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The sub-lethal effects of repeated cold exposure in insects

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The sub-lethal effects of repeated cold exposure in insects

(Thesis format: Integrated Article)

by

Katie Elizabeth <u>Marshall</u>

Graduate Program in Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

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Abstract

While insect cold tolerance has been well studied, the vast majority of work has focused on the effects of a single cold exposure. However, many abiotic environmental stresses, including temperature, fluctuate within an organism's lifespan. In this thesis I address two major questions. First, does frequency of cold exposure impose additional stress on insects? Second, how does this stress translate to performance and fitness? I first summarize the literature on the effects of repeated cold exposure in insects, critically examining experimental designs. I then address my questions experimentally using four insect species with contrasting life histories and responses to cold exposure. I examine the fitness costs of repeated cold exposure in the chill-susceptible fly *Drosophila melanogaster* by recording survival, generation time, and number of offspring in flies that had received five 2 h, one 2 h, or one 10 h exposure to -0.5 ℃. I found that *D. melanogaster* trades off immediate survival and reproductive output in response to repeated cold exposure. In the freeze-tolerant caterpillar *Pyrrharctia isabella*, repeated freezing did not deplete energy reserves, but did damage hemocytes and Malpighian tubules, and led to decreased survival in repeatedly-frozen caterpillars. Similarly, I found in the freeze-avoiding caterpillar *Choristoneura fumiferana* that frequency of exposure, independent of period, intensity, or duration of cold exposures significantly increased mortality, likely due to increased investment in cryoprotection at the expense of glycogen reserves. Finally, in the freeze-tolerant fly *Eurosta solidaginis*, the frequency of exposure, again independent of period, intensity, or duration of cold exposures led to a significant decline in egg production. Repeated cold exposure therefore imposes additional costs to insects, even when intensity and duration of cold are controlled for. This cost may either be a result of accrued damage, or may be energetic as individuals trade off investment in survival mechanisms for reproductive output. Given that many environments are not static, these effects indicate that investigating the effects of repeated stress exposure is important for understanding and predicting physiological responses in the wild.

Keywords

Insects, repeated stress, winter, freeze tolerance, cold tolerance, phenotypic plasticity

Co-Authorship Statement

A version of Chapter 2 is published in *Journal of Experimental Biology* with Dr. Brent Sinclair as a co-author. Dr. Sinclair contributed significantly to the ideas and discussion included in this chapter, and provided editorial comments on the manuscript.

A version of Chapter 3 is published in *Proceedings of the Royal Society B* with Dr. Brent Sinclair as a co-author. Dr. Sinclair aided in the design and provided funding and equipment for the experiment conducted in this chapter. Dr. Sinclair also contributed significantly to the ideas and discussion included in this chapter, and provided editorial comments on the manuscript.

A version of Chapter 4 is published in *Journal of Experimental Biology* with Dr. Brent Sinclair as a co-author. Dr. Sinclair aided in the design and provided funding and equipment for the experiment conducted in this chapter. Dr. Sinclair also contributed significantly to the ideas and discussion included in this chapter, and provided editorial comments on the manuscript.

Chapter 5 is currently in preparation for submission to *Journal of Animal Ecology* with Dr. Brent Sinclair as a co-author. Dr. Sinclair aided in the design and provided funding and equipment for the experiment conducted in this chapter. Dr. Sinclair also contributed significantly to the ideas and discussion included in this chapter.

Chapter 6 is currently in preparation for submission to *The American Naturalist* with Dr. Brent Sinclair as a co-author. Dr. Sinclair aided in the design and provided funding and equipment for the experiment conducted in this chapter. Dr. Sinclair also contributed significantly to the ideas and discussion included in this chapter.

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"…many places you would like to see are just off the map and many things you want to know are just out of sight or a little beyond your reach. But someday you'll reach them all, for what you learn today, for no reason at all, will help you discover all the wonderful secrets of tomorrow." – Norton Juster, The Phantom Tollbooth

Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

acTAG: acetylated triacylglycerol

AIC: Akaike's information criterion

AMP: Adenosine monophosphate

ANCOVA: Analysis of covariance

ANOVA: Analysis of variance

ATP: Adenosine triphosphate

AWG: American wire gauge

BHT: butylated hydroxytoluene

C.I.: confidence interval

CTmin: critical minimum temperature

DF: degrees of freedom

FFA: free fatty acids

FTR: fluctuating thermal regime

GFP: green fluorescent protein

Hsp: Heat shock protein

lcTAG: long-chain triacylglycerol

λmax_{em}: Maximum emission wavelength

λmax_{ex},: Maximum excitation wavelength

MANCOVA: Multivariate analysis of covariance

MANOVA: Multivariate analysis of variance

NAO: North Atlantic oscillation

PCA: Principle components analysis

r: Intrinsic rate of population increase

RCE: Repeated cold exposure

RCH: Rapid cold hardening

RFT: Repeated freeze-thaw

R_o: Reproductive output

SCP: Supercooling point

SE: Standard error

T_g: Generation time

TLC-FID: Thin-layer chromatography coupled to flame-ionization detection

Chapter 1

1   Introduction

1.1   Overview of thesis

Temperature varies on multiple temporal and spatial scales in terrestrial environments. Insects are integral parts of every terrestrial ecosystem on the planet and their abundance and distribution is shaped by temperature. In these systems, insects face the multiple stressors of winter, and adjust their life histories around this important season. During winter, cold temperatures and freezing can induce physiological damage, which can lead to mortality. Insects have a variety of biochemical and behavioural mechanisms to prevent and repair this damage. These mechanisms are elicited during both long-term seasonal acclimation and short-term rapid hardening responses to temperature changes. Following a low temperature event, there are measurable changes to an individual insect's physiology, which may have consequences for that individual's response to subsequent exposures. While responses to a single cold exposure are relatively well-explored, natural temperature regimes fluctuate, and individual insects are exposed to repeated cold exposures during their lifespan. In this thesis, I link physiological responses to fitness consequences of repeated low temperature stress in insects using four species that represent a broad range of life histories and physiological responses to cold. This integrative approach allows me to explore the mediators of the relationship between low temperature exposure and fitness.

In this introduction, I describe the physiology of insect cold hardiness, the climatology of temperature variation, and the costs and benefits of low temperature adaptation. I summarize that: 1) temperature is highly variable within an individual insect's lifetime, 2) low temperature impacts insect fitness through its effects on physiology, and 3) that insects rapidly respond to low temperature in both adaptive and maladaptive ways. Given these traits, I argue that understanding the effects of repeated cold exposure on ectotherms is an important aspect of thermal biology and may lead to better predictions of

insect population and range dynamics. I end by describing the structure of my thesis, and the study systems I used to explore the effects of repeated low temperature exposure.

1.2 Insect populations and life history in temperate environments

Insects dominate terrestrial ecosystems in terms of both biodiversity and abundance (Scheffers *et al.*, 2012). Their presence and activity impacts ecosystems on the landscape scale, regulating boreal forest dynamics (McCullough *et al.*, 1998) and soil nutrient cycling (Brussaard, 1998). Insects are also key providers of ecosystem services such as pollination, control of pest species, and decomposition, with one estimate placing the annual value of these insect-provided services at $57 billion a year in the United States (Losey and Vaughan, 2006). In addition, the introduction of invasive insect species such as the emerald ash borer *Agrilus planipennis* (Coleoptera: Buprestidae) and the gypsy moth *Lymantria dispar* (Lepidoptera: Ereibidae) has caused wide-spread damage through both direct effects such as herbivory of native plant species and indirect effects such as interspecific competition and spread of introduced diseases (Kenis *et al.*, 2008). Therefore understanding the ways that insect population dynamics are regulated is a key component to understanding whole ecosystem function.

In temperate environments, unfavorable conditions in the winter months require insects to adapt their life histories. Winter is a key driver of insect populations through its effects on temperature regimes. Many insects spend the winter in dormancy in characteristic life stages, and in many species overwintering survival is aided by the process of diapause. Due to the central role of winter in insect life histories, understanding the responses of insects to wintering conditions is an important aspect of predicting population effects under climate change.

1.2.1 The importance of winter to insects in temperate regions

Insects are found on every continent, and thus have had to adapt to the seasonality of these environments. Within temperate environments, winter is a season of stress and low resource availability, and insect life histories have therefore evolved to compensate for

the seasonality of temperate thermal regimes (Bradshaw *et al.*, 2004; Danks, 2006a; Schmidt *et al.*, 2005). Insects in temperate climates spend a large portion of their lives in dormancy during which growth and development cannot occur due to unfavourable conditions like reduced temperature and decreased food availability (Danks, 2006b; Hahn and Denlinger, 2011; Koštál, 2006; Leather *et al.*, 1995). These stresses have large effects on individual insects, and consequently populations.

As small ectotherms, the body temperature of insects varies directly with the temperature they experience, and so environmental temperature is of great importance for influencing survival, growth, and reproduction. Summer maximum temperatures do not vary greatly with latitude, but winter minimum temperatures do (Sunday *et al.*, 2011). In many species, the northern range limit is set by the minimum temperature experienced in a particular location while summer maximum temperatures are of lesser importance (Calosi *et al.*, 2010; Gray, 2008; Stahl *et al.*, 2006). Consequently, winter temperatures can drive species range edges. For example, the population growth rate of butterfly populations in the Northeastern United States is strongly influenced by the overwintering life stage, rather than by growing season characteristics such as host plant or habitat specialization (Breed *et al.*, 2012). Similarly, the date of spring snow melt explains the majority of variation in population growth in the butterfly *Speyeria mormonia* (Lepidoptera: Nymphalidae), (Boggs and Inouye, 2012). Therefore understanding the processes that drive individual fitness through the winter is an important aspect of understanding how species ranges are set.

1.2.2 Overwintering temperature regimes in temperate climates

Temperatures in temperate environments vary on multiple, interacting temporal scales that can have profound consequences on the physiology and fitness of the animals that encounter them. In addition, environments vary in their temperature predictability, which can alter the return time of low temperature exposures. Finally, the effects of air temperatures on insects are filtered through the microclimate that those insects experience, and can either buffer or exacerbate low temperature stress. These critical factors of temperature regimes drive the experiences of individual insects in their environments.

On very short time scales, diurnal fluctuations in air temperature in temperate environments can be 20 ºC or more (Irwin and Lee, 2003; Kelty and Lee, 2001; Storey and Storey, 1986). Insects can respond physiologically to temperatures on timescales of one hour or less through rapid cold-hardening (Lee *et al.*, 1987; Lee *et al.*, 2006; Michaud and Denlinger, 2006; Ransberry *et al.*, 2011). Thus it is clear that even brief fluctuations in temperature are ecologically relevant. On slightly longer time-scales, temperature regimes can be altered week-to-week by the occurrence of weather fronts which can alter the severity of diurnal extreme events (Stenseth and Mysterud, 2005). On annual time-scales, temperatures seasonally warm from winter to summer and back again predictably with changes in photoperiod, which induces insects to enter dormancy (Danks, 2005; Storey and Storey, 1986; Williams and Lee, 2005). Finally, on broader time scales, continent-wide patterns like the North Atlantic Oscillation (NAO) can alter seasonal and weather frontal patterns by either mitigating or exacerbating variability, which makes the NAO index a relatively good predictor of population shifts in some species (Stenseth and Mysterud, 2005). The temperature fluctuations that individuals experience are driven by broader climatic conditions that occur on multiple timescales.

Natural thermal regimes also vary in predictability, meaning that the time between stressful events can be more or less predictable for individual insects (Vasseur and Yodzis, 2004). In the Southern Hemisphere, environments are generally less predictable than in the Northern Hemisphere due to oceanic influences (Sinclair *et al.*, 2003a). Noise in environmental variables can be measured by the sum of variance at different frequencies of return time (Vasseur and Yodzis, 2004). "White" noise describes the situation when the variance of every frequency of return time is the same, while "red" noise is dominated by long-frequency cycles that are autocorrelated. In terrestrial systems, the variation in temperate environment minimum temperatures is generally red-shifted, meaning that current minimum temperature can help predict future minimum temperature (Vasseur and Yodzis, 2004). However, Vasseur and Yodzis (2004) measured predictability on a monthly scale, and smaller-scale variations in temperature can be important for insects. Depending on the environment, the predictability of these small-timescale fluctuations can differ substantially (Chown and Sinclair, 2010). This reduced predictability of warm, ocean-dominated temperature regimes in the Southern

Hemisphere has been suggested as an explanation for the predominance of freeze tolerant insect species in this environment (Sinclair *et al.*, 2003a). Predictability impacts the ability of insects to anticipate stressful conditions, which can have important consequences depending on the physiological status of the insect experiencing them.

Dynamic air temperatures can be dampened or exacerbated through the filter of microclimate. Overwintering insects can be found in a broad range of microhabitats such as under bark (Vermunt *et al.*, 2012b), in leaf litter beneath snow cover (Marshall and Sinclair, 2012), or in galls suspended above snow level (Irwin and Lee, 2003). Microclimate selection can have profound influences on the temperatures experienced by the insect during the overwintering period, and thus on the insect's physiology and subsequent fitness. For example, snow cover insulates the ground beneath, which both increases the mean temperature over the course of the winter and buffers the soil from temperature extremes (Henry, 2008; Marshall and Sinclair, 2012). These effects can work together to increase overall metabolic rate in insects that overwinter beneath the snow relative to individuals above the snow, which can manifest in smaller sizes and lower fecundity in the spring (Irwin and Lee, 2003; Marshall and Sinclair, 2012). Similarly, temperatures underneath tree bark are significantly impacted by aspect, with the north side of trees generally colder than the south side (Vermunt *et al.*, 2012a). Other factors such as shade, rock size, soil type, and slope can all influence the thermal regime and produce vastly different overwintering conditions for different species (Layne, 1991; Pincebourde and Woods, 2012; Sinclair, 2001a; Sinclair, 2001b; Sinclair *et al.*, 2013). Fluctuations in air temperatures in concert with the effects of microclimate determine the temperature stress that individual insects experience.

1.2.3 Diapause in northern temperate insects

To prepare for the adverse conditions of winter, many insects enter diapause: an adaptive, programmed interruption of morphological development with several phases during which a series of gradual physiological changes occur (Danks, 2006a; Koštál, 2006; Ragland *et al.*, 2011). The first step is the induction phase during which the reception of token stimuli such as photoperiod cue an individual to switch state from direct development to diapause (Bale and Hayward, 2010; Bradshaw *et al.*, 2004; Koštál, 2006).

Most species require the reception of these token stimuli, but many species have an obligate diapause whereby the diapause program is triggered by a particular life stage (Danks, 2006a). Induction frequently occurs well before the onset of adverse conditions (Tauber *et al.*, 1986).

Following induction, the diapause program is initiated (Koštál, 2006). The initiation phase includes a broad array of physiological adjustments that prepare the individual for the onset of winter. These adjustments may include suppression of metabolic rate (Williams *et al.*, 2012), accumulation of large quantities of energetic reserves (Hahn and Denlinger, 2011), production of compounds that aid in stress resistance (Rinehart *et al.*, 2007; Storey and Storey, 1986), and cessation of development (Koštál, 2006). This phase occurs well ahead of adverse conditions, in late summer or early fall (Tauber *et al.*, 1986). Diapause is then maintained, despite the occurrence of favourable development conditions, for weeks or months until termination (Ragland *et al.*, 2011; Régnière, 1990). In some species, this diapause stage can be maintained for years or decades, as in the yucca moth *Prodoxus y-inversus* (Lepidoptera: Prodoxidae) (Powell, 1989). After the reception of appropriate stimuli (which can include events such as chilling, shortened photoperiod, or depletion of energetic reserves), diapause is terminated (Koštál, 2006; Ragland *et al.*, 2011; Régnière, 1990). This phase frequently occurs in early or mid-winter, well before the cessation of adverse conditions (Hodek, 2002). The insect spends the remainder of winter in quiescence until favourable conditions re-occur and development can resume. Different insect species produce different physiological adaptations for winter as part of their diapause program, which then alters the effects of temperature regimes.

Insect species vary in the number of generations each year, and as a result are more or less sensitive to environmental cues to time their diapause. Species such as the pitcher plant mosquito *Wyeomyia smithii* (Diptera: Culicidae) are bivoltine or multivoltine (Bradshaw *et al.*, 2004), passing through multiple generations every year. These species are poised to receive environmental cues such as photoperiod that force one of the generations to halt development and prepare for the low temperature stress of winter (Hodek, 2002). Other species such as the eastern spruce budworm *Choristoneura*

fumiferana (Lepidoptera: Tortricidae) are univoltine (Han and Bauce, 1998), relatively inflexible in their development, and pass each winter in a dormant state in a particular life stage (Régnière, 1990).

The life histories and overwintering stage of individual species helps determine their response to overwintering. Insects can overwinter in every life stage from egg to adult, but which life-stage overwinters is highly species-specific (Sinclair *et al.*, 2003a). Depending on the life stage that a species overwinters in, the life history consequences can greatly vary. For example, species that overwinter in later life stages such as pupae or adults must conserve nutrients acquired in the larval stage for use in the spring, while species that overwinter as eggs or early-instar larvae have the opportunity to replace overwintering nutrient consumption in the spring (Hahn and Denlinger, 2011). Therefore, studies of the effects of low temperatures in winter must incorporate knowledge about individual life histories.

1.2.4 Predicting insect population dynamics and distribution under climate change

The central role of winter in the ecology of temperate insects is especially important to consider in the context of climate change. Over the past 40 years, winter temperatures in northern regions have warmed by 2-4 ℃, while summer temperatures have increased much less (Serreze *et al.*, 2000; Soja *et al.*, 2007). Consensus model predications indicate winter will warm by 5-7 ℃ in northern temperate regions by 2099, with summers warming by only 3-4 ℃ (IPCC, 2007). In addition to warming, the occurrence of extreme low temperature events and minimum temperatures is also being impacted (Easterling *et al.*, 2000; Kodra *et al.*, 2011). This is further complicated by changes in precipitation patterns, which alter snow cover, and have led to a general increase in the number of soil-freeze thaw cycles over the past 50 years in Canada (Henry, 2008). These temperature changes have led to increases in voltinism in European butterflies (Altermatt, 2010), as well as documented northward range shifts in scores of terrestrial ectotherms (Sunday *et al.*, 2012). These dire predictions of winter-biased change, combined with the impact that winter already has on insect populations, indicates the importance of

understanding how insects respond to winters, and will be a critical part of predicting range shifts in insect populations.

The effects of climate change are seen on broad geographical and temporal scales, and understanding the resultant effects on insect populations must be similarly broad. In recent years there has been a call to reunify the fields of macroecology and physiology through the establishment of a macrophysiology that seeks to understand the variation of physiological traits over broad geographical scales (Chown and Sinclair, 2010; Gaston *et al.*, 2009). Some of the work in this field has attempted to address the question of how species live where they do by correlating laboratory-measured thermal tolerance of ectotherms with range extent (Addo-Bediako *et al.*, 2000). In the case of low temperature tolerance, two studies have found that many species ranges extend into regions that are colder than those species can tolerate in lab-based tests (Sánchez-Fernández *et al.*, 2012; Sunday *et al.*, 2012). It is difficult to ascertain from these types of studies whether this is a result of microclimate buffering, the capacity of these species to increase their cold tolerance, or inadequate laboratory measurement of potential plasticity in the field.

Predictive models of species ranges under climate change fall into two categories: bioclimatic envelope models and mechanistic eco-physiological models. Bioclimatic envelope models correlate species' presence and abundance data with measures of climatic variables within a species' range (Chevin *et al.*, 2010). Predicted changes in climatic variables are then used to extrapolate from the current range extent to a predicted distribution under climate change. These models can be extremely complicated and integrate large quantities of information about the realized niche of a species (Gray, 2008), but they do not take into account information about the physiological mechanisms that set range boundaries. They assume that the processes that set range limits remain inflexible, and they may have poor predictive power in novel climates (Buckley *et al.*, 2010; Kearney and Porter, 2009).

Mechanistic models, by contrast, are built from the "ground-up": they incorporate laboratory-gathered data about thermal limits and reaction norms with climatic data to

predict population growth and persistence (Kearney and Porter, 2009). These models may offer more robust predictions in novel temperature conditions such as climate change since they do not rely on current temperature regimes (Buckley *et al.*, 2010; Kearney and Porter, 2009). In addition, mechanistic models can incorporate fine-scale temporal variability (Régnière *et al.*, 2012), and explicit tests of the effects of these small fluctuations have shown they can have large effects on model outputs (Kearney *et al.*, 2012; Marshall and Sinclair, 2012; Williams *et al.*, 2012). However mechanistic models generally do not incorporate the plasticity in physiological parameters caused by seasonal acclimation or RCH (but see Régnière *et al.*, 2012; Williams *et al.*, 2012).

Modelling species' development times and population growth is not a purely academic exercise. This type of work plays a central role in decision making for management of pest species in the agriculture and forestry industries (Knight, 2007; Régnière *et al.*, 2012). To provide more accurate predications of population dynamics under climate change, investigating the fitness impacts of and physiological responses to repeated cold exposure is an important first step to more accurate modelling.

1.3   Physiological effects of low temperatures

Through the winter months, decreasing temperature induces a range of direct and indirect effects on insect physiology. During the occurrence of low temperature episodes, there is the potential for freezing to occur, which imposes additional stressors. Winter is also a time of low nutrient availability, so insects frequently fuel overwintering metabolism on the energetic resources that they gathered through the growing season. Since metabolic rate is directly dependent on body temperature, increased temperatures can increase resource use. Both low and high temperatures during overwintering can therefore be stressors for insects.

1.3.1 Cold-induced damage

Since insect body temperature lowers linearly with decreasing environmental temperature, rates of chemical reactions within their bodies decline with decreasing environmental temperature following Arrhenius kinetics (Hochachka and Somero, 2002). This temperature dependence has important consequences for insects at low

temperatures: the biological activities of important macromolecules such as phospholipids and enzymes slow in cold conditions. At low enough temperatures, this can disrupt the function of these macromolecules, and lead to chill coma and chilling injury (Chen and Walker, 1994; Lee, 2010; MacMillan and Sinclair, 2011a). Chill coma is the reversible cessation of movement in an insect that occurs when temperatures drop (MacMillan and Sinclair, 2011a), and the temperature this occurs at (the CT_{min}) is plastic on both evolutionary (Huey, 2010) and individual (Ransberry *et al.*, 2011) time-scales. Chilling injury is the damage, unrelated to freezing of water, induced by low temperature exposure, and may be divided into two categories based on the speed of damage accumulation (Lee, 2010). Direct chilling injury occurs on the scale of minutes, while indirect chilling injury may take days or weeks to accumulate. Damage caused by either type of chilling injury usually accumulates exponentially over time, with increasing intensity, or with the interaction between the two (Nedvĕd *et al.*, 1998).

The mechanisms of both direct and indirect chilling injury remain under active investigation (Koštál and Tollarová-Borovanská, 2009; Lee, 2010; MacMillan and Sinclair, 2011b; Powell and Bale, 2004). Proteins can be denatured following low temperature exposure, such as in Antarctic notothenoid fish (Todgham *et al.*, 2007). Similarly, membrane fluidity decreases at lower temperatures, which can result in disrupted cell homeostasis (Chown and Terblanche, 2007; Hochachka and Somero, 2002; Koštál *et al.*, 2007a). Together, cold inactivation or denaturation of membrane proteins and decreased membrane fluidity can severely disrupt ion homeostasis (Koštál *et al.*, 2004; Koštál *et al.*, 2007a; MacMillan and Sinclair, 2011a). This disruption of ion homeostasis may cause neuromuscular damage or loss of function. For example, cold shock damages behaviour (and likely nervous system tissue) in the flesh fly *Sarcophaga crassipalpis* (Diptera: Sarcophagidae), (Yocum *et al.*, 1994). At low temperatures, antioxidant enzymes can be less effective and allow damage from reactive oxygen species to accumulate, causing oxidative damage (Lalouette *et al.*, 2011; Rojas and Leopold, 1996). Cold-induced oxidative damage is problematic for many biologically important macromolecules. For example, in the intertidal mussels *Mytilus galloprovincialis* and *M. californianus* (Mytiloidea: Mytilidae), low temperature exposure causes both single and double-stranded breaks in DNA (Yao and Somero,

2012). Similarly, low temperature exposure can oxidize the double bonds within unsaturated lipids within living insects (Lalouette *et al.*, 2011). There is much less known about the mechanisms of indirect chilling injury. This type of chilling injury takes much longer to accrue, and is often associated with mortality that occurs much later in development (Rojas and Leopold, 1996). While many of the mechanisms appear to be similar between direct and indirect chilling injury, selection for survival of direct chilling injury in *Drosophila melanogaster* (Drosophila: Drosophilidae) does not confer resistance to indirect chilling injury (Chen and Walker, 1994). Despite the ongoing research in this area, the majority of studies have only focused on the physiological effects of a single exposure to low temperature (Chown and Sinclair, 2010).

1.3.2 Freezing-induced damage

Pure water is capable of supercooling (cooling below its melting point without the nucleation of ice) to -40 ℃ (Zachariassen and Kristiansen, 2000; Zachariassen *et al.*, 2004). Due to their small size and high concentration of solutes, even very cold-intolerant insects do not risk freezing until well below 0 ℃, despite having relatively high melting points (Lee, 2010; Lee and Costanzo, 1998; Renault *et al.*, 2002; Strachan *et al.*, 2011; Zachariassen, 1985). Ice forms in insects in one of two ways: either by heterogeneous or homogeneous nucleation (Zachariassen, 1985; Zachariassen *et al.*, 2004). Homogeneous nucleation occurs when, at low enough temperatures, water molecules aggregate spontaneously into a lattice structure. By contrast, heterogeneous nucleation occurs when water molecules aggregate around another substance (Zachariassen *et al.*, 2004). Regardless of the method of nucleation, ice formation in insects can cause lethal damage to biological tissues.

Formation of ice crystals can induce mechanical damage to membranes and proteins (Storey and Storey, 1988). In addition, ice is often nucleated extracellularly (Zachariassen and Kristiansen, 2000), and as liquid water joins the growing ice lattice, both intra and extracellular solutes concentrate (Lee, 2010). This concentration increase can also denature proteins which disrupts metabolism. Reduction of cell volume in response to efflux of cellular water content can also induce additional damage to cytoskeleton and membranes (Storey and Storey, 1988). These effects can significantly

disrupt ion homeostasis (Boardman *et al.*, 2011), and damage nervous tissue (Collins *et al.*, 1997). In addition, animals are hypoxic while frozen (Joanisse and Storey, 1996). During thawing, reperfusion of oxygen can generate reactive oxygen species, and therefore insects and may face oxidative damage during reperfusion during thawing (Storey and Storey, 2010). Despite study of these effects, in insects that can survive freezing it remains unclear which process results in the most damage: the freezing event itself, the physiological effects of remaining frozen, or the events during thawing.

1.3.3   Overwintering energetics

Another important stress during overwintering is energetic drain. Nutrient resources are usually unavailable through the winter months due to the senescence of plant tissues in temperate environments as well as the dormancy of prey species. As a result, insects must overwinter on the lipid and carbohydrate resources they have stored during the growing season (Hahn and Denlinger, 2011). Since body temperature is directly linked to environmental temperature, and metabolic rate is directly related to body temperature, temperature regimes can therefore greatly alter the nutrient usage of overwintering insects. For example, increased overwintering temperature raises the metabolic rate of *Eurosta solidaginis* (Diptera: Tephritidae), which results in fewer eggs per individual in the spring (Irwin and Lee, 2003). Many species deeply depress their metabolic rates during diapause and thereby reduce energetic demand (Hahn and Denlinger, 2011). In addition to the effects of increased mean temperature, increased variation in temperature can also increase metabolic rate due to the nonlinear relationship between metabolic rate and temperature ("Jensen's Inequality"; Ruel and Ayres, 1999). As a result, some species have adapted by reducing the sensitivity of their metabolic rate to temperature (Williams *et al.*, 2012). While the predominant overwintering energy source for insects is triacylglycerols (Hahn and Denlinger, 2011), some species such as *C. fumiferana* fuel overwintering metabolism with glycogen reserves (Han and Bauce, 1998). Since carbohydrate cryoprotectants such as glycerol and sorbitol are synthesized from glycogen (Storey and Storey, 1983), this could lead to a conflict between the need to conserve glycogen and the need to synthesize cryoprotectants in these species (Sinclair *et al.*, 2013).

1.4   Insect cold hardiness

Insect responses to cold exposure can be grouped into several broad categories based on the relationship between their lower lethal temperature and supercooling point (SCP; the temperature at which the supercooling of an insect's body fluids stops Lee, 2010; Sinclair, 1999). Species that have lower lethal temperatures significantly higher than their SCP are "chill-susceptible". Species that are killed by ice formation, but not otherwise injured by chilling are "freeze avoiding", and species with lower lethal temperatures lower than their SCP that can survive freezing are "freeze tolerant" (Lee, 2010; Sinclair, 1999). Most cold-hardy species spend the winter exhibiting one of these responses to cold, although individuals can alter their response from season to season (Williams and Lee, 2005), and species can switch strategies from freeze tolerance to freeze avoidance on a year-to-year basis (Régnière and Bentz, 2007). Despite differences in the ability to survive cold and freezing, insects in each category utilize similar molecular mechanisms of low temperature tolerance.

1.4.1 Insect responses to low temperature

Chill susceptible species die of chilling injury unrelated to freezing. These species include well-known species such as *D. melanogaster*, the fall field cricket *Gryllus pennsylvanicus* (Orthoptera: Gryllidae), and the active life stages of most insects (Addo-Bediako *et al.*, 2000; MacMillan and Sinclair, 2011b; Strachan *et al.*, 2011). Based on a phylogeny of *Drosophila* species, Strachan *et al.* (2011) concluded that chill-susceptibility is likely the basal state for *Drosophila* larvae, and it may be for insects generally. By contrast, freeze avoidant species such as the emerald ash borer *A. planipennis* (Crosthwaite *et al.*, 2011) and the gall moth *Epiblema scudderiana* (Lepidoptera: Tortricidae), (Joanisse and Storey, 1996) can survive very low temperature exposure (<-20 ℃) as long as they remain unfrozen (Bale and Hayward, 2010). These species have a suite of adaptations that enhance their ability to supercool and increase their ability to survive chilling (Lee, 2010). By contrast, freeze tolerant species such as the woolly bear caterpillar *Pyrrharctia isabella* (Lepidoptera: Arctiidae), (Layne *et al.*, 1999) can survive freezing of their body water. These species promote the nucleation of internal ice at relatively high temperatures (*ca.* -8 ℃), which is believed to aid the control

and speed of ice formation (Lee, 2010; Sinclair *et al.*, 2009). Generally, tolerance of internal ice formation is restricted to extracellular freezing only, although a very few species such as *E. solidaginis* can survive intracellular freezing of specific cell types (Sinclair and Renault, 2010). Freeze tolerance has evolved independently in many insect lineages, including Diptera, Lepidoptera, and Coleoptera, leading to the suggestion that it is a derived state in insects (Sinclair *et al.*, 2003a; Vernon and Vannier, 2002).

The broad overlap in biochemistry between species that are freeze tolerant and freeze avoiding, the frequent evolution of freeze tolerance in the insect lineage, and the fact that freeze tolerant and freeze avoiding species can overwinter in identical microhabitats (Churchill and Storey, 1989) produces a puzzle: what constrains a species to be freeze tolerant or avoiding? There are several hypotheses that address this problem. It is unlikely that phylogenetic constraint is the solution, since freeze tolerance has evolved independently in several insect lineages (Sinclair *et al.*, 2003a; Vernon and Vannier, 2002). Voituron *et al.* (2002) produced an energetics model predicting the most adaptive strategy, and found that the freeze avoiding strategy is favoured when pre-winter energy stores are high and the cost of supercooling is low. By contrast, freeze tolerance is favoured when the pre-winter energy level is low and when prolonged freezing and freeze-thaw cycles are not overly stressful. Evidence for this explanation is mixed. The metabolic rates of many freeze tolerant species are significantly lower when frozen, which also reduces their rate of water loss (Irwin and Lee, 2002; Sinclair *et al.*, 2004). However, freeze-thaw can induce substantial costs in freeze tolerant animals by damaging gut tissues such as in the sub-Antarctic caterpillar *Pringleophaga marioni* (Lepidoptera: Tineidae), (Sinclair and Chown, 2005a), and increasing metabolism during freezing and thawing processes in the frog *Rana sylvatica* (Anura: Ranidae), (Sinclair *et al.*, 2013). Another explanation centers on the significantly increased frequency of freeze tolerant species in the Southern Hemisphere relative to the Northern Hemisphere (Sinclair *et al.*, 2003a). It has been suggested that freeze tolerance is more prevalent in Southern Hemisphere conditions due to the unpredictability of the risk of nucleation, while in the Northern Hemisphere freeze tolerance is advantageous during very low temperature episodes of long duration (Sinclair and Chown, 2005b). Despite these investigations, it remains unclear why freeze tolerance or avoidance might be favoured.

1.4.2 Biochemical mechanisms of low temperature survival

Insects have evolved a suite of biochemical adaptations to survive low temperatures. These can be divided into two groups by molecular weight. First, the low molecular weight carbohydrate or polyol cryoprotectants are frequently seasonally accumulated in large quantities. The amino acid proline has recently been identified as another important low molecular weight cryoprotectant. Secondly, several classes of proteins are important in cold and freeze tolerance. Heat shock proteins can be upregulated following heat shock, and aid in stabilizing enzymes at low temperature. Antifreeze proteins either prevent freezing or reduce recrystallization following freezing. Ice nucleating proteins are important for controlling the initiation and spread of ice. In addition, there are several enzymes that may repair or prevent other forms of cold or freezing injury such as dehydrins and aquaporins. Finally, lipid compounds are receiving attention as potential cryoprotectants. This broad array of mechanisms together allows insects to survive very cold temperature conditions.

1.4.2.1 Cryoprotectants

The most commonly-explored mechanism for survival at low temperatures is the production of low molecular weight cryoprotectants. These may be polyols (*e.g.* glycerol and sorbitol), or sugars (*e.g.* trehalose), which are usually synthesized from glycogen stores (Lee, 2010; Storey and Storey, 1983). These compounds are highly soluble in water, are generally of low toxicity when accumulated in large quantity, and relatively efficiently synthesized (Storey and Storey, 1990a). These cryoprotectants can reach extremely high concentrations mid-winter (as much as 6 M, Sformo *et al.*, 2010; Zachariassen, 1985), and are generally produced in the early fall well before low temperature exposure (*e.g.* Crosthwaite *et al.*, 2011; Storey and Storey, 1986), and are recycled back into glycogen in the spring or directly metabolized (Storey and Storey, 1990b). Some species, such as *E. solidaginis* have dual cryoprotectant systems whereby they accumulate one component seasonally, and a second following low temperature exposure (Koštál *et al.*, 2007b; Storey and Storey, 1983). Almost all cold-hardy insects in every category of cold hardiness accumulate these cryoprotectants, although their roles vary depending on the insect's response to cold. In freeze avoiding insects, accumulation

of cryoprotectants decreases the amount of freezable water, and colligatively depresses the SCP by almost double the colligative depression of melting point (1.86 °C/Osm, Zachariassen, 1985). In freeze tolerant insects, increased supercooling capacity due to accumulation of cryoprotectants is unlikely due to the retention of ice nucleators. In these species, cryoprotectants serve to depress melting point, which helps reduce the amount of ice formed at a given temperature (Lee, 2010). In addition, cryoprotectants prevent osmotic stress during freezing by reducing the amount of water that flows out of cells into extracellular ice thereby minimizing cell volume changes, and bind to proteins and cell membranes producing a hydration shell to prevent damage (Lee, 2010; Storey and Storey, 1988). Even in relatively small quantities (~0.25 M), carbohydrate and polyol compounds can still impart cryoprotection (Koštál *et al.*, 2007b).

The most well-studied cryoprotective molecules are low molecular weight polyols or sugars, but there have been several studies that show the importance of free amino acids (particularly proline) in freeze tolerance (Storey, 1997). For example, the free amino acid pool significantly increases in the winter in the freeze tolerant alpine weta *Hemideina maori* (Orthoptera: Anostostomatidae), and this is largely a result of accumulation of proline (Ramløv, 1999). Similarly, feeding freeze tolerant larvae of *Chymomyza costata* (Drosophila: Drosophilidae) proline-enriched diet significantly increases survival at -196 °C (Koštál *et al.*, 2011). Most remarkably, freeze tolerance was induced in larvae of the chill susceptible *D. melanogaster* by subjecting them to fluctuating temperatures and feeding them free proline (Koštál *et al.*, 2012). Proline is believed to aid in freeze tolerance by stabilizing cell membrane phospholipid head groups (Storey, 1997), or by promoting vitrification (the solidification of water into an amorphous, rather than crystalline, solid, Koštál *et al.*, 2011).

Another group of putative cryoprotective molecules are the heat shock proteins. First described in *D. melanogaster* in response to heat stress, these proteins act as molecular chaperones and bind to other proteins, helping prevent stress-induced denaturation (Lindquist and Craig, 1988). Individuals of many insect species significantly upregulate heat shock proteins such as Hsp70, Hsp90, and small heat shock proteins during

diapause, suggesting that they be helpful for surviving low temperature stress (Rinehart *et al.*, 2007). In addition, the freeze tolerant Antarctic midge *Belgica antarctica* (Diptera: Chironomidae) significantly upregulates *hsp70* expression in response to repeated freeze-thaw cycles, suggesting a cryoprotective role for Hsp70 (Teets *et al.*, 2011). However this response is not universal—for example the freeze tolerant gall fly *E. solidaginis* does not upregulate heat shock proteins in response to low temperature stress (Lee *et al.*, 1995). In addition, a few species appear to upregulate small heat shock proteins (<30 kDa) during diapause (Joplin *et al.*, 1990; Yocum *et al.*, 1991). While these proteins may also function as chaperones, it has been suggested that small heat shock proteins may function to arrest the cell cycle during periods of low temperature stress (Joplin *et al.*, 1990).

1.4.2.2 Control of ice

Control of the initiation of ice crystallization is essential for both freeze tolerant and freeze avoiding species (Zachariassen and Kristiansen, 2000). This requires precise control by the insect over the retention of substances that can nucleate ice: removing them in the case of freeze avoiding insects, and retaining or producing them in the case of freeze tolerant insects (Duman, 2001). Gut contents can also nucleate ice, and therefore are seasonally cleared by freeze avoiding insects (Zachariassen, 1985). Freeze avoiding species may also prevent inoculation by external ice by the accumulation of cuticular waxes (Crosthwaite *et al.*, 2011), avoiding contact with water by behavioural means (Larsen and Lee, 1994), or reducing the volume of freezable water in their bodies (Han and Bauce, 1993). By contrast, nucleation is adaptive in freeze tolerant insects, ensuring that ice nucleation happens extracellularly and at a high enough temperature to promote slow freezing (Duman, 2001; Sinclair *et al.,* 2009; Zachariassen and Kristiansen, 2000). Some insects are only freeze tolerant when inoculated by ice crystals (Gehrken *et al.*, 1991). Nucleation is induced by both the fat body cells as well as calcium phosphate spherules found in the Malpighian tubules in the freeze tolerant *E. solidaginis* (Mugnano *et al.*, 1996). Nucleation can also be promoted by proteins. These proteins can be synthesized by the insects themselves as in the case of the freeze tolerant queens of the hornet *Vespula maculata* (Hymenoptera: Vespidae), (Duman *et al.*, 1984), or by bacteria

such as *Pseudomonas syringae* (Pseudomonadales: Pseudomonadaceae), (Widehem and Cochet, 2003), and can nucleate ice at relatively high temperatures.

Another group of proteins interact with water and ice, binding to ice crystals to keep them small, stabilizing supercooling solutions, or preventing inoculation (Duman, 2001). These "antifreeze proteins" are also known as thermal hysteresis agents, since they lower the freezing point of solutions below the melting point in a non-colligative manner (Duman, 2001). They are found in a broad range of organisms, including fish, bacteria, plants, and several groups of terrestrial arthropods (reviewed in Duman, 2001). In insects, these proteins are extremely effective, producing thermal hysteresis between 5-10 ºC in the case of the spruce budworm *C. fumiferana* (Tyshenko *et al.*, 1997). Antifreeze proteins, while very diverse among taxa, generally are relatively small, threonine- and cysteine-rich in insects, and may function by adsorbing to ice crystal surfaces, preventing their growth (Duman, 2001). Antifreeze proteins may be produced by freeze avoiding species like *C. fumiferana* to prevent inoculation (Tyshenko *et al.*, 1997; Zachariassen and Husby, 1982), or more rarely by freeze tolerant species such as the beetle *Dendroides canadensis* (Coleoptera: Pyrochoidae), (Duman *et al.*, 1998) to prevent recrystallization after freezing. Antifreeze proteins can also increase low temperature survival in freeze tolerant animals (Tursman and Duman, 1995). The roles of antifreeze proteins are diverse, but significant for low temperature survival.

