MOLECULAR PHYSIOLOGY OF INSECT LOW TEMPERATURE STRESS RESPONSES

DISSERTATION

In partial fulfillment of the requirements for the degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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The Ohio State University
2007

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ABSTRACT

Insect responses to low temperature can be broadly-categorized into two groups: freeze-tolerant and freeze-intolerant. Freeze-tolerant insects respond to low-temperatures by allowing their body tissues to freeze, and damage is typically mitigated in this mechanism by limiting ice formation to the extracellular spaces. Freeze-tolerant species accomplish this by increasing the amount of intracellular solutes and promoting ice formation in the extracellular spaces, which limits damage to membranes. Freeze-intolerant insects suffer damage from cold at temperatures above the point at which body tissues freeze. These organisms prevent damage from low-temperature by increasing the concentrations of polyols in the hemolymph and repair the damage from low-temperature by the production of heat shock proteins. None of what is previously known about insect low-temperature survival is complete, especially at the molecular level. Other than the production of heat shock proteins during recovery from low-temperature damage, no genes have definitively been identified as functionally upregulated to promote low-temperature survival. In addition, the body of knowledge concerning cytoskeletal, membranal, and metabolic changes in insects exposed to low-temperatures is sparse, but all three of these are hypothesized to be involved in low-temperature survival in other organisms. It is the purpose of this work to identify previously unknown molecular mechanisms by which insects deal with low temperatures. This exploration should
encompass transcriptional, membranal, and metabolic methods in order to glean the maximal amount of information. The freeze tolerant midge, Belgica antarctica, and the freeze intolerant flesh fly, Sarcophaga crassipalpis, were chosen as model organisms for testing.

A gas chromatography-mass spectroscopic analysis of the membrane lipids from flesh flies revealed that diapause and rapid cold-hardening both dramatically altered the composition of fatty acids in cell membranes. Both physiological conditions increased oleic acid levels, which promotes cellular survival to low-temperatures by widening the window by which cell membranes maintain their liquid crystalline state. In addition, a thin-layer chromatographic analysis of phospholipid head groups revealed that phosphatidycholines were replaced by phosphatidylethanolamines, which also lowers the temperature window by which cell membranes maintain homeostasis. In short, membrane restructuring appears to contribute to low-temperature survival in the freeze-intolerant flesh fly.

A metabolomic analysis of whole-body metabolites isolated from flesh flies in diapause and rapid cold-hardening revealed wide-spread alterations in metabolism. Rapid cold-hardening produced the predictable increase in glycerol concentration, but this increase was also coupled with the increase of another polyol, sorbitol. Rapid cold-hardening also produced increases in many other metabolites that have been previously unknown to be increased, most notably alanine, glutamine and pyruvate. In an environment with these metabolic changes, flesh flies experiencing rapid cold-hardening are well-equipped to survive low-temperature stress because they have increased polyols to protect proteins and membranes, and glutamine is present should the need for heat
shock proteins arise during recovery. Increases in glycerol, alanine, and pyruvate were also seen for diapause, but unlike rapid cold-hardening, diapause produced a metabolic profile consistent with a disruption of Krebs cycle activity. Not only does this cellular environment promote cold survival, but it also explains the previous observation that oxygen consumption is very low during flesh fly diapause.

A metabolomic analysis of the freeze tolerant midge, Belgica antarctica, revealed that freezing increased a number of different polyols in whole-body extracts, including glycerol, mannitol, and erythritol. Freezing also increased alanine, asparagine, and glycine. In addition, the frozen midge larvae accumulated Krebs cycle intermediates, indicating that aerobic respiration is considerably slowed. Membrane involvement in freezing was not conclusively supported, although there was a reduction in oleic acid levels. A comparison of the metabolic responses of this midge to heat, freezing and desiccation revealed that freezing and desiccation produced similar results, supporting the hypothesis that the cellular response to these two stressors is related.

In conclusion, a number of physiological mechanisms by which insects survive low-temperatures have yet to be discovered, but the richness of these mechanisms is clearly displayed in the present work using only two model species. From the present study, it appears that freeze-tolerant and freeze-intolerant species employ similar mechanisms to survive low-temperatures at the molecular level since their responses to low-temperature were similar. These mechanisms include polyol increases, alterations in the amino acid pool, and Krebs cycle shutdown. The differences in response between
these insects (e.g. membrane involvement, type of polyol used) most likely is explained by the difference in environmental conditions between these insects and their phylogenetic separation.
Dedicated to my grandmother, Reba McHugh, and my grandfather, Mike Mihalow.
ACKNOWLEDGMENTS

Thanks to Dr. David L. Denlinger for his support, his tutelage, and his expert editorial and scientific contribution to this work.

Thanks to my wife, Erica D. Michaud, for her infinite patience and clerical support to this text.

Thanks to thank Richard Sessler of the Campus Chemical Instrument Center of The Ohio State University for his advice on chromatographic technique and Amelia Brown for her technical support in chemical separation.

Thanks to Dr. Scott Hayward and Dr. Dan Hahn for their consultations on lipid analysis and Dr. John Wenzel for his assistance in multivariate statistics.

Thanks to the Ohio Agricultural Research and Development Center, The Ohio State Graduate School, and the Department of Entomology for their financial support.

Thanks to the National Science Foundation for their research support to this work.
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CHAPTER 1
INTRODUCTION

Molecular modalities of insect cold survival: current understanding and future trends.

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Abstract. Insect cold survival has drawn increased attention in recent years, prompting a new classification of survival phenotypes that reflect the ecological and physiological differences in the manner in which insects survive the winter. In addition, more is known about the nature of cold injury and its effects on an insect system from the whole body to the sub-cellular level, although this area of research is still preliminary.

Other than the detection of misfolded protein by the heat shock response, signal transduction of low temperatures by insects is largely unknown, although recent advances in *Drosophila* and a few vertebrate systems reveal the potential
importance of transient receptor potential ion channels in low temperature signal transduction. Membrane fluidity also possibly plays a role in the detection of low temperatures by insects.

Physiological changes that occur in insect cells as a result of low temperatures or in anticipation of low temperatures include the induction of a suite of heat shock proteins, metabolic polyol synthesis, antifreeze protein production, and the induction of extracellular ice nucleation. In addition, a variety of new genes are now known to be induced upon cold exposure, although the roles of these molecules in cold survival awaits experimentation.

Keywords: cold shock; Insecta; heat shock protein; molecular response; cytoskeleton cryobiology

1.1. Introduction

The field of insect cryobiology has advanced considerably in recent years due to its potential applicability in pest control, its contribution to understanding evolutionary trends in adaptation to abiotic stress, and its potential role in understanding effects of global climate change. A number of strategies, mechanisms, and phenotypes have been elucidated for the 250+ species that have been studied thus far, weaving a convoluted tapestry that has underscored the great variability of insect systems, but these studies have shed light on common adaptive strategies arising independently over several insect lineages [1].
Surviving the cold can encompass a number of abiotic and biotic variables. The lowest temperature which the insect experiences, the length of time the environment remains at that temperature, the rate of cooling, and the presence of water all influence survival at the abiotic level and have been proposed to influence certain adaptive strategies, as well [2]. Biotic variables are even more widespread. The detection of token stimuli with subsequent employment of cryoprotective mechanisms (e.g. diapause) greatly influences the ability of an insect to survive the upcoming winter. In addition, insects can benefit from tracking the conditions of the natural environment in real-time and adjusting their physiology accordingly (e.g. rapid cold-hardening). Finally, an insect can influence its survival by employing reparative mechanisms that ameliorate the damage sustained from a cold insult when conditions return to normal (e.g. heat shock proteins).

Each of these variables implies a molecular mechanism, and even though many of these pathways have been studied in detail, evidence suggests that there are pathways and molecular species yet undiscovered. In this review we briefly discuss cold survival phenotypes, the nature of cold injury, and then provide an overview of the molecular aspects of insect cold hardiness.

1.2. Cold hardiness phenotypes

Insects in temperate and cooler climates employ various mechanisms to survive temperatures of 0°C or lower. These mechanisms fall into two broad categories: freeze-tolerance and freeze-avoidance [3]. Freeze-tolerant insects are
characterized by their ability to raise their supercooling point to freeze extracellular spaces, preventing damage to the cell membranes from encroaching ice crystals [4]. The second category, freeze-avoidance, covers a broad range of insects that survive the cold by preventing the formation of ice crystals, although the cold survival phenotype of these species and the mechanisms employed vary considerably.

Because of this variation, Bale [5,6] has proposed that freeze-avoidance should be further subcategorized into four groups based on the observation that freeze-avoidance falls into four distinct survival phenotypes. The first of these categories, freeze-avoidance (sensu stricto), applies only to insects that do not experience significant mortality until the body begins to freeze. These insects benefit from a lowering of the supercooling point alone. On a worldwide scale, the numbers of insect species that fall into freeze tolerance and freeze avoidance (sensu lato) categories is comparatively small [5].

The next three categories are based on the observation that many insects begin to experience mortality long before the supercooling point is reached. The first of these, chill-tolerance, refers to species that experience mortality only after long exposures (days) to temperatures below 0°C but above the supercooling point. A similar category, chill-susceptibility, describes insects that experience mortality after short (minutes or hours) exposure to temperatures similar to those above. Even though the dividing line between these two groups is not clearly defined, acknowledgment of these two categories reflects the physiological
distinction between flesh flies (Sarcophaga crassipalpis) that are chill-tolerant in the diapause state but chill-susceptible during the remainder of their life cycle [7].

The final category, opportunistic survival, refers to insects that are incapable of employing preventative cold hardening mechanisms. To survive, these insects must avoid low temperatures altogether by seeking thermally favorable microhabitats [5].

This new sub-categorization of freeze-avoidance conforms to natural trends [2] and encompasses the entire range of observed responses in species from the tropics to the poles [6].

1.3. The nature of low-temperature injury

Cold injury falls into three categories based on the exposure temperature and its relation to the freezing point of water and the freezing point of the insect itself. If the insect suffers damage from positive temperatures at or near 0°C, usually over a period of time expressed in days, then the insect is said to have suffered from indirect chilling injury [4]. Direct chilling injury, or cold shock, describes damage from temperatures that are below 0°C but above the supercooling point [8]. Cold shock injury arises from low temperature exposures of minutes, hours, or days, depending on the insect species under study and the intensity of the exposure. Finally, insects that suffer damage because of intracellular ice crystal formation (due to temperature depression below the supercooling point) suffer from freezing injury [9]. For freeze-tolerant insects, freezing injury occurs well-
below the supercooling point if the insect is physiologically prepared for winter [10], but freeze-avoiding insects experience this injury at their supercooling point [4].

1.3.1 Injury at the whole-body level

A few studies have concentrated on the effects of cold exposure at the whole-body level. The flesh fly, *S. crassipalpis*, can survive for up to 25 days when exposed to 0°C [11], but flies that have suffered from indirect chilling injury at 2°C for ten days are slow to emerge from the puparium, provided they are capable of opening the puparium at all. Many that fail to emerge after this exposure are also unable to retract the ptilinum if the flies are removed from the puparium artificially. The coordination of muscular movement is preserved in this particular instance, but the amplitude of such movement is reduced to one-third the level of untreated controls, hence the impairment of eclosion. Many flesh flies that successfully emerge have wing abnormalities from failure to properly expand their wings after eclosion [12]. Developmental abnormalities have been reported from indirect chilling injury in other species as well [13,14] Study of the blood-sucking hemipteran, *Panstrongylus megistus*, revealed that short exposures (1h) to 5°C or 0°C greatly reduces molting incidence, but survival is only affected by 0°C exposures, with 96.7% mortality reached after 12 hours without induced cryotolerance [15].

Non-diapausing *Sarcophaga crassipalpis* pupae exposed to a -10°C cold shock (16°C above their supercooling point) for 45 minutes fail to emerge 50% of
the time and die soon afterwards. These flies also have disrupted emergence patterns and reduced coordination of extrication movements, although the amplitude of muscular coordination is unaffected [12]. Cold-shocked flesh flies have reduced motor neuron conductivity and decreased activity at the neuromuscular junction [16]. Even more important to overall viability, the fecundity of these flies is also affected, showing a reduction of viable eggs after a one hour cold shock at -10°C. Fecundity reduction from cold shock was caused by a dramatic reduction in male mating frequency [17]. In contrast, a study in the house fly, *Musca domestica*, has shown that cold shock reduces total egg production in females, but the effect on males was not measured [18].

By definition, freezing injury often leads to immediate, catastrophic consequences for the survival of freeze-avoiding insects. Often the freezing injury begins in the gut where potential ice nucleators reside; this is one reason why *S. crassipalpis* possesses a lower supercooling point (SCP) in its non-feeding stages [7]. Freeze-tolerant species can only withstand low temperatures to a certain point below their SCP, often a few degrees below the SCP in the Southern hemisphere and tens of degrees below the SCP in the Northern hemisphere [1]. When this critical point is reached, the freeze-tolerance mechanism loses its ability to stem the increasing growth of ice crystals, and the cells lethally freeze.

1.3.2 Injury at the tissue level and below

At the tissue and cellular level, cold injury is poorly understood, although research in this area is beginning to attract more attention. It is generally accepted
that damage from cold shock at the cellular level results from the loss of cell function due to denaturation, the formation of insoluble protein aggregates, and the loss of membrane homeostasis from phase transitions [19]. Vital dyes and direct microscopic examination have been applied to insect cells to determine what effects are the most common after cold exposure. In Panstrongylus megistus, exposure to sequential temperatures of 5°C and 0°C leads to a reduction in apoptotic events for Malphigian tubule epithelial cells compared to control. Concurrently, the level of necrosis increases when compared to controls, leading to the hypothesis that cold shock injury favors necrosis either through direct apoptotic suppression or indirectly by ATP depletion [15].

Vital dye studies of fat body cells from the freeze-tolerant species, Eurosta solidaginis, have demonstrated that after freezing, these cells have much larger lipid droplets but fail to show signs of membrane damage [20]. A subsequent Eurosta investigation using fluorescent vital dyes revealed increased freeze injury susceptibility (>50% cell mortality) for integumentary muscle, hemocytes, tracheae, the distal portion of the Malphigian tubules, and the fat body after treatment for 10 days at -80°C. The tissues least susceptible to damage from this treatment were the gut, proximal portion of the Malphigian tubules, and the salivary gland [9].

Ultrastructural analysis of lethally frozen brain tissues from Eurosta larvae demonstrated intermembranous expansions of the nuclear envelope and clumping of chromatin in the brain with associated rough endoplasmic reticulum expansion, and increased presence of autophagic bodies in the cytoplasm. Muscles studied in
this manner had missing Z-lines as a result of myofilament breakdown, and the
nuclei went from a structured peripheral array of chromatin to a diffuse
organization. Finally, the Malpighian tubules of lethally frozen *Eurosta* had
expanded mitochondria with broken cristae and an increased number of
autophagic bodies [10].

All of the evidence above reveals tissue vulnerability to cold damage in
nervous tissues and muscles, both of which are heavily reliant on membrane
integrity, cytoskeletal structure, and energy for normal functioning. To further
complicate matters, energy production may be hampered by tracheal damage and
mitochondrial disruption, although no immediate change in oxygen consumption
has been reported from cold shock damage in *S. crassipalpis* [8]. Changes in
nuclear organization also seems to be a commonality associated with damage to
insects from the cold [10,15], although the evidence is preliminary. In future
studies of cold damage at the tissue level or below, it would be useful to see the
differences between the types of cold injury for each of the five groups of low-
temperature survival phenotypes. Such work may reveal the cellular benefits of
the various insect cold survival strategies based on differences in observed tissue
damage or intracellular phenomena after low-temperature insult.

1.4. Detection of low-temperature stimuli

For an insect to initiate protective mechanisms that aid in low-temperature
survival without seasonal acclimation, the cell must be able to detect low
temperatures directly or to detect subtle changes in the cell composition or
architecture that accompany reduction in temperature. For insects, this area of research is still in its infancy, although work in other model systems may illuminate potential targets for study.

1.4.1 Detection of denatured protein

As previously discussed, cold shock damage is thought to lead to the denaturation of many different protein species within the cell [21]. As a result, these proteins often lose their hydrophobic/hydrophilic interactions and fall out of the cytoplasmic solution. When this occurs, the Heat-shock-cognate 70 (Hsc70) protein, which normally is heterodimerized with the Heat-shock transcription factor (HSF), dissociates from HSF in favor of binding to the newly-denatured protein [22]. The free HSF molecule then trimerizes with other free HSF molecules and subsequently enters the nucleus to bind with promoter regions of DNA called heat shock elements (HSE’s) [23].

By far, the most notable group of molecules to contain HSE’s in their promoter regions are the various heat shock proteins (Hsps), which, upon an HSF trimer binding to the HSE, are then transcribed and translated to protect the cell from further damage and to repair the damage already sustained (Section 5.2). Studies of heat shock in Drosophila melanogaster have not only shown the presence of most of these molecules in the Drosophila genome, but have also drawn functional conclusions for many of these molecules [24], including HSF [25]. The fact that heat shock proteins (including Hsc70) are highly-expressed
during recovery from cold shock in *Sarcophaga crassipalpis* [26, 27, 28, 29, 30] is the best indicator that the HSF cascade is involved in cold shock recovery, but as of yet, this has not been confirmed experimentally.

Although this method of low-temperature detection and repair is highly-conserved throughout all kingdoms [22], it is not proactive; it is reactive. Other cellular methods of low-temperature detection may be involved in priming the cell for cold hardiness long before damage is sustained, a response strategy arguably more favorable than a damage-dependent response.

### 1.4.2 Transient receptor potential ion channels

When faced with a temperature gradient, insects tend to move to a point in the gradient that is optimal for their physiology. This behaviour must be controlled by a reception system that can detect small differences in temperature in order to “know” what temperature is ideal. For adult insects, cold-sensing cells in the antennae called “cold cells” generate action potentials in response to low-temperature [31]. Recent work in *Drosophila* larvae has identified the terminal organs for cold reception using Ca$^{2+}$ influx as a marker for neural activity [32]. These neurons, which have the same physiological characteristics of “cold cells” [33], can sense changes in temperature as small as 0.3°C, beginning at temperatures below 18°C [34].

