Profile of FoxO Proteins and MnSOD in Two Cold-hardy Insect Species Exposed to Low Temperature

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A Thesis Submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of

Master of Science

Department of Chemistry

Carleton University
Ottawa, Ontario, Canada

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ISBN: 978-0-494-33721-9

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ABSTRACT

Expression of FoxO genes and proteins in two insects with different overwintering strategies were studied with Western blotting and RT-PCR. *Epiblema scudderiana*, a freeze-avoiding gall moth, and *Eurosta solidaginis*, a freeze-tolerant gall fly were used as model organisms to assess the role of FoxO transcription factors and one of their downstream proteins, manganese superoxide dismutase (MnSOD) in insect cold hardiness. FoxO3a gene and protein levels, as well as MnSOD protein, were analyzed in *Epiblema scudderiana* over time courses of exposure to 5°C or -15°C. Significant changes in total Foxo3a protein, the amount of phosphorylated inactive FoxO3a, FoxO3a mRNA levels, and MnSOD content were identified under different conditions. For example, reduced phospho-FoxO3a (Ser 253) content in larvae exposed to -15°C suggested enhanced transcriptional activation of genes under FoxO3a control at subzero temperatures whereas MnSOD content increased in larvae exposed to 5°C. Sequencing of FoxO3a mRNA from *Epiblema scudderiana* showed that the transcription factor was highly conserved as compared with mammals. FoxO3a, FoxO1 and MnSOD protein levels were also measured in the freeze tolerant insect *Eurosta solidaginis* subjected to 5 and -15°C exposures as well as a time course of thawing after freezing at -15°C for 1 week. The results implied that FoxO3a and FoxO1 functioned oppositely during cold, freezing and thawing of the larvae. Expression of MnSOD increased with 5°C exposure, remained elevated when larvae were frozen at -15°C, and decreased after thawing. Differential expression of FoxOs and MnSOD in both species suggest that MnSOD may not be transcriptionally controlled by FoxO3a or FoxO1 in cold hardy insects.

Acknowledgements

I would like to express my deep thanks to my thesis supervisor Dr. Kenneth B. Storey, who not only accepted me to do my graduate studies in his lab, but also encouraged me constantly so that I could pass the hardest time during study. He gave me a chance to learn new knowledge and techniques in Biology, thus opened another door for my career except for Chemistry. Thanks also give to Dr. Storey because of the multiple academic and social activities provided in his lab, from which I not only got academic experiences but also learned Canadian Culture.

Sincerest thanks are given to Janet Storey, not only for her perfect editing, insightful suggestions and comments on my writing, but also for her hard work with me on my animal. From insect collecting, to experiment design and sampling, without her help, I would not be able to accomplish my project. Also thanks for her kindness, patience and understanding at all time.

Many thanks to my friends and lab colleagues for their support, help, and fun – Lin, Jeremy, Mamady, Chris D., Anastasia, and all other lab members. Thanks also to all those who inspired me and encouraged me during my studies in Carleton University.

Finally, thanks to my husband Yuxiao and my daughter Xinyi for their understanding and support of my studies.

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List of Abbreviations

ADP	adenosine diphosphate
APS	ammonium persulfate
ATP	adenosine triphosphate
CAT	catalase
cDNA	complementary deoxyribonucleic acid
<i>daf-16</i>	dauer abnormal formation-16
DEPC	diethylpyrocarbonate
dFOXO	<i>Drosophila</i> Forkhead box Other
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis(β -aminoethyl)-N,N,N',N'-tetraacetic acid
FOX	forkhead box
FoxO	forkhead Box Other (transcription factor family)
G6P	glucose-6-phosphate
G6Pase	glucose-6-phosphatase
G6PDH	glucose-6-phosphate dehydrogenase
GR	glutathione reductase
GSH	glutathione
GST	glutathione-S-transferase
GSTPox	selenium-dependent glutathione peroxidase
GTPase	guanosine triphosphate hydrolase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IGF	insulin-like growth factor
IGFBP-1	insulin-like growth factor binding protein-1
JNK	c-Jun N-terminal kinase
kDa	kilodalton

MnSOD	manganese-dependent superoxide dismutase
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
p130 ^{Rb2}	retinoblastoma 2-related protein 130
p27 ^{kip1}	kinase inhibition protein 1-related protein 27
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEPCK	phosphoenolpyruvate carboxykinase
PFK	phosphofructokinase
PKB(Akt)	protein kinase B
PMSF	phenylmethylsulfonyl fluoride
PtdIns3-K; PI3K	phosphatidylinositol 3-kinase
PVDF	polyvinylidene fluoride
Ral	Ras-related Ral protein
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SCPx	sterol carrier protein-x
SDS	sodium dodecyl sulfate
SEM	standard error of mean
Skp2	S-phase kinase-associated protein 2
TBST	Tris-base, SDS, Tween
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane

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CHAPTER ONE

Introduction

1.1 Cold-hardy insects

Cold hardiness is the ability to resist injury during exposure to low temperatures. As ectotherms, insects that live in seasonally cold environments have to have a mechanism to deal with subzero temperatures in winter. Many species are able to avoid exposure to temperatures below 0°C by retreating to underground sites that are well below the frost line or by wintering in deep water that will not freeze to the bottom. Many other insect species have developed behavioural, physiological, and biochemical adaptations that allow survival at temperatures below the normal freezing point of their body fluids. Several examples of extreme cold hardiness exist. For example, dehydrated larvae (desiccated to a water content of 8%) of the African chironomid, *Polypedilum vanderplanki* (Diptera) can survive submersion in liquid helium (-270°C) for up to 5 min (Hinton, 1960). The lowest temperature survived by an insect in a nonanhydrobiotic state is -196°C by the prepupae of the sawfly, *Trichiocampus populi* (Tanno, 1968). The species able to survive the lowest natural temperatures for adaptive reasons is the Arctic carabid beetle, *Pterostichus brevicornis*. In laboratory tests, the winter adult of this carabid beetle tolerated temperatures as low as -87°C (Miller, 1969).

For this thesis, cold hardy insects are defined as those that are able to resist injury when exposed to subzero temperatures below the equilibrium freezing point of their body fluids. I focus on two species of insects, *Epiblema scudderiana* and *Eurosta solidaginis*, that are commonly found in the Ottawa area and that survive over the winter in goldenrod galls exposed to ambient temperature. In Ottawa, winter temperatures stay below 0°C for

many weeks at a time and temperatures of -30°C or lower can occur for a few days. The two insects use different fundamental strategies to overwinter: freeze tolerance by *Eurosta solidaginis* and freeze avoidance by *Epiblema scudderiana*.

1.2 Two insects, Two strategies

Freeze avoidance - *Epiblema scudderiana*

Epiblema scudderiana can remain liquid even at temperatures that are well below 0°C ; in fact, its midwinter supercooling point is about -38°C . This strategy is called freeze avoidance. Water does not always freeze at 0°C . In reality, both plain water and the water in an animal's body can often be cooled to much lower temperatures before ice forms. Indeed, microscopic droplets of very pure water can be cooled to -40°C before they freeze. Water "normally" freezes at 0°C because the growth of ice crystals is "seeded" by the presence of some particle or surface that helps to line up the water molecules into the crystal shape. Once an initial microscopic nucleus of ice is formed, more and more water molecules quickly join the crystal and high speed crystal growth is triggered. The trick, therefore, is to keep the body fluids from coming into contact with ice crystals themselves or with molecules or particles that can act as nucleators to seed crystal growth.

Epiblema scudderiana is good at this trick of supercooling - staying liquid when its body temperature is below 0°C . A number of factors are involved in successful supercooling. These factors include the small body size of the larvae, since small volumes of water supercool better than larger volumes, as well as a partial dehydration of the larvae

during the winter (about 25% of total body water is lost). The larvae gain external waterproofing from a silk cocoon and a strong, waxy cuticle; both help to prevent external ice from coming into contact with body fluids (Storey and Storey, 2004). However, the main adaptations that increase an insect's ability to supercool and avoid freezing are: (a) antifreeze proteins, and (b) cryoprotectants. Antifreeze proteins function by binding to microscopic ice crystals as they start to form in the insect's body fluids. Binding prevents the crystal from growing any larger. *Epiblema scudderiana* also produces glycerol, a 3-carbon polyhydric alcohol, as a cryoprotectant. This is the most common cryoprotectant in insects. The freezing points and supercooling points of water and body fluids decrease in proportion to the concentration of dissolved substances so the more cryoprotectant that is packed into a solution, the lower the temperature at which it will freeze. The key advantages of glycerol as a cryoprotectant are that it is highly soluble in water, it passes easily across cell membranes, it is not toxic to metabolism, and it can be easily synthesized by the insect. The concentrations of glycerol can be so high in some insect species in winter that as much as 20-25% of the insect's total body mass is glycerol! Glycerol also has another action and that is that it is very good at binding to water and, for insects that live in exposed sites such as in trees, this helps the animals to avoid death due to desiccation over the long winter months when they are exposed to dry, cold air.

The freeze avoidance strategy has two main risks: (1) supercooling capacity is exceeded; e.g. if an insect only resists freezing above -20°C and temperature drops to -25°C , then the insect freezes and dies, and (2) an insect can only stay supercooled if it can

avoid contact with ice crystals; e.g. even if antifreeze capacity is good to -20°C , if an insect touches ice crystals, its body fluids will be seeded and an insect will freeze and die. So, the freeze avoidance strategy is a gamble -- insects gamble that temperatures won't fall too low and that they are sufficiently protected so that they won't come in contact with external ice.

Freeze tolerance - *Eurosta scudderiana*

Freeze tolerant insects can endure the freezing of body fluids and not die. However, ice is allowed to grow only outside of cells, never inside the cells. Ice forms within the insect's body, surrounding all the internal tissues. In most freeze tolerant species about 65% of the insect's total body water freezes whereas the rest stays liquid inside cells. Ice that grows inside of cells kills them because the crystals cause extensive damage to the microstructure of the cells. (Storey and Storey, 2004)

Most insects are naturally good at supercooling but if an animal begins to freeze after it has supercooled to a low temperature (say -10°C or lower), the speed of ice formation is so high that the insect may be killed or severely injured. So, freeze tolerant insects use nucleators to help them "seed" their body fluids so that they start to freeze somewhere between 0 and -10°C . Sometimes they do this by letting their bodies be seeded through contact with external ice in their environment and sometimes they produce special proteins (called ice nucleating proteins) that trigger ice formation.

Freeze tolerant insects also have antifreeze proteins. This may seem contradictory but the antifreeze proteins are used to manage recrystallization. This is the process by which small ice crystals, which are generally harmless to cells, regroup over time when the insect is frozen into increasing bigger crystals that can do some serious damage. Antifreeze proteins are used to keep small crystals small and thereby limit physical damage from ice during long term freezing.

To keep the inside of cells liquid, freeze tolerant insects pack them with glycerol or other cryoprotectants. So, instead of preventing the entire body from freezing, like freeze avoiding insects do, freeze tolerant insects only focus on protecting the insides of their cells with cryoprotectants. When ice crystals grow in extracellular spaces, they set up an osmotic stress on cells that sucks water out of the cells so that the amount of water inside cells decreases and the cells shrink in size. The high concentrations of cryoprotectants inside cells help to hold onto intracellular water and prevent cells from shrinking below a critical minimum volume.

In insects oxygen is delivered to each tissue and cell by tiny tubes called trachea that lead inwards from small holes (spiracles) that are all over the insect's body. When an insect freezes, oxygen delivery stops for 2 reasons: (a) the muscles that help to keep air flow moving through the trachea freeze, and (b) the extracellular fluid at the end of the trachea freezes. So a frozen insect has to live without oxygen for the whole time that it is frozen. Furthermore, freezing of the hemolymph (the insect equivalent of blood) blocks the delivery of fuels between tissues as well as the removal of waste products. Hence,

freezing is an anoxic/hypoxic (no/low oxygen) and an ischemic (hemolymph flow halted) state and metabolic adjustments must be undertaken to maintain long term cell viability under these stresses.

1.3 Winter needs and stresses

Polyols Synthesis

Both insects use cryoprotectants to prevent the freezing of all body fluids (freeze avoiding insect) or intracellular fluids only (freeze tolerant insect). In *Epiblema scudderiana* glycerol levels rise to over 2 M or about 18% of total body mass whereas *Eurosta solidaginis* accumulates both glycerol and sorbitol in lower amounts that are maximally around 300 mM and 150 mM, respectively (Storey and Storey, 2004). To produce these huge amounts of polyols, it is obvious that major preparations are needed. These include the accumulation of huge amounts of glycogen in the fat body (the liver-like organ of insects) during summer/autumn feeding and the synthesis of increased amounts of key enzymes. Once preparatory measures are made, the actual initiation of polyol synthesis relies on low-temperature triggers that are positioned such that polyol accumulation can be complete before the insect experiences subzero temperatures. Glycerol synthesis in *Epiblema scudderiana* is initiated by exposure to $\sim 5^{\circ}\text{C}$ (in nature three to four consecutive nights with lows in this range are needed), and accumulates at the highest rate at temperatures between 0 and -5°C (the insects are not frozen in this range because their supercooling points in the absence of polyols are typically around -10°C or

lower). In *Eurosta solidaginis*, however, synthesis of the two cryoprotectants are triggered independently; glycerol is triggered by exposures to around 15°C whereas sorbitol synthesis is triggered at 5°C (Storey and Storey, 2004).

Biosynthesis of glycerol and sorbitol uses glycogen as the substrate. The pathways involved are shown in Figure 1.1 (Storey and Storey, 2004). Glycerol production can be accomplished by two routes but is likely mainly via the pentose phosphate pathway because this produces both the 3-carbon sugar (glyceraldehyde) and the NADPH needed to reduce glyceraldehyde to glycerol. The sorbitol synthesis pathway is shorter but involves the same initial steps as for glycerol followed by the conversion of glucose-6-P to glucose (by glucose-6-phosphatase) and then NADPH-linked glucose reduction to sorbitol (by polyol dehydrogenase) (Storey and Storey, 2004). For carbon and NADPH balance during glycerol synthesis some of the glucose-6-phosphate has to be processed via glycolysis and therefore requires ATP input at the fructose-6-P $\rightarrow$ fructose-1,6-P₂ reaction (Storey and Storey, 1991). Because of the ATP requirement, glycerol synthesis should be optimal under aerobic conditions and may be severely limited if low oxygen and/or freezing conditions prevail. Energetic considerations may therefore be the reason for the pattern of polyol synthesis seen in *Eurosta* where glycerol builds up early during the autumn months before the larvae freeze. Production of the C6 sorbitol has no ATP requirement and so can go forward under oxygen-limited conditions. This may be one reason why sorbitol came into use as a cryoprotectant by freeze tolerant insects (Storey and Storey, 1991).

Polyol synthesis is an enormously important event in the cold hardening of insects. Activities of key enzymes including glycogen phosphorylase which provides the carbon for all polyol synthesis and glucose-6-phosphatase which is involved in the synthesis of sorbitol are increased when insects were exposed to 5°C or lower temperature (Storey and Storey, 1988).

In nature, insects that have only one generation a year (univoltine) often show an obligatory acquisition of cold hardiness with the transition to the overwintering life stage (e.g. in *Eurosta* this is the third instar larva). Acquisition of cold hardiness is often linked with photoperiod cues and is accelerated by decreasing environmental temperatures. Cold hardiness and/or freeze tolerance is virtually always completed before subzero temperatures are experienced. We probably could not call the seasonal cryoprotectant synthesis in the two insects a stress condition. However, when we acutely transferred the freeze avoiding and freeze tolerant insects from 15°C to 5°C and further to -15°C in the laboratory environment to see their gene responses, we created stresses on the two insects that they had to act fast to rectify.

