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**METABOLISM DURING OVERWINTERING IN TWO SPECIES OF COLD-
HARDY GOLDENROD GALL INSECTS**

by

DENIS R. JOANISSE, B.Sc. (Hons)

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

Department of Biology

Ottawa-Carleton Institute of Biology

Carleton University

Ottawa, Ontario

April 1995

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HARDY GOLDENROD GALL INSECTS"**

submitted by

DENIS R. JOANISSE, B.Sc. (Hons)

in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Chair, Department of Biology

Thesis Supervisor

External Examiner

Carleton University

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ABSTRACT

Metabolism was examined in the larvae of two cold-hardy goldenrod gall insects, the freeze tolerant fly *Eurosta solidaginis* (Fitch) (Diptera, Tephritidae) and the freeze avoiding moth *Epiblema scudderiana* (Clemens) (Lepidoptera, Olethreutidae) during overwintering. Acclimatization resulted in specific changes in enzyme activities and cellular metabolites. Enzyme activity data suggested metabolic shifts concurrent with the requirements of both species. Larvae shifted metabolism towards cryoprotectant synthesis in the fall, as witnessed by increased activities of glycerol and sorbitol producing enzymes, and towards the removal of these polyols in the spring with increased activities of catabolic enzymes. Measured increases in lipid unsaturation in the fall helped to maintain membrane fluidity during cold-exposure. Decreased activities of enzymes of the tricarboxylic acid cycle paralleled the reduced demands on oxidative metabolism over the winter. In *Eurosta solidaginis*, decreased oxidative metabolism and the anoxic state of the animal when frozen further resulted in the lack of lipid reserve depletion over the winter, whereas freeze avoiding *Epiblema scudderiana* larvae appeared to use lipid reserves to support basal metabolism over the winter. Decreased activities of antioxidant enzymes in *Eurosta solidaginis* over the winter suggested that these larvae do not experience increased reactive oxygen species formation during freezing and thawing. Increasing antioxidant enzyme activities in *Epiblema scudderiana* over the winter suggested that these larvae may be subject to oxidative stress, perhaps due to the maintenance of aerobic metabolism during cold exposure.

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No mere words can offer the thanks I must give my parents, Marcel and Louise. If parents can be a person's best friends, then mine truly are. Nevertheless, they will surely be happy to see me go (finally!).

Finally to Lynn, my heart and soul.

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LIST OF ABBREVIATIONS

%a	percent of glycogen phosphorylase in the active <i>a</i> form
<i>a+b</i>	total glycogen phosphorylase activity (active <i>a</i> plus inactive <i>b</i>)
6PGDH	6-phosphogluconate dehydrogenase
ATP-CL	ATP-dependent citrate lyase
BHBDH	β -hydroxybutyrate dehydrogenase
CAT	catalase
CoA	coenzyme A
CPT	carnitine-palmitoyl transferase
CS	citrate synthase
DBI	double-bond index
DHAP	dihydroxyacetonephosphate
EDTA	ethylenediaminetetraacetic acid
F1,6P₂	fructose-1,6-bisphosphate
F6P	fructose-6-phosphate
FBPase	fructose-1,6-bisphosphatase
FP	freezing point
G1P	glucose-1-phosphate
G6P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
GAP	glyceraldehyde-3-phosphate
GDH	glutamate dehydrogenase
GPox	Se-dependent glutathione peroxidase
GR	glutathione reductase
GSH	γ -glutamyl-cysteinyl-glycine; glutathione, reduced form
GSSG	glutathione dimer, disulfide linked; oxidized glutathione
GST	glutathione-S-transferase
GSTPox	selenium-independent glutathione peroxidase
gwm	gram wet mass
HK	hexokinase
HOAD	hydroxyacyl-CoA dehydrogenase
IDH	isocitrate dehydrogenase
INP	ice nucleator protein
IU	international unit
LDH	lactate dehydrogenase

m:v	mass:volume
ME	malic enzyme
NAD⁺, NADH	oxidized and reduced forms of nicotinamide adenine dinucleotide
NADP⁺, NADPH	oxidized and reduced forms of nicotinamide adenine dinucleotide phosphate
PDHald	Glyceraldehyde-utilizing polyol dehydrogenase
PDHgluc	Glucose-utilizing polyol dehydrogenase
PEP	phosphoenolpyruvate
PFK	phosphofructokinase
PGI	phosphoglucoisomerase
PGK	Phosphoglycerate kinase
PK	pyruvate kinase
PGM	phosphoglucose isomerase
PMSF	phenylmethylsulfonyl fluoride
ROS	reactive oxygen species
SCP	supercooling point
SOD	superoxide dismutase
SoDH	sorbitol dehydrogenase
TBARS	thiobarbituric acid reactive substances
THP	thermal hysteresis protein
U	unit of enzyme activity (see IU)
V_{max}	maximal enzyme velocity
XDH	xanthine dehydrogenase
XO	xanthine oxidase

CHAPTER I
GENERAL INTRODUCTION

“Often in winter the end of the day is like the final metaphor in a poem celebrating death: there is no way out.”

Agustin Gomez-Arcos, *A Bird Burned Alive*

Ectothermic animals which live at high latitudes or altitudes have, over evolutionary time, developed strategies to cope with the cold temperatures they must face. Locally, a typical Ottawa winter will see temperatures fall to well below -30°C routinely, often staying at low subzero temperatures for days or weeks at a time. Some animals have chosen to avoid the cold altogether. This is best exemplified by the migration of the Monarch butterfly or of many species of ducks and geese, which travel thousands of kilometers to escape the cold Northern winter climate. Other species will remain in the general area, but will select hibernacula which protect them from the extreme cold. Thus, animals that winter under water (*e.g.* aquatic larvae and amphibians) or burrowing species gain enough insulation so that they need only endure temperatures in the 0 to 5°C range. More fascinating, perhaps, are those cold-hardy species that have selected to stand and face the cold with little or no thermal buffering. These organisms have evolved behavioural, physiological, and biochemical adaptations that allow the survival of temperatures below 0°C . Two fundamental strategies of overwintering are generally recognized: freeze avoidance and freeze tolerance.

Freeze avoidance

Freeze avoiding species (also called freeze susceptible) use physiological and biochemical adaptations to deal with low subzero ambient temperatures by extensive supercooling of body fluids (Storey and Storey, 1988; Zachariassen, 1985). This form of cold hardiness is commonly used by arthropods and many other invertebrates, and relies on the principle of supercooling (or undercooling)(Storey and Storey, 1989). Perfectly pure water, if cooled slowly, can remain liquid to temperatures as low as -40°C . This is a metastable state, however, and as soon as a nucleator (a structure which resembles an ice

crystal or a surface which promotes the rearrangement of water molecules into an ice-like configuration) is added to the supercooled liquid, spontaneous ice formation will occur at any temperature below 0°C. To use supercooling as a strategy for overwintering is somewhat risky, therefore, and the possibility of nucleation must be minimized to ensure survival.

A number of strategies are used by freeze avoiding insects to avoid spontaneous nucleation of internal body fluids or inoculation of body fluids by contact with external ice while allowing for extreme supercooling. These may include: 1) choosing a hibernaculum which limits potential exposure to nucleators (e.g. environmental ice) and/or forming a protective barrier such as a silk cocoon to prevent contact with external ice, 2) eliminating internal nucleators, notably by emptying the contents of the gut prior to cold exposure (fecal matter can contain many kinds of nonspecific nucleators), 3) the synthesis of thermal hysteresis proteins, 4) dehydration, concentrating the remaining body fluids which colligatively decreases their freezing point, and 5) the accumulation of high concentrations of cryoprotectants (colligative antifreezes), usually polyhydric alcohols, which serve to decrease the freezing and supercooling points of organisms and their cells (Storey and Storey, 1989; Zachariassen, 1985).

The freezing point of water is a colligative property, and is therefore controlled by the concentration of osmotically active molecules in solution. The addition of osmotically active solutes will decrease not only the freezing point temperature (FP), but also the supercooling point (SCP: the temperature below the FP at which spontaneous ice nucleation occurs). However, high concentrations of solutes must be added for significant depression of the FP and the SCP. The addition of 1 mole of osmotically active solute leads to a depression of the FP by -1.86°C; the corresponding SCP is depressed one to three times the FP depression (Hochachka and Somero, 1984). A 1 molal solution of glycerol will thus depress the FP of water from 0°C to -1.86°C, and the SCP may decrease to -5.58°C. For molecules to be useful as colligative cryoprotectants they must

then be of low molecular weight and highly water soluble (high molecular weight compounds cannot be accumulated in high enough concentrations to fulfill this function), and they must not be toxic to the cell at high concentrations (*i.e.* they are 'compatible solutes'). The polyhydroxy alcohols and some sugars are such compounds, and are used extensively where high concentrations are required (Hochachka and Somero, 1984; Yancey *et al.*, 1982). Glycerol is the most commonly utilized colligative cryoprotectant among freeze avoiding insects (Sømme, 1982). Other commonly found colligative cryoprotectants include sorbitol and in some cases mannitol. Inositol, ethylene glycol, threitol, erythritol, fucitol, arabitol, xylitol, rhamitol, ribitol, and inositol may also be elevated in the winter, but in some cases the observed increases are not sufficient to warrant their classification as colligative antifreezes (Duman *et al.*, 1991). An interesting case is that of the bark beetle *Ips acuminatus*, which accumulates 2.7 M ethylene glycol as a colligative antifreeze (Gehrken, 1984); this is the polyol we use in car radiators, and possesses some well known toxic effects on higher animals. The haemolymph sugars trehalose and to a lesser extent glucose and fructose may also be elevated in winter, normally with a concurrent increase in polyols (Duman *et al.*, 1991). Multicomponent systems consisting of a number of polyols and/or sugars are also known. Colligative antifreezes are usually synthesized from the breakdown of glycogen stores in insect fat body (Storey and Storey, 1988)

Thermal hysteresis proteins (THPs; also known as antifreeze proteins), which decrease the FP of water in a non-colligative manner, have been identified in over 26 species of insects (Duman *et al.*, 1991). These proteins depress the freezing point, but not the melting point, of water, and hence produce a thermal hysteresis between FP and MP. These proteins can prevent the freezing of an aqueous solution in the presence of a seed ice crystal to as much as 9°C below the freezing point of the solution. THPs are believed to function by adsorption onto the small seed ice crystals through hydrogen-bonding, inhibiting further growth (Raymond and DeVries, 1977; Raymond *et al.*, 1989).

Freeze tolerance

Contrary to freeze avoiding insects, freeze tolerant species survive the cold of winter by permitting the formation of ice within their bodies. Freeze tolerance is used by numerous types of animals to survive the winter cold, and is quite common in the insect world (Storey and Storey, 1992; Duman *et al.*, 1991). Freezing, however, is not a process which is left to chance, for highly supercooled tissue is likely to suffer ice nucleation in both extracellular and intracellular loci (Mazur, 1984) and intracellular ice formation is invariably lethal in Nature. Instead, these animals initiate, control, and limit ice formation to extracellular spaces.

Nucleation in extracellular spaces is achieved by the synthesis of ice nucleator proteins (INPs). These initiate ice formation at high temperatures, usually above -10°C , even in the presence of high concentrations of colligative solutes (Duman *et al.*, 1991). As the growing extracellular ice crystals are made up of pure water, the remaining extracellular liquid surrounding them becomes more concentrated. This then places an osmotic stress on cells, which causes an efflux of water and an influx of solutes. The cell volume decreases and intracellular fluids then become more concentrated, and as a consequence the freezing point of the cytoplasm decreases by colligative action. In essence a freeze tolerant system then consists of frozen extracellular spaces surrounding unfrozen cells.

Another important limitation to freeze tolerance is damage to cells resulting from water loss to the extracellular ice. Beyond a critical minimal volume compression stress on the membrane bilayer structure becomes too great and damage to the cell results. Freeze tolerant animals solve this problem by synthesizing colligative cryoprotectants, much like those of freeze avoiding animals, which limit the amount of shrinking a cell undergoes by increasing the intracellular and extracellular solute concentrations. In addition, membrane stabilizers such as the carbohydrate trehalose (a glucose dimer) can

help to further reduce membrane damage by stabilizing the membrane lipids into a more stable state in the cold.

Metabolic depression

Often associated with both freeze avoidance and freeze tolerance in insects is entry into a state of facultative metabolic rate depression, or metabolic arrest (Storey and Storey, 1990). This coordinated slow-down of all aspects of metabolism serves to limit or prevent metabolic damage (e.g. energy depletion under unfavorable conditions), and also prolongs the survival time of the non-feeding animal under those conditions. Many insects overwinter in a state of diapause, which is also associated with a decrease in metabolic rate (Danks, 1987). Oxygen consumption, largely used as an indicator of overall metabolic rate, falls to unmeasurable or extremely low levels in diapausing and overwintering insects, usually to less than 5% of the normal rate (Danks, 1987; Storey and Storey, 1987).

Objectives and approach

Much research has been published on the biochemical changes that occur in association with cold-hardening and overwintering goldenrod gall insects, including notably metabolite, enzyme activity, and enzyme purification and kinetic analysis studies (Storey and Storey, 1991, 1992; Holden and Storey, 1993, 1994a, b; Churchill and Storey, 1989a, b, c, d). However, most of this work has been accomplished using laboratory acclimated larvae and are generally limited in scope, focusing on one particular aspect of metabolism. What is missing is a picture or an understanding of the events which occur during larval acclimatization, that is the response to natural environmental change over the winter months. This present research attempts to fill that gap in our understanding of the mechanisms of winter survival of these larvae. This will not only provide an understanding of the acclimatization events which are characteristic

of overwintering, but will also serve as a point of comparison to verify that past acclimation studies and *in vitro* experiments do in fact mimic those events which occur in Nature.

The research is based on the hypothesis that changing environmental conditions lead to changing metabolic needs of animals and that, therefore, a modification of the metabolic machinery of an animal is necessary to answer those needs. As organisms enter extended periods of metabolic change (such as metabolic depression over the winter) their relative metabolic needs change. Flux through pathways once dominant may slow or cease, and other previously minor pathways may become major routes of activity. For cold-hardy insects, this latter situation is best exemplified by pathways of cryoprotectant synthesis in the autumn, which must respond to a need for the conversion of stored carbohydrate into molar levels of polyols. These changes are reflected in the activity of the engines that drive metabolism, *i.e.* the enzymes, and in the changes in the levels of metabolites, the substrates and products of metabolic flux. The present research is particularly geared toward the study of those systems which have been implicated in the synthesis of cryoprotectants, those which may be involved in metabolic depression, and those which may serve to protect the animals from specific damage during overwintering. These are then key mechanisms for survival.

The key to controlling metabolism lies in the modulation of the enzymes from which pathways are made. To change pathway flux involves modifying the activities of the enzymes which comprise it. There are basically three methods for modifying the rate of reaction (turnover) of an enzyme: coarse control, fine control, and covalent modification (Price and Stevens, 1989).

Coarse control is defined as the regulation of the amount of an enzyme, such as by enzyme induction or repression, or changes in the rate of enzyme biosynthesis or proteolysis. In essence, the total number of catalytic molecules changes, such that more or

less flux can occur for this reaction in a given period of time under identical conditions. Coarse control is generally held to be suitable for long term regulation.

Fine control and covalent modification of enzymes affect the activity of enzymes already present within the cell; that is, they modulate the catalytic capacity of the enzymes leading to changes in the rate of reaction (turnover). These mechanisms allow rapid responses to changes in cellular conditions or extracellular stimuli. The catalytic activity of allosteric enzymes can be modified by the binding of one or more metabolites at regulatory sites that are distinct from the active site of the protein. These metabolites, known as effectors (modifiers, modulators), may be activating or inhibiting of enzyme activity. A conformational change of the enzyme upon binding of an effector is responsible for the change in enzyme activity. Studies of the control of enzyme activity by fine control are generally performed on purified enzymes under *in vitro* conditions. The present research will not deal with this type of control.

Covalent (post-translational) modification involves changes to the structure of the protein, leading to changes in the catalytic ability of the enzyme. These changes can be irreversible or reversible. Examples of irreversible changes include proteolytic cleavage of a zymogen to yield an active enzyme, as seen for many proteases (trypsin, chymotrypsin) and thrombin in the blood coagulation cascade. Reversible covalent modification of proteins takes on many forms, including phosphorylation, ADP-ribosylation, adenylation, methylation, acetylation, tyrosinolation, and sulphation. Phosphorylation is the most common form of reversible covalent modification. In addition to being enzyme catalyzed, the reversible nature of these modifications allows efficient control of enzyme activity by the cell. Also, these are rapid processes, leading to great changes in enzyme activity within minutes or even seconds.

The model

These studies examine the maximal ($V_{\max}$) activities of a number of enzymes of metabolism, as well as the levels and nature of various metabolites as indicators of metabolic flux and change in overwintering insect larvae. Enzyme $V_{\max}$ activity has been shown to be a reasonable indicator of overall metabolic flux through a pathway, and a definite indicator of changes in maximum possible flux (Newsholme and Crabtree, 1986). Metabolite level studies allow a more direct observation of the result of flux, showing changes in the substrate and product content. These changes will provide a picture of metabolism during overwintering in two larval insects.

Larvae of the gall fly *Eurosta solidaginis* and of the gall moth *Epiblema scudderiana*, respectively freeze tolerant and freeze avoiding, both winter inside galls on the woody stems of goldenrod (*Solidago* spp.), and frequently galls of both species can be found on the same plant. These species offer a unique comparative model for the study of the differences in the biochemical adaptations which underlie the two cold-hardiness strategies. Although much information has been gathered on the biochemistry underlying freeze avoidance and freeze tolerance in the goldenrod gall forming insects, many basic questions remain. Notably, little information has been gathered about the role of acclimation on the metabolic makeup of these larvae during an overwintering period. The studies presented in this thesis will examine this role.

CHAPTER II
ENZYME ACTIVITY PROFILES IN AN OVERWINTERING POPULATION OF
FREEZE TOLERANT LARVAE OF THE GALL FLY, *EUROSTA SOLIDAGINIS*

SUMMARY

The activity of some enzymes of intermediary metabolism, including enzymes of glycolysis, the hexose monophosphate shunt, and polyol cryoprotectant synthesis, were measured in freeze tolerant *Eurosta solidaginis* larvae over a winter season and upon entry into pupation. Flexible metabolic rearrangement was observed concurrently with acclimatization and development. Profiles of enzyme activities related to the metabolism of the cryoprotectant glycerol indicated that fall biosynthesis may occur from two possible pathways: 1. glyceraldehyde-phosphate $\rightarrow$ glyceraldehyde $\rightarrow$ glycerol, using glyceraldehyde phosphatase and NADPH-linked polyol dehydrogenase, or 2. dihydroxyacetonephosphate $\rightarrow$ glycerol-3-phosphate $\rightarrow$ glycerol, using glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase. Clearance of glycerol in the spring appeared to occur by a novel route through the action of polyol dehydrogenase and glyceraldehyde kinase. Profiles of enzyme activities associated with sorbitol metabolism suggested that this polyol cryoprotectant was synthesized from glucose-6-phosphate through the action of glucose-6-phosphatase and NADPH-linked polyol dehydrogenase. Removal of sorbitol in the spring appeared to occur through the action of sorbitol dehydrogenase and hexokinase. Glycogen phosphorylase activation ensured the required flow of carbon into the synthesis of both glycerol and sorbitol. Little change was seen in the activity of glycolytic or hexose monophosphate shunt enzymes over the winter. Increased activity of the α -glycerophosphate shuttle in the spring, indicated by greatly increased glycerol-3-phosphate dehydrogenase activity, may be key to removal and oxidation of reducing equivalents generated from polyol cryoprotectant catabolism.

INTRODUCTION

Larvae of gall fly *Eurosta solidaginis* (Fitch) (Diptera, Tephritidae) have been used extensively as a model system of insect freeze tolerance (Storey and Storey, 1992; Baust and Nishino, 1991). Because of their relative abundance and availability, larvae of this species have become the primary model of the biochemical events which underlie insect freeze tolerance. Freeze tolerance in this species results from a number of behavioral, physiological and biochemical adaptations. As in many other freeze tolerant species this includes the accumulation of cryoprotectant molecules and entry during the cold period into a state of metabolic depression in order to preserve fuel reserves for pupariation and mating the next spring and summer.

The third instar larvae of *Eurosta solidaginis* overwinter in ball-shaped stem galls on goldenrod plants. The galls themselves are poor insulators (Layne, 1991) and hence the insects must endure temperatures that are often well below the freezing point of their body fluids. Supercooling is limited to about -7 to -10°C in this species, and glycerol and sorbitol are accumulated as colligative cryoprotectants. These polyols provide protection against a freeze-induced concentration of cellular contents produced as the result of the osmotic stresses created by extracellular freezing. Survival of the larvae is limited to a maximum of 65% of extracellular water as ice (Storey and Storey, 1992).

Synthesis of polyol cryoprotectants by *Eurosta solidaginis* shows a marked temperature dependence (Baust, 1983; Storey and Storey, 1983; Rojas *et al.*, 1983). Glycerol accumulates to levels reaching 0.5 to 0.6 M (Morrissey and Baust, 1976; Storey *et al.*, 1981), its synthesis initiated around 15°C, slowing below 10°C and essentially stopping below 5°C (Storey *et al.*, 1981). Below 5°C, sorbitol synthesis begins as the result of a temperature dependent metabolic switch (Storey and Storey, 1983; Storey *et al.*, 1981; Baust and Lee, 1982; Rojas *et al.*, 1983). Sorbitol can accumulate to 0.2 M in haemolymph (Morrissey and Baust, 1976). Acclimation studies have shown that the control of and differential temperature effects on glycogen phosphorylase and

phosphofructokinase are the cause of the distinct profiles of biosynthesis of the two polyols, and hexose monophosphate shunt activity likely essential as the source of NADPH for the enzymes of cryoprotectant synthesis (Storey, 1982; Storey and Storey, 1988). The inverse relationship between glycogen disappearance and polyol accumulation, as well as the pattern of glycogen phosphorylase activation at low temperature, suggests that this carbohydrate is the major precursor for polyol synthesis in *Eurosta solidaginis* (Storey *et al.*, 1981; Storey and Storey, 1983, 1991), as in other insects (Wyatt, 1967).