Molecular temperature sensors for insects have yet to be convincingly identified, but evidence is emerging that *painless*, a transient receptor potential (TRP) gene, is responsible for detection of supraoptimal temperatures in
Drosophila larvae [35]. Painless is related to vertebrate TRPV1, the protein most responsible for thermoreception in vertebrates [36]. In addition, research in mammals has also uncovered a TRP Ca2+ channel that is responsible for the reception of cold as well as menthol [37]. A BLAST search through Genbank has uncovered a similar gene in both the Drosophila melanogaster (3e-76) and Anopheles gambiae (8e-77) genome, although both genes await study.

1.4.3 Membrane fluidity and histidine kinase 33

Exciting research in plants has recently revealed the role of membrane fluidity in cold reception. When the ambient temperature drops, decreasing membrane fluidity leads to membrane transport perturbations [19]. To counter this, plants cells have developed a system by which the histidine kinases, Hik33 and Hik19, act in concert with the transcription regulator, Rer1, in response to decreasing fluidity [38,39]. The cell’s response to this cascade features the induction of membrane desaturase genes (desA, desB, oleI) as well as other cold-acclimating genes [19,40,41,42]. Membrane desaturase genes increase the cell’s membrane fluidity, restoring transport functions [19]. A GenBank BLAST search reveals that the Anopheles genome has two ESTs with weak similarity to the Hik33 and Hik19 genes in Synechocystis sp. (7e-11, 1e-10). Drosophila Hsp83 and Gp93 chaperones have a histidine kinase-like ATPase domain, thus further supporting the possibility that this pathway is involved in insect cold tolerance.

Changes in membrane fluidity in response to short-term low-temperature exposure have not been well-studied in insects, although a reduction in saturated
fatty acids is typical of winter preparation [44,45]. One study in the arctiid moth, *Cymbalophora pudica*, found extensive lipid composition changes in the brain and gut in response to a one-day 4°C acclimation. Membrane fluidity for these two tissues was unaltered. In addition, the degree of lipid desaturation increased in the fat body, the silk glands, and the body wall, although these increases were moderate. This moth does not have pronounced cold hardiness even with acclimation, thus a moderate change in lipid composition might be expected for this species [46]. Although no genes were implicated in this study, insects such a *Drosophila* and many moths have multiple desaturase genes which could potentially be responsible for altering membrane fluidity [47], especially since de novo lipid synthesis from fat stores was not found in the arctiid study [46].

1.5. **Established mechanisms of cold-hardiness**

The insect cryobiology literature reveals four main mechanisms for insect cold hardiness. Any one of these mechanisms or any combination of these mechanisms may be utilized by an insect to survive suboptimal temperatures. Generally, the more mechanisms employed or the greater the induction is for a particular mechanism, the more cold-hardy the insect becomes.

1.5.1 *Polyols and other low-molecular weight substances*

Polyhydric alcohols (polyols) and other low-molecular weight compounds are manufactured by many insects either in response to or in preparation for a low temperature insult. Seasonally, these compounds are manufactured in the fat body
where they are released into the hemolymph to colligatively (0.2 M or greater concentration) protect the entire body from low-temperature damage by either reducing the supercooling point or increasing the osmolarity of the cell [4,48]. Some insects upregulate low-molecular weight compounds to levels far below 0.2 M and still increase cold-hardiness [48,49]. Non-colligative effects of polyols include stabilization of nascent proteins, increased repair of damaged protein by molecular chaperones [50], and membrane stabilization [51]. Low-molecular weight compounds that are known to protect insects from cold include glycerol, sorbitol, mannitol, trehalose, and proline [4, 52, 53].

Seasonally, insects upregulate polyols and other low-molecular weight compounds in response to shortening daylength and decreasing temperatures over a period of days or weeks [4,24]. Seasonal increases in these compounds may be attributed to freeze-tolerance [48], freeze-avoidance, or diapause [4]. Additionally, some insects can upregulate these compounds on a time scale from twenty minutes to eight hours in a process known as rapid cold hardening (RCH), although the level of polyol upregulation is lower than that seen for seasonal acclimation [49,54,55]. Rapid cold hardening may not be a strategy found in freeze-tolerant insects [56].

The mechanism of induction for glycerol involves the temperature-dependent activation of glycogen phosphoylase, which, upon its induction, frees glucose molecules to be cleaved into glycerol [52]. Glycerol synthesis may be further enhanced by the diversion of carbon flow to the pentose phosphate pathway [57,58]. In the rice stem borer, *Chilo suppressalis*, glycerol production
results from the inhibition of fructose 1,6 bisphosphate and pyruvate kinase. Glycogen phosphorylase, glyceraldehyde-3-phosphatase and polyol dehydrogenase are all activated in the stem borer during seasonal acclimation [58]. Inhibition of protein phosphatase-1 during glycerol synthesis is found in both freeze-tolerant and freeze-avoiding insects [60]. In the goldenrod moth, *Epiblema scudderiana*, hexokinase is strongly activated (300%) in the presence of glycerol-3-phosphate and low temperatures [61]. For the freeze-tolerant *Eurosta solidaginis*, glycerol is made utilizing the pentose phosphate pathway, but after temperatures drop below 5°C, phosphofructokinase is strongly inhibited, diverting the insect’s metabolism from glycerol to sorbitol synthesis in a one-step process [62,63].

Clearly, more research needs to be done to match the identity of the cryoprotective compound with cold-hardy insect species, measure rates of induction, and identify enzymes involved in these pathways. The information thus gleaned would serve to further define the relationship between these compounds and low-temperature survival, possibly highlighting common mechanisms that occur within an insect lineage or across lineages.

### 1.5.2 AFPs and INAs

Antifreeze proteins (AFPs) and ice nucleating agents (INAs) are used by some insects to prevent injury from ice crystal formation. INAs are produced in freeze-tolerant insects to drive the crystallization temperature of the hemolymph upward, forcing ice crystals to form in the extracellular spaces at higher sub-zero
temperatures. Ice crystals pull water out of solution extracellularly, driving the osmolarity of the cells higher and higher because water flows from the intracellular space to the extracellular space to compensate for water lost to ice [64]. In the end, the insect is quite well protected from intracellular ice formation. This attribute is well developed in Northern hemisphere insects that are known to be able to survive freezing to several tens of degrees below their supercooling points [1]. INAs have not been observed in freeze-avoiding insects.

Some insects, particularly beetles, produce AFPs in preparation for overwintering [65]. AFPs protect the insect by preventing the growth of ice crystals that have nucleated by adhering to the surface of the crystal, hindering the addition of new water molecules to the ice lattice. AFPs lower the freezing point of insect hemolymph up to 8.5°C without affecting the melting point in a condition known as thermal hysteresis [66]. Fish have five different classes of AFPs, each with preferential binding to a specific surface of the growing ice crystal lattice [67]. Insect AFPs have not been subdivided into as many classes, but evidence shows that fish and insect AFPs bind to ice with comparable affinity and greater effectiveness as measured by the degree of thermal hysteresis [68]. AFP activity is enhanced by protein enhancers [69], polyols, and organic anions [70].

1.5.3 Heat shock proteins

In cases where cold tolerance is not enough to sustain an insect through a particularly strong low-temperature insult without injury, Hsp5s must be manufactured to repair, contain, or remove damaged proteins. Thus far, investigations into Hsp expression after cold exposure in insects have been limited
to *Sarcophaga crassipalpis* [27,28,29], *Drosophila* (four species) [24,71], and the Colorado potato beetle (*Leptinotarsa decemlineata*) [29]. In all of these cases, heat shock protein transcripts are upregulated from 1 to 12 hours at recovery temperatures following the cold shock.

The most commonly studied transcript is the inducible form of heat shock protein 70, *hsp70*. This protein (~70 kDa) product of this transcript is highly conserved (>50%) across all kingdoms of organisms and is expressed after cold shock in all six of the above insects. Hsp70 aids in stress survival by refolding damaged protein, redissolving insoluble proteins [72], and tagging irreparable protein for degradation [73], although the specific function of insect Hsp70 for low-temperature survival has not been studied. However, induction of *S. crassipalpis* Hsps by heat shock and diapause has been correlated with increased survival to cold [7,27,74]. Stronger cold shocks induce stronger and longer induction of *hsp70* transcripts for *S. crassipalpis*, but the induction is delayed when compared to milder cold shocks [27]. Acclimation to lower developmental temperatures reduces the threshold of *hsp70* expression in *Drosophila triauraria*, but this has not been observed in the other two *Drosophila* species tested for *hsp70* expression thresholds [71].

The constitutive form of heat shock protein 70 (Hsc70) normally does not change its expression profile in response to stress, but *Sarcophaga crassipalpis hsc70* transcripts are upregulated in response to cold shock [27]. The role of Hsc70 in
stress signalling is important in that it is primarily responsible for the release of heat shock transcription factor (HSF) [22], although the purpose of Hsc70 upregulation after cold stress remains unclear.

Small heat shock proteins (sHsps) are a diverse family of proteins (12 to 40 kDa) that are identified by the presence of an alpha-crystalline domain. Their role in stress tolerance involves refolding local domains of damaged proteins, tagging aberrant protein in the early stages of denaturation for other chaperones to fold, and inhibition of proteolysis [22,75]. Also, sHsps are effective in preserving the actin cytoskeleton in mammalian hypothermia, which is particularly important for cold tolerance because cold is known to break down the cytoskeleton in vitro [76,77]. In *S. crassipalpis*, *hsp23* transcripts are upregulated in response to cold shock, heat shock, and during pupal diapause [26,29], although what benefit such upregulation confers has not been verified.

The 90 kDa family of heat shock proteins in flesh flies are also upregulated in response to cold shock [26], but not diapause [28]. Normally, *hsp90* transcripts are expressed at low constitutive levels but are induced quickly after cold shock (1 hour), peak after 4 hours and remain expressed at high levels even after 12 hours post-cold shock [28]. Hsp90 recognizes and repairs damaged proteins that are bound by Hsc70, sequesters HSF in the same manner as Hsc70, and targets protein for degradation in the ubiquitin-dependent proteosome pathway [78]. Hsp90 is also known to activate the ecdysone receptor-ultraspiracle complex. This may be the reason it is not expressed, like other Hsps, during diapause, a stage dependent on the absence of ecdysteroids [28,78].
Even though heat shock proteins have been correlated with cold survival, there is little direct evidence in the literature to confirm this. Fortunately, new RNA interference (RNAi) technology allows for the functional study of transcripts in a variety of ectothermic organisms, notably insects [23], and our laboratory’s unpublished data indicates a loss of cold tolerance when Hsps are knocked down using RNAi.

1.6. Frontiers in cold-hardiness mechanisms

1.6.1 The role of the cytoskeleton

Studies of plant responses to cold have emphasized the increasing importance of the plant cytoskeleton in conferring tolerance to low temperatures. In addition, cold tolerance of the cytoskeleton has been documented in the Atlantic cod [79]. Cold tolerance may be acquired through two cytoskeletal methods: depolymerization of cytoskeletal elements trigger cellular responses to cold, and microtubules may be assembled in a cold-stable configuration in anticipation of low-temperature. In the former process, calcium channels are attached to the depolymerising actin cytoskeleton, and consequently the ability to prevent $\text{Ca}^{2+}$ from entering the cell is lost at low temperature [80]. The ensuing $\text{Ca}^{2+}$ cascade leads to an undetermined signalling mechanism that results in cold-stable cells [81,82]. In the latter mechanism, post-translational modification of tubulin polymers [83], addition of cold-stabilizing proteins [76, 84] such as Hsps [83,85], or tubulin amino acid substitutions [79,86] are added to the growing
microtubules to increase the stability of the cytoskeleton. A cold-stable cytoskeleton presumably will maintain the structure, function, and organization of cells upon exposure to potentially damaging low temperatures.

<table>
<thead>
<tr>
<th>Name</th>
<th>Species/Description</th>
<th>Observations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp70</td>
<td><em>Drosophila</em> (4 species), <em>Sarcophaga crassipalpis</em>, <em>Leptinotarsa decemlineata</em></td>
<td>Upregulation during recovery from cold shock</td>
<td>[71]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Upregulation during diapause and recovery from cold shock</td>
<td>[27,30]</td>
</tr>
<tr>
<td>Hsc70</td>
<td><em>S. crassipalpis</em></td>
<td>Upregulation during diapause, recovery from cold shock</td>
<td>[27]</td>
</tr>
<tr>
<td>Hsp23</td>
<td><em>S. crassipalpis</em></td>
<td>Upregulation during diapause, recovery from cold shock</td>
<td>[22,29]</td>
</tr>
<tr>
<td>Hsp90</td>
<td><em>S. crassipalpis</em></td>
<td>Recovery from cold shock</td>
<td>[22,28]</td>
</tr>
<tr>
<td>Dca</td>
<td><em>D. melanogaster</em></td>
<td>Upregulation from 15°C acclimation</td>
<td>[88]</td>
</tr>
<tr>
<td>Fst</td>
<td><em>D. melanogaster</em></td>
<td>Upregulation during recovery from a 0°C cold shock</td>
<td>[89]</td>
</tr>
<tr>
<td>Hsr-omega</td>
<td><em>Drosophila</em> (3 species)</td>
<td></td>
<td>[90]</td>
</tr>
<tr>
<td>Svp</td>
<td><em>Bombyx mori</em></td>
<td>Transcription factor upregulated from chilling</td>
<td>[91]</td>
</tr>
<tr>
<td>Sorbitol</td>
<td></td>
<td>Upregulated after chilling for 50 days</td>
<td>[91]</td>
</tr>
<tr>
<td>dehydrogenase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Samui</td>
<td><em>B. mori</em></td>
<td>Chill-inducible protein that transmits low temperature signals</td>
<td>[92,93]</td>
</tr>
<tr>
<td>c-fos-like</td>
<td><em>Galleria mellonella</em></td>
<td>Induced in the brain during 3h chilling at 0°C</td>
<td>[94]</td>
</tr>
<tr>
<td>Cp1, Cp2</td>
<td><em>Spodoptera exigua</em></td>
<td>Proteins induced after 2h treatments at 0°C &amp; 5°C</td>
<td>[95]</td>
</tr>
<tr>
<td>Hik19</td>
<td><em>Synechocystis</em> sp. (algae)</td>
<td>Cytoplasmic, induces desaturases upon membrane fluidity loss, similar gene in insects</td>
<td>[40,43]</td>
</tr>
<tr>
<td>Hik33</td>
<td><em>Synechocystis</em> sp. (algae)</td>
<td>Membranal, induces desaturases upon membrane fluidity loss, similar gene in insects</td>
<td>[40,43]</td>
</tr>
<tr>
<td>cmr1</td>
<td><em>Rattus rattus</em> (rat)</td>
<td>Responsible for cold signaling in neurons, similar gene in insects</td>
<td>[37]</td>
</tr>
<tr>
<td>CIRP</td>
<td><em>Mus musculus</em> (mouse)</td>
<td>Upregulated during hypothermia, similar gene in insects</td>
<td>[96]</td>
</tr>
</tbody>
</table>

Table 1.1. Transcripts and proteins upregulated from cold treatments.
The role of the cytoskeleton for low-temperature survival has not been investigated in insects, although it is known that sHsps associate with the cytoskeleton in *Drosophila melanogaster* [87]. Preliminary results from cross-species microarrays (*S. crassipalpis* cDNA on *Drosophila* arrays) indicate changes in tubulin transcripts as a result of cold treatments, especially short-term acclimation (our unpublished data).

### 1.6.2 Insect genes upregulated by cold

A number of proteins and transcripts are upregulated as a result of low-temperature treatment in insects, although none of these genes have been the object of low-temperature studies at the functional level. Table 1 highlights many of the genes identified thus far. Included in the table are transcripts found in other model systems with similarity to known insect ESTs on GenBank.

### 1.6.3 Genome-wide studies in other organisms

Recent advances in functional genomics have provided valuable tools in the forms of cDNA macro- and microarrays. Through the use of this technology, thousands of genes can be screened for a particular treatment, even across species. Certainly, the field of insect cryobiology may benefit from the application of this technology to the various cold treatments and strategies, including recovery from cold shock, rapid cold hardening, seasonal acclimation, and freeze tolerance.

Microarray analyses of low-temperature treatment in other model systems have revealed a great variety of genes related to cold. *Arabidopsis thaliana*
exposed to 24 hours of 4°C increases expression of 40 transcription factors, 11 osmoprotective genes, 24 cellular metabolic genes, 20 carbohydrate metabolic genes, 4 heat shock genes, and 6 detoxification enzymes [97]. Similar results were obtained from rice treated in the same manner, although the number of genes tested was fewer [98]. Of note in these studies was the upregulation of beta tubulin in *Arabidopsis* and actin in rice, indicating changes in the cytoskeleton during cold acclimation. Glutathione-S-transferase was upregulated in *Arabidopsis* during cold acclimation, which was also noted on microarrays of freeze-injured yeast [99]. The only low-temperature transcriptome study in animals was performed on macroarrays of the channel catfish, *Ictalurus punctatus*, revealing considerable changes in ribosomal protein constituents and an increase in *hsp70*, calmodulin-inhibitor, and beta actin due to acclimation from 24°C to 12°C [100].

Our laboratory’s unpublished work on *S. crassipalpis* relies on cross-species hybridization with *Drosophila melanogaster* EST’s. Genes that appear to be upregulated in the flesh fly as a result of cold treatments include 11 defensive genes (including glutathione-S-tranfersase), 16 metabolic genes, 21 cytoskeletal genes (including both α- and β-tubulin), 36 genes related to transcription and translation (especially spliceosomal elements), and 13 signal transduction molecules (especially calcium-binding and proteins related to G-protein signalling). Although none of these genes have been confirmed by Northern blot
hybridization at present, it is clear that microarray studies such as this will enable
the field to make a quantum leap forward by allowing the identification of major
gene networks not previously known to be involved in temperature responses.