Nutrient Depletion and Diapause: The larvae of the two insects stay in the gall for the whole time before they become adults. The gall has a complex structure with a hard corky exterior and interior of specialized nutritive tissues. These latter tissues are the gall insects' only food, as they do not feed as adults. In the fall, as the plants, stems, and galls senesce and turn brown, the larva have to live only on the fat and glycogen they stored

during summer and face the stress of nutrient limitation. Given limited endogenous fuel reserves (the two species studied here will not eat again in their life cycle), the insects must rebalance ATP-utilizing and ATP-producing reactions to ensure energy conservation for long-term survival. To conserve energy and gain life extension, many organisms respond to environmental or physiological stresses with an arrest of the cell cycle and an overall strong metabolic rate suppression. In insects a programmed state of developmental arrest is called diapause, under which metabolic rates are typically <10% of normal values at the same temperature (Storey and Storey, 2004). Food signals also play a role in the formation of a developmentally arrested stage such as the dauer larva in *Caenorhabditis elegans*. Food supply influences both the entry into and exit from the dauer state. A dauer larva was induced by a pheromone when food supply was limited at higher temperatures, whereas an increase in the food supply could inhibit dauer larva formation and enhance its recovery (Golden and Riddle, 1984).

Both *Eurosta* and *Epiblema* have an obligatory diapause that lasts at least 3 months during midwinter. The food signals (the senescence of the plant galls in which the two insects live) alone might not be enough to trigger the insects' transition to a stage of diapause. However, it is likely that the two insects might also take cues from changes in photoperiod (days shorten), and the decrease in autumn temperatures.

Antioxidant Defenses: Most oxygen in cells undergoes tetravalent reduction to form H₂O in the cytochrome oxidase reaction, the final enzymatic step in the mitochondrial

electron transport chain. However, as an unavoidable consequence of oxygen-based metabolism, all cells also continuously produce reactive oxygen species (ROS); this results from the univalent reduction of O₂ in the respiratory chain under some conditions as well as from various other oxidase reactions in cells. ROS can damage many cellular macromolecules and in some cases the damage is severe enough to cause cell death. To protect themselves, living organisms possess a defense system consisting of both enzymatic and nonenzymatic antioxidants that scavenge ROS. Nevertheless, under some specific situations, the rate of generation of ROS exceeds that of their removal and oxidative stress occurs (Morales et al., 2004). A number of environmental factors can lead to oxidative stress including anoxia/ischemia, exposure to toxins, heavy metals and UV irradiation, and food deprivation. Studies in mammalian systems have linked a substantial portion of ischemic damage to organs, not to the period of oxygen deprivation itself, but to the reperfusion phase when oxygen is reintroduced and a burst of oxyradical production can temporarily overwhelm the antioxidant defenses of an organ (Storey and Storey, 2004). Mitochondria are a main intracellular source of reactive oxygen species (ROS). Mitochondrial ROS generation dramatically increases during oxygen reperfusion because the electrons released by the respiratory chain (which becomes highly reduced under anoxia) can be donated directly to the newly supplied oxygen (Domenicali et al., 2001). Starvation has also been reported to have pro-oxidant effects in mammals and was associated with significantly greater lipid and protein oxidative damage. Food deprivation was also associated with greater mitochondrial oxidative injury in rat livers exposed to

warm ischemia-reperfusion, and the extent of oxidative damage in mitochondria increased with the length of food deprivation (Domenicali, Caraceni et al., 2001). Glutathione (GSH) is a main representative of antioxidant systems in mitochondria; this reduced tripeptide is a potent intracellular antioxidant that reduces oxygen free radicals and detoxifies these highly toxic compounds (Deneke and Fanburg, 1989); glutathione has antioxidant effects on its own or it can act a substrate of antioxidant enzymes including glutathione *S*-transferase and glutathione peroxidase. Starvation reduces GSH concentration up to 50% in liver tissue of adult rodents due to the utilization of hepatic and intestinal GSH stores as cysteine reservoirs during food deprivation; this is associated with a striking depletion of antioxidant systems in the whole liver (Robinson et al., 1997; Domenicali et al., 2001).

Both *Epiblema scudderiana* and *Eurosta solidaginis* may suffer nutrient limitation during overwintering. The larvae rely on the breakdown of fatty acids and glycogen to support their fuel needs and glycogen is also used to synthesize cyoprotectants. It is not known whether nutritional limitation in the overwintering larva can trigger oxidative stress in their cells. However, a previous study in our lab did show that the GSH content in *Epiblema scudderiana* collected in March had decreased to 70% of the September value. Furthermore, activities of most antioxidant enzymes: CAT (catalase), GSTPox (selenium-dependent glutathione peroxidase), and GST (glutathione-S-transferase) increased over the winter (Joanisse and Storey, 1996). Since *Epiblema scudderiana* remain liquid throughout the winter, they are not faced with oxidative stress associated

with oxygen reperfusion following an ischemic or anoxic event such as freezing. Therefore, there is a possibility that oxidative stress in the freeze avoiding larvae during overwintering was associated with the food shortage. Furthermore, for lipid depots to remain fluid and metabolizable at subzero temperatures, they need a high content of polyunsaturated fatty acids. These are highly susceptible to free radical attack on their double bonds resulting in lipid peroxidation. Hence, oxidative damage in freeze avoiding insects may also be associated with the winter storage of the large reserves of lipids that are then mobilized in the spring to fuel metamorphosis. Another suspicion was also put forth to explain the induction of oxidative stress in overwintering larvae, that is that low temperature decreased the effectiveness of antioxidant systems and required an increase of the antioxidant enzymes to compensate (Joanisse and Storey, 1996).

With 65% of total body water freezing into extracellular ice, the freeze tolerant insect *Eurosta solidaginis* undergoes hypoxic/anoxic conditions during winter freezing episodes because the delivery of oxygen to cells is halted. Fluctuating temperatures over day/night or longer cycles will result in numerous freeze-thaw cycles over the winter for the larvae. Oxygen reperfusion occurs when extracellular ice thaws and each thaw would likely result in a burst of ROS formation and induce the oxidative stress in cells. However, in contrast to the freeze avoiding species, studies in our lab have shown that GSH levels did not change in *Eurosta solidaginis* over the winter. In addition, the activities of most antioxidant enzymes such as CAT (catalase), GST (glutathione-S-transferases), and GR (glutathione reductase) decreased in the larva over the winter (Joanisse and Storey, 1996).

These data suggest that oxidative stress may not be a major problem for the freeze tolerant insect during the winter.

1.4 Gene Expression in Cold Hardy Insects

Gene expression responses associated with cold hardening can typically occur on two time frames. The first is seasonal and/or cold acclimation responses that accrue over days or weeks prior to exposure to subzero temperatures. Other factors can also come into play over this time frame such as changes in food availability, temperature and photoperiod. The second type of response is rapid responses triggered within minutes to hours when temperature drops (cold shock) or freezing begins (Storey and Storey, 2004). In general it seems that there are relatively few gene and protein changes needed to achieve cold hardiness (Storey and McMullen, 2004); that is, a wholesale reorganization of metabolism is not needed. However, selected specific genes with key roles to play should be up-regulated by cold or freezing exposures. This would predictably include the FoxO proteins that have diverse cellular functions in metabolic regulation and diapause regulation.

1.4.1 Forkhead Box O Transcription Factor Family

Forkhead, the founding member of the entire family (now classified as FOXA), was originally identified in *Drosophila* as a gene whose mutation resulted in ectopic head structures that looked like a fork. Forkhead proteins are also sometimes referred to as

“winged helix” proteins because X-ray crystallography revealed that the DNA-binding domain features a 3D structure with three alpha-helices flanked by two characteristic loops that resemble butterfly wings (Carter and Brunet, 2007). The family is characterized by a conserved DNA-binding domain (the “Forkhead box”, or FOX) and comprises more than 100 members in humans, classified from FOXA to FOXR on the basis of sequence similarity. Forkhead box O (Forkhead box ‘Other’) genes were first identified in humans because three family members (FOXO 1, 3, and 4) were found at chromosomal translocations in rhabdomyosarcomas and acute myeloid leukemias (Carter and Brunet, 2007). In mammals, the class O of forkhead box transcription factors (FOXO) now consists of four members: FOXO1, FOXO3, FOXO4 (previously known as FKHR, FKHL1 and AFX, respectively) and FOXO6. In invertebrates, there is only one FOXO gene identified to date which is termed *daf-16* (Dauer Abnormal Formation-16) in the nematode worm *Caenorhabditis elegans* and dFOXO in the fruit fly *Drosophila melanogaster* (Carter and Brunet, 2007).

FoxO proteins contain a unique insert of five amino acids within the region of the DNA binding domain (alpha-helix 3) that is directly involved in sequence-specific interaction with DNA binding sites. Whereas consensus sequences for binding most Forkhead proteins include a highly conserved common core sequence (AAACA), the sequences flanking this core vary (Barthel et al., 2005). The consensus binding sequence for FoxO proteins [(T/C)(G/A)AAACAA] diverges from that of other Forkhead proteins, providing a mechanism by which FoxO proteins can preferentially interact with a distinct

set of target sites in the genome.

1.4.2 Diverse Functions of FOXO Transcription Factors in Metabolism, Cell Development, Stress Resistance

FoxO proteins are ancient, evolutionarily conserved targets of insulin/insulin-like growth factor signalling that are important in regulating metabolism in response to changes in nutrient availability and environmental conditions (see Fig.1.2). Early studies focusing on the regulation of insulin-like growth factor binding protein-1 (IGFBP-1) provided evidence that signalling through phosphatidylinositol 3-kinase (PtdIns3-K; PI3K) and protein kinase B (PKB; also known as Akt) is necessary and sufficient to mediate the sequence specific effects of insulin on gene expression (Barthel et al., 2005; O'Brien et al., 1990; Cichy et al., 1998). Subsequent studies in *Caenorhabditis elegans* provided genetic evidence that FoxO proteins are major targets of insulin-like signaling (Ogg et al., 1997; Lin et al., 1997). In mammals, FOXO factors function to mediate cell cycle arrest, DNA repair and apoptosis, promote cellular differentiation, and also play a part in protection of cells against genotoxic and environmental stresses. The following are some of the potential roles that FoxO proteins may have in the metabolism of the cold-hardy insects.

Glucose Metabolism: Insulin functions as the prime regulator of glucose homeostasis by stimulating the uptake of glucose and its subsequent metabolism (e.g. glycolysis versus glycogen synthesis, etc.). FoxOs regulate the gene expression of

enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase, which are important enzymes in gluconeogenesis (the conversion of carbon from other sources such as lactate and amino acids back to glucose) (Barthel et al., 2001; Nakae et al., 2001). In addition, FoxO can suppress the expression of genes that are involved in glycolysis, the pentose phosphate pathway and lipogenesis (Zhang et al., 2006). Therefore, FoxO activity induces a metabolic switch that is similar to low glucose and fasting conditions. The function of FoxO transcription factor makes it a candidate that might play a role in polyol synthesis in cold hardy insects because the production of extremely high levels of polyols requires a reorganization of carbohydrate metabolism to feature pathways that are very different from the common routes of carbohydrate use in cells.

Cell Cycle Arrest: Insulin–PI3K signalling stimulates cell-cycle progression and, in part, this relies on the repression of FoxO-induced cell-cycle inhibition through regulating factors such as p27^{kip1} and p130^{Rb2} (Kops et al., 2002; Medema et al., 2000). Oppositely, in the absence of insulin signalling (e.g. during food starvation), FoxOs induce cell-cycle arrest and entry into cellular quiescence (Kops et al., 2002) which is reminiscent of the dauer switch that is induced by DAF-16 in *C. elegans*. Both freeze avoiding and freeze tolerant insects enter diapause states over the winter when food is no longer available and temperature is low. Whether the diapause state was triggered by FoxO transcription factor and whether nutrition and/or temperature signals play roles in

the activation of FoxO genes/proteins in the two cold-hardy insects remain largely unknown.

Stress Resistance: FOXO proteins play a part in protection of cells against genotoxic and environmental stresses. Recent studies have identified a number of FOXO-regulated genes involved in a variety of cellular stress responses, ranging from defence against oxidative and caloric stresses to DNA repair (Greer and Brunet, 2005). In particular, FOXO3a enhances the expression of the antioxidant enzymes mitochondrial manganese superoxide dismutase (MnSOD) and catalase, which are scavengers of oxygen-free radicals (Kops et al., 2002). Cells lacking MnSOD have greatly reduced oxidative damage protection, demonstrating the critical role of this mitochondrial antioxidant in the oxidative stress defence pathway. Consistent with this, enhanced expression of FOXO3a increases both hydrogen peroxide scavenging and oxidative stress resistance. This is further demonstrated by FOXO-mediated upregulation of the fatty acyl-CoA carriers sterol carrier protein-x (SCPx) and SCP2 (Dansen et al., 2004), which probably protect unsaturated fatty acids from oxidative damage to ensure that they are properly processed.

1.4.3 Regulation of FOXO Proteins by Phosphorylation

The activity of FOXO proteins is carefully controlled at multiple levels, including initiation of gene expression, subcellular localization, protein degradation and most prominently, signal-induced post-translational modifications (Horst and Burgering, 2007).

PKB-dependent phosphorylation disables FOXO proteins by inhibiting the FOXO nuclear translocation signal and exporting these transcription factors from the nucleus, thereby inhibiting their transcriptional competence. Phosphorylated FOXO proteins are retained in the cytoplasm by the 14-3-3 chaperone proteins. Another consequence of PKB phosphorylation is that phosphorylated FOXO proteins are targeted for proteosomal degradation through interaction with specific ubiquitin E3 ligases such as Skp2 (Huang et al., 2005).

Conversely, it has been reported that phosphorylation of cytoplasmic FOXO by the stress-activated JNK (c-Jun N-terminal kinase) microtubule-associated protein reverses the nuclear–cytoplasmic shuttling in response to PI3K/PKB activation. For instance, reactive oxygen species generated as a consequence of oxidative stress induced the activation of the small GTPase, Ral, which in turn activates JNK. JNK dependent phosphorylation of FOXO proteins results in nuclear accumulation and enhanced transcriptional activity, leading to increased expression of antioxidants such as MnSOD (Essers et al., 2004). JNK has also been reported to phosphorylate 14-3-3 proteins, resulting in the release of bound FOXO transcription factors (Sunayama et al., 2005). However, the ability of JNK to relocate FOXO to the nucleus appears to be dependent on the simultaneous repression of PKB activity, at least in breast cancer cells (Lam et al., 2006).

1.4.4 FoxO Proteins in Invertebrates

Studies of the nematode, *Caenorhabditis elegans*, have provided insights into the conservation of signaling pathways from lower invertebrates to the highest vertebrate groups. The invertebrate homologue of mammalian FOXO is DAF-16. Of the three genes of the mammalian FOXO family: FOXO3a, FOXO1, and FOXO4, Daf-16 shares the highest identity (76%) with FOXO1 in the forkhead DNA-binding domain which consists of about 100 amino acids. In *C. elegans*, the Daf-2 gene encodes the nematode homologue of the insulin/IGF-1 receptor; the Age-1 gene is the homologue of PI 3-kinase, and the two Akt genes are homologous to mammalian Akt. Mutants in DAF-2 and AGE-1 arrest development at the dauer stage in *C. elegans* (Riddle, 1997). Mutations of the Daf-16 gene prevent Daf-2, Age-1, and Akt mutants from entering the dauer stage (Ogg et al., 1997). This observation suggests that Daf-16 is a negative regulator of the Daf-2/Age-1/Akt signalling pathway, just as FOXO1 is a negative regulatory of insulin/PI 3-kinase/Akt signalling in mammals. Daf-16 has four consensus Akt phosphorylation sites, raising the possibility that Daf-16 may be a direct substrate of Akt (Nakae et al., 1999).

