kinase A, G or C demonstrating their susceptibility to reversible phosphorylation control. This is the same mechanism that regulates the suppression of ion motive ATPases (and other ATP-expensive cell functions) in other systems of hypometabolism (Storey and Storey, 2004) and hence the data on the two cold-hardy insect species expand our understanding of the use of reversible phosphorylation as a global mechanism of stress-induced metabolic rate depression. Furthermore, the stable cold-induced changes in the kinetic properties of SERCA that were observed (reduced $V_{\max}$ in both species and increased K_m ATP in *E. scudderiana*) are both consistent with phosphorylation effects on enzymes. An additional intriguing element of SERCA control was suggested by the identification of phospholamban transcripts and protein in *E. scudderiana*. The amount of phosphorylated phospholamban decreased over the winter months, implying an opposite increase in the amount of the dephosphorylated form that inhibits SERCA and paralleling the changes in SERCA activities. Hence, the data provide good evidence that a protein inhibitor of SERCA is elevated over the winter. Therefore, at least two methods – direct phosphorylation of SERCA and regulation by phospholamban inhibitor protein (itself controlled by reversible phosphorylation) – appear to contribute to the control of SERCA in cold hardy *E. scudderiana*.

Reversible mechanisms of protein control, such as by phosphorylation and

dephosphorylation, have the key advantage of being rapidly implemented to modify the activities of existing enzymes as well as being very inexpensive compared with protein synthesis/degradation as a means of regulating net enzyme/protein activity. However, adaptive responses to environmental stress do not all involve modification of existing cellular proteins and enzymes. Changes in gene expression that bring about either altered protein levels or even the synthesis of all new protein types are an additional important part of adaptation. Indeed, the synthesis of selected unique proteins (e.g. thermal hysteresis proteins, ice nucleating proteins) may constitute a crucial difference between stress-tolerant and stress-intolerant systems. To continue the search for specific proteins that help to confer cold hardiness in insects, three different strategies were used to examine the contribution of differential gene expression to either cold or hypoxia tolerance. The construction and screening of a cDNA library identified two cold-induced genes in *E. solidaginis*, both probably coding for proteases. One appeared to be an aspartyl protease and the other was weakly similar to a metalloprotease. This is the first known association of protease upregulation with insect cold hardiness. Possible functions for upregulation of these genes may include a low degradation of larval tissue over the winter as part of diapause development in preparation for the transition to the pupal stage in the spring or degradation of proteins that are not required during diapause or that are liabilities for cold hardiness.

The use of mouse-specific cDNA macroarrays allowed for the simultaneous screening of about 1200 genes in *E. scudderiana* during cold-stress. Even though this was heterologous probing, I obtained a high percentage of cross-reactivity. The effects of two different temperature shifts were compared: (a) from warm to cold (+15°C to +4°C,

and (b) from cold to subzero temperature ($+4^{\circ}\text{C}$ to -5°C). About 3 times as many genes were identified as being putatively upregulated by the latter subzero temperature exposure which suggests that there are a variety of “last minute” changes to metabolic organization and regulation that are required when insects are first exposed to subzero temperatures. This supports the idea that cold hardening has two components – anticipatory changes that occur well in advance of the first cold exposures and acutely triggered changes that respond directly to subzero temperatures. Another key observation that could be made from examining the macroarray results (the same also applies to the microarray results) is the fact that very few of the putatively upregulated genes that were identified have known links to any of the well-described “macro” adaptations that are well-known components of cold hardiness: e.g. cryoprotectant biosynthesis, ice management, etc. Several of the genes identified had links with responses to hypoxia or with inhibition of apoptosis and the identification of phospholamban as putatively upregulated again points to the regulation of SERCA as being important to cold hardiness. One point that has become clear from the microarray screenings is that solute and ion channels must play a bigger role in cold-hardiness or hypoxia tolerance than previously imagined. Every cold stress or hypoxic stress investigated by microarray screening with either *E. scudderiana* or *E. solidaginis* identified several membrane transporters as being putatively upregulated. These included both plasma membrane and mitochondrial membrane transporters. Hence, the array screening data provides the important insight that cold hardiness may involve not only the “macro” cryoprotective adaptations but also subtle modifications of many different cell functions. A wealth of new information was generated from these array screening experiments and a lot of

follow up work will be required to validate and assign roles in stress response to all the putatively upregulated genes. Furthermore, another large data set is also available from the array screening identifying genes that are strongly downregulated in response to cold or hypoxia stress. None of these have been examined to date but clearly this group of genes may provide additional important insights into the adaptive processes of stress tolerant animals. Hence, the leads generated from the present array screening studies will open up a new generation of studies of the molecular mechanisms of cold hardiness and freeze tolerance.