Although much is known about various aspects of metabolism and specific enzyme characteristics of *Eurosta solidaginis* larvae, an overall picture of how the enzymatic make-up changes or reorganizes during winter acclimatization is missing. In this study I investigated seasonal and developmental changes in metabolic organization by assessing changes in enzyme complements including glycolysis, the hexose monophosphate shunt, cryoprotectant synthesis and other key enzymes of intermediary metabolism. This was achieved by measuring the maximal ($V_{\max}$) activities of enzymes, which relates directly to the potential throughput at these metabolic loci (Newsholme and Crabtree, 1986). These results reflect changes brought about as a result of coarse control or covalent modification, as the enzymes are assayed away from their cellular environment, i.e. after the removal of metabolites which could act as allosteric modifiers of activity. The results were correlated with previous data in order to establish the relative contribution of the various metabolic pathways to overall metabolism during overwintering and early pupation in *Eurosta solidaginis*.

MATERIALS AND METHODS

Chemicals and Animals

All biochemicals and coupling enzymes were purchased from Sigma Chemical Co., St. Louis, Mo., or Boehringer Mannheim, Montréal, Québec.

Galls containing larvae of *Eurosta solidaginis* were collected from goldenrod plants in fields around Ottawa during the fall of 1989 and kept outdoors in lightly coloured cloth sacs hanging above the snowline. At each sampling date, a few sacs containing galls were brought indoors and placed in incubators set to the outdoor temperature for that day. Table 2.1 shows mean outdoor temperatures for the month (30-31 days) and week (7 days) preceding the sampling dates as an indicator of larval acclimatization. Galls were removed from the sacs, quickly opened, and larvae were removed. Larvae assessed to be alive by visual inspection were quickly dropped into a container of liquid nitrogen. Larvae were then transferred to plastic scintillation vials for storage at -80°C until use.

Sample preparation for enzyme assays

Whole larvae were homogenized 1:5 (m:v) in ice-cold 20 mM imidazole pH 7.2, 5 mM EGTA, 5 mM EDTA, 10 mM 2-mercaptoethanol, and 50 mM NaF with a few crystals (~0.1 mM) of PMSF (a protease inhibitor) using either a glass/glass homogenizer for volumes smaller than 1.5 mL or a Tekmar Ultra-Turrax homogenizer for larger volumes. Buffer additions (EDTA, EGTA, NaF) prevented any changes in enzyme activities *in vitro* as a result of protein kinase or protein phosphatase action. Homogenates were then centrifuged at 20,000 × g for 20 min, and the supernatant was removed and desalted by centrifugation through a Sephadex G-25 column (1 × 5 cm) pre-equilibrated in homogenization buffer. Samples for glycogen phosphorylase activity determinations were

homogenized as above but were not subject to centrifugation or a spun column. Instead, settled homogenate was used as the source of enzyme.

Coupled Enzyme Assays

All enzyme activities were measured at 25°C using a Gilford 240 recording spectrophotometer. Optimal conditions were determined for each enzyme and used to measure the $V_{\max}$ activities based on the assays of Storey and Storey (1981) and Storey and Bailey (1978a, b). Controls for non-specific activity (measured in the absence of substrate or enzyme) were run for all assays and any blank value was subtracted to yield final activity values. The linearity of the assay was tested by measuring activity with two different volumes of homogenate, and the coupling system was tested with substrate for the coupling enzymes. Fructose-6-phosphate and fructose-1,6-bisphosphate solutions were tested for contamination by fructose-2,6-bisphosphate by verifying with acid treatment. One unit of enzyme activity is defined as the amount of enzyme that will convert 1 μ mole of substrate per min at 25°C. Activities linked to NAD(H) or NADP(H) are monitored at 340 nm ($\epsilon = 6.22 \text{ mM}^{-1}$). Optimal assay conditions for maximal activities were determined to be:

Glycogen phosphorylase (GPase; EC 2.4.1.1) (total $a+b$) 50 mM potassium phosphate buffer (pH 7.0), 4 mg/mL glycogen, 5 μ M glucose-1,6-P₂, 0.2 mM NADP⁺, 2 mM AMP, 15 mM MgSO₄, and 0.5 IU each of phosphoglucomutase and NADP⁺-dependent glucose-6-P dehydrogenase. The active form of the enzyme (a) was measured in the absence of AMP.

Hexokinase (EC 2.7.1.1) 20 mM imidazole-HCl buffer (pH 7.2), 2 mM glucose, 2 mM ATP, 0.5 mM NADP⁺, 10 mM MgSO₄, and 0.5 IU NADP⁺-dependent glucose-6-phosphate dehydrogenase.

Glucokinase (EC 2.7.1.2) same conditions as for hexokinase except for 100 mM glucose. Hexokinase activity was subtracted as the blank.

Phosphofructokinase (PFK; EC 2.7.1.11) 20 mM imidazole-HCl buffer (pH 7.2), 10 mM fructose-6-phosphate, 30 mM glucose-6-phosphate, 4 mM ATP, 0.15 mM NADH, 50 mM KCl, 5 mM MgSO₄, and 0.5 IU aldolase, 0.5 IU triosephosphate isomerase, and 2 IU glycerol-3-phosphate dehydrogenase.

Glycerol-3-phosphate dehydrogenase (G3PDH; EC 1.1.1.8) 20 mM imidazole-HCl buffer (pH 7.2), 1 mM dihydroxyacetonephosphate (DHAP), and 0.15 mM NADH.

Pyruvate kinase (PK; EC 2.7.1.40) 20 mM imidazole-HCl buffer (pH 7.2), 5 mM phosphoenolpyruvate, 2 mM ADP, 0.15 mM NADH, 50 mM KCl, 10 mM MgSO₄, and 1 IU lactate dehydrogenase.

Lactate dehydrogenase (LDH; EC 1.1.1.27) 20 mM imidazole-HCl buffer (pH 7.2), 2 mM pyruvate, 0.15 mM NADH.

NADP⁺-dependent isocitrate dehydrogenase (NADP-IDH; EC 1.1.1.42) 20 mM Tris-HCl (pH 8.0), 5 mM D,L-isocitrate, 0.2 mM NADP⁺, and 5 mM MgSO₄.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) 20 mM imidazole-HCl (pH 7.2), 20 mM 3-phosphoglycerate, 1 mM ATP, 0.15 mM NADH, 5 mM MgSO₄, and 1 IU phosphoglycerate kinase.

Phosphoglycerate kinase (PGK; EC 2.7.2.3) 20 mM imidazole-HCl (pH 7.2), 10 mM 3-phosphoglycerate, 1 mM ATP, 0.15 mM NADH, 5 mM MgSO₄, and 1 IU glyceraldehydophosphate dehydrogenase.

Aldolase (EC 4.1.2.13) 20 mM imidazole-HCl (pH 7.2), 0.1 mM fructose-1,6-P₂, 0.15 mM NADH, and 0.5 IU triosephosphate isomerase and 2 IU glycerol-3-phosphate dehydrogenase.

Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) 20 mM imidazole-HCl (pH 7.2), 1 mM glucose-6-phosphate, 0.2 mM NADP⁺, and 5 mM MgSO₄.

6-Phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44) 20 mM imidazole-HCl (pH 7.2), 1 mM 6-phospho-D-gluconate, and 0.2 mM NADP⁺.

Phosphoglucomutase (PGM; EC 2.7.5.1) 20 mM imidazole-HCl (pH 7.2), 1.5 mM glucose-1-phosphate, 0.02 mM glucose-1,6-P₂, 0.2 mM MgSO₄, 0.2 mM NADP⁺, and 0.5 IU glucose-6-phosphate dehydrogenase.

Phosphoglucoisomerase (PGI; EC 5.3.1.9) 20 mM imidazole-HCl (pH 7.2), 2 mM fructose-6-phosphate, 0.2 mM NADP⁺, 5 mM MgSO₄, and 0.5 IU glucose-6-phosphate dehydrogenase.

Fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11) 20 mM imidazole-HCl (pH 7.2), 0.05 mM fructose-1,6-P₂, 5 mM MgSO₄, 0.2 mM NADP⁺, and 0.5 IU each of phosphoglucoisomerase and glucose-6-phosphate dehydrogenase.

Glyceraldehyde kinase (GAK; EC 2.7.1.28, triokinase) 20 mM imidazole-HCl (pH 7.2), 10 mM D-glyceraldehyde, 1 mM ATP, 1 mM PEP, 0.2 mM NADH, 5 mM MgSO₄, and 0.5 IU each of pyruvate kinase and lactate dehydrogenase.

Sorbitol dehydrogenase (SoDH; EC 1.1.1.14) 20 mM imidazole-HCl (pH 7.2), 500 mM D-fructose, and 0.15 mM NADH.

Polyol dehydrogenase, glucose activity (PDHgluc) 20 mM imidazole-HCl (pH 7.2), 250 mM D-glucose, and 0.1 mM NADPH.

Polyol dehydrogenase, glyceraldehyde activity (PDHald) 20 mM imidazole-HCl (pH 7.2), 4 mM D-glyceraldehyde, and 0.1 mM NADPH.

Phosphatase Assays

The activity of phosphatase enzymes was assayed by the method of Atkinson *et al.* (1973). Larvae were homogenized 1:5 (m:v) in ice-cold imidazole-HCl buffer (pH 7.2) in the presence of a few crystals of PMSF in a glass/glass homogenizer. Non-specific background was decreased by desalting homogenates by passage through Sephadex G-25 columns (1 × 5 cm) equilibrated in homogenization buffer.

Solutions of homogenate and appropriate reagents were incubated for 30 minutes at 25°C. The enzyme reactions were then stopped by the addition of 2 volumes of acid molybdate reagent (10:25:65 mix of 5% Lubrol wax:2% ammonium molybdate in 1.8 M H₂SO₄:distilled water). Precipitated protein was removed by a 1 min spin in an Eppendorf centrifuge at 13000 × g. Blanks contained all components of the assay mixture, but were stopped at time zero. Standard curves of phosphate solutions (0-0.8 mM) containing the reaction substrate were run simultaneously. Colour development was monitored as absorbance at 390 nm on a Pye-Unicam SP8-100 spectrophotometer. Assay conditions used were:

Glucose-6-phosphatase (G6Pase, EC 3.1.3.9) 20 mM imidazole-HCl (pH 7.2), 5 mM glucose-6-phosphate, and 10 mM MgSO₄.

Fructose-6-phosphatase (F6Pase) 20 mM imidazole-HCl (pH 7.2), 5 mM fructose-6-phosphate, and 10 mM MgSO₄.

Glycerol-3-phosphatase (G3Pase) 20 mM imidazole-HCl (pH 7.2), 10 mM D,L-glycerol-3-phosphate, and 10 mM MgSO₄.

Glyceraldehyde-3-phosphatase (GAPase) 20 mM imidazole-HCl (pH 7.2), 5.6 mM glyceraldehyde-3-phosphate, and 10 mM MgSO₄.

Metabolite assays

Individual pre-weighed larvae were extracted in 500 μ L 6% perchloric acid containing 1 mM EDTA (Storey *et al.*, 1981); an aliquot of the homogenate was retained for glycogen determination. Homogenates were centrifuged at 18,000 $\times g$ at 4°C and then the supernatant was removed and neutralized with 3 N KOH/0.4 M TRIS/0.3 M KCl and recentrifuged. Neutralized supernatants were frozen at -70°C until use. Metabolites were quantified by coupled enzyme assay using a Pye-Unicam SP8-100 spectrophotometer and the methods of: glycogen (Keppler and Decker, 1974), glycerol (Eggstein and Kuhlman 1974), sorbitol (Bergmeyer *et al.*, 1974), glucose and fructose (Lowry and Passonneau, 1972).

Statistical analysis

All enzyme data are reported as mean $\pm$ SEM for n=4 samples with 1 or 2 larvae per sample. Metabolite levels are mean $\pm$ SEM for n=4 to 6 samples, 1 larva per sample. Data were analyzed with a one-way analysis of variance. When a significant *F* ratio was found a Student-Neuman-Keuls test was performed to examine the difference between means; *P*<0.05 was considered statistically significant.

RESULTS

Figure 2.1 shows cryoprotectant and associated metabolite levels in *Eurosta solidaginis* over the 1989-1990 winter season. Data are for third instar larvae except for the final (28 April) sampling point when larvae had pupated. Glycerol and sorbitol accumulated during the fall to reach maximal levels during the mid-winter months (190-230 $\mu\text{mol}/\text{gram}$ wet mass for glycerol, 140 $\mu\text{mol}/\text{gwm}$ for sorbitol). Glycerol levels remained high and constant throughout the winter into April, at which point they began to drop. Levels decreased to 55.5 ± 5.1 $\mu\text{mol}/\text{gwm}$ by 12 April and to 41.3 ± 8.5 $\mu\text{mol}/\text{gwm}$ by 21 April. Sorbitol levels decreased by mid-March to 9.5 ± 0.9 $\mu\text{mol}/\text{gwm}$, but rebounded transiently in late March to 48.4 ± 4.5 $\mu\text{mol}/\text{gwm}$. The temperatures corresponding to these periods show that the preceding 7 day average minimum and maximum were substantially higher in mid- than late-March (Table 2.1). Sorbitol levels resumed their decline in April upon return of warmer temperatures such that by late April pupae contained little of the polyol. Changes in fructose levels paralleled those of sorbitol, whereas glucose levels followed a pattern opposite to that of sorbitol. Pupae of *Eurosta solidaginis* (28 April) contained little glycerol (5.9 ± 1.7 $\mu\text{mol}/\text{gwm}$) or sorbitol (0.57 ± 0.16 $\mu\text{mol}/\text{gwm}$).

Synthesis of polyol cryoprotectants occurs via the mobilization of glycogen reserves in *Eurosta solidaginis* (Storey *et al.*, 1981) and Figure 2.1 concurs with this. Glycogen decreased by ~ 210 $\mu\text{mol}/\text{gwm}$ as glucose equivalents over the course of cryoprotectant synthesis in the fall, accounting for 82-89% of the total carbon equivalents for glycerol and sorbitol production. The use of glycogen is dependent on the activity of glycogen phosphorylase. Figure 2.2 shows, as expected, that total glycogen phosphorylase activity increased significantly from September to November during maximal cryoprotectant synthesis. Activity subsequently remained high through to 21 April, but had decreased by half when larvae pupated. The percentage of glycogen phosphorylase in the active α form was also highest (>90%) during the period of autumn

cryoprotectant synthesis (November) but remained at >60% *a* over the remainder of the time course.

Two possible pathways for glycerol biosynthesis have been proposed, leading from either dihydroxyacetonephosphate (DHAP) or glyceraldehyde-3-phosphate (GAP) at the triosephosphate isomerase branch point of glycolysis (Figure 2.3; Storey and Storey, 1981). From DHAP, the enzymes involved are glycerol-3-phosphate dehydrogenase (G3PDH) and glycerol-3-phosphate phosphatase (G3Pase) whereas from GAP the route is via glyceraldehyde-3-phosphatase (GAPase) and polyol dehydrogenase (PDHald). Figure 2.4 shows the status of these enzyme activities over the winter months. In overwintering *Eurosta solidaginis*, G3Pase activity did not change significantly over the study period (Figure 2.4). However G3PDH activity increased from January to a peak in March (a 2-fold increase), and then declined to reach initial levels again by 31 March. An increasing trend was seen for G3PDH activity from this date into the pupal stage. The activity of GAPase, required for glycerol synthesis from GAP, remained low and constant throughout the larval stage, but increased significantly upon puparium formation (Figure 2.4). The activity of PDHald increased during the fall, and subsequently decreased to September values by 19 March. The activity then increased significantly into the pupal stage. Figure 2.4 also shows the activity of glyceraldehyde kinase, an enzyme required for the reentry of glycerol into central metabolism. GAK activity increased 7-fold in March, preceding the decrease in glycerol levels, and subsequently declined to September levels by 4 April and remained low into puparium formation. The alternative route of glycerol catabolism requires glycerol kinase. However, we failed to detect activity of this enzyme at any time over the fall, winter, or spring. Similarly, glycerol dehydrogenase, another enzyme that might participate in glycerol metabolism, was not detected at any time.

Sorbitol synthesis has been proposed to occur via G6P, involving the dephosphorylation of the sugar phosphate and then reduction of glucose via polyol dehydrogenase (Figure 2.3; Storey and Storey, 1981). Changes in the activities of G6Pase

and PDHgluc (Figure 2.5), needed for the synthesis of sorbitol from glucose-6-phosphate, paralleled the profile of sorbitol synthesis almost exactly. Activities of both enzymes increased significantly in the fall, by 4.5-fold for PDHgluc and 2-fold for G6Pase, during the period of active sorbitol synthesis. G6Pase activity decreased slightly during the winter, whereas PDHgluc activity decreased significantly from November into spring. Both activities rose again in late March, coinciding to the transient increase in sorbitol synthesis in response to an early spring cold spell (Figure 2.1 and Table 2.1). The activities of F6Pase and sorbitol dehydrogenase (SoDH)(Figure 2.5), representing potential sorbitol synthesis from fructose-6-phosphate, did not follow the pattern of sorbitol accumulation in *Eurosta solidaginis*. Although F6Pase activity was highest during sorbitol synthesis in the fall, changes in SoDH activity paralleled the course of sorbitol removal upon spring warming. SoDH activity remained low and constant into November, after which it began to increase to peak by 19 March, a 10-fold increase. The activity of SoDH decreased during late March when a cold spell induced a transient sorbitol synthesis, but then remained high until larvae entered the pupal stage. F6Pase activity largely paralleled G6Pase activity, possibly suggesting that both activities originated from a single broadly-specific hexose-phosphate phosphatase.

Hexokinase (Figure 2.2), which is required for the uptake of hexose sugars into glycolysis, is a key enzyme needed for the return of sorbitol equivalents into intermediary metabolism. The activity of hexokinase decreased approximately 5-fold from September to November, during maximal sorbitol synthesis. However, hexokinase activity then increased steadily from November into the spring. The activity of glucokinase, a high K_m glucose-specific kinase, was also lowest in the fall, and increased steadily from September to 3 March, with a slight decline in activity during the mid-March cold spell-induced sorbitol accumulation. Taken together, the activities of these two kinases were maximal during sorbitol removal in the spring, and minimal during sorbitol synthesis in

the fall. Entry into the pupal stage was preceded by significant decreases in the activities of both enzymes.

Figure 2.6 shows the activities of several enzymes of glycolysis over the winter months. The flow of carbon from glycogen into cryoprotectant synthesis is dependent upon key enzymes of glycolysis. The maximal activity of the two irreversible enzymes which regulate the metabolism of hexose phosphates, PFK and FBPase, were relatively constant during the fall (Figure 2.6). FBPase activity was lowest during maximal cryoprotectant synthesis (November), 5-fold lower than that of PFK, reflecting the glycolytic (vs. gluconeogenic) metabolic state of the larvae. FBPase activity increased slightly in January, and subsequently remained constant until 21 April when the activity increased upon entry into the pupal stage. PFK activity peaked in early March but then decreased 2-fold upon entry into the pupal stage, such that the ratio of PFK to FBPase activities approached 0.8 in pupae compared to a ratio of 0.25 in September. Aldolase activity increased in March and remained elevated until entry into the pupal stage at which point activity decreased to values equivalent to those seen in the fall. PGI activity was elevated in November, as seen for other enzymes related to sorbitol synthesis, but decreased to reach a minimum by April. PK activity was constant throughout the winter, but decreased in the spring to reach a minimal value upon entry into the pupal stage (28 April). LDH activity remained constant from September through January but had transiently increased by March. Except for a transient increase on 19 March (30.0 ± 1.3 U/g wet wt.), PGK activity was not significantly different from 15 September activity (21.1 ± 1.0 U/g wet wt.) throughout the winter (data not shown). GAPDH and PGM activities did not change over the time course from 15 September values of 47.3 ± 2.1 and 25.0 ± 0.4 U/g wet wt. respectively (data not shown).

The activity of the two initial enzymes of the hexose monophosphate shunt, G6PDH and 6PGDH, remained relatively constant during the overwintering period (Figure 2.7). Cryoprotectant synthesis must rely on the activity of the HMS to provide

NADPH for polyol dehydrogenase activity. The activity of 6PGDH was 3 to 4-fold less than that of G6PDH during overwintering, the difference in activities narrowing to 2-fold upon entry into pupation due to a decrease in G6PDH activity. This suggests a regulatory function for 6PGDH in the flux of carbon through the HMS. NADP-IDH activity, key to providing NADPH for fat synthesis and potentially also for cryoprotectant synthesis, nearly doubled from 4 January to 3 March, and remained high even after a slight decrease by 31 March. A slight increase in NADP-IDH activity was also seen upon puparium formation.

Table 2.1 Mean Ottawa monthly (30-31 days) and weekly (7 days) temperatures immediately preceding gall insect collection dates for the winter of 1989-1990. Numbers were calculated from weather summaries obtained by the Land Resource Research Center (Agriculture Canada).

Collection date	Preceding month (°C ± SD)		Preceding week (°C ± SD)	
	max	min	max	min
Sept. 15	23.5±3.1	13.1±3.9	22.7±4.6	14.7±4.1
Nov. 1	14.4±6.2	4.1±3.4	21.1±0.6	6.0±3.1
Jan. 4	-10.8±6.6	-20.0±6.3	-6.0±9.2	-17.4±9.3
Mar. 3	-1.9±6.2	-11.6±6.5	-4.4±6.7	-14.3±7.1
Mar. 19	-0.1±7.4	-9.7±8.5	7.4±2.4	1.4±1.2
Mar. 31	2.2±6.1	-6.4±7.1	0.3±3.1	-8.0±2.2
Apr. 12	4.8±3.7	-1.9±4.0	5.2±2.3	-1.9±2.3
Apr. 21	5.3±4.3	-2.3±4.2	9.5±3.3	0.6±3.6
Apr. 28	9.9±8.2	0.7±5.3	22.9±6.0	7.6±4.1

Figure 2.1 Metabolite levels in *Eurosta solidaginis* over the 1989-90 winter season.

Animals were larvae throughout except for the final sampling date (28 April) when all had pupated. Metabolites were measured enzymatically as described in Materials and Methods. Glycogen values are expressed as μmol glucose after enzymatic hydrolysis. Results are mean $\pm$ SEM for $n=4$ to 6 samples (1 larva per sample) at each point. ^s, significantly different from 15 September values. *, significantly different from previous sampling date. Where error bars are not seen these are within the dimensions of the symbol used.

Figure 2.2: Activities of enzymes responsible for substrate entry into glycolysis in *Eurosta solidaginis* over the 1989-90 winter season. Animals were larvae throughout except for the final sampling date (28 April) when all had pupated. Activities are U/gwm, mean $\pm$ SEM for n=4 to 6 samples (1 or 2 larvae per sample). ^s, significantly different from Sept. 15 values. ^{*}, significantly different from previous sampling date. For glycogen phosphorylase (GPase) both total (upper panel) and active (%a) are shown. Where error bars are not seen these are within the dimensions of the symbol used.