The *Drosophila* genome encodes a single FOXO ortholog, dFOXO, whose sequence includes three Akt phosphorylation consensus sites similar to those found in mammalian FOXOs and nematode Daf-16. As in these proteins, phosphorylation of dFOXO is stimulated by Akt activation in response to insulin, and this, in turn, results in its cytoplasmic localization and transcriptional inactivation (Puig et al., 2003). Studies demonstrated that overexpression of dFOXO or mammalian FOXO proteins in developing

Drosophila tissues leads to a significant reduction in growth. Importantly, more severe phenotypes are obtained by expression of FOXO proteins lacking their Akt phosphorylation sites, or by coexpression of wild type dFOXO with an inhibitory version of PI3-kinase. The degree of growth suppression by dFOXO also increases in response to nutrient deprivation (Jünger et al., 2003), which has been shown to reduce the levels of insulin-like protein expression. Together these results provide in vivo support for the idea that FOXO proteins are negative regulators of growth in response to conditions of low insulin signalling.

Hypotheses

1. Changes in the expression of FoxO proteins occur in response to cold and/or freezing exposures in *Epiblema scudderiana* and *Eurosta solidaginis* and are one of the regulatory mechanisms of cold hardiness.
2. Changes in the expression of FoxO proteins at low temperatures will stimulate the expression of downstream genes to enhance the expression of antioxidant enzymes in the larvae.

Summary

FoxO proteins are transcription factors that mediate gene expression in response to diverse cellular stresses that might be triggered during insect cold-hardening. The objectives of this study were to analyze the expression of FoxO genes and downstream

proteins in response to cold temperatures and/or freezing exposures in freeze avoiding and freeze tolerant insects. Gene and protein expression were tested separately in the two insect species. Chapter 3 evaluates FoxO3a gene and protein expression as well as MnSOD protein expression in the freeze avoiding insect, *Epiblema scudderiana*, in response to time courses of exposure to 5°C and -15°C. Chapter 4 reports the expression of FoxO3a, FoxO1 and MnSOD proteins in the freeze tolerant species, *Eurosta solidaginis*, under the same cold exposures as well as in response to thawing after long term freezing.

Figure 1.1 Pathways of polyol synthesis in insects. Enzyme abbreviations are: GP, glycogen phosphorylase; G6PDH, glucose-6-phosphate dehydrogenase; G6Pase, glucose-6-phosphatase; PDH, polyol dehydrogenase; PFK, phosphofructokinase. From Storey and Storey (2004).

Fig.1.1

Figure 1.2 Insulin signalling pathway of FOXO transcription factors. FOXO proteins are major targets of insulin/insulin-like growth factor signalling. The transcriptional activity of FOXO proteins is controlled by PI3K and Akt (PKB) through phosphorylation. In the absence of insulin or growth factors, FOXO transcription factors are activated through dephosphorylation and translocate to the nucleus, where they specify target gene expression. For example, FOXO factors regulate glucose metabolism by upregulating Glucose-6-Phosphatase in response to low insulin levels; promote the cell-cycle arrest by upregulating the cell cycle inhibitor protein 27(kip1) and protein 130(Rb2) to induce G1 or G0 arrest. FOXO factors are also involved in reactive oxygen species detoxification via upregulation of two antioxidant enzymes catalase and MnSOD. Picture was from *Current Biology* (Carter and Brunet, 2007).

Fig.1.2

Current Biology

CHAPTER TWO

Methods and materials

2.1 Animal treatments

Goldenrod galls containing last instar larvae of *Eurosta solidaginis* and *Epiblema scudderiana* were collected in October, 2006 from fields around the Ottawa area. Galls of both insects were put in cardboard boxes and either acclimated at 15°C (for those collected when outdoor temperature was around 15°C) or 5°C (for galls collected when outdoor temperature well below 15°C but still above 0°C) for 3 weeks. Some of the galls were then opened and sampled as controls (15°C control and 5°C control). The rest of galls were used as follows: (a) the ones acclimated at 15°C were used for the 5°C time course, and (b) the ones acclimated at 5°C were used for the -15°C time course.

For *Epiblema scudderiana*, galls acclimated at 15°C or 5°C were divided into groups and then transferred acutely (short term, in vitro) to 5°C (from 15°C) or -15°C (from 5°C), respectively. Galls of each group were opened and sampled after set time periods at the new temperature (see Fig. 2.1).

For *Eurosta solidaginis*, the time course of exposure to 5°C was done the same way as above for *Epiblema scudderiana*. That is that galls acclimated at 15°C were divided into groups, transferred to 5°C and sampled after set time intervals. However, for the -15°C time course, galls acclimated at 5°C were first opened and the larvae were removed, divided into groups, and each group was put in a Petri plate with damp filter paper. This method was used because there is a high rate of parasitism of *Eurosta solidaginis* galls so removing larvae to Petri plates ensures an adequate number at each time point. Petri plates were held at 5°C for a further 24 h and then any damaged larvae were removed before the plates were transferred acutely (short term, in vitro) from 5°C to -15°C. For sampling, one plate was removed at each time point of freezing exposure and recovery (see Table 2.1). Some *Eurosta solidaginis* larvae were recovered to 5°C for certain times after being frozen at -15°C for 1 week.

For sampling, galls were opened quickly and larvae were flash-frozen in liquid nitrogen and then stored at -80°C before using. The animal experiments and the sample points for the two insect species are outlined in Figure 2.1 and Table 2.1, respectively.

Selected insects were checked for survival after the 5°C to -15°C switch by warming the frozen or supercooled insects at room temperature ($\sim 20^{\circ}\text{C}$) for about one hour. Both species survived the subzero exposure.

2.2 Sample Preparation

2.2.1 Isolation of Total Soluble Protein

For each sample point, 6 larvae (~ 0.3 g) and 0.5 ml homogenizing buffer were used to isolate total protein. Larvae and the volume of homogenizing buffer can be multiplied depending on the total amount of larvae. Five independent extracts were made for each sample point. Sample vials containing frozen larvae were held in liquid nitrogen until use. Larvae were quickly crushed in a mortar and then transferred to a test tube and homogenized in homogenizing buffer with the Polytron homogenizer for 1-2 minutes. The homogenizing buffer contained: 20 mM HEPES, 400 mM NaCl, 20% v:v glycerol, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM NaF, 10 mM β -glycero-phosphate, and 1 mM Na_3VO_4 . An aliquot of Sigma protease inhibitor cocktail, 1:1000 (v:v), and a full microspatula tip of phenylmethylsulfonyl fluoride (PMSF) were added just before homogenizing to prevent degradation of the proteins by proteases. The homogenate was centrifuged at 12,000 xg at 4°C for 15 minutes and the supernatant ("white" protein extract) was transferred to a fresh tube and chilled in ice.

2.2.2 Measurement of Protein Concentration

Total protein concentrations in each sample were determined by the Coomassie blue dye-binding method using the Bio-Rad prepared reagent with bovine serum albumin as the

standard. The Bio-Rad reagent was diluted 5-fold with distilled water and protein extracts were diluted 1:60 (v:v). For protein assay, 10 μ l of diluted protein extracts were added to 190 μ l of Bio-Rad reagent in microplate wells. For blanks 10 μ l of distilled water was added instead. Samples were mixed and color was developed for 5 min followed by measurement of absorbance at 595 nm. Each sample was assayed 3 times (n=3). Blank values were subtracted and protein concentration was determined from a pre-programmed standard curve.

2.2.3 Preparation of Blue Protein Samples

After measuring protein concentrations, “white” protein extracts were diluted to a concentration of 8 μ g/ μ l with homogenizing buffer. Then a measured volume of “white” extract was mixed with an equal volume of 2X SDS buffer which contained 100 mM Tris-HCl pH 6.8, 4% w:v SDS, 20% v:v glycerol, and 0.4% w:v bromophenol blue with 10% v:v 2-mercaptoethanol added just before use. This gave final protein concentrations in “blue” extracts of 4 μ g/ μ l. The protein mixtures were then boiled in a water bath for 5 minutes and stored at -80°C.

2.3 Western Blotting

Western blot analysis is based on antigen-antibody interaction that allows probing for proteins of interest. Briefly, proteins are separated by electrophoresis in SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes. The membrane is blocked with nonfat milk and probed first with antibody specific to the protein of interest and then with a secondary antibody, an anti-immunoglobulin linked to an enzyme (horseradish peroxidase; HRP). Detection is performed using ECL reagents (Immobilon™ Western Detection Reagents, Millipore Corporation, U. S. A.).

2.3.1 SDS-Polyacrylamide Gel Electrophoresis

“Blue” protein samples that contained 10-20 µg total protein (depending on the protein of interest) were loaded into the SDS-polyacrylamide gel. The concentration of the gel varied from 8% to 15% (8-15% acrylamide, 0.4 M Tris pH 8.8, 0.1% SDS, 0.1% APS, 0.06-0.04% TEMED) depending on the size of the protein of interest. Each sample point had 5 independent trials which were loaded into 5 individual wells. The protein samples from stress conditions were always run with the corresponding control sample on one gel. An aliquot of Kaleidoscope prestained protein standard (Bio-Rad) was also loaded in one well in order to show the size of proteins. Electrophoresis was run at 180 V in 1X running buffer (3.03 g Tris base, 14.4 g glycine and 1g/l SDS, pH ~8.3) till the tracking dye in the protein sample reached the bottom of the gel.

After electrophoresis, proteins were electroblotted onto polyvinylidene difluoride (PVDF) membrane by wet transfer with transfer buffer (25 mM Tris pH8.5, 192 mM glycine, 20% v:v methanol) at 4°C, 160 mA for 75-100 min (depending on the size of the protein). In some cases, complete protein transfer from the gel was confirmed by staining the gel with Coomassie blue dye (0.25% w/v Coomassie brilliant blue R, 50% v/v methanol, 10% v/v acetic acid) followed by destained in 25% v/v methanol, and 10% v/v acetic acid until background levels were acceptable. Almost complete transfer was ensured when there were no blue protein bands shown on the gel.

2.3.2 Antibody Incubations

Before antibody incubations, the blots were blocked with non-fat milk in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20). The concentration of non-fat milk (0.5%-2.5%) for blocking and the blocking time (10-60min or not block at all) were optimized and were shown in Table 2.2. The blots were quickly washed 3 times with TBST after blocking and then incubated with primary antibody at 4°C overnight followed by the secondary antibody incubation for 1.5 hours at room temperature. The concentration of primary antibody was optimized with the source that an antibody came from and the protein detected (see Table 2.2). Secondary antibodies were normally diluted to 1:2000 (v:v) with TBST.

Protein band of interest was confirmed with blocking peptide when it was available. Briefly, the protocol for peptide competition for an antibody was done as follows: prepare a membrane that contains at least two lanes of equal amount of the protein of interest, cut the membrane so that each half of the membrane contains the lane(s) of the protein of interest. One of the half membranes was incubated with the primary antibody against the protein of interest at an optimized concentration; another half membrane was incubated with the mixture of primary antibody and blocking peptide. The concentration of the primary antibody in the mixture solution was made same as the former primary antibody solution by splitting the stock primary antibody solution into two aliquots. One was added blocking peptide and mixed for 1-2 hours at room temperature to make the antibody/peptide mixture solution. Both antibodies (with and without peptide) were used for Western blotting. The ratio of primary antibody versus blocking peptide (1:5-1:50) in the mixture solution was optimized so that one protein band on the membrane incubated with the mixture solution disappeared compared to the band on another membrane incubated with primary antibody solution without peptide. The disappeared protein band was the band of protein of interest.

2.3.3 Protein Detection and Quantification

Detection of the protein signal used the enhanced chemiluminescence reagent (Immobilon™ Western Detection Reagents, Millipore Corporation, U. S. A.) following the manufacturer's protocol. Light signals of immunoblotted protein bands were visualized and saved using a ChemiGenius (Syngene, MD, USA). The membranes were stained thereafter with Coomassie blue staining solution (see 2.3.1). Stained blue protein bands were also visualized and saved by ChemiGenius.

Intensities of immunoblot bands and selected Coomassie-stained bands were quantified using the GeneTools program (Syngene, MD, USA). Intensity of the immunoblot band of interest in each lane was normalized against the corresponding Coomassie-stained bands in the same lane. The intensity ratio of the two type of signals (light-emitting intensity and absorbance) eliminated the loading variations among lanes and the normalized band intensity values were used to do further statistical analysis.

Statistically significant differences between control and stress conditions (both $n=5$) were evaluated using the Student's t-test with a probability level of $P<0.05$ accepted as significantly different.

2.4 RT-PCR

2.4.1 RNA Isolation and Purity Assessment

Tubes, tips and water used for RNA isolation were treated overnight with 0.1% (v/v) diethylpyrocarbonate (DEPC) solution at room temperature and later autoclaved. Facilities were washed with 0.1% DEPC solution before use.

For gene quantification, 5 parallel isolations were done for each sample point with 2-3 larvae (~0.1 g) pooled per trial. Larvae were crushed into a powder under liquid nitrogen in a mortar prior to being homogenized in 1 ml Trizol™ reagent (Gibco-BRL, Bethesda, MD).

Homogenates were allowed to stand for 5 min at room temperature followed by the addition of chloroform (0.2 ml/ml of Trizol). Samples were shaken for 15 sec, held at room temperature for an additional 2-3 min and then centrifuged at 11,000 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 shaken for 30 sec. The mixture was held for 10 min at room temperature to allow RNA precipitation before being centrifuged at 12,000 x g for 15 min at 4°C. The pellet was washed with 1 ml of 70% ethanol and mixture was centrifuged at 12,000 x g for 5 min at 4°C. The total RNA pellet was dried for 5min at room temperature and resuspended in DEPC-treated water and stored at -80°C until further use.

The quantity and quality of the total RNA were assayed spectrophotometrically at 260 nm and 280 nm. The ratio of A260/A280 was measured to determine RNA quality. A value of 1.6 or higher indicated that the RNA samples were relatively pure.

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

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

2.4.2 First Strand Synthesis

First strand cDNA synthesis was performed following the manufacturer's instruction (M-MLV reverse transcriptase, Invitrogen). That is, 1-5 µg of total RNA in 9.5 µl of DEPC-treated water was placed in a 0.5 ml tube to which was added 2.5 µl (200 ng/µl) of Oligo dT primer (5'-TTTTTTTTTTTTTTTTTTTTTV-3'; V=A or G or C) (Sigma Genosys) and 1 µl of dNTPs (10 mM, Invitrogen). The tube was heated in a 68°C water bath for 5 min then quickly chilled on ice for >1 min. To this was added 2 µl DTT (0.1 M, Invitrogen), 4 µl of 5X first strand buffer, and 1 µl M-MLV reverse transcriptase (200 Units, Invitrogen) for a total volume of 20 µl. The mixture was incubated at 37°C for 60 min and then at 70°C for 15min. cDNA was stored at -20°C.

A 5 µl aliquot of first strand cDNA product was mixed with 45 µl DEPC-treated water to produce 5 fold dilution, the procedure was repeated to make 25, 125, and 625 fold cDNA dilutions as compared with the stock cDNA samples.