The completion of many genome-sequencing projects combined with advancements in cDNA microarray technology has let to a wealth of gene expression data being generated in a short period of time. Large databases are being created with expression data from numerous animal systems under a variety of condition or stresses. Global analysis of theses database is defining the roles of many genes faster than ever before.

Gene chip information along with data presented by myself and previously by Denis Joannis indicate that the mitochondrion is dynamic organelle during cold and hypoxic stress. Furthermore, mitochondrial responses to these stresses appear to differ between freeze tolerant and freeze avoiding species. This probably reflects a need for natural hypoxia tolerance by the freeze tolerant insect in order to endure periods of extended hypoxia/ischemia during winter freezing episodes. By contrast, the freeze avoiding insect, *E. scudderiana*, may have little or no natural experience of hypoxia and may actually show characteristics of a hypoxia sensitive species. The present studies of mitochondrial metabolism indicated that maximal activities of mitochondrial enzymes,

such as COX, are actively regulated by both freeze-tolerant and freeze-avoiding insects; the activity of COX was reduced in response to cold shock and throughout the winter in both species. However, the two species differed in their responses to hypoxia exposure with COX activity significantly depressed in *E. scudderiana*, whereas hypoxia did not alter COX activity in *E. solidaginis*. The two species also showed evidence of differences in the fate of mitochondria over the winter months. Levin *et al.* (2003) reported previously that the number of mitochondria were decreased over the winter in the freeze tolerant *E. solidaginis* (as assessed by mitochondrial DNA content) whereas my similar analysis of *E. scudderiana* mitochondrial DNA content indicated that the number of mitochondria did not change significantly throughout the winter in the freeze avoiding species. These differences may be explained by the different energy producing pathways employed by these species. Because *E. solidaginis* spends much of the winter frozen, it must rely on anaerobic metabolism to supply much of its energy needs whereas the supercooled *E. scudderiana* relies on the aerobic oxidation of fats, a process that occurs in the mitochondria, to generate ATP. A decrease in mitochondrial numbers in *E. scudderiana* could impair its aerobic capacity. It is also possible that mitochondria may be susceptible to freeze/thaw injury and that a reduction in mitochondrial numbers in *E. solidaginis* might actually minimize the risk of damage to these organelles over the midwinter months that may be spent largely in a frozen state.

Stress-induced upregulation of the aforementioned genes has no insignificance to cells and organs if the transcripts are not actually translated into functional proteins. However, protein synthesis is an energy consuming process that is very sensitive to the available energy. In fact, it is been well documented that protein synthesis is strongly

suppressed during periods of energy depletion and decreased oxygen availability such as in hibernation, hypoxia, and diapause (Joplin and Denlinger, 1989; Land et al., 1993; Larade and Storey, 2002). In addition, the amount of energy made available for protein synthesis is decreased in hypometabolic states as the cells shift the priorities around to stretch energy reserves. For example, the percentage of total ATP turnover used for protein synthesis fell from 24.4% in normoxia to just 1.6% in anoxia in turtle hepatocytes (Land et al., 1993). This reorganization of cellular energetics is a rapid proactive process to conserve energy rather than a reactive process responding to depleted energy charge (Larade and Storey, 2002; Churchill and Storey, 1996; Storey and Storey, 2004).

There are two main methods to inhibit protein synthesis: (1) by limiting mRNA substrate availability, and (2) specific inhibition of the translational machinery. The data available from gene chip analysis indicated that only about 5% of genes were actually downregulated in response to hypoxia or cold stress in the two insect species. Studies of other hypometabolic systems largely concur; transcript levels of very few genes are actually reduced during hypometabolism (Storey and Storey, 2004). Clearly, this indicates that substrate availability is not a major factor in limiting protein synthesis during diapause, hypoxia, or cold-induced-quiescence in *E. scudderiana* or *E. solidaginis*. Primary regulation of protein synthesis must then result from regulation of the translational machinery. Work from our lab and others have identified two conserved methods that are used to regulate translation: (1) reversible phosphorylation of specific proteins making-up the ribosomal translational apparatus, and (2) the state of ribosome assembly (Storey and Storey, 2004).