Acknowledgements

This study was funded in part by NSF grant IBM-9728573.
References


CHAPTER 2

Oleic acid is elevated in cell membranes during rapid cold-hardening and pupal diapause in the flesh fly, *Sarcophaga crassipalpis*.

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Abstract

The integrity of cellular membranes is critical to the survival of insects at low temperatures, thus an advantage is conferred to insects that can adjust their composition of membrane fatty acids (FAs). Such changes contribute to homeoviscous adaption, a process that allows cellular membranes to maintain a liquid-crystalline state at temperatures that are potentially low enough to cause the membrane to enter the gel state and thereby lose its ability to maintain homeostasis. Flesh flies (Sarcophaga crassipalpis) were subjected to two experimental conditions that elicit low temperature tolerance: rapid cold-hardening and diapause. FAs were isolated and analyzed using gas chromatography-mass spectrometry. FAs changed in response to both rapid cold-hardening and diapause. In response to rapid cold-hardening (8h at 4°C), the proportion of oleic acid (18:1n-9) in pharate adults increased from 30% to 47% of the total fatty acid pool. The proportion of almost every other fatty acid was reduced. By entering diapause, pupae experienced an even greater increase in oleic acid proportion, to 58% of the total FA pool. Oleic acid not only promotes membrane fluidity at low temperature but also allows the cell membrane to maintain a liquid crystalline state if temperatures increase.

Keywords: Homeoviscous adaptation; Phospholipid; Fatty acid; Cell membrane
2.1. Introduction

Preventing cellular damage due to low temperatures (cold shock) is a major challenge for insects. If the cold shock is of sufficient intensity, proteins denature and the cellular membrane ceases functioning, leading to loss of homeostasis. For pharate adults of the flesh fly, *Sarcophaga crassipalpis*, cold shock damage is achieved within an hour of exposure to -10°C and is evidenced by a failure in adult eclosion as a consequence of reduced muscular coordination and contraction (Yocum et al. 1994). Even if the adult emerges after a cold shock, there may be a significant loss in fecundity as well as a possible breakdown of muscular coordination and control (Rinehart et al. 2000).

*S. crassipalpis* possesses two known mechanisms by which cold shock can be prevented or attenuated: by entering into a cold-hardy pupal diapause and by rapid cold-hardening (RCH), a quick response that can occur at any stage of the life cycle. Diapause for *S. crassipalpis* is a photoperiod-induced developmental arrest that features physiological changes that contribute to low temperature survival such as long-term upregulation of heat shock proteins (Hayward et al. 2005) and glycerol (Lee et al. 1987a). The heat shock proteins function in preventing or repairing protein denaturation, and the glycerol serves to lower the organism’s supercooling point and provide for the stabilization of membranes and proteins (Chen et al. 1987b, Tsvetkova and Quinn 1994, Tang and Pikal 2005). The total effect of all these physiological changes allows diapausing flesh flies to achieve cold hardiness that increases low temperature survival far beyond the capacity of their non-diapausing counterparts (Chen et al. 1987a).
RCH is a cold-hardening mechanism that flesh flies and other insects possess throughout their lifespan, although the degree of protection imparted by RCH varies with life stage (Chen et al. 1987b). RCH for the flesh fly is induced at ambient temperatures between 0°C and 10°C at the cellular level (Yi and Lee 2004) and can increase the survival of pharate adults from less than 60 minutes to over 120 minutes following exposure to -10°C (Chen et al. 1987b). The time of induction is short, ranging from a mild increase in survival after 20 minutes of RCH induction to an achievement of maximum benefit at 24 hours (Chen and Denlinger 1992). Physiologically, the whole-body glycerol content of S. crassipalpis increases three-fold during RCH, ostensibly protecting the insect in the same manner as diapause-induced glycerol upregulation (Chen et al. 1987b). However, the upregulation of glycerol probably does not fully explain the protection imparted by RCH because the final glycerol concentration after induction remains far below that shown to protect proteins and membranes in vitro, and some other insects that possess RCH ability do not have any detectable upregulation of polyols (Kelty and Lee 2001).

As temperatures decrease, cellular membranes with a static composition tend to increase rigidity until regions of the membrane transition from a liquid crystal to gel state and the membrane loses its ability to function as a barrier (Cossins 1983). To counter this effect, the membrane may change in composition to maintain the liquid crystalline state at lower temperatures, a process known as homeoviscous adaptation (Sinensky 1974). The best evidence for homeoviscous adaption is determined directly by the measurement of membrane viscosity at
fluctuating temperatures, but it can also be inferred by membrane compositional changes (Hazel 1995). These changes occur at low temperatures by an increase in points of unsaturation along phospholipid fatty acid chains, but can also be brought about by changes in other membrane composition characteristics such as increased cholesterol content or a change in phospholipid class distribution (Thompson 1983, Hazel 1995).

The protection from low temperature that diapause and RCH imparts to the insect may be positively influenced by changes in membrane composition. Diapause-induced alteration in membrane phospholipids has been demonstrated for several insect species in which the diapause program also features cold hardiness (Furusawa et al. 1994, Hodkova et al. 1999, Kostal et al. 2003, Bashan and Cakmak 2005). A correlation between lack of membrane changes and cold-susceptibility was demonstrated by Kostal and Simek (1998) in their study of a summer-diapausing arctiid moth which did not display widespread diapause-induced fatty acid changes. Membrane compositional change is yet to be studied in an insect with a cold-hardy pupal diapause, such as S. crassipalpis, although Sarcophaga bullata, a closely-related species, has been demonstrated to increase cell membrane fluidity in response to rapid cold-hardening (Lee et al., 2006). The only insect to be investigated for membrane compositional change in response to RCH is Drosophila melanogaster (Overgaard et al. 2005). When the flies were chilled, survival after a subsequent cold shock was greatly increased, and the fatty acids underwent a significant but modest change. In the present study we investigate the role of phospholipid compositional changes in S. crassipalpis
during RCH of pharate adults and diapause by chromatographic analysis of fatty acid constituents of phospholipids and by thin layer chromatography of phospholipid classes.

2.2. Materials and Methods

2.2.1. Insect rearing

The colony of Sarcophaga crassipalpis was maintained in the laboratory as described (Denlinger 1972). Flies used for rapid cold-hardening studies were kept in non-diapausing conditions (15L:9D photoperiod, 25°C) and allowed to develop into red-eye stage pharate adults before the experimental treatment. Parents of diapausing flies were kept at a photoperiod of 12L:12D and a temperature of 25°C until larviposition, and their offspring were reared at 20°C under the same photoperiod. Experiments used pupae that had been in diapause for 30 days. Non-diapausing pupae used as controls for the diapause experiments were maintained under long-day conditions (15L:9D) at 20°C and allowed to develop to the phanerocephalic stage, the developmental stage comparable to diapause.

2.2.2. Rapid cold-hardening

Approximately 120 female pharate adult flies were placed in an open petri dish in a 4°C walk-in cold room and five female flies (each ~100 mg) were removed from the petri dish after 0, 1, 2, 4, and 8 hrs and homogenized individually for lipid extraction. The puparium was not included in the lipid extraction.
2.2.3. Diapause

Groups of five pupae that had been in diapause for 30 days were homogenized for lipid extraction at room temperature (~22°C). Groups of five non-diapausing flies in the phanerocephalic stage of development were extracted in the same manner. To investigate the response of diapausing flies to further chilling, a group of five flies that had been in diapause for 30 days were placed at 4°C and sampled 8 hrs later.

2.2.4 Fatty acid methyl esters

Whole body lipids were extracted in two phases by a modified Bligh and Dyer (1959) procedure. To improve yield and encourage efficient phase separation in a small volume (approximately 5ml), a vacuum filtration step was added immediately after two phases were formed, and the emulsion was allowed to sit for 15 min before the bottom, organic layer was removed. The chloroform extract containing the total lipid sample was evaporated to dryness by a gentle stream of nitrogen gas. The dry sample was resuspended in chloroform and subjected to solid phase extraction (800 mg silicic acid in the column) with 10 ml chloroform followed by 15 ml of 9:1 acetone:methanol. These eluates were discarded. Finally, the phospholipids were eluted with 10 ml of methanol and the eluate was evaporated to dryness under nitrogen. To remove the fatty acids from their phospholipid backbone and improve their chromatographic behavior, the phospholipids were transesterified in methanolic KOH. A 0.5 ml 1:1 methanol:toluene resuspension of the total sample of phospholipids was added to a capped test tube containing 0.5 ml 0.2 N KOH in methanol and heated to 40°C in a
water bath for 30 min. The solution was cooled to room temperature for 30 minutes and neutralized with 0.5 ml 0.2N acetic acid in molecular grade H₂O. This transesterification process was determined to produce fatty acid methyl esters from phospholipids as determined by GC/MS (data not shown). The volume of the solution was increased with an additional 2 ml of chloroform and 2 ml of H₂O, and the organic layer was subsequently removed and evaporated to dryness under a nitrogen stream. The sample was then dissolved in 200μl of hexane containing a 1ng/μl heptadecanoic acid methyl ester (17:0, not found in S. crassipalpis, as determined by preliminary results) internal standard. The sample was placed in an autosampler vial, capped under a nitrogen stream, and stored at -20°C until GC-MS analysis.

2.2.5 Gas chromatography – mass spectrometry

Hexane samples containing fatty acid methyl esters were loaded into a Finnigan Trace GC/MS and 1 μl of each sample was subjected to chromatographic analysis. The oven temperature ramped from 70 to 300°C and the temperature increment was 8°C/min, which produced a chromatograph of sufficient resolution to separate the peaks of all fatty acids observed. The column was a Restek 30 m fused silica column (I.D. 25 mm, 95% dimethyl siloxane, 5% diphenyl) with helium gas used as a carrier at a rate of 50 ml/min. After each sample was run, the oven remained at 300°C for 7 minutes to clean impurities from the column.
2.2.6 Thin-layer chromatography

To determine whether there were changes in phospholipid class, non-transesterified replicates of all samples included in the diapause GC experiment were also analyzed by thin-layer chromatography. Phospholipids were run on a boric acid-impregnated TLC plate for 2 hours with 30:35:7:35 chloroform:ethanol:water:triethylamine as described by LeRay et al. (1987) and visualized under UV light with 1% primuline. Spot areas were carefully calculated and converted into a percentage of total spot size per sample. As determined by Rf comparison with standards, only phosphatidylcholines (PC) and phosphatidylethanolamines (PE) were resolved above trace levels in samples of individual flesh flies, therefore only these two signal percentages are reported.

2.2.7 Data analysis and statistics

Identification of each fatty acid by GC-MS was attained by comparing the spectrum of each peak with multiple chemical libraries (Xcalibur software, Thermo). Fatty acid peaks identified with an SI value greater than 800 were considered identified with a reasonable degree of confidence and assigned a designation of X:Yn-Z where X represents the total number of carbons in the fatty acid chain, Y represents the number of double bonds, and Z represents the fatty acid series where (n-Z) is the name of the fatty acid series. In addition, methyl branched fatty acids were given the additional designation of (m). The peak areas were converted to percentages of total content of fatty acids within the same chromatogram (relative proportions) for each fatty acid detected and the arcsin transformations of these values were analyzed by a one-way ANOVA followed by
a Tukey-Kramer mean comparison test (Graphpad, Inc.) with a 95% confidence interval. Quantification was achieved through the use of external as well as internal fatty acid standards. The index of unsaturation was calculated as a ratio of the total proportion of unsaturated fatty acids over the total proportion of all saturated fatty acids. Means for each fatty acid series (n-3, n-6, etc.) are the sums of all unsaturated fatty acids that belong to the given series. For thin-layer chromatography, arcsin transformations of the mean percentages of PC and PE signal per sample were subjected to a one-way ANOVA with a Tukey-Kramer post-test.

2.3. Results

2.3.1 Fatty acid composition

All chromatographs of phospholipid fatty acids from *S. crassipalpis* were dominated by palmitic acid (16:0) and the three unsaturates, palmitoleic acid (16:1n-7), oleic (18:1n-9), and linoleic acid (18:2n-6). Together, these four fatty acids represented >80% of the total phospholipid fatty acids in non-diapauing flies raised at 25°C. Peaks that were of lesser abundance, but above trace level (2-5%) include arachidonic acid (20:4n-6), stearic acid (18:0), and eicosapentaenoic acid (20:5n-3). Fatty acids found in trace amounts across nearly all samples were 14:0, 14:0 (m, methyl branched), 15:0, 16:0 (m), 16:1n-9, 18:3n-3, 18:3n-6, 20:0, 20:2n-6, and 20:3n-9.
2.3.2 Rapid cold-hardening

When red-eye pharate adults of *S. crassipalpis* were exposed to the RCH-inducing condition of 4°C for 0-8 hours, oleic acid (18:1n-9) levels in the phospholipid pool increased moderately by two hours and rose dramatically from 4-8 hrs (Fig. 1). After 8 hours at 4°C, the total proportion of oleic acid changed from 30% to 47%, a highly-significant increase (Tukey’s t-test, n=5, p<0.0001). The most substantial decrease occurred in the proportion of palmitoleic (16:1n-7) acid, which dropped from 13% to 4% after 8 hours of exposure to RCH-inducing conditions, especially after the 2 hour time point (Fig. 1). Palmitic acid (16:0) and linoleic acid (18:2n-6) proportions showed no significant change. All other detectable fatty acids were reduced or were found in levels too close to the GC-MS detection limits for confident analysis (Fig. 1B-D).

Overall, the large increase in oleic acid levels due to RCH shifted the entire phospholipid fatty acid pool to favor the n-9 (Tukey Kramer t-test, n=5, p<0.0001) series at the expense of the n-7 and n-6 series (Fig. 2A). Oleic acid, which has its single double bond in the middle of the length of the fatty acyl chain, is the primary constituent of the n-9 series in *S. crassipalpis*. The index of unsaturation was not affected by exposure to 4°C for 8 h (Fig. 2B), although a slight decrease was evident after 4 h (Tukey Kramer, n=5, p<0.05).

2.3.3 Diapause

The induction of pupal diapause caused oleic acid (18:1n-9) proportions within the phospholipid fatty acid pool to significantly increase from 43% in non-diapausing pupae to 58% in diapausing pupae (Tukey Kramer t-test, n=5,
p<0.0001) (Fig. 3A). Palmitic acid (16:0) and linoleic acid (18:2n-6) proportions were both reduced in diapause (Tukey-Kramer t-test, n=5, p<0.01). The proportion of palmitoleic acid (16:1n-7) was not significantly changed. Three fatty acids (18:3n-6, 20:4n-6, and 20:5n-3) were easily detectable above trace levels in non-diapause flies, but their proportions were substantially reduced in diapause (Fig. 3B). Other low-abundance fatty acids (<1%) had significant changes, but these peaks are too close to background levels (< 5X avg. noise) to draw conclusions with confidence.

Like rapid cold-hardening, diapause increased the proportion of n-9 (Tukey Kramer t-test, n=5, p<0.01) fatty acids in the whole-body phospholipid pool at the expense of n-6 and n-7 fatty acids (Tukey Kramer t-test, n=5, p<0.05) (Fig. 4). Phospholipid fatty acid change due to diapause additionally reduced the n-3 pool of fatty acids, but this was not the case for RCH. The ratio of unsaturated fatty acids to saturated fatty acids in the whole-body phospholipid pool of S. crassipalpis was considerably increased from 1.8 to 2.7 due to diapause (Tukey Kramer t-test, n=5, p<0.01) (Fig 5).

Chilling diapausing pupae at 4°C for 8 hrs did not increase the levels of oleic acid over that seen for diapause alone. In fact, the amount of oleic acid recorded was lower, but this difference was not significant. The only notable change in major fatty acids seen in response to chilling diapausing pupae was a rise in palmitoleic acid proportions (16:1n-7), however proportions of palmitoleic acid in chilled, diapausing pupae were not significantly different from levels observed in non-diapausing pupae.
2.3.4 *Shift in phospholipid head groups*

The data indicated an increase in the proportion of PE at the expense of PC proportion for both diapause and chilling at 4°C for 8 hrs (RCH). This was most evident when the PE proportions of nondiapausing *S. crassipalpis* pharate adults (44%) were compared with diapausing pupae that had been chilled at 4°C for 8 hrs (Tukey-Kramer, n=5, p<0.05) (Fig. 6). Diapause increased the proportion of PE (65%) to a higher level than that observed for RCH (Tukey-Kramer, n=5, p<0.05). Chilling a diapausing pupa at 4°C for 8 hours neither elicited a significant increase in PE beyond that seen for diapause (69%), nor did it result in a change in the index of unsaturation.