2.4.3 Gene Specific Primer Design and Synthesis

Degenerate primers were designed using DNAMAN 4.11 (Lynnon Biosoft) and Primer Designer software (Scientific and Educational Software). The sequence for the gene of

interest was retrieved from the NCBI Genbank for several species and then aligned and compared using DNAMAN. Highly conserved regions were identified and were used to design primers for the gene of interest using Primer Designer based on the variables including primer length, melting temperature, G/C content and 3'-end sequence. The designed primers were then synthesized by Sigma Genosys and were aliquoted into 300 pmol/ μ l stocks in DEPC-treated water and stored at -20°C.

2.4.4 mRNA Hybrid by RT-PCR

Reverse Transcription Polymerase Chain Reaction (RT-PCR) was used to amplify cDNA and quantify the relative levels of selected mRNA species in tissue extracts of control versus stressed larvae. The PCR reaction mixture of 25 μ l final volume was composed of 5 μ l of diluted cDNA, 1.25 μ l primer mixture (30 μ M forward and 30 μ M reverse primer, Sigma), 14 μ l of distilled water, 2.5 μ l 10X PCR buffer (Invitrogen), 1.25 μ l MgCl₂ (50 mM, Invitrogen), 0.5 μ l dNTP mixture (10 mM, Invitrogen), and 0.5 μ l Taq DNA Polymerase (kindly provided by Dr. W.G. Willmore). After mixing well and a quick spin, PCR reactions were performed in the thermocycler. The running cycles of PCR reaction and the annealing temperature of primer pairs were optimized before a specific gene was quantified. The optimized steps of PCR reaction are: (a) 94°C initiation for 2 min, (b) 35 cycles of running: 94°C for 45 sec (denaturation), 55°C for 45 sec (annealing) and 72°C for 45 sec (elongation), (c) final elongation at 72°C for 7 min, and (d) hold at 10°C.

The housekeeping gene β -Actin was amplified as an internal control. The primers for β -Actin were forward (5'-CACCAACTGGGACGACATGG-3') and reverse (5'-GTCGGCCAGCTCGTAGCTCT-3').

PCR products were separated on a 1% agarose gel by electrophoresis at 130 V for 30-50 min, and stained with ethidium bromide. DNA band intensities were visualized and quantified using the ChemiGenius Bio Imaging System (SynGene) and GeneTools program.

2.4.5 DNA Sequencing and Quantification

PCR products were sequenced to confirm that they encoded the target gene. The PCR product was excised from the agarose gel and purified with Sigma GenElute™ Gel Extraction Kit following the manufacturer's instructions. Briefly, the DNA band of interested gene was cut out from the agarose gel under UV light and weighed. Three gel volumes of Gel Solubilization Solution (3:1 v:w) was then added to dissolve the gel slice with incubation at 60°C for 10 min. One gel volume of 100% isopropanol was then added to the solubilized gel solution and mixed until homogenous. A binding column was prepared by adding 500 µl Column Preparation Solution to the column and centrifuged for 1 min. The gel solution was loaded into the column in 700 µl portions each time and washed through the column via centrifugation for 1 min. The column containing bound DNA was then washed with 700 µl of Wash Solution and then pure DNA was eluted off the column with 25 µl Elution Solution which was preheated to 65°C. The concentration of DNA sample was quantified by measuring the absorbance of the sample at 320 nm on the GeneQuant Pro spectrophotometer.

A mixture of 10 µl (10-20 µg DNA) of diluted pure DNA sample and 5 µl of single primer (5 mM, forward or reverse) was sequenced by Ontario Genomics Innovation Centre (Ottawa, ON, Canada). The sequence was verified as the sequence expected via the program BLASTN (<http://www.ncbi.nlm.nih.gov/blast>) at the National Institutes of Health, Bethesda, MD.

The amount of cDNA present in control versus stressed 1st strand samples was normalized (standardized) by using the housekeeping primers (β -Actin). This is important so that mRNA levels of target genes in stress conditions can be compared with that in control

accurately. This was achieved by running parallel RT-PCR reactions using primers of a housekeeping gene (β -Actin) on each of the dilution sets of stress and control cDNA samples. The concentration of cDNA samples of control and stress may be adjusted so that the band intensities of β -Actin in control and stressed samples were expressed relatively consistent.

mRNA values are analyzed as a ratio of the signal from the gene of interest in the selected linear amplification cycle divided by the β -Actin signal in the same condition. The relative mRNA levels for stress condition and control were calculated as the division of gene under stress (or control) over β -Actin under stress (or control).

The Student's t-test was used to evaluate a significant difference between normalized experimental and control samples at $P < 0.05$.

Figure 2.1. Flow chart for insect experiments and sampling

Fig.2.1

Table 2.1. Insect experiments and their corresponding sample points

Experiment	Sample points
1	1
2	2
3	3
4	4
5	5
6	6
7	7
8	8
9	9
10	10
11	11
12	12
13	13
14	14
15	15
16	16
17	17
18	18
19	19
20	20
21	21
22	22
23	23
24	24
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78	78
79	79
80	80
81	81
82	82
83	83
84	84
85	85
86	86
87	87
88	88
89	89
90	90
91	91
92	92
93	93
94	94
95	95
96	96
97	97
98	98
99	99
100	100

Table 2.1

<i>Epiblema scudderiana</i>									
Insect Experiments	Sample points								
15°C to 5°C	1h	4h	1d	3d	1w				
5°C to -15°C	1h	2h	4h	12h	1d	1w			
<i>Eurosta solidaginis</i>									
15°C to 5°C				4h			1d		1w
5°C to -15°C	30m	1h	2h	4h	8h	12h	1d	3d	1w
-15°C to 5°C recovery		1h		4h			1d	3d	

Table 2.2. Primary and Secondary antibodies and their applying conditions

Table 2.2

Primary antibodies	Sources	Blocking	Primary antibody dilutions	Secondary antibody dilution
FoxO3a	Cell Signalling	1% milk 30min	1:500	Antirabbit 1:2000
Phospho-FoxO3a-ser253	Cell Signalling	1% milk 30min	1:500	Antirabbit 1:2000
Phospho-FoxO3a-ser318/321	Cell Signalling	1% milk 30min	1:500	Antirabbit 1:2000
FoxO1	Cell Signalling	1% milk 30min	1:1000	Antirabbit 1:2000
Phospho-FoxO1-ser256	Cell Signalling	1% milk 30min	1:750	Antirabbit 1:2000
MnSOD	Stressgen	1% milk 30min	1:2000	Antirabbit 1:2000

CHAPTER THREE

Profile of FoxO Genes and Proteins in a Freeze Avoiding Insect, *Epiblema scudderiana*, Exposed to Low Temperature

3.1 Introduction

Epiblema scudderiana (Clemens) (Lepidoptera, Olethreutidae) is the caterpillar of the goldenrod gall moth. It lives inside elliptical galls on the stems of goldenrod. The gall is camouflaged with wood-grain like scars on the outside but frequently the presence of the gall makes the plant above it split out into a more bushy appearance so you can often find the galls by looking at the appearance of the plant. The final instar larva overwinters. It moves out of the main gall cavity and down into the hollowed out stem below where it fits snugly and vertically in a head up position. The caterpillar lines the interior of the stem and gall with silk which probably helps to act as a barrier to water or ice penetration.

The strategy used by *Epiblema scudderiana* for cold hardiness is freeze avoidance. By mid-winter, larvae in the Ottawa area can supercool to -38°C . Triggered when autumn temperatures fall to $\sim 5^{\circ}\text{C}$, the insects convert their huge stores of glycogen to glycerol to keep from freezing. Glycerol reaches levels of over 2 M, representing as much as 18% of the total body mass of the larvae. High glycerol plus the production of antifreeze proteins results in a suppression of the insect's supercooling point from -14°C in September to -38°C by December, a value well below the lowest expected environmental temperature. Freeze avoidance is also aided by a decrease in total body water content and by the presence of a waterproof silk cocoon that lines the interior of the stem cavity to prevent inoculative freezing by contact with environmental ice (Storey and Storey, 2004).

Acquisition of cold hardiness is often linked with photoperiod cues and is accelerated by decreasing environmental temperatures. The actual initiation of polyol synthesis relies on low-temperature triggers that are positioned such that polyol accumulation can be complete before the insect experiences the subzero temperatures that

require cryoprotection. For species like *Epiblema scudderiana* that produce only glycerol, synthesis is initiated by exposure to $\sim 5^{\circ}\text{C}$ (in nature three to four consecutive nights with lows in this range are needed), and glycerol accumulates at the highest rate at temperatures between 0°C and -5°C (insects are not frozen in this range because supercooling points of cold hardy species are typically -8°C or lower even in summer).

Insects that have only one generation a year (univoltine) often show an obligatory acquisition of cold hardiness with the transition to the overwintering life stage as well as an obligatory period of diapause (arrested development) before they can resume growth and development (Storey and Storey, 2004). The seasonal acquisition of cold hardiness involves changes in the expression of multiple genes. Gene expression responses can occur on two time frames: (1) seasonal and/or cold acclimation responses that accrue over days or weeks prior to exposure to potentially damaging subzero temperatures, and (2) rapid responses triggered within minutes to hours when temperature drops (cold shock) or freezing begins. Other factors can also come into play such as changes in food availability; goldenrod gall forming insects also seem to take cues from the senescence of the plant galls in which they live (Storey and Storey, 2004)

The known roles of FoxO proteins in other systems include some actions that could be used to help organisms achieve winter cold hardiness. As documented in Chapter 1, their diverse cellular functions are at the interface of crucial cellular processes, orchestrating programs of gene expression that regulate cell cycle arrest, glucose metabolism, and stress responses (e.g. nutrient depletion, oxidative defenses). Locked inside goldenrod galls over the winter, *Epiblema scudderiana* have to face multiple environmental stresses such as low subzero temperatures, desiccation (cold air is very dry

and the dead plant quickly dries out), lack of nutrients, and oxidative stress as well as maintain a diapause state. I hypothesized, therefore, that changes in FoxO gene and protein expression would occur in response to cold exposure of *Epiblema scudderiana* and would trigger enhanced expression of downstream genes regulated by FoxOs.

This chapter tested the responses of two members of the FoxO transcription factor family: FoxO3a (also called FKHRL1) and FoxO1 (or FKHR), as well as one of the downstream proteins controlled by FoxO3a: manganese superoxide dismutase (MnSOD). FoxO1 shares the high identity (76%) with Daf-16 from *Caenorhabditis elegans* in the forkhead DNA-binding domain (Nakae et al., 1999), and FoxO3a is an orthologue of dFOXO in *Drosophila*. The basic molecular pathway of insulin signaling to DAF-16/FoxO is identical among worms, flies and mammalian cells and, therefore, is apparently conserved throughout evolution (Van der Horst et al., 2007). To maintain sufficient ATP generation under low glucose and fasting conditions, cells rely on fatty acid breakdown and oxidative phosphorylation. To scavenge the ensuing oxygen free radicals produced in peroxisomes and mitochondria, FoxOs upregulate several antioxidant enzymes such as MnSOD (Kops et al., 2002).

3.2 Materials and methods

3.2.1 Antibodies and western blotting

Polyclonal antibodies against human FoxO3a and FoxO1a as well as two phospho-FoxO3a antibodies recognizing phosphorylation at serine 318/321 and serine 253 and one phospho-FoxO1a antibody recognizing phosphorylation at serine 256 were purchased from Cell Signaling Technology Inc. The MnSOD antibody was from Stressgen

Biotechnologies Corp.

The FoxO3a antibody detects exogenous and endogenous levels of total FoxO3a protein, and gave a broad band at molecular weight 85-97 kDa on Western blots separating *Epiblema* proteins. Phospho-FoxO3a (Ser253, Ser318/321) antibodies detect FoxO3a only when phosphorylated at serine 253 or serine 318/321 and both gave a single band at 97 kDa. The FoxO1 antibody detects endogenous levels of total FoxO1 protein and FoxO4, and gave two bands at 78-82 kDa (FoxO1a) and one band at 65-70 kDa (FoxO4) with *Epiblema* extracts. Phospho-FoxO1 (Ser256) antibody detects endogenous levels of FoxO1 only when phosphorylated at serine 256 and gave two bands at 82 kDa. The MnSOD antibody gave a specific single band at 25 kDa.

The conditions for Western blotting and the concentrations of antibodies used were as shown in chapter 2 Table 2.2. For analysis of FoxO3a, FoxO1 and MnSOD, 10% SDS-polyacrylamide gels were used. Membranes containing FoxO3, FoxO1 and MnSOD proteins were blocked with 1% non-fat milk for 30 min prior to primary antibody incubation.

3.2.2. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

FoxO3a mRNA from *Epiblema scudderiana* was amplified via RT-PCR using primers that were designed from the consensus sequence of mammalian FoxO3a genes. Forward and reverse primer sequences for FoxO3a were 5'-GCTCTTGGTGGATCATCAAC-3' and 5'-TCCAGCAGGTCGTCCATGAC-3', respectively. Amplified FoxO3a cDNA was purified and sent to Ontario Genomics Innovation Centre (Ottawa, ON, Canada) for sequencing. The cDNA sequence of FoxO3a

in *Epiblema scudderiana* was confirmed by blasting the sequence of FoxO3a at the NCBI website (<http://www.ncbi.nlm.nih.gov>) via the BLASTN program.

Relative transcript levels of FoxO3a mRNA were evaluated by RT-PCR from larvae under 3 conditions: 15°C controls, and 5°C cold exposures for 1 and 4 hours. Total RNA was extracted from n=4 samples of larvae from each condition. Total RNA (~5 µg) was used to synthesize cDNA. Serial dilutions of cDNA were prepared, each being a 5-fold dilution of the previous sample; final sample dilutions were 5, 25, 125, and 625 fold as compared with the stock cDNA samples. Each diluted cDNA sample was subjected to PCR using both FoxO3a and β -Actin primers at optimized annealing temperature for both primers (56°C). β -Actin was used as an internal control to normalize FoxO3a mRNA levels in each trial to allow comparison between control and stress samples. FoxO3a relative mRNA levels were obtained from the most dilute cDNA samples in the series of cDNA dilutions that gave visible PCR bands for both FoxO3a and β -Actin. This ensured that the cDNA amplification was within the linear amplification range of the progress logarithmic curve (the exponential phase PCR signal strength for both FoxO3a and β -Actin gene at 35 cycles). Expression ratios of FoxO3a mRNA versus β -Actin were calculated for control and experimental samples, and the mean ratio of 4 trials with SEM error bars were plotted. Significant differences were tested using the Student's t-test.

3.3 Results

3.3.1 FoxO protein profile during cooling and subzero exposures of *Epiblema scudderiana*

Cooling exposure at 5°C: Western blots showing FoxO3a protein levels in

Epiblema scudderiana over a time course of cooling at 5°C for 1 hour, 4 hours, 1 day, 3 days, and 1 week (n=5) are shown in Fig. 3.1A. Comparable protein bands of phospho-FoxO3a (Ser253) in the same series of samples are shown in Fig. 3.1B. Mean total FoxO3a and phosphorylated FoxO3a protein expression are shown as a histogram in Fig.3.1C in comparison to control values at 15°C that are set to a mean of 1.0. Compared to 15°C controls, levels of total FoxO3a protein decreased significantly to 61% of the control after larvae were exposed to 5°C temperature for 1 hour. Total FoxO3a protein levels rose back to the control level when cooling time was extended to 4 hours and 1 day but was reduced again to 57% of the control level after larvae were cooled at 5°C for 3 days. However, control values were re-established again after 5°C exposure for 1 week. The relative amount of phosphorylated FoxO3a (Ser 253) in the larvae remained constant over the whole time course of 5°C cooling exposure compared to controls. Another phosphorylated form of FoxO3a at serine 318/321 was assessed in both control and experimental samples but was not detectable. This may indicate that insect FoxO3a does not have these phosphorylation sites or that the antibody used does not recognize the sequence around these sites in the insect protein.