The inhibition of translation in hibernating ground squirrels was first linked to

phosphorylation of the alpha subunit of eukaryotic initiation factor (eIF2), which is responsible for the introduction of the initiating methionyl residue to the 40S ribosomal subunit. This has now been identified as a mechanism of translational suppression conserved across many species. A small amount of phosphorylated eIF2 α results in large-scale suppression of protein synthesis by blocking the initiation of peptide growths (Rhoads *et al.*, 1993; Mikulits *et al.*, 2000). I propose that this system of control would also be conserved in cold hardy insects. Reversible phosphorylation control over two of the major energy-consuming functions in cells (ion motive ATPases and protein synthesis) could provide a major portion of the reduction in cellular ATP use in metabolic rate depression. Indeed, preliminary analyses indicate that both *E. scudderiana* and *E. solidaginis* suppress protein synthesis by phosphorylation of eIF2 α (data not shown). Future studies will more fully explore the signals and the signal transduction cascades involved eIF2 α control in insects as well as the responses and regulation of other initiation and elongation factors to cold or hypoxia stresses.

If protein synthesis is suppressed during hypometabolism, then how are the selected transcripts that are upregulated translated into proteins? The answer is most likely a message selection system based on an internal ribosome entry site (IRES). These are sequences on an mRNA that give them preferential docking to ribosomes ahead of mRNAs that lack this sequences (Gingras *et al.*, 1999). IRESs have been known to exist in mammals for a few years but they have just recently been identified in insects, specifically *Drosophila melanogaster* (Ronfort *et al.*, 2004). It would be interesting to search the genes that were identified as cold or hypoxia upregulated on the gene chips for the presence of IRES sequences in their transcripts. For example, HIF-1 α is a well-

known example of a gene that contains an IRES and that can be translated under energy-limited stress conditions that suppress the translation of most other proteins. It is likely that genes that are upregulated during entry into hypometabolic states or states where ATP production would be limited (e.g. during freezing or under oxygen limitation) could be selected for translation by this IRES system or a similar mechanism.

Other future studies that should be done involve looking at the aggregation state of the ribosomes under stress conditions in both *E. scudderiana* and *E. solidaginis*. Active translation occurs on polysomes, which are a cluster of many ribosomes moving along a single strand of mRNA. Conversely, monosomes are typically translationally silent. It would be interesting to see how the proportion of polysome to monosome changes in these insects during stress.

Two of the three MAP kinase superfamilies, p38 and ERK, were evaluated in *E. scudderiana* and *E. solidaginis* to analyze their possible involvement in cold hardiness and/or hypoxia tolerance. The activities of the members of these families are determined by their level of phosphorylation, the phosphorylated form being the active one. In *E. scudderiana*, p38 was phosphorylated (activated) in response to cold temperatures but dephosphorylated in response to hypoxic conditions whereas in *E. solidaginis* p38 was phosphorylated in response to both conditions. Activation of p38 typically results from stress stimuli such as osmotic shock, heat, hypoxia, radiation and inflammatory cytokines. Freezing constitutes both an osmotic stress on cells and a hypoxic insult; hence, it is not surprising that p38 was activated by cold/freezing in *E. solidaginis*. Further studies will need to identify the downstream targets of p38 action which may include genes that address cell volume regulation or hypoxia/ischemia resistance. ERK1

(p44) was also activated in the insects in the cold winter months as compared to late summer and spring (April) whereas ERK2 did not change significantly throughout the winter in either species. Again, future studies will need to address the downstream targets of ERK1 action to determine what metabolic effects ERK1 is mediating in the cold hardy larvae. Finally, the role of the JNKs, the third MAPK module, in insect cold hardiness needs to be evaluated. JNKs often trigger cell death by apoptosis in response to stresses that cause severe damage to cells. Given that the results from the SuperArrayTM analysis of genes related to the NF- κ B pathway revealed upregulation of several genes that could be anti-apoptotic, it might be predicted that the JNK pathway would be inhibited during cold exposure in these cold hardy species. This may be a key difference between cold hardy and nonhardy systems. Nonhardy organisms may respond to cold stress by activating apoptosis whereas hardy species may employ mechanisms to inhibit cell death at the same time that they are activating mechanisms of cryoprotection.