2.4. **Discussion**

2.4.1 *Fatty acid composition of cell membranes in Sarcophaga crassipalpis*

The fatty acid components of phospholipids in the cell membranes of *S. crassipalpis* were similar to those in other Diptera, but the proportion of palmitoleic acid (16:1n-7) was slightly lower (Downer 1985). On the other hand, palmitic acid (16:0) proportions were higher than reported for other Diptera. The 20-carbon fatty acids, particularly arachidonic acid (20:4n6) were found in amounts that are markedly greater than the trace levels seen in most terrestrial insects (Ogg and Stanley-Samuelson 1992), possibly a consequence of this fly’s lipid-rich diet of beef liver. Nonetheless, 20-carbon fatty acids did not exceed 13% of the phospholipid fatty acid pool.
2.4.2 Rapid cold-hardening

Our experiments provide strong evidence for restructuring of cell membranes during RCH in the flesh fly. Fatty acid changes were detectable within 2 hrs of exposure to 4°C for some fatty acid species and were statistically significant for practically all detectable fatty acids after 8 hrs. Within 8 hours at 4°C, oleic acid dramatically increased from 30% to 47% of the total phospholipid fatty acid pool, and it did so at the expense of practically all other fatty acids, with the notable exception of 16:0, which remained at the same proportion. This is a change in magnitude one order higher than the linoleic acid (18:2n-6) increase noted during RCH in *Drosophila melanogaster* (Overgaard et al. 2005), the only other study of fatty acid changes during RCH. *S. crassipalpis* is considerably more cold-hardy than *D. melanogaster*, which may account for the greater response noted in the flesh fly. Neither of these two investigations of RCH in flies produced a significant increase in the ratio of unsaturated fatty acids to saturated fatty acids. A few other studies of insect membrane compositional change also report a response without an increase in the unsaturated to saturated fatty acid ratio (Ohtsu et al. 1998, Hodkova et al. 1999). The main reason that RCH fails to increase the unsaturated to saturated fatty acid ratio in *S. crassipalpis* is due to the concomitant (albeit slight) rise in palmitic acid (16:0) levels. It should be noted that the 17% oleic acid increase observed in this study would cause the proportions of other fatty acids to be reduced markedly as an artifact of
the use of proportional data. The slight upregulation of a single fatty acid species against such a drastic change could be masked or appear as no change at all while the rest of the fatty acid species appear to be reduced.

In addition to the boost in oleic acid we noted in association with RCH, we also observed a restructuring of membrane phospholipid head groups to favor PE over PC. This finding is consistent with observations that have been made in many organisms that have been investigated for homeoviscous adaptation (Hazel 1995, Hodkova 1999, McCoy 2003). The headgroup restructuring that favors PE serves as an “emergency response” that reduces torsional stress on the outer hemilayer of the membrane as temperatures drop (Thompson 1983). The inverted wedge shape of PE makes this possible by limiting membrane phase transitions by curtailing lateral packing of the phospholipid molecules as temperatures drop (Weislander 1980).

Glycerol upregulation is also a feature of RCH in S. crassipalpis and follows the same chronological scale as fatty acid changes seen here (Lee et al. 1987b). Glycerol may be acting in concert with changes in phospholipid composition to ensure membrane stability at lower temperatures. The direct membrane changes (fatty acids, phospholipid class, etc.) help to maintain proper barrier chemical characteristics while the glycerol serves to stabilize membrane proteins and prevent ice formation by competing with water for hydrogen bonding with the membrane (Jensen 2001). This is in addition to other protective effects of glycerol that are not associated with cell membranes.
2.4.3 Diapause

Unlike the RCH response that is observed in nondiapausing individuals, diapause is a seasonal arrest in development programmed well in advance of the onset of diapause. *S. crassipalpis* overwinters in a cold-hardy pupal diapause brought about by short-day photoperiodic signaling early in embryonic and larval development (Denlinger 1971). Cold hardiness is a component of the diapause program in this species, and diapausing pupae readily survive temperatures as low as -17°C for many weeks (Lee and Denlinger 1985), whereas nondiapausing pupae can only survive 1-2 hrs at -10°C (Lee et al. 1987a). The increased cold tolerance during diapause in *S. crassipalpis* has been previously correlated with the expression of heat shock proteins (Flannagan et al. 1998) and glycerol production (Lee et al. 1987b). Even though glycerol can stabilize cell membranes (Tsvetkova and Quinn 1994), the level of protection conferred to *S. crassipalpis* from upregulation of glycerol to <0.1 M has not been demonstrated to protect membranes directly.

From the present investigation, it is clear that membrane phospholipid changes are an important component of the diapause program in *S. crassipalpis* and may act in concert with heat shock protein and glycerol upregulation to confer cold hardiness. Phospholipid alterations have been associated with diapause in a number of other insects as well (Kostal and Simek 1998, Hodkova et al. 1999, Kostal et al. 2003, Bashan and Cakmak 2005, Tang and Pikal 2005), but this is the first report for an
insect with a cold hardy, pupal diapause. In this study we also demonstrate clearly that the change in membrane fatty acids is a component of the diapause program rather than the effect of low temperatures.

As observed for RCH, oleic acid is the primary fatty acid upregulated during diapause in *S. crassipalpis*, and the order of the change is roughly the same as noted for RCH (10-20% increase). However, diapause-induced phospholipid alterations in the flesh fly differ from RCH in that palmitic acid (16:0) is downregulated while palmitoleic acid (16:1n-7) levels remain unchanged (16:0 was unchanged in RCH, while 16:1n-7 was downregulated). The effect of this difference is enough to increase the unsaturated to saturated fatty acid ratio of diapausing flies from 1.8 to a significant 2.8. Our preliminary analysis of phospholipid headgroup distribution shows that PE was favored over PC in amounts that were significantly greater than the proportional change measured in *S. crassipalpis* during RCH. Diapausing pupae of the flesh fly are far more cold hardy than nondiapausing flies that have been rapid cold-hardened, thus it is perhaps not surprising that phospholipid change was more pronounced during diapause.

Chilling diapausing pupae for 8 hours at 4°C had very little overall effect on the fatty acid proportions of phospholipids beyond that already observed for diapause, however, there was an even greater shift to palmitoleic acid, but this time the change occurred at the expense of oleic acid. Shorter-chain fatty acids are thought to contribute more to membrane fluidity than longer chain fatty acids, thus this
subtle shift could be further enhancing the ability of the membrane to handle low temperature (Thompson 1983). Chilling diapausing pupae for 8h at 4°C did not significantly alter the proportions of PE and PC.

2.4.4 Oleic acid vs. linoleic acid

The primary fatty acid that increased during RCH and diapause in *S. crassipalpis* was oleic acid (18:1n-9), a monoene of the n-9 series. There are several possible reasons why it may be beneficial to upregulate this particular fatty acid in response to or in preparation for low temperatures. The double-bond in the acyl chain of oleic acid is located in the center of the chain, which is thought to be the best location for a single double-bond to maximize lateral displacement of the end of the chain and contribute to overall membrane disorder, i.e. fluidity (Barton and Gunstone 1975). The primary monoene upregulated in *Drosophila* cold selection experiments was palmitoleic acid (16:1n-7), which, like oleic acid, has its single double-bond in the center of the acyl chain (Overgaard et al. 2005). In this study, diapausing pupae of *S. crassipalpis* did not change the proportion of palmitoleic acid, but the remaining fatty acids were reduced in an environment of massive oleic acid increase. The effect of increased oleic acid would be maximized if the fatty acid were to be located at the sn-2 position of the glycerol backbone of the molecule. The fatty acid at the sn-2 position has a greater effect on membrane fluidity than a fatty acid located at the sn-1 position because the fatty acid at the sn-2 position is thrust deeper into the hydrophobic portion of the membrane by virtue of the angle of the glycerol molecule in relation to the plane of the membrane. Indeed, Kostal et al. (2003) found that the sn-2 position of cold-
changed phospholipids in the drosophilid, *Chymomyza costata*, had an unsaturated 18 carbon fatty acid in the sn-2 position and an unsaturated molecule at the sn-1 position. The present study does not address fatty acid location, but such information would be useful for further study of the role of low-temperature membrane restructuring in insects.

Many studies that examine changes in phospholipids due to cold acclimation or diapause in insects report that linoleic acid (18:2n-6) is the fatty acid that is increased for winter climates (Hodkova et al. 1999, Kostal and Simek 2003, Overgaard et al. 2005). Only 14 of 40 insects investigated possess Δ\(^{12}\) desaturase, the enzyme needed for adding a double bond to oleic acid to manufacture linoleic acid (Cripps et al. 1986). Insects that do not have a Δ\(^{12}\) desaturase gene or cannot regulate its expression may utilize the Δ\(^9\) desaturase instead to initiate membrane fatty acid changes. Both desaturases have been shown to be transcribed and activated at low temperatures (Wodtke and Cossins 1991, Macartney et al. 1994, Batcabe et al. 2000, Hseih and Kuo 2005). A measurement of the expression and activity levels of these desaturase genes may provide a clear reason why low-temperature phospholipid changes in certain insects favors one fatty acid over another.

Oleic acid and linoleic acid share many biological properties, including the ability to provide a wide temperature window for growth, as demonstrated for bacteria (McElhaney 1974). Oleic acid provides the best environment for critical membrane proteins such as membrane ATPases. This enzyme functions at optimum levels when oleic acid is present in the cell membrane (Starling et al.
1993), thus an increase in oleic acid in response to or in preparation of low-temperature may provide proper fluidity of the membrane without sacrificing the delicate balance needed to keep sensitive membrane proteins from maintaining optimum function. *Eurosta solidaginis* upregulates oleic acid when it acquires freezing tolerance (Bennett et al. 1997), and two diapausin hemipterans were also found to increase oleic acid levels during diapause (Bashan and Cakmak 2005).

Oleic acid is energetically more favorable to manufacture than linoleic acid (one less double bond), thus insects that upregulate oleic acid rather than linoleic acid in preparation for low temperatures may be preserving finite energy reserves while still gaining the benefit of a wide window of fluidity. For a temperate insect entering diapause, such as *S. crassipalpis*, oleic acid may be important for unexpected warm temperatures during the winter because the insect does not need to continuously readjust its membrane fatty acids to maintain fluidity.

**Acknowledgements**

This work was supported in part by NSF Grant 10B-0416720 and by OARDC and OSU Presidential Fellowships to M.R.M. Special thanks to Richard Sessler in the Ohio State Chemical Instruments Center for advice and guidance with the analysis of fatty acid samples. Additional thanks to Amelia Brown and Jonathan Ho for laboratory assistance and to the reviewers of this article for their thoughtful comments and suggestions.
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Figure 2.1. Change in individual fatty acids (FA) as a result of chilling is consistent with the induction of rapid cold-hardening. *Sarcophaga crassipalpis* pupae were subjected to 4°C for a period of 0-8 hours and their fatty acids (as methyl esters) were analyzed with GC-MS. Each data point represents the mean ± standard error (n=5). It is clear from the above data that oleic acid (18:1n9) is dramatically increased (17.6% total increase) between 4 and 8 hours at the expense of many other fatty acids (one-way ANOVA with Tukey’s post-test).
Figure 2.2. Change in fatty acid series (A) and index of unsaturation (B) of flesh fly phospholipids during conditions that induce rapid cold-hardening. Fatty acids were isolated from non-diapausing Sarcophaga crassipalpis pupae exposed to 4°C for 0-8 hours. Bars represent mean ± standard error (n=5). Letters above the bars represent statistical groupings compared with their respective 0 hour controls (Tukey’s t-test). Bars in (A) without a letter are not statistically different than their 0 hr counterpart. From these data, it is clear that fatty acids of the n-9 series are favored during chilling at the expense of n-7 and n-6 fatty acids, but the index of unsaturation does not consistently trend in the same direction.
Figure 2.3. Change in major (A) and minor (B) fatty acid (FA) proportions due to diapause and chilling in the flesh fly, *Sarcophaga crassiplapis*. Fatty acids (as methyl esters) from non-diapausing pupae (ND) were compared with 30 day diapausing pupae (D) and 30 day diapausing pupae that had been chilled at 4°C for 8 hours (D+C). Bars represent means ± standard error (n=5). Significance for each fatty acid peak was determined by one-way ANOVA, and significant peaks were assigned statistical groupings (a,b,c) by Tukey’s post-ANOVA t-tests.
Figure 2.4. Change in proportions of fatty acids (FA) from each series in diapausing and chilled flesh flies. *Sarcophaga crassipalpis* pupae in a non-diapause state (ND) were compared with 30 day diapausing pupae (D) and 30 day diapausing pupae that had been chilled at 4°C for 8 hours (D+C). Significance for each fatty acid series was determined through a one-way ANOVA, and significant peaks (mean ± standard error, n=5) were assigned statistical groupings (a,b,c) by Tukey’s post-ANOVA t-tests.
Figure 2.5. Change in overall phospholipid fatty acid saturation due to diapause and chilling in the flesh fly, *Sarcophaga crassipalpis*. Fatty acids (as methyl esters) from non-diapausing pupae (ND) were compared with 30 day diapausing pupae (D) and 30 day diapausing pupae that had been chilled at 4°C for 8 hours (D+C). Bars represent mean ± standard error (n=5). T-tests reveal the marked rise in index of unsaturation due to diapause, but chilling had no additional effect beyond diapause-induced fatty acid unsaturation.
Figure 2.6. Change in phospholipid class due to rapid cold-hardening and diapause in the flesh fly, *Sarcophaga crassipalpis*. Phosphatidylethanolamines (PE) are upregulated at the expense of phosphatidycholines (PC) due to rapid cold hardening (p<0.05) and diapause (p<0.01), but chilling a fly in diapause does not enhance this response beyond that induced by diapause alone. Letters above the bars (mean ± standard error, n=5) represent statistical groupings (Tukey’s post-ANOVA t-test).
CHAPTER 3

Shifts in the carbohydrate, polyol, and amino acid pools during rapid cold-hardening and diapause-associated cold hardening in flesh flies (*Sarcophaga crassipalpis*): a metabolomic comparison.

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**Abbreviations**: ANOVA (analysis of variance); GC-MS (gas chromatography–mass spectrometry); h (hour); NMR (nuclear magnetic resonance); PCA (Principal component analysis).
Abstract

Flesh flies can enhance their cold hardiness by entering a photoperiod-induced pupal diapause or by a temperature-induced rapid cold-hardening process. To determine whether the same or different metabolites are involved in these two responses, derivatized polar extracts from flesh flies subjected to these treatments were examined using gas chromatography-mass spectrophotometry. This metabolomic approach demonstrated that levels of metabolites involved in glycolysis (glycerol, glucose, alanine, pyruvate) were elevated by both treatments. Metabolites elevated uniquely in response to rapid cold-hardening include glutamine, cystathionine, sorbitol, and urea while levels of beta-alanine, ornithine, trehalose, and mannose levels were reduced. Rapid cold-hardening also uniquely perturbed the urea cycle. In addition to the elevated metabolites shared with rapid cold-hardening, leucine concentrations were elevated during diapause while levels of a number of other amino acids were reduced. Pools of two aerobic metabolic intermediates, fumarate and citrate, were reduced during diapause, indicating a reduction of Krebs cycle activity. Principle component analysis demonstrated that rapid cold-hardening and diapause are metabolically distinct from their untreated, nondiapausing counterparts. We discuss the possible contribution of each altered metabolite in enhancing the overall cold hardiness of the organism, as well as the efficacy of GC-MS metabolomics for investigating insect physiological systems.

Keywords: insect, diapause, amino acid, polyol, metabolism
3.1 Introduction

Low temperature poses one of the greatest challenges to insect survival, and consequently, insects have developed a range of behavioral and physiological mechanisms for preventing low temperature injury. For the flesh fly, *Sarcophaga crassipalpis*, low temperature injury can be countered by two distinctly different physiological mechanisms, rapid cold-hardening and a photoperiod-induced overwintering pupal diapause.

Rapid cold-hardening, as the name implies, is a quick physiological response (minutes to hours) that allows insects to survive exposure to low temperatures that would normally cause mortality in the absence of rapid cold-hardening (Lee et al. 1987a). For the flesh fly, rapid cold-hardening is achieved within a temperature window of 0 to 11°C, either through normal daily thermocycles or in the laboratory, and is evidenced by a 2-3 fold elevation of glycerol and a dramatic enhancement of survival at -10°C (Chen et al. 1987, Lee et al. 1987a). The effects of rapid cold-hardening can be seen after exposing the fly to 0°C for only 20 min, but maximal benefit is imparted after 8 h (Chen et al. 1991).

Even though glycerol elevation in flesh flies has been strongly correlated with survival in rapid cold-hardening, increased survival is not likely to be explained by the result of glycerol alone. The millimolar titres of glycerol in the hemolymph of *S. crassipalpis* cannot affect the melting point of the whole insect beyond 1°C colligatively (Lee at al. 1987b), and the non-colligative effects of
glycerol on protein and membrane stabilization require higher concentrations of glycerol, as well (Tsvetkova and Quinn 1994, Tang and Pikal 2005). It thus appears likely that rapid cold-hardening exploits more than one mechanism. Supporting evidence for additional players involved in rapid cold-hardening is provided by the recent finding that rapid cold-hardening alters biological membrane structure in flies (Overgaard et al. 2005, Michaud and Denlinger 2006).

Diapause in *S. crassiplapis* is a programmed developmental arrest at the phanerocephalic stage of pupal development, brought about by short daylengths and low temperatures (Denlinger et al. 1972). As with rapid cold-hardening, glycerol levels increase during flesh fly diapause (Lee at al. 1987b), and heat shock proteins are elevated (Flannagan et al. 1998, Hayward et al. 2000). A diapausing fly pupa can survive exposures of -17°C for days and -10°C for weeks at a time, whereas a non-diapausing flesh fly, at best, can tolerate these temperatures for only minutes or hours (Lee and Denlinger, 1985).

As a chemical chaperone, glycerol may act synergistically with the heat shock proteins to generate protection (Diamant et al. 2001, Chattopadhyay et al. 2004). The slow metabolic rate of diapausing flies may also increase survival by reducing the need for cellular energy and metabolic efficiency in an environment where enzymes may be damaged by low temperature and need repair. It is not known whether metabolites other than glycerol accumulate during diapause in flesh flies, but discovery of such metabolites would further our understanding of the metabolic events associated with diapause and highlight additional substances that could potentially enhance cold hardiness.
Metabolomics, along with metabolic profiling and metabonomics, is an emerging technology for quantitatively sampling the entire suite of detectable metabolites in a biological system to thus provide a holistic view of physiological processes and conditions (Dunn and Ellis 2005, Weckworth and Morganthal 2005, Kopka 2006). The advantage of metabolomics for analyzing changes in a biological context is the unbiased nature of the analysis; all detectable metabolites are quantified to eliminate \textit{a priori} determination of analytical targets. Also, metabolomics is the only field of “–omics” that addresses the terminal target of the central dogma of molecular biology, the substrate. The limitations to this technology, which chiefly employs detection of metabolites through gas chromatography-mass spectroscopy (GC-MS) or nuclear magnetic resonance (NMR), is the lower limit of detection and the ability to properly identify detected metabolites (Kopka 2006).