Subzero exposure at -15°C: *Epiblema scudderiana* larvae were further exposed to -15°C after being cooled at 5°C for 1 week. FoxO3a and phospho-FoxO3a (Ser 253) content were assessed after -15°C subzero exposure for 1 hour, 2 hours, 4 hours, 12 hours, 1 day, and 1 week (Fig 3.2A,B). Histograms summarizing the changes in total FoxO3a and phosphorylated FoxO3a (Ser 253) levels compared with 5°C controls are shown in Fig. 3.2E. Total FoxO3a protein levels in the larvae remained constant after -15°C exposure for

1 hour, 2 hours and 4 hours. After longer times, total FoxO3a protein dropped significantly; levels fell to 62%, 55% and 54% of the control value after 12 hours, 1 day and 1 week, respectively. Phosphorylated FoxO3a (Ser 253) levels were largely unchanged over most of the time course but had decreased significantly to 22% of the control level when larva was acutely exposed to -15°C for 1 day.

Western blotting to detect total FoxO1 and phosphorylated FoxO1 (Ser 256) protein was also carried out with *Epiblema scudderiana* samples, but neither of the two protein forms was detectable.

3.3.2 FoxO3a mRNA and its transcript levels in *Epiblema scudderiana*

FoxO3a mRNA and protein sequences: With the primers designed from the consensus sequence of FoxO3a in mammals, a 480 bp fragment of FoxO3a cDNA (23.7% of human and mouse FoxO3a mRNA) from *Epiblema scudderiana* was amplified by RT-PCR. Nucleotide sequencing showed that it encoded a portion of the FoxO3a protein. The nucleotide sequence of FoxO3a cDNA from *E. scudderiana* is shown in Fig. 3.3A aligned with the human, mouse, and pig sequences. A homology tree comparing FoxO3a nucleotide sequences from the four species is shown in Fig. 3.3B. The nucleotide sequence of *Epiblema* FoxO3a cDNA shows high homology with that in mammals (Fig. 3.3A) and was 89% identical to mouse and 91% identical to human and pig sequences over the amplified region (Fig. 3.3B).

The deduced amino acid sequence of FoxO3a cDNA aligned with its nucleotide sequence is shown in Fig 3.4. Translation of the *Epiblema* cDNA sequence into protein revealed a partial sequence of 152 amino acids. Alignment of the *Epiblema* amino acid

sequence with that of human, mouse and pig is shown in Fig. 3.5A. The alignment showed that FoxO3a protein was highly conserved among invertebrate (*Epiblema*) and vertebrate (human, mouse and pig) species. The homology tree of FoxO3a protein in Fig. 3.5B shows that *Epiblema scudderiana* shared 94% identity with FoxO3a protein from mammals. Consideration of the phylogenetic distance between moths and mammals, FoxO3a protein is highly conserved over the amplified region through evolution.

FoxO3a transcript levels: Since the protein level of FoxO3a was reduced significantly after larvae were exposed to 5°C for 1 hour, FoxO3a mRNA levels were tested at the same time point (1h) and the time point afterward (4h) to see if the protein was regulated at the transcriptional level. RT-PCR was used to amplify FoxO3a cDNA and the cDNA of an internal control, β -Actin, from a series of cDNA dilutions (5, 25, 125 and 625 fold diluted) of 15°C control and 1 and 4 hours cooling exposure at 5°C for *Epiblema scudderiana* (n=4). PCR bands of FoxO3a with an internal control β -Actin in *Epiblema scudderiana* for 1 and 4 hours cooling exposure at 5°C are shown in Fig. 3.6A and Fig. 3.6B respectively. The bands obtained from 25-fold dilution of stock cDNA samples of control and stress samples were used to quantify the FoxO3a transcript levels. A histogram summarizing the data was shown in Fig. 3.6C. Transcript levels of FoxO3a did not change significantly when larvae exposed to 5°C for 1 hour, but FoxO3a transcript levels decreased significantly to $32\% \pm 2\%$ of the control value after for 4 hours exposure at 5°C.

3.3.3 MnSOD protein profile during cooling and subzero exposures of *Epiblema scudderiana*

Cooling exposure at 5°C: Western blots of MnSOD protein levels in *Epiblema* larvae are shown in Fig.3.7A for cooling exposure at 5°C for 1 hour, 4 hours, 1 day, 3 days, and 1 week (n=4-5) versus 15°C controls and Fig. 3.7B summarizes the data as a histogram. MnSOD relative protein levels did not change compared to controls until larvae were exposed to 5°C for 4 hours; protein level was 1.3 ± 0.1 fold of control after 4 hours exposure. Subsequently, MnSOD levels were lowered again down to control levels and remained at that level for extended exposure times at 5°C.

Subzero exposure at -15°C: MnSOD protein levels in *Epiblema scudderiana* during -15°C subzero exposure for 1 hour, 2 hours, 4 hours, and 12 hours are shown in Fig. 3.8A and summarized in a histogram in Fig. 3.8B. MnSOD protein levels were largely unchanged over the -15°C subzero exposure except for small but significant decreases in MnSOD content after for 4 hours and 1 day of exposure at -15°C; MnSOD levels were reduced to 83% and 72% of the value at 5°C, respectively.

3.4 Discussion

3.4.1 FoxO genes and proteins

FoxO proteins are ancient, evolutionarily conserved targets of insulin/insulin-like growth factor signaling in mammals. Various cellular functions of insulin are mediated through insulin-mediated inhibition of FoxOs. FoxO transcriptional activity is tightly controlled by direct phosphorylation through different signaling pathways. FoxO proteins

are retained in an inactive state in the cytoplasm when phosphorylated via the insulin-mediated phosphatidylinositol 3-kinase (PI3K) -Akt/PKB pathway; the mechanism of this effect is FoxO binding interactions with 14-3-3 proteins. In the absence of insulin signaling, however, FoxO proteins can be activated by dephosphorylation and translocated into the nucleus, where they stimulate transcription of negative regulators of growth (Horst et al., 2007). For example, in the absence of insulin signaling, FoxOs induce cell-cycle arrest and entry into cellular quiescence through regulating factors such as p130^{Rb2} (Kops et al., 2002).

FoxOs also regulate the gene expression of enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), which are essential regulators of gluconeogenesis. Therefore, FoxO activity induces a metabolic switch that is similar to low glucose and/or fasting signals (Barthel et al., 2001). In addition, FoxOs upregulate several antioxidant enzymes such as catalase and MnSOD to scavenge reactive oxygen species in peroxisomes and mitochondria (Kops et al., 2002). In general, a lack of insulin, low glucose, or increased oxidative stress result in FoxO-mediated cell cycle arrest in the G1–G0 stage, increased gluconeogenesis, and protection against cellular oxidative stress.

Larvae of the freeze avoiding insect, *Epiblema scudderiana*, initiate the production of their cryoprotectant, glycerol, when temperature falls to ~5°C, and glycerol accumulates at the highest rates at temperatures between 0 and -5°C. For optimal glycerol synthesis, a number of enzymes that are involved in the biosynthetic pathway for glycerol are activated. Previous studies in our lab showed that the activities of these enzymes rose in late autumn, including as glycogen phosphorylase (GP), glucose-6-phosphate

dehydrogenase (G6PDH), and phosphofructokinase (PFK) (Joannis and Storey, 1995). Oxidative stress may also be induced when the larva have to break down fatty acids to generate sufficient ATP to supply energy for this process. In addition, *Epiblema* are known to enter a hypometabolic or dormant state, called diapause, in the autumn after feeding ceases. Hence, processes which are typically insulin-mediated (growth, development) halt in the late autumn and the larvae settle into the diapause state over the winter. Given the known role of FoxO proteins as positive regulators of cell cycle and growth arrest (that are also negatively regulated by insulin), I postulated that FoxO proteins could have important roles to play in the development of cold hardiness in *Epiblema scudderiana*. The results in Fig 3.1C and Fig 3.2E supported this to some extent. Levels of phosphorylated (inactive) FoxO3a (Ser 253) were unchanged over the time course of 5°C exposure. However, when larva were acutely (short term, in vitro) transferred from 5°C to -15°C, phosphorylated FoxO3a (Ser 253) levels significantly decreased after 1 day at subzero exposure. The strong reduction in phospho-FoxO3a levels implies that the transcription factor was dephosphorylated and activated when the larvae were subjected to the greater subzero stress (-15°C exposure), as compared to the 5°C cooling exposure. After 1 week at -15°C, however, phospho-FoxO3a (Ser 253) levels had returned to control values suggesting that FoxO3a-mediated changes in gene transcription had been accomplished between 1 day and 1 week at -15°C.

FoxO3a transcript levels did not change after 1 hour of exposure at 5°C but were reduced significantly after 4 hours of 5°C exposure in *E. scudderiana*. However, protein levels at the same sample points changed differently. FoxO3a protein was decreased significantly after 1 hour of cold exposure but rose again to control levels after 4 hours

exposure at 5°C. These differential responses by FoxO3a mRNA and protein levels during cold exposure suggest that the observed change in FoxO3a protein was not regulated at the transcriptional level.

Analysis of *Epiblema* FoxO3a nucleotide and amino acid sequences with the homologous gene and protein sequences of mammalian species showed that the transcription factor was highly conserved through evolution; nucleotide and amino acid sequence identities were 91% and 94%, respectively, as compared with the human sequences.

Previous studies indicated that the invertebrate FoxO analogue, Daf-16 in *Caenorhabditis elegans*, shared the highest sequence identity (76%) with mammalian FoxO1 (Van der Horst et al., 2007). However, FoxO1 and phosphorylated FoxO1 were not detected in *Epiblema scudderiana* using polyclonal antibodies that were produced with a synthetic peptide (KLHcoupled) derived from the sequence of human FoxO1. Furthermore, I was also unable to detect and amplify FoxO1 mRNA from *Epiblema* using primers designed from the consensus sequence of FoxO1 in mammals. However, FoxO3a protein and mRNA was detected in *E. scudderiana* larvae and considering the reports that invertebrates appear to have only one FoxO gene (Daf-16 in nematodes and dFOXO in the fruit fly) (Carter and Brunet, 2007), my results would suggest that only one member of the FoxO transcription factor family exists in goldenrod gall moth larvae and that this FoxO member is FoxO3a.

3.4.2 MnSOD

MnSOD is a direct downstream gene target of the FoxO3a transcription factor and

is upregulated under FoxO3a control in response to cellular oxidative stress (Kops et al., 2002). Experimental results over a time course of cooling exposure at 5°C for *E. scudderiana* (Fig 3.7B) showed that MnSOD protein levels in the larvae increased significantly after a 4 hour cold exposure compared to 15°C controls. However, prolonged exposure at 5°C for up to 1 week had no further effect on MnSOD protein levels. When the larvae were further exposed to subzero temperatures, the levels of MnSOD fluctuated over time (decreased significantly after 4 hours and 1 day of -15°C exposure) but stabilized again after 1 week at -15°C. Thus, both low temperature stresses resulted in changes to MnSOD levels after 4 hours of exposure at either 5°C or -15°C, and this suggests that a rapid change in temperature is associated with a cellular oxidative stress that appears quite rapidly, within 4 hours of cold exposure. However, the oxidative stress does not seem to be sustained for long since MnSOD protein levels returned to control levels after 1 d at 5°C.

Overall, then, the results presented here for protein expression of FoxO and MnSOD in *Epiblema scudderiana* supported the idea that the insect has been well prepared for cold hardiness before being subjected to subzero temperatures.

Figure 3.1.

Foxo3a and phospho-Foxo3a (Ser253) protein levels in *Epiblema scudderiana* over a time course of exposure to 5°C. Western blotting was used to detect proteins.

A.

Protein bands of Foxo3a in 15°C control (C) *Epiblema* and after acute transfer to 5°C with sampling after 1 hour, 4 hours, 1 day, 3 days, and 1 week.

B.

Protein bands of Phospho-Foxo3a (Ser253) in 15°C control (C) *Epiblema* and after acute transfer to 5°C with sampling after 1 hour, 4 hours, 1 day, 3 days, and 1 week.

C.

Histogram showing relative levels of Foxo3a protein and Phospho-Foxo3a (Ser253) in *Epiblema* after transfer from 15°C to 5°C. Data are expressed relative to 15°C control values. Data are mean $\pm$ SEM, n =4-5 independent samples. Stars show values that are significantly different from the 15°C control, as determined by the Student's t-test, $P < 0.05$.

Figure 3.1

A.

B.

Figure 3.1

C.

Figure 3.2.

Foxo3a and phospho-Foxo3a-Ser253 protein levels in *Epiblema scudderiana* over a time course of exposure to -15°C. Protein levels were measured by Western blotting.

A.

Protein bands of Foxo3a in 5°C control (C) *Epiblema* and after acute transfer of insects from 5°C to -15°C with sampling after 1 hour, 2 hours, 4 hours, 12 hours, 1 day, and 1 week at -15°C.

B.

Protein bands of Phospho-Foxo3a (Ser253) in 5°C control (C) *Epiblema* and after transfer of insects from 5°C to -15°C with sampling after 1 hour, 2 hours, 4 hours, 12 hours, 1 day, and 1 week.

C.

Example membrane of Western blot of FoxO3a in *Epiblema* for subzero exposures of 12 hours and 1 day at -15°C

D.

Example membrane of Coomassie-stained Western blot of FoxO3a in *Epiblema* for subzero exposures of 12 hours and 1 day at -15°C

E.

Histogram showing relative protein levels of Foxo3a and Phospho-Foxo3a (Ser253) over the time course of exposure to -15°C. Data are expressed relative to 5°C control values. Data are mean $\pm$ SEM, n =4-5. Stars show statistically significant differences from the 5°C control values.

Figure 3.2

A.

B.

Figure 3.2

C.

D.

Figure 3.2

E.

Figure 3.3.

cDNA sequence and homology tree of Foxo3a in *Epiblema scudderiana*

A. Partial cDNA sequence of Foxo3a in *Epiblema scudderiana* (23.7% of the complete human FoxO3a mRNA) aligned with human (*Homo sapiens*, Genbank accession number BC021224), mouse (*Mus musculus*, Genbank accession number NM_019740 XM_001000298), and pig (*Sus scrofa*, Genbank accession AB281134). Dashes (-) represent the identical bases in the human, mouse, and pig sequences. Dots indicate where a base is not present in one of the species.

B. Homology tree of Foxo3a mRNA in *Epiblema*, human, mouse, and pig showing the percent identities of nucleotide sequences in four species.

Figure 3.3

A.