This thesis provides new insights into the biochemical adaptations that confer cold and hypoxia-tolerance in insects. Much work is needed to fully elucidate the complex network molecular intricacies that mediate stress-tolerance in insects but a good foundation has been initiated with the work presented here.

Appendix 1: List of putatively upregulated genes from *E. solidaginis* when screened on human 19K cDNA microarrays [Control (+15°C) versus -4°C, 1 hour].

Fold increase	Gene Description
3.41	22543:R21587:Sapiens, clone IMAGE-5288750, mRNA::10::0:31299
2.95	262036:AA156879:similar to Zinc finger protein 268 (Zinc finger protein HZF3)
2.57	Hs.143087:N49616:hypothetical protein
2.36	Hs.31819:H51444:HT014:HT014:11:1p36.11:0:179498
2.27	Hs.21902:R19050:hypothetical protein MGC42530:MGC42530
2.25	Hs.405044:R08689:karyopherin beta 2b, transportin:TRN2:19:
2.23	3:113616
2.21	Hs.302030:H55873:ATP-binding cassette, sub-family G (WHITE), member 8
2.09	Hs.8719:T77421:hypothetical protein MGC1136:MGC1136:8:8p12:0:23896
2.04	Data not found:R92320:::0:195875
2.01	Hs.114762:R12176:Homo sapiens transcribed sequences::4::0:25484
2	In multiple clusters:T79902:::0:24927
1.97	Hs.78890:R60109:numb homolog (Drosophila):NUMB:16:14q24.3:0:
1.95	Hs.137476:R00760:paternally expressed 10:PEG10:7:7q21:2:123837
1.95	Hs.13775:BG720392:homeodomain-only protein:HOP:3:4q11-q12:10:
1.93	Hs.313:R97904:secreted phosphoprotein 1 (osteopontin, bone sialoprotein I)
1.91	Hs.288968:H23370:RAB22A, member RAS oncogene family:
1.91	Hs.7138:H18866:cholinergic receptor, muscarinic 3:CHRM3:1:
1.9	Hs.43107:T74351:KIAA0555 gene product:KIAA0555:5:5q32:3:22582
1.85	Hs.323583:H23385:hypothetical protein DKFZp434L142:DKFZp434L142
1.82	Hs.84981:BM471778:X-ray repair complementing defective repair in CHC 5
1.82	0:234760
1.82	Hs.97927:N94043:similar to MOB-LAK:LOC148932:1:1p34.1:0:293457
1.82	Hs.446325:H12045:hypothetical protein FLJ31810:FLJ31810:9:9p21
1.81	Hs.35140:T94961:serine/threonine kinase 4:STK4:13:20q11.2-q13.2:2:
1.81	Hs.376593:H16933:Sapiens cDNA FLJ34825 fis, c
1.8	Hs.13775:R15222:homeodomain-only protein:HOP:3:4q11-q12:0:29649
1.79	Hs.7047:H23443:Sapiens mRNA; cDNA DKFZp566B064
1.79	Hs.233044:AA029330:LCHN protein:LCHN:7:7q34:0:366763
1.78	Hs.179657:T75241:plasminogen activator, urokinase receptor:PLAUR:
1.78	Hs.250618:BG675590:UL16 binding protein 2:ULBP2:6:6q25:3:36353
1.77	Hs.356325:AJ271379:hypothetical protein LOC169981:LOC169981
1.77	Hs.278871:H19015:hypothetical protein FLJ30921
1.76	Hs.34789:BQ070537:dolichyl-diphosphooligosaccharide-protein
1.75	Seq_Ver:In multiple clusters:H16921:::0:50472
1.74	Hs.81737:H54150:palmitoyl-protein thioesterase 2:PPT2
1.73	Hs.1298:AA203237:membrane metallo-endopeptidase
1.72	Hs.254129:R13758:SPHK1 (sphingosine kinase type 1) interacting protein
1.72	Hs.30965:H10128:neuronal Shc adaptor homolog:SLI:19:19p13.3:0:46843
1.71	Data not found:R83567:::10:295200
1.71	Hs.433452:H12679:KIAA1237 protein:KIAA1237:3:3q21.2:0:148639
1.7	Hs.107537:AA005415:chromosome 7 open reading frame 2:C7orf2:7:7q36:0:428328