Metabolomic techniques have been applied to physiological studies of plants (Weckworth et al. 2004), clinical screening of humans (Zytkovicz et al. 2001), and the detailed study of metabolism in bacteria (Buchholz et al. 2002). In spite of its limitations, metabolomics promises to provide useful insights for insect physiology, especially if coupled with information derived from other “–omics” studies. We are aware of only one published NMR-based metabolomic study on an insect system (Malmendal et al. 2006). In this study, we use GC-MS metabolomic techniques to identify metabolites that are altered by rapid cold-hardening and the overwintering pupal diapause in \textit{S. crassipalpis}. Our goal is to
identify a more complete spectrum of metabolites elevated by cold hardening and to determine if the same metabolites are elevated in these two forms of cold-hardening.

3.2. Materials and methods

3.2.1 Insect rearing

Colonies of *Sarcophaga crassipalpis* were maintained in the laboratory as described (Denlinger 1972). Flies used for rapid cold-hardening studies were reared in non-diapause conditions (15L:9D photoperiod, 25°C) and allowed to develop into red-eye stage pharate adults before being used for experiments. The maximal low temperature survival effect from rapid cold-hardening is observed during the red-eye stage. Parents of diapausing pupae were kept at a photoperiod of 12L:12D and a temperature of 25°C until larviposition, and the offspring were reared at 20°C under the same photoperiod. Experiments used pupae that had been in diapause for 30 days (mid-diapause). Non-diapausing pupae used as controls for the diapause experiments were maintained under long-day conditions (15L:9D) at 20°C and sampled at the phanerocephalic stage, the developmental stage equivalent to diapause.

3.2.2. Sampling

For rapid cold-hardening, female pharate adult flies were placed in an open petri dish and transferred from 25°C to 4°C. After 8 h, n=6 groups of three female flies were homogenized for 1 min in a 3.8 ml cold chloroform:methanol:water (1:2:0.8) mixture using a hand-operated glass homogenizer. The puparium was
not included in the homogenization. Each fly used in the experiment weighed approximately 85-100 mg, which is a robust but common size for S. crassipalpis. Six groups of three flies held continuously at 25°C were sampled as untreated controls. For the diapause experiment, six groups of three pupae that had been in diapause for 30 days at 20°C were homogenized. Six groups of three non-diapausing, phanerocephalic pupae were extracted as untreated controls.

3.2.3. Separation and derivatization

One ml of dH$_2$O containing 25 µg xylose (internal standard not normally detected in S. crassipalpis) and 1 ml chloroform were added to each sample, and the sample was vortexed for 30 sec. The resultant two-phase mixture was allowed to sit for 30 min and then vacuum-filtered through a glass filter to remove particulate matter and precipitates. The filter was washed with 1 ml chloroform:methanol:water (2:1:0.8) to remove soluble trace metabolites from the filter matrix. After filtration, the two-phase mixture was separated into an aqueous phase containing polar metabolites (sugars, polyols, amino acids) and an organic phase containing non-polar metabolites (lipids). The organic phase was discarded because identified compounds in the organic phase represented only 10% of the total number of observed peaks, and many peaks could not be adequately separated.

The aqueous phase was then placed under a gentle nitrogen stream and evaporated at 22°C-25°C to dryness. The dried sample was then immediately methoxyaminated by adding 1.0 ml methoxyamine hydrochloride in pyridine, incubated for 1 hr at 60 °C (turns clear as metabolites dissolve), followed by a 16
h incubation at room temperature. The sample was then trimethylsilylated by the addition of 300 µl MSTFA and incubated at 50°C for 30 min. 150 µl of the trimethylsilylated sample was added to a glass insert within an auto-sampler vial and analyzed with GC-MS. Aqueous samples processed in this manner produced distinctive and repeatable chromatographic peaks that differed across samples by 5 to 60%, within the expected range for this type of experiment.

3.2.4. Chromatographic analysis

One microliter of each sample was anto-injected into a Finnigan-Trace GC-MS and subjected to chromatographic analysis (50-450 m/z). The injector temperature was held at 280°C. The oven temperature ranged from 50 to 300°C, and the temperature increment was 5°C min⁻¹, which produced a chromatograph of sufficient resolution to separate ~175 chromatographic peaks per sample. The column was a Restek 30 m fused silica column (I.D. 25 mm, 95% dimethyl siloxane, 5% diphenyl) with helium gas used as a carrier at a rate of 50 ml/min. Samples were run using splitless mode. After each sample was run, the oven remained at 300°C for 10 min to clean impurities from the column.

3.2.5. Quantification and data analysis

Individual integrated peak areas were converted to response ratios in relation to the internal standard (xylulose) by dividing the peak area of the metabolite by the peak area of the internal standard. One-way ANOVA with an acceptable significance level of p ≤ 0.01 was performed on each metabolite to determine which individual metabolites were affected by rapid cold-hardening or diapause with respect to their controls. Peak identities were determined where
possible by comparison of retention times with amino acid, sugar, and polyol standards prepared in the same manner as the samples. Matches of spectral peaks with the National Institute of Standards and Technologies (NIST) and Wiley Chemical Structural libraries further confirmed or established metabolite identities provided the match carried an RSI value $\geq$700. For principal component analysis (PCA), peak areas of metabolites (or combined peak areas of metabolites with more than one peak) were normalized by dividing each peak area value by the mean peak area for that compound, and the resultant normalized data were entered into MetaGeneAlyse 1.7 (Max Planck Institute of Molecular Plant Physiology, Berlin, Germany) and separated by their principal components.

3.3. Results

3.3.1. General observations

Separation and derivativization of polar metabolites from pupae and pharate adults of *S. crassipalpis* yielded peaks that were distinct and generally consistent across samples. A representative portion of a chromatograph is seen in Fig. 1. Within any treatment group, peak height relative to the internal control did not vary more than 40% for any peak whose area was $>10X$ background. Across all samples, an average of 62 of 159 peaks could be identified as metabolites with a reasonable amount of certainty, a value typical for GC-MS metabolomics (Shauer et al. 2005). The first half of the chromatograph contained amino acids, phosphate, and urea while the last half of the chromatograph contained mostly carbohydrates and polyols. This pattern is similar to that of other metabolomic
studies utilizing GC-MS (Bino et al. 2004). Metabolic intermediates of the glycolytic and respiratory chain were found throughout the chromatograph. A list of identified metabolites recorded from *S. crassipalpis* is enumerated in Table 1. Of these 62 metabolites, 14 were amino acids, 14 were sugars or polyols, 8 were metabolic intermediates, 4 were small molecules, and an additional 22 representing a collection of diverse metabolites.

3.3.2. Rapid cold-hardening

When the response index of peaks from red-eye pharate adults of *S. crassipalpis* exposed to 4°C for 8 h (rapid cold-hardening) was compared with the response index of flies held continuously at 25°C (ANOVA, df=10, p≤0.01), a number of altered metabolite concentrations were identified (Fig. 2). Seven of the identified metabolites were elevated in response to rapid-cold hardening and four were reduced. Glycerol, the only polyol previously known to be accumulated during flesh fly rapid cold-hardening, increased 2-3 fold (p=0.0038). Another polyol, sorbitol, increased 1.6 fold (p=0.0032). Among the amino acids, alanine and glutamine increased nearly 2 fold (each p<0.001), while levels of β-alanine and ornithine dropped to half of the control value (p=0.01 and p≤0.001, respectively). Other metabolites upregulated were cystathionine (p=0.005), glucose (p=0.0018), pyruvate (p=0.014), and urea (p=0.0012). Mannose (p=0.0011) and trehalose levels were reduced (p=0.0017). 15 unidentified metabolites also changed in response to rapid cold-hardening (9 increased, 6 decreased).

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Multivariate PCA analysis of the normalized raw peak areas in a metabolomic experiment gives a measure of the separation of two different biological samples, based on the contribution of a set of metabolites, or principal components, in relation to the overall variance of the experiment. Plotting the principal components of each sample on an x-y plot from two physiologically distinct treatments should reveal a treatment-dependent clustering of samples in two dimensional space. When PCA analysis was applied to peaks from rapid cold-hardened flies and peaks from flies sampled at 25°C, the resultant principal component plot showed a loose, but definable, clustering effect along the first principal component, or PC1 (Fig. 3). PC1 for the rapid cold-hardening experiment included glycerol, proline, glucose, ornithine, and free phosphate and accounted for 36% of the variance in the experiment. Therefore, the levels of metabolites that contributed the most to the variance across samples also were deterministic with respect to the treatment groups. This implies that rapid cold-hardened flies are in a physiological state distinctively different from that of their non-hardened counterparts.

3.3.3. Diapause

A number of altered metabolite concentrations were noted when metabolite peaks from diapausing pupae were compared to peaks observed in their nondiapausing counterparts (Fig. 4). ANOVA analysis (df=10, p≤0.01) confirmed 5 identifiable elevated compounds and 8 identifiable compounds that were less abundant during diapause. The only metabolite previously known to be elevated during diapause in this species, glycerol, quadrupled in concentration (p=0.0034).
The greatest increases were in the glucose and pyruvate pools, which were elevated 30-fold and 10-fold, respectively (p<0.0001 for each). Among the amino acids, alanine increased 9-fold (p=0.0004), and leucine slightly increased (p<0.0066). Levels of aspartate (p=0.0012), glycine (p<0.0001), phenylalanine (p<0.0001), proline (p<0.0001), and tyrosine (p<0.0001) were lower during diapause. In addition to the reduction in amino acids, the metabolites sorbitol (p=0.0003), fumarate (p<0.0001), and isocitrate (p<0.0001) were significantly reduced. An additional 15 metabolites were altered by diapause (5 increased, 10 decreased), but these compounds were not identifiable with any degree of certainty.

PCA analysis of metabolite peaks derived from samples in the diapause experiment revealed an unambiguous clustering effect along the first principal component, PC1 (Fig. 5). The first principle component in this experiment consisted of cystathionine, isocitric acid, proline, and trehalose and accounted for 46% of the total variance in this experiment. Thus, the physiological state of diapause is biologically distinct from that of non-diapausing pupae at the same temperature (20°C) and this distinction may be defined (in part) by the peak intensities of these four metabolites. This separation is greater than that seen between rapid cold-hardened individuals and those that were not rapid cold-hardened (Fig. 4).
3.4. Conclusions

3.4.1 Efficacy of GC-MS insect metabolomics

Metabolomics has proven to be a powerful hypothesis-building tool in a variety of advanced biological fields, but has not been used extensively for insects, with the exception of a single study concerning heat shock in *Drosophila* (Malmendal et al. 2006). In that experiment, NMR-based technology detected 51 different compounds, with 16 being positively identified in both untreated and heat-treated flies. In the present study, GC-MS metabolomics were employed to derivatized samples and the resultant metabolic profiles produced 62 identifiable peaks out of 159, with subsequent multivariate analysis successfully separating samples into their respective treatment groups. The ability to enhance chromatographic behavior through derivativization and the availability of extensive mass spectral library searches most likely contributed to the disparity in the number of metabolites detected and identified between these two methods. Nevertheless, metabolomics by either method has successfully defined altered physiological states during high-temperature, low-temperature, and diapause and identified target compounds for subsequent comparison with genetic, proteomic, or transcriptomic data.

3.4.2 Rapid cold-hardening metabolic phenotype

When red-eye pharate adults of *S. crassiplapis* are chilled at 4°C, glycogen is converted to glucose by phosphorylase A (Chen and Denlinger 1990) and is presumed to be used either as an energy reserve or is converted to glycerol, a polyol previously linked to rapid cold-hardening (Lee et al. 1987a). In the present
study, both glycerol and glucose increased in response to rapid cold-hardening, confirming previous results. Glycerol is a polyhydric alcohol, or polyol, possessing unique chemical properties that allows it to protect membranes and proteins and promote supercooling of body fluids (Lee 1991). Metabolomic analysis of rapid cold-hardened flies in this study reveals another elevated polyol, sorbitol, although it is elevated to a lesser degree than glycerol. Sorbitol presumably contributes to low temperature survival in the same manner as glycerol, as it is a well-known cryoprotectant found in other insects (Salvucci 2000, Wang and Kang 2005). The goldenrod gall fly, Eurosta solidiginis, also accumulates sorbitol at low temperatures through glucose overloading from glycogen reserves, coupled with activation of the hexose monophosphate shunt (Joanisse and Storey 1995). The production of sorbitol is thought to be a direct response to low temperatures rather than a response in anticipation of low temperatures (Storey and Storey 1983), thus explaining why an increase in sorbitol was not observed in diapausing flesh flies at 20°C but was detected after 8 h at 4°C (Fig. 6). Even though this metabolomic experiment was not designed to determine metabolic rates or rate constants of biochemical pathways, it is easy to see how, in an environment of greater glucose availability, both polyols can be synthesized and contribute to cold survival during rapid cold-hardening.

This study also revealed that the end product of the glycolytic pathway, pyruvate, was elevated after rapid cold-hardening. This condition would occur either from overproduction from the glycolytic pathway, or from the inactivation of enzymes downstream of pyruvate, such as pyruvate dehydrogenase. Evidence
of the former is inferred from the high rate of production of glycerol and sorbitol (both shunted from the glycolytic pathway), the overloading of glucose from glycogen stores, and the lack of detectable differences in Krebs cycle intermediates between treatments in this study. The observed reduction in levels of mannose and trehalose seen during rapid cold-hardening may result from the transformation of these sugars into glucose equivalents for use in the glycolytic pathway, although this suggestion is speculative.

GC/MS metabolomics also reveals an elevation of alanine during rapid cold-hardening. Alanine is synthesized directly from pyruvate, therefore its elevation may be a by-product of increased pyruvate levels, alanine dehydrogenase activation, or a combination of both. Alanine upregulation has been correlated with a number of insect cold-hardy states (e.g. Fields et al. 1998, Rivers et al. 2000, Goto et al. 2001), including diapause in the flesh fly (Kukal et al. 1991). The colligative effectiveness of alanine is roughly the same as that of glycerol. In addition, studies in plants suggest that a shift to alanine synthesis from pyruvate is preferable to its alternative, lactic acid (for animals), because alanine is less toxic to cells (Touchette and Burkholder 2000). The observed reduction in β-alanine seen during rapid cold-hardening possibly results from conversion of this metabolite into L-alanine.

An intriguing observation from this study is the elevation of glutamine during rapid cold-hardening. Glutamine accumulation in response to low temperature has been documented for crickets (Tomeba et al. 1988), and this amino acid is increased in association with diapause in the silk worm Bombyx.
*mori* (Osanai and Yonezawa 1986), Colorado potato beetle *Leptinotarsa decemlineata* (Yi and Adams 2000), and the aphid *Drepanosiphum platanoidis* (Douglas 2000). In addition to contributing to the overall osmolality of the organism, glutamine has the non-colligative potential of increasing the responsiveness of heat shock proteins (Phanvijhitsiri et al. 2005) and suppressing apoptosis (Fuchs and Bode 2006). Heat shock protein transcripts are expressed after cold shock in the flesh fly (Rinehart et al. 2000), thus glutamine possibly elevates this response. Glutamine has a positive effect on low temperature survival at concentrations as low as 2 mM and increases survival of rat cells if present before the onset of cold shock (Kruuv et al. 1988).

Urea levels in *S. crassipalpis* were elevated slightly, but significantly, in response to rapid cold-hardening. In frogs, urea has a colligative cryopreservative effect that is similar to glycerol and sucrose (Costanzo and Lee 2005). The increase in urea levels seen in *S. crassipalpis* may also be the result of high alanine levels, which are known to perturb the urea cycle (Hensgens and Meijer 1980). The rise in alanine is coupled with a reduction in ornithine, another feature consistent with perturbation of the urea cycle during rapid cold-hardening.

Cystathionine is also elevated during rapid cold-hardening, but it is not clear how this metabolite contributes to cold hardiness other than by elevating osmolarity. Cystathionine increase has also been noted in the overwintering beach pea (Chinnasamy and Bal 2004), but its increase has not been correlated with any particular cold-hardiness mechanism.
Several recent studies have increased our understanding of the rapid cold-hardening process in flesh flies. The initial observation that glycerol elevation characterizes this process (Lee et al. 1987a) has been expanded to demonstrate an increase in membrane fluidity (Lee et al. 2006), membrane phospholipids restructuring (Michaud and Denlinger 2006), and now the presence of additional metabolites that presumably contribute to this process. The overall pattern of changed metabolites indicates a rapid increase in glycolytic activity from glucose “front-loading” coupled with an increase in amino acids and polyols derived from glycolytic intermediates. Krebs cycle activity does not appear to be altered by rapid cold-hardening, although there is an indication that the urea cycle is perturbed to favor urea production. Most of the altered metabolites we have noted potentially contribute to cold hardiness in a variety of ways, not all of which are colligative. Rapid cold-hardening (and cold hardiness in general) has long been suspected to involve a suite of biochemical changes, and our current analysis clearly supports this hypothesis.

3.4.2. Diapause metabolic phenotype

Flesh flies spend the winter months in pupal diapause. During this time, heat shock protein transcripts increase (Hayward et al. 2005) and glycerol is elevated in the body tissues (Lee et al. 1987a); both of these physiological responses render the diapausing pupae much more cold hardy than any other life stage. Diapausing flesh flies can survive months at sub-zero temperatures without ill effects (Lee et al. 1987a).
The metabolomic techniques applied in this study confirm the previously observed elevated levels of glycerol associated with diapause in this species. Glycerol is elevated during diapause in a number of insect species (e.g. Chino 1958, Yi and Bai 1991, Stanic et al. 2004) and is assumed to have a cryoprotective role in these organisms, utilizing the same mechanisms mentioned in the previous section. The glycerol elevation due to diapause in this experiment is the result of the insect’s response to a token stimulus (photoperiod), rather than a response to low temperature. The change in biochemical equilibrium favoring glycerol may be the result of direct effects on an existing enzyme or the result of enzyme synthesis from an alternative allele or locus.