1.4.2.3   Other cryoprotective molecules

In addition to these major groups of molecules, several key classes of proteins have been implicated in response to cold stress. Aquaporins are membrane proteins that control the flow of water in and out of cells. An important component of freeze tolerance is the control of water leaving the cell and glycerol entering. Insect aquaporins have been investigated for their ability to facilitate this transport in relation to freeze tolerance. The function of water transport is essential for survival of freezing in the larvae of the rice stem borer *Chilo suppressalis* (Izumi *et al.*, 2006). Aquaporins that are seasonally regulated and aid in water transport during freezing have been reported from the freeze tolerant flies *E. solidaginis* and *B. antarctica* (Goto *et al.*, 2011; Philip *et al.*, 2011). The

role of dehydrins, proteins that aid in retaining water, during overwintering is now also beginning to be elucidated as well (Lee, 2010).

In addition, modifications to lipids or novel lipid compounds can also increase cold tolerance. Many species of insect desaturate fatty acid components of triacylglycerols and phosopholipids to increase fluidity at low temperature (Bennett *et al.*, 1997; Michaud and Denlinger, 2006; Pruitt and Lu, 2008). In addition, a non-protein antifreeze consisting of a sugar backbone and fatty acids that can produce thermal hysteresis of 3.7 °C was described from the Alaskan beetle *Upis ceramboides* (Coleoptera: Tenebrionidae), (Walters *et al.*, 2009), and has since been found in many other taxa (Walters *et al.*, 2011). Finally, an unusual class of lipid (acetylated triacylglycerols) is accumulated in large quantities by *E. solidaginis* prior to overwintering and appears to be related to intracellular freeze tolerance (Marshall *et al.*, submitted).

1.4.2.4   Costs of cold tolerance

Insects reorganize their biochemistry and life history around adapting to the cold, and the mechanisms required for surviving low temperature exposure are energetically costly. For example, both the synthesis and degradation of glycerol are ATP-demanding, and thus investment into high glycerol concentrations represents a net energetic loss (Storey, 1997). Similarly, the costs of Hsp synthesis have been extensively investigated in both natural and genetically-manipulated *D. melanogaster*, and larvae with increased expression of Hsps have reduced growth rates (reviewed in Feder and Hofmann, 1999). Other potentially costly mechanisms include repair of the disruption of ions following low temperature (MacMillan *et al.*, 2012) or freezing exposure (Boardman *et al.*, 2011). By contrast, it appears that insects have suppressed metabolism following cold and freezing events (Layne and Leszczynski, 2008; MacMillan *et al.*, 2012; Sinclair *et al.*, 2004), and metabolic rate of frozen animals is generally greatly depressed (Irwin and Lee, 2002; Sinclair *et al.*, 2013). These savings can greatly impact the size and fecundity of freeze tolerant insects following overwintering (Irwin and Lee, 2003; Marshall and Sinclair, 2012). Understanding the costs and benefits of cold hardiness strategies are particularly pertinent in the case of insects overwintering since feeding to replenish

depleted energy stores is usually impossible. In addition, these considerations are particularly important for those species that overwinter in later life stages that do not have the opportunity to feed following overwintering.

1.4.3 Plasticity of insect cold tolerance

Individual insects respond to the multiple temporal scales of temperate fluctuations in temperate environments by modifying their cold hardiness. These modifications are generally organized into two categories. The first is the longer-term acclimatization that happens as part of the diapause program and prepares the insect for a prolonged period of stress. The second, called "Rapid Cold Hardening" (RCH), happens on very short time-scales. Plastic responses in anticipation of low temperature are reliant on the previously-described biochemical mechanisms.

Seasonal acclimatisation to low temperatures includes mechanisms at many scales of biological organization. At the whole animal level, these preparations include suppression of metabolism to conserve energy through the winter (Williams and Lee, 2005; Williams *et al.*, 2012) and depression of supercooling point in freeze avoiding species (Crosthwaite *et al.*, 2011). At a biochemical scale, these include increases in cryoprotectant concentrations (Han and Bauce, 1995), upregulation of heat shock proteins (Rinehart *et al.*, 2007), and remodelling of lipid membranes to increase fluidity (Pruitt and Lu, 2008). Seasonal acclimation responses can also be induced in many species by shortening photoperiod and gradually reducing temperature (Hoffmann, 1995; Hoffmann *et al.*, 2003; Wang *et al.*, 2006). These seasonal shifts in cold hardiness are essential for survival in cold conditions.

Insects can also increase their cold hardiness over very short time-scales (30 min - 2 h). First described in the flesh fly *S. crassipalpis,* the rapid cold hardening (RCH) response is the ability of insects subjected to a brief low temperature exposure to rapidly increase their hardiness to subsequent cold exposures (Lee *et al.*, 1987). Since its discovery, RCH has been described in a broad range of both diapausing and non-diapausing insects, including *D. melanogaster* (Czajka and Lee, 1990) and freeze tolerant insects such as *B. antarctica* (Lee *et al.*, 2006). RCH can also be induced at ecologically-relevant cooling

rates and in the field in *D. melanogaster* (Kelty and Lee, 1999; Overgaard and Sørensen, 2008; Sinclair *et al.*, 2003b). As a result, the RCH response likely plays an important role in natural conditions. The mechanisms of RCH remain under active investigation. Induction of additional cryoprotectant synthesis may be important (Lee and Denlinger, 2010; Overgaard *et al.*, 2007; but see MacMillan *et al.*, 2009). Membrane remodelling to increase fluidity (including desaturation of fatty acid tails and swapping of phospholipid head groups) may also aid survival of subsequent exposures (Michaud and Denlinger, 2006; Overgaard *et al.*, 2006; but see MacMillan *et al.*, 2009). Finally, additional heat shock protein may be synthesized (Lee and Denlinger, 2010).

Both RCH and acclimation increase cold hardiness, but there is evidence that they do so through differing mechanisms. RCH can increase freeze resistance even in acclimated *B. antarctica* (Lee *et al.*, 2006), and RCH is effective at increasing cold tolerance in *Drosophila* species where cold acclimation is not (Strachan *et al.*, 2011). In addition, the benefits of acclimation and RCH have been found to work additively in *D. melanogaster* (Rajamohan and Sinclair, 2009; Shintani and Ishikawa, 2007). Inhibition of protein synthesis decreases basal resistance to cold shock in *D. melanogaster*, but does not reduce the ability to rapidly cold-harden (Misener *et al.*, 2001). Similarly, RCH does not appear to come at a fitness cost, although seasonal acclimation does (Powell and Bale, 2004). Taken together, it appears that insects operate with dual systems for increasing cold hardiness: a long-term acclimation and a short-term hardening response.

1.5 Temperature fluctuations, trade-offs, and the importance of repeated cold stress

The mechanisms, patterns, and processes of insect cold hardiness have been explored for decades. However, the majority of studies of insect cold hardiness have focused on the effects of a single cold exposure or basal seasonal acclimation. It is unclear how well these studies of cold hardiness translate to fitness effects in nature. In addition, insects spend their winters in a thermally-variable, nutrient-limited state. These demanding environments impose low temperature damage on insects, which can induce immediate fitness costs. Alternatively, insects can invoke biochemical responses to repair or prevent this damage, which have energetic costs. The combination of nutrient limitation and

temperature stress during overwintering suggests that trade-offs between somatic investment and fecundity might become important mediators of the relationship between low temperature and fitness in insects (Zera and Harshman, 2001).

The effects of repeated cold exposures on insects are interesting for several reasons. First, temperatures during overwintering do not remain static: they fluctuate on multiple, interacting timescales. Secondly, even very short-term low temperature exposures can induce chilling injury or freezing (if low enough). Thirdly, it is clear that insects increase their cold hardiness after very short-term low temperature exposures (RCH). This hardening can be additive with seasonal acclimation. Therefore, when insects experience subsequent cold exposures, the insect is not naïve to low temperature exposure and may have initiated physiological changes that mitigate these exposures. We might expect then that repeated low temperature exposures would induce a different fitness response than single cold exposures for one of two reasons: unrepaired damage could accumulate causing increased injury or resources devoted to repair and preparation for subsequent exposures could reduce injury, but potentially invoke trade-offs due to the nutrient-limiting state of overwintering (Figure 1.1). Testing whether these trade-offs exist, as well as understanding some of the physiological mechanisms that might underlie them, is an essential part of predicting population dynamics and range shifts in future climate conditions. The existing research on the impacts of repeated cold exposure is reviewed in Chapter 2.

1.6   Summary and Objectives

Temperature regimes in temperate environments are characterized by temperature fluctuations. Insects in temperate environments have adapted to the seasonality of their environments by building their life histories around the unfavourable growing conditions of winter. They spend winter in a state of dormancy, which in some species includes a diapause. Winter survival and energetics drives individual fitness, which then drives population dynamics and the extent of species ranges. Understanding of the dynamics and mechanisms of overwintering can therefore aid predictions of population effects
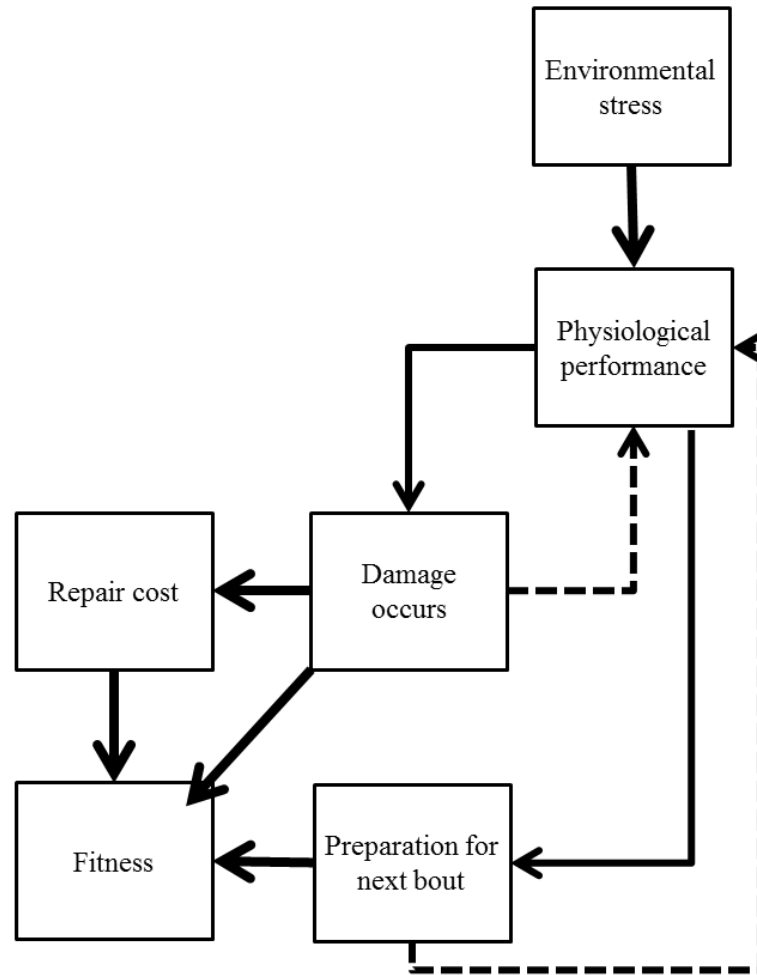
Figure 1.1 A conceptual model linking repeated low temperature stress to fitness, mediated by physiology. Solid lines indicate impacts of low temperature stress, while dashed lines indicate physiologically-mediated feedbacks. Low temperature stress can either cause damage, or induce preparation for subsequent bouts. Damage can also induce repair costs, and fitness can be impacted by investment either in repair or preparation for following bouts. In addition, damage can also induce direct fitness costs. Following a single low temperature bout, the organism may be damaged and less equipped to survive subsequent bouts, or alternatively has induced preparatory mechanisms that reduce damage induced by low temperature stress.

under differing climate conditions. The mechanisms of insect low temperature survival include a broad suite of biochemical adaptations that aid in the control of ice formation, prevent and repair damage to proteins, and aid in the function of membranes at low temperatures. Species differ in their ability to survive cold and/or freezing, but the mechanisms of low temperature survival are similar among responses to cold. Despite extensive research on these mechanisms, the majority of studies have focused on the effects of a single cold exposure, despite the fact that insects experience repeated cold exposures through their lifespans.

In this thesis I address two major questions: 1) Does frequency of cold exposure impose additional stress on insects? and 2) What are the sub-lethal components of this stress? To answer these questions, I structured my thesis with the following objectives, each addressed with different insect species that overwinter in southern Ontario and represent the range of responses to low temperature. My first objective was to understand how repeated cold exposure impacts survival in the fruit fly *Drosophila melanogaster* (Diptera: Drosophilidae), and whether increased survival would be costly. My second objective was to find out whether freeze-thaw events imposed additional damage above the cost of being frozen, and whether repair of this damage was possible, which I addressed in the freeze tolerant woolly bear caterpillar *Pyrrharctia isabella* (Lepidoptera: Arctiidae). My third objective was to explore how other low temperature parameters including intensity of exposure, number of exposures, and period between exposures in addition to frequency of exposure impacted energetics and survival of freeze avoiding insects, which I addressed using the eastern spruce budworm *Choristoneura fumiferana* (Lepidoptera: Tortricidae). Finally, my fourth objective was to address how these additional low temperature parameters impacted a freeze tolerant insect (where the cost of freeze-thaw events could be a factor), as well as understand potential fecundity trade-offs. To answer these questions I used pre-pupae of the goldenrod gall fly *Eurosta solidaginis* (Diptera: Tephritidae).

1.7 Thesis overview

While it would be ideal to study the sub-lethal impacts of repeated cold exposure in a broad array of insect species that would allow for the application of a phylogenetically-

independent approach (Garland *et al.*, 2005), practicalities require that a few species are chosen to focus on. To this end, I selected four insect species, all found in Ontario, that offered contrasting strengths as models, and allowed for the study of each of the three principal cold tolerance responses (freeze tolerance, freeze avoidance, and chill-susceptibility). Each study was prepared as a separate manuscript for publication. Chapters 2 – 4 are published, and Chapter 5 and 6 are currently being prepared for publication.

In chapter 2 ("The impacts of repeated cold exposure in insects"), I review published work that focused on repeated cold exposures, synthesize general responses, critique popular experimental designs, and offer recommendations for future studies on the topic. I find that while many studies report increased cold hardiness as a result of repeated cold exposure, few follow through to measure fitness effects, and many experimental designs fail to adequately address the questions posed.

In Chapter 3 ("Repeated stress exposure results in a survival-reproduction trade-off in *Drosophila melanogaster*"), I investigate how repeated cold exposure impacts metabolites, survival, and fertility in the chill-susceptible *D. melanogaster*. This species was chosen for the ability to rear large numbers of individuals, as well as the relative ease of measuring investment into reproductive output. *Drosophila melanogaster* likely overwinters as an adult, so this life stage was chosen for study (Izquierdo, 1991). I found that while repeated cold exposure decreases mortality due to chilling injury, it also reduces investment in female offspring. This investment seems to be mediated by a reduction in glycogen reserves. I conclude that repeated cold exposures can induce trade-offs between investment in somatic maintenance and reproductive output.

In Chapter 4 ("The sub-lethal effects of repeated freezing in the woolly bear caterpillar *Pyrrharctia isabella*"), I investigate how repeated freeze-thaw cycles impact the overwintering physiology of *P. isabella*. This species overwinters in the final larval instar. While difficult to acquire in large numbers, *P. isabella* is a useful model of freeze tolerance in a relatively large organism, which allowed for a broader range of

physiological techniques such as tests of immune function and low temperature respirometry. I found that repeated freeze-thaws reduce immediate survival, and this is correlated with increased damage to Malpighian tubules and hemocytes. In addition, repeated freeze-thaw increases glycerol concentration in woolly bears, but does not reduce glycogen or triacylglycerols. Surprisingly, caterpillars that received repeated freeze-thaw also have increased resistance to fungal infection. I conclude that freeze-thaw cycles induce mortality in caterpillars by directly damaging tissues, which cannot be repaired.

In chapter 5 ("Low temperature exposure during overwintering causes delayed mortality in the eastern spruce budworm"), I focused on the freeze avoiding eastern spruce budworm *C. fumiferana*, which is easily obtainable in large numbers from cultures maintained by Insect Production Services (a division of the Canadian Forest Service) in Sault Ste. Marie. This model was useful for studying the effects of repeated cold exposure on a freeze avoiding species, as well as providing enough individuals to follow through to longer-term effects on adult survival and body size. This species also overwinters in the 2nd instar, which means that I was able to study whether compensatory feeding following overwintering could mitigate the effects of repeated cold exposure. I found that repeated cold exposure induced delayed mortality relative to early season long cold exposures, and that compensatory feeding did not mitigate this impact. I also found that spruce budworm increased their glycerol concentration from their glycogen stores following repeated cold exposures, but this did not impact supercooling point.

In chapter 6, ("The four axes of stress: the impacts of intensity, duration, frequency, and period of an environmental stressor on physiology and fitness"), I focused on the goldenrod gall fly *E. solidaginis*. This species had the advantage of being available in large numbers, with cryobiology that is relatively well-explored from both a biochemical and ecological standpoint. In this species I was able to explore not only the energetic impacts of repeated freeze exposure (by measuring glycogen and lipid reserves), but also the subsequent fitness effects since the species is a capital breeder. I found that gall flies that experienced repeated freezing events increased their investment in acTAGs (a neutral lipid associated with freezing stress), sorbitol, and glycerol content, and this came at a

cost of future egg production. I conclude that repeated freezing can also induce trade-offs between investment in cryoprotection and future fecundity.

In the general discussion (Chapter 7), I synthesize the results from my four studies. I found that repeated cold exposure is stressful in all studied species, and can induce trade-offs that are not apparent from single cold exposure studies. I suggest that understanding the mechanisms by which environmental stress is transduced to population dynamics should include frequency of stress as a predictor.

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Chapter 2

2 The impacts of repeated temperature exposure on insects

Here I review previously-published work on the effects of repeated cold exposure in insects. I critically examine experimental designs, synthesize impacts, and provide recommendations for future work. This paper is adapted, with permission, from a version originally published in *Journal of Experimental Biology*[1].

2.1 Introduction

As small ectotherms, most insects experience changes in body temperature that reflect their thermal environment. Temperatures in the polar or temperate zones fluctuate at multiple, interacting timescales (Figure 2.1). Long-term fluctuations (which impart long-term selection pressure) range from climate change (Bale and Hayward, 2010) to multi-year cycles such as the El Niño Southern Oscillation (Mysterud *et al.*, 2001). At a scale relevant to the lifetime of individual insects, seasonal changes in temperature are generally predictable (at least in the Northern hemisphere; (Sinclair *et al.*, 2003; Figure 2.1D). These longer-term cycles are periodically interrupted by weather patterns on the scale of days to weeks (Kingsolver, 2000; Figure 2.1C). Finally, the most predictable of these timescales is the daily fluctuation between day and night that can lead to daily thermal ranges of 20 °C or more (Irwin and Lee, 2003; Kingsolver, 2000; Chapter 3; Figure 2.1 A,B).

Temperature regimes with large thermal ranges inevitably cross physiological thresholds (Sinclair, 2001; Figure 2.1A, B), meaning that many insects are exposed to cycles of cold stress that repeat on a daily basis, although in nature temperature and climate variation at longer timescales can also affect the frequency of cold exposure. Together these interact with the particular habitat an insect inhabits leading to the development of a

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[1] Marshall, K. E. and Sinclair. B. J. (2012). The impacts of repeated cold exposure on insects. *Journal of Experimental Biology* **215**, 1607-1613.
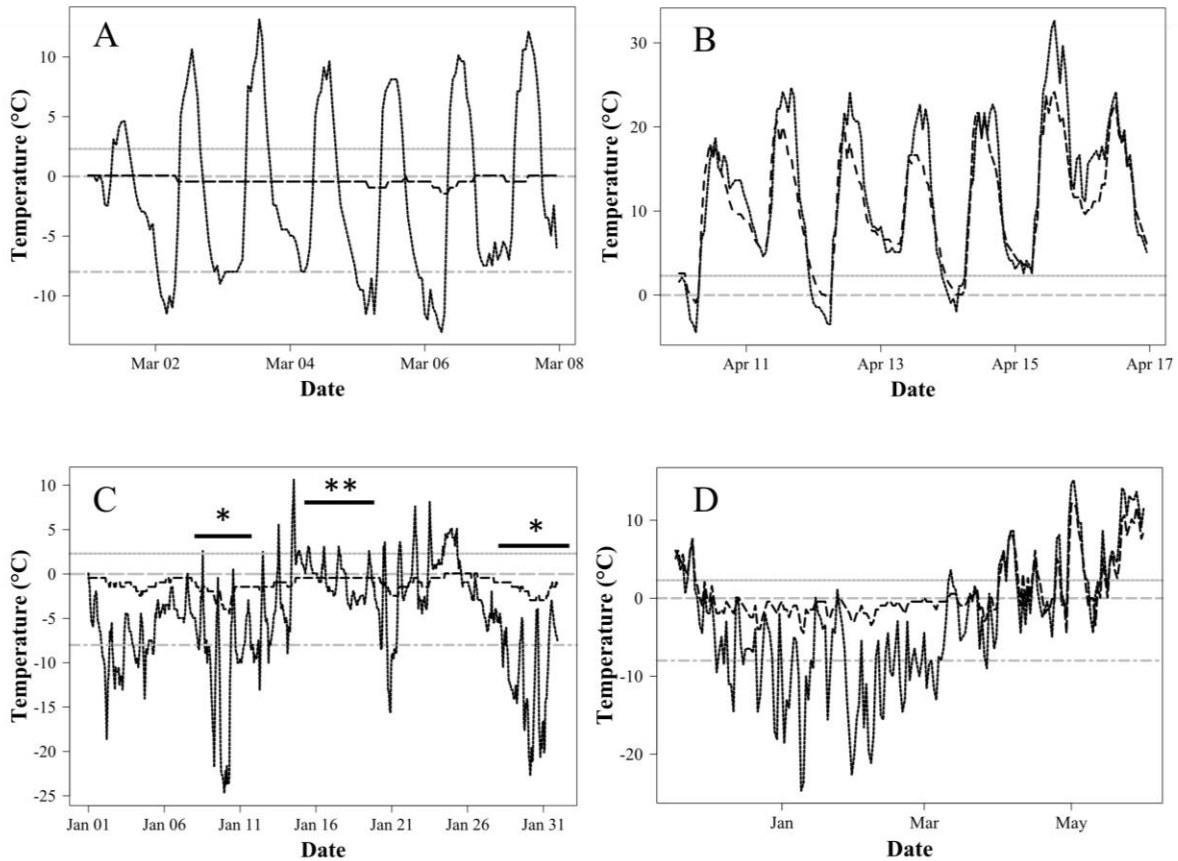
Figure 2.1 Hourly recordings of air (dotted line) and soil surface (dashed line) temperatures in London, Ontario, Canada during the winter of 2009-2010. A) Daily thermal variation in late winter with snow cover. B) Daily thermal variation in early spring without snow cover. C) The effect of frontal systems on winter temperatures in the presence of snow cover. Cold (*) and warm (**) fronts are marked with asterisks. D) Seasonal patterns of thermal variation. Horizontal lines indicate thresholds. Dotted line grey line: 2.3 °C (average chill-coma temperature of the chill sensitive cricket *Gryllus pennsylvanicus*, MacMillan and Sinclair 2011a), dashed grey line: 0 °C (the melting point of pure water), and dot-dash grey line -8 °C (the freezing point of the freeze tolerant caterpillar *Pyrrharctia isabella*; Chapter 4).

microclimate. For example, Sinclair (2001) predicted a New Zealand alpine cockroach would experience almost daily freeze-thaw cycles in an El Niño year, but relatively few freeze-thaw cycles in a year with greater snow cover. Thus, timing and frequency of cold exposure can be affected directly by microclimate, and indirectly by variation caused by climate shifts at larger temporal and spatial scales. Snow buffers thermal variability in microhabitats on the ground (Irwin and Lee, 2003; Figure 2.1A, B), but the extent of snow cover in many regions is declining with climate change (Déry and Brown, 2007), which could lead to increased freeze-thaw cycles in soil microhabitats. Similarly, aspect and exposure to solar radiation can lead to cycles in microhabitat temperatures of much greater magnitudes than seen in air temperatures (*e.g.* Sinclair *et al.*, 2003b). If these cycles cross physiological thresholds, such as those that induce stress responses (Le Bourg, 2011), chill-coma (MacMillan and Sinclair, 2011a), mortality (Sinclair *et al.*, 2003a), or freezing (Teets *et al.*, 2011), then they are expected to impact upon the fitness of insects. The majority of laboratory-based studies of insect stress responses focus on a single exposure, so a pertinent question is whether these repeated stresses have impacts that are distinct from those of a single stress exposure. Here, I will address this question, focusing on the impacts of repeated cold exposures (RCE) on insects.

Insects risk freezing when exposed to temperatures below the melting point of their body fluids. Globally, individuals of most insect species die of chilling injury before they freeze (Lee, 2010). However, freeze avoiding insects depress the temperature at which ice forms (the supercooling point, SCP) and survive as long as they do not freeze, while freeze tolerant insects can withstand the formation of internal ice (Lee, 2010). In both cold tolerance strategies, the production of low-molecular weight cryoprotectants (*e.g.* glycerol), the control of ice crystal growth with antifreeze agents, and the manipulation of the SCP are important physiological components of cold hardiness. Insect cold tolerance is plastic on evolutionary (*e.g.* Strachan *et al.*, 2011), inter-annual (*e.g.* Horwath and Duman, 1984) and seasonal scales (*e.g.* Pio and Baust, 1988). Seasonal cold-hardening in temperate insects is well-characterized: insects increase their cold hardiness from a summer chill-susceptible state by accumulating carbohydrate cryoprotectants, synthesizing antifreezes, reordering lipid membranes, and either retaining (in freeze tolerant insects) or removing (in freeze avoiding insects) ice nucleators (Lee, 2010).

Insect cold tolerance can also vary on relatively short timescales. For example, there are daily cycles in the SCPs of freeze-avoiding Antarctic Collembola (Sinclair *et al.*, 2003c), and chill-susceptible *Drosophila melanogaster* adults are more cold tolerant at the low points of daily temperature cycles (Kelty, 2007; Kelty and Lee, 1999). In the laboratory, many chill-susceptible insects show a rapid cold-hardening (RCH) response, whereby a short exposure to a mild temperature improves tolerance to a subsequent exposure to a more severe temperature (RCH; Lee, 2010; Lee *et al.*, 1987). Rapid cold-hardening can occur in minutes (Lee *et al.*, 1987), does not appear to depend on *de novo* protein synthesis or transcription (Misener *et al.*, 2001; Sinclair *et al.*, 2007), and is variously associated with changes in apoptotic pathways (Yi *et al.*, 2007), membrane composition (Overgaard *et al.*, 2006; but see MacMillan *et al.*, 2009) and cryoprotectant concentrations (Chen *et al.*, 1987; Overgaard *et al.*, 2007, but see MacMillan *et al.*, 2009). Thus, I can conclude two things. First, insects can respond to thermal fluctuations in their environment almost as quickly as the environment itself changes. Second, following cold exposures, insects appear to be in an altered physiological state, which influences their response to subsequent cold exposures.

Recently, there has been growing interest in the role of ecophysiological models for predicting organismal responses to a changing environment (Buckley *et al.*, 2010). This has precipitated a general recognition of the contrast between most laboratory studies (which examine a single stress exposure) and the repeated exposures experienced by insects in the field. Together, these have led to a surge of interest in the effects of repeated stress – particularly cold – exposure in insects. Here, I first examine the design and interpretation of RCE experiments in insects, before reviewing the general impacts of RCE on insect fitness and physiology. Finally, I will identify patterns in insect responses to RCE, gaps in knowledge, and recommend some future directions and experimental designs.

2.2   Design of repeated cold exposure studies

In defining RCE experiments, I excluded studies focused on the effects of thermal variability that do not use potentially damaging temperatures (*e.g.* Paaijmans *et al.*, 2010), because temperature regimes that vary without crossing a physiological threshold

investigate different sorts of questions than RCE experiments, which focus on the effects of repeated physiological threshold crossing. Since most insect species enter chill-coma (which is a clear physiological threshold) at or below 10 °C (Goller and Esch, 1990), I chose crossing below this temperature to define a "repeated cold exposure". I also exclude fluctuating thermal regimes (FTR, *e.g.* Colinet *et al.*, 2006), which are defined by a long period of cold exposure broken up by short periods of warm exposure (*e.g.* 22 h cool: 2 h warm; Colinet *et al.*, 2006). While I will touch on some major conclusions from FTR experiments, their focus on the repair mechanisms during the warm phase of the cycle is distinct from studies that focus on repeated exposure to a stressful temperature. I have identified more than twenty studies across five insect orders that use cold shocks at a low temperature ($\leq$ 10 °C) expected to cause low temperature stress, and where the cold shocks are relatively short compared to the warmer temperature ($\leq$ 50% of the experiment time; Appendix A Table A.2). In the case of freeze tolerant insects, it is important to distinguish between studies of repeated freeze-thaw (RFT) on its own (*e.g.* Brown *et al.*, 2004; Chapter 4), and those that contrast RFT to RCE (*e.g.* Sinclair and Chown, 2005; Teets *et al.*, 2011, Appendix A Table A.3).

Experiments investigating RCE are generally designed with reference to the ecology, tolerances and lifespan of the study organism. Thus, experiments vary in the intensity of cold shocks, their number and duration, and the length and temperature of warm period between shocks (Appendix A Table A.1). The most commonly-used design involves a RCE group that is compared to controls kept at a relatively warm maintenance temperature (which may be an average temperature of the RCE group) that does not cause low temperature stress ("RCE *vs* Warm", *e.g.* Brown *et al.*, 2004; Le Bourg *et al.*, 2009; Sinclair and Chown, 2005; Figure 2.2A). A second design contrasts an RCE group to both a warm control and a stressful prolonged low temperature ("RCE *vs.* Cold", *e.g.* Wang *et al.*, 2006; Yocum, 2001; Figure 2.2B). This design is similar to FTR designs where the total time spent at the stressful low temperature is greater than in the repeatedly exposed group. Finally, "RCE *vs.* Matched Cold", compares a RCE group to both a warm control and a stressful prolonged low temperature that is matched for total time spent by the RCE group at the stressful low temperature (*e.g.* Chapter 3; Teets *et al.*, 2011; Figure 2.2C).
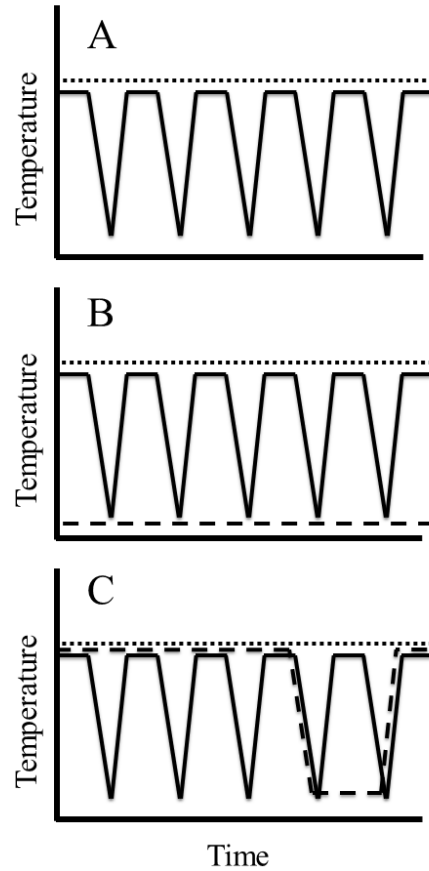
Figure 2.2 Schematic of common study designs in RCE experiments, incorporating "warm" non-stressful temperatures and "cold" stressful low temperatures, the exact values of which will depend on the study species. Time on the x-axis is usually in days, although studies have used regimes that vary from minutes to weeks (Table A.1). Temperature ramps between warm and cold temperatures are shown, although many studies use sudden exposures (Table A.1). A) RCE *vs*. Warm design. Repeated cold exposure (RCE): solid line; Constant warm: dotted line. B) RCE *vs*. Cold design. RCE: solid line, Constant cold: dashed line, Constant warm: dotted line. C) RCE *vs*. Matched Cold design. RCE: solid line, Constant warm: dotted line, group exposed to cold temperatures for the same total time as the RCE group: dashed line. In all cases, lines are slightly offset for clarity.

In RCE *vs*. Warm designs, it is not possible to distinguish between the effects of cold exposure in general or RCE in particular. While this is useful in some ecological contexts (*e.g.* stable subnivean versus fluctuating exposed microclimates; Irwin and Lee, 2003), it is not a useful design for identifying RCE-specific responses. However, RCE *vs*. Warm designs do allow evaluation of the potential for recovery from cold stress (and in that way are similar to FTR experiments). Similarly, RCE *vs*. Cold designs mismatch the 'amount' of stress they deliver between experimental groups. In RCE *vs*. Cold designs, RCE groups always receive less total time at the stressful temperature than the group kept only at low temperatures, so detection of RCE effects is confounded by the reduced stress on that group. I believe that RCE *vs*. Matched Cold designs are most appropriate for disentangling the impacts of RCE from other effects of cold exposure because they match the total amount of stress applied in the prolonged cold and RCE experimental groups.

The difficulty of choosing the appropriate intensity and duration of exposure is a limitation of the RCE *vs.* Matched design: the sum of the repeated exposures must fall within the range that the insect can survive. As a result, RCE *vs*. Matched designs may be forced to use RCE treatments that do not reflect the most extreme exposures possible. This constraint is particularly pertinent when working with species that accumulate lethal chilling injury with chronic cold exposures, since there is an interaction between the effects of time and intensity of cold exposure on recovery time from chill coma that may extend to effects on survival (see MacMillan and Sinclair, 2011b). For example, the cold exposures used by in Chapter 3 were based on the survival of *Drosophila melanogaster* after 10 hours at -0.5 °C, whereas these flies can survive acute exposures down to -5 °C (Nyamukondiwa *et al.*, 2011). While the temperatures used are important, other variables to consider in a RCE study include frequency, number, and duration of cold shocks (Appendix A Table A.1).

Despite the various timescales of temperature fluctuations (Figure 2.1), the majority of studies focus on daily temperature cycling (Appendix A Table A.1), although there have been a few studies showing that frequency of exposure is an important predictor of survival (*e.g.* Bale *et al.*, 2001, Brown *et al.*, 2004). Depending on the life history of the insect (particularly its generation time) it may be appropriate to incorporate cold cycles on other timescales (*e.g.* Chapter 5 and 6). Similarly, despite the majority of studies utilizing an immediate transfer between temperatures (Appendix A Table A.1), in the field, insects generally do not experience abrupt shifts of 10 – 15 °C (Sinclair, 2001b), so an appropriate cooling rate must be chosen (Chown *et al.*, 2009). Indeed, some of the negative effects of RCE and RFT may be due to rapid cooling, although with the limited number of studies it is difficult to parse the effect of cooling rate from the overall effect of RCE and RFT.

Another under-appreciated aspect of the design of RCE studies is the effect of aging on the physiology of cold hardiness. This is particularly problematic in *D. melanogaster* where age decreases reproductive output (Chapter 3) and up-regulates immune function after cold exposure on the scale of days (Le Bourg *et al.*, 2009). Aging is less of a concern in univoltine insects, although response to RCE in *Eurosta solidaginis* depends on month of collection and may be linked to diapause status, although this may be complicated by prior cold exposures in field conditions (Pio and Baust, 1988). The problem of age effects has a simple solution—age-matching of experimental groups (*e.g.* Zhang *et al.*, 2011).

Although the cold tolerance and life history of the insect will be the primary determinant of the thermal regime chosen, the non-linear effects of temperature on many biological processes need to be considered when designing RCE experiments. In some experiments (*e.g.* Petavy *et al.*, 2001; Yocum, 2001), the stable cold stress group received the same mean temperature as the fluctuating insects. However, if stress and temperature have a nonlinear relationship (as is seen in many biochemical and metabolic processes), Jensen's Inequality dictates that the mean of the response to the fluctuating temperature will be

different than the mean of a response to a stable temperature due to simple mathematics (see Ruel and Ayres, 1999 for discussion).

2.3   Fitness impacts of repeated cold exposure

The effects of RCE on survival are relatively straightforward – when compared to any prolonged cold exposure, RCE almost always results in higher survival (Appendix A Table A.2, with the exception of the lepidopterans *Aglais urticae* and *Inachis io*, in which RCE-exposed caterpillars show similar or decreased survival, but greatly increased mass loss, compared with constant low temperature groups; Pullin and Bale, 1989). Increasing recovery time between repeated exposures increased survival to the end of the experiment in repeatedly-frozen *Hydromedion sparsutum* (Bale *et al.*, 2001), but other studies exploring the impact of recovery duration are lacking. Experiments with freeze tolerant species that involve repeated freezing generate more mixed results (Appendix A Table A.3). Two studies found decreased survival of repeated freeze-thaw (RFT) relative to a period of prolonged freezing in a hoverfly (Brown *et al.*, 2004) and a caterpillar (Chapter 4). By contrast there were no differences in mortality between caterpillars that received RCE or RFT (Sinclair and Chown, 2005), and midge larvae exposed to RFT had increased survival relative to those that received a matching, sustained freeze exposure, but decreased survival compared to RCE (Teets *et al.*, 2011). So while it seems clear that RCE benefits survival, the effects of RFT are much more mixed.

Although mortality prevents reproduction, and therefore has rather large fitness consequences, most impacts of the environment on insects are mediated through sub-lethal effects, reducing performance, reproductive output and therefore Darwinian fitness. In many insects, fitness-related parameters like growth, development and reproductive output can be measured with relative ease. The observed effects of and responses to RCE suggest that there should be sub-lethal fitness consequences of RCE for insects. For example the energetic costs of repair of chilling or freezing injury, especially during winter, when insects often do not feed (Bale and Hayward, 2010) could lead to decreased investment in reproduction. Similarly the immediate physiological responses include pathways with demonstrated fitness costs, for example, the production of heat shock proteins with RCE (Silbermann and Tatar, 2000). Repeated cold exposure decreases

development time in *D. melanogaster* (Petavy *et al.*, 2001), which could indicate a fitness benefit. In *D. melanogaster*, although RCE increases survival relative to matched time in a single cold exposure, it decreases subsequent fitness through a male-biased sex ratio (Chapter 3). Thus, when only survival is measured, it is possible to conclude that RCE has a positive effect on fitness when sub-lethal effects could accumulate to a net depression of fitness. Disentangling the trade-offs between fitness components and how RCE impacts these trade-offs is a topic of clear importance for future research (see Chapters 3 and 6).

The studies reported here all focus on experiments conducted in the laboratory, where thermal regimes may easily be manipulated. In nature, variation in temperature depends greatly on snow cover, altitude, aspect, distance to ocean, and other geographical parameters. There are also potential interactions between seasonal acclimation and the additional acclimation that appears to occur during RCE. To date, only Pio and Baust (1988) appear to have addressed this concern, showing that repeated cold exposure only increases cold hardiness in *E. solidaginis* during the months of December and January – early winter in temperate North America. To add to this complexity, snow cover in temperate environments may only be present during a portion of the winter, so soil freeze-thaw may occur more frequently in spring and fall when snow is absent. These interactive effects remain poorly understood.

2.4   Physiological impacts of (and responses to) repeated cold exposure

Repeated cold exposures appear to have fitness impacts on insects that are distinct from the impact of a single cold exposure. These differences could arise from either a cumulative effect of the physiological impact of repeated cold and warm cycles, or because repeated exposure initiates a set of responses that set the insect on a different physiological path to that followed after just a single exposure. Microarrays indicate that the transcriptomic responses of *D. melanogaster* to single and repeated exposures are divergent (Zhang *et al.,* 2011) and these responses could well underlie the increasingly well-documented physiological responses that are unique to repeated cold.

Both the RCH response and acclimation responses lead to an expectation that RCE will improve cold tolerance in insects, and this appears broadly true (Appendix A Table A.2). Low temperature tolerance in *D. melanogaster* is improved after RCE relative to controls kept at room temperature (Le Bourg, 2007). Similarly, repeated freezing lowered the supercooling points (Bale *et al.,* 2001; Brown *et al.,* 2004) and increased cryoprotectant concentrations (Chapter 4; Teets *et al.,* 2011) of freeze-tolerant larvae. However, RCE in (chill-susceptible) *Orchesella cincta* did not decrease chill-coma recovery time relative to individuals held at a constant low temperature (van Dooremalen *et al.,* 2011). It is possible that lowered supercooling points and perceived strategy switches may result from artificial selection on individuals with lower SCPs, resulting in individuals with lower cold hardiness being removed from the experiment at the first exposure. This is likely an issue with any measurements of mortality, and measures of cold hardiness made apart from the cold exposures that constitute the experimental treatments should be made to avoid the potential bias of the death of less cold-hardy individuals.