1.69 Hs.90798:R18935:Clone 23695 mRNA sequence::6::0:33182
 1.68 Hs.20104:BF431222:likely ortholog of mouse Sds3:SDS3:12:12q24.23:3:253268
 1.68 Hs.20529:R13038:similar to RIKEN cDNA 1200015A19:na:12:1p36.32:0:27699
 1.68 Hs.102548:T81296:glucocorticoid receptor DNA binding factor 1:GRLF1
 1.68 Hs.166161:R54537:dynamin 1:DNM1:9:9q34:0:39086
 1.68 Hs.380875:R60474:similar to *S. cerevisiae* SSM4:TEB4:5:5p15.2:0:43058
 1.67 In multiple clusters:N42662:::0:271793
 1.67 Hs.3353:R13572 beta-1,3-glucuronyltransferase 1; glucuronosyltransferase P
 1.67 Data not found:R18526:::1:30698
 1.67 Hs.22584:R61564:prodynorphin:PDYN:5:20pter-p12:0:37687
 1.67 Hs.20879:R11682:hypothetical protein LOC51058:LOC51058:1:1p34.1:2:25285
 1.67 Hs.77462:H09054:DNA (cytosine-5-)-methyltransferase 1:DNMT1:19:19p13
 1.66 Hs.114949:AA127873 protein ref-NP_055301.1 (*H.sapiens*) neuronal thread protein
 1.66 Hs.154295:W32135:Sp3 transcription factor:SP3:2:2q31:0:320763
 1.66 Hs.20252:AA011707:ras homolog gene family, member U
 1.66 Hs.306051:BF056538:wingless-type MMTV integration site family, member 5B
 1.66 Hs.125036:H23044:tumor endothelial marker 7 precursor:TEM7:17:17q21.1:0:52058
 1.66 Hs.433700:R14129:protein kinase (cAMP-dependent, catalytic) inhibitor alpha:PKIA
 1.66 Hs.418650:BE879779:ferritin, heavy polypeptide 1:FTH1:11:11q13:10:36332
 1.66 Hs.13285:H11972:potassium channel, subfamily V, member 1:KCNV1
 1.66 Hs.137476:W95845:paternally expressed 10:PEG10:7:7q21:3:111124
 1.66 Hs.288862:H20904:DNA segment on chromosome 10 (unique)
 1.66 Hs.73454:AA151104:troponin T3, skeletal, fast:TNNT3:3:11p15.5:0:505182
 1.66 In multiple clusters:R11999:::2:25347
 1.65 Hs.55501:W37267:Homo sapiens transcribed sequences::8::1:321841
 1.65 Hs.274344:AA045905:forkhead box P1:FOXP1:3:3p14.1:0:488623
 1.65 Hs.352382:N24261:PI-3-kinase-related kinase SMG-1:SMG1:16:16p13.11:0:261580
 1.65 Hs.154332:W03258:ER degradation enhancing alpha mannosidase-like:
 1.65 Hs.240845:R56723:DKFZP434D146 protein:DKFZP434D146:3:3q25.2:3:40462
 1.65 Hs.295059:H71742:Sapiens cDNA FLJ35329 fis, clone PROST2013873:::0:213734
 1.64 Hs.432896:W79862:aldo-keto reductase family 1, member A1 (aldehyde reductase)
 1.64 Hs.27047:H58344:chromosome 14 open reading frame 101
 1.64 Hs.203402:H23112:Sapiens cDNA FLJ38454 fis, clone FEBRA2019690:::3:0:51968
 1.64 Hs.13467:AA011648:STARD3 N-terminal like:STARD3NL:7:7p14-p13:0:429666
 1.62 Hs.129952:H88465:likely ortholog of mouse aquarius:AQR:15:15q13.3:0:252858
 1.62 In multiple clusters:N42889:::10:271158
 1.62 Hs.171734:BI545796:protein phosphatase 2, regulatory subunit B (B56), gamma
 1.61 Hs.166184:R84711:intersectin 2:ITSN2:2:2pter-p25.1:0:275579
 1.61 In multiple clusters:W23477:::0:305406
 1.61 Hs.25450:R55445:solute carrier family 29 (nucleoside transporters), member 1
 1.61 Hs.29344:W24566:TIR domain containing adaptor inducing interferon-beta:TRIF:7
 1.61 Hs.356416:BE531094:chromobox homolog 7:CBX7:22:22q13.1:3:40562
 1.61 Hs.348963:BM543848:zinc finger protein 275:ZNF275:X:Xq28:3:197287
 1.61 Hs.183:BI819401:Duffy blood group:FY:19:1q21-q22:3:159421
 1.61 Hs.15519:W23476:oxysterol binding protein-like 2:OSBPL2:X:20q13.3:0:305402
 1.6 Hs.10336:H62237:Homo sapiens transcribed sequences::8::0:205381
 1.6 Hs.153546:BE892625:CDC23 (cell division cycle 23, yeast, homolog)
 1.6 Hs.3454:N79205:rab11-family interacting protein 4:RAB11-FIP4:17:0:292295
 1.59 Hs.347349:H39129:cell cycle progression 2 protein:CPR2:7:7p14-p13:1:192365
 1.59 Hs.166846:N53193:polymerase (DNA directed), epsilon:POLE:12:12q24.3:0:281485