Glucose and pyruvate also increased during diapause in the flesh fly, indicating that the glycolytic cycle is active during this period. Glucose elevation was huge, nearly 30-fold, thus greatly increasing the availability of substrate entering the glycolytic pathway. The glucose increase is presumed to be derived from glycogen reserves in a manner similar to that seen during rapid cold-hardening (Chen and Denlinger 1990). Glucose is upregulated for diapause in the Colorado potato beetle, *Leptinotarsa decemlineata* (Lefevere et al. 1989) and a tropical beetle, *Stenotarsus rotundus* (Pullin and Wolda 1993). Interestingly, the tropical beetle accumulated glucose during diapause in the absence of any temperature stimuli, in a manner similar to that observed in this study. Glucose has a positive effect on cold hardiness (Costanzo and Lee 2005) and is also
moderately effective in inhibiting development in embryos of *Bombyx mori* (Horie et al. 2000). Also, glucose carbons may be converted into a number of different polyols and amino acids through the glycolytic pathway and its branches.

Pyruvate levels also increased during diapause. Pyruvate was previously detected in larvae of the diapausing rice stem borer, *Chilo suppressalis*, and its level was inversely proportional to levels of lactic acid when the insects were exposed to anoxia (Tsumuki and Kanehisa 1980). The increase of pyruvate during diapause in the flesh fly is most likely the result of low activity in the Krebs cycle (evidenced by a reduction in fumarate and citrate during diapause in this study) while the glycolytic pathway remains active. There are several potential effects of increased levels of pyruvate during diapause. First, pyruvate may be used at the end of diapause as a readily available energy source for the Krebs cycle, a pathway known to be depressed during diapause (Kageyama and Ohnishi 1971). Pyruvate is widely recognized as an accessible energy source, a feature that is commonly exploited medically during liver transplantation (So and Fuller 2003). In addition, the presence of pyruvate could serve as a building block for the synthesis of amino acids, especially alanine.

As with rapid cold-hardening, our metabolomics experiments demonstrated that alanine levels increased when the flies entered diapause. This was also previously observed during diapause in this species (Kukal et al. 1991, Rivers et al. 2001), as well as in a number of other species (e.g. Osanai and Yonezawa 1986, Morgan and Chippendale 1983, Li et al. 2001). High alanine levels have a synergistic, colligative effect with other solutes and thus contribute
to cold tolerance in species such as *Eurosta solidaginis* (Churchill and Storey 1989), but alanine also may serve as an alternative and less toxic by-product to lactic acid in the glycolytic cycle as seen for ethanol protection in plants (Touchette and Burkholder 2000).

The amino acid leucine also accumulated during diapause. To the best of our knowledge, the only other report of leucine accumulation during insect diapause is in the integument of the diapausing embryo of *Bombyx mori* (Dordea et al. 1987), but leucine levels are also elevated during diapause in a copepod (Wang et al. 2005). Although there are no reported non-colligative mechanisms of cold-hardiness inherent to this amino acid, leucine also accumulates in response to cold in both the grain beetle, *Sitophilus granarius* (Fields et al. 1998) and in the plant, *Arabidopsis thaliana* (Kaplan et al. 2004). Leucine is synthesized from glycolytic intermediates, thus further supporting the hypothesis that glycolysis is active during diapause in the flesh fly. A number of other amino acids (aspartate, glycine, phenylalanine, proline, and tyrosine) are reduced as a result of diapause in the flesh fly, but it is unclear what benefit the pupa may gain from a reduction in these amino acids. Proline, a well-known energy source and cryoprotectant in some insects (Okazaki and Yamashita 1981, Storey 1997), does not appear to be important in the diapause of flesh flies. Proline levels were also reduced during diapause in the embryo of *Bombyx mori* (Osanai and Yonezawa 1986) and in chilled, diapausing larvae of the grass stem borer, *Chilo suppressalis* (Goto et al. 1998). Cold-induced reductions of amino acids, as seen in this study (except
phenylalanine), have been reported for snails (Churchill and Storey 1996), frogs (Storey and Storey 1986), and the grain beetles, *Sitophilus granaries* and *Cryptolestes ferrugeneus* (Fields et al. 1998).

The metabolite changes we have noted indicate that both diapause and rapid cold-hardening induce the glycolytic pathway, generating glucose to produce pyruvate, glycerol, and alanine (Fig. 6). Where rapid cold-hardening and diapause differ is in the aerobic portion of cellular respiration (Krebs cycle), which, by inference from metabolite concentration, is unaltered for rapid cold-hardening but slowed for diapause. This observation is supported by previous research showing that oxygen consumption is greatly reduced for flesh flies during diapause (Denlinger et al. 1972, Slama and Denlinger 1992) and by the reduced Krebs cycle enzyme activity noted during diapause in other insects (Kageyama and Ohnishi 1971). Diapause also differed from rapid cold-hardening in that glutamine was elevated by rapid cold-hardening, but not by diapause. However, if glutamine production is related to the modulation of heat shock proteins after a cold shock (Phanvijhitsiri et al. 2005), then such upregulation would not be necessary during diapause because heat shock protein transcripts are already present during diapause (Hayward et al. 2005) but not during rapid cold-hardening (unpublished data). While rapid cold-hardening (induced by low temperature) and diapause (induced by short daylength) in the flesh fly share a number of metabolic similarities, there are enough differences, especially in downregulated metabolites, to indicate that control of these two mechanisms of cold hardening are distinct.
References


### Table 3.1. Metabolites identified in *Sarcophaga crassipalpis* metabonomic samples.

<table>
<thead>
<tr>
<th><strong>Amino acids</strong></th>
<th><strong>Sugars and polyols</strong></th>
<th><strong>Other metabolites</strong></th>
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</thead>
<tbody>
<tr>
<td>Asparagine</td>
<td>Glucoson</td>
<td>2-butanoic acid</td>
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<tr>
<td>Glutamate</td>
<td>Glycerol</td>
<td>2-methyl propanoic</td>
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<tr>
<td>Glutamine</td>
<td>Inositol</td>
<td>acid</td>
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<tr>
<td>Glycine</td>
<td>Mannitol</td>
<td>2-propenoic acid</td>
</tr>
<tr>
<td>Leucine</td>
<td>Myo-inositol-1-phosphate</td>
<td>5-amino pentanoic</td>
</tr>
<tr>
<td>N-glycyl alanine</td>
<td>Sorbitol</td>
<td>acid</td>
</tr>
<tr>
<td>Ornithine</td>
<td>Trehalose</td>
<td>Citrulline</td>
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<tr>
<td>Phenylalanine</td>
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<td>Dihydouracil</td>
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<td>Proline</td>
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<td>Galactouronic acid</td>
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<tr>
<td>Serine</td>
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<td>Gamma amino isobutyric acid</td>
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<td>Threonine</td>
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<td>Glucopyranoside</td>
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<td>Tyrosine</td>
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<td>Glutaric acid</td>
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<td>Valine</td>
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<td>Hydroxybutane</td>
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<td>β-alanine</td>
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<td>Lactone pentonic acid</td>
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<tr>
<td><strong>Sugars and polyols</strong></td>
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<td>Metanephrine</td>
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<td>3-ketoglucose</td>
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<td>M-hydroxymandelic</td>
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<td>5-ketoglucose</td>
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<td>acid</td>
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<td>Erythritol</td>
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<td>N-butylamine</td>
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<td>Erythrose</td>
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<td>Nonadecanoic acid</td>
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<td>Fructose</td>
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<td>Norvaline</td>
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<td>Galactose</td>
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<td>Oxalic acid</td>
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<td>Glucose</td>
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<td>Putrescine</td>
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<tr>
<td><strong>Metabolic intermediates</strong></td>
<td></td>
<td>Trihydroxybutyric acid</td>
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<tr>
<td>Acetic acid</td>
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<td>Xylopyranose</td>
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<td>Cystathionine</td>
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<td>α-glycerophosphoric acid</td>
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<td>Isocitric acid</td>
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<td>Urea</td>
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<td><strong>Small molecules</strong></td>
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<tr>
<td>Free sulfate</td>
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Figure 3.1. Representative GC-MS chromatograph depicting the metabolic profile of *S. crassipalpis* during pupal diapause (day 30). Identities of some of the peaks are provided, but this represents only a small portion of the total identified metabolites. In most samples, ~62 metabolites out of 159 are identifiable by comparison with mass spectral libraries as well as authentic external standards.
Figure 3.2. Metabolites altered by rapid cold-hardening in *Sarcophaga crassipalpis*. GC-MS peaks of derivatized metabolites from whole-body extracts of *S. crassipalpis* held at 25°C (black bars) were compared with peaks from *S. crassipalpis* held for 8 h at 4°C (white bars), a treatment that generates maximum cold-hardening through the rapid cold-hardening mechanism. Two amino acids and 5 other metabolites accumulated in response to rapid cold-hardening, while 2 amino acids and 2 other metabolites were reduced in abundance. Substances listed here were significant beyond the p=0.01 interval (t-test, p≤0.01, n=6).
Figure 3.3. Principal component analysis (PCA) of metabolomic samples from *Sarcophaga crassipalpis* during maximum rapid cold-hardening. Raw peak areas from samples were normalized, analyzed by PCA, and plotted on an x-y graph in accordance with their first two principal components (PC1 and PC2). From the graph, it is clear that samples taken from rapid cold-hardened pharate adults held at 4°C for 8h are physiologically distinct from samples taken from flies held at 25°C as evidenced by clustering along the PC1 axis.
Figure 3.4. Metabolites altered by diapause in *Sarcophaga crassipalpis*. GC-MS peaks of derivatized metabolites from whole-body extracts of nondiapausing pupae of *S. crassipalpis* at 20°C (black bars) were compared with metabolite peaks from pupae that had been in diapause at 20°C for 30 days (open bars). Two amino acids and 3 other metabolites were elevated in response to the photoperiod-induced diapause, while levels of 5 amino acids and 3 other metabolites were reduced. Differences in the levels of substances listed here were significant beyond the p=0.01 interval (t-test, p≤0.01, n=6).
Figure 3.5. Principal component analysis (PCA) of metabolomic samples from diapausing and non-diapausing pupae of *Sarcophaga crassipalpis*. Raw peak areas from samples were normalized, analyzed by PCA, and plotted on an x-y graph in accordance with their first two principal components (PC1 and PC2). From the graph, it is clear that samples taken from diapausing pupae are physiologically distinct from their non-diapausing counterparts as evidenced by clustering along the PC1 axis.
Figure 3.6. Venn diagram representation of metabolites that are altered in abundance by rapid cold-hardening (left) and diapause (right) in *Sarcophaga crassipalpis*. Metabolomic analysis (GC/MS) reveals that both cold-hardy phenotypes of the flesh fly share 4 different identifiable upregulated metabolites (top circles), but share no common downregulated metabolites (bottom circles). Several metabolites are distinct to one or the other form of cold-hardening.
CHAPTER 4

Metabolomics reveals unique and shared metabolic changes in response to heat shock, freezing, and desiccation in the Antarctic midge, *Belgica antarctica*.

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Abstract
The midge, Belgica antarctica is subjected to numerous environmental stressors during its two-year life cycle on the Antarctic Peninsula, and in response it has evolved a suite of behavioral, physiological, and life-cycle changes to counter these stressors, but thus far only a limited number of biochemical adaptations have been identified. In this study, we use a metabolomics approach to obtain a broad overview of changes in energy metabolism, amino acids, and polyols in response to three of the midge’s major stresses: heat, freezing, and desiccation. Using GC-MS analysis, a total of 75 compounds were identified. Freezing (-10°C for 6h) and desiccation (50% water loss) elicited similar increases in succinate, isocitric acid, and glycerol. Freezing increased multiple polyols, but glycerol was the only polyol elevated by desiccation. Heat shock (30°C for 1h) increased the pools of gamma amino butyric acid (GABA) and α-ketoglutarate. A number of metabolic changes, especially those in the sugar and polyol pools, are adaptations that have potential to enhance survival during both cold and desiccation. The pools of free fatty acids and hydrocarbons were affected most by desiccation and heat; many of these alterations resulted in shifts toward lipid metabolism, possibly generating desiccation resistance. An increase in nonanoic acid (9:0) was common to all three stresses, but the function of this molecule remains unknown.

Keywords: cold, metabolism, polar, insect
4.1 Introduction

The Antarctic midge, *Belgica antarctica*, is the most southerly-distributed free-living insect and is spatially dispersed throughout the Antarctic Peninsula and its offshore islands (Sugg et al. 1983). This remarkable insect is active during the austral summer among pockets of primitive vegetation growing in nutrient-rich substrate near seal wallows and penguin rookeries (Usher and Edwards, 1984). The chironomid has a two-year life cycle and overwinters as larvae (all four instars represented) in the frozen substrate (Sugg et al. 1983). The adults eclose, mate, lay eggs, and die within a 7-10 d period during the summer.

Though air temperatures on the Antarctic Peninsula routinely drop below -15°C, the temperature in its hibernaculum, beneath the cover of ice and snow, remains fairly stable, in the range of 0°C to -7°C (Baust and Lee 1981). Desiccation is an additional abiotic stress during the winter because all of the available water is tied up as biologically-inactive ice, and during the summer high winds and periods of drought generate a highly desiccative environment. Furthermore, larvae are routinely exposed to heat stress during the summer; solar radiation generates substrate temperatures that far exceed the prevailing air temperatures (>20°C). The Antarctic midge, not surprisingly, is quite intolerant of high temperature; larvae die within a week at 10°C (Sugg et al. 1983, Baust and Lee 1987, Lee et al. 2006) and survive only a few hours at 30°C (Rinehart et al. 2006).

A few studies of *B. antarctica* have elucidated some of the physiological mechanisms involved in stress tolerance. In response to low temperature, this species accumulates three cryoprotective compounds: erythritol, glucose, and trehalose, in a manner dependent on temperature and geographic location (Baust and Edwards 1979, Baust and Lee 1983). Since this insect is freeze-tolerant, it is assumed that these three cryoprotective substances contribute
to low-temperature survival by colligatively decreasing the amount of ice that forms internally (Baust and Edwards 1979) and by hydrogen-bonding to proteins and membranes, rendering them less vulnerable to denaturation. In addition to these known cryoprotectants, larvae of *B. antarctica* constitutively produce a suite of heat shock proteins (Hsps) known to enhance both high and low temperature tolerance by preventing or repairing thermal damage to proteins (Rinehart et al. 2006). This potent combination of low molecular weight cryoprotectants and constitutive Hsp expression presumably enables *B. antarctica* to survive the rapid temperature fluctuations that characterize the fringes of the austral summer and to tolerate freezing and/or cryoprotective dehydration during the long polar winter.

In contrast to what is known about low-temperature stress in the Antarctic midge, there is little information available on the physiological mechanisms used by this species to survive heat and desiccation. Even though desiccation tolerance is quite pronounced in this species (can survive 70% water loss, Hayward et al., 2007), few of the physiological adjustments that accompany such a loss in body water have been identified. Using enzymatic assays, we previously determined that glycerol and trehalose accumulate in *B. antarctica* in response to desiccation, and a GC-MS analysis of surface hydrocarbons from desiccated larvae indicated that the hydrocarbon pool on the cuticle of this organism shifts to longer-chain hydrocarbons as it dehydrates (Benoit et al. 2007).

Metabolomics, an emerging field concerned with the study of an organism’s physiological state at the substrate level, offers a means to obtain a comprehensive view of the changes in the abundance of numerous compounds simultaneously (Dunn and Ellis 2005, Weckworth and Morgantal 2005). Sampling is accomplished by extracting small molecules (a.k.a. metabolites) with general solvents and derivatizing them to improve chromatographic
behavior. Chromatographic analysis is accomplished either through gas chromatography/mass spectrophotometry or by nuclear magnetic resonance (Dunn and Ellis 2005). Lastly, data analysis for metabolomics is accomplished through normal peak-by-peak analysis between treatments, followed by a multivariate principle component analysis (PCA) to determine whether the treatments did indeed produce different physiological states and to determine which metabolites contribute the most to that change in state (Weckworth and Morganthal 2005). The disadvantages of metabolomics are the semi-quantitative nature of the data and the limitations of chromatographic peak identification, but these disadvantages are outweighed by the unbiased nature of the data gathering and statistics, the ability to easily compare these data with other –omics data, and the vast amount of data and hypotheses generated from this avenue of research (Kopka 2006). Metabolomics has been used for physiological studies of plants (Weckworth et al. 2004), clinical screening of humans (Zytkovicz et al. 2001), and the detailed study of metabolism in bacteria (Buchholz et al. 2002), but thus far the technique has not been applied extensively to insect studies (c.f. Malmendal et al. 2006, Michaud and Denlinger 2007). In this study we use this approach to monitor changes in *B. antarctica* elicited by heat shock, freezing, and desiccation.

### 4.2 Materials and Methods

#### 4.2.1 Insect collection and storage

Larvae of *Belgica antarctica* were collected in January 2006 from islands near Palmer Station, Antarctica (64°46’S, 64°43’W) and maintained in the laboratory at 4°C and 100% relative humidity in their natural substrate. In early February, the larvae were shipped to our research laboratory (The Ohio State University) where they were held for two weeks prior to
experimentation. Larvae were of mixed stages, but the majority was fourth (final) larval instar and the others were third instars. Larvae maintained in this manner readily survived >6 mo in our home laboratory.