Figure 2.3: Proposed pathways for glycerol and sorbitol metabolism in overwintering *Eurosta solidaginis* larvae. The enzymes are: 1, Glycogen phosphorylase. 2, Glucose-6-phosphatase. 3, Hexokinase. 4, Glucokinase. 5, Glucose-utilizing polyol dehydrogenase. 6, Sorbitol dehydrogenase. 7, Fructose-6-phosphatase. 8, Phosphofructokinase. 9, Fructose bisphosphatase. 10, Aldolase. 11, Glycerol-3-phosphate dehydrogenase. 12, Glycerol-3-phosphatase. 13, Glyceraldehyde-3-phosphatase. 14, Glyceraldehyde kinase. 15, Glyceraldehyde-utilizing polyol dehydrogenase. 16, Glucose-6-phosphate dehydrogenase. 17, 6-Phosphogluconate dehydrogenase.

Figure 2.4: Activities of enzymes of glycerol metabolism in overwintering *Eurosta solidaginis* larvae. Animals were larvae throughout except for the final sampling date (28 April) when all had pupated. Activities are U/gwm, mean $\pm$ SEM for n=4 to 6 samples (1 or 2 larvae per sample). ^s, significantly different from Sept. 15 values. *, significantly different from previous sampling date. Where error bars are not seen these are within the dimensions of the symbol used. Abbreviations are: G3Pase, Glycerol-3-phosphatase. G3PDH, Glycerol-3-phosphate dehydrogenase. GAPase, Glyceraldehyde-3-phosphatase. PDHald, Glyceraldehyde-utilizing polyol dehydrogenase. GAK, Glyceraldehyde kinase.

Figure 2.5: Activities of enzymes of sorbitol metabolism in overwintering *Eurosta solidaginis* larvae. Animals were larvae throughout except for the final sampling date (28 April) when all had pupated. Activities are U/gwm, mean $\pm$ SEM for n=4 to 6 samples (1 or 2 larvae per sample). ^{*}, significantly different from Sept. 15 values. ^{*}, significantly different from previous sampling date. Where error bars are not seen these are within the dimensions of the symbol used. Abbreviations are: G6Pase, Glucose-6-phosphatase. PDHgluc, Glucose-utilizing polyol dehydrogenase. SoDH, Sorbitol dehydrogenase. F6Pase, Fructose-6-phosphatase.

Figure 2.6: Glycolytic enzyme activities in overwintering *Eurosta solidaginis* larvae.

Animals were larvae throughout except for the final sampling date (28 April) when all had pupated. Activities are U/gwm, mean $\pm$ SEM for n=4 to 6 samples (1 or 2 larvae per sample). ^s, significantly different from Sept. 15 values. *, significantly different from previous sampling date. Where error bars are not seen these are within the dimensions of the symbol used. Abbreviations are: PGI, Phosphoglucosomerase. PFK, Phosphofructokinase. FBPase, Fructose bisphosphatase. PK, pyruvate kinase. LDH, Lactate dehydrogenase.

Figure 2.7: Activities of enzymes involved in NADPH production in overwintering *Eurosta solidaginis* larvae. Animals were larvae throughout except for the final sampling date (28 April) when all had pupated. Activities are U/gwm, mean $\pm$ SEM for n=4 to 6 samples (1 or 2 larvae per sample). †, significantly different from Sept. 15 values. *, significantly different from previous sampling date. Where error bars are not seen these are within the dimensions of the symbol used. Abbreviations are: 6PGDH, 6-Phosphogluconate dehydrogenase. G6PDH, glucose-6-phosphate dehydrogenase. NADP-IDH, NADP-dependent isocitrate dehydrogenase.

DISCUSSION

Overall, the activities of many enzymes changed substantially over the course of the winter season in *Eurosta solidaginis* larvae. Many of the changes observed correlated with the synthesis and/or degradation of polyol cryoprotectants. Others appeared to be developmentally related since significant changes in the activities of 8 enzymes occurred with the larval to pupal transition in late April. Clearly, metabolic reorganizations take place throughout the winter in the larvae with several different patterns seen. In the fall, activities of many enzymes associated with cryoprotectant synthesis increased significantly. During the winter diapause, corresponding to November to January sampling dates in this study, relatively few changes in enzyme activities were seen; however, some enzymatic activities were changing during this time, reflecting the dynamic nature of metabolism even in dormant larvae at low ambient temperatures. Between January and March, numerous changes in enzyme activities appeared to be preparatory for the spring warming and resumption of development. Thus, the activities of enzymes associated with polyol catabolism increased to allow cryoprotectant catabolism as ambient temperatures warmed. Entry into the pupal stage was also characterized by specific changes in enzyme activities. Taken together, these results show a complex and flexible system of enzymatic rearrangement in this species in response to acclimatization or developmental needs.

The seasonal pattern of cryoprotectant accumulation and clearance by overwintering *Eurosta solidaginis* seen in this study was essentially the same as that previously reported for this species (Storey and Storey, 1986; Morrissey and Baust, 1976). Glycerol accumulated early in the fall, followed by sorbitol upon arrival of colder temperatures. Both cryoprotectants were synthesized at the expense of glycogen. As such, the activity of glycogen phosphorylase in both total units ($a+b$) and the percent active ($\%a$), was found to be maximal in the fall during active cryoprotectant synthesis (Figure 2.2). The importance of GPase activity to the synthesis of cryoprotectants was further

illustrated by the correlation between high GPase %a and periods of active sorbitol synthesis. The increase in sorbitol levels on 31 March, resulting from a late winter cold spell, was correlated with an increase in the active (%a) form of GPase. The minimum and maximum temperature averages of the week preceding 31 March were well below the 5°C trigger temperature believed to be key for the initiation of sorbitol synthesis (Table 2.1; Baust, 1983; Storey and Storey, 1983; Rojas *et al.*, 1983). This also reinforces the importance of this polyol as an easily reusable cryoprotectant, one that is readily interconverted with glycogen and that is energetically inexpensive to synthesize.

Glycerol synthesis by cold hardy insects has been proposed to occur by two possible pathways, shown in Figure 2.3 (Storey and Storey, 1988). In the first pathway GAP would be dephosphorylated to glyceraldehyde, which would then be reduced to glycerol by PDHald using NADPH, presumably produced by the hexose monophosphate shunt. The second pathway relies on the activity of G3PDH to make G3P from DHAP (with NADH), which would then be dephosphorylated by G3Pase. Evidence supporting glycerol synthesis via the first pathway includes the continuous presence of GAPase over the winter and an increase, during the period of maximal cryoprotectant synthesis in the fall, in PDHald activity. However, the concurrent presence of G3PDH and G3Pase activities could also allow the second pathway to operate in the synthesis of glycerol. In fact, the lowest activity enzymes in both pathways, G3Pase and GAPase, were present in nearly identical levels during the period of cryoprotectant synthesis indicating near equal metabolic potential of both pathways. The accumulation of G3P upon the cessation of glycerol biosynthesis (Storey and Storey, 1981; Storey *et al.*, 1981) has previously been used to argue for the routing of synthesis through G3PDH and G3Pase. However, the relatively high potential activity of the HMS in both *Eurosta solidaginis* and other cold-hardy insects (Storey *et al.*, 1991) has suggested that polyol synthesis is NADPH-linked and therefore implicates PDHald in glycerol synthesis. Indeed, of the various sugar substrates that are recognized by insect polyol dehydrogenases, relative maximal activity

and affinity for glyceraldehyde is the highest by far (Faulkner, 1957; Chino, 1960; Sømme and Velle, 1968; Takahashi *et al.*, 1974). Furthermore, radiolabelling studies have shown that carbon flow through the HMS increases relative to glycolytic flux as temperature decreases (Tsumuki *et al.*, 1987). The $V_{\max}$ activity of 6PGDH was considerably lower than that of G6PDH (Figure 2.6), suggesting a regulatory function for this enzyme in the HMS. Indeed, the kinetic properties of 6PGDH from *Eurosta solidaginis* have been shown to be geared towards maximal hexose monophosphate shunt and NADPH synthesis during cryoprotectant synthesis (Holden and Storey, 1994a). Carbon shuttling through the HMS also leads to GAP and F6P outputs from the cycle, suggesting that both GAP and NADPH products of the cycle may be channeled into glycerol synthesis. However, it has been calculated that although most of the glucose-6-phosphate from glycogenolysis must pass through the HMS to produce sufficient reducing equivalents for glycerol synthesis, only 20% of the carbon skeletons are retrieved as triose sugars (GAP and DHAP)(Storey and Storey, 1988). The remaining 80% exits as fructose-6-phosphate, and must be converted to fructose-1,6-bisphosphate (by PFK) to be used for glycerol production. Glycerol biosynthesis is then dependent on ATP supply.

The present study also changes previous concepts of how glycerol is catabolized by cold-hardy insects. The failure to detect glycerol kinase activity in the larvae at any time indicates that the catabolism of glycerol cannot follow the path of glycerol to G3P to DHAP as previously suggested (Storey and Storey, 1992, 1988, 1981). Instead, the increase in glyceraldehyde kinase activity in early March correlated well with the decrease in glycerol levels, suggesting that the removal of glycerol occurs via its reconversion to glyceraldehyde (via PDHald) followed by phosphorylation to produce GAP (via glyceraldehyde kinase). To my knowledge, this is the first report of the use of this pathway in cryoprotectant catabolism. The spring decline in glycerol does not occur with resynthesis of glycogen pools (Figure 2.1 and Storey and Storey, 1986). One

possible explanation for the fate of glycerol carbon is the conversion of glycerol to acetyl-CoA for energy production in the TCA cycle or for the production of lipid reserves. Increasing mitochondrial enzyme activity (Joanisse and Storey, 1994a) and increased NADP-IDH activity in the spring (Figure 2.6) could correspond to such metabolic arrangements (NADPH produced from NADP-IDH is utilized for fat synthesis). Elevated G3PDH activity in the spring might also suggest an increase in the activity of the α -glycerophosphate shuttle, used by insects to transfer reducing equivalents into the mitochondria (Sacktor, 1976). Indeed, G3PDH from *Eurosta solidaginis* has properties geared towards the operation of this cycle (Storey and Storey, 1982). NADPH produced from PDHald conversion of glycerol to glyceraldehyde could be converted to NADH by cellular transhydrogenases and utilized by the α -glycerophosphate shuttle. GAP from glycerol catabolism could also be converted to DHAP by triosephosphate isomerase, and utilized directly in the shuttle. This shuttle could be key to the removal of reducing equivalents liberated by the catabolism of polyols. The spring increase in G3PDH also correlates with the general increase in mitochondrial enzyme activities (Chapter V; Joanisse and Storey, 1994a); the α -glycerophosphate shuttle, required for aerobic carbohydrate oxidation in insects, is then closely linked with the increased aerobic scope of the larvae in the spring. Alternatively, NADPH from PDHald and glycerol carbon could be used directly in the synthesis of lipids; this may be especially true in *Epiblema scudderiana* since glycerol appears to be converted to lipids in the spring in these larvae (see Chapter IV).

An absence of change in the maximum activity of PFK over the winter suggests that coarse control or post-translational modification of the enzyme do not contribute in creating the required shift in metabolism from glycerol to sorbitol production at low temperatures. Instead, the shut down at PFK appears to result from the effects of temperature and temperature-modifier interactions, the enzyme having a Q_{10} of 3.64

between 10 and 0°C, and in the cold a decreased affinity for F6P, decreased activation by AMP, and increased inhibition by ATP (Storey, 1982).

Sorbitol synthesis from glycogen could occur through the glycolytic intermediates G6P or F6P in *Eurosta solidaginis* (Figure 2.3) but the data from this study support the routing via G6P. This is evidenced by the activity profiles of PDHgluc and SoDH. PDHgluc activity peaked in the fall, and was 8-fold higher than SoDH activity. The predominance of carbon shuttling through the HMS and not glycolysis during sorbitol synthesis (Tsumuki *et al.*, 1987) also couples the NADPH produced in this pathway to PDHgluc, which requires this nucleotide as a specific cofactor for the reduction of glucose. The removal of sorbitol in the spring might also proceed by either of two routes. Both glucokinase and the non-specific (glucose or fructose utilizing) hexokinase activities increased in the spring, suggesting that the catabolism of sorbitol could be channeled into either G6P or F6P. However, by springtime the activity of SoDH had become greater than that of PDHgluc, suggesting this is probably the pathway for sorbitol degradation. In addition, equilibrium constants and affinity constants (K_m values) for both PDHgluc and SoDH support the use of PDHgluc for anabolism and SoDH for catabolism of sorbitol (Jeffrey and Jörnvall, 1988; Yaginuma and Yamashita, 1979; Goil and Harpur, 1978; Leissing and McGuinness, 1983; O'Brien *et al.*, 1983). These interpretations also agree with previous precursor-product studies that indicated that sorbitol synthesis resulted from G6P via PDHgluc whereas the clearance of sorbitol was routed via SoDH to fructose and then F6P (Storey and Storey, 1981, 1983). Increased SoDH activity correlated with sorbitol catabolism has also been seen in other insect species (Yaginuma and Yamashita, 1979; Yamashita *et al.*, 1988).

Gluconeogenesis would be expected to contribute little to metabolism in overwintering *Eurosta solidaginis*, as metabolic flux is primarily directed to carbohydrate conversion into polyol cryoprotectants. This is reflected in the PFK/FBPase activity ratio. Throughout the winter PFK activity was 5-fold greater than that of FBPase. However,

pupation was characterized by an increase in FBPase activity and a decrease in PFK, so that the activity ratio approached unity (~ 0.8). This suggests a greater gluconeogenic flux as a part of pupal reorganization.

The constant activities of PK indicate that the total potential shuttling of carbon into mitochondria from glycolysis through this enzyme remains unchanged over the winter. However, as with PFK, temperature effects have been shown to affect strongly the activity of the enzyme. Thus, cold temperatures decrease the catalytic activity of PK, shutting down flow from glycolysis to the TCA cycle (Storey and Storey, 1983). Mitochondrial enzyme activities have been shown to decrease dramatically by mid-winter and to subsequently recover in the spring (Chapter V; Joanisse and Storey, 1994a), reflecting the reduced dependence of the larvae on aerobic metabolism during dormancy and freezing.

An overall metabolic picture in overwintering *Eurosta solidaginis* begins to take shape from the present and previous studies. As fall progresses, enzymes of glycolysis and the HMS channel glucose equivalents down to DHAP and GAP, where they are used for the massive production of the cryoprotectant glycerol, which probably occurs primarily via GAPase and PDHald but also via G3PDH and G3Pase (Figure 2.3). Colder temperatures shut down PFK, blocking the flow of hexose monophosphates through the glycolytic pathway, shifting the flow of G6P into sorbitol via G6Pase and PDHgluc (Figure 2.3). Upon the return to warmer temperatures in the spring, SoDH and hexokinase activities lead to sorbitol removal (back to glycogen). Flexible activities of G6Pase and PDHgluc ensure the resynthesis of sorbitol in the spring in the event of a cold spell. As spring temperatures increase and the chance of freezing decreases, glycerol removal begins through PDHald and GAK. Increased α -glycerophosphate shuttle enzyme activity ensures the transfer of reducing equivalents from cryoprotectant catabolism into mitochondria for oxidation, mitochondrial enzyme activity increasing concurrently (Chapter V; Joanisse and Storey, 1994a). Glyconeogenic enzyme activities (FBPase,

hexokinase and glucokinase) remain low during cryoprotectant synthesis, but increase in the spring for the return of glucose equivalents to glycogen or other metabolic fates.

CHAPTER III
ENZYME ACTIVITY PROFILES IN AN OVERWINTERING POPULATION OF
FREEZE AVOIDING GALL MOTH LARVAE, *EPIBLEMA SCUDDERIANA*

SUMMARY

The maximal activities of enzymes of intermediary metabolism, notably glycolysis, the hexose monophosphate shunt, and polyol cryoprotectant synthesis, were measured over a winter season in freeze avoiding larvae of *Epiblema scudderiana*. Changes in enzyme activities were found to reflect metabolic events associated with different and changing requirements of the larvae for survival as winter progressed and ended. Activities of enzymes associated with the cryoprotectant glycerol indicated two possible pathways for its synthesis: 1. glyceraldehyde-phosphate $\rightarrow$ glyceraldehyde $\rightarrow$ glycerol, via glyceraldehyde phosphatase and NADPH-linked polyol dehydrogenase, or 2. dihydroxyacetonephosphate $\rightarrow$ glycerol-3-phosphate $\rightarrow$ glycerol, via glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase. Glycogen phosphorylase activation in the fall supplied carbon equivalents required for glycerol synthesis from glycogen. Hexose monophosphate shunt enzyme activity was high, reflecting the role of this pathway in supplying NAD(P)H for glycerol synthesis. Spring clearance of glycerol appeared to occur via polyol dehydrogenase and glyceraldehyde kinase. Increasing fructose-bisphosphatase activity into late winter and spring was found to increase the gluconeogenic potential, needed for cryoprotectant removal. Increased activity of glycerol-3-phosphate dehydrogenase in the spring, possibly reflecting increased α -glycerophosphate shuttle activity, may be key to the removal of reducing equivalents generated from glycerol removal.

INTRODUCTION

Late instar larvae of the moth *Epiblema scudderiana* (Clemens) (Lepidoptera, Olethreutidae) overwinter in spindle-shaped galls on stems of goldenrod plants (*Solidago* spp.), often on the same stem as *Eurosta solidaginis* galls. Here they must face low ambient winter temperatures, as the gall is usually above the snowline and offers no thermal buffering (Layne, 1991). These larvae maintain a liquid state throughout the winter, depressing their supercooling point to below -38°C . Survival of the larvae is maintained as long as freezing does not occur: a silk cocoon lining the gall cavity may prevent inoculative freezing. Reduced metabolic rates (quiescence and/or diapause) ensure sufficient fuel reserves for overwintering and pupation the following spring. Key to *Epiblema scudderiana* survival is an accumulation of the cryoprotectant glycerol to $2000\text{ }\mu\text{mol/g}$ wet mass, representing almost 19% of the body mass of the insect (Rickards *et al.*, 1987).

The synthesis of glycerol in *Epiblema scudderiana*, as in other insect species, is supported by the breakdown of glycogen accumulated prior to the winter season (Storey and Storey, 1991; Wyatt, 1967). In *Epiblema scudderiana* synthesis of glycerol is rapidly stimulated below 5°C , maximal rates occurring between 0 and -10°C (Kelleher *et al.*, 1987; Storey and Storey, 1988). Cold activation of glycogen phosphorylase is key in the initiation of glycerol biosynthesis (Churchill and Storey, 1989a, 1989b), likely from temperature modulation of the phosphorylation cascade that activates the enzyme (Storey and Storey, 1991; Ziegler *et al.*, 1979; Hayakawa and Chino, 1982).

A number of studies have examined specific aspects of metabolism and the regulation of various enzymes of polyol metabolism in *Epiblema scudderiana*. For example, the initiation and control of polyol biosynthesis is well documented (Churchill and Storey, 1989b) as are the properties of key regulatory enzymes such as phosphofructokinase (Holden and Storey, 1993), glucose-6-phosphate dehydrogenase (Storey *et al.*, 1991) and glycogen phosphorylase (Holden and Storey, 1994b). However,

as for *Eurosta solidaginis* (Chapter II), a complete study of enzymatic changes and control over a winter season is missing. In this study I investigated seasonal changes in metabolic organization in the larvae by monitoring changes in the enzyme complements of glycolysis, the hexose monophosphate shunt, cryoprotectant synthesis and other pathways of intermediary metabolism. This was accomplished by relating maximal ($V_{\max}$) activities of enzymes to potential throughput at metabolic loci (Newsholme and Crabtree, 1986). When correlated with other data a picture of overwintering metabolism in these larvae is formed.

MATERIALS AND METHODS

Chemicals and Animals

All biochemicals and coupling enzymes were purchased from Sigma Chemical Co., St. Louis, Mo., or Boehringer Mannheim, Montréal, Québec. Galls containing larvae of *Epiblema scudderiana* were collected from goldenrod plants in fields around Ottawa during the fall of 1989 and larvae were prepared as described in Chapter II.

Sample preparation for enzyme assays

Larvae were homogenized and enzymes assayed as described in Chapter II.

Coupled Enzyme Assays

Enzyme activities were measured as described in Chapter II. Optimal assay conditions for maximal enzyme activities were the same as in Chapter II with the following exceptions:

GPase (EC 2.4.1.1) (Total *a+b*) 50 mM potassium phosphate buffer (pH 7.0), 4 mg/mL glycogen, 5 μ M glucose-1,6-P₂, 0.2 mM NADP⁺, 1 mM AMP, 15 mM MgSO₄, and 0.5 IU each of PGM and NADP⁺-dependent G6PDH. The active form of the enzyme (*a*) was measured in the absence of AMP.

G3PDH (EC 1.1.1.8) 20 mM imidazole-HCl buffer (pH 7.2), 0.5 mM DHAP, and 0.15 mM NADH.

NADP⁺-IDH (EC 1.1.1.42) 20 mM imidazole-HCl (pH 7.2), 10 mM D,L-isocitrate, 0.2 mM NADP⁺, and 5 mM MgSO₄.

PGI (EC 5.3.1.9) 20 mM imidazole-HCl (pH 7.2), 4 mM fructose-6-phosphate, 0.2 mM NADP⁺, 5 mM MgSO₄, and 0.5 IU G6PDH.

FBPase (EC 3.1.3.11) 20 mM imidazole-HCl (pH 7.2), 0.1 mM fructose-1,6-P₂, 5 mM MgSO₄, 0.2 mM NADP⁺, 0.5 IU PGI, and 0.5 IU G6PDH.

PDHald 20 mM imidazole-HCl (pH 7.2), 80 mM D-glyceraldehyde, and 0.1 mM NADPH.

Phosphatase Assays

Assays were performed as described in Chapter II, except for the following optimal conditions:

G6Pase (EC 3.1.3.9) 20 mM imidazole-HCl (pH 7.2), 10 mM glucose-6-phosphate, and 10 mM MgSO₄.

Metabolite assays

Assays were performed as described in Chapter II.

Statistical analysis

All enzyme data are reported as mean $\pm$ SEM for n=4 samples with 1 or 2 larvae per sample. Metabolite levels are mean $\pm$ SEM for n=4 samples, 1 larva per sample. Data were analyzed with a one-way analysis of variance. When a significant *F* ratio was found a Student-Neuman-Keuls test was performed to examine the difference between means; *P*<0.05 was considered statistically significant.