The synthesis of additional cryoprotectants in response to RCE (Churchill and Storey, 1989; Teets *et al.,* 2011) would be expected to deplete other energy stores, particularly in species that do not feed during winter; however, this does not consistently appear to be the case for cold-hardy insects. Repeated freezing did not change metabolic rate or energy reserves in *P. isabella* (Chapter 4). Similarly, there was no change in adenylate energy charge in the freeze intolerant caterpillar *Epiblema scudderiana* after RCE (Churchill and Storey, 1989). However, although RFT decreased energy reserves in larvae of the Antarctic midge *Belgica antarctica*, RCE without freezing appeared to increase energy reserves (Teets *et al.,* 2011). By contrast, in laboratory colonies of *D. melanogaster,* both glycogen stores (Chapter 3) and starvation resistance (Le Bourg, 2007) were decreased after RCE, even though food was not limiting during the warm parts of the cycles. It is possible that these conflicting results are due to cycling of energetic reserves to other, unmeasured, components, or that changes in energetic reserves do not result directly from RCE.

The acquisition of energy reserves is a key component of the performance of the juvenile stages of many holometabolous insects, so observations of decreased feeding rates after

RCE in caterpillars (Sinclair and Chown, 2005) could imply a mechanism that links physiological impacts to fitness costs. Cellular damage in Malpighian tubules of *P. isabella* larvae was greater following repeated freezing relative to sustained freezing or controls (Chapter 4), and repeatedly-frozen *Belgica antarctica* had lower cell survival in their midgut relative to those frozen in a single period (although RCE without freezing improved cell survival relative to a single long chill; Teets *et al.,* 2011). Thus, the full extent of the relationship between RCE, gut damage, feeding performance and subsequent fitness remain to be determined, and likely varies considerably among species.

Recently, several lines of evidence indicate that RCE may result in an enhanced immune response. Repeated cold exposure increased survival of infection by entomopathogenic fungi in both the chill-susceptible *D. melanogaster* (Le Bourg *et al.,* 2009) and freeze tolerant *P. isabella* (Chapter 4). Immune-related genes were upregulated in response to cold in *D. melanogaster*, but not differentially so in response to RCE (Zhang *et al.,* 2011). These observations indicate that there may be complex interactions with pathogens over winter. It is not clear whether RCE in these cases is priming the immune response in preparation for potential attack, if RCE causes damage that directly elicits the immune response, for example, to encapsulate damaged tissue or respond to gut flora that escape into the hemocoel (*e.g.* MacMillan and Sinclair, 2011b), or whether the cold exposure is increasing the pathogenicity of existing microbes. Given that many insects overwinter in environments in direct contact with soil, this topic appears to be of direct importance to not only studies of RCE effects but of insect overwintering in general.

2.5   Future directions

The driving force of RCE studies is the observation that natural temperature regimes vary. In response to this observation, researchers have hypothesized that insect physiological responses to cold depend on the frequency and timing of cold exposure. This hypothesis has been supported by three decades of study, and mechanisms of these differing responses are beginning to be investigated. From these beginnings, I have

identified several areas that I believe are particularly important for understanding the effects of RCE.

Reproductive output and development time in insects are nearly as important to population growth as the survival of individuals. Yet the effects of RCE on these parameters have barely been investigated, and translation of population effects from the laboratory to natural settings is complicated by the costs and benefits of phenotypic plasticity (Driessen *et al.,* 2011). Given also that gene expression patterns change significantly between RCE and prolonged cold exposure (Zhang *et al.,* 2011), it seems likely that extrapolation of laboratory studies of cold exposure to field settings will require a more thorough understanding of RCE in the context of the complexity, frequency, and number of ecologically-relevant cold exposures (addressed in Chapters 5 and 6).

Of the cold exposure studies reported here, the majority deal with either a single species, or comparisons between two species that are unlikely to directly interact (*e.g. E. solidaginis* and *Epiblema scudderiana*; Churchill and Storey, 1989). However there are clearly differences between insect species in cold hardiness and strategy even within the same environment, and this difference seems likely to hold between broader taxonomic classes. Thus, a clear next direction in repeated cold exposure research seems to be the integration of several trophic levels, including both insects and their pathogens and parasites. Similarly, studies on community assemblages could be a fruitful area of research since it might be important to understand individual species' responses and how those play out in a community setting. These studies will be complex to plan and perform, but there are simple systems that could be employed. Microarthropod mesocosms have already been used to investigate the community-level impacts of RCE (Sjursen *et al.,* 2005), but other possibilities could include single pathogen-host interactions or further investigations of the goldenrod gall system (particularly the parasite assemblage associated with *E. solidaginis*; Judd, 1953).

I believe the greatest unknown is why the effects of RCE and RFT differ so greatly from single cold exposures. There must be a mechanistic reason for this difference, yet choice

of study design and experimental animal can create difficulties comparing impacts between studies, and understanding of the integration of fitness and physiological impacts of RCE remains relatively unexplored. Given that these differences can be traced from gene expression through to fitness effects, it should be possible to determine the connecting physiological links. Signaling pathways, enzymes and intermediates involved in carbohydrate metabolism, wounding responses and vitellogenesis are likely candidates for some of the observed effects.

I have several recommendations for the design of future studies of repeated cold exposure. First, experimental designs should reflect the research question. I recommend RCE *vs*. Matched Cold design for isolating the effects of RCE, but other questions may require different sets of controls. Second, designs must acknowledge both the thermal biology and life history of the study species. Species that have multiple generations per year may need age-controls in the design, but may also facilitate detection of cross-generational effects. Third, prior to embarking on a matched design I strongly suggest performing a pilot study to determine the maximum survivable single prolonged exposure for the study organism. This pilot study allows the selection of appropriate temperature and time of exposures throughout the experiment. Finally, I encourage measurement of sub-lethal impacts. Few studies have linked how immediate physiological changes impact long-term fitness, yet this question underpins the purpose of laboratory studies of RCE.

2.6   Acknowledgements

2.7 References

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Chapter 3

3 Repeated stress exposure results in a survival-reproduction trade-off in *Drosophila melanogaster*

In this chapter I examine the impacts of repeated cold exposure on survival and subsequent reproduction in the chill-susceptible fly *Drosophila melanogaster*. This paper is adapted with permission from a version originally published in *Proceedings of the Royal Society B*[2].

3.1   Introduction

Abiotic environmental factors such as light availability, precipitation, and temperature fluctuate on a broad range of temporal scales (Ozgul *et al.*, 2009; Ruel and Ayres, 1999; Sinclair, 2001). As a result, many organisms are faced with cycling of environmental conditions within their lifespan. These environmental conditions can produce important physiological stresses upon organisms, and, given limited resources, force trade-offs of survival and maintenance with reproduction (Zera and Harshman, 2001).

Life history trade-offs have been intensely investigated with the model species *Drosophila melanogaster* due to its tractability in the laboratory, and its easily-measured fitness proxies (Hoffmann *et al.*, 2003; Zera and Harshman, 2001). Both wild-type and laboratory-selected lines have been used to investigate correlations among life history traits. For example, stress resistance (desiccation resistance and heat resistance) is positively correlated with longevity (Lin *et al.*, 1998) and negatively correlated with fecundity in *D. melanogaster* (Chippindale *et al.*, 1996; Chippindale *et al.*, 1998; Luckinbill *et al.*, 1984; Partridge *et al.*, 1991). While modelling efforts suggest that stress encountered in early life may trade-off with performance later in life (Mangel,

2008), trade-offs induced by early fluctuating stress remain under active investigation. For example, while repeated non-lethal high and low temperature stress both positively impact later longevity and heat resistance, only repeated mild heat stress reduces later fertility (Hercus *et al.*, 2003; Le Bourg, 2007).

Low temperatures are a significant source of stress for many insect species, and are thought to limit fitness and consequently distribution (Bale, 2002; Sinclair *et al.*, 2003). Low temperatures can cause delayed development and death through chilling injuries such as denaturation of proteins, membrane phase transitions, hypoxia, and loss of membrane potential in insects (see Chen and Walker, 1994; Koštál *et al.*, 2007a; Storey and Storey, 1988). Similarly, temperature induced changes in gene expression and protein production can reduce fitness by decreasing the viability of eggs laid by treated adult *D. melanogaster* (Silbermann and Tatar, 2000). However, while the majority of these studies report on the results of a single stress exposure, it is clear that, in nature, animals are exposed to thermal stress on a regular and repeated basis (Sinclair, 2001b), and understanding the consequences of these repeated stresses is essential for interpreting and predicting climatic change effects in the natural world (Helmuth, 2009).

Fluctuating thermal regimes (FTR; long-term cold exposure broken by repeated short warm temperature exposures) increase cold survival in many chill-susceptible insects (discussed by Koštál *et al.*, 2007a). FTR induce upregulation of metabolic pathways in *Aphidius colemani,* which is consistent with repair of cold injuries occurring during the warm period of the temperature cycle (Colinet *et al.*, 2007). Similarly, populations of *D. melanogaster* and *D. simulans* exposed to fluctuating temperatures maintain development at temperatures that would be lethal under a constant low temperature regime (Petavy *et al.*, 2001). While multiple cold exposures appear to be beneficial for chill susceptible insects, freeze tolerant insects appear to incur fitness consequences with repeated cold exposure (Bale *et al.*, 2001; Sinclair and Chown, 2005).

This previous work on fluctuating temperatures is limited because, first, the multiple cold exposure groups experienced less net time at the lower temperature, even though the net amount of time spent at low temperatures may drive the accumulation of injuries (Petavy

et al., 2001). Second, the binary responses of survival or emergence versus death in the short term do not account for longer-term fitness (Partridge *et al.*, 1991), so there is a need to quantify the sub-lethal effects of multiple cold exposure.

Here I address the impacts of repeated stress exposure from a life-history trade-off perspective using a *D. melanogaster* model system. *Drosophila melanogaster* is chill-susceptible: while the lower lethal temperature for a two hour exposure in adults is -5 °C, freezing does not occur until -20 °C (Czajka and Lee, 1990). The sub-lethal costs of multiple cold exposures were quantified through fecundity (both total number of offspring produced and intrinsic rate of population increase—*r*) and measures of energy reserves (Dillon *et al.*, 2007; Djawdan *et al.*, 1998). I tested three competing hypotheses on the fitness costs of cold exposure as measured by *r*. First, chill accumulation: fitness cost is based on the total amount of time spent cold, regardless of warming intervals. Under this hypothesis, I predict that multiple and sustained exposure groups will incur similar costs. Second, repair: warming intervals allow for repair to take place. I predict that the sustained exposure group will incur the most cost. Finally, increased cost: the opportunity to repair is costly. In this case, I predict that the multiple exposures group will incur the most cost.

3.2   Methods

3.2.1   Fly collection and rearing

The experimental population was established by combining 35 isofemale lines of *Drosophila melanogaster* collected six months previously from London (43°00′N 81°15′W) and Niagara on the Lake (43°04′N 79°04′W). Flies were reared on cornmeal-agar medium in 35 mL plastic vials at low density (Markow and O'Grady, 2006). For flies reared for experimentation, 60 vials of adult flies (~100 flies each) were emptied into each population cage. Eggs were collected from a Petri dish of food medium that was left in the cage for 8 h, and placed into new 35 mL medium vials at a density of 100 eggs/vial. After 10 days, virgin females were collected under CO_2 anesthesia and placed in new 35 mL medium vials in groups of 15. Virgin males were also collected at this

time, also placed in new 35 mL medium vials at a density of ~30/vial, and maintained at 22 °C. All experiments started on the third day after sorting to avoid CO_2 effects on cold tolerance (Nilson *et al.*, 2006). Any vial that subsequently showed signs of larvae was discarded, since it indicated that the female fly had mated.

3.2.2   Experimental Design

All cold treatments were conducted in inverted food vials placed into an incubator (MIR153, Sanyo, Bensenville, Il.) set to -0.5 °C (± 0.25 °C as measured in medium vials). There are potential interactions between age and cold tolerance (Czajka and Lee, 1990) and age and fecundity (Dillon *et al.*, 2007), so several controls were required to isolate the effects of multiple cold exposure. Four main experimental groups were created: (1) a control group maintained at 22 °C; (2) a sustained cold group, exposed to -0.5 °C for 10 h; (3) a multiple cold group: 2, 3, 4 or 5 two hour exposures to -0.5 °C, each separated by 22 h at 22 °C and all ending at 7 days of age; and (4) a single short cold group that received a single 2 h exposure to -0.5 °C. To control for the potential interaction of age and cold tolerance, sustained and single short treatments were conducted 3, 5, and 7 days after sorting. Control flies were sampled at 3, 5, and 7 days after sorting.

3.2.3   Mortality and fertility assays

Immediately after the final treatment for a group of flies, 50 females per age × treatment combination were narcotized with CO_2 and placed individually into new food vials with two untreated virgin males of the same age. After two days, the triads were transferred to new vials. Females were examined daily for four days following treatment, and were recorded as "dead" if unable to right themselves. After four days, all adult flies were removed from their vials and vials were monitored daily for emergence of offspring, which were removed, counted and sexed each day until eclosion ceased.

3.2.4   Energy reserves and cryoprotectant content

Immediately after cold treatment, as well as 24, 48, and 72 h later, groups of fifteen flies were snap frozen in liquid nitrogen and stored at -80 °C until analysis. Flies in groups of 5 were homogenized in 0.05% Tween 20 using 1 mm diameter glass beads in a Bullet Blender (Next Advance Inc., Averill Park, NY, USA). Samples were centrifuged at $16,000 \times g$ for 1 min and the supernatant stored at -20 °C (Gefen *et al.*, 2006).

Triglyceride, glycerol, and glycogen content were measured in triplicate according to Gefen *et al.* (2006), with the following modifications for the glycogen assay: liquid glucose reagent was added to the samples and free glucose determined. Then 10 µL *Rhizopus* amyloglucosidase was added, and the plate left overnight at 20 °C to allow conversion of glycogen to glucose, which was determined as the difference between free glucose and the glucose in the digested sample.

3.2.5   Data analysis

Each treatment group (multiple, control, sustained, or single short) was first examined separately for the effects of age. If age was a non-significant factor, data from flies of all ages for a treatment were combined together. Mortality was compared within and then between treatments by generalized linear model with binary error distributions and a logit link function in SPSS. Fecundity (sex ratio, total number of offspring, and intrinsic rate of population increase) was analyzed by separate general linear models with treatment and age as factors, and Tukey *post-hoc* tests. Energy reserves and cryoprotectant (triglyceride, glycogen, and glycerol) assays were compared by separate general linear models with treatment and time after treatment as factors and Tukey *post-hoc* tests. Sex ratio was calculated as the proportion of females out of total offspring per vial. Intrinsic rate of population increase (r) was calculated as per Dillon *et al.* (2007) with the following modifications: instead of only including surviving females, the probability of survival for each individual each day for each treatment was calculated, and then used as the l_x term in the calculation of net reproductive value (R_o). Only vials where both males survived and there were no escapees were counted and only the non-iterative technique

was used to calculate r (Birch, 1948). Net reproductive value was calculated for each treated fly as:

$$R_o = \sum_{x=1}^{4} l_x m_x \qquad (1)$$

where x is time after treatment in days, l_x is the probability of being alive at time after treatment x, and m_x is the number of female offspring produced by that female on that day. Generation time (T_g) was then estimated by calculating the mean development time of the female's offspring by:

$$T_g = \frac{\sum_{x=1}^{4} x l_x m_x}{\sum_{x=1}^{4} l_x m_x} \qquad (2)$$

These two calculations allowed estimation of intrinsic rate of population increase (r, Birch 1948) by:

$$r = \frac{\ln R_o}{T_g} \qquad (3)$$

Analyses were conducted in R using the base package (R Development Core Team 2008) and SPSS (version 17.0). Because of the high power of this study, α was set *a priori* to 0.01.

3.3   Results

I monitored survival for four days post-cold exposure in a total of 50 female flies in each of 15 age × treatment interaction groups. Overall mortality in the experiment was 13%, and number of cold exposures did not affect mortality in multiply-exposed flies (Wald χ^2 = 1.645, df = 3, p = 0.649). Similarly, age did not significantly affect survival in control (Wald χ^2 = 0.603, df = 2, p = 0.740), sustained (10 h) (Wald χ^2 = 6.257, df = 2, p = 0.044), or single short (2 h) cold exposure flies (Wald χ^2 = 10.693, df = 4, p = 0.030).

66

Therefore, all age interaction groups were pooled within each treatment, as were all treatments within the multiple group and a significant difference was found between groups (Wald $\chi^2 = 23.941$, df = 3, p < 0.001). Multiply-exposed and single short exposed flies had similar survival to controls, while sustained exposure flies had higher mortality (Figure 3.1).
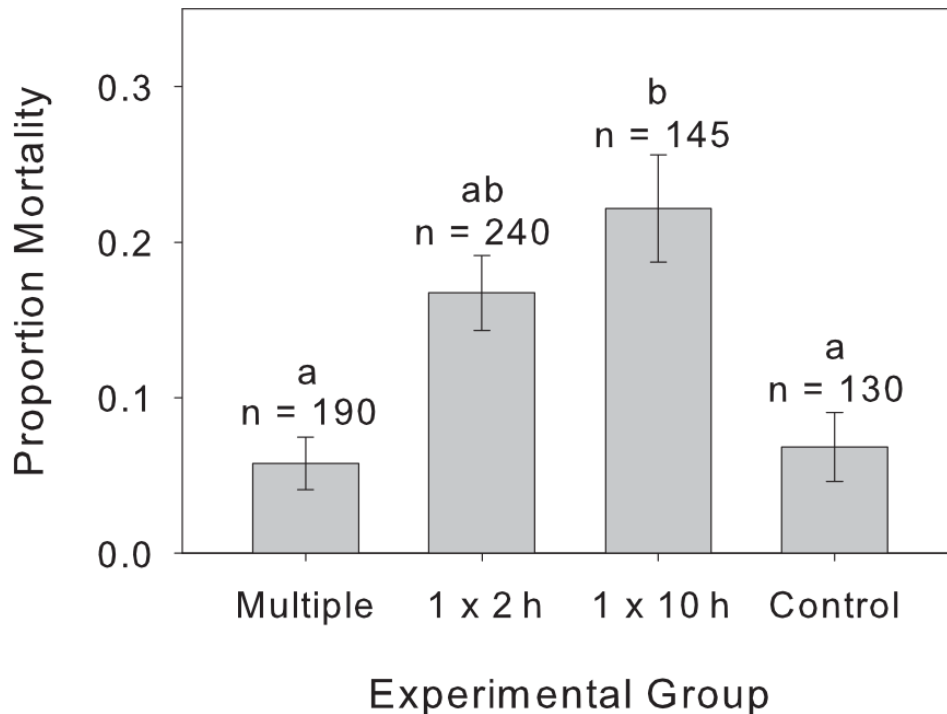
Figure 3.1 Mortality four days after cold treatment at -0.5 ºC in female adult *Drosophila melanogaster*. Treatments with the same letter are not significantly different (p < 0.01) in a generalized linear model. Error bars are binomial standard error, and sample sizes are indicated on the figure.

3.3.1   Total offspring produced

Increased number of cold exposures decreased the number of offspring produced in the multiply exposed flies ($F_{3,195}$ = 10.407, p < 0.001, Figure 3.2A). Age also affected total number of offspring produced by control flies ($F_{2,147}$ = 10.828, p < 0.001), sustained exposure (1 × 10 h) flies ($F_{2,147}$ = 7.969, p < 0.001), and single short exposure (1 × 2h) flies ($F_{4,242}$ = 12.534, p < 0.001, Figure 3.2A), In general, multiple and sustained (10 h) exposure flies produced significantly fewer offspring than single short (2 h) or control flies (Figure 3.2A; $F_{3,742}$ = 60.102, p < 0.001).

3.3.2   Sex ratio

Number of cold exposures did not significantly affect offspring sex ratio in the multiple exposures group, nor did age of exposure (p > 0.07 in all cases, see Table 3.1). However, when all age interaction groups were pooled within each treatment, as were all treatments within the multiple group, the multiply-exposed group had a significantly male-biased sex ratio (mean ± SE = 0.457 ± 0.006, $F_{3,705}$ = 8.615, p < 0.001) compared to other groups (mean ± SE = 0.501 ± 0.009).

3.3.3   Intrinsic rate of population increase (*r*)

Increasing numbers of short cold exposures significantly decreased *r* in multiply-exposed flies (Figure 3.3, $F_{3, 189}$ = 93.309, p < 0.001). Similarly, younger flies had higher values of *r* than older flies in control flies ($F_{2, 142}$ = 116.360, p < 0.001), sustained cold (10 h) flies ($F_{2, 128}$ = 25.640, p < 0.001), and single short exposure (2 h) flies ($F_{4,227}$ = 105.080, p < 0.001, Figure 3.2B). There was a significant effect of treatment when all ages were pooled within each treatment and compared with the five 2 h exposures group ($F_{3,459}$ = 63.512, p < 0.001). Flies with five 2 h exposures had the lowest *r* in every case except one: there was no significant difference between the five 2 h exposure group and the oldest sustained (10 h) exposure flies, although the five 2 h exposure group had a lower mean *r* (Figure 3.2B).

Table 3.1 Number and sex ratio of offspring produced by female flies after cold treatment at -0.5 ºC as a function of treatment in *Drosophila melanogaster.* Values with the same letter within a treatment are not significantly different at $\alpha = 0.01$. Bolded values indicate sex ratios that are significantly different from 0.5 by two-sided one-sample t-test at $\alpha = 0.01$.

Treatment	Age at first treatment (days post eclosion)	Mean ± SE offspring produced	Mean ± SE proportion female offspring
1×2 h	3	432 ± 34^a	0.48 ± 0.02
	4	309 ± 29^{bc}	0.51 ± 0.02
	5	427 ± 28^{ab}	0.51 ± 0.01
	6	301 ± 22^c	0.49 ± 0.02
	7	218 ± 13^c	0.53 ± 0.02
Multiple (2 × 2 h)	6	243 ± 11^a	**0.46 ± 0.01**
Multiple (3 × 2 h)	5	197 ± 15^a	**0.47 ± 0.02**
Multiple (4 × 2 h)	4	206 ± 11^{ab}	**0.47 ± 0.01**
Multiple (5 × 2 h)	3	150 ± 11^b	**0.44 ± 0.01**
1×10 h	3	115 ± 16^a	0.54 ± 0.03
	5	179 ± 12^{ab}	0.50 ± 0.01
	7	200 ± 19^b	**0.47 ± 0.01**
Control	3	246 ± 16^a	0.51 ± 0.01
	5	403 ± 26^b	0.50 ± 0.02
	7	369 ± 31^b	0.50 ± 0.02
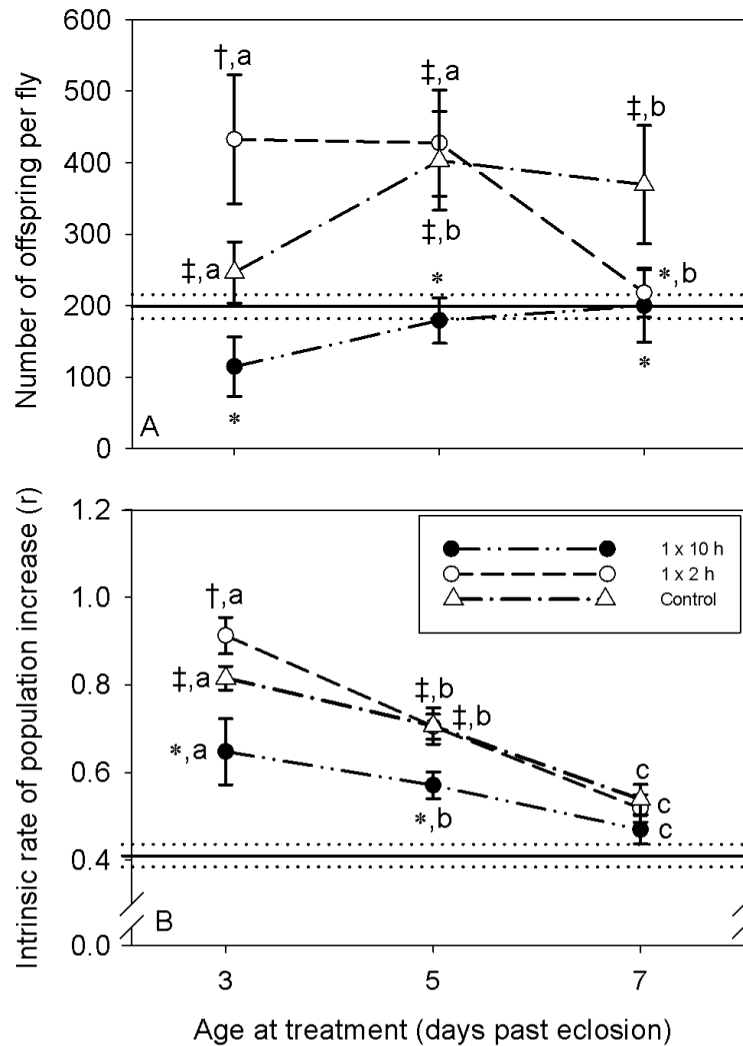
Figure 3.2 Fecundity effects of cold exposure at -0.5 °C in *Drosophila melanogaster*.
A) Total number (mean ± 99 % C. I.) of offspring produced in a four day period after
cold treatment at -0.5 °C by female adult *Drosophila melanogaster*.   Points with the
same superscript symbols do not significantly differ between treatments at a given age at
p < 0.01. Points with the same lower-case letters are not significantly different within a
treatment at different ages at p < 0.01. Solid horizontal line indicates mean number of
offspring of multiple treatment group (two to five 2 h treatments), with dotted lines
indicating 99 % C.I. N= 42-50 females per point. B) Intrinsic rate of population increase
(mean ± 99 % C.I.) after cold treatment at -0.5 °C as a function of treatment and age in
female adult *Drosophila melanogaster*. Points with the same superscript symbols are not
significantly different between treatments at a given age at p < 0.01. Points with the
same lower-case letters are not significantly different within a treatment at different ages
at p < 0.01. Solid horizontal line indicates intrinsic rate of increase of the multiple
treatment group that received five 2 h treatments, with dotted lines indicating 99 % C.I. N
= 42-50 females per point.

3.3.4   Energy reserves

There was no effect of either age cohort or time after treatment on glycogen content in multiply exposed, sustained exposed (10 h) or control flies. In single short (2 h) exposure flies, the youngest age cohort (3 days old at treatment) had significantly less glycogen than 5 or 6 day old flies ($p < 0.001$ for each). Multiply exposed flies contained less glycogen than other cold exposure groups but not controls when treatments were compared ($F_{3,252} = 106.654$, $p < 0.001$, Figure 3.4A).

Number of cold treatments did not affect triglyceride content in multiply exposed flies, although there was a significant effect of time after cold exposure in each group where triglyceride content was low immediately after exposure, then climbed following cold exposure ($F_{3, 59} = 8.221$, $p < 0.001$). There were significant effects of age, time after treatment, and the interaction between them on triglyceride mass on single short (2 h) cold treatment flies ($p < 0.001$ in each case), however *post hoc* comparisons reveal that this was driven by only the significant difference between the 7 day old and 5 day old flies at 72 h after exposure ($p < 0.001$). Similarly, there were significant effects of age, treatment, and the interaction between them on triglyceride content in sustained (10 h) exposure flies ($p < 0.001$ in each case), however the only significant difference between ages was between the 7 day old and 3 day old flies immediately after exposure ($p < 0.001$). Finally, the interaction between age and time after treatment significantly affected control flies ($F_{6,46} = 4.059$, $p = 0.002$), however there were no significant *post-hoc* differences. Therefore all ages were pooled in all treatments. The interaction between treatment and time after treatment had a significant effect on triglyceride mass of flies ($F_{9,271} = 5.8653$, $p < 0.001$). Immediately after cold treatment, multiply-exposed flies had significantly lower triglyceride content than any other treatment group ($p < 0.001$, Figure 3.4B).
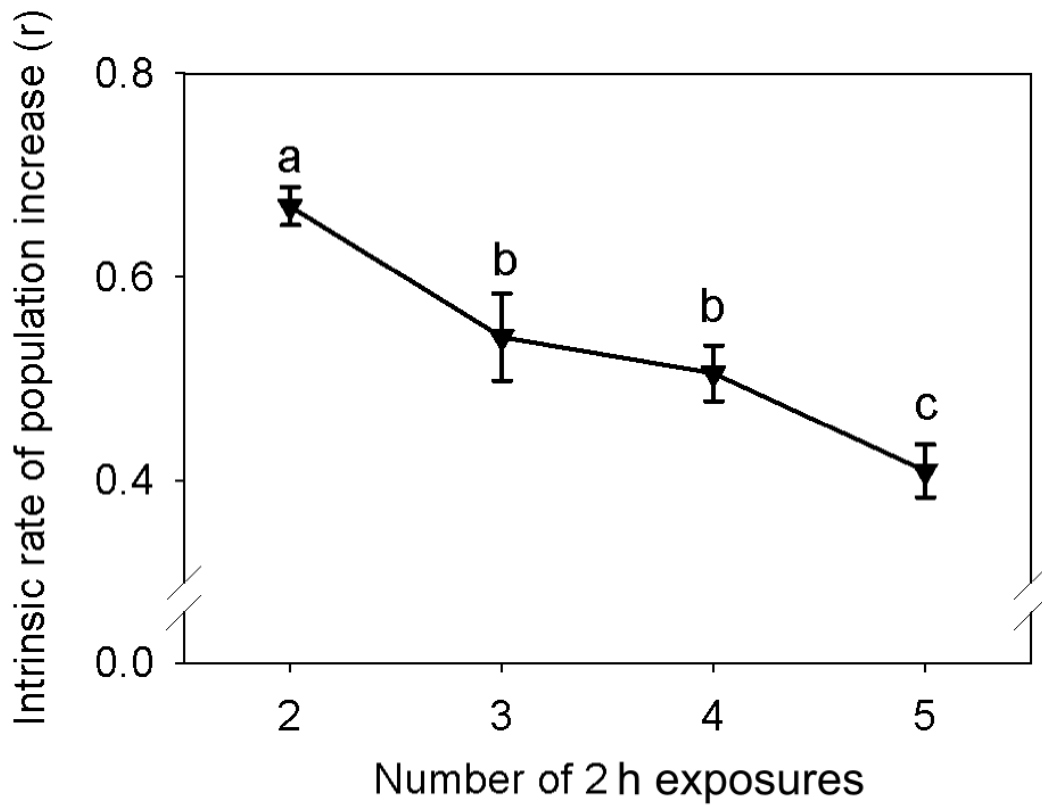
Figure 3.3 Intrinsic rate of population increase (mean ± 99 % confidence interval) after cold treatment at -0.5 °C as a function of number of 2 h treatments in female adult *Drosophila melanogaster*. Points with the same lower-case letters are not significantly different at p < 0.01.
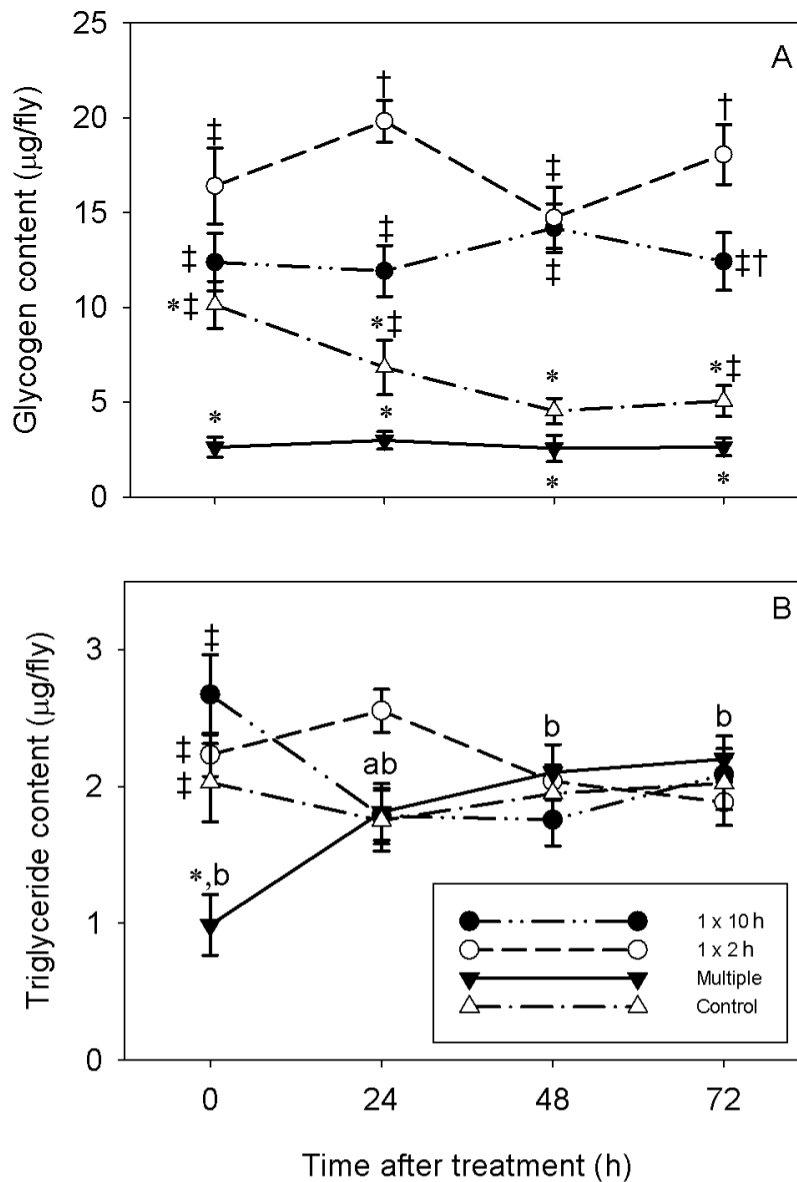
Figure 3.4 Effects of cold treatment on energy reserves in *Drosophila melanogaster*.
A) Glycogen mass (mean ± standard error) after cold treatment at -0.5 °C as a function of
treatment and time after treatment in female adult *Drosophila melanogaster*.   Points with
the same superscript symbols are not significantly different between treatments at a given
time after treatment at p < 0.01.   N = 12 - 20 samples of five flies per point. B)
Triglyceride mass (mean ± standard error) after cold treatment at -0.5 °C as a function of
treatment and time after treatment in female adult *Drosophila melanogaster*. Points with
the same superscript symbols are not significantly different between treatments at a given
time after treatment at p < 0.01.   Points with the same lower case letters are not
significantly different within the multiples group only between time points. N = 13 - 20
samples of five flies per point.

There was no effect of either age cohort or time after treatment on glycerol content in flies that received a single short (2 h) exposure, a sustained exposure (10 h), or control flies. Therefore all ages were pooled in all treatments. The number of 2 h exposures did not affect glycerol content in flies that received multiple exposures, but there was a significant increase in glycerol content immediately following cold exposure ($F_{3,73}$ = 24.94, p < 0.001). When all treatment groups were compared with each other, there was a significant interaction between treatment type and time after exposure on glycerol content ($F_{9,291}$ = 14.43, p < 0.001, Figure 3.5). Flies that received repeated 2 h exposures had significantly higher glycerol content immediately following a cold exposure than flies in any other exposure group (Tukey's *post-hoc* test, p <0.001 in all comparisons).

3.4   Discussion

While providing a warming period between cold exposures decreases mortality in several insect species (Colinet *et al.*, 2007; Koštál *et al.*, 2007a; Petavy *et al.*, 2001), our study provides the first evidence of a trade-off between immediate survival and future fitness as a result of a multiple stress response. Regardless of the age of the flies, I found that multiply exposed flies had lower mortality than flies exposed to cold for the same amount of time without opportunity for repair. However, I also found that this improvement in survival is costly: flies exposed to cold on multiple occasions had a lower intrinsic rate of population increase than any other cold treatment. Therefore, our increased cost hypothesis was supported: there is a fitness consequence to repeated cold exposure.

My study is the first to quantify a long-term negative fitness impact of repeated cold injury. This response was found only in multiply exposed flies, and not in response to a single stress. I therefore believe this response is likely not due to the rapid cold-hardening (RCH) response (Czajka and Lee, 1990) because it is not found in our single 2 h exposure group, which is an equivalent treatment to RCH. While Overgaard *et al.* (2007) showed a significant reduction in fertility in *D. melanogaster* for eight hours after a RCH treatment, Kelty and Lee (1999) showed no decrease in egg-laying in a five day period after RCH. Also, RCH only depressed fertility compared with controls, and provided a beneficial effect when compared with an acute cold shock
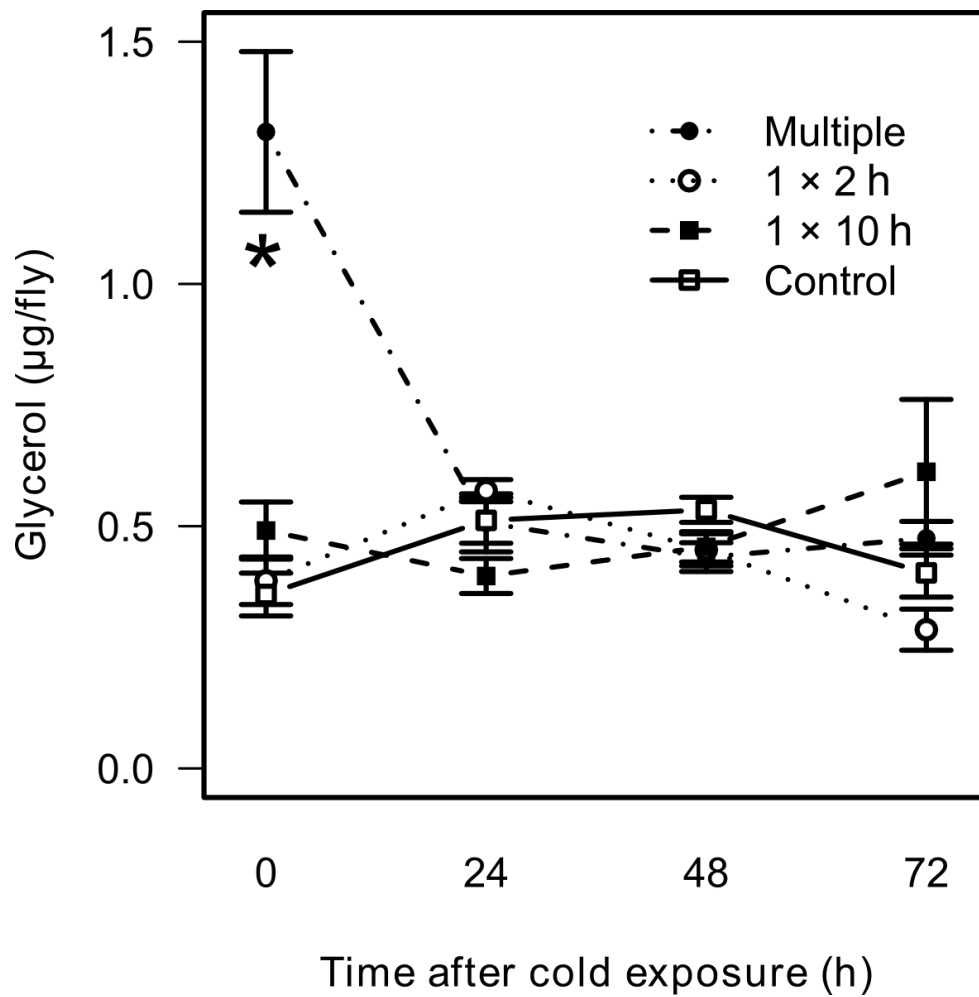
Figure 3.5 Effects of cold treatment on glycerol content in *Drosophila melanogaster*. Glycerol mass (mean ± standard error) after cold treatment at -0.5 °C as a function of treatment and time after treatment in female adult *Drosophila melanogaster*. N = 12 - 20 samples of five flies per point. Asterisk indicates significant difference between flies that received multiple 2 h exposures and flies that received all other experimental treatments. Note: this figure does not appear in the original published manuscript.

(Overgaard *et al.,* 2007). Similarly, a 1-3 day mild low temperature stress (18 °C) caused a decrease in fertility in *D. melanogaster* during the stress period, but a return to normal temperatures allowed for compensation in egg production, and no overall depression of fitness was detected (Dillon *et al.,* 2007).

According to Trivers and Willard (1973), individuals in poor condition should produce more female offspring because females have a more uniform number of offspring than males. While this hypothesis is well-supported in mammals and *D. melanogaster* appears to follow the assumptions of this hypothesis (Burke and Little, 1994), multiply exposed flies in our work had a male-biased offspring sex ratio. Since the cold exposures in our study occurred before mating, this result is not due to differential retention of sperm. Possibly female offspring are more costly to produce, or are more sensitive during development. There is evidence that *D. melanogaster* can adaptively bias sex ratios: females mated with older males (9 to 13 days post-eclosion) are more likely to produce female offspring, which Long and Pischedda (2005) interpreted as possible compensation for the fact that sons of older male flies fare poorly in competitive mating assays. By contrast, I found that after repeated stress, *D. melanogaster* females bias sex ratio in an apparently maladaptive fashion. There are two possible interpretations for this result: either female offspring are more costly to produce, or the sex ratio change is adaptive. During the experiment, I noted large numbers of offspring of the multiple exposures flies that failed to completely eclose and died in their pupal cases. While I did not sex these flies, it is possible that female offspring of the multiple exposure group were unable to properly complete development. Further work on the performance of the F1 offspring will be necessary to determine the consequences of, and perhaps mechanisms, underlying this bias.