4.2.2. Stress exposure

All stress exposures (plus the untreated controls) were performed using 5 groups of 25 larvae held in Eppendorf 1.7 ml microcentrifuge tubes (VWR Scientific, West Chester, PA). Tap water (500µl) was added to each tube, with the exception of those used in the desiccation experiment. Untreated controls were homogenized at 4°C, 1 h after removal from the substrate. For the heat-shock treatment, tubes containing the larvae were submerged for 1 h in a 30°C bath (Brinkmann Instruments, Westbury, NY) containing 50% ethylene glycol, and the larvae were homogenized immediately thereafter. For the freezing treatment, tubes were placed for 6 h at -10°C in a 50% ethylene glycol bath, and the larvae were homogenized after removal. For the desiccation treatment, larvae were washed and placed for 6 d at 4°C in a ventilated tube within a plastic desiccator containing an environment of 98.5% RH that resulted in a 50% loss of total body water (Benoit et al. 2007). Following desiccation, the larvae were immediately homogenized at 4°C.

4.2.3. Separation and derivatization

One ml of dH2O containing 25 µg heptadecanoic acid (internal standard not normally detected in polar samples of B. antarctica) and 1 ml chloroform were added to each sample, and the sample was vortexed for 30 sec. The resultant two-phase mixture was allowed to sit for 30 min and then vacuum-filtered through a glass filter to remove particulate matter and precipitates. The filter was washed with 1 ml chloroform:methanol:water (2:1:0.8) to remove soluble trace metabolites from the filter matrix. After filtration, the two-phase
mixture was separated into an aqueous phase containing polar metabolites (sugars, polyols, amino acids) and an organic phase containing non-polar metabolites (lipids). The organic phase was used in a different experiment. The aqueous phase was placed under a gentle stream of nitrogen and evaporated to dryness at room temperature. The dried sample was immediately methoxyaminated by adding 1.0 ml methoxyamine hydrochloride in pyridine, incubated for 1 hr at 60°C (turns clear as metabolites dissolve), followed by a 16 h incubation at room temperature. The sample was then trimethylsilylated by the addition of 300 µl MSTFA and incubated at 50°C for 30 min. 150 µl of the trimethylsilylated sample was added to a glass insert within an auto-sampler vial and analyzed with GC-MS. Aqueous samples processed in this manner produced distinctive and repeatable chromatographic peaks that differed across samples by 5 to 60%, within the expected range for this type of experiment.

4.2.4. Chromatographic analysis

One microliter of each sample was auto-injected into a Finnigan-Trace GC-MS and subjected to chromatographic analysis (50-450 m/z). The injector temperature was held at 280°C. The oven temperature was ramped from 50 to 300°C, and the temperature increment was 5°C min⁻¹, which produced a chromatograph of sufficient resolution to separate ~175 chromatographic peaks per sample. The column was a Restek 30 m fused silica column (I.D. 25 mm, 95% dimethyl siloxane, 5% diphenyl) with helium gas used as a carrier at a rate of 50 ml/min. Samples were run using the splitless mode. After each sample was run, the oven remained at 300°C for 10 min to clean impurities from the column.

4.2.5. Quantification and data analysis

Individual integrated peak areas were converted to response ratios in relation to the internal standard (heptadecanoic acid) by dividing the peak area of the metabolite by the peak
area of the internal standard. One-way ANOVA with an acceptable significance level of $p \leq 0.05$ was performed on each metabolite to determine which individual metabolites were altered with respect to the controls. Peak identities were determined where possible by comparison of retention times with amino acid, sugar, and polyol standards prepared in the same manner as the samples. Matches of spectral peaks with the National Institute of Standards and Technologies (NIST) and Wiley Chemical Structural Libraries further confirmed or established metabolite identities, provided the match carried an RSI value $\geq 700$.

For principal component analysis (PCA), peak areas of metabolites were normalized by dividing each peak area value by the mean peak area for that compound, and the resultant normalized data were entered into MetaGeneAlyse 1.7 (Max Planck Institute of Molecular Plant Physiology, Berlin, Germany) and separated by their principal components. In addition to PCA, hierarchal clustering was performed using the same statistical software to determine overall relatedness between the physiological responses of *B. antarctica* to heat, freezing, and desiccation.

### 4.3. Results

#### 4.3.1. General observations

Extracted and derivatized samples isolated from *B. antarctica* produced chromatographic peaks with fairly consistent retention times (within a few hundreds of a minute) and excellent resolution (92% of peaks were singular). The first half of each chromatograph contained mostly amino acids, small fatty acids, and small molecules (e.g. free phosphate), and the latter half of the chromatograph featured sugars, polyols, and longer fatty acids and hydrocarbons, although some overlap occurred (e.g. glycerol was found on
the first half of the chromatograph. Metabolites from the glycolytic pathway and the Krebs cycle were found throughout the chromatographs. Each peak within a treatment group varied 5% to 60% in area when compared to peak area of the internal standard (heptadecanoic acid), indicating a moderate level of total variation for this experiment. The treatment that produced the most variation was the heat shock treatment of 1 h at 30°C.

A total of 75 metabolites out of 141 total peaks could be identified across all samples in this experiment; this is a high proportion of identified peaks for a metabolomics experiment (Shauer et al. 2005). Some (~30) of the peaks in the “unidentified” category had high RSI values (>700) but could not be assigned a biological function based on the available literature, thus these compounds will not receive further comment at this time, but may be revisited as the data base of spectral libraries increases. The 75 identified compounds included 14 amino acids, 13 sugars and polyols, 8 metabolic intermediates, 4 small molecules, 8 fatty acids and hydrocarbons, and 28 metabolites that did not fall into the above categories (Table 1).

4.3.2. Response to heat-shock

The response ratios of a number of metabolites from B. antarctica larvae were significantly altered (ANOVA, df=8, p≤0.05) by heat shock (30°C for 1 h) relative to larvae continuously maintained at 4°C (Fig. 1). Concentrations of four total metabolites were elevated by heat shock (Fig. 1A), and six were reduced (Fig. 1B). The amino acids aspartate (p=0.019), glycine (p<0.001), and serine (p<1x10^-7) all were reduced (30-90%) in response to heat shock, but some derivatives of amino acids, gamma amino butyric acid (GABA, p<0.01), norvaline (p=0.019), and putrescine (p=0.031) were elevated 2-3 fold. Two fatty acids, nonanoic acid (9:0, p<0.001) and tetradecanoic acid (14:0, p=0.049) increased 2-3 fold
in the heat-shocked midges. Three known cryoprotective sugars and polyols, sorbitol, glucose, and glycerol (p<0.001 for all) were dramatically decreased to 30% or less of their levels in response to heat shock when compared to untreated midges. Two intermediates of cellular respiration, glycerol-3-phosphate (p=0.017) and α-ketoglutarate (p=0.031) increased in response to heat shock, but the response of α-ketoglutarate, though significant, was subject to considerable variation (standard deviation is >40% of the mean for heat-shocked midges). In addition to the aforementioned metabolites, free phosphate was reduced in response to heat shock (p<0.0001). A total of 24 “unknown” metabolites also were altered in heat-shocked larvae; 19 of these were elevated, and 5 were reduced (data not shown).

### 4.3.3. Response to freezing

A comparison between the response ratios of chromatographic peaks from midges that had been frozen 6 h at -10°C and midges held continuously at 4°C (ANOVA, df=8, p≤0.05) revealed a total of 16 metabolites that changed in concentration in response to freezing. Twelve of these metabolites increased (Fig. 2A) and four decreased (Fig. 2B). Three constituents of the amino acid pool (alanine [p<1x10^{-6}], asparagine [p<0.01], and glycine [p<1x10^{-5}]) were elevated 2-3 fold by freezing, but serine decreased to less than 10% of its concentration in untreated larvae. Two amino acid derivatives, norvaline (p<0.01) and putrescine (p<0.01), increased in peak intensity with respect to their untreated controls.

Among the cryoprotective polyols and sugars, sorbitol levels were reduced to approximately 40% of the levels seen in untreated midges, but this reduction was countered by a 2-5 fold elevation of three other polyols: erythritol (p=0.005), glycerol (p<1x10^{-7}), and mannitol (p<1x10^{-5}). Among the fatty acids, oleic acid (18:1, p<0.01) decreased in response to freezing, but the volatile fatty acid, nonanoic acid (9:0, p<10^{-5}), increased three-fold. Peaks
of dodecane, a short hydrocarbon found on the cuticular surface of the midge (Benoit et al., 2007), declined threefold in response to freezing (p=0.0044). Two Krebs cycle intermediates, isocitrate (p<0.01) and succinate (p<0.001), doubled in peak intensity. An additional 24 “unknown” metabolites changed in response to this treatment; 21 increased and 3 decreased (data not shown).

4.3.4. Response to desiccation

ANOVA analysis (df=8, p≤0.05) applied to the response ratios of peaks isolated from midges held at 98.5% RH for 6 d (50% loss of body water) and midges held continuously at 4°C, 100% RH revealed a total of 14 metabolite peak alterations. Seven metabolites were elevated (Fig. 3A), and six were reduced (Fig. 3B). Within the amino acid pool, three amino acids (aspartate [p<0.005], glycine [p=0.03], serine [p<1x10^-8]) declined and one amino acid, asparagine (p=0.018), increased. Among the sugars and polyols, glycerol concentrations doubled in response to desiccation (p=0.023), and two molecules, sorbitol (p<0.0005) and glucose (p=0.019), decreased to slightly less than half the levels seen in fully-hydrated midges. Octadecanoic acid (18:0, p=0.028) and nonanoic acid (9:0, p<1x10^-5), two free fatty acids, increased 2-fold and 4-fold, respectively. Among intermediates of cellular respiration, desiccation resulted in the accumulation of glycerol-3-phosphate (p=0.04), isocitrate (p<0.005), and succinate (p=0.03), all of which were elevated 2-3 fold. Free sulfate levels were reduced by desiccation, but this reduction was modest (p=0.05). A total of 26 “unknown” metabolic peaks were altered in intensity in response to desiccation. Of these, 15 were elevated and 11 were reduced.
4.3.5. Principle component analysis and clustering

Multivariate PCA statistics of normalized peak areas from all samples in this metabolomic experiment gives a measure of the degree of separation of each treatment group (or metabolic state) based on the contribution of sets of metabolites, or principal components, to the overall variance within the data set. Plotting the principle components of each sample within a treatment group yields a cluster of points that can be circumscribed spatially, and the resultant area can be compared with areas generated by other treatment groups to determine if these treatments are physiologically distinct from one another (if they do not overlap). For this experiment, none of the areas generated by the four treatment clusters (untreated, heat-shock, freezing, desiccation) overlapped (Fig. 4A). Also, an orthogonal axis may be drawn through all of the sample clusters with each cluster occupying a distinct space along that axis, indicating excellent separation. Of course, this orthogonal can only be mathematically described in terms of both principle components, PC1 and PC2. Two of the five samples produced by heat shock (1 h at 30°C) deviated considerably from the bulk of their cluster, but this variation did not preclude physiological distinctiveness for this stressor. Deviation in these two samples is likely due to some inability of *B. antarctica* to survive a severe heat shock at 30°C (Rinehart et al. 2006).

The metabolites comprising the first principle component, PC1, contained α-ketoglutarate, glycerol-3-phosphate, serine, proline, aspartate, glycerol, and sorbitol, accounting for 38% of the total variation in this experiment. Metabolites that comprised the second principle component PC2 (52% of total variation) included α-ketoglutarate, proline, and hydroxyhexadecane. Hierarchal clustering using normalized response ratios of peak areas across all samples (Fig. 4B) demonstrated that the metabolic response of *B. antarctica*
to freezing and desiccation was the most similar. The midges responded to heat shock in a manner that is considered to be an out-group with respect to the untreated control and the responses to freezing and dessication.

4.4. Discussion

4.4.1. Metabolic response to heat shock

*B. antarctica* constitutively expresses a suite of heat shock proteins throughout its larval life (Rinehart et al. 2006). The heat shock regime used in our current study (1 h at 30°C) does not affect heat shock expression nor is it lethal, but a 2 h exposure to this temperature shuts down heat shock protein transcript expression, and a 3 h exposure to this temperature kills >50% of the larvae (Rinehart et al. 2006). Although we anticipated that our 1 h heat shock at 30°C would be relatively mild, the varied response we observed in this metabolomic experiment suggests that this treatment caused a major physiological disruption.

In response to heat shock, *B. antarctica* increased concentrations of α-ketoglutarate more than 5-fold. In addition, GABA levels more than doubled. These two metabolites are linked in that α-ketoglutarate serves as both a Krebs cycle intermediate and a precursor to the amino acid biosynthesis of glutamine and glutamate. Glutamate may then be converted into GABA, which, in turn, can be converted into another downstream Krebs intermediate (succinate) to completely bypass a section of the Krebs cycle that may be slowed due to stress (Bown and Shelp 1997). Alternatively, in insects GABA serves as an excitatory or inhibitory neurotransmitter, depending on the cell type in question (Gisselmann et al. 2004). Thus, the increase in α-ketoglutarate and GABA may be an indicator that the Krebs cycle is
disrupted and must be bypassed to allow the midge to continue cellular respiration during a heat shock, or GABA synthesis may be related to the elevation of some essential neural activity (e.g. avoidance behavior). Although the GABA bypass has not been reported in insects, it has been identified in bacteria (Green et al. 2000), plants (Bown and Shelp 1997), and mammals (Gonzales et al. 1987). GABA is also known to be elevated in heat-stressed plants (Kinnersley and Turano 2000) and to be involved in neural protection of anoxia-exposed turtles (Lutz and Milton 2004). In addition, adding α-ketoglutarate to glucose-starved mouse cells leads to increased thermotolerance (Gomes et al. 1985).

Another notable metabolite elevated by heat shock in *B. antarctica* was a 3-fold increase in putrescine, a polyamine derived from the amino acid arginine. An elevation of putrescine due to stress is also seen in plants (Renaut et al. 2005) and worms (Hamana et al. 1995). Putrescine is used as a marker for stress in plants (Renaut et al. 2005) because stressed plants activate the enzyme, arginine decarboxylase, leading to putrescine conversion from arginine (Galston and Sawnhey 1990, Borrell et al. 1996). In animals, the conversion of arginine to putrescine is initially accomplished through the action of arginase. Arginase shows activation plasticity in response to stress for humans (Lange et al. 2004) and crabs (Reddy and Bhagyalakshmi 1994). Putrescine also can regulate the response of heat shock proteins (Basra et al. 1997, Koenigshofer and Lechner et al. 2002) and change the topology of DNA in a manner that promotes survival (Tkachenko et al. 1998).

Glycerol-3-phosphate, an intermediate of glycolysis, was elevated 3-fold in the Antarctic midge in response to heat shock. This molecule may accumulate due to a reduction in the activity of a downstream enzyme (e.g. phosphoglycerate mutase, enolase) or it may be present in high amounts to reflect extensive membrane restructuring (Avelange-Macherel et
The concomitant 3-fold increase in tetradecanoic acid (14:0) and nonanoic acid (9:0) lends further evidence for membrane alterations in response to heat shock in *B. antarctica*, as is well known for numerous organisms (Cossins 1983).

Norvaline, an amino acid derivative of leucine, increased more than 2-fold in response to heat shock. The function of this molecule for thermal stress is unclear, but it is known that norvaline can thermo-stabilize enzymes (Kachenko et al. 1998) and can antagonize arginase activity (Wheatley et al. 2004).

*B. antarctica* exhibited an overall pattern of metabolite reduction in response to heat shock. We observed a reduction in numerous amino acids (glycine, serine, aspartate) and polyols (glycerol and sorbitol). Glycine and serine are biochemically related amino acids that are both products of a pyruvate precursor, indicating that the activity of serine-pyruvate aminotransferase may be reduced during heat shock. The reduction of glycerol and sorbitol points to temperature-dependent inhibition of the aldose reductase enzyme. Glucose reduction seen in this study could be driving all of these glycolysis-related changes in amino acids and polyols by reducing the abundance of substrates available for glycolysis, thus reducing the rate of all downstream processes.

4.4.2. Metabolic response to freezing

Seasonal studies tracking whole-body concentrations of cryoprotectants in *B. antarctica* previously reported that glycerol, erythritol, sorbitol, glucose, and trehalose concentrations increased in the spring and fall, when compared to the summer (Baust 1980, Lee and Baust 1981). These concentrations are thought to be reliant upon diet and temperature, but not photoperiod (Baust and Edwards 1979, Baust and Lee 1983).
In the present study, metabolomics identified a number of known and previously unknown metabolic changes in response to freezing at -10°C. Confirming previous results, erythritol and glycerol increased in response to freezing. To our knowledge, *B. antarctica* is the only insect known to accumulate erythritol as a cryoprotectant. Another polyol previously found in *B. antarctica*, mannitol (Block 1982), increased in response to freezing, but this is the first time that this polyol has been found to change in response to a stressor in this species. Sorbitol declined in response to freezing, a result that runs counter to previous studies (Baust 1980); however, the sorbitol loss detected in this study is on a short time scale (hours) in comparison to previous studies (days). Overall, three polyols increased and one decreased in response to freezing. All of the polyol increases in this experiment likely contribute to cold survival by decreasing the amount of ice that forms in the larvae. In addition, polyols can contribute non-colligatively to survival by hydrogen-binding to proteins to prevent low-temperature denaturation (Tang and Pikal 2005) and by prevention of membrane damage (Tsvetkova and Quinn 1994). Glucose and trehalose did not change in response to our freezing regime, although both were previously reported to change in response to seasonal acclimation (Lee and Baust 1981, Baust and Lee 1983).

The free amino acid pool was considerably altered by freezing. Alanine, glycine, and asparagine increased while serine levels decreased. Glycine and serine are linked in the same gluconeogenic pathway, thus an increase of glycine at the expense of serine indicates that the enzyme in the interconversion of glycine and serine, serine transhydroxymethylase, is affected by freezing to favor glycine production. Glycine levels are also elevated in a number of insects in response to cold (Hanzal and Jegorov 1991, Storey et al. 1993), while serine levels were reduced in overwintering *Ostrinia furnacalis* (Goto et al. 2001). Glycine
serves as a precursor to glycine betaine, an important cryoprotectant in plants (Weibing and Rajashekar 2001), and glutathione, a molecule essential for mitigating damage to oxidative stress (Senthilkumar et al. 2004). Glycine also has a cryopreservative effect on proteins in vitro (Carpenter and Crowe 1988). Injection of glycine into mammals (Senthilkumar et al. 2004), as well as the addition of this amino acid to mammalian cell cultures (Marsh et al. 1993), aids cold survival.