RESULTS

Cryoprotectant and associated metabolite levels of *Epiblema scudderiana* for the 1989-1990 winter season are shown in Figure 3.1. Glycerol accumulated during the fall to reach maximal levels during mid-winter, rising 67-fold. Glycerol levels had begun to decrease by early March and by 21 April the levels were not significantly different from pre-overwintering levels. Sorbitol levels increased 63-fold by 1 January, but the maximal content of $7.6 \pm 0.8 \mu\text{mol/gwm}$ was clearly inconsequential in terms of colligative cryoprotection when compared to the content of glycerol reaching $2155 \pm 274 \mu\text{mol/gwm}$. By 21 April sorbitol levels had also decreased to pre-overwintering levels. Changes in glucose and fructose levels paralleled those of glycerol and sorbitol, except for an apparent increase in their levels in late April. This increase was not statistically significant due to large variance.

Figure 3.1 also shows that the depletion of glycogen reserves paralleled the increase in polyol cryoprotectant levels. Glycogen was accumulated to $800 \pm 40 \mu\text{mol/gwm}$ (in glucose equivalents) by 1 November, and decreased by $756 \mu\text{mol/gwm}$ to $44 \pm 18 \mu\text{mol/gwm}$ by 4 January. Only a portion of cryoprotectant clearance in the spring could be accounted for by reconversion to glycogen: the increase of $382 \mu\text{mol/gwm}$ of glycogen in the spring accounted for only 36% of removed cryoprotectant. GPase activity, required to initiate cryoprotectant synthesis, peaked in mid-winter, total activity ($a+b$) increasing almost 3-fold during this period (Figure 3.2). The active form ($\%a$) also peaked at this time, increasing from near 0% on 1 November to almost 60% by 4 January. Both total and active form activities of the enzyme returned to September values in the spring. The changes in GPase activity exactly paralleled the winter accumulation and spring removal of glycerol in the larvae.

Pathways for glycerol biosynthesis, shown in Figure 3.3, arise from the triose phosphates of glycolysis, DHAP or GAP, as described in Chapter II. The enzymes required for both routes were found in *Epiblema scudderiana* larvae, and their activities

are shown in Figure 3.4. G3PDH activity increased over 3-fold in the fall and remained relatively constant for the remainder of the study period. G3Pase activity remained constant throughout the fall into late winter, but increased 2-fold in mid-March; subsequently activity decreased to a constant level for the remainder of the study period. PDHald activity increased over 7-fold from September to November, and then remained constant for the rest of the study period. GAPase was constant into late winter, and increased transiently in mid-March. GAK activity, required for the reconversion of glycerol into GAP, increased over 2-fold beginning in the fall to peak by 2 March, and subsequently decreased to September values by 21 April. Neither glycerol kinase nor glycerol dehydrogenase, both enzymes that could also act in the catabolism of glycerol, were detected in these larvae at any time.

The activities of sorbitol metabolizing enzymes are shown in Figure 3.5. G6Pase and SoDH activities were constant throughout the study period, SoDH activity being very low. F6Pase activity was highly variable, with transient decreases in November, early March, and mid-April. The activity of polyol dehydrogenase utilizing glucose as substrate (tested with 250 mM glucose), required for the conversion of glucose to sorbitol, was not detected in the larvae at any time during the study period.

The activities of hexokinase and glucokinase, involved in providing carbon for intermediary metabolism from hexoses, are shown in Figure 3.2. Hexokinase activity decreased slightly from September to November, remained constant into mid-March, and decreased transiently to return to September values by late April. Glucokinase activity remained constant throughout the fall and winter and increased 4-fold starting 19 March to 21 April.

The FBPase/PFK maximal activity ratio, representing relative potential of the gluconeogenic versus glycolytic pathways, was <1 during the period of cryoprotectant accumulation in the fall (Table 3.1). By 4 January the ratio had reached 3.5, and it continued increasing into the late winter and spring to peak at 6.8 on 3 March, during

cryoprotectant removal. This represented a 31-fold increase in the ratio from 15 September to 3 March. The increased ratio was largely due to a 14-fold increase in FBPase activity (Figure 3.6). The activity of PFK, the regulatory enzyme of the first half of glycolysis, decreased in the spring (except for a transient increase on 19 March) during maximal cryoprotectant removal (Figure 3.6). The activities of other enzymes of the top half of glycolysis are also shown in Figure 3.6. Aldolase activity increased 7-fold from September to January and remained high for the remainder of the study. PGM activity was constant until entry into April, when it increased significantly. PGI activity was constant except for a transient decrease from 19 March to 31 March, activity returning to September values by 12 April.

The activities of the enzymes of the lower half of the glycolytic pathway are shown in Figure 3.7. GAPDH and PGK increased 2.8- and 3.6-fold from September into the spring. PK activity remained constant to 19 March, when it increased transiently by 1.3-fold. LDH activity did not change significantly throughout the study period. This is consistent with previous work which has shown no accumulation of lactate during cold exposure (Churchill and Storey, 1989c; Kelleher *et al.*, 1987) and reflects the maintenance of a largely aerobic state of the larvae during undercooling.

The activity of G6PDH and 6PGDH, the first two enzymes of the HMS required for the generation of NADPH, did not change significantly from their September values (Figure 3.8). NADP-IDH activity, also involved in NADPH production, increased 1.6-fold from 15 September to 1 November and remained constant to 12 April, where activity peaked at 11.2 ± 0.8 U/gwm after a second 1.7-fold increase (Figure 3.7). Overall this represented a 2.7-fold increase in NADP-IDH activity from 1 September to 12 April.

Table 3.1. Relative glycolytic versus gluconeogenic potential expressed as the ratio of fructose bisphosphatase / phosphofructokinase activity in overwintering *Epiblema scudderiana* larvae. Values are the ratio of the final $V_{\max}$ means for each enzyme activity at each sampling date.

Date	Sept. 15	Nov. 1	Jan. 4	Mar. 3	Mar. 19	Mar. 31	Apr. 12	Apr. 21
<u>FBPase</u>								
<u>PFK</u>	0.2	0.9	3.5	6.8	2.5	4.2	1.9	2.1

Figure 3.1: Metabolite levels in larval *Epiblema scudderiana* over the 1989-90 winter season. Glycogen values are expressed as μmol glucose after enzymatic hydrolysis. Results are mean $\pm$ SEM for $n=4$ samples (1 larva per sample) at each point. ^s, significantly different from 15 September values. ^{*}, significantly different from previous sampling date. Where error bars are not seen these are within the dimensions of the symbol used.

Figure 3.2: Activities of enzymes responsible for substrate entry into glycolysis in *Epiblema scudderiana* larvae over the 1989-90 winter season. Activities are U/gwm, mean $\pm$ SEM for n=4 to 6 samples (1 or 2 larvae per sample). ^s, significantly different from Sept. 15 values. *, significantly different from previous sampling date. Where error bars are not seen these are within the dimensions of the symbol used. For glycogen phosphorylase (GPase) both total (upper panel) and active (%a) forms are shown.

Figure 3.3: Proposed pathways for polyol metabolism in overwintering *Epiblema scudderiana* larvae. The enzymes are: 1, Glycogen phosphorylase. 2, Glucose-6-phosphatase. 3, Hexokinase. 4, Glucokinase. 5, Fructose-6-phosphatase. 6, Sorbitol dehydrogenase. 7, Phosphofructokinase. 8, Fructose bisphosphatase. 9, Aldolase. 10, Glycerol-3-phosphate dehydrogenase. 11, Glycerol-3-phosphatase. 12, Glyceraldehyde-3-phosphatase. 13, Glyceraldehyde kinase. 14, Glyceraldehyde-utilizing polyol dehydrogenase. 15, Glucose-6-phosphate dehydrogenase. 16, 6-Phosphogluconate dehydrogenase. Note the absence of polyol dehydrogenase converting glucose to sorbitol (PDHgluc) and of glycerol kinase.

Figure 3.4: Activities of enzymes of glycerol metabolism in overwintering *Epiblema scudderiana* larvae. Activities are U/gwm, mean $\pm$ SEM for n=4 to 6 samples (1 or 2 larvae per sample). ^s, significantly different from Sept. 15 values. ^{*}, significantly different from previous sampling date. Where error bars are not seen these are within the dimensions of the symbol used. Abbreviations are: G3Pase, Glycerol-3-phosphatase. G3PDH, Glycerol-3-phosphate dehydrogenase. GAPase, Glyceraldehyde-3-phosphatase. PDHald, Glyceraldehyde-utilizing polyol dehydrogenase. GAK, Glyceraldehyde kinase.

Figure 3.5: Activities of enzymes of sorbitol metabolism in overwintering *Epiblema scudderiana* larvae. Activities are U/gwm, mean $\pm$ SEM for n=4 to 6 samples (1 or 2 larvae per sample). ^s, significantly different from Sept. 15 values. ^{*}, significantly different from previous sampling date. Where error bars are not seen these are within the dimensions of the symbol used. Abbreviations are: G6Pase, Glucose-6-phosphatase. SoDH, Sorbitol dehydrogenase. F6Pase, Fructose-6-phosphatase.

Figure 3.6: Enzyme activities of the top half of glycolysis in overwintering *Epiblema scudderiana* larvae. Activities are U/gwm, mean $\pm$ SEM for n=4 to 6 samples (1 or 2 larvae per sample). ^s, significantly different from Sept. 15 values. *, significantly different from previous sampling date. Where error bars are not seen these are within the dimensions of the symbol used. Abbreviations are: PGM, Phosphoglucomutase. PGI, Phosphoglucoisomerase. PFK, Phosphofructokinase. FBPase, Fructose biphosphatase.

Figure 3.7: Enzyme activities of the bottom half of glycolysis in overwintering *Epiblema scudderiana* larvae. Activities are U/gwm, mean $\pm$ SEM for n=4 to 6 samples (1 or 2 larvae per sample). ^s, significantly different from Sept. 15 values. ^{*}, significantly different from previous sampling date. Where error bars are not seen these are within the dimensions of the symbol used. Abbreviations are: GAPDH, Glyceraldehyde-3-phosphate dehydrogenase. PGK, Phosphoglycerate kinase. PK, Pyruvate kinase. LDH, Lactate dehydrogenase

Figure 3.8: Activities of enzymes involved in NADPH production in overwintering *Epiblema scudderiana* larvae. Activities are U/gwm, mean $\pm$ SEM for n=4 to 6 samples (1 or 2 larvae per sample). ^s, significantly different from Sept. 15 values. ^{*}, significantly different from previous sampling date. Where error bars are not seen these are within the dimensions of the symbol used. Abbreviations are: 6PGDH, 6-Phosphogluconate dehydrogenase. G6PDH, glucose-6-phosphate dehydrogenase. NADP-IDH, NADP-dependent isocitrate dehydrogenase.

DISCUSSION

Epiblema scudderiana larvae enter a diapause state which extends from late November to sometime in February (Rickards *et al.*, 1987). However, even during diapause or the subsequent winter quiescence maintained by cold temperatures and dormancy, the larvae remain metabolically active, changing the activities of individual enzymes and the ratios of enzymes in metabolic pathways. This dynamic system responds to the metabolic needs of the larvae for winter survival.

The seasonal accumulation and removal of the cryoprotectant glycerol in the larvae used in this study was identical to that previously reported for this species (Storey and Storey, 1986; Rickards *et al.*, 1987). Glycerol accumulated in the fall at the expense of glycogen. In support of this biosynthesis the activity of GPase, both total and active (%a), was found to be maximal at times of maximal cryoprotectant synthesis, as was also previously described for laboratory acclimation studies (Churchill and Storey, 1989a). The well-documented low temperature activation of GPase in insects (Ziegler *et al.*, 1979; Hayakawa and Chino, 1982) and specifically in *Epiblema scudderiana* (Churchill and Storey, 1989a) are key in initiating cryoprotectant synthesis. GPase activity was low up to early November, and then activated by colder fall temperatures (Table 2.1). The reverse occurred in the spring, with warmer temperatures paralleled by a decrease in glycerol content of the larvae and decreased glycogen phosphorylase activity.

Activity profiles of the enzymes of glycerol biosynthesis suggest two possible pathways for the accumulation of this polyol in *Epiblema scudderiana*, as shown in Figure 3.3. Both pathways begin at the triose-phosphate branchpoint of the glycolysis, and require NADH or NADPH as a source of reducing equivalents. The first pathway, from DHAP, requires activities of G3PDH and G3Pase. The possible use of this route is supported by the steady increase in G3PDH activity in the fall to peak in mid-winter and the constant activity of G3Pase throughout the same time period. Also supporting this route are metabolite studies that showed a decrease in G3P upon cold-initiation of

glycerol synthesis and G3P accumulation upon cessation of glycerol synthesis in this species (Churchill and Storey, 1989b). The second possible pathway involves GAPase and PDHald activities. Although GAPase activity was low and constant during the fall, PDHald activity increased over 7-fold from 15 September to 1 November, strongly supporting the use of this enzyme in glycerol biosynthesis. Unfortunately, no corroborating metabolite data are available for this pathway.

Reducing equivalents for glycerol synthesis are produced by the activity of the first two enzymes of the HMS. These produce NADPH that can be used by PDHald whereas transhydrogenase reactions can make these reducing equivalents available to G3PDH. It has been calculated that at least 86% of glycogenolytic carbon must flow through the HMS to support the NAD(P)H requirement for glycerol synthesis (Storey and Storey, 1988). Indeed, high rates of HMS activity have previously been shown to accompany cold-induced cryoprotectant accumulation in insects (Wood and Nordin, 1980; Tsumuki *et al.*, 1987). The activities of the NADPH producing enzymes of the HMS, G6PDH and 6PGDH, were high and constant throughout the study period (Figure 3.7). G6PDH activity in this study was comparable to that found in a previous study (Storey *et al.*, 1991), which also showed this activity to be 16-17 fold greater than in a species that does not produce glycerol, *Tenebrio molitor*. The maximal activity of 6PGDH was slightly lower than that of G6PDH (Figure 3.7), suggesting a possible regulatory function for this enzyme in the HMS of *Epiblema scudderiana*.

A surprising result of this study was our failure to detect any glycerol kinase activity at any time. This suggests that glycerol clearance upon spring warming cannot occur via this enzyme, as previously suggested (see Storey and Storey, 1992). Instead, the present data suggest glycerol catabolism occurs via the route glycerol to glyceraldehyde to glyceraldehyde-3-phosphate. The first step involves a reversal of the PDHald reaction, regenerating NADPH that may be used for biosynthesis by the larvae. The second step is catalyzed by an ATP-dependent glyceraldehyde kinase and reintroduces the carbon

skeleton into the glycolytic pathway. This is analogous to events in the freeze tolerant gall fly *Eurosta solidaginis* (Chapter I.; Joannis and Storey, 1994b).

Of the total glycerol cleared in the spring only 36% can be accounted for by reconversion to glycogen (Figure 3.1). This is consistent with previous work on this species (Rickards *et al.*, 1987), showing most of the glycerol must have other metabolic fates. Some clues may be provided by this study. As suggested for the freeze tolerant gall fly *Eurosta solidaginis* (Chapter II; Joannis and Storey, 1994b), alternative fates of the polyol occur from the triose phosphates (GAP and DHAP) arising from glycerol clearance back into the glycolytic pathway. These can include conversion of GAP to acetyl-CoA and its subsequent use in either fat synthesis or energy production via the TCA cycle. The NADPH produced by PDHald during glycerol clearance could be used directly in fat synthesis. Increasing NADP-IDH activity in the spring, an important enzyme in the generation of NADPH for fat synthesis, also suggests increased fat production. Increased G3PDH activity in the spring could serve to supply glycerol-3-phosphate required for glyceride synthesis. Elevated G3PDH and increased activities of mitochondrial enzymes in the spring (Chapter V; Joannis and Storey, 1994a) also suggest increased operation of the α -glycerophosphate shuttle, used by insects for the transfer of reducing equivalents into mitochondria for ATP production (Sacktor, 1976). The operation of this shuttle could be key to the removal of reducing equivalents resulting from polyol clearance. Increased mitochondrial enzyme activities in the spring (Joannis and Storey, 1994a) also suggest an increased potential for energy production by the oxidation of carbon from glycerol clearance in the TCA cycle.

The increase in the ratio of FBPase/PFK activity as the winter progresses (Table 3.2) indicates an increasing potential for gluconeogenesis into the spring, and precedes the removal of glycerol in late winter and early spring. This increased gluconeogenic potential may explain the observed ability of mid- and late-winter larvae to resynthesize glycogen from glycerol upon exposure to 16°C (Churchill and Storey, 1989a). These

authors have shown that glycerol accumulation did not cease in cold-acclimated (-4°C) fall larvae when they were subsequently exposed to alternating temperatures of 16°C and -4°C . From December on, however, the same cycling procedure led to the catabolism of glycerol in each 16°C period. The present results suggest that FBPase activity is kept low in the fall to ensure the efficient production of cryoprotectant, and to prevent the needless and energy expensive conversion-reconversion of the polyol to glycogen (ATP is required at PFK in the biosynthesis of glycerol and at GAK in the degradation of the polyol). In addition, low aldolase activity in the fall (Figure 3.6) may further serve to limit the return of glycerol into glycogen. The increased activity of aldolase paralleling the potential for the return of glycerol to glycogen indicates that this enzyme may also play a role in the control of carbon flux in the interconversion of glycerol and glycogen. Glycolytic versus gluconeogenic flux is also controlled by allosteric effectors affecting different enzymes in *Epiblema scudderiana*. Cold exposure leads to activation of 6-phosphofructo-2-kinase (PFK2) and increased levels of fructose-2,6-bisphosphate (F2,6P₂) paralleling glycerol accumulation (Churchill and Storey, 1989b). F2,6P₂ is a potent inhibitor of FBPase and activator of PFK, and its high levels during cryoprotectant synthesis help to ensure flux is unidirectional during cryoprotectant synthesis. In the event of cold temperatures during the spring, i.e. during the usual period of cryoprotectant clearance, an increase in F2,6P₂ and low temperature modulation of FBPase could serve to shut down gluconeogenesis, preventing further decreased levels of the cryoprotectant. In addition to FBPase, control of flux at PDHald and GAK are also likely to be important in the spring clearance of the cryoprotectant.

Glycerol is the only polyol of any consequence accumulated by *Epiblema scudderiana*, although various other species accumulate sorbitol or other hexose-derived polyols. The lack of substantial sorbitol accumulation in *Epiblema scudderiana* can be related directly to the characteristics of polyol dehydrogenase in this species. The absence of a glucose utilizing polyol dehydrogenase activity contrasts sharply with what is

observed in the freeze tolerant *Eurosta solidaginis* (Chapter II; Joannis and Storey, 1994b). In these fly larvae, which accumulate sorbitol to levels reaching 0.2 M in haemolymph (Morrissey and Baust, 1976), the activity of this enzyme was found to parallel closely sorbitol synthesis. This strongly suggests that glucose utilizing polyol dehydrogenase is key in the synthesis of sorbitol as a cryoprotectant. Since the kinetic behaviour of SoDH favors the conversion of sorbitol to fructose, little sorbitol accumulation can occur from this enzyme. The observed small increase in sorbitol in *Epiblema scudderiana* may be explained by the increased fructose levels (although these were not statistically significant due to large variance, the increase is 2-fold) pushing the equilibrium of SoDH towards sorbitol in the fall.

From this and other studies we can begin to form a picture of metabolism in *Epiblema scudderiana* larvae during overwintering. Accumulated in early fall, glycogen is broken down under stimulation by cold temperatures in late autumn and carbon is channeled through the hexose monophosphate shunt and glycolysis to produce triose phosphates (dihydroxyacetonephosphate and glyceraldehyde phosphate) for glycerol synthesis. Reducing equivalents supplied from the hexose monophosphate shunt support this synthesis. Removal of glycerol in the spring proceeds via glyceraldehyde-utilizing polyol dehydrogenase and glyceraldehyde kinase, producing glyceraldehyde phosphate. Increasing gluconeogenic (aldolase, fructose-1,6-bisphosphatase) and glyceraldehyde-3-phosphate dehydrogenase activities provide routes for the removal of the triose phosphates arising from glycerol catabolism. Increased α -glycerophosphate shuttle enzyme activity in the spring allows removal of reducing equivalents from glycerol catabolism, by transferring them to mitochondria for oxidation.

CHAPTER IV
FATTY ACID CONTENT AND ENZYMES OF FATTY ACID METABOLISM IN
OVERWINTERING COLD-HARDY GALL INSECTS

SUMMARY

Fatty acid content and enzymes of fatty acid metabolism were studied in overwintering larvae of two cold-hardy gall insects, the freeze tolerant fly *Eurosta solidaginis* and the freeze avoiding moth *Epiblema scudderiana*. Both species increased the content of unsaturated fatty acids during the winter, consistent with the homeoviscous adaptation theory. Whereas total lipid content did not change in *Eurosta solidaginis*, a decrease in total lipids over the winter in *Epiblema scudderiana* suggested the use of fat reserves to maintain basal metabolism. Changes in the activities of enzymes of fat oxidation correlated with these observations: hydroxyacyl-CoA dehydrogenase, carnitine-palmitoyl transferase, and thiolase activities all decreased during overwintering in *Eurosta solidaginis*, whereas, respectively, these activities were constant, decreased, or increased in *Epiblema scudderiana*. Lipid content in *Epiblema scudderiana* increased again by early spring, possibly indicating this pool as the fate of carbon derived from the spring clearance of the cryoprotectant glycerol pool. Decreased activities of malic enzyme and ATP-citrate lyase indicated decreased potential for fatty acid synthesis in both species over the winter, consistent with the cessation of feeding in the fall. The potential for ketone body metabolism, measured as the activity of β -hydroxybutyrate dehydrogenase, increased greatly in both species during overwintering; however, levels of β -hydroxybutyrate remained less than 0.35 $\mu\text{mol/g}$ wet mass throughout the study period.

INTRODUCTION

Temperature effects on the properties of molecules and on rate processes represent a major challenge to animals that must survive at low subzero temperatures. Adaptations for the preservation of membrane structure and function in cold-hardy animals are believed to be essential for the maintenance of a viable state both during and after cold-exposure. The homeoviscous adaptation theory predicts that membrane restructuring is needed to maintain fluidity, and hence preserve structural and functional integrity upon exposure to low temperature conditions (Sinensky, 1974). This may occur through changes in the content or the distribution of fatty acid types, such as an increase in fatty acid unsaturation. Longer and more saturated fatty acids are generally less fluid, possessing higher melting temperatures (Hochachka and Somero, 1984). Lipids may also serve as a source of energy to maintain basal metabolism over the winter in cold-hardy insects, since diapausing or quiescent insects do not feed. However, fats can only be used after mobilization and under aerobic conditions. Hence, we predicted differences in the use of lipids as metabolic fuel between freeze tolerant and freeze avoiding insects. The freezing of extracellular spaces in freeze tolerant species such as *Eurosta solidaginis* leads to a state of anoxia or ischemia, as evidenced by the accumulation of anaerobic end-products and decreasing ATP levels and energy charge (Storey and Storey, 1985). This would prevent the oxidation of lipids in the frozen state and, as well, frozen haemolymph would serve as a barrier to the transport of lipids. The freeze avoiding *Epiblema scudderiana* larvae face no such limitations to the utilization of lipids during overwintering.