One life history aspect I did not address in this study is the potential trade-off between offspring size and number predicted by theory (Stearns, 1992). This trade-off assumes that the "decision" to allocate resources to total reproductive output is independent of the decision to allocate resources to individual offspring. However, one of the first mathematical models testing this assumption found a link between the optimal number of offspring and total reproductive output (Winkler and Wallin, 1987). This was empirically

tested in *D. melanogaster* selected for egg size by Schwarzkopf *et al.* (1999) who found that that while egg size significantly responded to selection, relative fecundity remained unchanged. This suggests that offspring size and number are related in *D. melanogaster* and that calculations of intrinsic rate of increase correspond closely to total reproductive allocation in this species.

Triglyceride reserves significantly decline immediately after cold exposure only in multiply exposed flies, and this decline was consistent for all multiple exposure groups (2, 3, 4, and 5 two hour exposures). Glycogen stores in multiple exposure flies were also significantly lower than any other cold treatment at every time interval after exposure. Similarly, glycerol concentration is significantly increased immediately following low temperature exposure. Thus, the multiple exposure flies appear to have a different physiological response to injurious cold exposure than either the single short (2 h) or sustained (10 h) exposure flies. Given that there was no effect of number of cold exposures on triglyceride mass, glycogen mass, or glycerol mass in flies that experienced between two and five 2 h exposures, this different response appears to be primed by a single 2 h exposure, then activated by a second. While the increase in glycerol content was not sufficient to have colligative effects on freezing point, it is possible that the increase was sufficient to serve cryoprotective functions protecting membranes and proteins during cold exposure (Koštál *et al.*, 2007b). Our flies had access to high quality diet following each cold exposure, which may explain the increase in glycogen stores in single short (2 h) and sustained (10 h) exposure flies. While access to the diet was identical for each experimental group, it is possible that cold injury caused a decrease in feeding rates or assimilation efficiency. Investigation of the mechanism of the multiple cold exposure response should include this possibility.

With predicted increases in temperature variability (Easterling *et al.*, 2000), understanding the physiological and ecological effects of fluctuating temperatures becomes more urgent, particularly when attempting to model climate effects (Helmuth, 2009). While the impacts of single low temperature exposures on insects are well-studied (Bale, 2002), only recently have investigators focused on the effects of repeated injurious

cold exposure on insect populations (Bale *et al.*, 2001; Colinet *et al.*, 2007; Koštál *et al.*, 2007a; Petavy *et al.*, 2001; Sinclair and Chown, 2005). Our study points to two important aspects of repeated cold exposures. First, that interpreting the results of FTR studies is far more complex than simply assessing mortality since I have shown that multiple cold exposure induces a trade-off between survival and future reproduction. Second, that multiple cold exposure can produce non-linear changes in energy reserves that may characterize a new physiological response to fluctuating temperature conditions. These effects can also produce consequences such as changes in offspring sex ratio that can impact fitness. Taken together, these results imply that laboratory studies without fluctuating temperatures may deviate significantly from field situations, reducing the utility of interpreting the results of lab studies in a field context (*i.e.* Kristensen *et al.*, 2008). In addition, I point out that many other stresses such as desiccation, high temperatures, and exercise affect organisms on a repeated basis in the field. Investigation of the impacts of repeated exposure to these stressors is vital when interpreting and predicting the physiological responses of organisms in the wild.

3.5   Acknowledgements

3.6   References

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Chapter 4

4 The sub-lethal impacts of repeated freezing in the woolly bear caterpillar *Pyrrharctia isabella*

In this chapter I examine how repeated freeze-thaw events impact the physiology and subsequent survival of the freeze tolerant caterpillar *Pyrrharctia isabella*. This paper is adapted with permission from a version originally published in *Journal of Experimental Biology*[3]

4.1 Introduction

The frequency of extreme events is useful for predicting organismal responses to the abiotic environment (Gaines and Denny, 1993). One physiologically- and ecologically-relevant event is the freezing transition in insects (Lee, 2010; Sinclair *et al.*, 2003a). Freezing occurs when the temperature of an individual, which tracks microhabitat temperature closely in small ectothermic insects, drops below its supercooling point. The supercooling point (SCP) is the lowest temperature an animal reaches before freezing (Lee, 2010). To survive freezing, freeze tolerant insects often synthesize carbohydrate cryoprotectants and antifreeze proteins which protect cells and inhibit recrystallization (Bale, 2002; Lee, 2010). Many species also promote ice nucleation at higher temperatures through ice nucleating agents or selection of more benign overwintering sites (Bale, 2002; Storey and Storey, 1988).

While the benefits of freeze tolerance also include reduced metabolic and water loss rates during freezing (Irwin and Lee, 2002), there is a potential energetic cost to both preventing and repairing freeze-induced damage (Bale, 2002). Damage from freezing may be a result of mechanical damage to tissues (particularly the nervous system), disruption of cellular membranes and proteins, osmotic shock, or hypoxia (see reviews in

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[3] Marshall, K. E. and Sinclair, B. J. (2011) The sub-lethal impacts of repeated freezing in the woolly bear caterpillar *Pyrrharctia isabella*. *Journal of Experimental Biology*. **214**, 1205-1212.

Lee, 2010; Storey and Storey, 1988). In addition to physical damage, freeze tolerance may carry energetic costs from the active re-establishment of ion gradients (Churchill and Storey, 1989a), or if cryoprotectants are synthesized from glycogen reserves before or after freezing (Churchill and Storey, 1989a; Churchill and Storey, 1989b; Lee, 2010). Allocation of energy reserves to either prevent or repair of cold-induced damage may then consume resources that could otherwise be invested in reproduction.

Modelling of the energetic benefits and costs of the freeze tolerant and freeze avoidant strategies indicates that freeze tolerance is optimal when there is low cost to prolonged freezing and the freezing event itself is not costly (Voituron *et al.*, 2002). This suggests that understanding the costs of multiple freezing is an important key to understanding the selection pressures leading to a particular cold tolerance strategy. In another possible scenario, Sinclair *et al.* (2003b) suggest that freeze tolerance may be favoured in environments where the likelihood of freezing is unpredictable. Nevertheless, the physiological trade-offs that drive the "choice" of the freeze tolerance strategy remain largely unexplored.

Despite nearly three centuries of research establishing the biochemical and behavioural traits leading to freeze tolerance, the majority of research has focused on the effects of a single freeze exposure (Bale, 2002; Sinclair *et al.*, 2003a; Sømme, 2000). Multiple freeze-thaw cycles in a single winter are common in temperate latitudes (Henry, 2008; Sinclair, 2001), and could produce additional stress for individuals that experience low temperatures. In addition, the number of these cycles in a winter in temperate latitude soil is predicted to increase as a result of declining snow cover (Henry, 2008).

There have been few, but contradictory, studies on the effects of multiple freeze-thaw cycles in freeze tolerant insects. For example, freeze tolerant larvae of *Eurosta solidaginis* were repeatedly frozen at -16 ℃, which led to decreased energy availability (reflected in ATP:AMP) with no recovery of adenylate charge between cold cycles (Churchill and Storey, 1989a). Two studies on freeze tolerant insects found that with successive freeze-thaw cycles some individuals lost their ability to survive freezing, apparently switching to a strategy of freeze avoidance (Bale *et al.*, 2001; Brown *et al.*,

2004). By contrast, there was no evidence of a strategy switch after multiple freezes in the freeze tolerant sub-Antarctic tineid caterpillar *Pringleophaga marioni,* although multiple cold exposures caused a long-term depression of feeding regardless of whether individuals froze or not, suggesting that chilling injuries may be more important than freezing *per se* in this species (Sinclair and Chown, 2005).

Previous experimental designs have failed to control for the total amount of time spent frozen which may drive the accumulation of injuries (Bale *et al.*, 2001; Brown *et al.*, 2004; Churchill and Storey, 1989a; Sinclair and Chown, 2005). In addition, the sub-lethal effects of multiple freeze exposure have not been well elucidated (but see Churchill and Storey, 1989a; Sinclair and Chown, 2005), because binary responses (survival or emergence versus death) in the short term do not account for sub-lethal effects (Layne and Peffer, 2006). These shortcomings limit the capacity to predict potential climate change impacts in insects that overwinter in temperate zones.

The arctiid moth *Pyrrharctia isabella* Smith (Lepidoptera: Arctiidae) overwinters as a freeze tolerant final instar caterpillar under leaf litter in the USA and southern Canada (Goettel and Philogene, 1978; Layne *et al.*, 1999). During the fall, *P. isabella* move to diapause locations and cease feeding (Goettel and Philogene, 1978; Layne *et al.*, 1999). At this time they accumulate large quantities of glycerol as a cryoprotectant and their winter-acclimatized SCP varies between -6 and -8 °C (Layne *et al.*, 1999). The overwintering microhabitat of *P. isabella*, its susceptibility to inoculative freezing (Layne *et al.*, 1999) and its relatively high SCP suggests that individuals of this species would likely experience repeated freezing events through the winter in southern Ontario.

I used *P. isabella* to investigate the sub-lethal effects of multiple freeze-thaw cycles on three sub-lethal measures likely to affect post-winter fitness. First, multiple freeze-thaw cycles could affect aspects of caterpillar overwintering and freeze tolerance such as SCP, cryoprotectant concentration, and survival, as caterpillars physiologically adjust to freezing events. Secondly, repeatedly-frozen caterpillars could use more energy reserves that would be predicted to impact future fitness since overwintering energy reserves are directly tied to spring reproductive ability in univoltine insects (*e.g.* Irwin and Lee, 2003).

Finally, given that immune function is costly to many organisms (Zuk and Stoehr, 2002), immune function could be compromised if energy is prioritized to cryoprotectant compounds or repairing freeze-induced damage and away from immune system components.

I test three competing hypotheses. First, if there is no cost to freezing *per se* (Sinclair and Chown, 2005), then fitness will not differ between individuals exposed to multiple freeze-thaw cycles or a single sustained freezing event. If there is a cost to the freezing and/or thawing processes (Churchill and Storey, 1989a), then repeated freezing will reduce fitness compared to the sustained freezing and control treatments. Finally, if lowered metabolic rate experienced by frozen animals results in increased fitness and overwinter energy use is a prime determinant of subsequent fitness (Irwin and Lee, 2002), then caterpillars that experienced sustained freezing and caterpillars that experienced multiple freeze-thaw cycles would have similar fitness, and both would have higher fitness than control individuals.

4.2　Methods

4.2.1　Animal collection and maintenance

Pyrrharctia isabella caterpillars were collected from fields and pathways in London, Ontario, Canada (43°00′ N, 81°15′ W) and Niagara on the Lake, Ontario, Canada (43°04′ N, 79°04′ W) during September and October 2007, 2008, and 2009. Caterpillars were maintained individually in plastic containers containing pinto bean diet (Goettel and Philogene, 1978) until feeding ceased (mid-November) and food was removed. Incubator temperatures followed weekly climate maximum and minimums for London, Ontario, obtained from Environment Canada (www.weatheroffice.ec.gc.ca) until 0 °C was reached in the incubators. Relative humidity was maintained at 70 % by the addition of a tray of salt solution in the incubator. Caterpillars were then maintained at 0 °C, and experiments were conducted after at least three weeks at 0°C. These methods were employed each year with the following modifications: In 2007-2008 caterpillars were maintained on grass until feeding ceased, and in 2009-2010, temperatures were set to fluctuate daily from 0 °C to 2 °C (12:12 L:D).

4.2.2   Freezing treatments

In all experiments, caterpillars were assigned to one of three treatment groups: control (maintained at 0 °C), frozen for a single bout of 35 hours (1×35 h), or five bouts of 7 hours (5×7 h). Caterpillars receiving the 5×7 h freeze-thaw treatment were frozen every fifth day. Due to inter-annual variability in SCP, to ensure that all caterpillars froze the cold exposure used varied between years: -12 °C in 2007-2008 and 2009-2010, and -14 °C in 2008-2009. To reduce the effect of unequal freezing time between the 1×35 h and 5×7 h freeze groups due to cooling rate, in all years, caterpillars were cooled at 0.5 °C min^{-1}. Freeze treatments were performed by placing caterpillars in 35 ml plastic vials in contact with the tip of a 36-AWG copper-constantan thermocouple (Omega, Laval, Quebec, Canada) held in place with a foam stopper. These vials were placed into wells in an aluminum block cooled by either methanol or propylene glycol circulated by either a programmable Proline 3530C refrigerated bath (Lauda, Wurzburg, Germany) or programmable VWR Signature 1157P refrigerated circulator (VWR Missisauga, Ontario, Canada).

Thermocouples were connected to Picotech TC-08 thermocouple interfaces and temperatures were recorded using PicoLog software for Windows (Pico Technology, Cambridge, UK). Freezing was detected and SCP determined from the exotherm (Lee, 2010). After freezing, caterpillars were warmed to 0 °C at 0.5 °C min^{-1}, and returned to their incubator at 0 °C. Supercooling points in repeatedly frozen caterpillars were compared between 2008-2009 and 2009-2010, as well as within each year for effects of freezing interval using a repeated-measures two-way ANOVA conducted in R (R Development Core Team, 2008).

4.2.3   Survival

Survival was defined as a curling defensive response to stimulus from a pointed probe 24 h after removal from freeze treatment. Control caterpillars were set aside when the 5×7 h treatments were started and then survival was tracked for the duration of the experiments (25 days). Survival was compared between caterpillars in the three treatment

groups (control, 1×35 h and 5×7 h) with a generalized linear model with binomial error and logit link in R.

4.2.4   Metabolic rate

Metabolic rate measurements were conducted during the winter of 2007-2008. Eighteen hours after thawing, caterpillars from each treatment (n = 5 per treatment) were placed a glass respirometry chamber suspended in a propylene glycol VWR refrigerated bath set to 5 °C and allowed to acclimate for 6 hours before metabolic rate measurements (approximated from CO_2 production). CO_2 production by caterpillars was then measured using flow-through respirometry system with dried CO_2-free air flowing at 12 ml·min^{-1} (Sable Systems International, Las Vegas, NV, USA) connected to a Li-Cor 7000 CO_2 differential infrared gas analyzer for a continuous recording of 6 h (Li-Cor Biosciences, Lincoln, NB, USA, method modified from Williams *et al.* (2010). For CO_2 production traces that exhibited discontinuous gas exchange, data from an entire cycle was selected. For traces that exhibited continuous gas exchange, a section of data that exhibited no upward or downward trends, with no detectable caterpillar movement, was analyzed. The rate of CO_2 production was compared among groups using an a nonparametric Kruskal-Wallis test conducted in SPSS Statistics 17.0 (SPSS Inc., Chicago, Illinois, USA) with caterpillar mass immediately before respirometry as a covariate.

4.2.5   Quantification of metabolic reserves and cryoprotectants

Twenty-four hours after cold exposure, ten caterpillars from each treatment group in winter 2008-2009 were flash frozen in liquid nitrogen and stored at -80 °C. Caterpillars were then weighed (fresh mass), dried at 60°C for one week, then weighed again (dry mass). Water mass was calculated as the difference between wet mass and dry mass. Whole caterpillars were homogenized in 10 mL 0.05 % Tween 20, then diluted $10\times$ in additional 0.05 % Tween 20. Subsamples of 1 mL (in duplicate for each caterpillar) were centrifuged at $16,000 \times g$ for 1 minute, and the supernatant removed and stored at -20 °C. Total protein, glucose, glycogen, glycerol and glycerol from triglyceride in the supernatant were assayed in triplicate using spectrophotometric assays as described by Gefen *et al.* (2006). Differences in energy reserve mass, cryoprotectant concentration,

and protein mass among treatment groups were investigated with ANCOVAs with dry mass as a covariate in R.

4.2.6   Immune challenge

Immune challenge experiments were conducted during the fall of 2009. A fungal challenge was chosen because entomopathic fungi are common in *P. isabella* overwintering habitat (Bidochka *et al.,* 1998), and I had previously noted fungal infection in laboratory-maintained caterpillars. Cultures of *Metarhizium anisopliae*, (Hypocreales: Clavicipitaceae, strain number 2575, from USDA collection, Ithaca, NY) a broadly entomopathic fungus (Bidochka *et al.*, 1998), were obtained from Michael Bidochka (Brock University, St Catharine's, ON, Canada). Spores were plated onto potato-dextrose agar plates and cultured at 25 °C in darkness for 14 days. After sporulation, plates were frozen at -20 °C until use. To obtain standardized spore suspensions, spores were suspended in a 0.01 % solution of Triton-X in distilled water. Spore concentration was determined using an improved Neubauer counting chamber (CA Hausser & Son, Philadelphia, PA, USA), and the suspension was diluted to 1×10^6 spores·mL^{-1}.

Twenty-four hours after thawing, five microlitres of spore suspension was injected into the ventral surface of caterpillars between the first and second pair of prolegs, immediately below a proleg to avoid piercing the gut. A 20 µL Hamilton syringe with a 30.5-gauge needle was used for all injections. Control caterpillars received a sham injection of 5 µL 0.01 % Triton-X. The needle and syringe were sterilized with bleach between injections, and the order of injections was randomized. Caterpillars were then placed on moistened vermiculite in parafilm-sealed 100 mL plastic vials. Vials were placed into an incubator at 26 °C. Containers were opened to check caterpillar survival and allow for fresh air circulation daily. Mortality was compared between control and spore-injected caterpillars within each freeze treatment group after 8 days using Fisher's Exact Test in SigmaStat.

4.2.7 Tissue damage

Five caterpillars from each group were dissected 24 h after their final cold exposure in 2009-2010. First, a 10 μL sample of hemolymph was obtained by pricking the caterpillar on the ventral surface between the first and second thoracic segment with an insect pin then sampling from the beaded hemolymph with a pipette. The caterpillar was then dissected from the ventral surface, and a small portion of Malpighian tubules and fat body was removed from the abdomen.

Live/dead staining was conducted with modifications to the method of Yi and Lee (2003). Tissue and hemolymph samples were incubated at room temperature for 10 min in 30 μL of a 1:10 solution of SYBR 14 (in DNA-bound complex λmax_{ex} = 475 nm, λmax_{em} = 516 nm) in Coast's solution on a glass slide (Coast, 1988; Yi and Lee, 2003). Then 30 μL of a 1:20 solution of propidium iodide (in DNA-bound complex λmax_{ex} = 540 nm, λmax_{em} = 617 nm, both dyes obtained from Live/Dead Sperm Viability Kit, Invitrogen Canada Inc, Burlington, Ontario, Canada) in Coast's solution were added and the cells or tissues were incubated for a further 10 min. Slides were then visualized under 50 × magnification using an Axio Observer Z1 microscope (Carl Zeiss MicroImaging GmbH, Goettingen, Germany) using GFP (excitation wavelengths = 430-510 nm, emission wavelengths = 474-575 nm) and Rhodamine (excitation wavelengths = 534-568 nm, emission wavelengths = 575-640 nm) filters for the SYBR 14 and propidium iodide dyes respectively. SYBR 14 dyes live cells and fluoresces green, while propidium iodide stains dead cells and fluoresces red.

Image analysis was conducted with ImageJ software (Abramoff *et al.*, 2004). Contrast of images was enhanced and standardized by setting the percentage of saturated pixels to 0.4 % and equalizing and normalizing the histogram. The threshold for each image was set at a level that minimized background noise, and only area identifiably part of the tissue in question was included. Finally, the area of the image covered by fluorescence was calculated separately for red (propidium iodide) and green (SYBR 14) channels. One-way ANCOVA with total fluorescing area as a covariate was used to compare amounts of damaged tissue between freeze treatment groups for each tissue in R.

4.3   Results

4.3.1   Survival

In every year, caterpillars that were repeatedly frozen had higher mortality than control and caterpillars that had a single prolonged freeze exposure. Although the difference was not statistically significant in every year, when pooled across years within treatment groups, mortality was significantly higher in caterpillars repeatedly frozen at 29.7 % compared to caterpillars that experienced a single prolonged freeze exposure and control caterpillars at 10.3 % and 13.1 % respectively (Wald χ^2 = 12.685, df = 2, p = 0.002, n = 69 – 111 in each group, Figure 4.1). Cumulative survival in caterpillars repeatedly frozen decreased linearly with increased freeze-thaw cycles (R^2 = 0.981, n = 5, p < 0.001, total caterpillars tested = 111, Figure 4.2).

4.3.2   Supercooling points

Caterpillars that were frozen for the first time had supercooling points that ranged from -8.2 °C to -12.1 °C in 2008-2009 and from -3.8 °C to -12.1 °C in 2009-2010. Supercooling points in 2008-2009 (-10.7 ± 0.2°C) were significantly lower than in 2009-2010 (-7.6 ± 0.2 °C, $F_{1,538}$ = 207.82, p < 0.001, Figure 4.3). Within the winters of 2008-2009 mean supercooling points significantly increased by approximately 0.2 °C (from -10.5 ± 0.2 °C to -10.7 ± 0.3 °C) over five freeze-thaw cycles. By contrast, in 2009-2010 supercooling points declined 0.5 °C (from -7.1 ± 0.2 °C to -7.6 ± 0.2 °C) after 5 freeze-thaw cycles ($F_{1,538}$ = 4.83, p = 0.028).

4.3.3   Metabolic rate

Discontinuous gas exchange was exhibited by six out of 15 caterpillars (Williams *et al.*, 2010).   Neither log- nor square root-transformation improved normality of CO_2 production rate data, so a nonparametric Kruskal-Wallis test was used to compare CO_2 production rates between treatment groups. Caterpillars that experienced multiple freeze-thaw events did not have an increased metabolic rate 24 h after their freeze compared with caterpillars that had a single sustained freezing event (Kruskal-Wallis Test, H =

0.033, df = 2, p = 0.981, n = 5 in each group, Figure 4.4), nor was there a trend in metabolic rate with successive freezing events (data not shown).
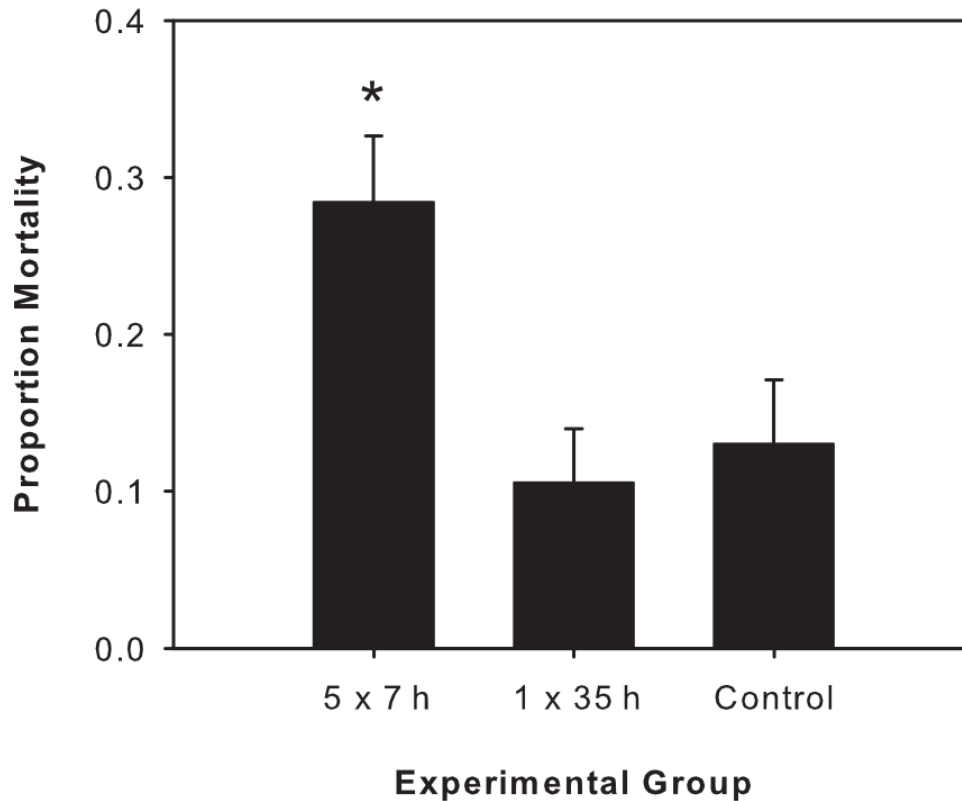
Figure 4.1 Mortality of *Pyrrharctia isabella* caterpillars 24 h after freeze treatments. Individuals are pooled over three winters of experiments differs significantly between treatment groups (control caterpillars were followed from the first freeze treatment in the 5 × 7 h freeze group through to 24 h after final freeze, Wald χ^2 = 12.685, df = 2, p = 0.002, n = 69 − 111 in each group). Control indicates maintained at 0 °C, "5 × 7 h" indicates five 7 hour freezes at -12 °C with 5 days between each freeze at 0 °C, while "1 × 35 h" indicates a single 35 h freeze at -12 °C. Error bars indicate standard error of the proportion, asterisk indicates a significant difference between caterpillars with multiple freeze-thaw exposures and other groups.
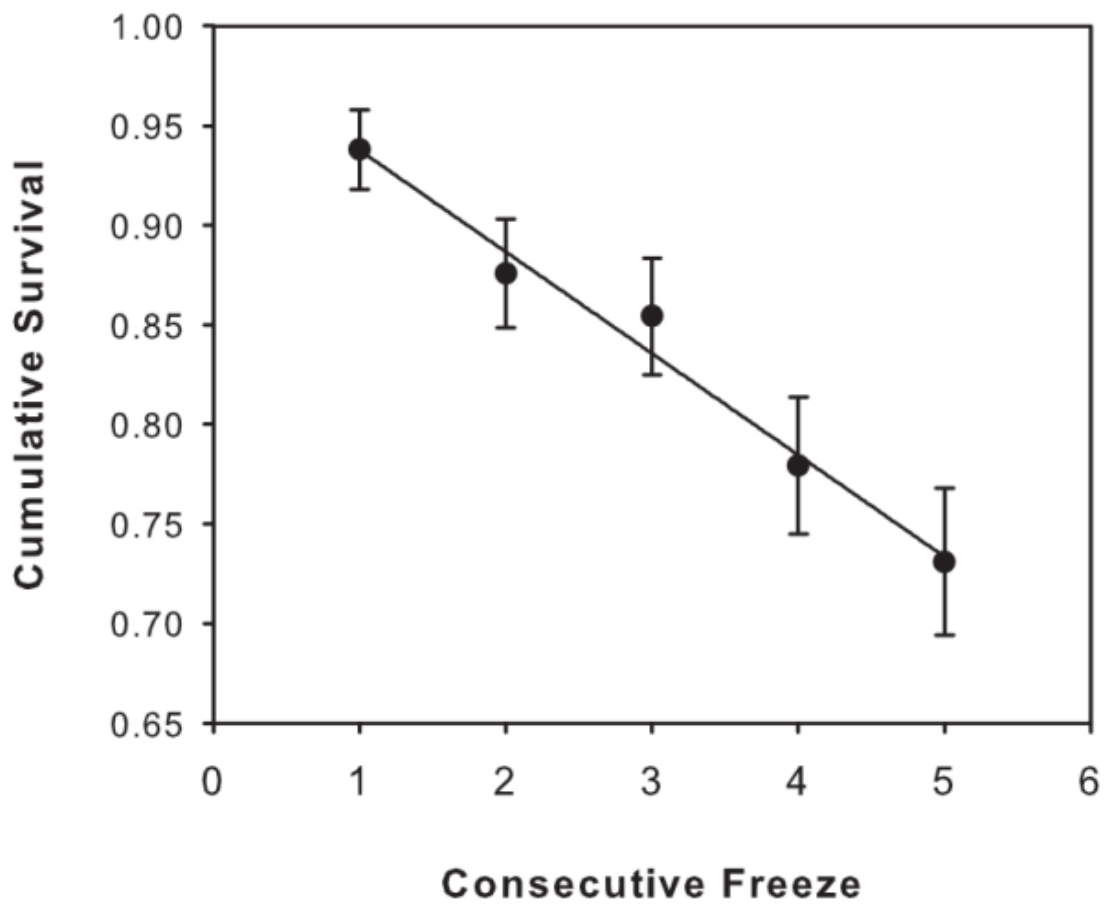
Figure 4.2 Cumulative survival in freeze tolerant *Pyrrharctia isabella* caterpillars after repeated freeze thaw cycles. Survival declines linearly with increasing freezes (R^2 = 0.982, n = 5, p < 0.001, total caterpillars tested = 111, error bars indicate standard error of the proportion).
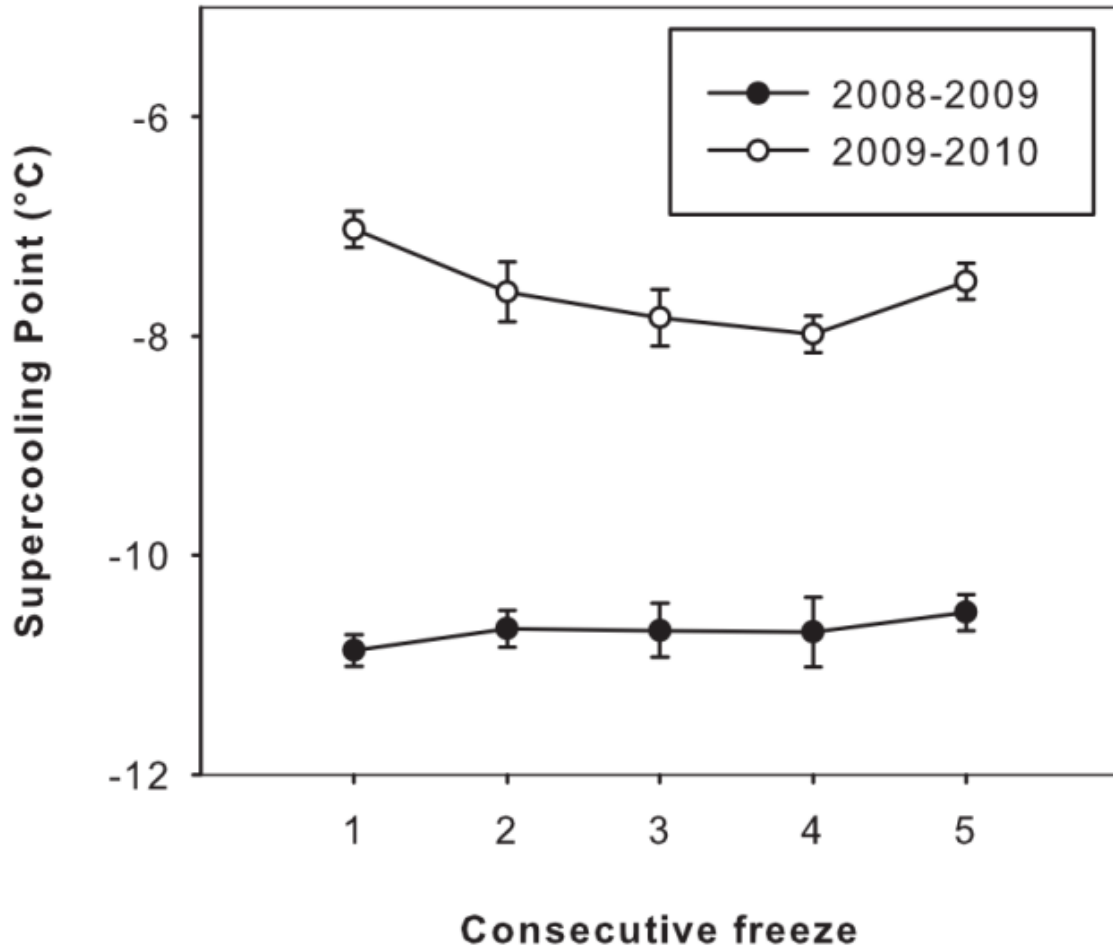
Figure 4.3 Supercooling points (mean ± SE) of freeze tolerant *Pyrrharctia isabella* caterpillars. Supercooling points declined only by -0.5 °C in 2009-2010 after repeated freeze-thaw cycles between -12 °C and 0 °C. In 2008-2009, supercooling points changed very slightly with increased freeze-thaw cycles between -14 °C and 0 °C. All caterpillars were frozen for 7 h every 5 days.
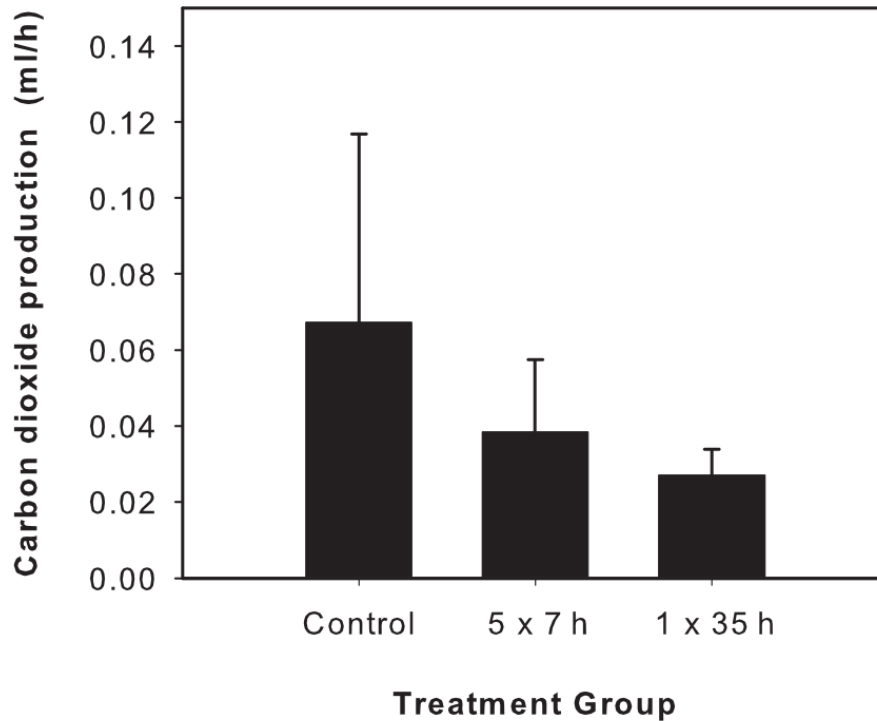
Figure 4.4 Carbon dioxide production rate (ml/h, mean ± SE) of *Pyrrharctia isabella* caterpillars. Carbon dioxide production rate at 5 °C does not differ between treatment groups 24 hours after freezing (Kruskal-Wallis Test, H = 0.033, df = 2, p = 0.981, n = 5 in each group). Control indicates maintained at 0 °C, "5 × 7h" indicates five 7 hour freezes at -12 °C with 5 days between each freeze at 0 °C, while "1 × 35h" indicates a single 35 hour freeze at -12 °C.

4.3.4   Energy reserves and cryoprotectants

Repeated freezing increased caterpillar glycerol concentration by 0.82 M compared with control caterpillars, while caterpillars that had experienced a sustained freeze had an intermediate concentration of glycerol (all statistics reported in Table 4.1). Other than this change in glycerol content, there were generally few differences in energy reserves or cryoprotectants between caterpillars with different freeze exposures (Table 4.1). Triglyceride stores did not differ between caterpillars with different freeze treatments, although there was a trend towards decreased quantities in caterpillars that had experienced repeated freezing. Repeated or prolonged freezing did not change caterpillar water content, protein content, or glucose mass. There was a near-significant interaction between dry mass and freeze treatment group on glycogen mass ($F_{2,29} = 3.39$, $p = 0.051$), however when the residuals of a regression of glycogen mass on dry mass were examined, there was no difference between caterpillars in different freeze treatment groups ($F_{2,29} = 0.258$, $p = 0.774$).

4.3.5   Immune challenge

Caterpillars injected with *M. anisopliae* spores died approximately five days after injection, and white mycelia were observed on their ventral surface. Injection with the spores greatly increased mortality in caterpillars relative to sham injections in both the control and 1×35 h freeze treatment groups (Fisher's Exact Test, $p < 0.03$ in both cases, n = 17-20 in each group). However, repeatedly frozen caterpillars had low mortality whether injected with spores or sham solution (Fisher's Exact Test, $p = 1.00$, n = 28 in each group, Figure 4.5).

4.3.6   Tissue damage

Repeated freeze-thaw caused increased damage to both Malpighian tubules ($F_{2,9} = 7.05$, $p = 0.014$, Figure 4.6) and hemocytes ($F_{2,9} = 11.18$, $p = 0.004$) compared to a single

Table 4.1 Energy reserves, cryoprotectant, and protein content (mean ± SE) of *Pyrrharctia isabella* caterpillars either frozen five times for 7 h at -12 °C, a single 35 h exposure at -12 °C, or control (maintained at 0 °C; n = 10 per group). F and p-values relate to comparing caterpillars in different treatment groups in an ANCOVA using dry mass as a covariate (except for dry mass, which was tested by one-way ANOVA). Similar letters indicate groups whose values are not significantly different in a Tukey *post-hoc* test.

Measure	Control	5 × 7 h	1 × 35 h	F (p)
Protein (mg/mg dry tissue)	0.0783 ± 0.0093	0.0675 ± 0.0093	0.0733 ± 0.0076	0.332 (p = 0.720)
Glucose (mg/mg dry tissue)	0.014 ± 0.006	0.016 ± 0.002	0.015 ± 0.002	1.08 (p = 0.355)
Glycogen (mg/mg dry tissue)	0.002 ± 0.0004	0.003 ± 0.0013	0.003 ± 0.0009	0.258 (p = 0.774)
Free glycerol (M)	1.8257 ± 0.2659[a]	2.6434 ± 0.2281[b]	2.5159 ± 0.2271[ab]	4.548 (p = 0.021)
Glycerol from triglyceride (µmol/mg dry tissue)	0.5968 ± 0.0845	0.4542 ± 0.0875	0.4981 ± 0.0894	0.125 (p = 0.883)
Caterpillar dry mass (mg)	255.166 ± 17.467	309.626 ± 12.552	293.449 ± 18.542	0.756 (p = 0.480)
Caterpillar water mass (mg)	286.188 ± 35.544	283.304 ± 27.008	252.433 ± 24.436	0.433 (p = 0.653)
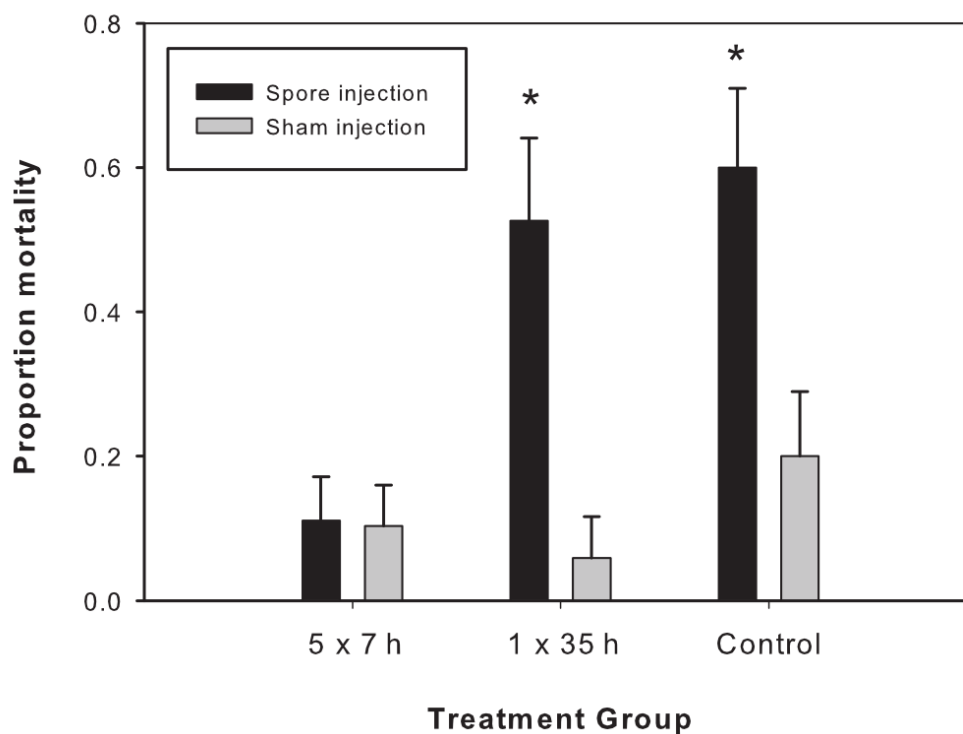
Figure 4.5 Survival of *Pyrrharctia isabella* caterpillars following freezing and injection with *Metarhizium anisopliae* spores.   Injection with *Metarhizium anisopliae* spores caused significantly increased mortality in control caterpillars and caterpillars frozen once at -12 °C for 35 hours, however caterpillars that experienced five repeated seven hour freezes at -12 °C had similarly low mortality whether injected with spore solution or a sham solution.   Asterisks indicate significant differences in mortality between injections within each freeze treatment. Error bars indicate standard error of the proportion.
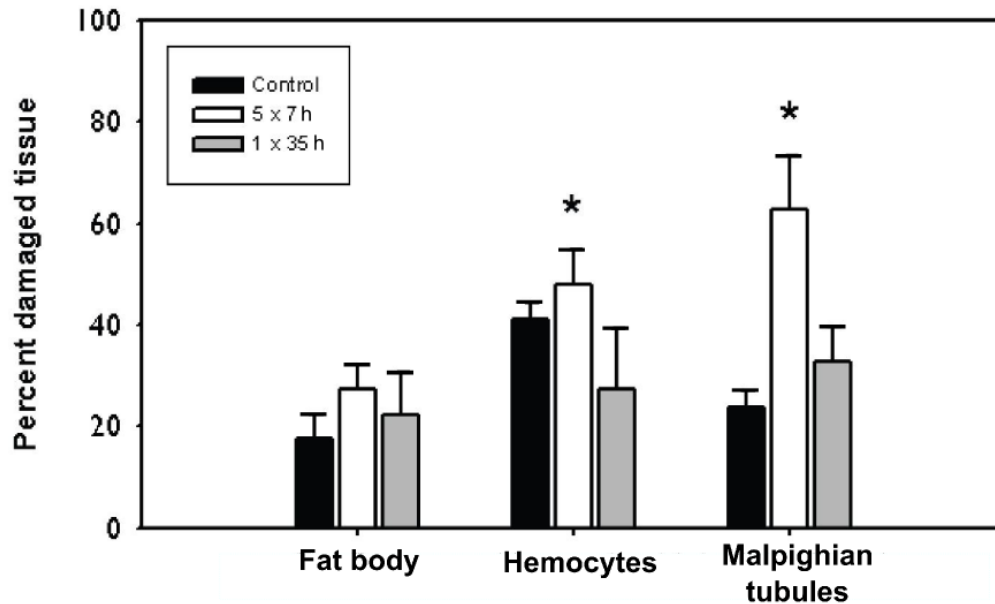
Figure 4.6 Tissue damage in *Pyrrharctia isabella* caterpillars caused by freezing at -12 ºC. Amount of tissue damage (calculated as area fluorescing red due to propidium iodide staining divided by total fluorescing area, mean $\pm$ SE, n = 5 caterpillars in each group) differed in *P. isabella* individuals between control, 5×7 h freeze, or 1×35 h freeze treatments in the Malpighian tubules and hemocytes. Percentage damaged area was similar between groups in fat body. Asterisks indicate significant differences between groups within tissues with *post-hoc* testing in an ANCOVA with total fluorescing area as a covariate.

sustained freeze in caterpillars. Viability of fat body cells did not differ significantly among caterpillars with different freeze treatments ($F_{2,9} = 0.09$, p = 0.933; Figure 4.6).