Another free amino acid, alanine, increased due to freezing in the midge. Alanine upregulation has been correlated with a number of insect cold-hardy states (e.g. Fields et al. 1998, Rivers et al. 2000, Goto et al. 2001), including diapause in the flesh fly (Kukal et al. 1991). This amino acid also protects proteins from cold inactivation (Carpenter and Crowe 1988) and may contribute to cold survival by providing a less toxic end-product than lactic acid in an environment where the Kreb’s cycle is slowed due to cold inactivation (as indicated by the accumulation of Krebs intermediates in this experiment). Studies in plants suggest that a shift to alanine synthesis from pyruvate is preferable to its alternative, lactic acid (for animals), because alanine is less toxic to cells (Touchette and Burkholder 2000). Indeed, lactic acid did not accumulate due to freezing in this study. The final free amino acid elevated by freezing in the Antarctic midge, asparagine, is also elevated in response to cold acclimation in two beetles (Fields et al. 1998) and due to desiccation in the blister beetle, *Cysteodemus armatus* (Cohen et al. 1986). Asparagine may serve as a storage molecule for nitrogenous waste, while concurrently serving as an osmolyte during osmotic stress (Cohen et al. 1986). Since GC-MS metabolomics also determined that urea levels increased due to freezing in the midge, an overall nitrogen cycle perturbation is likely. Perturbation of the nitrogen cycle was also observed when the flesh fly, *Sarcophaga crassipalpis*, was subjected
to low temperatures for the induction of rapid cold-hardening (Michaud and Denlinger 2007). However, urea apparently also functions as an osmoprotectant and cryoprotectant in freeze-tolerant ectotherms (Costanzo and Lee 2005). The similarity in responses we noted to freezing and desiccation in this study (Fig. 4A,B) further underscores the point that osmotic stress is a component of freezing in this species.

Subjecting larvae of *B. antarctica* to freezing temperatures also caused succinic and isocitric acid, both Krebs cycle intermediates, to accumulate. Elevation of both of these compounds suggests that the enzymes immediately downstream to these metabolites (succinate and isocitrate dehydrogenase) have been cold inactivated as seen in goldfish (Van Del Thillart and Smit 1984, Lahnsteiner et al. 1996). Previous research showed that *B. antarctica* does not have an aerobic compensatory mechanism for low temperatures (Lee and Baust 1982), as seen in some other species (Sommer and Poertner 2004, Siddiqui et al. 2006), therefore a loss of Krebs cycle activity is not surprising. Under these physiological circumstances, the frozen midge either has greatly reduced cellular energy requirements or is deriving its energy from a source other than aerobic respiration, most likely through glycolysis or gluconeogenesis. Considering the fact that this insect does not develop appreciably during the Antarctic winter (Sugg et al. 1983), the former explanation is more likely.

Oleic acid and dodecane were reduced during freezing in the Antarctic midge. Although it may be surmised that membrane restructuring and hydrocarbon deposition on the cuticle would result in alterations in the levels of these metabolites, the absence of any further changes in the fatty acid or hydrocarbon pool renders these conclusions uncertain.
Putrescine, norvaline, and nonanoic acid were also elevated in the midge in response to 6 h freezing at -10°C and presumably serve the same functions as noted during heat stress (see previous section).

4.4.3. Metabolic response to desiccation

The loss of 50% of the total body water in larvae changed the concentrations of a number of metabolites, but most of these changes were seen in either the heat or the freezing treatments, as well (Fig. 5). Similar to heat stress, desiccation caused the accumulation of glycerol-3-phosphate and nonanoic acid, but unlike heat stress this accumulation was coupled with an increase in tetradecane and octadecanoic acid. Both of these overall changes from heat and desiccation point towards shifts in lipid metabolism (membranes, surface hydrocarbons), although the species of long-chain lipid utilized is different for these two stresses. While tetradecanoic acid (14:0) was elevated by heat, octadecanoic acid (18:0) was elevated by desiccation. Accumulation of free fatty acids is correlated with desiccation survival in *Aedes egyptii* (Sawabe and Mogi 1999) but may also be a marker for cells that have endured membrane stress (Hoekstra et al. 2001). Further metabolic analysis using purified non-polar extracts would present a clearer picture of the changes in lipid metabolism of the Antarctic midge during desiccation.

The only amino acid that increased in response to desiccation was asparagine. The same response was observed in the blister beetle, *Cystodemus armatus* when subjected to desiccation (Cohen et al. 1986). Because *B. antarctica* did not alter levels of any other nitrogen storage or waste molecule, it is assumed that asparagine functions as an osmolyte during desiccation. In addition, aspartic acid decreased in response to desiccation, indicating that asparagine synthetase, the enzyme that regulates conversion from aspartate to
asparagine, favors asparagine synthesis during desiccation. Osmotic stress also regulates asparagine levels in plants (Braun and Fluckiger 1984, Kusaka et al. 2005) and crustaceans (Marangos et al. 1989).

Glycine and serine were reduced in response to desiccation. These two amino acids are synthesized in a complex enzymatic reaction that branches from the glycolytic intermediate, glycerol-3-phosphate. As determined in this study, desiccation increased glycerol-3-phosphate concentration in \textit{B. antarctica}, therefore one or more of the enzymes in the pathway of serine biosynthesis was inhibited during desiccation. To our knowledge, a reduction in serine or glycine has not been correlated previously with cellular survival to desiccation.

Unlike the multiple polyol upregulation seen in response to the freezing regime, the only detectable polyol that increased due to desiccation in \textit{B. antarctica} was glycerol. Glycerol protects membranes and proteins during desiccation in the same manner as during low temperature stress (see previous section). This important polyol protects cells against desiccation in plants (Eastmond 2004) and accumulates in response to desiccation in the flesh fly, \textit{Sarcophaga crassipalpis} (Yoder et al. 2005). An increase in glycerol content due to desiccation in \textit{B. antarctica} also has now been confirmed using an enzymatic assay (Benoit et al. 2007).

Desiccation in \textit{B. antarctica} larvae led to a reduction in free sulfate levels. Free sulfate participates in a number of biological functions, but the most notable is the involvement with the glutathione-S-transferase detoxification system. Reduction of free
sulfate in an environment of osmotic stress may also help to preserve membrane integrity if coupled with increases in other anions (Santarius 1986), but increases in other anions were not detected.

Desiccation, like freezing, elicited an accumulation of isocitric and succinic acid, indicating a general inhibition of aerobic metabolism. In addition, glucose levels dropped and the glycolytic intermediate, glycerol-3-phosphate, increased. Based on these two metabolite changes, it is likely that the glycolytic pathway is inhibited during desiccation, as well. The increase in glycerol levels may indicate that the gluconeogenic pathway remains active during desiccation. Because of the severity of the water loss (50% is enough to produce some mortality in *B. antarctica* larvae), it is difficult to determine whether the inhibition of central cellular metabolism is an adaptive response or indicative of a damaged physiological state.

4.4.4. *Summary*

GC-MS based metabolomics successfully identified a number of polyols, sugars, amino acids, and intermediates of cellular metabolism that were altered in response to heat shock, freezing, and desiccation in larvae of the Antarctic midge, *B. antarctica*. A Venn diagram summarizing all metabolic changes (Fig 5) shows that desiccation and freezing share the greatest number of elevated metabolites, but desiccation and heat shock share the greatest number of reduced metabolites. Principle Component Analysis and Heirarchal Clustering determined freezing and desiccation to be the most similar, most likely because both introduce osmotic stress. Nonanoic acid was the only metabolite elevated in response to all three stressors (Fig 5A), but, at this time, no function for this molecule can be surmised.
beyond a potential building block for synthesis of longer chain fatty acids. The same perplexity lies in the ubiquitous reduction of serine in response to all three stresses examined in this study.

Heat shock induced metabolites consistent with the GABA bypass (GABA, α-ketoglutarate). Freezing induced the production of multiple polyols (glycerol, erythritol, mannitol) and an inhibition of the Krebs cycle (isocitric acid, succinic acid). Frozen larvae also display evidence of shifting nitrogen equivalents (arparagine, urea) and active defense from oxidative stress (glycine). Desiccating the larvae reduced the activity of both the Krebs cycle (isocitric acid, succinic acid) and glycolysis (glycerol-3-phosphate, glycerol, glucose). All three stresses caused metabolites involved with lipid metabolism (glycerol-3-phosphate, tetradecanoic acid (14:0), oleic acid (18:1), octadecanoic acid (18:1), dodecane) to change, indicating that lipids play an important role in responding to these abiotic challenges. There appears to be no generalized response to abiotic stress in the Antarctic midge, but rather, the midge’s metabolic reaction to these stressors is highly-controlled and specifically tailored.

Acknowledgements: We are grateful to the staff at Palmer station for facilitating our work in Antarctica and to Richard Sessler of the Campus Chemical Instrument Center at The Ohio State University for guidance in the analysis of samples. This work was supported in part by grants OPP-0337656 and OPP-0413786 from the Polar Program of the National Science Foundation.
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Table 4.1. Selected metabolites identified in metabolomic samples from the Antarctic midge, *Belgica antarctica*.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Metabolic intermediates</th>
<th>Other metabolites</th>
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<tbody>
<tr>
<td>β-alanine</td>
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<td>2-butanoic acid</td>
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<tr>
<td>Asparagine</td>
<td>Cystathionine</td>
<td>2-methyl propanoic acid</td>
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<td>Fumaric acid</td>
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<td>Glutamine</td>
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<td>Malic acid</td>
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<td>Pyruvate</td>
<td>8-hydroxysclerodin</td>
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<tr>
<td>N-glycyl alanine</td>
<td>Succinic acid</td>
<td>α-glycerophosphoric acid</td>
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<td>Phosphonic acid</td>
<td>Metanephrine</td>
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<tr>
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<td>Free sulfate</td>
<td>Myo-inositol-1-phosphate</td>
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<td>Fructose</td>
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<td>Sorbitol</td>
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<td>Xylopyranose</td>
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<td>Fatty acids and hydrocarbons</td>
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<td></td>
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<td></td>
<td>Oleic acid (18:1)</td>
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<td>Tetradecanoic acid (14:0)</td>
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Figure 4.1. Metabolites elevated (A) or reduced (B) in larvae of the Antarctic midge, *Belgica antarctica* by 1 h heat shock at 30°C. GC-MS peaks of derivatized metabolites from whole-body extracts of larvae held at 4°C (white bars, untreated) were compared with peaks observed following heat shock (black bars). Four metabolites accumulated in response to heat shock while 3 amino acids and 3 other metabolites were reduced in abundance. All bars are means ± SD of five determinations. Substances listed here were significantly different beyond p=0.05 (ANOVA, p≤0.05, df=8).
Figure 4.2. Metabolites elevated (A) or reduced (B) in larvae of the Antarctic midge, *Belgica antarctica* by a 6 h freezing at -10°C. GC-MS peaks of derivatized metabolites from whole-body extracts of larvae held at 4°C (white bars, untreated) were compared with peaks observed following freezing (black bars). Twelve metabolites accumulated in response to freezing, including 3 amino acids, 3 polyols, 2 Krebs cycle intermediates, and 4 other metabolites. Four metabolites decreased. Substances listed here were significantly different beyond p=0.05 (ANOVA, p≤0.05, df=8). All bars are means ± SD of five determinations.
Figure 4.3. Metabolites elevated (A) or reduced (B) by desiccation (5 d at 98% RH, resulting in a 50% water loss). GC-MS peaks of derivatized metabolites from whole-body extracts of larvae held at 4°C (white bars, untreated) were compared with peaks from larvae held for 5 days at 98% RH (black bars). Eight metabolites increased in abundance, including 1 amino acid, 1 polyol, 3 Krebs cycle intermediates, 2 fatty acids, and 1 other metabolite. Six metabolites decreased (B). Substances listed here were significantly different beyond p=0.05 (ANOVA, p≤0.05, df=8). All bars are means ± SD of five determinations.
Figure 4.4. Multivariate analysis of metabolomic data derived from larvae of the Antarctic midge *Belgica antarctica* subjected to heat shock, freezing, and desiccation. Panel A illustrates that plotting the first two principle components of all samples of the stressed midges results in distinct treatment-dependent clustering along an orthogonal axis that includes both principle components (MetGenAlyse v. 1.7). Hierarchal clustering analysis (Panel B) demonstrates that the responses of the midge to cold and desiccation share the greatest similarity when compared to all other treatment types, and the clade containing these two stressors is more dissimilar to heat-shocked midges than it is to the untreated midges held continuously at 4°C.
Figure 4.5. Venn diagrams illustrating the overlap of elevated (above) and reduced (below) metabolites in response to three abiotic stressors in the Antarctic midge, *Belgica antarctica*. 

A. Elevated metabolites

- Heat (1 h 30°C)
  - tetradecanoic acid
  - α-ketoglutarate
  - GABA
- Cold (6 h -10°C)
  - alanine
  - urea
  - mannitol
  - glycine
  - erythritol
- Desiccation (50% water loss)
  - nonanoic acid
  - succinate
  - isocitric acid
  - glycerol
  - asparagine
  - tetradecane
  - octadecanoic acid

B. Reduced metabolites

- Heat (1 h 30°C)
  - glycercol
  - free phosphate
- Cold (6 h -10°C)
  - oleic acid
  - dodecane
- Desiccation (50% water loss)
  - serine
  - sorbitol
  - glycine
  - glucose
  - aspartate
  - free sulfate
Figure A.1. Survival of *Sarcophaga crassipalpis* injected with heat shock protein 23 (A) or heat shock protein 70 (B) double-stranded RNA. Flies during the wandering phase of larval development were injected with either 1 µl distilled water (dotted bars) or 1 µl of dsRNA derived from the sequence of the heat shock protein (black bars). The flies were allowed to develop into 7 day adults (25°C) and then exposed to experimental low-temperature treatments (CNT = no treatment [25°C], RCH = 2h at 0°C, CS = 20 min at -10°C, RCH + CS = 2h at 0°C followed by 20 min at -10°C). Survival was recorded after 24 hours of recovery at 25°C. The only treatment that showed significant difference from the water-injected sample were RCH+CS flies that had been injected with dsRNA from heat shock protein 23. Number above bars represent the total number of flies tested for each group in three separate trials. Bars represent the mean ± standard deviation for three separate trials. Asterisks represent significance beyond p<0.05 (t-test, df=4).
Figure A.1. Survival of *Sarcophaga crassipalpis* injected with heat shock protein 23 (A) or heat shock protein 70 (B) double-stranded RNA.
Appendix B

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Upregulated</th>
<th>Treatment</th>
<th>Sarcophaga probe</th>
<th>Northern Result</th>
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<td>3.37</td>
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Table B.1. Select genes detected as upregulated in microarrays of cold-shocked and rapid cold-hardened *Sarcophaga crassipalpis* and the results of Northern blot.
Figure C.1. Survival of cold-selected strains of *Sarcophaga crassipalpis* to cold shock (A) and rapid cold-hardening followed by a cold shock (B). Three colonies of flies that had been selected against a 1 h cold shock at -10°C (CS select) and three colonies of flies that had been selected against a 2 h cold shock at -10°C after rapid cold-hardening induction for 2 h at 4°C (RCH select) were compared to three colonies of flies that had not been selected (control) in their ability to survive a cold shock of 70 min at -10°C (A) or a 160 min cold shock after rapid cold hardening induction for 2 h at 4°C (B). After 10 rounds of selection (over 20 generations), both forms of selection (CS select and RCH select) produced flies with significantly (t-test, p<0.05, df=4) improved cold shock survival only if rapid cold-hardening is induced first. Selected colonies were tested for differences in weight, development, longevity, and sex ratio with no differences from unselected flies detected (data not shown in this text).
Appendix D

Figure D.1. Changes in fatty acid composition of phospholipids in *Belgica antarctica* following freezing (top panel) and desiccation/heat (bottom panel). Fatty acid methyl esters (FAMEs) isolated from larvae of *B. antarctica* held at 4°C were compared with FAMEs isolated from larvae frozen at -10°C for 6 h (frozen), frozen at 10°C for 6 h after being pretreated at -5°C for 2 h to induce rapid cold-hardening (RCH + frozen), desiccated at 98.5% RH for 6 d (desiccation), or heated to 30°C. All larvae were homogenized in a two-phase solvent and the phospholipids were isolated by solid-phase extraction and derivatized into fatty acid methyl esterases before measurement by gas chromatography-mass spectrometry. Peak areas of individual fatty acids (designated X:Y where X is the length of the hydrocarbon chain and Y are the number of double bonds) for all treatments were compared by ANOVA with a Tukey’s post-ANOVA t-tests (n=5, df=8, p<0.05). Letters above bars represent Tukey’s statistical groupings for each individual fatty acid. Bars represent mean ± standard deviation of 5 individual samples. From the data, it may be surmised that the delta-9 desaturase is involved in the restructuring of membranes due to many of these stress treatments, as evidenced by significant change in oleic acid (18:1[9]) levels and palmitoleic acid (16:1) levels. Only freezing affected linoleic acid (18:2), the most prominent fatty acid in this species.

(Figure on the following page)
Figure D.1. Changes in fatty acid composition of phospholipids in *Belgica antarctica* following freezing (top panel) and desiccation/heat (bottom panel).


Braun, S., Fluckiger, W., 1984. Increased population of the aphid *Aphis pomi* at the motorway 2: the effect of drought and deicing salt. Environmental Pollution Series A. Ecological and Biological 36, 261-270.


inhibitors, but not injury caused by calcium ionophores or oxidative stress. Hepatology 17, 91-98.


Rinehart, J.P., Yocum, G.D., Denlinger, D.L., 2000. Thermotolerance and rapid cold hardening ameliorate the negative effects of brief exposures to high or low