1.59 Hs.379090:R21466:glycoprotein M6B:GPM6B:X:Xp22.2:0:31251
 1.59 Hs.247478:H10318:HLA-B associated transcript 4:BAT4:6:6p21.3:0:46944
 1.59 Hs.39619:H74164:reticulocalbin 3, EF-hand calcium binding domain:RCN3
 1.59 Hs.88474:R96180:prostaglandin-endoperoxide synthase 1 (
 1.58 Hs.20104:H89297:likely ortholog of mouse Sds3:SDS3:12:12q24.23:0:253245
 1.58 Hs.71969:H88211:hypothetical protein LOC286148:LOC286148:X:8q22.1:0:252718
 1.58 In multiple clusters:H20645::::0:51426
 1.58 Hs.75313:AU143596:aldo-keto reductase family 1, member B1 (aldose reductase)
 1.58 Hs.270799:H48847:Homo sapiens transcribed sequences::::0:207172
 1.58 Hs.242947:R54194:diacylglycerol kinase, iota:DGKI:7:7q32.3-q33:10:41552
 1.58 Hs.247423:R49716:adducin 2 (beta):ADD2:2:2p14-p13:0:38754
 1.58 Hs.26981:H29410:amyotrophic lateral sclerosis 2 (juvenile) chromosome region
 1.58 Data not found:T75386::::0:23354
 1.57 Data not found:AA203654::::0:446752
 1.57 Hs.65425:H87934:calbindin 1, 28kDa:CALB1:5:8q21.3-q22.1:0:252663
 1.57 Hs.79101:W37850:cyclin G1:CCNG1:5:5q32-q34:0:322199
 1.57 Hs.348088:N79214: weak similarity to protein ref-NP_
 1.57 Hs.110736:H65116:solute carrier family 12 (sodium/potassium/chloride transporters)
 1.57 Hs.413760:W92000:chromosome 5 open reading frame 13:C5orf13
 1.57 Hs.22607:H02418:Homo sapiens transcribed sequences::10::0:151214
 1.57 Hs.455779:BF797625:similar to growth hormone-inducible soluble protein
 1.56 Hs.132863:H88884:Zic family member 2 (odd-paired homolog, Drosophila)
 1.56 Hs.77823:R19716:ring finger protein 38:RNF38:12::0:34496
 1.56 Hs.27191:H05863:promethin:LOC57146:3:16p12.3:43614
 1.56 Hs.279881:BI861012:mannosidase, alpha, class 1B, member 1:MAN1B1
 1.56 Hs.172350:H23459:HIR histone cell cycle regulation defective homolog A
 1.56 Hs.75478:W61184:ATPase, Class VI, type 11B:ATP11B:3:3q27:0:342260
 1.56 Hs.22930:H15482:Homo sapiens transcribed sequences::4::3:32102
 1.56 Hs.106674:W35227:BRCA1 associated protein-1
 1.55 Data not found:H86368::::10:222653
 1.55 Data not found:AA031572::::0:470666
 1.55 Hs.19673:W02319:homolog of yeast MAF1:MAF1:5:8q24.3:0:291972
 1.55 Hs.423343:R53638:Sapiens cDNA FLJ36937 fis, clone BRACE2005433
 1.55 Hs.420269:AL531751:collagen, type VI, alpha 2:COL6A2:21:21q22.3:3:134153
 1.55 Data not found:AW630978::::3:486095
 1.55 Hs.151385:AA098909:KIAA0564 protein:KIAA0564:13:13q13.3:0:489797
 1.55 Hs.10748:T75252:hypothetical protein
 1.54 Hs.2540:W94963:cholinergic receptor, nicotinic, alpha polypeptide 7