4.4   Discussion

I found that there is a fitness cost to freeze-thaw events in overwintering freeze tolerant *P. isabella* caterpillars that was independent of the amount of time spent frozen. Individuals that had experienced multiple freeze-thaw events had higher mortality and more damaged cells in their Malpighian tubules and hemolymph than individuals that either had no freezing events or a single freezing event. These fitness consequences also appeared to be independent of energetics: there was neither an increase in metabolic rate, nor a change in energy reserves observed between individuals that experienced multiple freezing versus single freezing events. Because I controlled for the amount of time spent frozen, in contrast to Sinclair and Chown (2005), our results indicate that the impact of freezing on freeze tolerant organisms like *P. isabella* depends on the number of freeze-thaw transitions that the animal experiences rather than the cumulative amount of time spent in the frozen state.

I also found that cumulative survival in caterpillars decreases linearly with increasing freeze-thaw cycles, indicating that the proportion of caterpillars that die increases with each successive freezing exposure (Figure 4.2). Thus, the probability of being killed by freezing increased with the number of freezing exposures *P. isabella* experienced. This implies that rather than a constant threat of mortality, each freezing event causes caterpillars to accumulate injuries that are not fully repaired and consequently increase the likelihood of being killed by subsequent freezing events. Similarly, in the fly *Delia radicum* and the moth *Mamestra configurata*, repair from extreme cold stress does not take place even with four weeks recovery time (Turnock *et al.*, 1983; Turnock *et al.*, 1985).

Similar to previous studies (Bale *et al.*, 2001; Brown *et al.*, 2004), I observed a statistically significant decrease in SCP with increased freeze-thaw cycles in one year (Figure 4.3). However this change was very small (0.5°C) and in an ecological context I believe it is of doubtful biological significance. After five freeze-thaw cycles, glycerol

concentration increased by 0.82 M, which would be expected to decrease SCP by approximately 1 °C (Zachariassen, 1985). Since SCP declined by only 0.5 °C, I suggest that SCP in this species is defended by the presence of ice nucleators. While survival was reduced in caterpillars that had experienced multiple freeze-thaw cycles, this does not appear to be a result of losing freeze tolerance as reported by Bale *et al.* (2001) and Brown *et al.* (2004) since many individuals were still able to survive freezing, but rather a result of accumulated freeze-thaw damage.

There was no significant difference in energy reserves or metabolic rate among any of the experimental groups 24 h after a freeze (Figure 4.4, Table 4.1). I interpret this to indicate that the response to being frozen does not demand a sustained investment of energy in *P. isabella*. There has been much speculation about the energetic costs of repair of freezing-induced damage and this has been used to conclude that reduced metabolic rate in the 4 h post-freezing indicates that repair has not occurred (Sinclair *et al.*, 2004), and (conversely) that elevated metabolism post-freezing indicates the cost of repair (Block *et al.*, 1998). I similarly observed a non-significant trend towards depression in metabolic rate 24 hours after a freezing event (Figure 4.4). However, I also observed residual tissue damage after 24 h. Since I do not know how long (if ever) that damage takes to be repaired, I cannot draw a conclusion about the relationship between metabolic rate and tissue damage, although the relationship could be resolved using the approach I describe to examine the process of recovery from freezing.

I found that *P. isabella* caterpillars collected in southern Ontario constitutively contained almost ten times the amount of glycerol previously reported from western Pennsylvania (Layne and Kuharsky, 2000; Layne *et al.*, 1999). As some broadly-distributed freeze tolerant insects vary their cold tolerance (and glycerol concentration) along latitudinal gradients (*e.g. E. solidaginis*; Williams and Lee, 2008), this large difference between glycerol concentrations may be at least partially due to greater cold hardiness in our more northerly population. Another possible explanation may be an effect of our overwintering treatment since dry conditions can increase glycerol concentration in *P. isabella* up to five-fold (Layne and Kuharsky, 2000).

I found that repeatedly frozen caterpillars increased their glycerol concentration by 0.82 M relative to control, while there was a non-significant increase after a single prolonged exposure (Table 4.1). Given that water content did not differ among experimental groups, and that feeding does not occur during winter, I suggest that this indicates that *P. isabella* synthesize glycerol in response to a freezing event. Churchill and Storey (1989a) found that with increased number of freeze-thaw cycles, pre-pupae of the freeze tolerant gall fly *Eurosta solidaginis* almost quadrupled their concentration of sorbitol and concomitantly decreased their glycogen content. Similarly, both freeze tolerant frogs (*Rana sylvatica*) and earthworms (*Dendrobaena octaedra*) mobilize glucose as a cryoprotectant during freezing (Calderon *et al.*, 2009; Storey and Storey, 1996).

Contrary to expectations, I observed no change in glycogen content corresponding to the increase in glycerol. However evidence that glycogen is the direct source of all glycerol produced during cold hardening is mixed. For example, during prolonged cold exposure in larvae of the moth *Epiblema scudderiana*, the amount of glycogen initially dips as glycerol is synthesized, then rebounds to control values over the course of two days even though glycerol concentration remains high (Churchill and Storey, 1989b). Similarly, while the rice stem borer *Chilo suppressalis* and the eastern spruce budworm *Choristoneura fumiferana* increase glycerol concentration during seasonal cold hardening, in both cases the amount of carbon in glycerol produced greatly outstrips the quantity of carbon depleted in glycogen (Han and Bauce, 1995; Li *et al.*, 2002). Given that glycogen content in overwintering Lepidopteran larvae is often very low (Han and Bauce, 1995; Li *et al.*, 2002) I suspect that glycerol was synthesized from other sources. While I found no significant difference between freeze treatment groups in glycerol released from triglycerides, there is a large trend, and I suggest that either the glycerol backbone or the free fatty acids produced could be the source. Another potential source for the glycerol is phospholipids, which can be an important energy source during starvation (reviewed in Fast, 1964).

Caterpillars that had experienced multiple freeze-thaw cycles were more likely to survive an immune challenge. To our knowledge, this is the first report of enhancement of the immune response in an insect as a result of freezing, and adds to the limited knowledge

of the interactions between ecological processes and immune function (Rolff and Siva-Jothy, 2003). Although research on the effects of temperature extremes on invertebrate immune function is limited, mild cold stress has been shown to improve survival of fungal infection in *D. melanogaster* (LeBourg *et al.,* 2009) whereas snails given repeated high temperature shocks have decreased immune function (Seppälä and Jokela, 2011). I found that, rather than trading off allocation to immune function for repairing freeze damage, *P. isabella* caterpillars have increased immune response to a fungal challenge after multiple freeze-thaw cycles. I did not investigate the mechanisms underlying this improvement in immune function, however I note that repeated freezing damages Malpighian tubules and hemocytes (Figure 4.6). This damage could potentially induce a wounding response leading to increased immune function through the phenoloxidase pathway (Cerenius and Söderhäll, 2004; Marmaras *et al.*, 1996). Alternatively, this increased immune function could be a result of a generalized stress response—for example, *Hsp70* is upregulated in many insect species after cold shock (Sinclair *et al.*, 2007) or as part of seasonal hardening (Rinehart *et al.*, 2007) and is also important in the immune responses in vertebrates (Pockley, 2003), suggesting a link between cold stress and immune responses in insects. Given that the spores of the species of fungus I used are found broadly and abundantly through Ontario (up to 70% of sites sampled, Bidochka *et al.*, 1998), and that entomopathic fungi in general are common (91% of sites sampled, Bidochka *et al.*, 1998), I suggest that overwintering immune function is an important, yet neglected, part of insect overwintering ecology.

The increased tissue damage in multiple freeze caterpillars occurred in the Malpighian tubules and hemocytes, but not in the fat body. Malpighian tubules and hemocytes are also among the most freeze-sensitive tissues in *E. solidaginis* (Yi and Lee, 2003). In contrast to our results, Yi and Lee (2003) also found that *E. solidaginis* fat body cells are severely damaged during prolonged freezes. However, unlike in other freeze tolerant insects, *E. solidaginis* fat body cells freeze at similar temperatures to the whole organism (Bennett and Lee, 1997). *P. isabella* fat body cells do not freeze at -10 °C (Layne and Blakeley, 2002), and they suggested that freezing of fat body is unrelated to survival in this species. Given the lack of increased tissue damage to fat body cells, as well as the

low water content of the fat body, it seems unlikely that *P. isabella* fat body cells freeze during a whole organism freezing event.

In summary, I found that multiple freeze-thaw events led to increased damage to the Malpighian tubules and hemocytes of *P. isabella*, and increased mortality compared to single prolonged freezes. I suggest that the stress of freeze-thaw events outweighs the stress of prolonged freezing, however, repeated freezing also appears to upregulate immune function. These effects indicate the potential for important trade-offs in overwintering insects in the field. Given that I found the response to multiple freezing events increased immune function enough to completely match survival of infected caterpillars to uninfected caterpillars (reduced mortality five-fold), yet only tripled freezing mortality, the advantages of increased immune function due to repeated freezing may interact with the increased risk of freezing mortality in a field context. Taken together our study demonstrates that important sub-lethal effects of repeated freezing are not predictable from the effects of single freezing event, and I suggest that these differential responses to multiple freezing events will become increasingly important in a changing climate.

4.5   Acknowledgements

4.6 References

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Chapter 5

5 Low temperature exposure during overwintering causes delayed mortality in the eastern spruce budworm

In this chapter I examine how increased period, duration, and decreased temperature impact the overwintering physiology and subsequent survival of the freeze avoiding eastern spruce budworm *Choristoneura fumiferana*. This chapter is in preparation for the *Journal of Animal Ecology*.

5.1 Introduction

The eastern spruce budworm (*Choristoneura fumiferana*, Lepidoptera: Tortricidae) is one of the most important natural pests of the Canadian boreal forest (Gray, 2008; Régnière *et al.*, 2012), and is found throughout the North American boreal forest, from Alaska to the island of Newfoundland. The eastern spruce budworm feeds on several boreal tree species, with balsam fir (*Abies balsamea*) as its preferred host (reviewed by Régnière *et al.*, 2010). Over the past several centuries *C. fumiferana* populations have exhibited extreme population fluctuations with a period of 30-40 years (Boulanger and Arseneault, 2004). For example, in the 1920s outbreak, *C. fumiferana* larvae killed 45% of available host trees in eastern Canada (Gray, 2008). In non-peak years *C. fumiferana* population densities are so low that there can be fewer than one individual larva per several hundred branches (Royama, 1992).

The eastern spruce budworm is strictly univoltine. Adults emerge in July and lay eggs over several weeks. Eggs hatch within two weeks, and the first instar larvae spin hibernacula and molt into second instar larvae without feeding. These second instar larvae enter an obligate diapause (which continues until the end of February; Régnière, 1990), and remain in their hibernacula all winter (six months in total, Han and Bauce, 1993). In the spring the larvae emerge from their hibernacula and feed on newly-grown buds. This period, when the larvae rapidly develop through an additional four instars before pupation in the spring, is when the defoliation that leads to tree death occurs (Régnière *et al.*, 2012).

Due to the length of the overwintering stage, as well as the fact that the eastern spruce budworm's range does not extend as far north as that of its host trees (Gray, 2008), there has been significant interest in the cold hardiness of the species (Han and Bauce, 1993; Han and Bauce, 1995b; Han and Bauce, 1998). More recently, models of the effects of climate change on future population distribution and outbreak cycles of the eastern spruce budworm have explicitly included the overwintering stage (Gray, 2008; Régnière *et al.*, 2012). These models have indicated that spring and summer population dynamics may be strongly mediated by fitness of the overwintering stage.

5.1.1   Cold hardiness

The eastern spruce budworm is a freeze avoiding species which survives the winter by depressing its supercooling point (SCP, the temperature at which supercooling of body fluids stops and the insect freezes, Lee, 2010). This is accomplished by accumulating *ca.* 0.8 M glycerol synthesized from glycogen reserves (Han and Bauce, 1995a; Han and Bauce, 1995b; Lee, 2010), expressing a suite of hyperactive antifreeze proteins (Qin *et al.*, 2007; Tyshenko *et al.*, 1997), and reducing body water to *ca.* 35% of fresh mass (Bauce and Han, 2001). Overwintering larvae are highly cold tolerant with SCPs as low as -35 ℃, although exposures to -15 ℃ for longer than ten days can induce mortality (Han and Bauce, 1995a). Their cold hardiness peaks during mid-diapause, while early and late diapause larvae have reduced cold hardiness (Han and Bauce, 1995a). By the end of winter glycogen reserves are nearly depleted while lipid reserves remain intact, suggesting that overwinter metabolism in *C. fumiferana* larvae appears to be fueled by glycogen reserves rather than lipids (Han and Bauce, 1993). Glycogen reserves therefore represent both fuel and potential cryoprotectant. In the spring glycogen reserves are depleted and thus the amount of glycerol that can be synthesized is also reduced, which suggests that cold snaps in spring may be an important source of mortality (Han and Bauce, 1998).

Low temperature exposure can induce chilling injury in insects, which leads to mortality unrelated to freezing as the duration and intensity of cold exposure increases (Chen and Walker, 1994; Nedvĕd *et al.*, 1998). There are two types of chilling injury, categorized by how quickly damage accumulates (Chen and Walker, 1994; Lee, 2010). Direct

chilling injury occurs within hours of exposure to very low temperatures, while indirect chilling injury occurs over longer periods (days or weeks) of exposure to more mild temperatures (Chen and Walker, 1994; Lee, 2010).   Indirect chilling injury can cause mortality long after the low temperature exposure (Rojas and Leopold, 1996). At low temperatures, lipid membranes have reduced fluidity or transition from fluid to gel phase (Quinn, 1985), and enzyme function slows (Hochachka and Somero, 2002). This disrupts homeostasis, which can lead to ion imbalances (Koštál *et al.*, 2004; MacMillan and Sinclair, 2011).   An alternative hypothesis is that chilling injury may be caused by oxidative stress, since antioxidant enyzmes are upregulated during chilling in the laboratory (Rojas and Leopold, 1996) and while overwintering in the field (Joanisse and Storey, 1996). In addition, low temperature exposure can denature proteins (Todgham *et al.*, 2007) and cause single- and double-stranded DNA breaks (Yao and Somero, 2012). There is support for many of these mechanisms underlying both indirect and direct chilling injury; however, it appears that the underlying causes of each type of injury may differ. For example, *Drosophila melanogaster* selected for survival of indirect chilling injury do not have improved survival of direct chilling injury (Chen and Walker, 1994). Since indirect chilling injury can cause delayed mortality, particularly during pupation, it is important to track survival of individuals until later life stages to fully quantify the effects of low temperature stress (Layne and Peffer, 2006; Renault, 2011).

Low temperature exposure can also be beneficial to overwintering insects. Many species have a chilling requirement before diapause can be terminated (Hodek and Hodková, 1988).   In addition, low temperature can reduce metabolic rate during the energy-depleting overwintering period since lower environmental temperature leads to reduced energetic requirements overwinter (Hahn and Denlinger, 2011; Hodek and Hodková, 1988; Irwin and Lee, 2003; Williams *et al.*, 2012a). While many of the effects of low temperature exposure are well documented, these studies have focused on the results of a single cold exposure.

5.1.2　Temperature fluctuations

The eastern spruce budworm overwinters in flower scars, under bud scales at the base of shoots, and under rough bark scales—all microenvironments that will vary in temperature in concert with changing air temperatures (Régnière and Duval, 1998). During the *C. fumiferana* overwintering stage, air temperatures in the boreal forest fluctuate repeatedly, and regularly drop below -20 ºC (Han and Bauce, 1998). Therefore extending the laboratory-measured impacts of a single low temperature exposure may not represent field conditions. For example, *C. fumiferana* that overwinter in colder, more variable field conditions have higher glycerol content and lower supercooling points than *C. fumiferana* overwintered in more stable, warmer lab conditions (Han and Bauce, 1998). Several studies have additionally shown that repeated low temperature exposure appears to increase cold hardiness relative to the effects of a single cold exposure (reviewed in Chapter 2), which may come at an energetic cost (Chapter 3; Teets *et al.*, 2011). In addition, exposure to warm temperature pulses following cold shock can prevent or allow repair of chilling injury in many species (Koštál *et al.*, 2007; Lalouette *et al.*, 2011). Ion homeostasis can be regained (MacMillan *et al.*, 2012), and ubiquitin and heat shock proteins can be synthesized to aid in removal or repair of damaged enzyme (Hochachka and Somero, 2002; Qin *et al.*, 2005). Alternatively, it is possible that damage from chilling is irreparable, and that damage from membrane phase transitions, oxidative damage, protein denaturation, and disruption of ion homeostasis, accumulates with each cold exposure. These processes of recovery from cold shock and preparation for subsequent shocks suggest that, while intensity and duration of low temperature exposure are important, the impacts of repeated cold exposure may differ from those of a single exposure.

If repeated cold exposures can induce differential physiological and fitness effects, then it is also possible that the period between these exposures can have an effect. Longer periods of time between exposures could allow for increased synthesis of cryoprotectant or antifreeze proteins, or allow for additional repair of chilling injury between subsequent exposures. Alternatively, increased time between exposures could reduce the ability of insects to entrain repair or preparatory responses (Bale *et al.*, 2001). Models of insect

populations currently assume that responses to low temperature remain static, despite previous exposure. But if this is not true, then models predicting how temperature exposure impacts individual survival and thus population dynamics should incorporate this information (Gray, 2008; Régnière *et al.*, 2012). In addition, tracking individuals until an ecologically-meaningful endpoint like eclosion can help link stress exposure at an early stage to subsequent fitness impacts (Calow and Forbes, 1998; Renault, 2011).

As winters rapidly warm, and regimes of low temperature exposure change (Bale and Hayward, 2010; IPCC, 2007; Shabbar and Bonsal, 2003), understanding the mechanisms that drive population dynamics in insect populations become increasingly important for accurate predictions (Buckley *et al.*, 2010; Helmuth *et al.*, 2005). It is clear from previous studies that cold hardiness in insects can be increased following repeated low temperature stress, and that chilling injury can be repaired between stresses. However, the impacts of period between exposures has been little explored, and never in combination with intensity and duration to allow for the investigation of interactions between these factors. In this study I investigate whether frequency, period, intensity, and duration of cold exposure, and their interactions, impact the overwintering energetics and long-term success of *C. fumiferana*. I predict that repeated cold exposure will produce a change in survival relative to individuals that receive a prolonged cold exposure, and this change will be reflected in changes in fuel reserves and cryoprotectant.

5.2   Methods

5.2.1   Animal source and rearing

I ordered pre-diapause 2nd instar *C. fumiferana* larvae (diapausing strain) from Insect Production Services at the Great Lakes Forestry Centre (Sault St. Marie, Ontario, Canada) in October 2010. Eggs are deposited on gauze at this facility, then 1st instars emerge, molt into 2nd instars, and spin hibernacula on the gauze prior to overwintering. Larvae in hibernacula were shipped on ice to the University of Western Ontario. Upon receipt, I placed larvae in an incubator at 2/0 °C 12/12 h, in constant darkness. After allowing a month of acclimation to these conditions, I extracted larvae from their hibernacula on a Petri dish filled with ice. I then placed larvae into 0.2 mL

microcentrifuge tubes with perforated lids in groups of 20 (for metabolite assays) or 24 (for supercooling point assays). I kept additional larvae (in groups of 50) for adult fitness assays in their hibernacula in 50 mL plastic bottles. All larvae were then allowed to acclimate to 2/0 °C 12/12 h, in constant darkness, for an additional month before low temperature exposures began.

5.2.2   Low temperature exposures

The experiments were conducted over a three month period from the final week of December 2010 and continuing until late March 2011. I conducted all low temperature exposures using an insulated aluminum block chilled by a Proline 3530C programmable refrigerated circulator (Lauda, Wurzburg, Germany) containing 50:50 methanol:water. I placed larvae (still in 0.2 mL Eppendorf tubes or on gauze) in 20 mL plastic tubes fitted into holes drilled into an aluminum block. I inserted 36 AWG Type T (copper-constantan) thermocouples (Omega Engineering Inc., Laval, Quebec, Canada) into several of the 20 mL centrifuge tubes to monitor the temperature during the exposure. The thermocouples were interfaced to a computer running PicoLog Software for Windows (Pico Technology, Cambridge, UK) by Picotech TC-08 thermocouple interfaces. Temperatures were recorded at 0.5 s intervals. All low temperature exposures began at 8 pm, started at 0 °C, and cooling rate was 0.1 °C/min to -5, -10, -15, or -20 °C (Figure 5.1, Figure 5.2). Larvae received low temperature exposures for either 12 h or 120 h, and then were rewarmed to 0 °C at 0.1 °C/min. For individuals given repeated low temperature exposures, three, six, or 10 exposures of 12 h were repeated at intervals of one, five, or 10 days. To control for the potential effects of diapause intensity, I gave individuals a single 120 h cold exposure at the beginning or end of the experimental period. I sampled control individuals at the beginning, middle, and end of the experimental period (early January, mid-February, and late March). After the conclusion of low temperature treatment, I placed all individuals back (still in their 0.2 mL microcentrifuge tubes or on gauze) in the incubator for recovery at 2/0 °C 12/12 h.

114
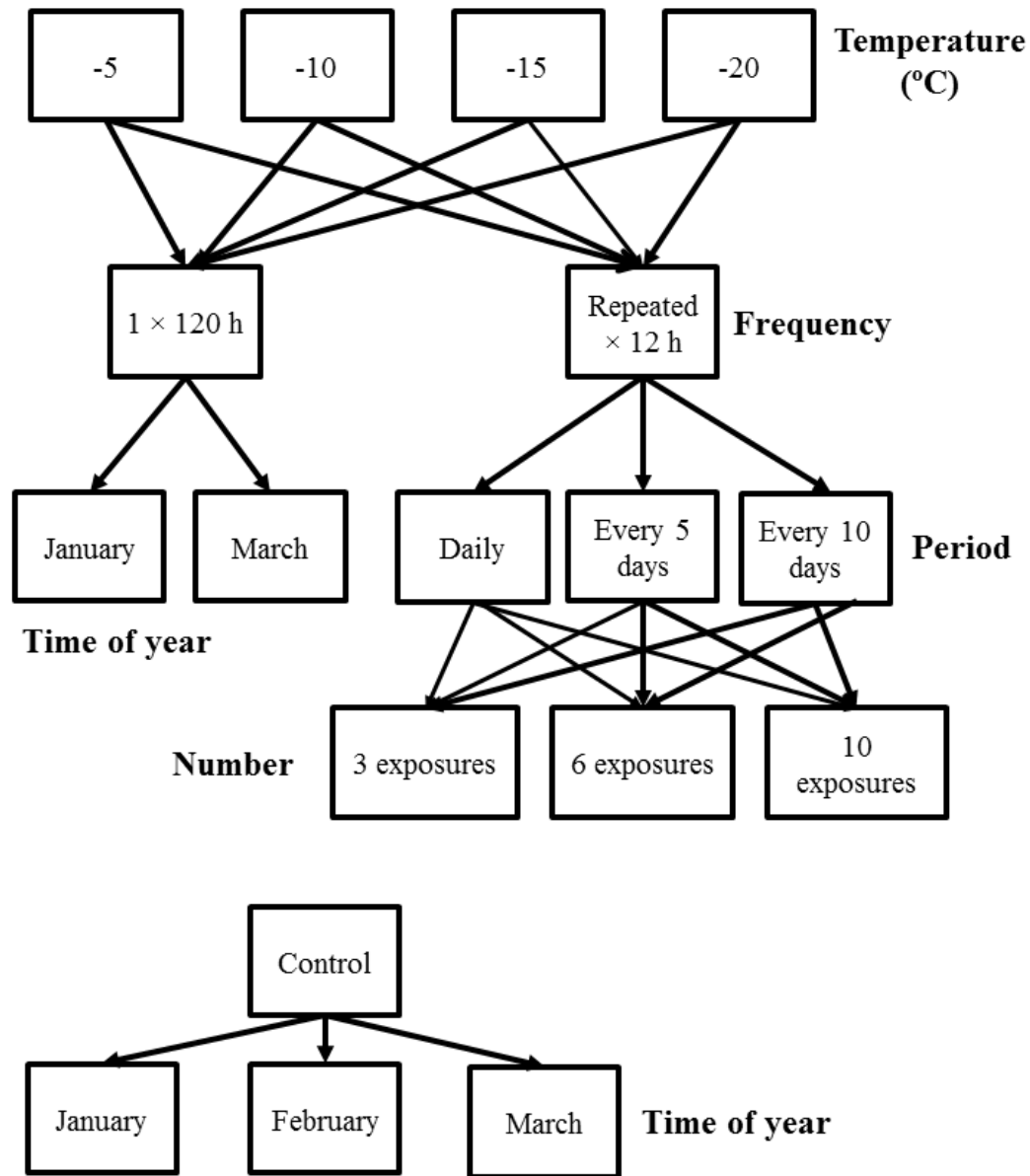
Figure 5.1. Experimental design for studying the effects of frequency, intensity, duration, and period of low temperature exposure on *Choristoneura fumiferana*. Terms in boldface indicate predictive terms used in statistical models.
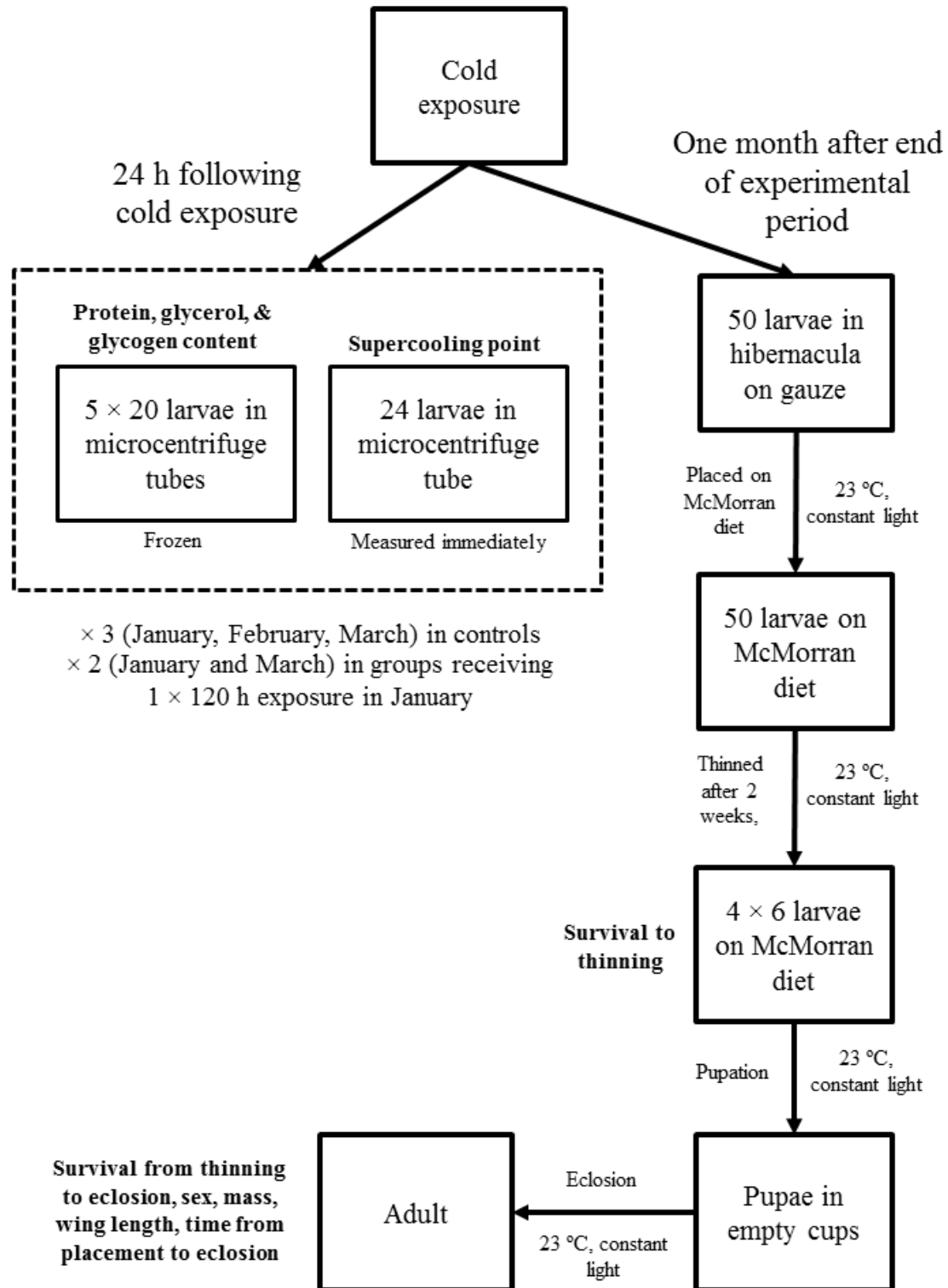
Figure 5.2 Sampling scheme for the experiment described in Figure 5.1. Terms in bold typeface indicate measures investigated statistically.

In freeze avoiding *E. scudderiana* pupae that received repeated low temperature exposures, there was little change in glycerol and glycogen concentration between two and 24 hours following exposure (Churchill and Storey, 1989). Therefore, to ensure adequate time for recovery prior to sampling, I allowed larvae a 24 h recovery at 2/0 °C 12/12 h following exposure (Figure 5.2). I snap-froze five sets of 20 individuals by direct immersion in liquid nitrogen vapour after transfer to 1.7 mL microcentrifuge tubes for a minimum of five minutes, then stored them at -80 °C. At this time, I also measured the supercooling point of 24 individuals. I returned individuals still on gauze to the incubator at 2/0 °C 12/12 h for later fitness assays. To measure the long-term effects of prolonged cold exposure on glycerol and glycogen stores, I also allowed an additional group of individuals that received 120 h of low temperature exposure in January to recover in the same incubator until late March, then sampled them at that time (Figure 5.2).

5.2.3   Supercooling points

I measured supercooling points as in Strachan *et al.* (2010). I placed individual larvae in 1.7 mL microcentrifuge tubes in contact with a 36 AWG Type T copper-constantan thermocouple (attached with vacuum grease) threaded through a hole in the top of the tube. Thermocouples were held in place by the addition of Sticky Tack (Amscan Canada Ltd., Dorval, Quebec, Canada) at the top of the microcentrifuge tube. I conducted supercooling point measurement runs by cooling the aluminum block from 0 °C to -45 °C at a rate of 0.1 °C/min, in the same apparatus as for low temperature exposures. I recorded supercooling point as the lowest temperature immediately prior to the exotherm representing the latent heat of crystallization (Lee, 2010).

5.2.4   Glycerol and glycogen assays

Larvae were homogenized in groups of 20 in a Bullet Blender (Next Advance Inc., Averill Park, New York, USA) in 50 µL of 0.05% Tween 20 with eight 1 mm glass beads as in Chapter 3. I then added an additional 450 µL of 0.05% Tween 20, and mixed the sample vigorously using a vortexer. I centrifuged the samples for 15 min at $15,000 \times g$, then removed and mixed the supernatant (~350 µL). I separated the supernatant into three equal aliquots and stored them at -80 °C until they were used in subsequent assays.

I measured protein, glucose, glycogen (from undiluted homogenate), and glycerol (diluted 1:9) content spectrophotometrically as in Gefen *et al.* (2006) using bovine serum albumin, glucose, Type II glycogen from oyster, and glycerol standards respectively. Briefly, soluble protein content was measured using a Bicinchoninic Acid Kit (BCA1, Sigma-Aldrich Canada Co., Oakville, Ontario, Canada). Glucose content was measured using a hexokinase-based glucose assay kit (GAHK20, Sigma-Aldrich Canada Co.), while glycogen content was measured using the same kit following overnight amyloglucosidase (A9228, Sigma-Aldrich Canada Co.) digestion at room temperature. Glycogen is expressed in glucose units throughout. Glycerol content was measured using Free Glycerol Reagent (F6425, Sigma-Aldrich Canada Co.).

5.2.5 Life history measures

After the conclusion of the low temperature treatments, I kept all remaining larvae (50 per group) in constant darkness at 2/0 °C 12/12 h for an additional month. After this period, I transferred the larvae (on gauze, 50/cup) to 30 mL plastic cups containing 22 mL McMorran diet (Insect Production Services) and placed them in an incubator in constant light at a constant 23 °C. To control for density, after two weeks I thinned larvae by placing them onto new McMorran diet in groups of six randomly chosen larvae. A total of four cups per experimental treatment were prepared, and additional larvae were counted and discarded (survival to this point constitutes the "survival to thinning" measure, Figure 5.2). I checked cups daily for pupae, which were immediately removed and placed into empty 22 mL plastic cups to allow for eclosion. Pupae were monitored daily and the date eclosed was noted (survival to this stage was scored as "survival from thinning to eclosion"). I also recorded sex and adult mass, and measured wing length using digital calipers (± 0.5 mm, Mastercraft, Toronto, Ontario, Canada).

5.2.6 Statistical analyses

In all statistical comparisons, I first fitted a Type II ANOVA model (implemented using the Anova function from the car package in R v.2.15.0, Fox and Weisberg, 2011, R Development Core Team 2012) with all possible terms and interactions. Then, using the step algorithm implemented in R (Venables and Ripley, 2002), I simplified to the model

with the lowest Akaike's Information Criterion (AIC) by sequentially removing the highest-order interaction terms regardless of p-value. Since the step algorithm will halt when removing a term causes an increase in AIC, even if that increase is non-significant (*i.e.* ΔAIC <2, Crawley, 2005), I compared AIC values between the best-fit model from the step function with the next-simplest model (*i.e.* best fit model from the step function with the highest-order interaction term removed) using the extractAIC function in R. If the increase in AIC was <2, I restarted the step algorithm with the next simplest model (Crawley, 2005).

In all analyses, larvae that had experienced repeated exposures were first compared using three-way ANOVA with period (length of time between exposures), intensity (temperature of cold exposure), and frequency (number of low temperature exposures) as factors. I also compared samples from larvae that experienced a single prolonged exposure using a one-way ANOVA to determine the effects of intensity and time of year, while I compared control larvae between sampling points using one-way ANOVA to determine the effects of time of year. If the period between exposures was not a significant predictor, I pooled all the larvae that received ten 12 h exposures regardless of period within each temperature. Similarly, if time of year was not a significant predictor in larvae that had experienced prolonged exposures or control conditions, these individuals were also pooled within temperatures. I then compared larvae that had received cold exposures using a two-way ANOVA with temperature and experimental group as predictors (where experimental group could include separate groups for repeatedly-exposed larvae with different periods of exposure and larvae that had experienced prolonged exposure at different times of year).

This was repeated for all analyses, with the following exceptions. To compare survival to eclosion and sex ratio, I used generalized linear models with a binomial distribution to statistically test differences. Glycerol, glucose, and glycogen analyses were conducted on a μmol per sample basis, with protein mass as a covariate. Means and SE are reported throughout. Alpha was set to 0.05 in all tests.

5.3   Results

5.3.1   Survival

A total of 1505 larvae (out of 2340 initially counted and placed on McMorran diet) emerged from their hibernacula, and were viable two weeks following placement. Intensity, duration, or period between exposures did not significantly impact survival to thinning of larvae that received repeated 12 h exposures (Table 5.1). The interaction between exposure temperature and time of year exposed significantly impacted survival in larvae that received 120 h cold exposure, (Table 5.2). This was driven by extremely low survival (1 larva out of 48 initially placed on McMorran diet) of larvae that received 120 h cold exposure to -20 ºC in March (Figure 5.3A). When survival was compared among larvae in all experimental groups (repeated 12 h exposures *vs*. one 120 h exposure), the only significant deviation from the overall high survival (64.3%) was found in those larvae that received prolonged exposure to -20 ºC in March (Figure 5.3A, Table 5.3). Similarly, intensity, duration, and period between cold exposures did not reduce survival from thinning to eclosion in larvae that received repeated low temperature exposures (Table 5.1). Larvae given a single 120 h cold exposure in March had significantly lower survival than larvae exposed to 120 h of cold in January (Figure 5.3B, Table 5.2). Frequency of cold exposure significantly impacted larval survival from thinning to eclosion (Figure 5.3B, Table 5.3). Larvae that received repeated exposures had significantly lower survival (difference in coefficients, p = 0.027) than those that received 120 h in January, and trended toward increased survival relative to larvae that received 120 h cold exposures in March (p = 0.068).
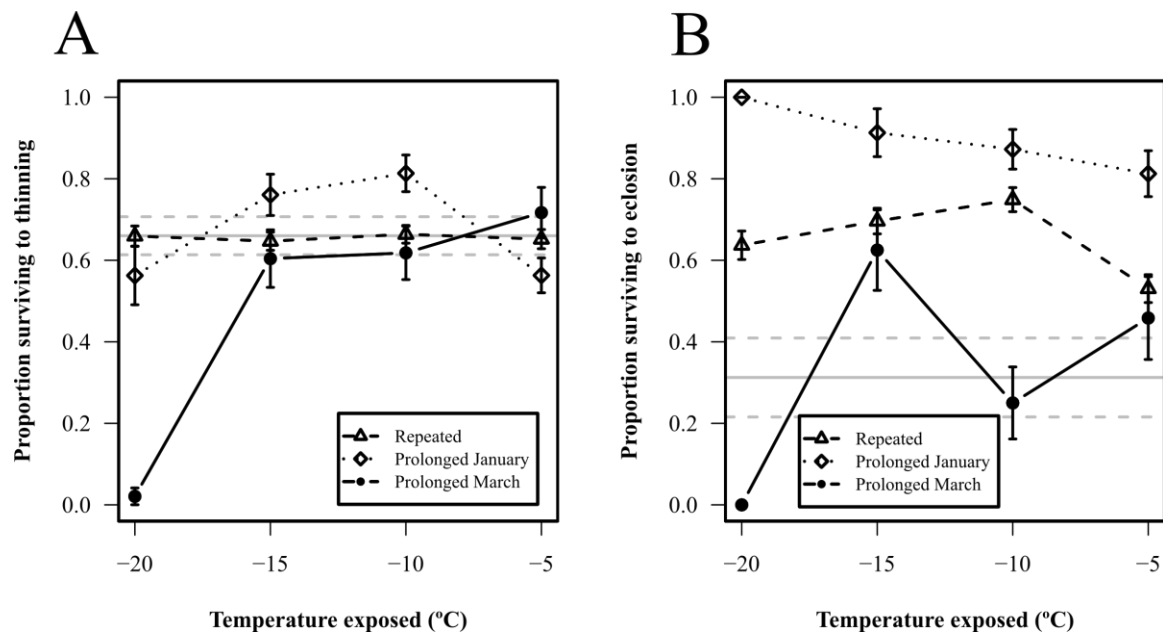
Figure 5.3 The effects of cold exposure on long-term survival of *Choristoneura fumiferana*. Accompanying statistics are presented in Table 5.1. "Intensity" = temperature of cold exposure, "Type" = either "Repeated" 12 h exposures, or a single "Prolonged" 120 h cold exposure. Prolonged exposures either occurred in early January, or late March. Solid and dotted horizontal grey lines indicate mean ± standard error of the proportion survival of controls, respectively. Error bars indicate standard error of the proportion. A) Survival from placement on McMorran diet to thinning after two weeks at 23 ℃. N = 48 − 185. B) Survival from thinning until eclosion as adults. N = 24 − 72 (N = 1 for larvae exposed to -20 °C for 120 h in March).