Epiblema_Foxo3a	CCCgNTCGGCGGGCTGTCTCCATGGACAACAGCAACAAGT	40
Human_Foxo3a	---cgg-----t-----	40
Mouse_Foxo3a	---cgg----t--g-----	40
Pig_Foxo3a	---cgg-----t-----	40
Epiblema_Foxo3a	ACACCAAGAGCCNCNGTTNNGATGCAGCCAAGAAGAAGGC	80
Human_Foxo3a	-t-----gtg-cc...gc-----	77
Mouse_Foxo3a	-----gag-cc...gg-----	77
Pig_Foxo3a	-t-----gtg-cc...gc-----	77
Epiblema_Foxo3a	AGCCCTGCAGACTGCCCCGGAGTCGGCAGACGACAGTCCC	120
Human_Foxo3a	-----a----c--a--a--t-----	117
Mouse_Foxo3a	g-----g-----a-----t	117
Pig_Foxo3a	-----a----c--a--a--t-----	117
Epiblema_Foxo3a	TCCCAGCTCTCCAAGTGGCCTGGCAGCCCCACATCACGCA	160
Human_Foxo3a	-----g-----	157
Mouse_Foxo3a	-----g--c----	157
Pig_Foxo3a	-----g-----	157
Epiblema_Foxo3a	GCAGTGATGAGCTGGATGCATGGACAGACTTCCGCTCACG	200
Human_Foxo3a	-----g----g-----t-----	197
Mouse_Foxo3a	----c--c-----g----c-----g--	197
Pig_Foxo3a	-----g----g-----t-----	197
Epiblema_Foxo3a	CACCAATTCCAATGCCAGCACAGTCAGCGGCCCGCTGTCA	240
Human_Foxo3a	-----t--c-----t-----g	237
Mouse_Foxo3a	-----c-----c--g-----g	237
Pig_Foxo3a	-----t--c-----t-----g	237
Epiblema_Foxo3a	CCCATCCTAGCAAGCACAGAGTTGGATGACGTCCAGGACG	280
Human_Foxo3a	-----a-g-----a-----	277
Mouse_Foxo3a	-----g-----g--c-----t-	277
Pig_Foxo3a	-----a-g-----a-----	277
Epiblema_Foxo3a	ATGACGCGCCGCTCTCCCCATGCTCTACAGCAGCTCAGC	320
Human_Foxo3a	----t-----t----g-----	317
Mouse_Foxo3a	----t-ga--c--g-----g-----t--	317
Pig_Foxo3a	----t-----t----g-----	317
Epiblema_Foxo3a	CAGCCTGTCACCCTCTGTGAGCAAGCCATGCACTGTGGAG	360
Human_Foxo3a	-----t--a--a-----g----g-----a	357
Mouse_Foxo3a	-----g-----c-----g--t-----	357
Pig_Foxo3a	-----t--a--a-----g----g-----a	357

Figure 3.3

Epiblema_Foxo3a	CTGCCACGGCTGACTGACATGGCGGGCACCATGAATCTGA	400
Human_Foxo3a	-----t-----a-----	397
Mouse_Foxo3a	--t--g-----g-----c-----	397
Pig_Foxo3a	-----t-----a-----	397
Epiblema_Foxo3a	ATGACGGGCTGGCTGACAACCTCATGGACGACCTGCTGGA	440
Human_Foxo3a	----t-----a----a-----	436
Mouse_Foxo3a	----t-----c--g-----	437
Pig_Foxo3a	----t-----a----a-----	43

B.

Figure 3.4.

Partial cDNA sequence of Foxo3a amplified from *Epiblema scudderiana* (23.7% of the complete human FoxO3a mRNA, top of each line) aligned with the translated amino acid sequence (bottom of each line). X in the amino acid sequence means no corresponding amino acid.

Figure 3.4

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1      NTGGANTGGGANGCGCCCCGNTCGGCGGGCTGTCTCCATGGACAACAGCAACAAGTACAC
1      W X G X R P X R R A V S M D N S N K Y T

61     CAAGAGCCNCNGTTNNGATGCAGCCAAGAAGAAGGCAGCCCTGCAGACTGCCCCGGAGTC
21     K S X X X D A A K K K A A L Q T A P E S

121    GGCAGACGACAGTCCCTCCCAGCTCTCCAAGTGGCCTGGCAGCCCCACATCACGCAGCAG
41     A D D S P S Q L S K W P G S P T S R S S

181    TGATGAGCTGGATGCATGGACAGACTTCCGCTCACGCACCAATTCCAATGCCAGCACAGT
61     D E L D A W T D F R S R T N S N A S T V

241    CAGCGGCCGCTGTACCCATCCTAGCAAGCACAGAGTTGGATGACGTCCAGGACGATGA
81     S G R L S P I L A S T E L D D V Q D D D

301    CGCGCCGCTCTCCCCCATGCTCTACAGCAGCTCAGCCAGCCTGTCACCCTCTGTGAGCAA
101    A P L S P M L Y S S S A S L S P S V S K

361    GCCATGCACTGTGGAGCTGCCACGGCTGACTGACATGGCGGGCACCATGAATCTGAATGA
121    P C T V E L P R L T D M A G T M N L N D

421    CGGGCTGGCTGACAACCTCATGGACGACCTGCTGGAA
141    G L A D N L M D D L L E

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Figure 3.5.

Protein sequence and homology tree of Foxo3a in *Epiblema*

A.

Protein sequence of Foxo3a in *Epiblema* scudderiana aligned with homologous proteins in human (*Homo sapiens*, Genebank accession number BC021224), mouse (*Mus musculus*, Genebank accession number NM_019740 XM_001000298), and pig (*Sus scrofa*, Genebank accession AB281134). Dashes (-) represent the identical amino acids in the human, mouse, and pig sequences. Dots indicate where an amino acid is not present in the coding region of one of the species.

B.

Homology tree of Foxo3a protein in *Epiblema*, human, mouse, and pig showing the percent identities of the amino acids in the four species.

Figure 3.5

A.

Epi_Foxo3a	WXGXRPXRRRAVSMDNSNKYTKSXXXDAAKKKAALQTAPES	40
human_Foxo3a	-r-----rgr.-----	34
Mouse-Foxo3a	-r-----rgr.-----a---	34
Pig_Foxo3a	-r-----rgr.-----	34
Epi_Foxo3a	ADDSPSQLSKWPGSPTSRSSDELDAWTDFRSRTNSNASTV	80
human_Foxo3a	-----	74
Mouse-Foxo3a	-----	74
Pig_Foxo3a	-----	74
Epi_Foxo3a	SGRLSPILASTELDDVQDDAPLSPMLYSSSASLSPSVSK	120
human_Foxo3a	-----m-----e-----	114
Mouse-Foxo3a	-----g-----	114
Pig_Foxo3a	-----m-----e-----	114
Epi_Foxo3a	PCTVELPRLTDMAGTMNLNDGLADNLMDDLLE.....	152
human_Foxo3a	-----te-----dnitlppsq	154
Mouse-Foxo3a	-----e-----d.....	146
Pig_Foxo3a	-----te-----d.....	146

B.

Figure 3.6.

Foxo3a transcript levels in *Epiblema scudderiana* at 5°C cooling exposure for 1 hour and 4 hours

A.

Bands of RT-PCR products of Foxo3a and β -Actin mRNA in the same series of dilutions in *Epiblema* 15°C control (C, n=4) and 5°C exposure for 1 hour (n=4). Abbreviation 1, 2, 3, 4 mean the concentrations of the cDNA samples used for PCR were 5 (5^1), 25 (5^2), 125 (5^3), and 625 (5^4) fold dilutions from stock cDNA samples.

B.

Bands of RT-PCR products of Foxo3a and β -Actin mRNA in the same series of dilutions in *Epiblema scudderiana* 15°C control (C, n=4) and 5°C exposure for 4 hours (n=3). Serial dilutions of 1:5 v:v were used. The abbreviations 1, 2, 3, 4 mean the concentrations of the cDNA samples used for PCR were 5 (5^1), 25 (5^2), 125 (5^3), and 625 (5^4) fold dilutions from stock cDNA samples.

C.

Relative mRNA levels of Foxo3a in *Epiblema scudderiana* after 5°C exposure for 1 hour and 4 hours versus 15°C control. mRNA levels were quantified from 25 fold dilution samples.

Figure 3.6

A.

B.

Figure 3.6

C.

Figure 3.7.

MnSOD protein levels in *Epiblema scudderiana* over a time course of exposure to 5°C.

Western blotting was used to detect proteins.

A.

Protein bands of MnSOD in 15°C control (C) *Epiblema* and after transfer of insects from 15°C to 5°C with sampling after 1 hour, 4 hours, 1 day, 3 days, and 1 week of cold exposure.

B.

Histogram showing relative protein levels of MnSOD in *Epiblema* at 15°C and over the time course of 5°C exposure. Data are shown relative to 15°C control values which are normalized to 1. Data are $\pm$ SEM, n = 4-5. Stars show statistically significant differences from the 15°C control values.

Figure 3.7

A.

B.

Figure 3.8.

MnSOD protein levels in *Epiblema scudderiana* over a time course of exposure to -15°C.

A.

Protein bands of MnSOD in 5°C control (C) *Epiblema* and after acute transfer of insects from 5°C to -15°C with sampling after 1 hour, 2 hours, 4 hours, 12 hours, 1 day, and 1 week at -15°C.

B.

Histogram showing relative protein levels of MnSOD in 5°C control (C) *Epiblema* and over the time course of -15°C exposure. Data are expressed relative to 5°C control values. Data are $\pm$ SEM, n = 4-5. Stars show statistically significant differences from the 5°C control values.

Figure 3.8

A.

B.

CHAPTER FOUR

Profile of FoxO Proteins and MnSOD in a Freeze Tolerant Insect, *Eurosta solidaginis*, Exposed to Low Temperature

4.1 Introduction

Eurosta solidaginis (Fitch) (Diptera, Tephritidae) is the larva of the goldenrod gall fly. Eggs are laid in the growing tips of goldenrod plants in the spring and when they hatch the larvae bore into the center of the stem and start to eat. Secretions of the larvae mimic plant hormones and cause the plant to form a ball gall around them and to stock the inside of the gall with cells that are high in nutrients which the larvae eat. Third instar larvae reach maximum size by early autumn. They bore a tunnel out to near the surface of the gall (leaving just the epithelium layer) and then settle back into the center of the gall to spend the winter. Once there, the larvae respond to autumn cues (shorter days, cooler temperatures, senescence of the plant) by preparing for winter. They accumulate two cryoprotectants, glycerol and sorbitol. Glycerol synthesis is triggered when ambient temperatures cool below 15°C and sorbitol by 5°C exposure. Moreover, the larvae elevate the supercooling point of their body fluids by adding ice nucleators that stimulate freezing of the larvae whenever temperature drops below about -8 to -10°C. Freezing is initiated at relatively high subzero temperatures so that the rate of ice formation is slow, crystal size is regulated, and ice growth is limited to extracellular and extraorgan spaces only. The larvae survive freezing and can endure the conversion of up to ~65% of their total body water in the extracellular ice. They endure multiple freeze/thaw cycles over the winter and can survive to at least -30°C in southern Canada. Known freezing survival times extend to 16 weeks at -15°C (Storey and Storey, 2004).

Multiple biochemical adaptations address issues associated with freezing in freeze tolerant insects. These include:

1. Ice Management: Ice nucleating agents (often specific proteins) are used to

trigger and regulate ice crystal formation in extracellular compartments. Antifreeze proteins help to manage crystal size and prevent recrystallization.

2. Cell Volume Reduction: The growth of ice (a crystal of pure water that excludes solutes) in extracellular spaces creates a powerful osmotic force that sucks water out of cells and causes a major decrease in cell volume and a large increase in intracellular ionic strength. Volume changes and the structural pressure that they place on cellular membranes must be managed. Two types of cryoprotectants are employed for this. Low molecular weight polyols (glycerol, sorbitol) offer colligative resistance to water loss from cells whereas membrane protectants (e.g. trehalose, proline) interact with phospholipids to stabilize the bilayer structure of the membrane against the compression stress imposed as cell volume shrinks.

3. Ischemia: The freezing of hemolymph (the blood equivalent in insects) halts circulation and the delivery of blood-borne fuels. Oxygen delivery through trachea is also interrupted during freezing. Hence, survival requires good anoxia tolerance as well as antioxidant defenses that prevent damage due to the formation of reactive oxygen species when oxygen is reintroduced upon thawing. Mechanisms of metabolic rate depression may also be employed to minimize cellular energy requirements and, hence, maximize survival time in the frozen state.

Eurosta accumulate two cryoprotectants, glycerol and sorbitol. Synthesis of both is derived from glycogen and the pentose phosphate cycle is used to produce the NADPH that reduces sugars (glyceraldehyde, glucose) to the respective polyol (Figure 1.1). As discussed in Chapter 1, sorbitol is derived from glycogen in four enzymatic steps: glycogen phosphorylase, phosphoglucomutase, glucose-6-phosphate phosphatase

(G6Pase), and polyol dehydrogenase. G6Pase is an important enzyme in this pathway for it commits G6P into the pathway of sorbitol synthesis. Previous studies in our lab have shown that G6P levels in *Eurosta* rose 2-fold within 1 h of exposure to 3°C and remained elevated over the first 3 days at 3°C; similarly, glucose levels rose more than 13-fold within the 2 h of 3°C exposure and continued to rise rapidly over 3 days at 3°C while sorbitol was rapidly accumulated (Storey and Storey, 1983). Furthermore, G6Pase activity was elevated by 2-fold in larvae collected in November as compared with larvae earlier in the autumn when glycerol synthesis was high. The FoxO1 transcription factor has been reported to regulate G6Pase in H4IIEC3 rat hepatoma cell line (H4 cells). Overexpression of FKHR (FoxO1) markedly increased the expression of the catalytic subunit of G6Pase (by about 2.5-fold above basal levels) suggesting a specific function for FKHR (FoxO1) in the regulation of hepatic gluconeogenesis at the level of G6Pase (Barthel et al., 2001). Studies with cultured mouse kidney epithelial cells (LLC-PK1-FBPase+) also showed that FoxO1 mediated the activity of G6Pase in response to insulin signaling (Nakae et al., 2001).

Freeze-tolerant insects such as *Eurosta solidaginis* also show well-developed anoxia tolerance. In laboratory trials *Eurosta* survived an 18-day anoxia exposure. Anoxic conditions brought about by ice formation in extracellular spaces and the subsequent return to aerobic conditions parallel the phenomenon of ischaemia-reperfusion in mammals. Oxidative stress may also be induced in the cells of *Eurosta solidaginis* in response to glucose starvation (food shortage in winter), which probably requires a shift from glycolysis to gluconeogenesis and the use of fatty acids and/or oxidative phosphorylation as in mammalian cells (Van der Horst et al., 2007).

As an insect that has only one generation a year (univoltine), *Eurosta solidaginis* also shows an obligatory acquisition of cold hardiness with the transition to the overwintering life stage (e.g., in *Eurosta* this is the third instar larva) as well as an obligatory period of diapause (arrested development) before it can resume growth and development. The larvae need a minimum of ~3 months of cold exposure before they can break diapause and will not resume development if removed from cold storage earlier. After diapause ends (usually in early February in Canada), the larvae remain in a quiescent state until warming temperatures in the spring facilitate pupation (Storey and Storey, 2004).

The FOXO transcription factor FOXO3a was reported to protect quiescent cells from oxidative stress in DLD-1 human colon carcinoma cells by directly increasing their quantities of manganese superoxide dismutase (MnSOD) messenger RNA and protein (Kops et al., 2002). The FOXO homologue Daf-16 in the nematode, *C. elegans*, similarly promotes entry into the dauer state, a reversible condition in which metabolic rate is reduced and life-span is markedly enhanced when the nematodes are exposed to harsh environmental conditions, including nutrient depletion. By contrast, mutation of the *daf-16* gene disrupts entry into dauer and the associated effect on life span. Daf-16a protein is 65% identical (78/120) to human FOXO1 (Ogg et al., 1997).