Most studies of the cold-hardy gall insects *Eurosta solidaginis* and *Epiblema scudderiana* have focused on cryoprotectant metabolism and its control. Recent studies have shown how metabolic rearrangements at the enzymatic level modulate the activity of different pathways and how these may be controlled (Chapters II and III; Joannis and Storey, 1993, 1994a, 1994b; Holden and Storey, 1993, 1994a, b; Storey, 1982). For

example, the activity profiles of glycogen phosphorylase over the winter and the kinetic properties of the enzyme have been shown to be optimized for the rapid and massive mobilization of glycogen for the production of the cryoprotectant glycerol in *Epiblema scudderiana* (Chapter III; Joannisse and Storey, 1994c; Holden and Storey, 1994b). However, little or no information is available on lipids or fatty acid metabolism and their modulation or role over the winter months in these or other cold-hardy species. In this study we investigate seasonal changes in lipid content, fatty acid composition, and the maximal activities of various enzymes of fatty acid metabolism in an effort to understand the role of these in insect cold-hardiness and the possible differences between freeze tolerant and freeze avoiding insects.

MATERIALS AND METHODS

Chemicals and animals

All biochemicals and coupling enzymes were purchased from Sigma Chemical Co., St. Louis, MO, or Boehringer Mannheim, Montréal, Qué. Galls containing larvae of *Epiblema scudderiana* or *Eurosta solidaginis* were collected from goldenrod plants in fields around Ottawa during the fall of 1990 and larvae were prepared as described in Chapter II.

Lipid analyses

Lipids were isolated from the larvae by chloroform-methanol extraction (Bligh *et al.*, 1957). The recovered lipid from each sample was dissolved in chloroform and transferred to pre-weighed glass vials, the chloroform removed by drying under nitrogen gas, and the total lipid measured by mass. Fatty acids were separated from other lipids by transesterification with 1.0 M methanolic acid (Christie, 1982). The resulting fatty acid methyl esters were separated with a Varian 3400 gas chromatograph fitted with a J&W Scientific Co. model DB-23 (Folsom, CA) capillary column (30 m × 0.25 mm i.d.) and detected by flame ionization. The column temperature was initially 160°C and was increased to 210°C at a rate of 4°C per minute. Helium at a flow rate of 4 mL/min was used as the carrier gas. Data were collected and analyzed using a Varian GC Star Workstation and reported as the percentage of total transesterified fatty acids. Fatty acids were identified by comparison with known standards run under the same conditions. The double-bond index, representing the average number of double bonds per fatty acid molecule of the entire mixture, was calculated as in Richardson *et al.* (1961) using:

$$\frac{\sum \%n \times (\text{double bonds in } n)}{100}$$

where n represents each class of fatty acid identified from GC analysis.

2

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PRECISIONSM RESOLUTION TARGETS

Enzyme studies

Whole larvae were homogenized 1:5 (m:v) in either ice-cold 50 mM TRIS (pH 8.0) containing 5 mM EDTA, 5 mM EGTA and 50 mM NaF (for malic enzyme, β -hydroxybutyrate dehydrogenase, hydroxyacyl-CoA dehydrogenase, and ATP-dependent citrate lyase) or 50 mM Imidazole (pH 7.4) containing 0.2% v/v Triton X-100 (for carnitine-palmitoyl transferase and acetoacetyl-CoA thiolase). A few crystals of phenylmethylsulfonyl fluoride (~0.1 mM) were added to each sample prior to homogenization in Eppendorf tubes with a glass hand-held homogenizer. All homogenates were then sonicated for 10 seconds with a Kontes Micro-Ultrasonic Cell Disrupter set to ~75% of maximum tune and power. Homogenates were centrifuged at $15000 \times g$ in a Biolab 13 centrifuge for 15 minutes. The clear supernatant was used directly for assays of CPT, thiolase, and ATP-CL activity, or was desalted by centrifugation through a Sephadex G-25 column (1 $\times$ 5 cm) pre-equilibrated in homogenization buffer to decrease non-specific background for ME, BHBDH and HOAD assays.

Enzyme assays

All activities were measured at 25°C using a Gilford 240 recording spectrophotometer. Controls for non-specific activity (in the absence of substrate or enzyme) were run for all assays and any blank value subtracted to yield final activity values. Activity is reported as units/gram wet mass, where 1 unit is the amount of activity which catalyzes the utilization 1 μ mole of substrate or the production of 1 μ mole of product in 1 minute. The assays were essentially as in Ballantyne and Berges (1991) or Storey and Bailey (1978b), and optimum conditions for maximal activities in the present species were determined to be:

Malic enzyme (ME) (EC 1.1.1.40): 50 mM Tris, pH 8.1, 0.8 mM MnCl_2 , 0.4 mM NADP^+ , and 2 mM L-malate.

ATP-Citrate lyase (ATP-CL) (EC 4.2.3.8): 50 mM Tris, pH 8.0, 20 mM MgCl_2 , 10 mM GSH, 0.15 mM NADH, 20 mM citrate, 10 mM ATP, 0.4 mM CoASH, and 1 IU porcine heart malate dehydrogenase.

3-Hydroxyacyl-CoA dehydrogenase (HOAD) (EC 1.1.1.35): 50 mM Tris, pH 8.1, 0.1 mM (*Epiblema scudderiana*) or 0.025 mM (*Eurosta solidaginis*) acetoacetyl-CoA, 0.1 mM NADH.

Carnitine palmitoyl transferase (CPT) (EC 2.3.1.21): 50 mM imidazole, pH 7.5, 5 mM L-carnitine, 1 mM DTNB, and 50 μM palmitoyl-CoA.

Acetoacetyl-CoA thiolase (Thiolase) (EC 2.3.1.9): 50 mM Imidazole, pH 7.2, 5 mM MgCl_2 , 0.12 mM CoA, 70 μM acetoacetyl-CoA, measured at 303 nm ($\epsilon=22 \text{ mM}^{-1}$).

β -Hydroxybutyrate dehydrogenase (BHBDH) (EC 1.1.1.30): 50 mM Tris, pH 8.1, 2 mM NAD, 10 mM D,L-hydroxybutyrate.

Statistical analysis

Data were analyzed with a one-way analysis of variance. When a significant *F* ratio was found a Student-Neuman-Keuls test was performed to examine the difference between means; $P<0.05$ was considered significantly different. Percentage data were transformed using $\arcsin\sqrt{y}$ prior to analysis.

RESULTS

The total lipid content of freeze tolerant *Eurosta solidaginis* larvae was constant throughout the study period (Table 4.1). In larvae of *Epiblema scudderiana*, the freeze avoiding moth, the total lipid content decreased by 32% from September to December, but returned to 90% of the September level by March (Table 4.1). The degree of fatty acid unsaturation increased from September to December in both insect species as reflected in the double-bond index (DBI; Table 4.2). The DBI remained constant from December to March in *Eurosta solidaginis*, but decreased slightly in *Epiblema scudderiana* (although remaining higher than September values).

The analysis of fatty acids from transesterified lipids of both *Eurosta solidaginis* and *Epiblema scudderiana* show that body lipids consisted almost entirely of 6 fatty acids (Figures 4.1 and 4.2): palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2) and α -linolenic (18:3). Some unidentified minor peaks were present in some samples, but since these represented less than 0.5% of the total fatty acid methyl ester content at any time they were not reported.

The winter increase in unsaturation (increased DBI) in *Eurosta solidaginis* resulted from a decrease in the content of the saturates palmitic acid and stearic acid (by 2.7% and 2.6% of total fatty acid methyl esters) with a concurrent increase in oleic acid content by 7% of the total fatty acid content (Figure 4.1). Linoleic acid content of the total pool decreased slightly (by 0.7%) from September to December. Palmitoleic and α -linolenic acid levels did not change over the study period. Oleic acid was the dominant fatty acid at any given time, accounting for over 50% of the total fatty acid content. This agreed with a previous study using NMR analysis which had suggested the predominance of oleic acid in whole larvae and chloroform extracts of *Eurosta solidaginis* (Buchanan and Storey, 1983).

The DBI increase in winter *Epiblema scudderiana* was accounted for by a significantly decreased content of palmitic (by 9% of total fatty acid methyl esters) and

stearic (2.1%) acids, as in *Eurosta solidaginis*, but with a concurrent increase by 7% in palmitoleic acid content (Figure 4.2). Oleic and linoleic acid content did not change significantly during the study period. Linolenic acid content increased by 1.3% from September to January, and decreased to approach September levels by March. The most common fatty acids in *Epiblema scudderiana* were the monounsaturates palmitoleic and oleic acid, each accounting for approximately 40% of the total fatty acid content.

Activities of 2 enzymes associated with fatty acid synthesis, malic enzyme and ATP-CL, decreased from September to December in both species (Figures 4.3 and 4.4), indicating a reduced potential for fatty acid synthesis during the winter. In *Eurosta solidaginis* ME activity decreased gradually to 64% of September values by early February, but returned to September activities by 20 March (Figure 4.3). The activity of ATP-CL decreased to 48% of September values by late December, and subsequently increased slightly to 62% of September values by 20 March (Figure 4.3). In *Epiblema scudderiana* the activity of ME decreased to 55% and ATP-CL to 40% of the September activity by December, and remained low for the remainder of the study period (Figure 4.4). Activities for both enzymes were, in all cases, greater in *Eurosta solidaginis* indicating a greater potential for fatty acid synthesis in this species.

The potential for fatty oxidation, as indicated by the activities of HOAD, CPT, and thiolase, decreased in *Eurosta solidaginis* from September into the winter, and remained low into spring (Figure 4.5). HOAD activity decreased to 56% of September values by November 12, and subsequently increased to a range of 67-85% of September values for the remainder of the study period. CPT activity decreased gradually to 63% of September values by December, and then gradually increased to 83% of September values by 20 March. Thiolase activity decreased to 48% of September activities by November 12, and remained low until the late winter when slight increase in activity was seen leading to 60% of the September activity by 20 March.

The state of enzymes of fatty acid oxidation in *Epiblema scudderiana* was different than that found in the freeze tolerant species (Figure 4.6). In *Epiblema scudderiana* there was a 31% increase in the activity of HOAD from September to March, with a transient drop to September values in February. Thiolase activity increased to 229% of the September value by December, and then decreased slightly to reach 165% of the September activity by 20 March. As in the freeze tolerant species, CPT activity in *Epiblema scudderiana* decreased from September to November (by 35%) and remained low for the duration of the study period. The activities of all three enzymes were two- to four-fold higher in *Epiblema scudderiana*, indicating a greater potential for fatty acid oxidation in the freeze avoiding species.

The potential for ketone body metabolism, as measured by BHBDH activity, increased dramatically from September to January in both species. In *Eurosta solidaginis*, the activity increased from 0.0024 ± 0.0024 U/g wet mass in September to 0.066 ± 0.004 U/g wet mass in December, an increase of 27.5-fold, and remained high for the duration of the study period (Figure 4.5). In *Epiblema scudderiana* the activity increased from 0.052 ± 0.004 U/g wet mass in September to 0.34 ± 0.02 U/g wet mass by 5 February, a 6.44-fold increase, and subsequently decreased to a value 3.27-fold higher than the September value by 20 March (0.17 ± 0.02 U/g wet mass; Figure 4.6). Although the activity of BHBDH increased dramatically in both species over the winter, quantification of β -hydroxybutyrate levels in the larvae (using a microplate reader adaptation of Sigma kit 310-A) showed that these remained below the limit of detection of the assay throughout the study period, at less than $0.35 \mu\text{mol/g}$ wet mass. As with enzymes of fatty acid oxidation, the activity of BHBDH was consistently higher in *Epiblema scudderiana* indicating a greater potential for ketone body metabolism in this species than in *Eurosta solidaginis*.

Table 4.1: Total lipid content of cold-hardy goldenrod gall larvae expressed as a percentage of wet mass. All values are n=3 (19-22 animals per group) and given as mean $\pm$ SEM. ^s, significantly different from the September value; ^{*}, significantly different from the December value.

Date	<i>Epiblema scudderiana</i>	<i>Eurosta solidaginis</i>
13 September	13.1 $\pm$ 0.3	18.5 $\pm$ 0.5
27 December	8.9 $\pm$ 0.3 ^s	17.1 $\pm$ 0.3
20 March	11.6 $\pm$ 0.6 ^{s,*}	16.9 $\pm$ 0.2

Table 4.2: Double-bond indices (DBI) of fats from overwintering cold-hardy gall insects. All values are $n=3$ (19-22 animals per group) and given as mean $\pm$ SEM. s , significantly different from the September value; * , significantly different from the December value.

Date	<i>Epiblema scudderiana</i>	<i>Eurosta solidaginis</i>
13 September	1.02 $\pm$ 0.01	0.86 $\pm$ 0.01
27 December	1.17 $\pm$ 0.01 s	0.92 $\pm$ 0.01 s
20 March	1.09 $\pm$ 0.01 $^s,^*$	0.92 $\pm$ 0.01 s

Figure 4.1: Whole larvae fatty acid composition (as a percentage of the total pool) of *Eurosta solidaginis*. The fatty acids are palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and α -linolenic acid (18:3). Values are mean $\pm$ SEM, for n=3 groups, 19-22 larvae per group. ^s, significantly different from September values.

Figure 4.2: Whole larvae fatty acid composition (as a percentage of the total pool) of *Epiblema scudderiana*. The fatty acids are palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and α -linolenic acid (18:3). *, significantly different from previous time point. ^s, different from September values. [†], trace amounts.

Figure 4.3: Activities of enzymes of fatty acid synthesis during overwintering in larvae of *Eurosta solidaginis*. Values are mean $\pm$ SEM for n=5-6 homogenates, 1 larva per homogenate. ^s, significantly different from Sept. 14 values. ^{*}, significantly different from previous sampling date. ATP-CL, ATP-dependent citrate lyase.

Figure 4.4: Activities of enzymes of fatty acid synthesis during overwintering in larvae of *Epiblema scudderiana*. ^s, significantly different from Sept. 14 values. ^{*}, significantly different from previous sampling date. ATP-CL, ATP-dependent citrate lyase.

Figure 4.5: Activities of enzymes of fatty acid oxidation and ketone body metabolism during overwintering in larvae of *Eurosta solidaginis*. Values are mean $\pm$ SEM for n=5-6 homogenates, 1 larva per homogenate. ^s, significantly different from Sept. 14 values. *, significantly different from previous sampling date. Abbreviations are: HOAD, Hydroxyacyl-CoA dehydrogenase. CPT, Carnitine-palmitoyl transferase. BHBDH, β -Hydroxybutyrate dehydrogenase.

Figure 4.6: Activities of enzymes of fatty acid oxidation and ketone body metabolism during overwintering in larvae of *Epiblema scudderiana*. ^s, significantly different from Sept. 14 values. *, significantly different from previous sampling date. Abbreviations are: HOAD, Hydroxyacyl-CoA dehydrogenase. CPT, Carnitine-palmitoyl transferase. BHBDH, β -Hydroxybutyrate dehydrogenase.

DISCUSSION

With the arrival of colder autumn temperatures, the cold-hardy gall insects must initiate adaptations that will provide defenses against the upcoming onslaught of winter. The larvae stop feeding in the fall, and enter a period of metabolic depression (diapause and/or quiescence) in which they survive on stored fuel reserves until emergence in the spring. The low, subzero temperatures faced by larvae of freeze avoiding *Epiblema scudderiana* and freeze tolerant *Eurosta solidaginis* lead to a dramatic decrease in metabolic rate, and survival necessitates various physiological and biochemical adaptations. In this study I examined the changes in fatty acid composition and fatty acid metabolism over the winter months in these cold-hardy insects, focusing on the role of lipids as a metabolic fuel for overwintering and the role, if any, of homeoviscous adaptation for survival.

The absence of lipid accumulation during overwintering (Table 4.1) is consistent with the behavior and metabolic state of the larvae of both species. Since the larvae do not feed during the winter, there is no ingestion of precursor for lipid (and fatty acid) synthesis and the process is metabolically decreased. The total lipid content of both species (Table 4.1) is also consistent with the lipid content of most phytophagous insects (>10%; Fast, 1964). Significant decreases in the activities of two enzymes of fatty acid synthesis, ME and ATP-CL, were observed in both species (Figures 4.3 and 4.4), concurrent with the absence of lipid accumulation and cessation of feeding. Such large decreases in the activity of multiple enzymes of a common pathway are not novel in these cold-hardy insects (see Chapter V; Joannis and Storey, 1994a). These results reinforce the notion of a great plasticity in enzyme activities in these larvae during overwintering, and strongly suggest an important role for enzyme turnover despite the extended period of metabolic suppression in the diapause state.

The homeoviscous adaptation theory, which defines changes in membrane fluidity in response to changing temperatures (Sinensky, 1974), predicts that poikilothermic

animals will change the composition of their lipids upon entry into colder seasons to decrease the temperature at which the liquid crystalline to gel state transition occurs. Also, depot fats must be fluid to be metabolizable (Irving *et al.*, 1957). One mechanism that can be used to increase lipid fluidity is to raise the degree of unsaturation of the component fatty acids. The fatty acid and DBI analysis of *Eurosta solidaginis* and *Epiblema scudderiana* clearly showed that both species increased their contents of unsaturated fatty acids between the fall and winter (Figures 4.1 and 4.2). This permits the maintenance of a fluid state of lipids as the larvae enter periods of colder temperature. The DBI values we observed were higher than those found in mammalian non-hibernators, but not as high as those found in the depot fats of hibernating ground squirrels *Spermophilus beldingi* (Frank, 1991), consistent with increased unsaturation as an adaptation required for successful cold-tolerance. It is interesting to note that *Epiblema scudderiana* larvae contained a much higher percentage of palmitoleic acid and a lower percentage of palmitic acid than reported in Fast (1964) for a number of lepidopteran species. Furthermore, a comparison of *Eurosta solidaginis* fatty acids to others listed by Fast (1964) showed an unusually high percentage of oleic acid. Both of these may be key for overwintering in these species, although the acclimatization state and the cold-hardiness of the species listed in Fast (1964) are unknown.

Providing that the increase in the DBI represents a fairly accurate picture of the status of membrane fatty acids (with the caveat that most of the fatty acids extracted would have originated from storage lipids), it is apparent that the observed adaptive changes would help to ensure the maintenance of sufficiently fluid membranes in the cold as predicted by the homeoviscous adaptation theory. Also, the metabolizable state of depot lipids would be ensured by maintaining their fluidity at cold temperatures. Studies with bacteria, plants, and poikilothermic animals have all shown that the proportion of unsaturated fatty acids in membranes is increased with low temperature acclimation as a compensation mechanism to maintain normal membrane function (Hazel and Prosser,

1974; Cossins, 1983). The present data suggest a role for seasonal desaturation of fatty acids in cold-hardy insects. Since both larval species stop feeding in the fall when the plant dies, the increase in fatty acid unsaturation from September to December cannot be due to a change in diet and/or selective fatty acid uptake or retention and must be the result of desaturase activity. In *Epiblema scudderiana*, the decrease in total body lipid content into the winter (Table 4.1) might also suggest a preferential oxidation of saturated fatty acids during cold-hardening which would have the additional result of increasing the level of unsaturation of remaining lipids. The mechanisms of activation and control of desaturase activity in these insects are unknown.

The data also show an interesting difference in the lipid metabolism of the two species during overwintering. Since total lipid content does not change over the winter, freeze tolerant larvae of *Eurosta solidaginis* apparently do not utilize fats as metabolic fuel for winter survival (Table 4.1), and this was correlated with decreased activities of the fatty acid oxidation enzymes HOAD, CPT, and thiolase (Figure 4.5) over the winter. This agrees with previous work which showed constant levels of glycerides over the winter in this species (Storey and Storey, 1986). Freeze avoiding *Epiblema scudderiana* larvae, on the other hand, do deplete their body lipid reserves over the winter, presumably by oxidation as fuel (Table 4.1). This result correlates with the decrease in glyceride content per gram dry mass observed by Rickards *et al.* (1987). These two different strategies for the use of lipid reserves during insect hibernation have been described previously (see Fast, 1964 and references therein). Our results add to previous information, however, by showing that the decrease in lipid content in *Epiblema scudderiana* was correlated with increased winter activities of enzymes of fat oxidation (HOAD and thiolase), although CPT activity decreased (Figure 4.6). Also, it is noteworthy that all of these activities were much higher in the freeze avoiding species when compared to the freeze tolerant species. One contributing factor to this interspecies difference in lipid utilization may be the physical state of the larvae during most of the

winter. *Eurosta solidaginis* larvae will spend many weeks of the winter frozen. In this condition, fats cannot be mobilized through the haemolymph or oxidized by mitochondria due to lack of oxygen. *Epiblema scudderiana* larvae, on the other hand, do not face these challenges to the utilization of lipids, and like many other species experiencing long term dormancy, they rely on lipid reserves to fuel metabolism.

The fate of glycerol carbon during the spring clearance of cryoprotectant in *Epiblema scudderiana* has always been somewhat of a mystery, since only 20-36% of the cryoprotectant could be accounted for by reconversion to glycogen (Chapter III; Rickards *et al.*, 1987; Joannis and Storey, 1994c). However, the 2.7 % increase in lipid content of the larvae from December to March may provide a partial answer to this dilemma. If all of this new lipid was made from glycerol carbon, this would account for up to 42% of the cryoprotectant lost in the spring (calculations based on a starting glycerol content of 2000 $\mu\text{mol/g}$ wet mass and assuming palmitate as the final product). These accumulated lipids might then be utilized to fuel pupation and adult life. The NADPH required for fatty acid synthesis would also be a product of glycerol clearance, by the reversal of the dehydrogenases involved in the synthesis of the cryoprotectant (Chapter III; Joannis and Storey 1994c).

Both species increased the activity of BHBDH during cold hardening indicating a possible increased use of ketone bodies as a metabolic fuel over the winter. This would parallel the situation in vertebrates where ketone body metabolism increases during starvation. However, the larval concentration of β -hydroxybutyrate never exceeded 0.3 $\mu\text{mol/g}$ wet mass, suggesting the use of ketone bodies to be minor in the cold-hardy larvae or a steady turnover of these metabolites.