Table 5.1 The effects of repeated 12 h cold exposure during the 2nd instar on adult characteristics of *Choristoneura fumiferana*. Values are ANOVA statistics comparing body mass, survival, sex ratio, and development time among moths as a result of exposure temperature (either -5, -10, -15, or -20 °C), period between exposures (daily, every five days, or every 10 days), and number of exposures (three, six, or 10 exposures) as 2nd instar larvae. Terms with significant p-values ($p < 0.05$) are in bold typeface. If there were no significant effects (other than intercept), this is indicated by "null model".

Y	Initial model	Minimal adequate model	Terms from minimal adequate model	F	df	P
Female body mass =	wing length + exposure temperature × period between exposures × number of exposures	wing length + exposure temperature × period between exposures × number of exposures	**Wing length**	**265.37**	**1, 252**	**<0.001**
			Temperature	1.88	3, 252	0.133
			Period	0.66	2, 252	0.516
			Number	0.77	2, 252	0.467
			Temperature × Period	1.73	6, 252	0.115
			Temperature × Number	**2.84**	**6, 252**	**0.011**
			Period × Number	0.64	4, 252	0.636
			Temperature × Period × Number	**3.93**	**11, 252**	**<0.001**
Female development time =	exposure temperature × period between exposures × number of exposures	exposure temperature × period between exposures × number of exposures	**Temperature**	**1.97**	**3, 253**	**0.005**
			Period	**3.73**	**2, 253**	**0.025**
			Number	1.21	2, 253	0.360
			Temperature × Period	0.63	6, 253	0.706
			Temperature × Number	**5.75**	**6, 253**	**<0.001**
			Period × Number	1.82	4, 253	0.126
			Temperature × Period × Number	**2.32**	**11, 253**	**0.010**
Survival to thinning =	exposure temperature × period between exposures × number of exposures	null model				
Survival from thinning to eclosion =	exposure temperature × period between exposures × number of exposures	null model				
Sex ratio =	exposure temperature × period between exposures × number of exposures	null model				

Table 5.2 The effects of a single 120 h cold exposure during the 2nd instar on adult characteristics of *Choristoneura fumiferana*. Values are ANOVA statistics comparing body mass, survival, sex ratio, and development time among moths as a result of exposure temperature (-5, -10, -15, or -20 °C) or time of year exposed (January or March) after 120 h cold exposure while 2nd instar larvae. Retained terms with significant p-values (p < 0.05) are in bold typeface. In the cases of survival and sex ratio, a generalized linear model was fitted with a binomial distribution. If there were no significant effects (other than intercept), this is indicated by "Y = null model".

Y	Initial model	Minimal adequate model	Terms from minimal adequate model	F	df	P
Female body mass =	wing length + exposure temperature × time of year exposed	wing length + exposure temperature	**Wing length** **Temperature**	**94.14** **6.50**	**1, 84** **3, 84**	**< 0.001** **< 0.001**
Female development time =	exposure temperature × time of year exposed	exposure temperature × time of year exposed	Temperature **Time of year** **Temperature × time of year**	0.99 **5.35** **3.24**	3, 82 **1, 82** **2, 82**	0.399 **0.023** **0.044**
Survival to thinning =	exposure temperature × time of year exposed	time of year exposed	**Time of year**	**12.24**	**1, 3**	**<0.001**
Survival to thinning =	exposure temperature × time of year exposed	exposure temperature × time of year exposed	**Temperature** Time of year **Temperature × Time of year**	**5.30** 3.59 **7.86**	**3, 4** 1, 3 **3, 0**	**0.001** 0.058 **<0.001**
Survival from thinning to eclosion =	exposure temperature × time of year exposed	time of year exposed	**Time of year**	**8.45**	**1, 6**	**0.004**
Sex ratio =	exposure temperature × time of year exposed	null model				

Table 5.3 The effects of low temperatures exposure during the 2nd instar on adult characteristics of *Choristoneura fumiferana*.
Values are ANOVA statistics comparing body mass, survival, sex ratio, and development time among moths as a result of exposure temperature frequency (ten 12 h cold exposures or one 120 h cold exposure) as 2nd instar larvae. Retained terms with significant p-values ($p < 0.05$) are in bold typeface. In the case of survival and sex ratio, a generalized linear model was fitted with a binomial distribution. If there were no significant effects (other than intercept), this is indicated by "Y = null model".

Groups tested	Y	Initial model	Minimal adequate model	Terms from minimal adequate model	F	Df	P
Repeated: pooled **Prolonged**: pooled	Female body mass =	wing length + exposure temperature × exposure type	thorax width + exposure temperature	**Wing length** **Temperature**	**224.50** **6.22**	**1, 176** **3, 176**	**< 0.001** **<0.001**
Repeated: pooled **Prolonged**: time of year exposed	Female development time =	exposure temperature × exposure type	exposure type	**Exposure type**	**3.28**	**4, 176**	**0.013**
Repeated: pooled **Prolonged**: time of year exposed	Survival to thinning =	exposure temperature × exposure type	exposure temperature × exposure type	Temperature Exposure type **Temperature × Type**	0.81 2.48 **6.16**	3, 39 2, 37 **6, 31**	0.486 0.084 **<0.001**
Repeated: pooled **Prolonged**: time of year exposed	Survival to eclosion =	exposure temperature × exposure type	exposure type	**Exposure type**	**4.66**	**2, 40**	**0.009**
Repeated: pooled **Prolonged**: pooled	Sex ratio =	exposure temperature × exposure type	null model				

5.3.2   Life history measures

A total of 716 adult *C. fumiferana* (378 female, 338 male) eclosed successfully from all experimental groups, with overall survival from thinning (time from placement on new McMorran diet after two weeks in warm conditions) to eclosion of 65.4%. There were significant interactions between exposure temperature, number of exposures, and period between exposures on the mass of female moths that had received repeated 12 h exposures (Figures 5.4A-C). Increased number of exposures generally led to smaller moths (Figure 5.4A-C, Table 5.1). However, the effect of increasing period between exposures was not consistent (Figure 5.4A). Exposure temperature also impacted the mass of female moths that received prolonged exposures of 120 h as 2nd instar larvae, although this was driven by the small size of moths that were exposed to -5 ℃ in January (Figure 5.4D, Table 5.2). Frequency of exposure did not significantly affect mass in female moths, although increased exposure temperature significantly decreased adult mass (Figure 5.3E, Table 5.3). Exposure to any low temperature also significantly increased female mass relative to control moths (Figure 5.4E). Adult male mass followed similar trends to those observed in females (data not shown since female mass is likely more directly correlated to potential fitness, Appendix B Tables B.1-3).

Development time (the number of days between placement on McMorran diet and successful eclosion) in female moths was affected by the interactions among exposure temperature, number of exposures, and period between exposures in individuals that received repeated exposures in the 2nd instar (Figure 5.5A-C, Table 5.1).   There was no consistent effect of increased number, intensity, or period between exposures in any of these factors (Figure 5.5A-C). Female larvae that received prolonged low temperature exposure had increased development time if this exposure occurred in March, and this effect was more apparent after exposure to -5 ℃ (Figure 5.5D, Table 5.2). Finally, while there was a significant effect of exposure type on female development time (Table 5.3), there was no clear trend other than that low temperature exposure reduced development time (Figure 5.5E). Adult male development time followed similar trends to those observed in females (Appendix B Tables B.1-3).

5.3.3   Supercooling points

Supercooling points of individual larvae ranged from -43.9 ℃ in an individual that had been exposed to ten bouts at -5 ℃ every five days, to -21.8 ℃ in an individual that received 120 h at -10 ℃ in January, and averaged -32.3 ± 0.1 ℃ (Appendix B Figure B.1, Table 5.4).   There was no consistent direction in the effects of period, number, or temperature in supercooling points of larvae that received repeated 12 h exposures (Table 5.4), although there were significant interactions between each of these measures (Appendix B Figure B.2A-C, Table 5.4). Similarly, while supercooling point was altered by the interactive effects of exposure temperature and time of year exposed in larvae that received repeated 12 h exposures, this effect was not consistent (Appendix B Figure B.2D, Table 5.4).   In control larvae, the lowest supercooling points occurred in mid-February (-33.0 ± 0.5 ℃), and no individuals had supercooling points above -30 ℃ (Appendix B Figure B.2E, Table 5.4).   Finally, when the supercooling points of larvae that received repeated 10 h exposures were compared to those that received a single 120 h exposure, there was a significant interaction between exposure type and exposure temperature, but again no consistent effects of exposure type or temperature on supercooling points (Appendix B Figure B.2F, Table 5.4).

5.3.4   Glycerol and glycogen measures

There was no significant impact of exposure temperature on glucose mass in larvae that received repeated 12 h cold exposures, although there was a significant interaction between the number of and period between exposures (Table 5.5). This was driven by low glucose content in larvae that received 12 h exposures every five days three times (Appendix B Figure B.3A-C). Similarly, there was no effect of exposure temperature on glucose content in larvae that received a single 120 h exposure, but larvae that received their 120 h exposure in January and were sampled 24 h following low temperature exposures had significantly higher amounts of glucose compared to those exposed in January 3 months after exposure and then recovered for three months (Appendix B Figure B.3D, Table 5.6).   Glucose content significantly declined through the experimental period in control larvae (Appendix B Figure B.3E, Table 5.7).

Figure 5.4 The effect of cold exposure during the 2nd instar on adult mass of female *Choristoneura fumiferana.* All significant effects are indicated by the bold terms in each figure, and accompanying statistics are presented in Tables 5.1-3. "Period" = number of days between cold exposures (one, five, or ten), "Intensity" = temperature of cold exposure, "Number" = number of cold exposures (three, six, or ten). Solid and dotted horizontal grey lines represent mean $\pm$ SE of control individuals, respectively. Points represent mean $\pm$ SE. A) Moths that received ten 12 h exposures as 2nd instar larvae. B-C) Moths that received repeated 12 h cold exposures as 2nd instar larvae. D) Moths that received a single 120 h exposure as second instar larvae (no female moths eclosed after 120 h at -20 ºC in March). E) Comparison of moths that received repeated 12 h and repeated 120 h cold exposures.
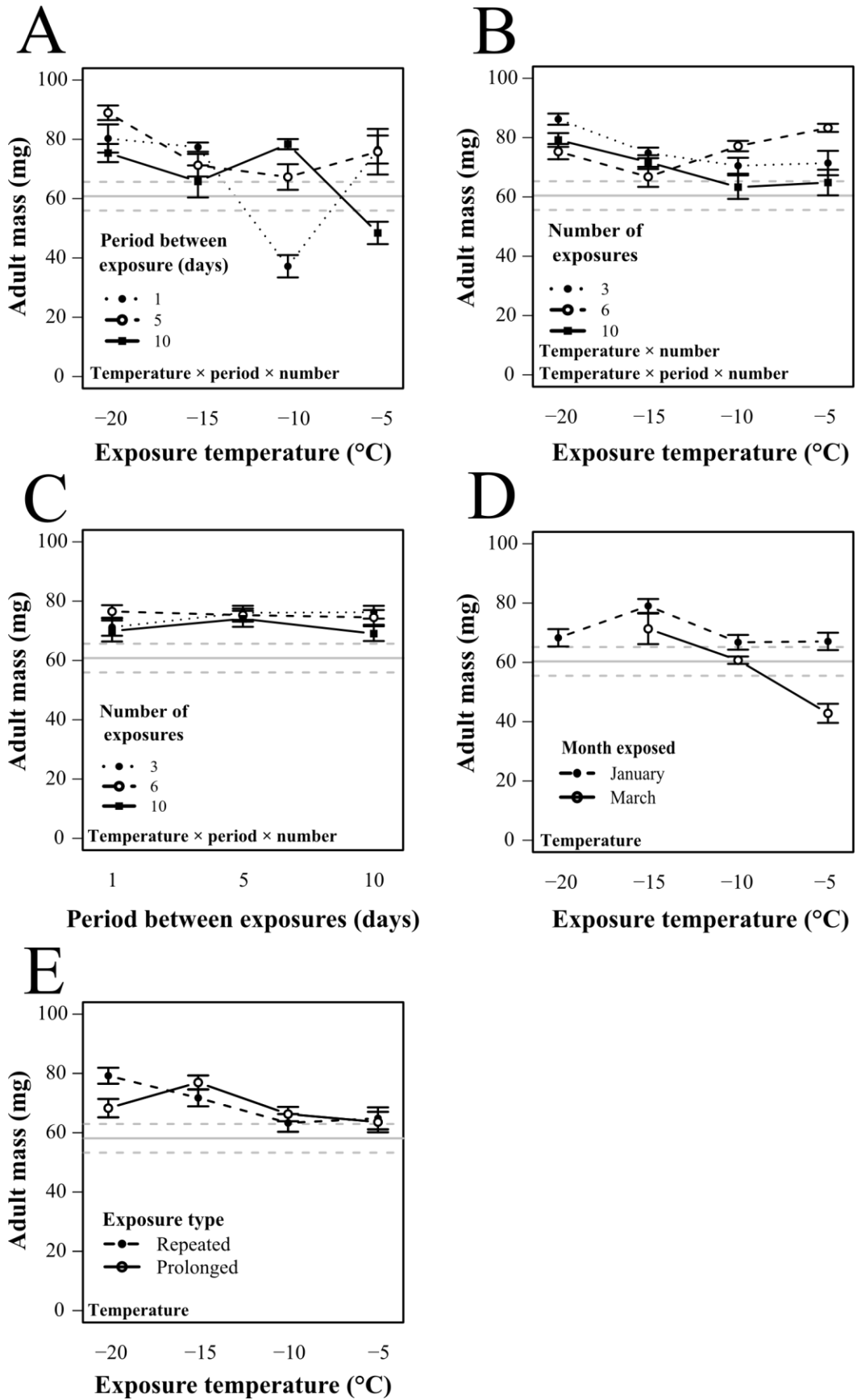
Figure 5.5 The effect of cold exposure during the 2nd instar on development time (from 2nd instar to adult) of female *Choristoneura fumiferana*. All significant effects are indicated by the bold terms in each figure, and accompanying statistics are presented in Tables 5.1-3. "Period" = days between cold exposure (daily, every five days, or every 10 days), "Intensity" = temperature of cold exposure, "Number" = number of cold exposures (three, six, or 10). Solid and dotted horizontal grey lines represent mean ± SE of control individuals, respectively Points represent mean ± SE. A) Moths that received 10 twelve hour exposures as 2nd instar larvae. B-C) Moths that received repeated 12 h cold exposures as 2nd instar larvae. D) Moths that received a single 120 h exposure as second instar larvae (no female moths eclosed after 120 h at -20 ℃ in March). E) Comparison of moths that received repeated 12 h and repeated 120 h cold exposures.
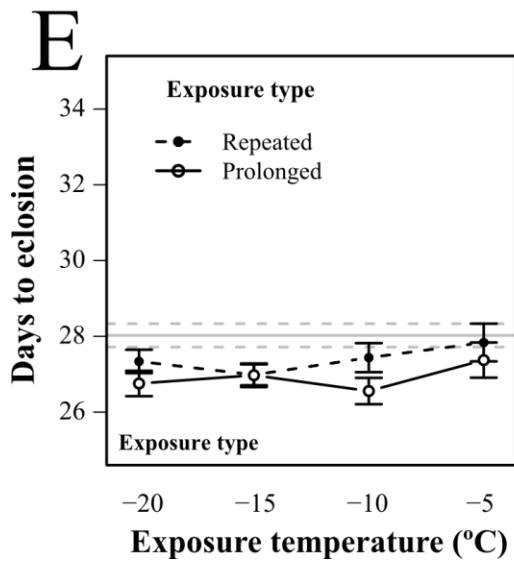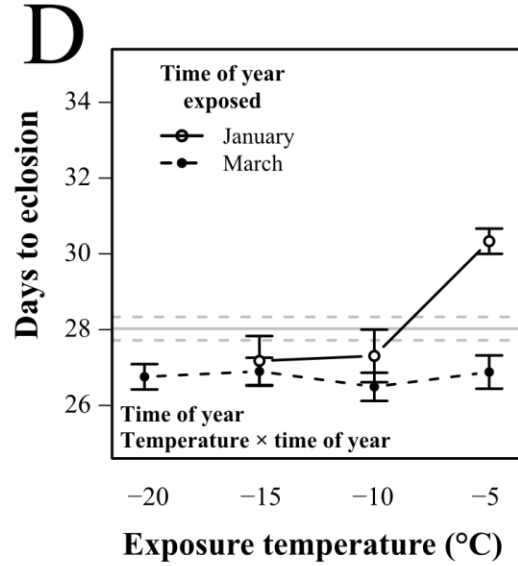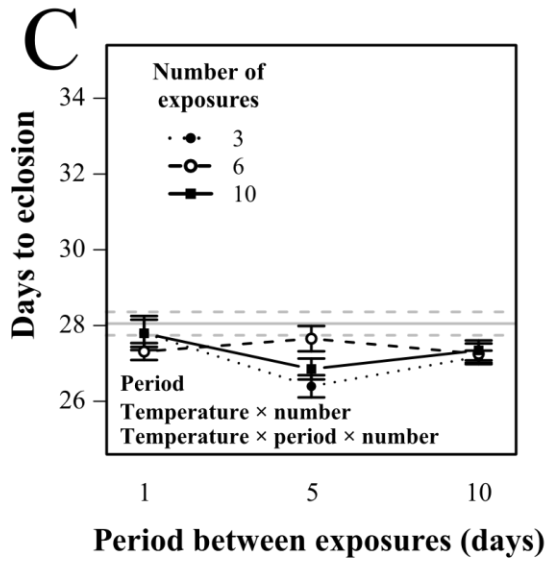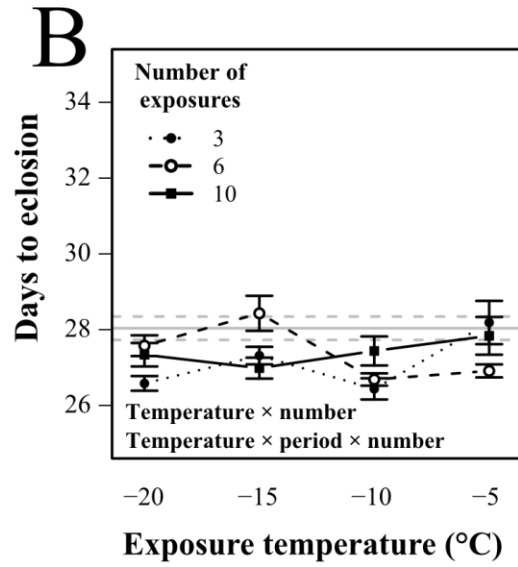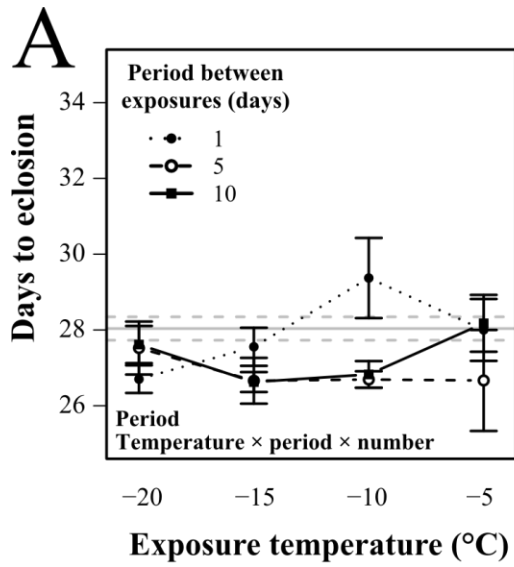
Table 5.4 The effects of low temperatures exposure on supercooling points of 2nd instar *Choristoneura fumiferana*. Values are ANOVA statistics comparing supercooling point (ºC) as a result of exposure. Initial models were first fitted containing all possible terms and interactions, then models were simplified based on Akaike's Information Criterion (AIC) to a minimal adequate model. Retained terms with significant p-values (p < 0.05) are in bold typeface.

Exposure group	Initial model	Minimal adequate model	Terms from minimal adequate model	F	df	P
Control	Y = time of year sampled	Y = time of year sampled	**Time of year**	**5.55**	**2,53**	**0.007**
Prolonged	Y = exposure temperature × time of year exposed and sampled	Y = exposure temperature × time of year exposed and sampled	Temperature	2.39	3, 182	0.071
			Time of year	0.54	2, 182	0.584
			Temperature × time of year	**3.42**	**4, 182**	**0.010**
Repeated	Y = exposure temperature × period between exposures × number of exposures	Y = period between exposures × number of exposures	**Period**	**6.30**	**2, 618**	**0.002**
			Number	**3.22**	**2, 618**	**0.041**
			Period × Number	**3.38**	**4, 618**	**0.010**
Repeated: All periods **Prolonged:** All times of year exposed and sampled	Y = exposure temperature × exposure type	Y = exposure temperature × exposure type	Temperature	1.90	3, 396	0.128
			Exposure type	**6.00**	**5, 396**	**<0.001**
			Temperature × Type	**2.65**	**13, 396**	**0.002**

Table 5.5 The effects of repeated 12 h low temperature exposures on glucose, glycerol, and glycogen content in 2nd instar *Choristoneura fumiferana*. Values are ANOVA statistics comparing glucose, glycogen, and glycerol content among *Choristoneura fumiferana* 2nd instar larvae as a result of exposure temperature (either -5, -10, -15, or -20 °C), period between exposures (daily, every five days, or every 10 days), and number of exposures (either three, six, or 10 exposures) as 2nd instar larvae. Retained terms with significant p-values ($p < 0.05$) are in bold typeface.

Y	Initial model	Minimal adequate model	Terms from minimal adequate model	F	df	P
Glucose =	protein mass + exposure temperature × period between exposures × number of exposures	protein mass + period between exposures × number of exposures	Protein mass	2.47	1, 164	0.118
			Period	0.93	2, 164	0.397
			Number	1.31	2, 164	0.272
			Period × number	**2.90**	**4, 164**	**0.024**
Glycogen =	protein mass + exposure temperature × period between exposures × number of exposures	protein mass + exposure temperature + period between exposures + number of exposures + exposure temperature × period between exposures + period between exposures × number of exposures	**Protein mass**	**163.54**	**1, 155**	**<0.001**
			Temperature	**6.85**	**3, 155**	**0.001**
			Period	2.89	3, 155	0.059
			Number	**69.11**	**2, 155**	**<0.001**
			Temperature × period	**4.69**	**6, 155**	**<0.001**
			Period × number	**11.99**	**4, 155**	**<0.001**
Glycerol =	protein mass + exposure temperature × period between exposures × number of exposures	protein mass + exposure temperature + period between exposures + number of exposures + exposure temperature × period between exposures + period between exposures × number of exposures	**Protein mass**	**41.73**	**1, 155**	**<0.001**
			Temperature	**6.54**	**3, 155**	**<0.001**
			Period	**7.91**	**2, 155**	**<0.001**
			Number	**5.06**	**2, 155**	**<0.001**
			Temperature × period	**2.94**	**6, 155**	**<0.001**
			Period × number	**12.30**	**4, 155**	**<0.001**

Table 5.6 The effects of a single 120 h low temperature exposures on glucose, glycerol, and glycogen content in 2nd instar *Choristoneura fumiferana*. Values are ANOVA statistics comparing glucose, glycogen, and glycerol content among *Choristoneura fumiferana* 2nd instar larvae as a result of exposure temperature (either -5, -10, -15, or -20 °C) and time of year exposed and sampled (January or March) after 120 h cold exposure while 2nd instar larvae. Retained terms with significant p-values ($p < 0.05$) are in bold typeface.

Y	Initial model	Minimal adequate model	Terms from minimal adequate model	F	df	P
Glucose =	protein mass + exposure temperature × time of year exposed and sampled	protein mass + time of year exposed and sampled	**Protein mass** **Time of year**	**10.55** **6.35**	**1, 56** **2, 56**	**0.002** **0.003**
Glycogen =	protein mass + exposure temperature × time of year exposed and sampled	protein mass + exposure temperature + time of year exposed and sampled	**Protein mass** **Temperature** **Time of year**	**39.81** **4.67** **123.19**	**1, 55** **1, 55** **2, 55**	**<0.001** **0.035** **<0.001**
Glycerol =	protein mass + exposure temperature × time of year exposed and sampled	exposure temperature × time of year exposed and sampled	Temperature **Time of year** **Temperature × time of year**	0.20 **34.92** **2.57**	3, 48 **2, 48** **6, 48**	0.897 **<0.001** **0.031**

Table 5.7 The effects of overwintering on glucose, glycerol, and glycogen content in 2nd instar *Choristoneura fumiferana*.
Values are ANOVA statistics comparing glucose, glycogen, and glycerol content among *Choristoneura fumiferana* 2nd instar larvae through overwintering (either in January, February, or March). Retained terms with significant p-values (p < 0.05) are in bold typeface.

Y	Initial model	Minimal adequate model	Terms from minimal adequate model	F	df	P
Glucose =	protein mass + time of year sampled	protein mass + time of year sampled	**Protein mass**	**18.76**	**1, 11**	**0.001**
			Time of year	**9.64**	**2, 11**	**0.015**
Glycogen =	protein mass + time of year sampled	protein mass + time of year sampled	**Protein mass**	**81.25**	**1, 11**	**<0.001**
			Time of year	**59.33**	**2, 11**	**<0.001**
Glycerol =	protein mass + time of year sampled	time of year sampled	**Time of year**	**7.86**	**2, 12**	**0.007**

Table 5.8 The effects of low temperature exposure on glucose, glycerol, and glycogen content in 2nd instar *Choristoneura fumiferana*. Values are ANOVA statistics comparing glucose, glycogen, and glycerol content among *Choristoneura fumiferana* 2nd instar larvae as a result of cold exposure type (ten 12 h exposures or one 120 h exposure) as 2nd instar larvae. Retained terms with significant p-values (p < 0.05) are in bold typeface.

Exposure type	Y	Initial model	Minimal adequate model	Terms from minimal adequate model	F	df	P
Repeated: All periods **Prolonged:** Time of year sampled and exposed	Glucose =	protein mass + exposure temperature × exposure type	protein mass + exposure type	**Protein mass** **Exposure type**	**13.02** **4.16**	**1, 113** **5, 113**	**<0.001** **0.002**
Repeated: All periods **Prolonged:** Time of year sampled and exposed	Glycogen =	protein mass + exposure temperature × exposure type	protein mass + exposure temperature × exposure type	**Protein mass** **Temperature** **Exposure type** **Temperature × type**	**76.55** **17.94** **75.56** **2.95**	**1, 109** **1, 109** **4, 109** **4, 109**	**<0.001** **<0.001** **<0.001** **0.024**
Repeated: All periods **Prolonged:** Time of year sampled and exposed	Glycerol =	protein mass + exposure temperature × exposure type	protein mass + exposure temperature × exposure type	Protein mass Temperature **Type** **Temperature × type**	0.80 2.33 **31.98** **2.22**	1, 95 3, 95 **5, 95** **15, 95**	0.374 0.093 **<0.001** **0.010**

When all experimental groups were compared, there was a significant effect of exposure type on glucose concentration (Appendix B Figure B.3F, Table 5.8), however this was driven by the significantly lower glucose concentration of control larvae relative to all other experimental groups.

The interactions between exposure temperature, period between exposures, and number of exposures significantly impacted glycogen mass in larvae that received repeated 12 h cold exposures (Table 5.5). Increased number of exposures significantly decreased glycogen mass, and this was exacerbated at longer periods between cold exposures (Figure 5.6A-B). Lower exposure temperature and longer period between exposures also reduced glycogen mass (Figure 5.6A-C). Similarly, exposure temperature and time of year sampled (regardless of whether the larvae were exposed in January or March) had a significant effect on glycogen mass (Figure 5.6D, Table 5.6). Lower exposure temperature, and sampling later in the experimental period reduced glycogen mass in larvae (Figure 5.6D). Glycogen mass significantly decreased in control larvae through the experimental period (from 0.52 µmol/sample to 0.07 µmol/sample over the three month period, Figure 5.6E, Table 5.7). When all experimental groups were compared, there were significant effects of both exposure temperature and exposure type on glycogen content (Table 5.8) whereby lower temperature exposure generally resulted in lower glycogen mass (Figure 5.6F). Larvae that received a prolonged exposure in January had significantly higher glycogen mass than any other group, while larvae that received repeated 12 h exposures either daily or every five days had significantly decreased glycogen relative to those that received prolonged exposure in January (Figure 5.6F).

Glycerol concentration was dependent on almost every aspect of low temperature exposure. Increased number of exposures at shorter periods and lower temperatures significantly increased glycerol concentration in larvae that received repeated 12 h cold exposures (Figure 5.7A-C, Table 5.5). Glycerol concentration was dependent on time of year only in larvae given a single 120 h cold exposure (Figure 5.7D, Table 5.6). In

Figure 5.6 The effects of cold exposure on glycogen content of 2nd instar *Choristoneura fumiferana* **larvae.** All significant effects are indicated by the bold terms in each figure, and accompanying statistics are presented in Tables 5.5-8. "Period" = days between cold exposure (daily, every five days, or every 10 days), "Intensity" = temperature of cold exposure, "Number" = number of cold exposures (three, six, or 10), "Time of Year" = time of year exposed or sampled. "Exposure Type" = either "Repeated" 12 h exposures, or a single "Prolonged" 120 h cold exposure. Samples are homogenates of 20 larvae. Upper solid and dotted horizontal grey lines represent mean ± SE of control larvae in February, respectively. Lower solid and dotted horizontal grey lines represent mean ± SE of control larvae in February, respectively. Points represent mean ± SE. A) Larvae that received 10 twelve hour exposures. B-C) Larvae that received repeated 12 h cold exposures as 2nd instar larvae. D) Larvae that received a single 120 h cold exposure, exposed in either early January or late March, and sampled either early January or late March E) Control larvae, sampled in early January, mid-February, or late March. F) All exposure types together.
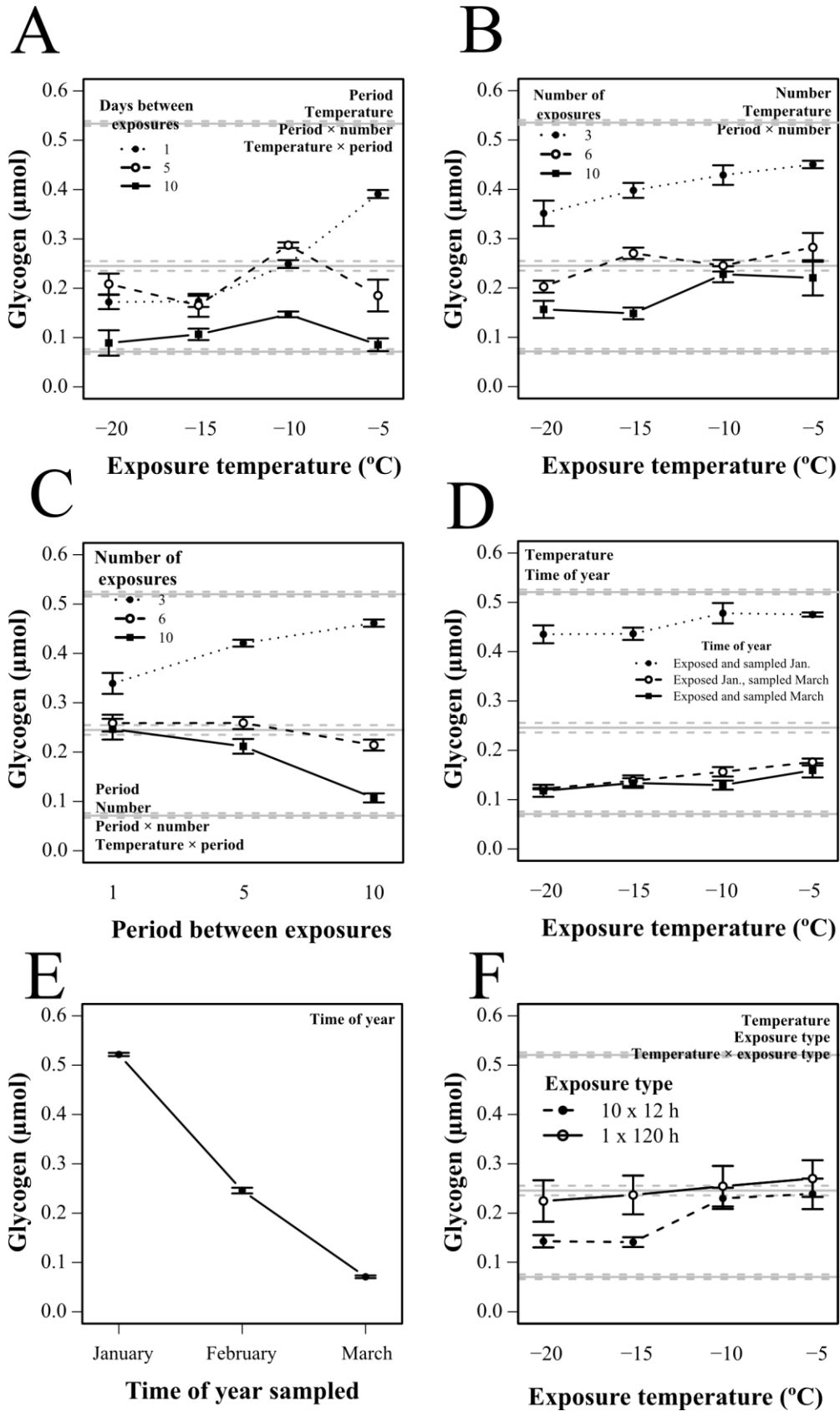
Figure 5.7 The effects of cold exposure on glycerol content of 2nd instar *Choristoneura fumiferana* **larvae.** All significant effects are indicated by the bold terms in each figure, and accompanying statistics are presented in Tables 5.5-8. "Period" = days between cold exposure (daily, every five days, or every 10 days), "Intensity" = temperature of cold exposure, "Number" = number of cold exposures (three, six, or 10), "Time of Year" = time of year exposed or sampled. "Exposure Type" = either "Repeated" 12 h exposures, or a single "Prolonged" 120 h cold exposure. Samples are homogenates of 20 larvae. Solid and dotted horizontal lines represent mean and standard error of all control larvae, respectively. Points represent mean ± SE. A) Larvae that received 10 twelve hour exposures. B-C) Larvae that received repeated 12 h cold exposures as 2nd instar larvae. D) Larvae that received a single 120 h cold exposure, exposed in either early January or late March, and sampled either early January or late March E) Control larvae, sampled in early January, mid-February, or late March. F) All exposure types together.
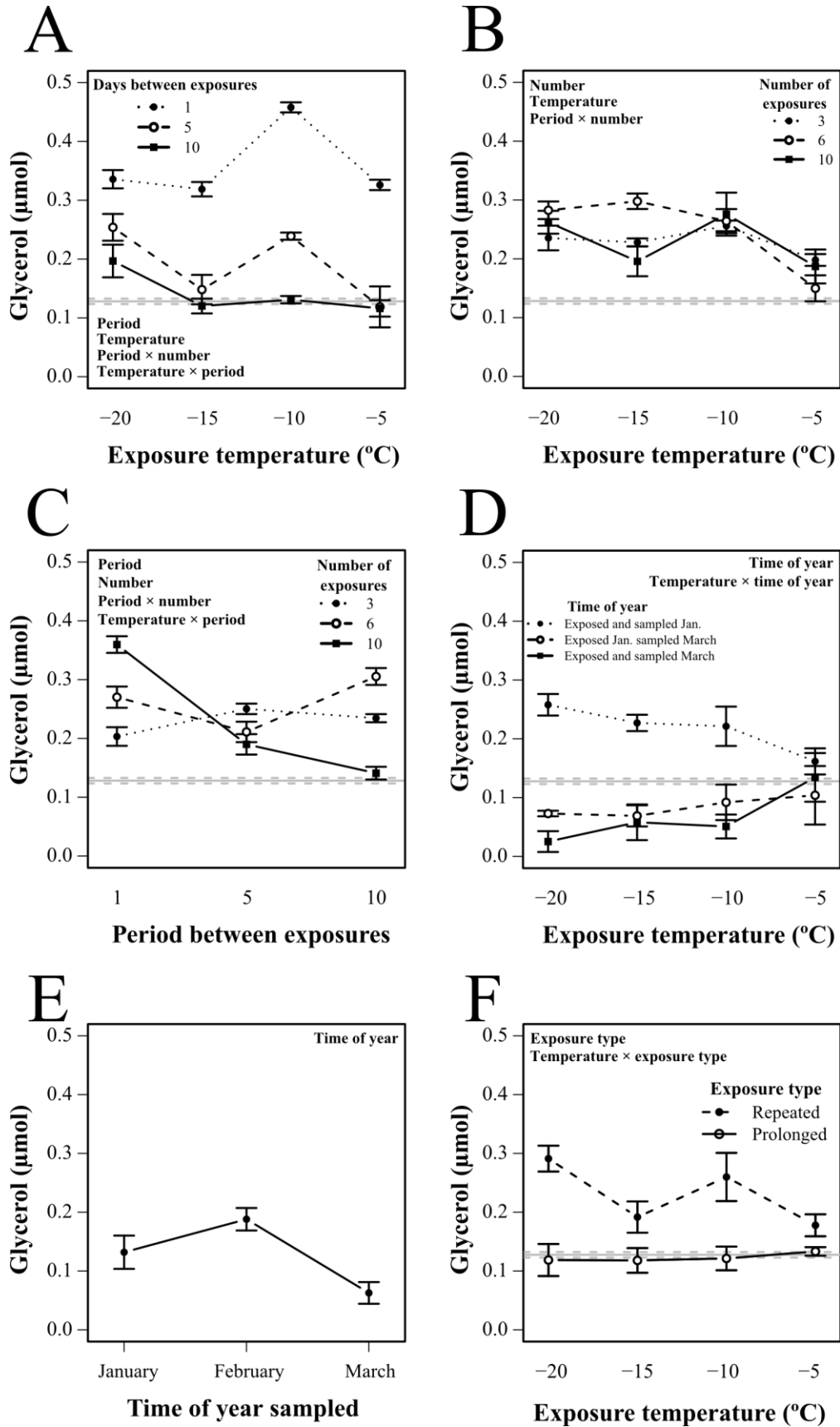
control larvae, glycerol concentration peaked in February, with lesser amounts in January and March (Figure 5.7E, Table 5.7). When all exposure groups were compared, larvae that received repeated daily exposures in January had the highest glycerol concentration, while larvae that received repeated exposures every five or ten days or a prolonged exposure in January had the next highest concentrations. Larvae that received prolonged exposures and were sampled in March had the lowest glycerol concentrations (Figure 5.7F, Table 5.8).

5.4   Discussion

Here I show that repeated cold exposure alters the relationship between applied temperature stress and survival in the eastern spruce budworm *Choristoneura fumiferana*. In addition, I found that the low temperature tolerance of the *C. fumiferana* is highly plastic through overwintering. Larvae that received a single 120 h cold exposure in early winter mobilized large quantities of glycerol (Figure 5.7D) from glycogen stores (Figure 5.6D), and experienced little mortality at any developmental stage following (Figure 5.5A-B). By contrast, larvae that received a prolonged cold exposure in March had significantly reduced ability to mobilize glycerol (Figure 5.7D) due to depleted glycogen stores (Figure 5.6D), which led to mortality later in development (Figure 5.5B). Repeated cold exposure (even with matched intensity and duration of cold exposure) evoked a different response: there was greatly increased investment in glycerol content (Figure 5.7A-C), but survival was still significantly impacted (Figure 5.5B). While sub-lethal measures of stress such as mass and development time were not affected by repeated cold exposure (Figure 5.3-4), suggesting these traits are relatively poor proxies of fitness in *C. fumiferana*, survival to eclosion was strongly impacted by the nature of low temperature exposure early in life (Figure 5.5). Taken together, this indicates that increased frequency of, rather than intensity, duration, or period between stresses drives the relationship between cold exposure and survival in *C. fumiferana.* In addition, this stress applied early in life (at the 2nd instar) affects later performance of *C. fumiferana*, despite the ability of the larvae to feed to replenish energetic outlay.

5.4.1   Causes of mortality

There was little evidence that the mortality I observed was driven by freezing injury or direct chilling injury driving the effects of repeated cold exposure I observed. The supercooling points I measured (mean = -32.3 ± 0.1 ℃, with no individual measurement > -21.8 ℃) were all significantly lower than the lowest temperature treatment I exposed larvae to (-20 ℃), which strongly implies that freezing mortality was not a factor in this experiment. While *C. fumiferana* is a very cold tolerant species, Han and Bauce, (1995b) showed that late in diapause there is reduced survival of prolonged exposure (>5 days) to -15 and -23 ℃. They measured survival as emergence from hibernacula, which corresponds roughly to my survival to thinning measure. I found that while five days of exposure at -20 ℃ in March caused substantial mortality prior to thinning, likely due to direct chilling injury. By contrast larvae with every other type of exposure had similar survival at this stage to controls. In these groups, mortality occurred in the period between thinning and eclosion.