These observations indicated that FoxO proteins are ancient, evolutionarily conserved targets of insulin-like signaling that are important in regulating metabolism in response to changes in nutrient availability and environmental conditions (Barthel et al., 2005). Therefore, I hypothesized that FoxO genes, FoxO proteins, and the downstream genes under their control would be up-regulated in freeze tolerant gall fly larvae in

response to nutrient depletion and low temperature exposure.

The genes studied in this chapter are two members of the FoxO transcription factor family: FoxO3a (also called FKHL1) and FoxO1 (or FKHR), and one of the downstream proteins encoded by FoxO3a: manganese superoxide dismutase (MnSOD).

4.2 Materials and methods

4.2.1 Antibodies and western blotting

Polyclonal antibodies raised against human FoxO3a and FoxO1a as well as two phospho-FoxO3a antibodies recognizing peptides containing phosphorylated Serine 318/321 or Serine 253, and one phospho-FoxO1a antibody recognizing phosphorylated Serine 256 were purchased from Cell Signaling Technology Inc. MnSOD antibody was from Stressgen Biotechnologies Corp.

The FoxO3a antibody detects exogenous and endogenous levels of total FoxO3a protein, and gave a broad band at a molecular weight of 85-97 kDa. Phospho-FoxO3a (Ser253, Ser318/321) antibodies detect endogenous levels of FoxO3a only when phosphorylated at Serine 253 or Serine 318/321 and gave a single band at 97 kDa. The FoxO1 antibody detects endogenous levels of total FoxO1 and FoxO4 protein, and gave two bands at 78-82 kDa (FoxO1) and one band at 65-70 kDa (FoxO4). Phospho-FoxO1 (Ser256) antibody detects endogenous levels of FoxO1 only when phosphorylated at serine 256 and gave two bands at 82 kDa. The MnSOD antibody gave a specific single band at 25 kDa. Western blotting used 10% SDS-PAGE gels and, after transfer, PVDF membranes were blocked with 1% non-fat milk for 30 min prior to primary antibody incubation. Antibody concentrations and other conditions of Western blotting were as

described in Chapter 2 and Table 2.2.

4.3 Results

4.3.1 FoxO3a profiles during cooling and subzero exposures of *Eurosta solidaginis*

FoxO3a protein levels were analyzed in *Eurosta solidaginis* over three experimental time courses: (a) cooling at 5°C for 4 hours, 1 day, and 1 week, (b) freezing at -15°C for 30min, 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 1 day, 3 days, and 1 week, and (c) thawing at 5°C after 1 hour, 4 hours, 1 day, and 3 days (after 1 week frozen at -15°C).

Cooling exposure at 5°C: Western blots showing changes in FoxO3a protein levels in the larvae over a time course of 5°C cooling exposure are shown in Fig. 4.1A. Western blots detecting phospho-FoxO3a (Ser253) in the same series of samples are shown in Fig. 4.1B. Histograms summarizing the data for n=5 samples are shown in Fig. 4.1C. Compared to 15°C controls, the levels of total FoxO3a protein did not change until larvae were exposed to 5°C for 1 day. Total FoxO3a protein increased significantly by 1.25, 1.37 and 1.23-fold compared with controls after 1 day, 1 week, and 3 week exposures at 5°C, respectively. Phosphorylated FoxO3a protein (Ser 253) in *Eurosta* remained constant over the time course of 5°C cooling exposure except for a 1.89-fold increase after 1 day exposure at 5°C. Another phosphorylated form of FoxO3a protein (Ser 318/321) was not detectable in either control or stress samples.

Freezing exposure at -15°C and thawing at 5°C: Larvae of *Eurosta solidaginis*

were acutely exposed to -15°C after being cooled at 5°C for 3 weeks. Western blots showing total FoxO3a protein levels in the larvae over the time course of -15°C exposure are shown in Fig.4.2A and a histogram summarizing the results is shown in Fig.4.2B. Levels of total FoxO3a protein fluctuated somewhat in the larva over the above time course. FoxO3a levels were first reduced significantly after exposure to -15°C for 1 hour but then returned to control levels at 2 and 4 hours of freezing exposure. Total FoxO3a levels decreased a second time after 8 hours of exposure, rose again after 12 hours, and then fell a third time after 1 day of freezing exposure before rising again. Total FoxO3a protein levels after 1 hour, 8 hours and 1 day exposures at -15°C were $82 \pm 4\%$, $81 \pm 0.6\%$, and $75 \pm 3\%$ of the 5°C control values, respectively.

After being frozen at -15°C for 1 week, some of the *Eurosta solidaginis* larvae were thawed at 5°C and sampled over a time course of 1 hour, 4 hours, 1 day, and 3 days (Figure 4.3). Total FoxO3a levels did not change compared to 15°C controls until larvae had been thawed for 3 days. At that time FoxO3a protein levels increased by 1.69 ± 0.17 fold as compared with controls.

Two phosphorylated forms of FoxO3a (Serine 253 and Serine 318/321) were not detected in the samples of larvae exposed to the time courses of -15°C freezing and 5°C thawing.

4.3.2 FoxO1 profiles during cooling and freezing of *Eurosta solidaginis*

FoxO1 protein levels were also tested in *Eurosta solidaginis* over the time courses of 5°C cooling, -15°C freezing exposures and subsequent 5°C thawing. Unlike the situation in *Epiblema scudderiana* (Chapter 3), FoxO1 was detected in the *Eurosta*

solidaginis. However, a phosphorylated form of FoxO1 (Serine 256) was not detected in the larvae under any conditions.

Cooling exposure at 5°C: Figure 4.4A shows Western blots detecting total FoxO1 protein over a time course of cooling exposure at 5°C and Figure 4.4B shows a histogram summarizing the results. Total FoxO1 protein dropped significantly ($P < 0.05$) to $68 \pm 5\%$ of the 15°C control value when *Eurosta solidaginis* larvae were exposed to 5°C for 4 hours. However, FoxO1 protein amount returned to control levels after longer times of cold exposure.

Freezing exposure at -15°C and thawing at 5°C: Larvae were further exposed to a subzero temperature (-15°C) after 3 weeks acclimated at 5°C (control) (Figure 4.5). Over the long freezing exposure ranging from 30 min to 1 week, total FoxO1 protein in the larvae was largely stable. The only significant change was a 1.48 ± 0.11 fold increase in FoxO1 content seen after 12 hours of freezing.

Figure 4.6 shows the results for FoxO1 levels in larvae thawed at 5°C after 1 week frozen at -15°C. Total FoxO1 was stable for the first 4 hours of thawing, then decreased to $66 \pm 3\%$ of control levels (-15°C 1 week) after 1 day thawed, but had increased again to control levels after 3 days thawed.

4.3.3 MnSOD protein profiles during cooling and freezing exposures of *Eurosta solidaginis*

Cooling exposure at 5°C: Western blots of MnSOD levels in *Eurosta*

solidaginis larvae over a time course of cooling exposure at 5°C are shown in Fig. 4.7A and relative protein levels are summarized in Fig. 4.7B. Relative protein levels of MnSOD were unchanged in the short term but increased significantly after the larvae had been exposed to 5°C for 1 day. Protein amounts of MnSOD remained high over the subsequent extended exposures of 1 week and 3 weeks at 5°C. MnSOD levels were 1.7 ± 0.2 , 1.5 ± 0.1 and 2.0 ± 0.3 fold higher than levels in 15°C controls, respectively.

Freezing exposure at -15°C and thawing at 5°C: Figure 4.8 shows MnSOD protein levels in *Eurosta solidaginis* over the time course of freezing exposure at -15°C temperature. MnSOD protein amounts were generally unchanged during freezing with only one short interval where MnSOD decreased over the whole freezing time course. MnSOD levels showed a reduced trend after 2 hours of freezing and decreased significantly to 28 ± 3 % of control values after 4 hours frozen. Values then returned to control levels over the remainder of the time course.

The effect of thawing on MnSOD levels in *Eurosta solidaginis* is shown in Figure 4.9. MnSOD levels during thawing recovery at 5°C changed quickly. Relative protein levels of MnSOD dropped significantly ($P < 0.003$) after 4 hours thawed and remained low through 3 days thawed. MnSOD levels after 4 hours, 1 day, and 3 days thawed were 52 ± 9 %, 47 ± 6 %, and 61 ± 9 % of the control (-15°C 1 week) values, respectively.

4.4 Discussion

4.4.1 FoxO proteins

The FoxO family of transcription factors is well-known to be regulated via insulin

signaling. Insulin signals plentiful fuels and stimulates growth and cell division. Insulin transmits signals via PI3K and Akt (PKB) resulting in the phosphorylation of FoxO proteins. This leads to retention of FoxO transcription factors in the cytoplasm by binding with the 14-3-3 chaperone proteins, and thereby down-regulating the transcriptional activity of specific FoxO-regulated target genes. The activity of FoxOs is tightly controlled by posttranslational modifications including Akt/PKB mediated phosphorylation, phosphorylation by other protein kinases, acetylation and ubiquitylation. Phosphorylated FoxO proteins are targeted for proteosomal degradation through interaction with specific ubiquitin E3 ligases such as Skp2 (an oncogenic subunit of the Skp1–Cul1–F-box protein ubiquitin complex) (Lam et al., 2006). Plas and Thompson (2003) also reported that Akt (PKB) activation causes proteasomal degradation of FoxO1 and FoxO3a, resulting in reduced levels of these proteins. By contrast, the addition of proteasome inhibitors stabilized the phosphorylated forms of FoxO proteins (Plas and Thompson, 2003).

In the absence of insulin signalling, however, FoxO transcription factors are active and their role seems to be as negative regulators of growth. FoxOs induce cell-cycle arrest and entry into cellular quiescence (Kops et al., 2002) and they are important in coordinating entry into hypometabolic states such as the dauer state in nematodes (Ogg et al., 1997).

Total FoxO3a protein increased significantly in *Eurosta solidaginis* larvae that were given cold exposure at 5°C for 1 day to 3 weeks (Fig. 4.1C) or that were thawed at 5°C after 3 days frozen at -15°C (Fig. 4.3B). However, during freezing itself, total FoxO3a was relatively constant or protein levels decreased (Fig. 4.2B). Under the same

cold exposure stresses, total FoxO1 protein levels in *Eurosta solidaginis* changed oppositely. Total FoxO1 showed a short term decrease when larvae were chilled at 5°C and then rebounded (Fig. 4.4B) and a similar decrease was noted when larvae were thawed for 1 day at 5°C (Fig. 4.6B). However, when larvae were frozen at -15°C, total FoxO1 showed a short term significant increase by 1.48-fold after 12 hours (Fig. 4.5B). Elevated levels of both proteins under different cold/freezing conditions suggest that regulation of FoxO proteins occurs in *Eurosta solidaginis* in response to cold stress. This could indicate important actions of FoxO proteins in the cold larvae. Two possible areas of FoxO effects are suggested from studies of FoxO actions in other systems. Firstly, studies with mammals reported that FoxO1 triggered enhanced expression of G6Pase (Barthel et al., 2001; Nakae et al., 2001). This enzyme in *Eurosta* produces glucose for sorbitol synthesis and G6Pase activity is known to increase in the late autumn when sorbitol naturally accumulates in the larvae. A sharp drop in temperature from 15°C to 5°C has also been shown to activate sorbitol synthesis in the larvae under laboratory conditions (Storey and Storey, 1983). Hence, sorbitol synthesis would also have been triggered by the 5°C exposure in the present experiments and changes in FoxO transcription factors might be involved in mediating enzymatic changes that supported sorbitol accumulation. FoxO3a was elevated at 5°C in *Eurosta* but FoxO1 that is known to up-regulate G6Pase in mammals not. However, how G6Pase is regulated in insects is unknown to date and further studies of the targets of FoxO signaling in cold hardy insects is clearly needed. A second possible action of FoxO transcription factors in cold hardy insects is their role in cell-cycle arrest and entry into cellular quiescence (Ogg et al., 1997; Kops et al., 2002). *Eurosta solidaginis* enter a programmed diapause state over the

midwinter months but, just like low temperature speeds up the rate of polyol accumulation, cold temperature would probably also speed up the changes in the metabolism of the larvae that are needed for successful diapause. This could be an important role for FoxO proteins in the larvae at cold and frozen temperatures.

It is also interesting that the phosphorylated forms of FoxO3a and FoxO1 were not detected under some of the cold exposure conditions. Since phospho-FoxO3a was detected in larvae switched from 15°C to 5°C, the antibody clearly crossreacts with *Eurosta solidaginis* extracts. The absence of phospho-FoxO3a (Ser 253) under the other low temperature exposures suggests, therefore, that the FoxO3a present in these states might not be phosphorylated or might be degraded after phosphorylation.

4.4.2 MnSOD protein

MnSOD is a direct downstream target that is transcriptionally regulated by FoxO3a in response to oxidative stress (Kops et al., 2002). It has been reported that phosphorylation of cytoplasmic FoxO3a by the stress-activated c-Jun N-terminal kinase (JNK) microtubule-associated protein reverses the nuclear–cytoplasmic shuttling of FoxO3a that occurs in response to PI3K/PKB activation. JNK-dependent phosphorylation of FOXO proteins results in their nuclear accumulation and enhanced transcriptional activity, leading to increased expression of antioxidant genes such as MnSOD (Essers et al., 2004).

Corresponding to the increase of total FoxO3a protein induced by the cold exposure at 5°C for 1 day to 3 weeks, MnSOD levels were also elevated significantly at the same time points (Fig. 4.7B). A similar correlation between FoxO3a and MnSOD

profiles also happened over the time course of freezing exposure at -15°C (Fig. 4.2B and Fig. 4.8B). Total FoxO3a protein levels first decreased significantly ($P<0.05$) after 1 hour exposure at -15°C , and the MnSOD levels dropped significantly ($P<0.05$) after 4 hours exposure at -15°C . Then the larvae were thawed at 5°C , total FoxO3a levels increased after 3 days whereas MnSOD level decreased after 4 hours and remained at a low level until 3 days (Fig. 4.3B and Fig. 4.9B respectively). The results demonstrated that MnSOD has a tight relationship with FoxO3a in responding to cold stress in the freeze tolerant insect.

MnSOD is typically synthesized in cells under oxidative stress. The increased amounts of MnSOD in the cold exposed larvae at 5°C (Fig. 4.7B) implies, therefore, that the oxidative stress occurs in the larvae at this time, which was also the same time when sorbitol synthesis was triggered in the freeze tolerant larvae. The data in Fig. 4.7B suggest, therefore, that the oxidative stress appeared when the larvae of *E. solidaginis* were exposed to 5°C for 1 day, and was sustained in the larva body through 3 weeks of cold exposure. Alternately, MnSOD may be up-regulated in the larvae at 5°C in order to have increased antioxidant defenses ready for when the larvae undergo subsequent freeze/thaw cycles. Indeed, MnSOD was largely maintained at elevated levels when larvae were exposed to freezing (-15°C) (Fig. 4.8B) but protein levels of the enzyme fell quickly after the larvae were thawed (Fig. 4.9B). This suggests that the need for antioxidant defense by MnSOD was either in the frozen state or within the very early minutes/hours after thawing when the rapid reintroduction of oxygen to reduced cellular systems could cause a rapid increase in reactive oxygen species generation. The results also provide further support for previous work in our lab that indicates that the

acquisition of cold hardiness and/or freeze tolerance in the freeze tolerant insect is nearly always completed before subzero temperatures are experienced (Storey and Storey, 2004).

Fig 4.1. Foxo3a protein levels in *Eurosta solidaginis* over a time course of exposure to 5°C. Western blotting was used to detect proteins.

A.