Appendix 2: Technical bulletin: Semi-quantitative PCR

Background information:

This is a technique done **in addition** to your regular RT-PCR (with tubulin, actin, etc. as reference material) in order to quantify your PCR template concentration. This is a way to approximate a numerical value for the number of gene-specific products found within a total RNA sample using RT-PCR. It is based on the rate of log phase growth of your PCR products. **The concentration of a PCR product is exclusively proportional to the template concentration.** In this technique, PCR is started as normal, but tubes are removed from the PCR machine at 2 cycle intervals at **the end of the 72°C extension step**. The PCR products are then run out on a gel and the slope of the amplification is calculated from the graph of intensity versus cycle number.

It is necessary to load a ladder on the gel because the amount of DNA in the ladders is known and can be used to normalize the intensity of the bands in the gel. The low DNA mass ladder from Invitrogen is ideal because the amount of DNA in each band is accurately known. Load 4 ul of this ladder on a gel. When analyzing the gel with GeneTools, set the value of the 400 bp band to 100. This will help to eliminate any erroneous data resulting from variations in staining, exposure times, and contrast levels.

Fragment size	2 ul	4 ul	8 ul
2000 bp	100 ng	200 ng	400 ng
120 bp	60 ng	120 ng	240 ng
800 bp	40 ng	80 ng	160 ng
400 bp	20 ng	40 ng	80 ng
200 bp	10 ng	20 ng	40 ng
100 bp	5 ng	10 ng	20 ng

Construction of calibration curve:

This technique is based on a parameter called the **cycle threshold (C_t)**. The C_t is the number of cycles required for the PCR product to be detected by your method. The C_t values of a standard dilution series plotted against the log of their known initial concentrations yields a straight line. The higher the initial concentration of a template, the lower the number of PCR cycles necessary to detect product. Conversely, the lower the amount of template, the higher the number of cycles needed to detect a product.

A calibration curve was created using kanamycin positive control RNA (Promega, cat. # C138A). This RNA was chosen because it was commercially available, the exact concentration was known, and it is polyadenylated so it was amenable to our two-step RT-PCR protocol. All conditions employed were the same as those used in our standard lab protocols. Primers were ordered from Sigma Genosys and diluted to a concentration of 15 μ M. Our standard PCR protocol (technical bulletin MB20) was employed. A 2X PCR reaction was prepared and aliquoted into 10 PCR tubes. Amplification yielded a 323 bp DNA product. The optimum annealing temperature is 60°C. Following PCR, the products were combined with 2 μ l DNA loading dye and 10 μ l of this mixture was run on a 0.8% agarose gel containing ethidium bromide. Each gel was also loaded with 4 μ l of the low DNA mass ladder. The gel images were captured using the Syngene and the images analyzed with GeneTools software. The 400 bp band of the DNA ladder was assigned the value of 100 so that all gels would be normalized relative to each other and that band. The arbitrary value chosen for the noise band (threshold) was 100 because this value should fall in the linear phase of the PCR growth curve.

Your task is identify the C_t for your PCR product and use the equation of the line from the graph above and solve for the initial concentration of your template.

Procedure outline:

- A.) PCR reaction stopped after many separate cycle intervals.
- B.) Construction of graph, intensity versus cycle #
- C.) Use equation of the line from part B and standard curve from above

($y = -3.3888x + 45.641$) to calculate original template concentration.