I found delayed mortality in individuals that received late season prolonged exposure, repeated exposures, and controls (all of which had relatively high survival early in development). In larvae that received 120 h of cold exposure in March, indirect chilling could have accrued that caused delayed mortality (Yocum *et al.*, 1994). This mortality may be due to reduced ability to mobilize glycerol since glycogen reserves are significantly depleted in March (3.5 ± 1.5 nmol/larva) relative to January (26.0 ± 3.5 nmol/larva, Figures 5.6-7). By contrast, larvae that received repeated low temperature exposures had lower survival to eclosion than larvae that received 120 h in Jan., but higher survival to eclosion than larvae that received 120 h in March (Figure 5.5). This may be due to either accrued indirect chilling injury or energetic depletion due to investment in glycerol.

Low temperature also reduces metabolic rate and thus energetic demands during diapause. However, when additional cryoprotectants are produced as a result of repeated low temperature stress the energetic saving could be reversed, particularly when metabolism and production of cryoprotectants are drawn from the same energetic reserve (Hahn and Denlinger, 2011). Glycerol production from glycogen (and subsequent

catabolism) is an ATP-demanding process that is not 100 % efficient (Storey and Storey, 1983). Thus the increased glycerol content seen in repeatedly-exposed larvae represents an irreversible energy expenditure. While *C. fumiferana* larvae are able to feed following exposure, it is possible that repeated exposure damages gut or other tissues that reduces feeding performance, as was observed in the sub-Antarctic tineid caterpillar *Pringleophaga marioni* (Sinclair and Chown, 2005). Similarly, the costs of re-establishing homeostasis can induce additional energetic costs due to processes such as repairing ion gradients (MacMillan *et al.*, 2012) or synthesizing other important compounds such as antifreeze proteins (Tyshenko *et al.*, 1997).

The delayed mortality observed in control larvae may be due to increased energetic drain during the overwintering period as a result of higher metabolic rate (Irwin and Lee, 2003; Williams *et al.*, 2012a), or it may be due to insufficient chilling leading to an incomplete break in diapause leading to damage during diapause termination (Hodek and Hodková, 1988). Energetic depletion can result in mortality in *C. fumiferana* overwintering in warm conditions (Han and Bauce, 1998). Further work is required to differentiate among these explanations. These experiments could include closer tracking of mortality at each molt, measurement of juvenile hormone titer, or the expression of enzymes associated with diapause termination (reviewed in Koštál, 2006) to determine whether diapause is broken. By contrast, increased energetic drain could be inferred by overwintering larvae in control *vs.* early cold exposure conditions and measuring glycogen and lipid reserves immediately following overwintering *sensu* Williams *et al.*, (2012b) or the time-course of energetic depletion.

5.4.2   Energetic reserves and cryoprotection

The seasonal production of glycerol from glycogen stores is well-characterized in *C. fumiferana* (Han and Bauce, 1995b), but here I show that glycerol production can also rapidly increase following short-term low temperature exposure. By contrast, the freeze avoiding moth *Epiblema scudderiana* does not increase glycerol concentration following repeated 12 h exposures to -16 ℃ (Churchill and Storey, 1989), although this may be confounded by catabolism of glycerol during overwintering (which I observed in *C.*

fumiferana, Figure 5.7D). While there have been no other studies on freeze avoiding species, many other insect species increase glycerol content following repeated cold exposure (reviewed in Chapter 2). I found that individuals that received a prolonged exposure in January had reduced glycerol mass if sampled in March (Figure 5.7D), and the interaction between period and number of exposures is likely due to catabolism of glycerol through the winter (Figure 5.7C). From my data I cannot determine whether this additional glycerol is directly metabolized or recycled back into other energetic pools. In *Eurosta solidaginis*, glycogen content increases in the spring while glycerol content decreases, but this exchange is far from stoichiometric, and other fates such as direct catabolism or incorporation into triacylglycerols for the increased glycerol concentration have been suggested (Storey and Storey, 1986). It is unclear whether the purpose of this increased glycerol production is to aid in cryoprotection, especially since there was no significant decrease in supercooling point. If the larvae are attempting to increase their cold hardiness, other mechanisms likely also have been upregulated, including antifreeze and heat shock proteins.

5.4.3   The importance of frequency of low temperature exposure

The 2nd instar larvae of *C. fumiferana* overwinter in thermally-exposed habitats that received repeated exposures to -20 ºC through the winter (Han and Bauce, 1998). Individual overwintering larvae are exposed to repeated low temperature stress on at least the order investigated by this study. Here I show that the frequency and time of winter of low temperature stress is more important than the duration and intensity for determining the overwintering survival of this species. This surprising result points to a cost of repeated low temperature events (whether direct damage, recovery, or preparation) that accumulates with each subsequent exposure, rather than with intensity of exposure. While the data in this study cannot differentiate between the costs of damage, recovery, or preparation, follow-up studies that examine gene expression following repeated cold exposure may help dissect these mechanisms (Zhang *et al.*, 2011). The mortality differences I observed could directly impact population dynamics, and future modelling efforts could incorporate these effects by tracking the number and duration of cold exposures that overwintering larvae experience (as in Chapter 2). These results also

indicate the importance of fine-scale (at least daily) temperature records for detecting low temperature events.

The northern range edge of *C. fumiferana* is correlated to the minimum temperature experienced (Gray, 2008). However in this study I found that minimum temperature does not determine survival, rather it is the number of low temperature exposures. It is possible that the correlation Gray (2008) found is because minimum temperature is also correlated with the number of low temperature exposures, although as of yet there is no work that attempts to correlate number of low temperature exposures with species range edges. In addition, overwintering survival in *C. fumiferana* is also driven by spring energetics which will be impacted by investment into cryoprotection during winter (Régnière and Duval, 1998; Régnière *et al.*, 2012). The responses I documented here to frequency of low temperature exposure could have important implications for predicting the spatial location of outbreaks due to geographic differences in temperature variability across Canada (Henry, 2008). Over the past 50 years, the number of winter cold spells in western Canada has significantly decreased, while they have increased in eastern Canada (Shabbar and Bonsal, 2003). If these trends hold, it is possible that future *C. fumiferana* outbreaks could be more severe in western Canada due to reduced frequency of low temperature events.

5.4.4   Conclusions

In a rapidly-changing world, models predicting species range shifts in response to climate change need to include relevant physiological data in models (Buckley *et al.*, 2010; Kearney and Porter, 2009). While there has been interest in building both correlative and mechanistic models of eastern spruce budworm population dynamics under climate change (Gray, 2008; Régnière *et al.*, 2012), these models (and many others) have assumed that intensity and duration of cold stress are the most salient variables predicting fitness effects. At the same time, it has become clear that insects respond to repeated cold stress differentially to prolonged cold stress—that duration and intensity of low temperature exposure are not sufficient predictors of subsequent fitness effects (reviewed in Chapter 2). In this study, I investigated the effects of frequency of and period between

cold exposures on the overwinter energetics and long-term survival of the eastern spruce budworm *C. fumiferana*. I found that repeated cold stress induces additional investment into cryoprotection at the cost of glycogen reserves as well as delayed mortality following low temperature exposure in *C. fumiferana*. This result is independent of intensity, duration, and period between low temperature exposures. Future models of population dynamics of *C. fumiferana* should incorporate the impacts of repeated low temperature stress to predict the timing, spatial location, and severity of future outbreaks.

5.5   Acknowledgements

5.6   References

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Chapter 6

6 The four axes of stress: the impacts of intensity, duration, frequency, and period of an environmental stressor on physiology and fitness

I examine how four different parameters (intensity, duration, frequency, and period) of low temperature stress impact the cryobiology and subsequent fitness of the goldenrod gall fly *Eurosta solidaginis*. This manuscript is in preparation for *American Naturalist*.

6.1   Introduction

The existence of species range limits is usually explained by biotic and abiotic factors that together impose physiological stress on individuals. A fundamental area of study in physiological ecology is the mechanisms by which species survive such stress (Calosi *et al.*, 2010; Gaston *et al.*, 2009; Sexton *et al.*, 2009). This stress can be divided into two components: the external abiotic and biotic factors (*e.g.* high temperature, parasitism) that are imposed upon an individual, and the physiological response of the individual that is subjected to that external factor (Bijlsma and Loeschcke, 2005). While biotic factors such as interspecific and intraspecific competition impose significant stresses, particularly near the equator, abiotic factors such as temperature, water and oxygen availability, and UV radiation have received the majority of attention in temperate and polar environments (Chown and Terblanche, 2007; Sexton *et al.*, 2009). Extreme values of these abiotic factors can reduce fitness in a particular environment, which drives local population dynamics (Feder *et al.*, 2000). However the mechanisms by which the physiological stress imposed by abiotic factors is translated through to fitness reductions remain under investigation (Feder *et al.*, 2000; Kingsolver and Huey, 2003; Moore and Hopkins, 2009).

Temperature is arguably the best-understood cause of abiotic stress in individual ectotherms (Chown and Terblanche, 2007). Climate change will modify the number and frequency of extreme temperature events which could impose physiological stress on individuals (Easterling, 2000; Hance *et al.*, 2007; Henry 2008). Thus, understanding

whether climate change effects on temperature regimes can increase physiological stress is of considerable interest (Chown *et al.*, 2010; Gilman *et al.*, 2006; Helmuth, 2009; Helmuth *et al.*, 2005). Extreme temperatures, whether high or low, generally reduce individual fitness in a dose-dependent manner whereby physiological stress increases with increasing duration and intensity of the stress-inducing temperature (Calow and Forbes, 1998; Hoffmann *et al.*, 2003; Nedvĕd *et al.*, 1998). This relationship, parameterized for the species of interest, underpins mechanistic models of alterations to species ranges following climate change (Buckley *et al.*, 2010; Kearney, 2012; Kearney and Porter, 2009; Régnière *et al.*, 2012).

Decreasing temperatures and increasing duration of cold exposure together decrease the survival of individuals (Han and Bauce, 1995; MacMillan and Sinclair, 2011; Nedvĕd *et al.*, 1998). Therefore mean monthly or annual environmental temperature is a valuable predictor of species ranges and population dynamics (Gray, 2008; Sunday *et al.*, 2012). However, many investigations find that incorporating the finer-grained temporal variation inherent in environmental temperature provides better predictive power in mechanistic models of individual or population response (Kearney *et al.,* 2012; Pincebourde *et al.*, 2012; Williams *et al.*, 2012). This is likely due to the occurrence of stressful temperatures that are captured in fine-scale temperature datasets. In insects, the physiological stress response may include the synthesis of molecular chaperones (Feder and Hofmann, 1999), membrane proteins, ubiquitination (Qin *et al.*, 2005), and glucose (Overgaard *et al.*, 2007). While mechanistic models account for the variation in environmental temperature that occurs on multiple, interacting timescales (Chapter 2), they do not account for the physiological stress response which could alter individual responses to subsequent extreme temperature exposures (Feder and Hofmann, 1999). They also do not account for the existence of discontinuities in the relationship between applied stress and fitness: crossing physiological thresholds can reduce fitness in a discontinuous manner (Marshall and Sinclair, 2012; Sinclair *et al*., 2013; Chapter 4).

Repeated low temperature stresses elicit distinct responses when compared to a single exposure in insects, even when the total duration of cold exposure is matched among treatments (Chapter 2). Survival of cold exposure is frequently higher when the same duration of cold exposure is experienced in repeated short bouts rather than a single bout (Chapter 2). This may be due to repair of damage incurred during cold exposure, or preparation for the next exposure that could reduce damage from subsequent exposures. By contrast, preparation for subsequent exposures, or re-establishing homeostasis (such as in the cricket *Gryllus pennsylvanicus*, MacMillan *et al.*, 2012) following exposures, could exact an energetic cost. Similarly, in insects that can survive freezing of body tissues, repeated freezing stress appears to exact a survival cost (Bale *et al.*, 2001; Chapter 4; Sinclair and Chown, 2005), likely due to mechanical or osmotic damage during freezing events causing tissue damage (Chapter 4). Therefore the frequency, independent of the duration or intensity, also affects the impacts of low temperature stress.

The frequency of low temperature stress alters the monophasic interaction between intensity and duration of low temperature of fitness through the effects of repair and preparation for subsequent exposures. As a result, the time between cold exposures—period—could also alter the relationship between stress and fitness. If the physiological processes that repair damage incurred during cold exposure or prepare insects for subsequent cold exposures take more time than the period between exposures, fitness could be reduced due to repeated cold exposures. It is also possible that longer periods could reduce the capacity for insects to entrain responses that could prevent future damage. For example, increased time between freezing exposures decreases survival in the hover fly *Syrphus ribesii* (Brown *et al.*, 2004). Taken together, it is possible that incorporating the effects of these four potential axes of environmental stress—intensity, duration, frequency, and period—can lead to fitness impacts through three processes: 1) direct effects of damage, 2) energetic costs of recovery and repair and 3) preparation for subsequent exposures. Intensity, duration, frequency and period could also interact with each other, leading to complex relationships between imposed temperature stress and fitness.

The effects of intensity and duration are reasonably well-parameterized in many species, but very few studies have explicitly investigated period and frequency (Chown and Terblanche, 2007; Chapter 2). Here I present the first experimental assessment of the fitness impacts of all four of axes of stress (period, intensity, duration, and frequency) in a design that allows for detection of interactive effects. This approach allows me to directly test the relative importance of each axis, as well as whether they are additive or interactive. Effects were measured in three separate categories: compounds associated with protection (glycerol and sorbitol), measures of negative effects (tissue damage and energetic reserves), as well as subsequent fitness effects (survival, development time, and number of eggs). Placing the physiology of low temperature stress in the context of long-term measures of survival and reproductive output allows me to directly test how abiotic stress is translated to fitness reductions.

6.2   Methods

6.2.1   Study system

Due to its abundance and wide geographical range, the goldenrod gall fly *Eurosta solidaginis* has been used as a model for low temperature physiology (Irwin and Lee, 2003; Storey and Storey, 1990a). The goldenrod gall fly is an obligate parasite of goldenrod plants (*Solidago spp.*), and spends the majority of its life as a diapausing pre-pupa within a senesced gall that it induces on the plant (Uhler, 1951). During this overwintering phase, the goldenrod gall fly is remarkably freeze tolerant, withstanding both intracellular and extracellular freezing of its body tissues down to -80 ºC (Lee *et al.*, 1993; Mugnano *et al.*, 1996; Yi and Lee, 2003). The pre-pupae experience relatively unbuffered air temperatures in their overwintering microclimate, and at temperatures *ca.* -8.5 ºC, their body water freezes (Layne, 1991; Mugnano *et al.*, 1996; Irwin and Lee, 2003). Since the adult flies cannot feed on gall tissue following the winter (Uhler, 1951), the remaining lipid stores are an important determinant of egg production (Irwin and Lee, 2003). The eggs produced within a few days of eclosion in the spring represent the entire potential reproductive output of a female (Uhler, 1951).

The biochemical correlates of freeze tolerance in the goldenrod gall fly, *Eurosta solidaginis* (Diptera: Tephritidae) are well understood (Storey and Storey, 1988; Storey and Storey, 1990b). In the early fall, *E. solidaginis* larvae accumulates large amounts of glycerol, synthesized from glycogen stores, as a cryoprotectant (Storey *et al.*, 1981). Exposure to temperatures below 5 ℃ induces the synthesis of sorbitol as a second cryoprotectant (Storey and Storey, 1988). Goldenrod gall flies also remodel lipid membranes to increase fluidity (Pruitt and Lu, 2008), decrease saturation in triacylglycerols (lcTAGs, Bennett *et al.*, 1997), and accumulate acetylated triacylglycerols (acTAGs, Marshall *et al.*, submitted) from late August until late October, all of which are associated with freeze tolerance in this species. Finally, aerobic metabolism is fueled through the winter by catabolism of glycogen and lipid resources that were accumulated during the summer growing season. This well-characterized low temperature physiology, the ease of collection, and understanding of basic life history makes *E. solidaginis* a useful model for studying the impacts of repeated cold exposure.

6.2.2 Insect collection and maintenance

I collected *Solidago canadensis* galls containing *E. solidaginis* from old-field habitats in London, ON, Canada (43°00′N, 81°15′W) in October and November 2009, following the senescence of the *S. canadensis* plant. I kept galls in an incubator at 15 °C until I extracted pre-pupae (< 2 weeks). I placed extracted pre-pupae in perforated 0.2 mL microcentrifuge tubes, in an incubator cycling (12 h:12 h) between the normal high and low for London, Ontario (Environment Canada, http://www.weatheroffice.gc.ca), adjusted weekly, in constant darkness until they reached a daily high of 2 °C and daily low of 0 °C (late November). This cycle was then maintained for the remainder of the winter. I combined pre-pupae from all collection locations, and chose 60 individuals randomly for each experimental group. All pre-pupae were acclimated to laboratory conditions for at least one month prior to low temperature exposures.

6.2.3   Experimental design

I conducted all low temperature exposures during a three month period beginning in late December 2009 and continuing until late March 2010.   I investigated the effects of intensity, duration, period, and frequency, both separately and in interaction with each other. I examined the effects of intensity by exposing pre-pupae to one of four different temperatures: -5, -10, -15, or -20 ºC.   I examined the effects of frequency of low temperature exposure was by either subjecting flies to ten 12-hour exposures at these temperatures, or a single 120-hour exposure. Period was examined by exposing flies to 12 h exposures on a daily, 5-day, or 10-day cycle. Duration was studied by exposing flies to three, six, or ten low 12-hour exposures. Finally, to control for the potential effects of diapause intensity, individuals were either given a single 120-hour low temperature exposure at the beginning or end of the experimental period. I also sampled control individuals at the beginning (late December), middle (mid-February), and end (late March) of the experimental period (experimental design in Figure 5.1, sampling scheme in Figure 6.1).

6.2.4   Cold exposures

A programmable Proline 3530C refrigerated circulator (Lauda, Wurzburg, Germany) containing 50:50 methanol:water was used to control temperature for all cold exposures. I placed groups of pre-pupae (still in 0.2 mL microcentrifuge tubes) in 50 mL centrifuge tubes, which were held upright in the circulator bath by an aluminum insert. The temperature within four of these tubes was monitored by 36 AWG Type T (copper-constantan) thermocouples (Omega, Laval, Canada) interfaced to a computer running PicoLog Software for Windows (Pico Technology, Cambridge, UK) by Picotech TC-08 thermocouple interfaces (Pico Technology, Cambridge, UK), and recording temperature at 0.5 s intervals. I began all low temperature exposures at 0 °C at 8 pm, and cooled at a rate of 0.1 °C/min to -5, -10, -15, or -20 °C. After the conclusion of a low temperature treatment, all individuals were placed back in the incubator for recovery at 12 h/12 h 0°C /2 °C. Twenty-four hours after each low temperature exposure concluded, I transferred 15 individuals into 1.7 mL microcentrifuge tubes, and snap-froze them in direct

immersion in liquid nitrogen for a minimum of 5 min. These were stored at -80 °C for later analysis. A subset (20) of each group were placed back in the incubator at 12 h/12 h 0°C /2 °C for later fitness assays. At this time I also measured the supercooling point of an additional 20 pre-pupae, as well as tissue damage in an additional five pre-pupae.

6.2.5   Survival, egg production, adult mass, and development time

After the conclusion of the low temperature treatments, all remaining pre-pupae (20 per group) were kept in constant darkness at 12 h:12 h 2 °C:0 °C until April 30 2010. I then transferred pre-pupae in groups of 20 from 0.2 mL microcentrifuge tubes to 100 mm diameter Petri dishes lined with moistened filter paper and placed them in an incubator (Sanyo Scientific MIR-153, Bensenville, Illinois, USA) in constant light at a constant 20 °C to induce pupation. Petri dishes were checked daily for eclosed adults, and females were placed into a new Petri dish to allow development of eggs (males were kept in separate Petri dishes, Irwin and Lee, 2003) and the date of eclosion was recorded. Three days following eclosion, all adults were frozen and stored at -20 °C until dissection. I then weighed all adults, measured thorax width and length of females under 15× magnification. I then dissected females, and counted the eggs in both ovaries (Irwin and Lee, 2003).

6.2.6   Supercooling points

I measured supercooling points after Strachan *et al.* (2010). I placed pre-pupae in 1.7 mL microcentrifuge tubes in contact with a Type T copper-constantan thermocouple threaded through a hole in the top of the tube. Thermocouples were held in place with Sticky Tack (Amscan Canada Ltd., Dorval, Quebec, Canada) placed on the top of the microcentrifuge tube. Temperature was recorded at 0.5 s intervals as in low temperature treatments. I placed the microcentrifuge tubes into an aluminum block cooled by a Lauda Proline bath circulating 50:50 methanol:water. Temperature was cooled from 0 °C to -25 °C at a rate of 0.1 °C/min. I recorded the supercooling point as the lowest temperature immediately prior to the exotherm indicating the latent heat of crystallization (Lee 2010).
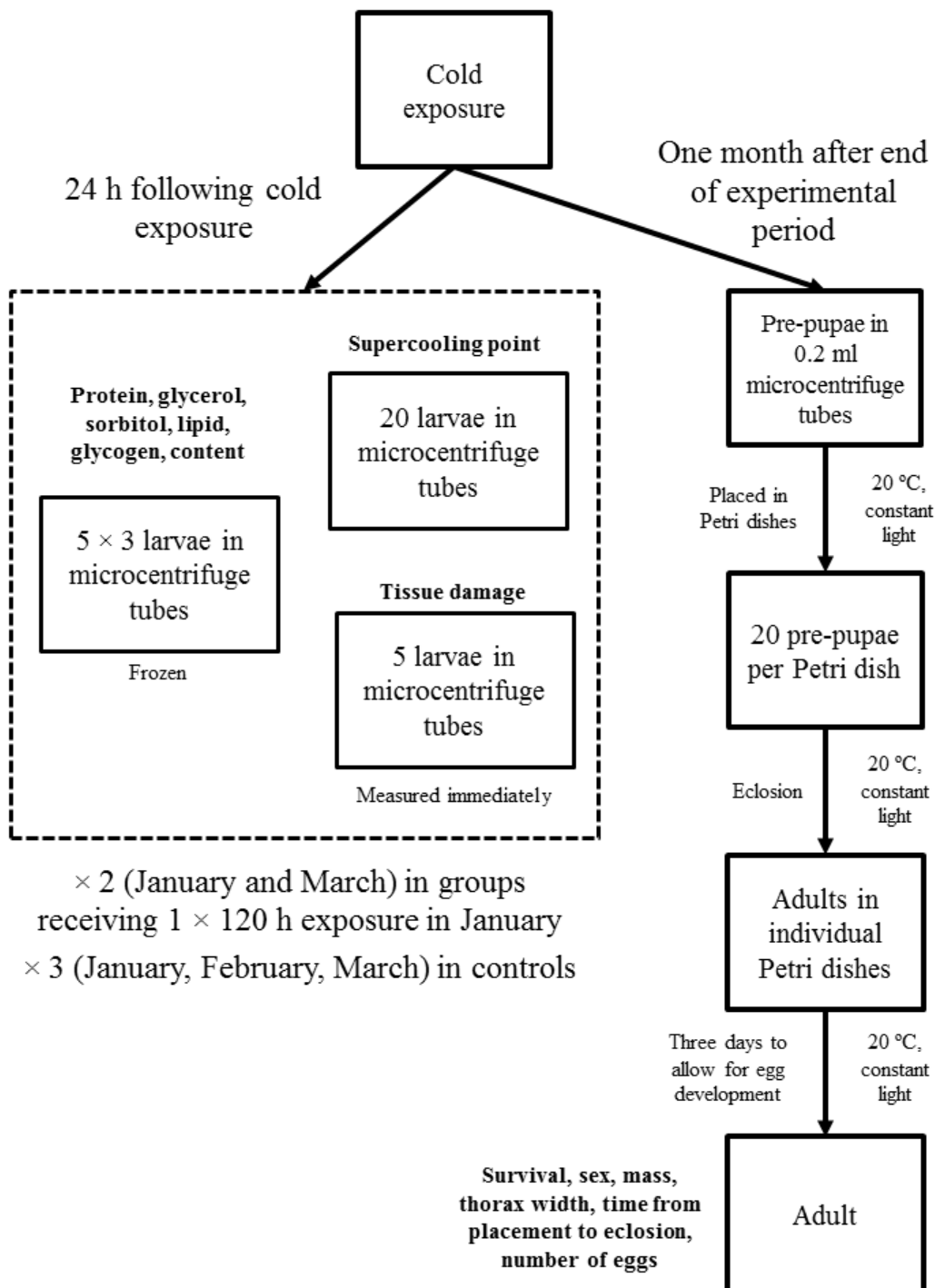
Figure 6.1 Sampling scheme for the experiment described in Figure 5.1. Terms in bold indicate measures investigated statistically.

6.2.7   Tissue damage

I quantified total living and dead cells in tissues as in Chapter 4 with a few modifications. All dissections were conducted on silicon elastomer-filled Petri plates as in Yi and Lee (2003). First, I used an insect pin to pierce live pre-pupae at the posterior end and collected a 10 µL sample of the resulting droplet of hemolymph. This hemolymph was immediately placed on a glass slide in 30 µL of a 1:100 solution of SYBR 14 (in DNA-bound complex λ_{maxex} = 475 nm, λ_{maxem} = 516 nm, obtained from a Live/Dead Sperm Viability Kit, Invitrogen Canada Inc., Burlington, ON, Canada) in Coast's solution (Coast, 1988). I then pinned the pre-pupa through the anterior end, and dissected it. I removed a small portion of Malpighian tubules and fat body, then placed both tissues on glass slides in 1:100 SYBR 14:Coast's solution. I then incubated all slides in the dark for 10 min under a coverslip, and added 30 µL of 1:100 propidium iodide (in DNA-bound complex λ_{maxex} = 540 nm, λ_{maxem} = 617 nm, obtained from a Live/Dead Sperm Viability Kit, Invitrogen) in Coast's solution, and I incubated the slides again for 10 minutes.

I visualized the prepared slides under 50× magnification using an Axio Observer Z1 microscope (Carl Zeiss MicroImaging, Goettingen, Germany) using green fluorescent protein (GFP, excitation wavelengths = 430–510 nm, emission wavelengths =474–575 nm) and Rhodamine (excitation wavelengths = 534–568 nm, emission wavelengths =575–640 nm) filters to detect the fluorescence from SYBR 14 and propidium iodide dyes, respectively. SYBR 14 dyes live cell nuclei and fluoresces green, whereas propidium iodide only stains nuclei of cells with compromised cell membranes and fluoresces red. Images of the available field of view at 50× magnification were captured. For hemolymph samples, cells in the images from the GFP and Rhodamine filters were counted separately by the built-in Analyze Particles function in ImageJ software (Abramoff *et al.* 2004). Counts were verified by inspection of the image to ensure that all flags assigned by the function corresponded with a hemocyte. For Malpighian tubule and fat body cells, GFP and Rhodamine images were merged and colour-coded in ImageJ. Individual cells in each tissue type were then flagged either red or green using the cell counter plugin in ImageJ.

All cell counts were $\log_{10}$-transformed to satisfy the assumption of normality, and ANCOVAs were conducted with total number of cells counted as a covariate (see below for detailed explanation of analyses). There was a strong relationship between the $\log_{10}$-transformed number of cells and $\log_{10}$ transformed number of cells stained red (slope = 0.64, R^2 = 0.463, df = 353, p < 0.001), so $\log_{10}$-transformed number of cells was retained as a covariate in all subsequent analyses.

6.2.8 Metabolite and energy reserve assays

I homogenized pre-pupae using an ice-cold 1 mL glass homogenizer in pools of three individuals in 0.1 % butylated hydroxytoluene (BHT) in 125 µL of ice-cold Coast's solution (Coast, 1988). I poured the homogenate into a 1.7 mL microcentrifuge tube, then rinsed the homogenizing tube with an additional four washes of 125 µL of Coast's solution. I mixed the sample vigorously, and removed a 70 µL aliquot that I froze at -80 °C for later lipid analysis. I centrifuged the remaining liquid for 15 min at 15,000 × g, then removed the supernatant (~420 µL) and mixed it thoroughly. I then split the supernatant into four 100 µL aliquots which were frozen at -80 ℃ for later metabolite analysis.

I measured sorbitol content in samples diluted 1:99 (with 0.05 per cent Tween 20) using a Sorbitol Assay Kit with sorbitol standards (Megazyme International Ireland, Bray, Ireland). Protein, glucose, glycogen (all diluted 1:99 with 0.05 per cent Tween 20), and glycerol (diluted 1:199 with 0.05 per cent Tween 20) content were all measured spectrophotometrically (after Gefen *et al.*, 2006) using bovine serum albumin, glucose, Type II glycogen from oyster, and glycerol standards respectively. Briefly, soluble protein concentration was measured using a Bicinchoninic Acid Kit (BCA1, Sigma-Aldrich, Oakville, Ontario, Canada). Glucose concentration was measured using a hexokinase-based glucose assay kit (GAHK20, Sigma-Aldrich Canada Co.), while glycogen mass was measured using the same kit following overnight amyloglucosidase (A9228, Sigma-Aldrich Canada Co.) digestion at room temperature. Glycerol concentration was measured using Free Glycerol Reagent (F6425, Sigma-Aldrich Canada Co.).

I extracted all lipids from undiluted homogenate as in Williams *et al.* (2011). I added a 50 µL aliquot of homogenate to 2.5 mL 0.1 % BHT in 2:1 chloroform: methanol, with an additional 100 µg of 1 mg/mL 1-stearoyl-*rac*-glycerol added as an internal standard. I centrifuged the samples at 2,000 × *g* for 10 minutes, then added 1 mL 0.25 % KCl. I heated the mixture at 70 °C for 10 minutes to allow separation of aqueous and organic layers. I then removed the lower organic layer (*ca*. 1.5 mL) and placed it into glass vials, and dried the samples under nitrogen at 70 °C. After resuspension in 800 µL chloroform, samples were stored at -20 °C until analysis.

To separate and quantify neutral lipid classes, I spotted 1.5 µL of each sample in triplicate using a glass syringe on silica-coated Chromarods that had been repeatedly blank-scanned in an Iatroscan MK-6 TLC-FID (thin-layer chromatography coupled to flame ionization detector, Shell-usa, Spotsylvania, Virginia, USA). I then developed rods in a mixture of 70:30:05 benzene: chloroform: formic acid for 35 min to allow separation of neutral lipid classes (Williams *et al.* 2011). I then removed rods from the solvent mixture and dried them at 70 °C for 5 min before they were scanned on the Iatroscan at a speed of 3 cm/s. I identified individual peaks by retention time compared to known standards, and peaks were quantified using PeakSimple software (SRI Instruments, Torrance, USA) and standard curves of known concentrations of lcTAGs and free fatty acids (FFAs) with a fatty acid profile of 60:20:20 oleic acid:palmitic acid:palmitoleic acid (Pruitt and Lu, 2008). Acetylated triacylglycerols were quantified with a standard curve of known concentration from acTAGs purified from other pre-pupae.

6.2.9   Statistical analysis

I began all statistical analyses by first examining the effects of period, number of exposure, and intensity of exposure in the individuals that received repeated 12 h exposures. Then the effects of month exposed and sampled and intensity of temperature exposure were compared in individuals that received a single 120 h exposure. Control individuals were compared among sampling months. A final model including all three exposure types was then used to investigate differences. Maximal models were first fit,

including all potential terms and interactions. Then, using the step() algorithm implemented in R (Venables and Ripley, 2002), I simplified to the model with the lowest Akaike's Information Criterion (AIC) by sequentially removing the highest-order interaction terms. Since the step function will halt when removing a term increases the AIC regardless of its significance (*i.e.* ΔAIC between the simplified and original model <2, Crawley 2005), I compared AIC values between the best-fit model from the step function with the next-simplest model (*i.e.* the best-fit model from the step function with the highest-order interaction term removed) using the extractAIC function in R. If the increase in AIC at this point was not significant (ΔAIC < 2), I restarted the step function with the next simplest model (Crawley 2005).

I first investigated covariance among measures of metabolites (long-chain triacylglycerol, free fatty acid, acetylated triacylglycerol, glycerol, sorbitol, protein, glycogen, and glucose) using principal components analysis (PCA) implemented using the prcomp command in R after scaling and centering to standardize variance. Principal components (PCs) that explained more than 15 % of the variance in the data set were retained, and PCs were defined based on variables that were strongly loaded on each PC (>0.3). The PCA showed that lipid metabolites accounted for the largest amount of variation. There was no PC that included significant loading of both lipid and carbohydrate/polyol metabolites, so in subsequent analyses I considered lipids and carbohydrate/polyol metabolites separately.

I then used MANCOVA to examine the effects of frequency, duration, period, and intensity on both metabolites and adult measures. I used the drop1.mlm function (W. Stahel, available at http://stat.ethz.ch/~stahel/courses/regcourse/regr.R) to remove highest-order interactions that did not cause a significant increase in AIC as above. I then split all data into three broad categories based on the pre-pupal low temperature exposure type first: 1) those that received repeated 12-hour exposures, 2) those that received a single 120-hour exposure, and 3) control flies (no low temperature exposure). I then compared individuals that had experienced repeated exposures using three-way

MANOVA with period (length of time between exposures), frequency (number of low temperature exposures), and intensity (temperature exposed to) as a covariate. Then I compared samples from individuals that experienced a single prolonged exposure using two-way MANOVA to determine the effects of intensity and time of year, while control flies were compared between sampling points using one-way MANOVA to determine the effects of time of year. If the period between exposures was not a significant predictor of the multivariate axis, then I pooled individuals that had then repeated exposures within each temperature. Similarly if time of year was not a significant predictor in pre-pupae that had experienced prolonged exposures or control conditions, I pooled these individuals within temperatures. I then compared pre-pupae using a two-way MANOVA with temperature and experimental group as predictors (where experimental treatment could include separate terms for repeatedly-exposed treatment groups with different periods of exposure and flies that had experienced prolonged exposure at different times of year). Finally, I compared adult measures in individuals among the three exposure frequencies (prolonged, repeated, or control).

To investigate differences among groups in lipid composition, I performed MANOVA on the mass of long chain triacylglycerols (lcTAGs), free fatty acids (FFAs), and acetylated triacylglycerols (acTAGs). Due to the large variability in the fat content, body mass is not an appropriate measure of body size. Therefore I investigated differences in the carbohydrate/polyol pools (glucose, glycogen, glycerol, and sorbitol) in a separate MANCOVA with soluble protein mass (to account for metabolizing tissue) as a covariate in all models using the same procedure as for lipids. Finally, I compared adult measures for females (egg production, development time, mass, thorax width, and thorax length) among experimental treatments using MANCOVA as described above. After MANCOVA, I used univariate ANCOVAs to compare among pre-pupae with different low temperature exposures. To compare survival to eclosion among flies that had received different low temperature exposures, I used generalized linear models with a binomial distribution. To test for differences in number of eggs among flies that had received different low temperature exposures, I used generalized linear models with a quasi-Poisson distribution. I conducted all cryoprotectant and metabolic fuel analyses on

a mass per individual fly basis after scaling up from the concentration of each aliquot then dividing by the three individuals in each sample. I report mean and SE throughout. For plots that represent data from models with covariates, the predict function in the base package of R was used to estimate response values that took into account the value of the covariate. Alpha was set to 0.05 in all statistical tests.

6.3   Results

6.3.1   Survival, egg production, adult mass, and development time

A total of 549 flies emerged from the 849 pre-pupae set aside for measuring adult characteristics (overall survival rate = 65%). Of these, a total of 247 were female (overall female:male sex ratio = 0.45). Since measures of adult fitness (development time, mass, and number of eggs produced) in female flies are expected to be correlated, MANCOVA (with thorax width as a covariate) was conducted on these measures to investigate the effects of low temperature exposure as pre-pupae. This analysis produces the multivariate axis that best discriminates among experimental groups. There was a significant interaction between number of exposures and period between exposures on the multivariate relationship of fitness proxies in a three-way MANCOVA on flies that had received repeated cold exposures as pre-pupae (Table 6.1). There was also a significant two-way interaction between period between exposures and temperature of exposure in flies that had received repeated exposures (Table 6.1). The highest coefficients for all terms were for the number of eggs, suggesting this measure drove the differences between experimental groups in the MANCOVA (Appendix C Table C.1). There was no effect of either exposure temperature or time of year exposed on the multivariate fitness axis in individuals that received a single prolonged exposure (Table 6.2). Finally, when all three frequency groups (1 × 120h exposure, 10 × 12 h exposures, and control) were compared in a one-way MANCOVA, there was a significant difference between the three exposure types (Table 6.3). *Post-hoc* testing (using Wilks' tests implemented by the testFactors function in the phia package for R, De Rosario-Martinez, 2012) showed there was a significant difference between individuals that had received repeated and prolonged exposures ($F_{3,260} = 3.64$, p = 0.013), as well as between control individuals and

repeatedly-exposed individuals ($F_{3,260} = 3.13$, p = 0.026), but not between individuals that had received a prolonged exposure and control individuals ($F_{3,260} = 1.90$, p = 0.130). The coefficients for this MANCOVA indicated that body mass and egg production were variables that accounted for the most amount of variation among exposure types, while development time had a lesser effect (Appendix C Table C.2), so I further examined all three fitness proxies using univariate analyses.

Adult mass was compared among female flies that received different low temperature exposures using univariate ANCOVA with thorax width as a covariate. While there were significant interactions between exposure temperature and period between exposures, as well as period between exposures and number of exposures on body mass (statistics in Appendix C Table C.3), there was no clear influence of any one variable on adult mass (Figure 6.2A-C). There was no impact of exposure temperature or time of year exposed on female mass in individuals that received a single 120 h cold exposure (statistics in Appendix C Table C.4, Figure 6.2D). Finally when all groups were compared against the control, there was no significant difference in adult female mass between any group and control flies (statistics in Appendix C Table C.5, p > 0.10 in Tukey's *post-hoc* tests in all cases, Figure 6.2E). Adult male flies showed similar trends in body mass to female flies (Appendix C Results, Appendix C Tables C.3-5).

The best predictor for number of eggs was the mass of the adult female fly (AIC = 1987.66, *vs*. 2095.30 or 2083.14 for thorax width or length). Therefore the number of eggs was compared among females with fly mass as a covariate. There was no effect of exposure temperature, number of exposures, or period between exposures on number of eggs produced in female flies that received a repeated exposure (Appendix C Table C.3, Figure 6.3A-C). Similarly, there was no impact of exposure temperature or time of year exposed on number of eggs in flies that received a prolonged exposure (Appendix C Table C.4, Figure 6.3D). Finally, when flies from all frequency of exposure groups (control, repeated, or prolonged) were compared, there was a significant difference between repeatedly-exposed flies and control flies, but not between flies that received a prolonged exposure and control flies (Appendix C Table C.5, Figure 6.3E). When repeatedly-exposed and control flies were compared, there was a significant difference

Table 6.1 MANCOVA statistics comparing adult characteristics (body mass, number of eggs, and development time) among female *Eurosta solidaginis* flies as a result of low temperature exposure as pre-pupae. For flies that had received repeated exposures, exposure temperature (either -5, -10, -15, or -20 °C), period between exposures (daily, every 5 days, or every 10 days), and number of exposures (either 3, 6, or 10 exposures) are investigated. For flies that received a prolonged exposure, exposure temperature (either -5, -10, -15, or -20 °C), and time of year exposed (exposed in January, exposed in March) are compared. Retained terms with significant p-values ($p < 0.05$) are in bold typeface.

Type of exposure	Initial model	Minimal adequate model	Terms from minimal adequate model	Wilk's λ	F	Df	P
Repeated (12 hour exposures)	Body mass + number of eggs + development time = thorax width + exposure temperature × period between exposures × number of exposures	= thorax width + temperature + period + number + temperature × period + period × number	**Thorax width**	**0.775**	**18.650**	**3, 193**	**< 0.001**
			Temperature	0.993	0.042	3, 193	0.988
			Period	0.960	1.326	6, 386	0.244
			Number	0.969	1.008	6, 386	0.420
			Temperature × period	**0.852**	**5.364**	**6, 386**	**<0.001**
			Period × number	**0.863**	**2.432**	**12,511**	**0.004**
Prolonged (1 × 120h exposure)	Body mass + number of eggs + development time = thorax width + exposure temperature × time of year exposed	= thorax width	**Thorax width**	**0.618**	**10.621**	**3, 50**	**< 0.001**
Prolonged (1 × 120 h exposure) Repeated (10 × 12 h exposure) Control	Body mass + number of eggs + development time = thorax width + exposure group	= thorax width + exposure group	**Thorax width**	**0.762**	**27.15**	**3, 260**	**<0.001**
			Exposure group	**0.930**	**3.183**	**6, 520**	**0.004**

between flies that experienced repeated and prolonged exposures (Appendix C Table C.5, p = 0.0289, Figure 6.3E).