A picture of lipid metabolism in cold hardy insects arises from the present data. Both species clearly increase fatty acid unsaturation in the winter which would help maintain membrane and depot lipid fluidity during the cold winter months, as predicted by the homeoviscous adaptation theory. In the freeze tolerant dipteran larvae, *Eurosta*

solidaginis, lipid stores do not appear to be used to fuel basal metabolism during overwintering and the decreased activities of enzymes of both fat synthesis and fat oxidation help to shut down these processes. By contrast, the freeze avoiding lepidopteran larvae of *Epiblema scudderiana* do consume lipids over the winter, and this was supported by decreased activities of fat synthesis enzymes plus changes in the activities of fat oxidation enzymes.

CHAPTER V
MITOCHONDRIAL ENZYMES DURING OVERWINTERING IN TWO
SPECIES OF COLD-HARDY GALL INSECTS

SUMMARY

The activities of 3 mitochondrial enzymes were measured in larvae of the cold-hardy gall insects *Eurosta solidaginis* and *Epiblema scudderiana* over a winter season. Citrate synthase, glutamate dehydrogenase, and NAD-isocitrate dehydrogenase activities all decreased by 50% over the mid-winter compared with autumn values. In *Eurosta solidaginis* all activities returned to initial values by mid-April, but decreased again in late April when the larvae pupated. In *Epiblema scudderiana*, activities of GDH and NAD-IDH increased in the spring, but CS activity remained relatively unchanged from mid-winter on. To determine the influence of temperature alone on enzyme activities, mid-October larvae acclimated to -4°C or 15°C were compared; results showed greater activity of NAD-IDH in *Eurosta solidaginis* acclimated to 15°C ($P < 0.05$ by the Student's t-test) and a lower activity of GDH in *Epiblema scudderiana* acclimated to 15°C ($P < 0.05$). Other enzyme activities were unchanged by acclimation. Reduced activities of mitochondrial enzymes in mid-winter are consistent with winter dormancy of these species, and could reflect lower mitochondrial numbers at this time. The regulation of these seasonal changes appears to be largely a response to cues other than short-term temperature acclimation.

INTRODUCTION

In addition to the effects of low temperature in reducing metabolic rate, most cold-hardy species enter a dormant state (quiescence and/or diapause) that allows them to minimize energy use over the winter months. Little is known about the status of mitochondria and mitochondrial enzymes during these periods of diapause/quiescence, although decreased oxygen uptake is well characterized in many diapausing insects (Danks, 1987). Some studies have shown decreased mitochondrial metabolic activity or decreased mitochondrial numbers correlated with reduced metabolic rate, presumably the result of a much reduced oxidative metabolism in the arrested state. For example, contents of cytochromes *b* and *a+a₃* were greatly reduced during pupal diapause in *Hyalophora cecropia* (Shappirio and Williams, 1957), and the high Arctic freeze-tolerant caterpillars *Gynaephora groenlandica* showed greatly decreased ratios of mitochondria to nuclei when cold acclimated (Kukal *et al.*, 1989). Whether such changes also characterize other species during states of reduced metabolism (during diapause, a freezing episode, or quiescence) is still unknown. To examine this question, I studied two cold hardy insects which adopt different strategies for successful overwintering survival.

Common to both freeze tolerance and freeze avoidance is a decrease in overall metabolic rate due to the cold, which appears to include a diapause that lasts until at least mid-winter followed by a quiescent period that is broken when ambient temperatures again rise to a level that can support renewed development. Thus, *Eurosta solidaginis* larvae warmed to room temperature at any time before the end of January do not resume development and pupate, but die after several weeks. Somewhat later in the winter, however, larvae will pupate when brought to room temperature with the time elapsed before pupation decreasing from 1 week or more in early March to ~24h in mid-April (J.M. Storey, unpublished observations). Behavioral and biochemical indices have also defined a diapause period for *Epiblema scudderiana* beginning sometime in October and

ending by February (Rickards *et al.*, 1987; Kelleher *et al.*, 1987). Diapause and quiescence due to cold both entail a reduced demand on mitochondrial systems, and suggest the possibility of adaptation of mitochondrial systems.

In this study I investigated seasonal and developmental effects on mitochondrial enzyme complement by assessing the maximal potential throughput of mitochondrial metabolism with measurements of the $V_{\max}$ activities of key enzymes (Newsholme and Crabtree, 1986). Changes in the activities of these enzymes, in response to acclimation to different temperature regimes and upon entry into the pupal stage (in *Eurosta solidaginis*), are correlated with what is known about oxidative metabolism of these insect species over the winter.

MATERIALS AND METHODS

Chemicals and Animals

All biochemicals and coupling enzymes were purchased from Sigma Chemical Co., St-Louis, Mo., or Boehringer Mannheim, Montréal, Qué.

Galls containing larvae of *Eurosta solidaginis* or *Epiblema scudderiana* were collected from goldenrod plants in fields around Ottawa during the fall of 1989 and larvae were prepared as described in Chapter II. The average outdoor monthly (30-31 days) and weekly (7 days) temperatures immediately preceding the collection dates are given in Table 2.1 as an indication of the acclimatization state of the larvae. Other groups of larvae for the acclimation studies were collected in mid-October, kept in their galls and placed in dark incubators at constant temperatures of 15 or -4°C for two weeks, and then sampled as above.

Enzyme preparation

Whole larvae were homogenized 1:5 (m:v) in ice-cold buffer (20 mM imidazole-HCl pH 7.2, 5 mM EGTA, 5 mM EDTA, 10 mM 2-mercaptoethanol, and 50 mM NaF) with the addition of a few crystals (~0.1 mM) of PMSF and using a glass/glass homogenizer. Homogenates were then centrifuged at 20,000 × g for 20 minutes and the supernatant was desalted by centrifugation through a G-25 Sephadex column (1 × 5cm) pre-equilibrated in homogenization buffer. The homogenization buffer for citrate synthase assays was as above minus 2-mercaptoethanol.

Enzyme assays

All enzyme activities were measured at 25°C using a Gilford 240 spectrophotometer. One unit of activity is defined as the amount of enzyme which utilizes 1 µmol of substrate in one minute at 22°C. Activities were measured at 340 nm for

glutamate dehydrogenase and NAD-isocitrate dehydrogenase and at 412 nm for citrate synthase. Optimal assay conditions for $V_{\max}$ rates were determined to be:

Citrate Synthase (CS; EC 4.1.3.7) 20 mM imidazole-HCl pH 7.2, 0.3 mM acetylCoA, 0.5 mM oxaloacetate, 0.1 mM 5,5'-dithio-bis-2-nitrobenzoic acid.

Glutamate Dehydrogenase (GDH; EC 1.4.1.2) 20 mM imidazole-HCl pH 7.2, 5 mM α -ketoglutaric acid, 400 mM (*Eurosta solidaginis*) or 200 mM (*Epiblema scudderiana*) ammonium acetate, 1 mM ADP, and 0.15 mM NADH.

NAD-dependent Isocitrate Dehydrogenase (NAD-IDH; EC 1.1.1.41) 20 mM imidazole-HCl pH 7.2, 10 mM D,L-isocitrate, 1 mM ADP, 5 mM MgSO_4 , and 1 mM NAD.

Statistical analysis

Enzyme data are reported as mean $\pm$ SEM for $n=4$ samples with 1 larvae per sample. Data for the winter time course was analyzed by one-way analysis of variance. When a significant F ratio was found a Student-Newman-Keuls test was performed to examine the difference between means; $P<0.05$ was considered statistically significant. Acclimation data were analyzed by a 2-tailed Student's t -test.

RESULTS

Figure 5.1 shows the changes in activities of mitochondrial enzymes over the winter season (September 15 to April 28) in *Eurosta solidaginis*. Note that at the final sampling point (April 28) the animals had pupated. Activities can be correlated with temperature data for the week (7 days) and month (30-31 days) preceding each collection date (Table 2.1). The activities of CS, NAD-IDH, and GDH all decreased significantly during the winter months, but then rose again during the spring. NAD-IDH and GDH reached their lowest activities in January at 53% and 43% of their initial (September) activities, respectively. CS activity was lowest in November (39% of the September value) and increased slightly by January to 59% of the September value. In the late winter and spring months, the activities of all 3 enzymes increased and reached levels not significantly different from September values by April 21. Entry into the pupal stage (April 28 animals) was characterized by a significant decrease in the activities of CS and NAD-IDH (compared to April 21 activities). CS activity in pupae was comparable to January 4 and early March levels in overwintering populations. Pupal NAD-IDH activity was also comparable to January 4 values. GDH activity in pupae was not significantly different from April 21 and September 15 values, although a decreasing trend was apparent from the April 21 value.

Figure 5.2 shows the enzyme activities in larval *Epiblema scudderiana* over the winter. Again, CS, NAD-IDH, and GDH activities all decreased significantly in mid-winter. NAD-IDH and GDH activities rebounded in the spring. Minimum activities for NAD-IDH and GDH were found in January, 40% and 39% of September values, respectively. CS activity was lowest in early March (58% of initial values), although this value was not significantly different from that found in January. NAD-IDH activity increased from late winter into the spring to reach a value not significantly different from the initial September value by mid- to late-March. GDH activity also increased during the

same period, but remained slightly lower than the September value. CS activities remained low from mid-winter with no significant increase into the spring.

Mid-October *Eurosta solidaginis* larvae acclimated in the laboratory to -4°C and 15°C proved to differ only in their activities of NAD-IDH, with significantly greater activity found in the 15°C animals (Table 5.1). The activities of CS and GDH were the same at both acclimation temperatures. Similarly acclimated mid-October *Epiblema scudderiana* larvae showed no differences in the activities of CS and NAD-IDH, but GDH activity was slightly higher in the -4°C animals (Table 5.1). The GDH values from acclimated larvae were slightly lower than those of the outdoor population, but do fall in a range corresponding to intermediate values between outdoor September and January levels.

Figure 5.1 Changes in the activities of mitochondrial enzymes over the winter in third instar larvae of *Eurosta solidaginis*, the freeze-tolerant gall fly. Values are U/gwm, and are given as mean $\pm$ SEM for n=4. ^s, significantly different from September 15 value. *, significantly different from previous time point. Abbreviations are: CS, Citrate synthase. GDH, Glutamate dehydrogenase. NAD-IDH, NAD-dependent isocitrate dehydrogenase.

Figure 5.2 Changes in the activities of mitochondrial enzymes over the winter in larvae of *Epiblema scudderiana*, the freeze avoiding gall moth. Values are U/gwm, and are given as mean $\pm$ SEM for n=4. ^s, significantly different from September 15 value. *, significantly different from previous time point. Abbreviations are: CS, Citrate synthase. GDH, Glutamate dehydrogenase. NAD-IDH, NAD-dependent isocitrate dehydrogenase.

Table 5.1 Effect of acclimation at two temperatures on mitochondrial enzyme activities in larvae of the freeze tolerant *Eurosta solidaginis* and the freeze avoiding *Epiblema scudderiana*. Larvae were collected in mid-October and kept at -4°C or 15°C for 2 weeks. Data are U/gwm, mean $\pm$ SEM for n=4. *, significantly different from value at -4°C by Student's t-test at $p < 0.05$

Enzyme	Activity U/gwm			
	<i>Eurosta solidaginis</i>		<i>Epiblema scudderiana</i>	
	-4°C	15°C	-4°C	15°C
Citrate synthase	1.83 $\pm$ 0.11	2.10 $\pm$ 0.18	5.94 $\pm$ 0.37	5.48 $\pm$ 0.36
Glutamate dehydrogenase	3.06 $\pm$ 0.26	3.29 $\pm$ 0.15	4.81 $\pm$ 0.19	4.11 $\pm$ 0.21*
NAD-Isocitrate dehydrogenase	0.36 $\pm$ 0.03	0.46 $\pm$ 0.03*	1.22 $\pm$ 0.13	1.26 $\pm$ 0.10

DISCUSSION

During periods of diapause and/or quiescence brought about by exposure to cold temperatures, or entry into the pupal stage (for *Eurosta solidaginis*), the activities of mitochondrial enzymes decreased in both insect species. Thus, mid-winter activities of CS, GDH, and NAD-IDH were found to decrease by about 50% from September 15 values, and generally rebounded in the spring. *Eurosta solidaginis* pupae (April 28 animals) displayed similar activities to those of mid-winter larvae, values having decreased significantly from the larval values on April 21. These changes could not result from alterations in allosteric effector concentrations because low molecular mass molecules were removed by the Sephadex G-25 column treatment before enzyme assay. The observed changes are also unlikely to result from post-translational modifications of the enzymes because these enzymes are not known to be subject to such controls in other species. These changes then probably reflect *de novo* enzyme synthesis or degradation, and would occur as the result of or in anticipation of periods of low oxidative metabolic activity. A decrease in oxygen uptake is well characterized for many insects upon entry and during pupation (Danks, 1987). The increasing activities of mitochondrial enzymes in the spring suggests an increased potential for the entry of metabolites into mitochondrial metabolism from glycolysis and other pathways upon resumption of activity at warmer temperatures.

The decrease in activities of mitochondrial enzymes during the winter could be the result of a change in total mitochondrial numbers or a decrease in enzyme activities within existing mitochondria. In either case the potential for aerobic mitochondrial metabolism, based on $V_{\max}$ enzyme activities, is decreased during these periods of diapause and/or quiescence. If there was a decrease in the numbers of mitochondria as winter progressed, the percentage loss of mitochondrial enzymes may be expected to follow the same pattern. Indeed, the large significant decreases in activities of all 3 mitochondrial enzymes, following near parallel time courses and resulting in about a two

fold change for each enzyme activity in both species, strongly suggests a concerted change in overall mitochondrial enzyme activity. Indeed, a decrease in mitochondrial numbers in response to cold acclimation has been shown in the high Arctic caterpillar, *G. groenlandica*, which spends approximately nine months in a dormant and frozen state (Kukal *et al.*, 1989). Mitochondria to nuclei ratios fell from 100:1 in 15°C acclimated *G. groenlandica* larvae to 0.04:1 or less at -15°C in brain and fat body. Upon emergence from winter hibernation there was a rapid replication of mitochondria in *G. groenlandica* and a dense packing of the organelles was achieved by the end of the month-long feeding period (Kukal *et al.*, 1989).

The present enzyme activity data would suggest that such a decrease in mitochondrial numbers may be possible in *Eurosta solidaginis* and *Epiblema scudderiana*. However, data on the activity of other mitochondrial enzymes in these species do not support this hypothesis. HOAD, CPT, thiolase and BHBDH activities did not decrease in *Epiblema scudderiana* larvae, and BHBDH increases in overwintering *Eurosta solidaginis* (Chapter IV). Hence the differential effects of winter cold exposure on mitochondrial enzymes are not consistent with an overall reduction in mitochondrial numbers, but suggests instead specific metabolic reorganization within existing mitochondria.

Although not frozen, larvae of *Epiblema scudderiana* have a reduced metabolic rate during the winter (even if by temperature effects alone), and therefore a reduced energy demand. This period is also believed to correspond to a diapause condition in *Epiblema scudderiana* larvae (Rickards *et al.*, 1987; Kelleher *et al.*, 1987). Fermentative metabolism likely does not occur in these insects over the winter, as evidenced by the relatively low and constant levels of lactate (Rickards *et al.*, 1987). Rapid temperature alternation experiments with *Epiblema scudderiana* larvae (alternating 24 hours at -16°C and 24 hours at 3°C) did not affect adenylate levels and energy charge, indicating that aerobic metabolism was maintained throughout (Churchill and Storey, 1989d). Larvae

acclimated to -20°C for 11 weeks accumulated no lactate, but did show increased L-alanine (about $20\text{ }\mu\text{mol g}^{-1}$), suggesting aerobic mitochondrial oxidative phosphorylation was partially compromised, but still functioning at these temperatures (Churchill and Storey, 1989c). On the other hand, *Eurosta solidaginis* larvae enter an anaerobic state upon freezing, characterized by accumulation of lactate (Storey *et al.*, 1981). The similar decrease in the activities of the mitochondrial enzymes in both species then suggests that this is the result of an adaptation to a reduced metabolic state, and is not a specific adaptation to either a freeze tolerant or freeze avoidance strategy of cold hardiness. However, the greater enzyme activities on a gram per wet mass basis in *Epiblema scudderiana*, indicative of a greater aerobic potential overall, could reflect the greater aerobic reliance of the Lepidopteran versus Dipteran larvae, or a difference in fuel storage and utilization between the two species. This is perhaps best exemplified by the oxidation of lipids by *Epiblema scudderiana* over the winter (Chapter IV), an aerobic process not observed in *Eurosta solidaginis*. The increases in enzyme activities during the spring months in the two species, although these insects do not resume feeding in the spring, are probably due to increased demand for oxidative ATP production to support renewed developmental activity.

Acclimation of mid-October *Epiblema scudderiana* larvae to 15°C and -4°C for two weeks produced changes only in GDH activity (Table 5.1). This would indicate that temperature is not, at least in the short term, a sufficient cue to explain all the changes in enzyme activities seen during overwintering. Similar acclimation of mid-October *Eurosta solidaginis* larvae produced changes in the activities of NAD-IDH. In comparison to enzyme activities in outdoor larvae, the change in NAD-IDH was such that September activities were found in 15°C acclimated larvae, and November levels in -4°C acclimated larvae (compare Figure 5.1 and Table 5.1). However, in a previous study, acclimation of early October *Eurosta solidaginis* larvae to -30°C (temperature reduced from 15°C by a $-1^{\circ}\text{C}/\text{day}$ regime) failed to induce any change in the activities of CS and NAD-IDH, but

did produce an increase in GDH activities (Storey and Storey, 1981). Furthermore, results from the present study for CS and NAD-IDH activities (Table 5.1) do not correspond to these acclimation studies. However, the larvae used in the 1981 study expressed NAD-IDH activity similar to that found during the winter months in the present outdoor population. These discrepancies may highlight differences in the acclimatization of larvae prior to experimentation, such as daily temperature variation. The acclimation regime used in this experiment was very short (2 weeks). Longer acclimation periods or other cues, such as thermoperiod, may be necessary to elicit the observed decreases in enzyme activities over the winter months. The observed decreases during entry into the pupal stage in *Eurosta solidaginis* provide further evidence that the cue may be a developmental, rather than environmental, one. Given that the lowest activity mitochondrial enzyme was found to be NAD-IDH in both insects, suggesting a possible regulatory function for this enzyme, the observed temperature acclimation effect on its activity may be of physiological importance for *Eurosta solidaginis*.

The results suggest that a decrease in mitochondrial enzyme activities during periods of reduced oxidative demand may be common in insects. Thus, in a state of reduced metabolism brought about by dormancy (diapause and/or quiescence) animals could rid themselves of 'unneeded' systems. This implies some benefit in the removal of these systems. The maintenance (turnover) and/or repair costs of mitochondria upon cold exposure may be greater than those involved in the resynthesis of these systems upon resumption of development. On the other hand, enzyme synthesis, involving many distinct steps (transcription, translation) and being energy expensive, could be more susceptible to cold temperature effects than enzyme degradation by proteolysis. This could create an imbalance favoring enzyme breakdown. However, the absence of changes in many non-mitochondrial enzymes of intermediary metabolism in these species over the winter (Chapters II and III) does not support this latter idea. The observed decreases in mitochondrial enzyme activity appear to correlate not only with a known decrease in

oxidative metabolism during larval quiescence/diapause, but also with the transition to the pupal stage in *Eurosta solidaginis*. These results then suggest that decreased mitochondrial enzyme activity may not be a response to a single specific environmental condition, but may rather reflect a more general adaptation to physiological states in which oxidative metabolism is reduced and these enzyme systems (or whole mitochondria) are excess to requirements.

In summary, decreases in mitochondrial enzyme activity appear to reflect a change brought on by a decrease in oxidative metabolism. The benefits of regenerating mitochondrial enzymes after long term reduced metabolic demand appear to outweigh the requirements for the protection or turnover of mitochondrial systems.

CHAPTER VI
OXIDATIVE STRESS AND ANTIOXIDANTS DURING OVERWINTERING IN
LARVAE OF COLD-HARDY GALI INSECTS

SUMMARY

Antioxidant and prooxidant systems were studied in overwintering larvae of two cold-hardy gall insects. Slightly increased glutathione in the oxidized form during the winter suggested oxidative stress in both species during the winter. Freeze tolerant *Eurosta solidaginis* larvae generally decreased activities of antioxidant enzymes in the winter indicating that these larvae do not face increased reactive oxygen species formation during the numerous freeze-thaw events they experience. Instead, existing defenses must be sufficient to prevent any damage. By contrast, increased antioxidant enzyme activities in freeze avoiding *Epiblema scudderiana* suggest that these larvae must defend against reactive oxygen species formation during the winter. This may result from the oxidative nature of metabolism in these larvae, as well as a dependence on lipid oxidation as their fuel over the winter. Dramatically decreased activities of the prooxidants xanthine dehydrogenase and xanthine oxidase in both species, likely the result of cessation of feeding in the fall, may contribute significantly to decreasing the rate of reactive oxygen species formation.

INTRODUCTION

The appearance of significant levels of free oxygen in our atmosphere some two billion years ago, and the resulting layer of ozone in the high atmosphere, allowed the evolution of complex aerobic organisms. Most animals require oxygen for the efficient production of energy, and thus to live.

Oxygen is so toxic, however, that all organisms exposed to a pure oxygen atmosphere die (Haugaard, 1968). The unique chemistry of oxygen is what lies behind this pathological effect. The electronic configuration of oxygen, containing two unpaired electrons in separate outer π orbitals with the same spin quantum number, largely restricts oxygen to reactions involving the transfer of only one electron at a time with other compounds (Halliwell and Gutteridge, 1989). This sequential univalent reduction of oxygen leads to the formation of radicals and other reactive species, collectively referred to as reactive oxygen species (ROS):

The discoveries of superoxide (O_2^-) by Gershman *et al.* (1954) and superoxide dismutase by McCord and Fridovich (1969) opened the way for what is now a broad and far reaching field of study involving the detection, characterization and analysis of the role of ROS in both normal and pathological processes of cellular metabolism.

The formation of ROS *in vivo* under normal conditions is now well established (Halliwell and Gutteridge, 1989). It is estimated that as much as 3-5% of the oxygen used by the electron transport chain in mitochondria leads to superoxide production, electrons 'leaking' from cytochrome oxidase and reducing ground-state oxygen (Turrens and Boveris, 1980). Other cellular mechanisms, including the autooxidation of small molecules (e.g. flavines, catecholamines), enzymic activities (e.g. xanthine oxidase,

NADPH oxidase, cytochrome P₄₅₀), and the redox cycling of xenobiotics can also lead to O₂⁻ and hydrogen peroxide (H₂O₂) production. Superoxide is relatively stable under physiological conditions of pH and ionic strength, but is even more so in organic solvents; thus it has been postulated that the diffusion of this nucleophile across membranes or into membranes may spread damage beyond the original site of radical formation (Fuller *et al.*, 1988; Valentine *et al.*, 1984). Hydrogen peroxide is itself a weak oxidizing agent but in the presence of transition metals (such as iron or copper) it can react to form the hydroxyl radical (OH[•]) which is extremely reactive and damaging to biological systems (Halliwell and Gutteridge, 1986). Damage to the cell occurs under oxidative stress, defined as any condition where the rate of ROS production surpasses the ability of antioxidant systems to absorb or repair the damage arising from these molecules. This oxidative stress has been demonstrated under numerous conditions, including notably ischemia-reperfusion, iron-overload, and increased oxidative metabolism (such as in exhaustive exercise).