Protein bands of Foxo3a in 15°C control (C) *Eurosta* and in larvae acutely transferred from 15°C to 5°C and sampled after cooling for 4 hours, 1 day, 1 week, or 3 weeks.

B.

Protein bands of phospho-Foxo3a (Ser253) in 15°C control (C) *Eurosta* and after cold exposure at 5°C for 4 hours, 1 day, 1 week, 3 weeks.

C.

Histogram showing mean relative protein levels of Foxo3a and phospho-Foxo3a (Ser253) in control *Eurosta* at 15°C and in larvae given cold exposure at 5°C. Data are means $\pm$ SEM, n = 4-5 and are expressed relative to 15°C control values which were set to 1.0. Stars show values that are significantly different from the 15°C control as determined by the Student's t-test, $P < 0.05$.

Fig. 4.1
A.

B.

C

Fig 4.2. Foxo3a protein levels in *Eurosta solidaginis* over a time course of freezing exposure at -15°C.

A.

Protein bands of Foxo3a in 5°C control (C) *Eurosta* and in larvae acutely transferred from 5°C to -15°C with sampling after 30 min, 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 3 days, and 1 week of freezing exposure.

B.

Histogram showing relative protein levels of Foxo3a in 5°C control *Eurosta* and larvae sampled over a time course of freezing at -15°C. Data are means $\pm$ SEM, n =4-5 and are expressed relative to 5°C control values which were set to 1.0. Stars show values that are significantly different ($P<0.05$) from the 5°C control values.

Fig 4.2

A.

Fig 4.2

B.

Fig 4.3. Foxo3a protein levels in *Eurosta solidaginis* during recovery at 5°C after 1 week frozen at -15°C.

A.

Protein bands of Foxo3a in control (-15°C 1 week, C) *Eurosta* and larvae sampled over a time course of thawing at 5°C for 1 hour, 4 hours, 1 day, and 3 days after -15°C freezing for 1 week.

B.

Histogram showing relative protein levels of Foxo3a in *Eurosta* during thawing recovery at 5°C. Data are means $\pm$ SEM, n = 4-5 and are expressed relative to control values which were set to 1.0. Stars show values that are significantly different ($P < 0.05$) from the control values.

Fig 4.3

A.

B.

Fig.4.4. Foxo1 protein levels in *Eurosta solidaginis* over a time course of cold exposure at 5°C.

A.

Protein bands of Foxo1 in 15°C control (C) *Eurosta* and in larvae transferred to 5°C and sampled over a time course of cooling for 4 hours, 1 day, 1 week.

B.

Histogram showing relative protein levels of Foxo1 in *Eurosta* over the time course of cooling at 5°C as compared with 15°C control values that are set to 1.0. Data are means $\pm$ SEM, n = 4-5. Stars show values that are significantly different ($P < 0.05$) from the 15°C control values.

Fig.4.4

A.

B.

Fig.4.5 Foxo1 protein levels in *Eurosta solidaginis* over a time course of freezing exposure at -15°C.

A.

Protein bands of Foxo1 in 5°C control (C) *Eurosta* and in larvae transferred to -15°C and sampled over a time course of freezing for 30 min, 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 3 days, and 1 week.

B.

Histogram showing relative protein levels of Foxo1 in *Eurosta* over the time course of freezing as compared to 5°C controls (set to 1.0). Data are means $\pm$ SEM, n = 4-5. Stars show values that are significantly different ($P < 0.05$) from the 5°C control values.

Fig.4.5

A.

B.

Fig.4.6 Foxo1 protein levels in *Eurosta solidaginis* over a time course of thawing recovery at 5°C after 1 week of freezing exposure -15°C.

A.

Protein bands of Foxo1 in control (-15°C 1 week, C) *Eurosta* and in larvae transferred back to 5°C after 1 week freezing at -15°C and sampled after 1 hour, 4 hours, 1 day, and 3 days of thawed recovery.

B.

Histogram showing relative protein levels of Foxo1 in *Eurosta* over a time course of thawed recovery at 5°C. Data are means $\pm$ SEM, n = 4-5. Stars show values that are significantly different ($P < 0.05$) from the control values.

Fig.4.6

A.

B.

Fig.4.7 MnSOD protein levels in *Eurosta solidaginis* over a time course of cold exposure at 5°C.

A.

Protein bands of MnSOD visualized by Western blotting in 15°C control (C) *Eurosta* and in larvae acutely transferred to 5°C and sampled after 4 hours, 1 day, and 1 week of cooling.

B.

Histogram showing variation of relative protein levels of MnSOD in *Eurosta* in response to cooling at 5°C as compared to 15°C controls that were set to 1.0. Data are means $\pm$ SEM, n = 4-5. Stars show values that are significantly different ($P < 0.05$) from the 15°C control values.

Fig.4.7

A.

B.

Fig.4.8 MnSOD protein levels in *Eurosta solidaginis* over a time course of freezing at -15°C.

A.

Protein bands of MnSOD assessed via Western blotting for *Eurosta* larvae transferred from 5°C to -15°C and sampled over a time course of freezing for 30 min, 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 1 day, 3 days and 1 week.

B.

Histogram showing the changes in relative protein levels of MnSOD in *Eurosta* over time during freezing at -15°C as compared to 5°C controls (C). Data are means $\pm$ SEM, n = 4-5. Stars show values that are significantly different ($P < 0.05$) from the 5°C control values.

Fig.4.8

A

Fig.4.8

B.

Fig.4.9 MnSOD protein levels in *Eurosta solidaginis* over a time course of thawing recovery at 5°C after -15°C freezing for 1 week.

A.

Protein bands of MnSOD in *Eurosta* over recovery from freezing for 1 hour, 4 hours, 1 day and 3 days as compared with values in control (-15°C 1 week, C) larvae.

B.

Histogram showing the change of relative protein levels of MnSOD in *Eurosta* over a time course of thawing recovery at 5°C as compared to control larvae at -15°C. Data are means $\pm$ SEM, n = 4-5. Stars show values that are significantly different ($P < 0.05$) from the control values.

Fig.4.9

B.

CHAPTER FIVE

General Discussion

5.1. FoxOs in Insects

FoxO transcription factors function to mediate cell cycle arrest, DNA repair and apoptosis, and promote cellular differentiation (Lam et al., 2006). Two FoxO transcription factors FoxO3a and FoxO1 were analyzed in this thesis, comparing their responses in freeze avoiding and freeze tolerant insects. The downstream transcriptional activity of FoxO3a was not activated in either insect exposed to 5°C, as assessed by changes in the relative amount of phospho-FoxO3a (Ser253). This is a temperature at which both glycerol synthesis in the freeze avoiding *Epiblema scudderiana* and sorbitol synthesis in the freeze tolerant insect *Eurosta solidaginis* are triggered. This suggests that the two insects accomplish cryoprotectant synthesis at 5°C without the need of regulation by FoxO3a.

When the two cold-hardy insects suffered supercooling or freezing exposures at -15°C, FoxO3a was activated in the freeze avoiding insect *Epiblema scudderiana* (ie. levels of phospho-FoxO3a [Ser253] decreased) implying that FoxO3a has a role to play in activating genes that aid subzero survival. The body fluids of the freeze avoiding species are still liquid at this time and so protein synthesis could occur at this subzero temperature to produce proteins that aid cold hardiness or diapause in the larvae. The expression of phospho-FoxO3a in *Epiblema* for -15°C exposure confirmed the expectation: the active form of FoxO3a (unphosphorylated form) increased after 1 day exposure at -15°C. The increased active form of FoxO3a imply that the transcriptional activity controlled by FoxO3a might occur under subzero temperature. As for the freeze tolerant fly *Eurosta*

solidaginis, its extracellular fluids are frozen at -15°C, but the fluid inside of cells is still liquid. Hence, FoxO3a should have the same function as it does in the freeze avoiding insect. However, the results did not support this idea. The amount of phosphorylated FoxO3a transcription factor (the active form) was too weak to be detected in the frozen larvae and thereafter in the thawed larvae. The reasons for this might be: 1) most of the FoxO3a was in the active form (dephosphorylated form) so that phospho-FoxO3a was too low to detect; 2) the phosphorylated FoxO3a was degraded; or 3) the FoxO3a was phosphorylated at other sites of its amino acid sequence.

FoxO1 protein was only found in the freeze tolerant *Eurosta solidaginis*. Because phosphorylated forms of both FoxO3a and FoxO1 were not detected in most *Eurosta solidaginis* samples (except FoxO3a [Ser253] in the 5°C exposure samples), I could not assess the changes in activity state of FoxO1 or FoxO3a under most cold exposure conditions. Total FoxO1 protein increased only in the larvae subjected to -15°C freezing but phospho-FoxO1 (Ser 256) was not found nor was phospho-FoxO3a (Ser 318/321). The antibodies used in these studies were raised against peptide sequences around the phosphorylation sites in human phospho-FoxO3a and phospho-FoxO1. It is possible that either these sites was migrated among the insect proteins or that the sequences around the phosphorylation sites in the insect proteins were not recognized by the antibodies used.

An interesting observation from the study of FoxO proteins in the two insect species is the type of FoxO proteins that exist. FoxO3a transcription factor was the only FoxO protein found in *Epiblema scudderiana*. FoxO1 was not detected in this species using

either RT-PCR to look for FoxO1 mRNA or immunoblotting to assess FoxO1 protein. However, both FoxO3a mRNA and protein were found in *Epiblema scudderiana* and sequence analysis of nucleotides and amino acids showed that the gene/protein was highly conserved as compared with mammals (human, pig, mouse). Both FoxO3a and FoxO1 protein was found in the freeze tolerant fly *Eurosta solidaginis* using antibodies raised against human FoxO3a and FoxO1. RT-PCR trials were also used to look for FoxO mRNA in *Eurosta solidaginis* but were successful in detecting only one gene: FoxO1 (unpublished data). Previous studies with other invertebrate species identified only one FoxO gene/protein to date in nematodes and *Drosophila* (Carter and Brunet, 2007) and this also agrees with my results for *Epiblema*. However, the results for *Eurosta* suggest that there may be at least two forms of FoxO proteins in the flies, similar to vertebrates. The different patterns of expression of FoxO3a and FoxO1 in response to cold exposure in *Eurosta* suggest that the two human antibodies being used must be crossreacting with different insect proteins even though the two proteins migrated similarly on gels (85 kD for FoxO3a and 78-82 kD for FoxO1). With the known importance of FoxO proteins in regulating the dauer state in nematodes, it will be important for future studies with insects to identify all the members of the FoxO family in insects and investigate their role in regulating the phenomena of cold hardiness and diapause in insects.

5.2. MnSOD Protein and Oxidative Stress in Two Insects

The mitochondrial antioxidant enzyme MnSOD (manganese superoxide dismutase)

scavenges superoxide radicals which are produced in the mitochondria, particularly dealing with superoxide that is formed as a byproduct by the electron transport chain. Interestingly, MnSOD protein levels were increased in both cold-hardy insect species during 5°C cold exposure but not during -15°C subzero exposure or during the 5°C thaw (*Eurosta solidaginis* only). This suggests that oxidative stress in the two insect species occurred only at the temperature (5°C) that is associated with triggering cryoprotectant synthesis (5°C triggers glycerol production in *Epiblema scudderiana* and sorbitol in *Eurosta solidaginis*). The length of cold exposure needed to trigger the MnSOD response was 4 hours for freeze avoiding moth larvae and 1 day for the freeze tolerant fly larvae which indicates a rather urgent need for enhanced antioxidant defenses. The pattern of MnSOD response also indicated that oxidative stress in *Epiblema scudderiana* was quickly released after the larvae were exposed to 5°C for 1 day but was sustained in *Eurosta solidaginis* for as long as 3 weeks of 5°C exposure. In *Eurosta*, the sustained higher MnSOD levels may also prepare the larvae for surviving the ischemia/reperfusion events associated with freeze/thaw exposures. Since supercooling at -15°C had little effect on the expression of MnSOD in freeze avoiding larva of *Epiblema*, it seems possible that the amount of MnSOD needed by *Epiblema* for winter survival is put in place at near 0°C temperatures well before the larvae experience subzero temperatures naturally.

Also interesting is the reduction in MnSOD expression in the freeze tolerant larvae when thawed at 5°C after long term freezing. Protein amounts of MnSOD were reduced when the larvae were thawed for 4 hours, 1 day, and 3 days. This reduction in

mitochondrial MnSOD suggests that there was no long term oxidative stress associated with the reperfusion of oxygen into the body of *Eurosta solidaginis* after thawing. However, high MnSOD appeared to be needed during freezing or within the first minutes/hours of thawing (levels were still high after 1 h thawed) but was then reduced with longer thawing. Hence, the freeze tolerant fly seems to coordinate its antioxidant defenses with the process of freeze/thaw in order to minimize or prevent oxidative stress.

5.3. Future Directions

In this thesis, two members of the FoxO transcription factor family, FoxO3a and FoxO1, were studied. The protein levels of FoxO3a and FoxO1 were analyzed via Western blotting and FoxO3a mRNA was amplified and sequenced from *Epiblema scudderiana*. Responses of the antioxidant enzyme MnSOD which is known to be regulated by FoxO3a were also evaluated in the two species. The results presented here add significantly to our knowledge of metabolic regulation in cold hardy insects but there are a variety of future directions that could be taken to continue this research. The following are some of them.

FoxO Genes: Protein levels of FoxO1 and FoxO3a were analyzed in the two insect species and FoxO3a mRNA from *Epiblema scudderiana* was sequenced. However, I was unable to amplify FoxO1 mRNA from *Epiblema scudderiana* and FoxO3a mRNA from *Eurosta solidaginis*. The expression of FoxO proteins from *Eurosta* suggests that there are two or more FoxO family transcription factors in the cold hardy insect species and so it

would be useful to try to find all the FoxO genes in these insects. For example, the primers used in my initial studies of FoxO1 were designed from homologous sequences of human and frog (*Xenopus tropicalis*) respectively and were used to amplify the mRNA of FoxO1 in insects. An RT-PCR band of FoxO1 in *Eurosta solidaginis* was obtained with primers designed from the frog, but the subsequent sequencing failed because of the nonspecific binding of primers to the FoxO1 cDNA in insects. Hence, future studies should probably use primers designed from the homologous sequences of FoxO1 from other insects such as the fruit fly (*Drosophila melanogaster*) or from other invertebrates such as the nematode, *C. elegans*. A similar redesign of FoxO3a primers should allow amplification and sequencing of *Eurosta solidaginis* FoxO3a mRNA. This should show which FoxO family members are present in the two cold-hardy insects.

Binding Activities of FoxO Genes: As the core DNA binding domain of FoxO genes is known from the literature, the binding activities of FoxO3a or FoxO1 in the two insects under stress conditions could be studied using EMSA (Electrophoretic Mobility Shift Assay). This would allow us to further monitor the transcriptional activities of FoxO3a or FoxO1 in insects during stress conditions. An increase in FoxO3a or FoxO1 DNA binding in response to cold stress would be good confirmation that genes under FoxO control are up-regulated.

Genes/proteins regulated by FoxO: In my thesis, I analyzed the expression of

MnSOD as an example protein that is under FoxO control. Other target genes of FoxO transcription factors are known in mammals and in *Drosophila* (Neufeld, 2003) and *C. elegans* (McElwee et al., 2003; Murphy et al., 2003) and the expression of some of these could be analyzed by Western blotting and/or RT-PCR during cold exposure in both insect species. The downstream genes/proteins that are related to cell cycle arrest and life extension in *C. elegans* or other species would be very interesting to study and might help long term survival of the insects over the winter months.

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