A. Quantifying new genes:

1. Optimize your annealing temperature, primer concentration, and cDNA concentrations as usual. It is a good idea for someone who is relatively new to PCR to run a standard curve of their own with the kanamycin RNA to see if you get a straight line with you're a similar slope to the standard curve I generated. Also, each PCR machine should be checked every 6 months or so to make sure the efficiency has not decreased.
2. Set up a 2X PCR reaction in order to give a final volume of 102.5 ul as follows:
 20 ul cDNA
 60 ul sterile water
 10 ul 10 x PCR buffer
 2.5 ul primer mix
 5.0 ul 50 mM MgCl₂
 2.0 ul dNTPs
 0.5 ul Taq
3. Aliquot the reaction mixture in 10 tubes each containing 10 ul of the PCR reaction.
4. Run 42 cycles of PCR with the following program:
 94°C for 30 seconds
 60°C for 1 min (primer annealing)
 72°C for 1 minute (extension)
5. Open the machine after the 72°C extension step and remove 1 tube every 2 cycles starting at the end of cycle 24 and finishing after cycle 42. **Note:** This is only a guide. You may have to start removing sample much earlier. It may be a good idea to do a preliminary run where you remove tubes every 5 cycles to identify the linear range of amplification. In order to avoid saturated band intensities, the first tube removed should not have no detectable PCR product
6. Add 2 ul of DNA loading dye to the samples and run 10 ul on a 0.8% agarose gel. Be sure to load **4 ul** of the **low mass DNA ladder** (Invitrogen). This ladder is in the freezer in room 431. Only use this ladder for quantification purposes because it is more expensive than the other ladders.
7. Take a picture of the gel on the Syngene and use Genetools to analyze the picture. Set the background correction to automatic. Use the "assign quantity" function to set the value of the **400bp band in the DNA ladder to 100**. This normalizes your gel to the same intensity values as the gels used to create the standard curve.
8. Use the intensities of the bands to generate a plot of **relative intensity (y-axis)**

versus number of cycles.

9. Calculate the slope of the graph in Excel. The rate of growth of your PCR products is related to the number of template molecules in the reaction mixture. Make sure you do not use bands whose intensities appear to be saturated.

10. B. Calculate the slope of the PCR amplification:

- export the normalized values to EXCEL.
- Hold down the Ctrl button and highlight the values for the x (# of cycles) and y (relative intensity) coordinates.
- Select the graphing function
- Select XY scatter
- Click on the data points and then right-click to select add trendline, go to options and click both "display equation on chart" and "display R-squared value on chart".

SAMPLE DATA:

cycle #	intensity
24	118.79
27	147.70
28	169.14
33	244.75
34	271.14
35	274.31
37	305.60
38	318.97

C. Solving for template concentration

The arbitrary noise band (threshold) has been set to 100. Use the equation of the line from the above data to solve for the cycle threshold for the intensity of 100 [$C_t(100)$]. From the equation of the line:

$$\begin{aligned}
 y &= 14.924x - 246.28 \\
 100 &= 14.924(\text{cycle \#}) - 246.28 \\
 \text{therefore:} \\
 \text{cycle \#} &= 100 - (-246.28)/14.924 \\
 \text{cycle \#} &= 23.20
 \end{aligned}$$

Now use the C_t calibration curve to calculate the original template concentration from your data. The equation of the line for the C_t calibration curve is $y = -3.3888x + 45.641$.

$$C_t(100) = -3.3888[\log(\text{copy \#})] + 45.641$$

$$\log(\text{copy \#}) = [C_t(100) - 45.641] / -3.3888$$

$$\log(\text{copy \#}) = (23.20 - 45.641) / -3.3888$$

$$\log(\text{copy \#}) = 6.62$$

$$\text{copy \#} = 10^{6.62}$$

$$\text{copy \#} = 4,168,694$$

PUBLICATION LIST

- Storey, K.B. and **McMullen, D.C.** (2004) Insect cold-hardiness: new advances using gene screening technology. Life in the Cold, Proceedings of the 12th International Symposium (Barnes, B.M. and Carey, H.V., editors).
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PRESENTATIONS AT SCIENTIFIC MEETINGS

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