All cellular components are susceptible to attack by ROS. Attack on proteins can lead to conformational changes or alterations in enzymatic activity, as well as crosslinking and peptide bond cleavage (Sies, 1986; Davies, 1987). DNA strand breaks and base modifications (leading to point mutations) also result from ROS attack (Sies, 1986). Polyunsaturated fatty acids appear to be particularly susceptible to radical attack. Lipid radicals formed by reaction with oxygen radicals can further form lipid hydroperoxides by reaction with ground-state oxygen, these hydroperoxides eventually breaking down to a series of low molecular weight products (such as alkanes, alkenes, hydroxy or epoxy derivatives, ketones, or polyhydroperoxides) which may themselves prove toxic to the cell (Fuller *et al.*, 1988; Gutteridge and Halliwell, 1990).

To deal with the potential dangers of ROS, a number of antioxidant defenses have arisen. Antioxidant systems can be divided into two general categories, primary and secondary. Primary antioxidants are involved in the direct removal of offensive species,

i.e. the ROS themselves. Secondary antioxidant are those which replenish primary antioxidants (e.g. glutathione reductase producing glutathione) or remove cellular components damaged as the result of ROS attack. Both enzymes and low molecular weight compounds make up the antioxidant defenses of the cell. Their function is to maintain low steady state levels of ROS and other radicals in the cell, a process involving precise regulation of their location and amount. Together, these form a concerted system involved in the detoxification of ROS and the removal of the products of their attack on the cell. The major antioxidant defense mechanisms are: 1) ROS scavenging (by superoxide dismutase, vitamin E, or other scavengers), 2) the reduction of hydroperoxides (glutathione peroxidases and catalase), and 3) the removal of transition metals (by proteins and chelating agents) (Halliwell and Gutteridge, 1989).

Antioxidant enzymes

The enzymes superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), Se-dependent glutathione peroxidase (GPox), Se-independent glutathione peroxidase (GSTPox) and the glutathione-S-transferases (GST) are recognized as a key interacting line of defense against ROS and their products of attack (Halliwell and Gutteridge, 1989; Figure 6.1). SOD dismutates superoxide anions directly (McCord and Fridovich, 1969), but in so doing produces potentially toxic hydrogen peroxide, H_2O_2 . This enzyme exists in a cytosolic Cu,Zn form as well as a mitochondrial Mn containing form. CAT and GPox (the selenium dependent glutathione peroxidase) act to remove peroxide. GPox is well known in all mammalian species examined (Mannervik, 1985) and is believed to be a key enzyme in vertebrate antioxidant defenses (Simmons and Jamall, 1988; Raes *et al.*, 1987), but this enzyme has only been found in very low activities in insects (Ahmad *et al.*, 1989; Aucoin *et al.*, 1991). Insects appear to rely instead on elevated activities of catalase and GSTPox for the removal of H_2O_2 and organic hydroperoxides, respectively, which result from ROS attack (Ahmad and Pardini,

1988, 1989). GSTPox activity is attributed to some multifunctional isozymes of GST, and has been shown to catalyze the reduction of a wide range of organic hydroperoxides, but not H_2O_2 (Mannervik, 1985; Flohé, 1982; Flohé and Gunzler, 1984). GST itself will conjugate GSH to, and help remove, nucleophilic xenobiotics or damaged cellular components from the organism. GR, an NADPH-dependent reductase, serves to replenish substrate for GPox, GSTPox, and GST by converting oxidized glutathione (GSSG) to its reduced form (GSH).

Low molecular weight antioxidants

A number of small molecules are involved in antioxidant systems, including notably the vitamins E and C, and glutathione. Glutathione (GSH) is the principal non-protein sulphydryl compound in tissues. In addition to its role as a substrate for GPox, GSTPox and GST, GSH is a scavenger of hydroxyl and singlet oxygen, can reactivate some enzymes inhibited under oxidizing conditions, and has also been shown to be involved in vitamin E regeneration (Halliwell and Gutteridge, 1989; Reddy *et al.*, 1982). An increase in oxidized glutathione (GSSG) at the expense of GSH is generally viewed as resulting from oxidative challenge to the cell.

Tocopherol (vitamin E) and ascorbate (vitamin C) both function as scavengers of ROS. The intramembrane localization of tocopherol has led to the suggestion that this antioxidant may be important in the prevention of membrane-bound polyunsaturated fatty acid peroxidation (Burton *et al.*, 1983). Ascorbate, in addition to its role as a ROS scavenger, has been shown to have a synergistic effect on the antioxidant function of vitamin E, maintains sulphydryl compounds in the reduced state (including GSH), and participates in many redox reactions (Chou and Khan, 1983).

Cold-hardiness and ROS

During the freezing of *Eurosta solidaginis*, anoxic conditions brought about by ice formation throughout the extracellular spaces lead to a shift towards anaerobic metabolism. During thawing, a return to aerobic metabolism may potentially lead to oxidative stress. ROS formation and resulting damage have been demonstrated in a number of systems during cold or warm ischemia/reperfusion (Halliwell and Gutteridge, 1989; Fuller *et al.*, 1988; Ruuge *et al.*, 1991). Although *Eurosta solidaginis* larvae may remain frozen for many weeks at time in winter, during other periods they may undergo freeze-thaw much more frequently, suggesting the possibility of repeated ROS insult. However, since these larvae survive this stress in Nature, they must defend themselves against the potential for damage from ROS. The options available for this include the prevention of ROS formation altogether, the interception of ROS before they can interact with and damage cellular components, and finally the possession of mechanisms by which to repair damaged cellular structures. *Epiblema scudderiana* larvae, on the other hand, are not faced with the problem of oxygen reperfusion following ischemia or anoxia. However, peroxidative damage is well known to occur in cold stored tissues, even down to -80°C temperatures (Whiteley *et al.*, 1992). In addition, the low steady oxidative metabolism that these larvae maintain during the winter will undoubtedly lead to a low steady state of ROS formation. In this work I examine possible changes to antioxidant systems, notably enzymes, and to the ROS generating xanthine dehydrogenase-xanthine oxidase system which may lead to decreased damage caused by ROS upon their formation during overwintering.

Figure 6.1: The antioxidant enzyme system.

MATERIALS AND METHODS

Chemicals

2-Vinylpyridine was obtained from Aldrich Chemical Co., Milwaukee, WI. All other biochemicals were obtained from Sigma Chemical Co., MO., or Boehringer Mannheim Corp., Montréal, Qué. Distilled deionized water was used in the preparation of all aqueous solutions.

Animals

Galls containing larvae were collected from goldenrod plants in fields around Ottawa during September 1990 and kept outdoors in cloth sacs. Larvae were sampled as described in Chapter II.

Preparation of extracts

Larvae were homogenized 1:10 (m:v) in 1.5 mL Eppendorf tubes using a glass, hand-held homogenizer. Two homogenization buffers were used, based on tests to determine which provided the best recovery of enzyme activity. Buffer A (1 mM EDTA in 50 mM potassium phosphate, pH 7.5) was used for SOD and total XDH+XO, whereas buffer B (buffer A + 10 mM dithiothreitol) was used for GST, GR, and CAT. All homogenizations were carried out in the presence of a few crystals of PMSF (~0.1 mM). Homogenates were centrifuged at $20000 \times g$ for 20 minutes, and the supernatant was removed and desalted by centrifugation through a G-25 spun column (0.5 $\times$ 6 cm; pre-equilibrated with the appropriate buffer). The clear eluate was used as the source of all enzymes. Samples for GSTPox were homogenized 1:5 m:v in buffer A, and were sonicated with a Kontes micro-ultrasonic cell disrupter for 15 seconds before centrifugation at full speed in a Brinkmann model 5412 centrifuge, and were then desalted through a spun-column.

Antioxidant enzyme assays

All assays were run on a Pye-Unicam SP8-100 or a Gilford 250 recording spectrophotometer at 25°C. All data is reported on a per mg soluble protein basis.

Total SOD activity (Cu,Zn-SOD and Mn-SOD) was measured by the method of Paoletti *et al.* (1986). This assay is based on the chemical formation of superoxide, which can be followed by the resulting oxidation of NADH monitored at 340 nm. The assay was run in 50 mM potassium phosphate (pH 7.4) containing 2.5 mM MnCl₂, 5 mM EDTA, 3.9 mM 2-mercaptoethanol (MeSH), and 0.27 mM NADH. Superoxide, formed from a chemical reaction between MnCl₂, EDTA, MeSH and oxygen, will oxidize NADH and decrease its absorbance at a superoxide concentration-dependent rate. In the presence of increasing SOD activity the rate of NADH oxidation slows due to decreasing O₂⁻ concentrations. One unit of SOD is defined as that amount of enzyme which will inhibit the oxidation of NADH by 50%, and is determined from an inhibition curve.

Catalase (CAT) activity was measured by the method of Aebi (1984) in the presence of 7.5 mM H₂O₂ in 50 mM pH 7.0 potassium phosphate. The removal of peroxide is measured at 240 nm ($\epsilon = 0.0394 \text{ mM}^{-1}$). One unit is defined as that amount of enzyme which catalyses the reduction of 1 μmole of peroxide per minute.

Selenium-independent glutathione peroxidase activity (GSTPox) was assayed in 50 mM potassium phosphate (pH 7.0) in the presence of 1 mM EDTA, 0.2 mM NADPH, 2 mM NaN₃, 1 IU GR, 5 mM GSH, and 1.2 mM cumene hydroperoxide. A blank without homogenate was run to account for the non-enzymatic oxidation of NADPH upon addition of cumene hydroperoxide. One unit is defined as the amount of enzyme which will catalyze the oxidation of 1 μmole of NADPH per minute, monitored at 340 nm. Selenium-dependent glutathione peroxidase (GPox), measured with H₂O₂ in place of cumene hydroperoxide, was not detected in the larvae at any time.

GST was assayed in 50 mM potassium phosphate (pH 6.5) containing 1 mM EDTA, 6 mM GSH, and 1 mM 1-chloro-2,4-dinitrobenzoic acid (CDNB) (Habig and

Jakoby, 1981). The reaction was initiated by the addition of CDNB after initial velocities were recorded to account for non-enzymatic conjugation of GSH and CDNB. One unit of activity is defined as that amount of enzyme which catalyses the formation of 1 μ mole of the conjugate *S*-2,4-dinitrophenylglutathione per minute, monitored at 340 nm ($\epsilon = 9.6 \text{ mM}^{-1}$).

GR was assayed by following NADPH oxidation at 340 nm in the presence of 5 mM GSSG, 0.2 mM NADPH, and 1 mM EDTA in 50 mM potassium phosphate buffer, pH 7.4 (Dillio *et al.*, 1983). GR activity was corrected for spontaneous reaction by measuring velocities prior to the addition of enzyme. One unit of activity is defined as that amount of enzyme which reduces 1 μ mole of NADPH per minute.

Xanthine dehydrogenase and oxidase assays

Total xanthine oxidase plus xanthine dehydrogenase was measured spectrophotometrically in the presence of 1 or 0.1 mM xanthine (*Epiblema scudderiana* and *Eurosta solidaginis*, respectively) and 6 mM NAD in 50 mM potassium phosphate pH 7.8 (Stirpe and Della Corte, 1969). One unit is defined as that amount of enzyme which catalyzes the reduction of 1 μ mole of NAD per minute.

Xanthine dehydrogenase and oxidase activities were also measured by the fluorometric assay of Beckman *et al.* (1989). Single pre-weighed larvae were homogenized 1:5 (m:v) with a hand-held glass homogenizer in Eppendorf tubes in ice-cold 50 mM KP_i (pH 7.0) containing 0.5 mM EDTA. Homogenates were centrifuged for 10 minutes at $15000 \times g$ in a Biolabs 13 microcentrifuge at 5°C. Supernatants were desalted by passage through a Sephadex G-25 spun column (0.5 x 6 cm). Pterine (40 μM final assay concentration) was added to desalted supernatant in 50 mM KP_i buffer (pH 7.0) for the determination of xanthine oxidase activity. Methylene blue was then added (20 μM final) to the same cuvette for the determination of total XDH+XO activity. Allopurinol, an inhibitor of XO and XDH, was then added to a final concentration of 40

μM (*Eurosta solidaginis*) or $120 \mu\text{M}$ (*Epiblema scudderiana*) to ensure the absence of non-specific activity. Finally an internal standard of isoxanthopterin ($\sim 0.1 \text{ nmol/cuvette}$) was added to calibrate the intensity readings. The exact concentration of the isoxanthopterin standard was determined spectrophotometrically at 336 nm ($\epsilon = 13.0 \text{ mM}^{-1}$) and the final concentration added to the assay cuvette was calculated. One unit of activity by this assay is defined as the amount of enzyme which catalyzes the formation of 1 nmole of isoxanthopterin per minute.

Determination of glutathione levels

Total (GSH+GSSG) and oxidized (GSSG) glutathione levels were measured using a modification of the method of Tietze (1969) by Griffith (1980). This assay monitored the change in absorbance at 412 nm in the presence of 0.6 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 0.21 mM NADPH and 0.5 IU/mL glutathione reductase in 125 mM Na-phosphate buffer containing 6.3 mM EDTA at a pH of 7.5. Single pre-weighed larvae were homogenized 1:3 (m:v) in N_2 -bubbled ice-cold sulfosalicylic acid in Eppendorf tubes using a hand-held glass homogenizer. After bubbling with N_2 for ~ 30 seconds, the homogenates were centrifuged in a Biofuge 15 microcentrifuge at $11000 \times g$ for 5 minutes at 5°C . The supernatants were divided into two aliquots for the measurement of total glutathione (reduced + oxidized) and oxidized glutathione. Total glutathione was measured directly in aliquots of the supernatant. To measure reduced glutathione a second aliquot of extract was first derivatized by incubation with 2-vinylpyridine (170 mM final concentration). Extracts neutralized to a pH between 5.5 and 7.0 with 1 M K_2P_4 (pH 7.3) were assayed for GSSG after 1 hour using the same assay as described for total glutathione. Both total and GSSG values in samples were obtained by comparison to a standard curve of varying GSH concentrations. All assays were corrected for spontaneous reaction in the absence of sample or glutathione reductase.

Protein assay

Soluble protein levels in larval extracts were determined using the Bio-Rad Assay kit which is based on the Coomassie Blue dye-binding method (Bradford, 1976) with a standard of bovine serum albumin.

Statistical analysis

All enzyme data are reported as mean $\pm$ SEM for $n=4-6$ samples with 1 larvae per sample. Metabolite levels are mean $\pm$ SEM for $n=5$ samples, 1 larva per sample. Data were analyzed with a one-way analysis of variance. When a significant F ratio was found a Student-Neuman-Keuls test was performed to examine the difference between means; $P<0.05$ was considered statistically significant.

RESULTS

The activities of antioxidant enzymes and their winter profiles differed greatly between the two species of larvae. The activities of the primary antioxidants, SOD and CAT, and of GST and GR were much higher (2 to 4-fold) in larvae of *Epiblema scudderiana* when compared to those of *Eurosta solidaginis*. Only GSTPox activity was similar in the two larvae. No activity of H₂O₂ detoxifying GPox was detected in either species.

With the exception of SOD, activities of all antioxidant enzymes in *Eurosta solidaginis* decreased over the winter (Figures 6.2 and 6.3). CAT activity decreased 2.5-fold from September to November, and subsequently increased stepwise to reach a March 20 value comparable to the initial September reading. Although not statistically significant due to high variance, GSTPox activity did follow a decreasing trend over the winter, the final March reading being 1.5-fold lower than the initial September value. GR activity decreased by 1.8-fold from September to November, and in a pattern mimicking that of CAT, increased stepwise to reach values similar to September readings by February and March. GST activity initially remained high in the fall, then decreased by 3.4-fold from November to December where it remained until a small increase was found in March. SOD activity remained essentially constant over the study period, with only a slight transient increase in February larvae. Overall, these results indicate a decrease in the potential for antioxidant activity in the organism during overwintering.

Total glutathione (GSH+GSSG) did not change during overwintering in *Eurosta solidaginis* (Table 6.1). However, the percentage of the total that existed as the oxidized form decreased 1.8-fold from September to November, but then increased 3.2-fold by December. Levels of GSSG then decreased stepwise to less than half of the September value by March.

In contrast to *Eurosta solidaginis*, activities of most antioxidant enzymes increased during overwintering in *Epiblema scudderiana* (Figures 6.4 and 6.5). CAT

activities increased 2-fold from September to December, the activity returning to September levels by February. GSTPox activity increased 3.2-fold from September to November and remained high for the remainder of the study. GST activity also increased from September to November (2-fold), but unlike GSTPox, decreased somewhat during the remainder of the study period to a final value 1.3-fold greater than the initial September reading. SOD activities remained essentially constant over the study period, with a slight transient decrease in November; although there was a 1.4-fold increase from February to March, neither value was significantly different to the initial September reading. Unlike activities of other antioxidant enzymes in *Epiblema scudderiana*, GR activity decreased in November (5.4-fold) and remained low and relatively constant until March.

Total glutathione content of *Epiblema scudderiana* larvae decreased slightly over the winter to a final March level of 70% of the September value (Table 6.2). The percentage of the total present as the oxidized form (%GSSG) decreased 4.8-fold from September to November, and then increased 2.5-fold and remained constant for the remainder of the study period. This resulted from both the decrease in GSH+GSSG and a slight increase in the content of GSSG from November.

Total XDH+XO activity decreased sharply from September to late winter in both species. In *Eurosta solidaginis* the activity decreased approximately 10- and 15-fold when measured spectrophotometrically versus fluorometrically, respectively (Figure 6.6). The activity of XO alone was below the limit of detection of either assay. In *Epiblema scudderiana* larvae the activity of total XDH+XO decreased steadily from September to activities below the limit of detection of either assay by March (Figure 6.7). XO activity was detectable fluorometrically in September and November, but the activity decreased to values below the limit of detection of the assay from December onwards.

Soluble protein content did not change significantly from September levels of 95.8 ± 2.3 and 110.4 ± 5.1 mg/gwm in extracts from *Eurosta solidaginis* and *Epiblema scudderiana* larvae, respectively, over the duration of the overwintering period.

TABLE 6.1: Glutathione status in overwintering larvae of *Eurosta solidaginis*. Data are mean $\pm$ SEM. ^s, ^{*}: significantly different from September and previous sampling date values, respectively.

	13 Sep	12 Nov	27 Dec	5 Feb	20 Mar
GSH+GSSG, mM	0.89 $\pm$ 0.03	0.91 $\pm$ 0.04	0.91 $\pm$ 0.04	0.80 $\pm$ 0.02	0.81 $\pm$ 0.04
GSSG, μ M	13.0 $\pm$ 0.3	7.5 $\pm$ 0.6 ^s	24.5 $\pm$ 2.7 ^{s*}	13.2 $\pm$ 4.5	5.6 $\pm$ 0.7 ^{s*}
% GSSG of total	2.93 $\pm$ 0.09	1.66 $\pm$ 0.11 ^s	5.38 $\pm$ 0.44 ^{s*}	3.44 $\pm$ 1.32 [*]	1.38 $\pm$ 0.16 ^{s*}

TABLE 6.2: Glutathione status in overwintering larvae of *Epiblema scudderiana*. Data are mean $\pm$ SEM. ^s, ^{*}: significantly different from September and previous sampling date values, respectively.

	13 Sep	12 Nov	27 Dec	5 Feb	20 Mar
GSH+GSSG, mM	1.2 $\pm$ 0.1	0.93 $\pm$ 0.06 ^s	0.92 $\pm$ 0.03 ^s	0.86 $\pm$ 0.08 ^s	0.83 $\pm$ 0.05 ^s
GSSG, μ M	31.8 $\pm$ 2.9	5.9 $\pm$ 0.5 ^s	14.6 $\pm$ 0.9 ^{s*}	15.4 $\pm$ 2.7 ^s	16.3 $\pm$ 2.2 ^s
% GSSG of total	6.2 $\pm$ 0.5	1.3 $\pm$ 0.2 ^s	3.2 $\pm$ 0.2 ^{s*}	3.5 $\pm$ 0.3 ^s	3.9 $\pm$ 0.3 ^s

Figure 6.2: Activities of superoxide dismutase (SOD), catalase (CAT), and Se-independent glutathione peroxidase (GSTPox) in overwintering larvae of *Eurosta solidaginis*. Data are mean $\pm$ SEM. ^s, ^{*}: significantly different from September and previous sampling date values, respectively.

Figure 6.3: Activities of glutathione reductase (GR) and glutathione-S-transferase (GST) in overwintering larvae of *Eurosta solidaginis*. Data are mean $\pm$ SEM. ^s, ^{*}: significantly different from September and previous sampling date values, respectively.

Figure 6.4: Activities of superoxide dismutase (SOD), catalase (CAT), and Se-independent glutathione peroxidase (GSTPox) in overwintering larvae of *Epiblema scudderiana*. Data are mean $\pm$ SEM. s, *: significantly different from September and previous sampling date values, respectively.

Figure 6.5: Activities of glutathione reductase (GR) and glutathione-S-transferases (GST) in overwintering larvae of *Epiblema scudderiana*. Data are mean $\pm$ SEM. δ , *: significantly different from September and previous sampling date values, respectively.

Figure 6.6: Activity of total xanthine dehydrogenase + xanthine oxidase measured spectrophotometrically (A) and fluorometrically (B) in overwintering larvae of *Eurosta solidaginis*. Data are mean $\pm$ SEM. ^s, ^{*}: significantly different from September and previous sampling date values, respectively.

Figure 6.7: Activities of total xanthine dehydrogenase + xanthine oxidase measured spectrophotometrically (A) and fluorometrically (B), and xanthine oxidase measured fluorometrically (C) in overwintering larvae of *Epiblema scudderiana*. Data are mean $\pm$ SEM. ^s, ^{*}: significantly different from September and previous sampling date values, respectively. nd: not determined, activity below the limit of detection of the assay.

DISCUSSION

Low temperatures alone are not sufficient to reduce ROS formation. Although Whiteley *et al.* (1992a, b) have shown that the rate of lipid peroxidation of frozen liver homogenates is reduced with decreasing temperatures, the lipid peroxide content measured as malondialdehyde production was found to increase more than 4-fold after 28 days when stored at -20°C and almost 2-fold in similarly treated homogenates kept at -70°C. This shows that peroxidation occurs readily at low temperatures, and cold-hardy animals, even with a greatly decreased metabolic rate, are susceptible to ROS generation and damage. They must therefore maintain sufficient antioxidant defenses throughout the winter months.

Changes to the activities of antioxidant enzymes in the two larvae were generally opposite over the winter. Whereas activities in *Eurosta solidaginis* largely decreased, *Epiblema scudderiana* antioxidant enzyme activities were for the most part increased during the winter months. These results imply that *Eurosta solidaginis* larvae face little challenge by ROS, allowing for the observed decrease in defenses, whereas *Epiblema scudderiana* larvae appear to require enhanced protection from these same damaging species.

I hypothesized that *Eurosta solidaginis* larvae, which undergo numerous freeze-thaw cycles as part of their freeze tolerance overwintering strategy, may suffer increased ROS formation as the result of oxygen reperfusion after thawing, as has been observed in many higher animal systems undergoing ischemic episodes (Halliwell and Gutteridge, 1989; Fuller *et al.*, 1988; Ruuge *et al.*, 1991). However, the observed changes in enzyme activities suggest this may not be the case. If there was a repeated insult of ROS after every thaw of the larvae, antioxidants would most likely have remained high during the winter. Their observed decrease then suggests that either the levels of defenses maintained are sufficient to deal with these ROS insults, or that there is little ROS formation at all. An interesting observation in those systems which undergo reperfusion

damage by ROS formation is that often there is both a severe depletion of energy charge and a rapid increase in aerobic metabolic rate. In *Eurosta solidaginis*, however, metabolism is always maintained at a low, steady rate, and the larvae are well adapted to long-term ischemia and show little depletion of energy charge under natural winter conditions. These may be key in preventing ROS formation and the resulting damage in naturally tolerant animals, and deserves more study.

Sustained aerobic winter metabolism, even if at a reduced rate, appears to be sufficient for the production of ROS at damaging levels in freeze avoiding *Epiblema scudderiana* larvae. A decreased effectiveness of antioxidant systems, possibly by temperature (Q_{10}) effects, may be responsible for the observed increase in antioxidant enzyme activities to compensate. The apparent dependence on lipid metabolism during overwintering (Chapter IV) may also predispose these larvae to ROS damage, as fatty acids are particularly susceptible to ROS attack.

There does not appear to be severe oxidative stress in *Eurosta solidaginis* or *Epiblema scudderiana* during the winter, as witnessed by the glutathione status of the larvae (Tables 6.1 and 6.2). Under oxidative stress there is usually an increase in GSSG as a fraction of the glutathione pool, as reduced GSH is consumed by antioxidant enzymes and as a radical scavenger in its own right. Also, a depletion of the total pool may be associated with long-term oxidative stress. However, during overwintering, the total pool of glutathiones did not change in the larvae. Although there was a slight increase in %GSSG of the total pool from November on in both species, these values were well within the normal range of other animals (Halliwell and Gutteridge, 1989) and of at least one other insect species, the flesh fly *Sarcophaga peregrina* (Sugiyama and Natori, 1994). In *Eurosta solidaginis*, this increase was lost as the winter progressed into spring. In *Epiblema scudderiana*, by contrast, the levels remained slightly elevated. Again this may relate to the largely aerobic and fat burning nature of this lepidopteran, predisposing the larvae to increased risk of ROS generation. In both larvae it is

interesting to note the high September levels of GSSG, especially when compared with November readings. An interesting possible explanation for this is found in the feeding state of the larvae. Phytophagous insects ingest various plant defense compounds, many of which have been identified as prooxidants. Studies have shown that insects which feed on plants containing higher levels of these compounds appear to possess greater antioxidant enzyme activities, apparently in order to overcome the plant's natural defense system (Aucoin *et al.*, 1991). The goldenrod gall insects, when feeding in the early fall, are then likely to be faced with such compounds. This would lead to increased oxidative stress, and may well explain the relatively high GSSG content. After cessation of feeding, oxidative stress may be reduced, explaining the decreased GSSG content by November.

XO activity has been implicated in ROS generation in a number of studies, notably during oxygen reperfusion (Terada *et al.*, 1991; Chiricolo *et al.*, 1991). This modified form of XDH uses oxygen as an oxidizing agent, leading to superoxide formation. The observed winter decrease in these activities during overwintering in the cold-hardy insect species studied here (Figures 6.6 and 6.7) shows that the potential for ROS from this source is greatly reduced. Although the larvae may benefit from this, a decrease in ROS formation is likely not the cause of these decreased enzyme activities. Instead, the decreasing activity of XDH in the autumn is likely a reflection of the physiological state of the larvae. Since insects generally excrete uric acid, the cessation of food intake in the autumn would lead to a reduced need for enzymes of the uric acid production pathway, which include XDH and XO.

Although Se-dependent glutathione peroxidase (GPox) is believed to be essential in the antioxidant strategy of most animals, the lack of any activity of a H₂O₂-removing peroxidase (GPox) in the two gall species was consistent with previous work in insects, especially phytophagous species. In many insect species including *Trichoplusia ni*, *Spodoptera eridania*, and *Papilio polyxenes* little or no activity of Se-dependent GPox was observed (Ahmad *et al.*, 1987, 1988a, b; Ahmad and Pardini, 1988; Pristos *et al.*,

1988a, b; Aucoin *et al.*, 1991). The inability to inactivate insect GPox activities by boiling homogenates for 1 hour (Ahmad *et al.*, 1989) casts some doubt on whether these were truly enzymic in nature. There does appear to be some GPox in *Musca domestica*, although a very low activity compared with mammalian species (Ahmad *et al.*, 1989). The absence of GPox in phytophagous species has been attributed to the fact that plants do not have a requirement for selenium, and therefore it is impossible for insects to accumulate the metal for the production of the enzyme (Ahmad *et al.*, 1989). This has led to the hypothesis that phytophagous species have evolved higher levels of catalase to deal with H_2O_2 . In support of this hypothesis, catalase has been shown to be found in all cellular compartments in insects, not restricted to peroxisomes as in mammalian tissue (Ahmad *et al.*, 1988a, b; Halliwell and Gutteridge, 1989). The high activity of GSTPox found in insects would also serve to compensate for the lack of GPox in removing organic hydroperoxides formed as the result of ROS attack.

CHAPTER VII
GENERAL DISCUSSION

The goldenrod gall insects *Eurosta solidaginis* and *Epiblema scudderiana* must face low temperatures as a natural part of their life history. As ectotherms, and given the lack of any protection from ambient temperature fluctuations in their chosen hibernacula, the challenge they face is daunting. Not only must they endure subzero temperatures, they must do so for prolonged periods of time, often for months. As well, since the larvae do not feed during the winter this leads to a need for sustained metabolic depression to prevent the depletion of on-board metabolic reserves. Survival of subzero temperatures is insured by freeze tolerance in the case of *Eurosta solidaginis*, and by freeze avoidance in *Epiblema scudderiana*. Both strategies are coupled with a state of depressed metabolic rate. Biochemical mechanisms which control essential aspects of these strategies, for example cryoprotectant accumulation and clearance, are necessary to maintain the integrity of the organism during the unfavourable conditions of winter.

The present studies allow us to form a picture of changes in potential metabolic flux through various pathways, however, on their own the data do not provide a picture of actual metabolism within the larvae at any time. Instead, when the data are interpreted in combination with that from *in vitro* enzyme kinetics, metabolite measurements and cross-over studies, as well as radiotracer studies, a clearer picture of the metabolic changes and their role for acclimatization is formed. For example, the observed changes in glycogen phosphorylase activity and activation state during the winter, coupled with the now well established cold-induced phosphorylation of the enzyme, allows us an understanding of the mechanism and its result for the organism during acclimatization, here glycogen carbon being diverted to cryoprotectant synthesis. The constant activity of PFK during the fall and winter in *Eurosta solidaginis* further suggests that the observed break in the Arrhenius plot for the enzyme measured *in vitro* (Storey, 1982), which occurs at the temperature at which the switch from glycerol to sorbitol production occurs in nature, is indeed key to the accumulation of dual cryoprotectants. Although many enzymes from these animals have been studied *in vitro*, and their kinetic behaviors and responses to

temperature are fairly well established, many key enzymes have not been studied in this manner. For those pathways of glycerol and sorbitol synthesis alone we can list the polyol dehydrogenases (PDHgluc and PDHald), SoDH, GAK, and GAPase as enzymes whose kinetic behavior is undoubtedly important in the formation of cryoprotectant.

From the work reported in this thesis both similarities and important differences between the two insects which relate to the overwintering strategy employed are found. Both species show seasonal variations in the enzyme activities required for the synthesis and clearance of polyol cryoprotectants, increasing activities of these in the fall and decreasing in the spring. Glycogen has been shown to be the source of carbon for cryoprotectant synthesis from the determination of changes in metabolite pools as well as cross-over analysis and enzyme activation studies in the gall larvae (Storey and Storey, 1992). Key to cryoprotectant synthesis is then glycogen phosphorylase activation in the fall, resulting from cold temperature effects on the phosphorylation cascade of the enzyme, leading to the shuttling of carbon into cryoprotectant synthesis from glycogen. The enzyme activity results indicate that both insect species possess two potential pathways for glycerol biosynthesis. Both routes arise from the triose phosphates of glycolysis: 1) from GAP, involving the activity of GAPase and PDHald, and 2) from DHAP, via G3PDH and G3Pase. High HMS activity during the fall, and the resulting NADPH_i production, suggest that the route from GAP may be preferred. The absence of a glycerol kinase activity in both species prevents glycerol catabolism (futile cycling) over the winter. Both species possess a common pathway of spring glycerol clearance via the sequential reactions of PDHald and GAK. The latter enzyme activity provides NADPH for anabolism as well as GAP which can be directed to various metabolic fates, including oxidation in the TCA cycle, fat synthesis, or gluconeogenesis. *Epiblema scudderiana* larvae start preparing for spring glycerol clearance as early as January, when the gluconeogenic potential increases following increased FBPase activity. The absence of PDHgluc activity in *Epiblema scudderiana* may be key to the lack of sorbitol

accumulation in this species, just as the presence of this same enzyme in *Eurosta solidaginis*, with G6Pase, allows sorbitol production from G6P. Spring clearance of sorbitol in *Eurosta solidaginis* occurs through the action of SoDH and hexokinase.

Metabolic depression in both species leads to decreased ATP demand, and this is reflected in the decreased activities of TCA cycle (CS, NAD-IDH) and associated enzyme (GDH) activities. In both species of larvae these activities decreased over 2-fold during the winter. The constant or increasing activities of other mitochondrial enzymes, such as BHBDH, show that the decrease in TCA cycle enzyme activities probably does not result from decreased mitochondrial numbers, as observed in at least one other cold-hardy insect (Kukal *et al.*, 1989). For the freeze tolerant *Eurosta solidaginis* the decreased oxidative capacity indicated by decreased winter TCA cycle enzyme activity of the larvae may also be linked to the necessary dependence on anaerobic metabolism during freezing for the production of metabolic energy.

The aerobic versus anaerobic nature of the two strategies leads to significant differences in overwintering metabolism in the two species. Whereas freeze avoiding *Epiblema scudderiana* larvae appear to rely on lipid oxidation for the provision of metabolic energy, this does not appear to occur in the freeze tolerant *Eurosta solidaginis* larvae. In *Epiblema scudderiana* fat synthesis is a probably major fate of glycerol cleared in the spring. This may serve to replenish lipid stores depleted over the winter that are necessary for pupation. Lipid content analyses indicated that this conversion of glycerol to lipid does not occur to any great extent in *Eurosta solidaginis*. Although the use of lipid metabolism differs between the two species over the winter, a common theme is an increase in the unsaturation of fatty acids during the fall. This suggests that homeoviscous adaptation occurs in these larvae.

Very little is known about insect antioxidant systems and their function, except in cases of plant prooxidant and pesticide resistance. This study has shown that these systems may play an important role in insect overwintering. The aerobic nature of

Epiblema scudderiana metabolism through the winter coupled with a reliance on fat oxidation may be responsible for the increased winter demand on antioxidant systems, witnessed by increased activities of antioxidant enzymes. The decreased activities of these same enzymes in freeze tolerant *Eurosta solidaginis* indicate a reduced need for protection from ROS in this species. This may result from the nature of the relatively slow thawing of the larvae, coupled with the maintenance of high energy charge during extended freezing and the low metabolic rate of the organism upon thawing and a return to oxidative metabolism. Interestingly, sugars and polyols are known to react quite readily with the hydroxyl radical (Halliwell and Gutteridge, 1989), and it is possible that cryoprotectants may serve as scavengers and help reduce oxidative damage in cold-hardy animals. This could add a novel aspect to the choice of polyols as colligative cryoprotectants in cold-hardy species.

The dynamic nature of the larval metabolism, even during extended periods of cold and metabolic depression, is evident from this study. Even under these conditions the larvae are still metabolically active, and changing, at least at the biochemical level. This is clearly shown when one looks at the alterations in enzymatic activities over the course of the winter. These changes reflect the needs of the organism at different times, best exemplified by changes in the enzyme activities related to cryoprotectant metabolism. They also show an anticipation of events to come, for example the increase of FBPase activity in mid-winter, for the removal of cryoprotectants later in the spring. What these studies show, then, is the result of a complex, highly regulated set of controls which operate to modify the biochemical complement of an organism during periods of quiescence and diapause. Interestingly, very little is known about how these controls operate in overwintering insects (or in insects overall). Cues for the initiation of these changes may include thermoperiod, programmed length of diapause, or perhaps photoperiod (although the level of light which makes its way into the gall is unknown).

The observed changes in the activities of some enzymes is intuitively logical, providing in a timely fashion the necessary machinery for a new metabolic requirement. For example, it is not surprising to find that the activities of enzymes of polyol cryoprotectant synthesis increase in the fall. Other enzyme activity increases may be needed to compensate for the effect of low temperature on their reaction rate (Q_{10} effects) in order to maintain a relatively high flux through a metabolic pathway. However, the decrease in other enzyme activities suggests that some benefit to the organism arises from this loss, or that there is some liability in keeping these activities at a high or constant level over the winter. In the case of the TCA cycle, for example, why not simply leave the enzyme activities at a constant level over the winter if they must return to fall values in the spring anyway? One possible explanation lies in the nature of enzyme turnover. An enzyme is not a permanent entity in the cell. Each enzyme has a limited lifetime, and in order to maintain steady state enzyme numbers the cell must continually replenish with new molecules the old ones that are constantly being broken down. Herein lies the challenge, for the synthesis of new protein is metabolically expensive. For an organism which must conserve energy stores for long term survival the use of metabolic energy for the synthesis of unneeded macromolecules is not a wise choice.

As very few of the enzyme activities examined in this study are known to be controlled by post-translational modification (such as by phosphorylation), it is inferred that most of the observed seasonal changes in enzyme activities arise from altering the balance between *de novo* enzyme synthesis and breakdown, *i.e.* coarse control. This then indicates a very significant role for enzyme synthesis and degradation, at the transcriptional/translational and proteolytic levels respectively, for long-term rearrangement of metabolism in the winter. How these mechanisms operate at low temperatures, and how they are regulated in these insects, is not known.

Although many changes which appear to be important for cold-hardiness have been shown in this work, practically nothing is known of the mechanisms by which a

change in environmental conditions initiates them. In fact, the environmental triggers which lead to the changes are themselves largely unknown. Likely candidates are thermoperiod and photoperiod, however the effects of photoperiod on larvae within galls have not been examined. Temperature has been argued to be a more reliable cue than photic signals, as it is indicative rather than predictive of environmental conditions (Baust, 1982). Joannis and Storey (1995) and Storey and Storey (1981) have shown that maintaining larvae at cold temperatures is sufficient for initiating some of the changes in enzyme activities during acclimatization, but is not sufficient for most changes observed. Other signals must then be involved. Interestingly, gall water content may serve such a purpose, as it has been shown to influence glycerol accumulation in *Eurosta solidaginis* (Rojas *et al.*, 1986), where glycerol content is inversely correlated with the water content of the gall as the plant dies and dries in the fall (the larvae themselves do not dehydrate). The neural or hormonal signals that operate in relating environmental change to biochemical change during acclimatization, as well as the role of their corresponding secondary messenger systems (*e.g.* protein kinases), are also largely unknown in gall insect acclimatization. In *Eurosta solidaginis*, changes in juvenile hormone titer have been shown to have some effect on the supercooling point as well as cryoprotectant synthesis (Rojas *et al.*, 1987; Hamilton *et al.*, 1986). However, these data cannot explain the observed changes in the larvae during acclimatization, and can only be used to suggest a possible as yet undefined role for juvenile hormone. Once the machinery for polyol cryoprotectant synthesis is in place, however, temperature cues in the form low temperatures in the fall and high temperatures in the spring appear critical for seasonal polyol synthesis and degradation, respectively (Storey and Storey, 1991).

The observed changes in enzyme activities from various pathways are not necessarily the result of cues for acclimatization to temperature, but instead may reflect changes brought about by the developmental state of the larvae. Hormonal signals for entry into diapause, which is suspected to last for the present larvae from sometime in

November to sometime in February, could then initiate some of the changes observed in enzyme systems. However, although many insects do enter a state of diapause during periods of low temperature exposure, there is no interdependence of insect diapause and cold-hardiness (Danks, 1987). Instead, the cues for cold-hardiness and diapause must be different or independent. The timing of the changes in enzyme activities reported in this work also support this independence. Changes in the activities of enzymes involved in cryoprotectant accumulation and removal (Chapters II and III), for example, largely occur before or after the apparent diapause of the gall larvae. This is also true for the TCA and related enzymes (Chapter V). This suggests that these changes relate not to entry into diapause, but to acclimatization. This also implies a function for these changes other than for those associated with diapause. For example, the decreased dependence on oxidative metabolism is not solely the result of the entry into a physiologically-induced state of reduced metabolism during diapause. The adaptations for cold-hardiness are then not simply an extension of diapause. Instead, they must have evolved from other existing mechanisms and for unique purposes. This is not to say that developmental effects do not influence the cold-acclimatization of the insects. Morrissey and Baust (1976) have shown that only third instar larvae (the overwinter stage) appear capable of glycerol synthesis. Shimada *et al.* (1993) have shown that 20-hydroxyecdysone treatment and the resulting metamorphosis from larvae to pupae in *Eurosta solidaginis* leads to a loss of nucleation ability and a switch to a freeze avoiding strategy for cold-hardiness. It would be enlightening to examine the response to 20-hydroxyecdysone and other hormones of larvae from different sampling times over the winter to see if they influence cold-hardiness. Puparium formation in late April *Eurosta solidaginis* undoubtedly leads to some changes in metabolic organization, but what these changes are is unknown at present.

The work presented in this study shows a dynamic system of metabolism which responds continuously to, and prepares for, environmental change. These alterations

occur even during stages of diapause and/or quiescence, showing that these are not states of metabolic inactivity. Instead, a complex system of metabolic change and its control operates during overwintering in these cold-hardy gall insects. The similarities between the cold-hardiness adaptations of insects as widely disparate as lepidoptera and diptera suggest that these adaptations have arisen from a common biochemical architecture, i.e. they have not required an extensive modification of the existing metabolic organization of the animal. Instead these larvae must have relied on relatively few changes to allow freeze tolerance or freeze avoidance. The similarity between the observed adaptations for freeze tolerance and freeze avoidance also suggest that there is relatively little difference in the arrangement of metabolism between the two strategies. For example, cryoprotectant metabolism and metabolic depression are largely identical in the two larvae. Sustainable anaerobic metabolism, the protection against ice damage to cells, and controlled ice nucleation may be key to freeze tolerance.

APPENDIX 1
PUBLICATION LIST

Published Research Articles

- Joanisse, D.R., and K.B. Storey. Temperature acclimation and seasonal responses by enzymes in cold-hardy gall insects. *Arch. Insect Biochem. Biophys.* 28:339-349.
- Joanisse, D.R., and K.B. Storey. Enzyme activity profiles in an overwintering population of freeze avoiding gall moth larvae, *Epiblema scudderiana*. *Can. J. Zool.*, 72:1079-1086.
- Joanisse, D.R., and K.B. Storey. (1994) Enzyme activity profiles in an overwintering population of freeze tolerant larvae of the gall fly, *Eurosta solidaginis*. *J. Comp. Physiol. B* 164:247-255.
- Joanisse, D.R., and K.B. Storey (1994) Mitochondrial enzymes during overwintering in two species of cold hardy gall insects. *Insect Biochem. Molec. Biol.* 24(2):145-150.
- Storey, K.B., T.A. Churchill, and D.R. Joanisse (1993) Freeze tolerance in hermit flower beetle (*Osmoderma eremicola*) larvae. *J. Insect Physiol.* 39(9):737-742.

Research Articles Submitted

- Joanisse, D.R., and K.B. Storey. Fatty acid content and enzymes of fatty acid metabolism in overwintering cold-hardy gall insects. Submitted to *Physiol. Zool.*

Research Articles In Preparation

- Joanisse, D.R., and K.B. Storey. Oxidative damage and antioxidant defenses in the freeze tolerant wood frog, *Rana sylvatica*.
- Joanisse, D.R., and K.B. Storey. Antioxidant Systems During Overwintering in Larvae of Cold-Hardy Gall Insects.
- Joanisse, D.R., and K.B. Storey. Oxidative damage and antioxidant systems during freezing- and anoxia-recovery in cold-hardy gall insects.
- Joanisse, D.R., and K.B. Storey. Cryoprotective effects of low molecular compounds on lactate dehydrogenase from cold-hardy goldenrod gall insects.

Communications Presented at Scientific Meetings

- Joanisse, D.R., and K.B. Storey (1994) Antioxidant systems and oxidative damage during freezing and thawing in the freeze tolerant frog *Rana sylvatica*.

Presented at the 37th meeting of the Canadian Federation of Biological Societies.

Joanisse, D.R., T.A. Churchill, and K.B. Storey (1992). Freeze tolerance in *Osmoderma eremicola*. Presented at the 29th meeting of the Society for Cryobiology. *Cryobiology* 29(6):752.

Joanisse, D.R., and K.B. Storey (1991). Enzyme activity profile during overwintering in two cold hardy gall insects. Presented at the 34th meeting of the Canadian Federation of Biological Societies.

Joanisse, D.R., and K.B. Storey (1990). Winter profile of enzymatic activities in freeze tolerant and freeze avoiding gall insects. Presented at the 27th meeting of the Society for Cryobiology. *Cryobiology* 27(6):672.

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