I recorded development time as the number of days required for *E. solidaginis* flies to eclose following placement of pre-pupae at 20 °C. In female flies that received repeated exposures, development time was significantly affected by the interactions between temperature and number of exposures, temperature and period between exposures, and number and period between exposures (Appendix C Table C.3). These interactions were driven by long development time in flies that received 10 exposures every 10 days at -20 °C as well in flies that received six exposures to -5 °C (Figure 6.4A). Therefore exposure temperature and period between exposures were retained as terms in the model comparing prolonged and repeated exposures. By contrast, there was no effect of month exposed or exposure temperature on development time of flies that received a single prolonged exposure (Appendix C Table C.4). When flies from all frequencies of exposure (control, repeated, and prolonged) were compared, there was a significant difference in development time among exposure types (Appendix C Table C.5). Flies that received repeated low temperature exposures had significantly longer development times at almost every exposure temperature (Figure 6.4E, Appendix C Tables C.3-5). In adult male flies, similar effects on development time were noted (Appendix C Table C.3-5).

I recorded survival as the successful eclosion of adult flies. A total of 549 flies emerged from the 849 pre-pupae set aside for measuring adult characteristics (overall survival rate = 65%). In flies that received repeated low temperature exposures, there was a significant two-way interaction between exposure temperature and period between exposures on survival (Appendix C Table C.3). Flies that received 12 h exposures every 10 days had increased survival after exposure to -20 °C relative to other periods of exposure, but flies that received exposures every five days had increased survival when exposed to -5 °C. In flies that received a prolonged exposure, flies that received their exposure in January had higher survival than flies that received their exposure in March (Appendix C Table C.4, Figure 6.5).

Figure 6.2 The effects of low temperature exposure during overwintering on adult female mass in *Eurosta solidaginis*. All significant effects are indicated by the bold terms in each figure, and accompanying statistics are presented in Appendix C Tables C.3-5. "Period" = time between cold exposures (one, five, or ten days), "Intensity" = temperature of cold exposure, "Number" = number of cold exposures (three, six, or ten days). Solid and dotted horizontal grey lines represent mean ± standard error of control flies, respectively. A) Flies that received ten 12 h exposures as pre-pupae. B-C) Flies that received repeated 12 h exposures as pre-pupae. D) Flies that received a single 120 h cold exposure. E) Comparison of flies that received repeated 12 h *vs*. a single 120 h cold exposure.

Figure 6.3 The effects of low temperature exposure during overwintering on the number of eggs produced by *Eurosta solidaginis*. All significant effects are indicated by the bold terms in each figure, and accompanying statistics are presented in Appendix C Tables C.3-5. "Period" = time between cold exposures (one, five, or ten days), "Intensity" = temperature of cold exposure, "Number" = number of cold exposures (three, six, or ten days). Solid and dotted grey horizontal lines represent mean ± standard error of control flies, respectively. A) Flies that received ten 12 h exposures as pre-pupae. B-C) Flies that received repeated 12 h exposures as pre-pupae. D) Flies that received a single 120 h cold exposure. E) Comparison of flies that received repeated 12 h *vs*. a single 120 h cold exposure.
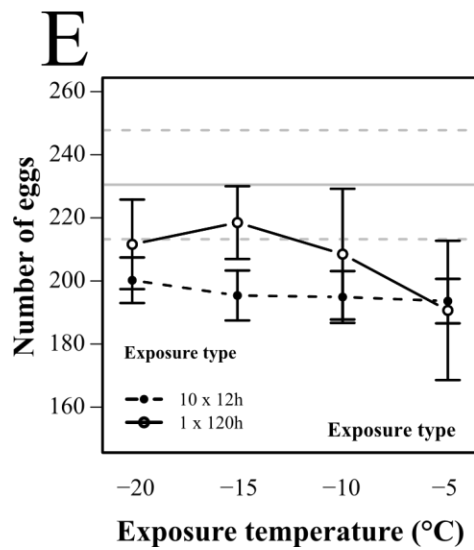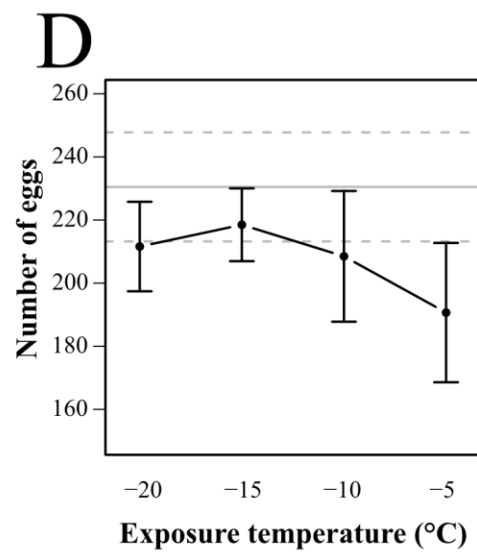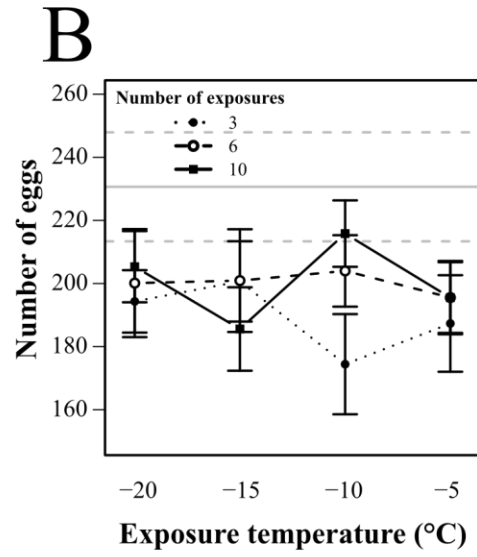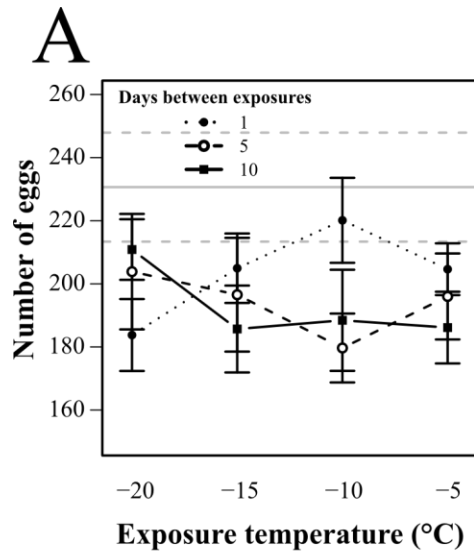
Figure 6.4 The effects of low temperature exposure during overwintering on the development time (number of days between the placement of pre-pupae at diapause breaking conditions to adult eclosion) of female *Eurosta solidaginis*. All significant effects are indicated by the bold terms in each figure, and accompanying statistics are presented in Appendix C Tables C.3-5. "Period" = time between cold exposures (one, five, or ten days), "Intensity" = temperature of cold exposure, "Number" = number of cold exposures (three, six, or ten days). Solid and dotted horizontal grey lines represent mean ± standard error of control flies, respectively. A) Flies that received ten 12 h exposures as pre-pupae. B-C) Flies that received repeated 12 h exposures as pre-pupae. D) Flies that received a single 120 h cold exposure. E) Comparison of flies that received repeated 12 h *vs*. a single 120 h cold exposure.

Figure 6.5 The effects of low temperature exposure during overwintering on the survival of *Eurosta solidaginis* to eclosion. Asterisk indicates significant difference between flies that received one 120 h exposure in January and all other exposure types ($p < 0.05$). All statistics are presented in Appendix C Tables C3-5.

Finally, when flies from all frequencies of exposure (control, repeated, and prolonged) were compared, there was a significant difference among the groups (Appendix C Table C.3), but this difference was due to the high survival of flies exposed for 120 hours in January (Figure 6.5).

6.3.2   Supercooling point

There was a significant interaction between number and period between exposures as well as between the exposure temperature and number of exposures on the supercooling points of repeatedly-exposed pre-pupae (Appendix C Table C.3, Appendix C Figure C.1). In flies that received a single prolonged exposure, flies that received their exposure in March had lower supercooling points than flies that received their exposure in January, regardless of whether their supercooling point was measured in January or March (Appendix C Table C.4), but there was no significant effect of exposure temperature, so time of year exposed was retained in subsequent tests. Supercooling point did not differ through the year in control flies (Appendix C Table C.4), so these individuals were pooled. Finally, flies that received a prolonged exposure in March had significantly lower supercooling points than any other exposure group (Appendix C Figure C.1).

6.3.3   Tissue damage

Increased number of exposures increased the number of damaged cells in pre-pupae that received repeated exposures (Appendix C Figure C.2, Appendix C Table C.8). Increased time following exposure increased the number of damaged fat body cells in pre-pupae that received a single 120 h exposure (Appendix C Table C.9). In control pre-pupae, there was a significant increase later in the season in number of damaged hemocytes, but all other tissues had similar numbers of damaged cells regardless of the time of year (Appendix C Table C.10, Appendix C Figure C.2). Finally, when each tissue type was compared among exposure groups (control, repeated, or prolonged exposures to -20 °C), there was no significant effect of exposure type on number of dead cells (Appendix C Table C.4, Appendix C Figure C.11).

6.3.4   Lipid reserves

The predominant energy reserves in *E. solidaginis* were lipids (8.61 ± 0.19 mg out of a total fresh body mass of 47.17 ± 0.51 mg), and these were split into three distinct classes of nearly equal masses: long chain triacylglycerols (lcTAGs, 2.50 ± 0.05 mg), free fatty acids (FFAs, 2.74 ± 0.05 mg), and acetylated triacylglycerols (acTAGs, 3.38 ± 0.07 mg, Marshall *et al.*, submitted).   An exploratory PCA of all metabolites (all lipid classes, glucose, glycogen, protein, glycerol, and sorbitol) showed there was little covariation of lipid metabolites and carbohydrate metabolites; no PC axis had significant loadings of both a lipid and a carbohydrate metabolite (Table 6.3). Therefore, in further multivariate analyses, I examined these two energy reserve classes separately.

The effects of intensity, duration, frequency, and period on lipid class content in *E. solidaginis* were examined using MANCOVA with total lipid mass as a covariate. Lipid class content was significantly impacted by exposure temperature and the period between exposures in pre-pupae that received repeated low temperature exposures (Table 6.4). The largest coefficients in this MANCOVA were generally acTAG mass, which suggests that variation in this lipid class drives the differences among pre-pupae that received differing exposures (Table 6.12). In pre-pupae that received prolonged exposures, there was a significant impact of the time of year exposed and sampled on lipid class content (Table 6.4).   Again, the impact of time of year exposed and sampled was driven by variation in acTAG mass (Appendix C Table C.12).   Finally, there was a significant multivariate effect of frequency of exposure (control, repeated, or prolonged) on lipid class content ($F_{6,200}$ = 4.19, p < 0.001), and *post hoc* comparisons showed that each exposure type was significantly different (p < 0.01 in all cases). As a result, acTAG and lcTAG mass were further investigated using univariate analyses (Figure 6.6-7).

Preliminary model exploration showed that total lipid mass was a better predictor of the mass of each neutral lipid component than protein mass (R^2 for model regression of total lipid mass = 0.846, for total protein content = 0.141), so total lipid mass was retained as a covariate in all analyses of lipid content. Increasing exposure temperature significantly increased acTAG mass in pre-pupae that received repeated 12 h exposures (Appendix C

Table C.16, Figure 6.6A-C). By contrast, there was no effect of exposure temperature on acTAG mass in pre-pupae that received prolonged exposures, nor was there an effect of time of year exposed or sampled (Appendix C Table C.17, Figure 6.6D). Finally, when all exposure groups were compared (while retaining the temperature term in for the pre-pupae with repeated exposures), there was a significant difference in acTAG mass in pre-pupae among the groups (Appendix C Table C.18). Pre-pupae that had repeated exposures had significantly larger acTAG mass than control pre-pupae ($p < 0.001$) or pre-pupae that received a prolonged exposure ($p = 0.041$). Pre-pupae that had received a prolonged exposure did not have significantly increased acTAG relative to control pre-pupae ($p = 0.180$).

Lower exposure temperature and increased number of exposures decreased lcTAG mass in pre-pupae that received repeated 12 h exposures (Appendix C Table C.16, Figure 6.7A-C). Similarly, time of year exposed and sampled and exposure temperature interacted to affect lcTAG mass in pre-pupae that received a single 120 h exposure (Appendix C Table C.17, Figure 6.7D). In control flies, lcTAG mass did not significantly change through the winter (Appendix C Table C.18, Figure 6.7E). Finally, there was a significant decrease in lcTAG mass in flies that received repeated exposures relative to flies that received prolonged exposures ($p = 0.014$, all other statistics in Appendix C Table C.19, Figure 6.7F).

6.3.5   Carbohydrates and cryoprotectants

Because the preliminary PCA showed correlations between carbohydrate and cryoprotectant concentrations (Table 6.4), I performed a MANCOVA with protein mass as a covariate to investigate the impacts of cold exposure on these in pre-pupae. There was a significant three-way interaction among exposure temperature, number of exposures, and period between exposures on this multivariate axis (Table 6.5). While that interaction was significant, it was a relatively weak effect compared to the effects of number and period between exposures (Appendix C Table C.14). The highest absolute values for coefficients were generally in glucose mass (Appendix C Table C.14), suggesting that variation in this measure drove differences among groups.   Exposure temperature and time of year exposed and sampled significantly impacted the

Figure 6.6 The effects of low temperature exposure on acetylated triacylglycerol (acTAG) mass in *Eurosta solidaginis* pre-pupae. All significant effects are indicated by the bold terms in each figure, and accompanying statistics are presented in Appendix C Tables C16-19. "Period" = time between cold exposures (one, five, or ten days), "Intensity" = temperature of cold exposure, "Number" = number of cold exposures (three, six, or ten days). Solid and dotted horizontal grey lines represent mean ± standard error of control flies, respectively. A) Flies that received ten 12 h exposures as pre-pupae. B-C) Flies that received repeated 12 h exposures as pre-pupae. D) Flies that received a single 120 h cold exposure in January, received a bout in January but were sampled in March, or received a single 120 h bout in March and were sampled immediately. E) Control flies maintained at 0/2 ºC all winter and sampled throughout. F) Comparison of flies that received repeated 12 h *vs*. a single 120 h cold exposure.

Table 6.2 Principle components for metabolites in overwintering *Eurosta solidaginis* pre-pupae.   Values are factor loadings for each metabolite. Variable values with significant loadings (>0.3) are in bold typeface.

Metabolite	PC1	PC2	PC3
lcTAG	**0.521**	-0.001	0.182
FFA	**0.513**	-0.110	0.183
acTAG	**0.512**	-0.100	0.219
Glucose	0.093	0.152	**-0.715**
Glycogen	0.239	**0.524**	-0.261
Protein	0.284	**0.455**	-0.169
Glycerol	0.146	**-0.513**	**-0.380**
Sorbitol	0.187	**-0.459**	**-0.367**
Variance explained	39.9 %	23.0 %	15.7 %

carbohydrate and cryoprotectant pool in pre-pupae that received a single 120 h exposure (Table 6.5).

Finally, there was no multivariate difference in carbohydrate or cryoprotectant content due to time of year in control pre-pupae (Table 6.5). Therefore, all possible explanatory terms were retained for pre-pupae that received repeated exposures (temperature and period between exposures) and pre-pupae that received prolonged exposures (temperature and time of year sampled and exposed), while control pre-pupae were pooled. The carbohydrate components of all exposure groups (prolonged at all temperature and time of year combinations, repeated including all temperatures and periods, and control pooled) were then compared using one-way MANCOVA, which showed a significant multivariate effect of exposure group on carbohydrates ($F_{72,325} = 3.325$, $p < 0.001$). Exposure frequency (repeated, prolonged, or control) was then investigated by one-way MANCOVA on data pooled within each group, which showed that exposure type significantly impacted carbohydrate pools ($F_{8,196} = 3.552$, $p < 0.001$). There was a multivariate difference in carbohydrates and cryoprotectant between pre-pupae that received repeated exposures and control pre-pupae ($p < 0.001$), as well as between pre-pupae that received repeated and prolonged exposures ($p = 0.027$). By contrast, there was no significant difference between pre-pupae that received a prolonged exposure and control pre-pupae ($p = 0.061$). There was no clear individual carbohydrate or cryoprotectant that had the largest absolute values for coefficients, so I then investigated each metabolite with univariate ANCOVAs (Appendix C Table C.15, Figure 6.8-9).

Glycerol content decreased with decreasing temperature in pre-pupae that received daily or every 10 day exposures, while pre-pupae that receive exposures every five days maintained high glycerol concentration (Figure 6.8, Appendix C Table C.16). In addition, increasing number of exposures decreased glycerol content at decreasing temperature (Figure 6.8A-C). In pre-pupae exposed in January and March that were sampled immediately, there was a negative relationship between exposure temperature and glycerol mass, while pre-pupae that were exposed in January but sampled in March have the same glycerol content regardless of exposure temperature (Figure 6.8D, Appendix C Table C.17). In control pre-pupae, there was no impact of time of year sampled on glycerol mass (Appendix C Table C.18). When pre-pupae from each exposure group
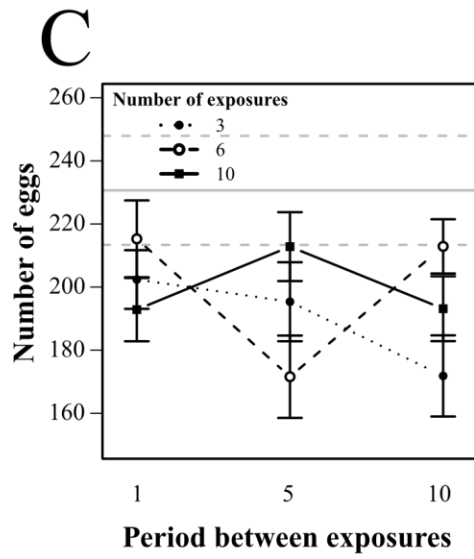
Figure 6.7 The effects of low temperature exposure on long-chain triacylglycerol (lcTAG) mass in *Eurosta solidaginis* pre-pupae. All significant effects are indicated by the bold terms in each figure, and accompanying statistics are presented in Appendix C Tables C.16-19. "Period" = time between cold exposures (one, five, or ten days), "Intensity" = temperature of cold exposure, "Number" = number of cold exposures (three, six, or ten days). Solid and dotted horizontal grey lines represent mean ± standard error of control flies, respectively. A) Flies that received ten 12 h exposures as pre-pupae. B-C) Flies that received repeated 12 h exposures as pre-pupae. D) Flies that received a single 120 h cold exposure in January, received a bout in January but were sampled in March, or received a single 120 h bout in March and were sampled immediately. E) Control flies maintained at 0/2 ℃ all winter and sampled throughout. F) Comparison of flies that received repeated 12 h *vs*. a single 120 h cold exposure.

Figure 6.8 The effects of low temperature exposure on glycerol mass in *Eurosta solidaginis* pre-pupae. All significant effects are indicated by the bold terms in each figure, and accompanying statistics are presented in Appendix C Tables C.16-19. "Period" = time between cold exposures (one, five, or ten days), "Intensity" = temperature of cold exposure, "Number" = number of cold exposures (three, six, or ten days). Solid and dotted horizontal grey lines represent mean ± standard error of control flies, respectively. A) Flies that received ten 12 h exposures as pre-pupae. B-C) Flies that received repeated 12 h exposures as pre-pupae. D) Flies that received a single 120 h cold exposure in January, received a bout in January but were sampled in March, or received a single 120 h bout in March and were sampled immediately. E) Control flies maintained at 0/2 ℃ all winter and sampled throughout. F) Comparison of flies that received repeated 12 h *vs*. a single 120 h cold exposure.

Figure 6.9 The effects of low temperature exposure on sorbitol mass in *Eurosta solidaginis* pre-pupae. All significant effects are indicated by the bold terms in each figure, and accompanying statistics are presented in Appendix C Tables C.16-19. "Period" = time between cold exposures (one, five, or ten days), "Intensity" = temperature of cold exposure, "Number" = number of cold exposures (three, six, or ten days). Solid and dotted horizontal grey lines represent mean ± standard error of control flies in February, respectively. A) Flies that received ten 12 h exposures as pre-pupae. B-C) Flies that received repeated 12 h exposures as pre-pupae. D) Flies that received a single 120 h cold exposure in January, received a bout in January but were sampled in March, or received a single 120 h bout in March and were sampled immediately. E) Control flies maintained at 0/2 ºC all winter and sampled throughout. F) Comparison of flies that received repeated 12 h *vs*. a single 120 h cold exposure.
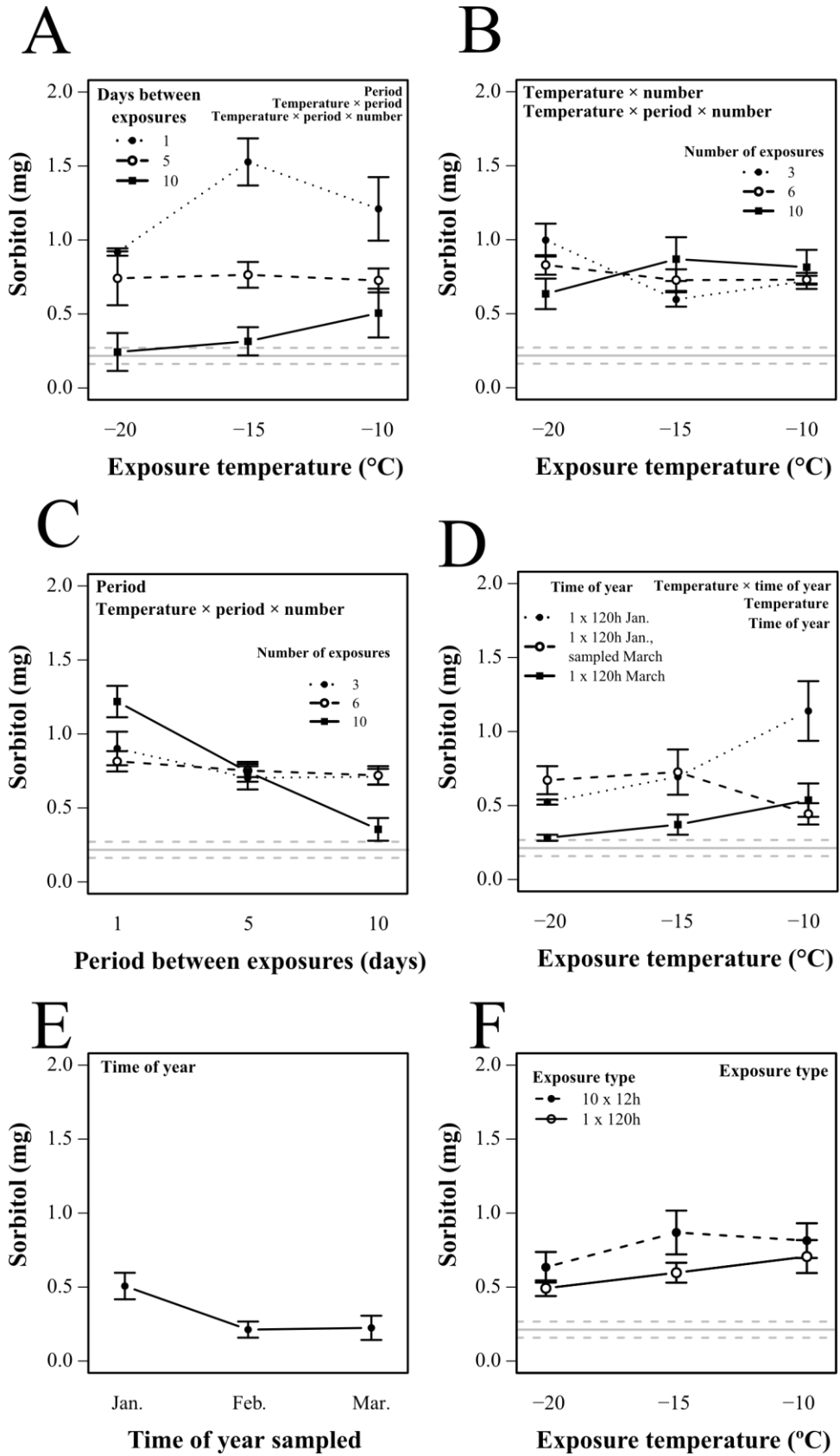
(repeated, prolonged, and control), there was a significant impact of exposure group on glycerol mass (Appendix C Table C.19, Figure 6.8F). This was further simplified, and pre-pupae were pooled within each exposure group. There was a significant impact of exposure group on glycerol mass ($F_{2,101}$ = 8.325, p <0.001). Pre-pupae that had received repeated exposures had significantly increased glycerol mass relative to pre-pupae that received prolonged exposures (p = 0.023) or control pre-pupae (p < 0.001), while pre-pupae that received prolonged exposures did not have significantly elevated glycerol mass relative to control pre-pupae (p = 0.153). Sorbitol content was reduced in pre-pupae that received exposures every 10 days for a total of 10 times (Figure 6.9A-C, Appendix C Table C.16), but at other numbers of exposures there were complicated interactions (Figure 6.9A-C). There was a significant interaction between exposure temperature and time of year exposed on sorbitol content in pre-pupae that received a prolonged cold exposure (Appendix C Table C.17). Pre-pupae exposed in January had higher sorbitol content than pre-pupae exposed in March at almost all exposure temperatures (Figure 6.9D), while in pre-pupae sampled immediately after a prolonged freeze, sorbitol mass decreased with decreasing temperature exposure (Figure 6.9D). Control pre-pupae had higher sorbitol content early in the year, and lower content in February and March (Appendix C Table C.18, Figure 6.9E). When all pre-pupae were compared against each other, there was a significant impact of exposure group on sorbitol mass, with pre-pupae that received repeated 12 h exposures having a higher sorbitol content than control and pre-pupae that had a prolonged exposure (Appendix C Table C.19, Figure 6.9F).

6.4   Discussion

Here I show that the frequency at which an insect receives a stress is a more important predictor of fitness than intensity, duration, or period of stress. I found that repeated stress exposure drives a trade-off between investment in cryoprotection (glycerol, sorbitol, and acTAG content) and egg production and development time in the goldenrod gall fly *Eurosta solidaginis*. This trade-off is only induced by repeated freezing events, which indicates that repeated freezing during overwintering increases stress in these flies, while a single freeze (matched for time and intensity) does not. This result demonstrates that aspects of stress exposure independent of intensity and duration can decrease fitness (Table 6.5). Studies of fitness costs of cold exposure

Table 6.3 MANCOVA statistics comparing lipid components (lcTAG, FFA, and acTAG mass) among *Eurosta solidaginis* pre-pupae as a result of low temperature exposure. Repeatedly-exposed flies were exposed to either -10, -15, or -20 °C, with periods between exposures of either daily, every 5 days, or every 10 days, and received either 3, 6, or 10 exposures as pre-pupae. Flies that received prolonged exposures either received their exposure at -10, -15, or -20 °C in January (sampled either immediately or in March) or March (sampled immediately). Retained terms with significant p-values (p < 0.05) are in bold typeface.

Exposure group	Initial model	Minimal adequate model	Terms from minimal adequate model	Wilk's λ	F	df	p
Repeated	lcTAG mass, FFA mass, acTAG mass = exposure temperature × period between exposures × number of exposures	= temperature + period + number + temperature × period between exposures	**Temperature**	**0.936**	**2.852**	**3, 125**	**0.0401**
			Period	0.955	0.979	6, 250	0.4402
			Number	0.921	1.741	6, 250	0.1120
			Temperature × Period	**0.884**	**2.656**	**6, 250**	**0.0163**
Prolonged	lcTAG mass, FFA mass, acTAG mass = exposure temperature × time of year exposed and sampled	= time of year exposed and sampled	**Time of year**	**0.715**	**2.431**	**6, 80**	**0.033**
Control	lcTAG mass, FFA mass, acTAG mass = time of year sampled	= null model					

Table 6.4 MANCOVA statistics comparing carbohydrate components (glucose, glycogen, glycerol, and sorbitol) among *Eurosta solidaginis* pre-pupae as a result of low temperature exposure. Repeatedly-exposed flies were exposed to either -10, -15, or -20 °C, with periods between exposures of either daily, every 5 days, or every 10 days, and received either 3, 6, or 10 exposures as pre-pupae. Flies that received prolonged exposures either received their exposure at -10, -15, or -20 °C in January (sampled either immediately or in March) or March (sampled immediately). Control flies were maintained at 0 °C all winter and were sampled in either January, February, or March. Retained terms with significant p-values ($p < 0.05$) are in bold typeface.

Exposure type	Initial model	Minimal adequate model	Terms from minimal adequate model	Wilk's λ	F	df	p
Repeated	Glucose mass + glycogen mass + glycerol mass + sorbitol mass = protein mass + exposure temperature × period between exposures × number	= protein mass + exposure temperature × period between exposures × number of exposures	**Protein**	**0.630**	**16.61**	**4, 113**	**<0.001**
			Temperature	0.938	1.187	4, 113	0.121
			Period	**0.689**	**5.795**	**8, 226**	**<0.001**
			Number	**0.836**	**2.650**	**8, 226**	**0.009**
			Temperature × period	**0.817**	**3.006**	**8, 226**	**0.003**
			Temperature × number	**0.696**	**5.607**	**8, 226**	**<0.001**
			Period × number	**0.699**	**2.692**	**16, 346**	**<0.001**
			Temperature × period × number	**0.683**	**2.870**	**16, 346**	**<0.001**
Prolonged	Glucose mass + glycogen mass + glycerol mass + sorbitol mass = protein mass + exposure temperature × time of year exposed and sampled	= protein mass + exposure temperature × time of year exposed and sampled	**Protein mass**	**0.427**	**11.751**	**4, 35**	**<0.001**
			Temperature	**0.594**	**5.983**	**4, 35**	**<0.001**
			Time of year	**0.465**	**4.079**	**8, 70**	**<0.001**
			Temperature × time of year	**0.586**	**2.677**	**8, 70**	**0.013**
Control	Glucose mass + glycogen mass + glycerol mass + sorbitol mass = protein mass + exposure temperature + time of year sampled	= null model					

Table 6.5 A summary of how intensity, duration, frequency, and period of low temperature stress affect the physiological processes of damage, repair, and preparation in _Eurosta solidaginis_, and how that in turn impacts fitness. "-" indicates no effect.

	Intensity	Duration	Frequency	Period
Damage	-	-	-	-
Repair	-	-	-	-
Preparation	Glycerol Sorbitol	Glycerol Sorbitol acTAG	Glycerol Sorbitol acTAG	Glycerol Sorbitol acTAG
Fitness	Development time	-	Development time Number of eggs	Development time

in insects have generally focused on the effects of intensity and duration, despite the fact that frequency, and period of temperature fluctuations also vary in natural environments (Chown and Terblanche, 2007; Chapter 2). While each of these four axes of stress can act independently, they also significantly interact as the animal responds physiologically to subsequent stressful events.

6.4.1 Fitness proxies

Although the goldenrod gall fly is very cold tolerant, and there was no impact of repeated cold exposure on survival, I was able to detect significant sub-lethal effects of cold stress. I found that repeated cold exposure decreased egg production and increased development time. *Eurosta solidaginis* larvae cease feeding in late summer and overwinter on the lipid and carbohydrate stores acquired through the feeding season, thus egg production in the spring is directly related to the overwintering conditions they experienced (Irwin and Lee, 2002; Irwin and Lee, 2003). In my study, the amount of time spent frozen (120 h) was short relative to the entire overwintering period in a natural environment. In an experiment conducted in a similar microclimate, based on temperature records and supercooling point, the woolly bear caterpillar *Pyrrharctia isabella* was predicted to spend a total of 362 h frozen through a five month winter (Marshall and Sinclair, 2012). While it might be expected that spending additional time at a low temperature would increase egg production due to the energetic savings (Irwin and Lee, 2002), I found no increase in number of eggs in flies that received prolonged low temperature exposure relative to controls (Figure 6.3D). By contrast, individuals that received cold exposures of 120 h (matched for duration and intensity), but in 12 h bouts, had reduced egg production (Figure 6.3E). Reduced production of female offspring was also observed in *Drosophila melanogaster* after repeated low temperature exposures (Chapter 3). This suggests that the phenotype of increased somatic investment at the cost of reproductive output might be a more general result of repeated low temperature stress.

There was no clear impact of any of the low temperature exposures on adult mass in either male or female flies. Similarly Irwin and Lee (2003) found that while egg production was impacted by overwintering microclimate, adult mass was not. This

suggests this trait is not plastic in this species. Development time, by contrast, appeared to be increased by intensity of exposure, period between exposures, and frequency of exposure, but not duration (Figure 6.4). While the observed changes in development time are relatively small (<2 days), the conditions used to measure development time in this study were *ca.* 10 °C warmer than spring conditions at that time of year in London, Ontario (Chapter 2). Since *E. solidaginis* is strictly univoltine, this will not affect generation time. However, disruption of spring phenology can be an important stress for individuals in species that must match their timing with a particular host plant (Visser and Holleman, 2001). In the case of *E. solidaginis*, eggs are laid on the growing shoots of goldenrod plants, and so their spring phenology must match that of *S. canadensis*. Disruptions in development time induced by repeated cold stress could produce a phenology mismatch that could have important population effects.

6.4.2   Freeze-induced damage

Low temperature exposure did not damage tissue in *E. solidaginis.* Yi and Lee (2003) found that hemocytes of *E. solidaginis* were particularly vulnerable to the effects of freeze exposure for ten days at -80 °C, while fat body cells and Malpighian tubules were less sensitive. I found that hemocytes were not damaged by a single 120 h freeze at -20 °C nor by even ten 12 hour freezing events at -20 °C, but that over the course of the winter there were increasing numbers of dead hemocytes in both control and low temperature exposure treatments. Survival to eclosion was higher in individuals that received a prolonged exposure early in winter than individuals that received prolonged exposures later in winter or repeated exposures. Similarly, *E. solidaginis* that overwinters in above-snow (and therefore unbuffered) microclimates has increased survival relative to individuals that overwinter beneath the snow, despite the occurrence of repeated freeze-thaw events (Irwin and Lee, 2003). By contrast, in *P. isabella*, repeated freezing induces damage to the Malpighian tubules and hemocytes and is correlated with increased mortality (Chapter 4), and repeated freezing appears to damage gut tissue in the sub-Antarctic caterpillar *Pringleophaga marioni* (Sinclair and Chown, 2005).   This indicates that the reduced egg production in flies that received repeated exposures is not a direct result of damage from repeated freezing events. Instead this

reduced egg production is likely a result of the (non mutually-exclusive) energetic costs of repair of damage (other than tissue damage) and/or preparation for subsequent low temperature events.

6.4.3 Cryoprotectants

Both glycerol and sorbitol concentrations significantly increased in response to repeated cold exposures. Surprisingly, there was less accumulation at lower temperature exposures relative to higher temperatures. Like Churchill and Storey (1989), I found increased sorbitol content in *E. solidaginis* pre-pupae that received repeated freeze-thaw cycles (Figure 6.9). By contrast to their study, I also found increased glycerol content, which may be due in part to their study design, which only examined the effects of a single temperature and did not examine whether glycerol could be catabolized. I found that increasing the period and number of exposures decreased glycerol mass (Figure 8), which could be due to catabolism of glycerol in glycolysis, since glycerol is not readily recycled into glycogen (Storey and Storey, 1986). By contrast, sorbitol can be easily recycled back into glycogen in *E. solidaginis* (Storey and Storey, 1983), which may explain why all exposure groups had similar glycogen concentration following cold exposure (Appendix C Figure C.3). Repeated freezing appears to increase glycerol concentration in the Antarctic midge *Belgica antarctica* (Teets *et al.*, 2011), and the Arctiid caterpillar *P. isabella* (Chapter 4), suggesting that this response to repeated freezing exposure might be relatively common. While the induction of increased cold hardiness following low temperature exposure ("rapid cold hardening") is well-characterized in chill susceptible insects (Bale, 2002; Lee *et al.*, 1987), it has been less-thoroughly investigated in freeze tolerant species (Lee *et al.*, 2006; Sinclair and Chown, 2003). This increased glycerol and sorbitol is likely not due to rapid cold hardening response like in *B. antarctica* (Lee *et al.*, 2006), because repeated freezing induces additional glycerol and sorbitol above the quantities accumulated following a single freeze, and since there appears to be a fitness cost to this repeated freezing.

6.4.4   Energetic costs

Repeated cold exposure did not induce energetic costs in *E. solidaginis*, as measured by glycogen and lipid reserves, which is in contrast to other studies on repeated freezing in insects (Churchill and Storey, 1989; Teets *et al.*, 2011).   There was, however, a shift in allocation of lipid classes as a result of repeated freezing. Flies that received repeated low temperature exposures had greater acTAG mass and less lcTAG mass than flies that received prolonged exposures. Acetylated triacylglycerols are lower in energy density than lcTAGs due to having only two fatty moieties rather than three (Durrett *et al.*, 2010), so it is possible that this translates to lower energy density in *E. solidaginis*.   This conversion may be especially important during metamorphosis, since acTAGs are rapidly converted back to lcTAGs during this life stage (Marshall *et al.*, submitted). In addition, there are other potential costs of repeated cold exposure (for example, *hsp70* expression increases with repeated freezing in *B. antarctica,* Teets *et al.*, 2011) that I did not measure that could contribute to reduced egg production.

The metabolite measurements in this experiment were all made during winter to obtain a snapshot during the overwintering period, so it is also possible that differences in energy reserves were not detectable until the end of winter.   The biochemistry of acTAGs remains under investigation, but it is clear that biosynthesis of glycerol and sorbitol from glycogen is not 100% efficient, which suggests that the increased energetic allocation to cryoprotection in pre-pupae that received repeated exposures represents a non-reversible ATP investment (Storey and Storey, 1990b). This increased expenditure could explain the reduced egg production in flies that received repeated exposures, and provides a mechanistic link between the stress induced by repeated low temperature exposures and the resulting reduction in fitness. In many insects, caloric restriction can induce a shift in investment from reproduction to survival, and this switch is believed to be mediated by insulin signalling, at least in *Drosophila melanogaster* (reviewed in Rion and Kawecki, 2007).   In overwintering insects with a limited energetic budget, the conditions for a trade-off between reproduction and survival are met (Van Noordwijk and De Jong, 1986; Zera and Harshman, 2001), although the signalling that mediates this transition is still unknown.

6.4.5   Cooling rate

One aspect of low temperature exposure that I did not study was the effect of the rate of onset of environmental stress on cryobiology and fitness. All individuals in this study were cooled at 0.1 ºC/min, which is relatively slow compared to the majority of studies of low temperature exposure (Bale *et al.*, 1989; Kelty and Lee, 1999), but faster than natural cooling rates (which can range between 0.003 ºC/min and 0.12 ºC/min in the environment that the individuals in this study were collected in, K.E. Marshall, unpublished observations). Fast cooling rates (*ca.* 0.35 º/min) reduce survival in freeze tolerant insects such as the beetle *Upis ceramboides* (Miller, 1978). Similarly, in *E. solidaginis*, cooling at rates of 0.1°C/min to -40 ºC results in mortality indistinguishable from control, while faster rates (10, 5, or 1 ºC/min) result in higher mortality (Bale *et al.*, 1989). By contrast, ecologically-relevant cooling and heating rates reduce the available thermal window for tsetse fly (*Glossina pallidipes*, Terblanche *et al.*, 2007), and it appears that the effect of cooling and/or heating rates can be dependent on other aspects of experimental design such as starting temperature (Rezende and Santos, 2012; Terblanche *et al.*, 2011). The impact of cooling rate likely depends on physiological aspects such as the individual organism's ability to alter cold hardiness during cooling (Kelty and Lee, 1999), or rate and location of ice formation. Further investigation of this variable at ecologically-relevant rates, in addition to the other four axes of stress (intensity and duration are likely the simplest candidates), is necessary to discern these potential interactions.

6.4.6   Extending the four axes

I present four axes of stress (intensity, duration, frequency, and period) in the context of low temperature exposure in a freeze tolerant insect. I propose that this paradigm could be extended to other abiotic stresses. Many other abiotic variables can also vary in frequency, intensity, duration, and period of return time of stressful events, including water availability, pH, UV radiation, wind, and wave energy (Chown and Terblanche, 2007; Gaines and Denny, 1993). I recommend that modelling the effects of these factors on populations should, when possible, include all four axes. This involves two distinct steps. The first includes lab studies linking fitness to stress exposure that determine the

impacts of frequency of and period between induced stress and fitness. This step is not always possible or practical, and correlative approaches that estimate the impacts of these axes on species distributions could be used instead. In the case of low temperature, using predictors like number and period between low temperature exposure in addition to maximum, mean, and minimum temperature in species distribution models. The second step would include incorporating environmental data with sufficient time resolution to detect potentially stressful events into these models (Kearney *et al.*, 2012).

This study of manipulating the frequency and period of an easily-manipulated variable (temperature), in a species whose fitness proxies and physiology are relatively easily measured demonstrates three important points: 1) laboratory measures of the capacity of phenotypic plasticity following a single stress exposure or acclimation are large underestimates (Churchill and Storey, 1989; Lee *et al.*, 2006; Teets *et al.*, 2011), 2) stress accumulates along axes other than only intensity and duration, and 3) this stress can mediate life history trade-offs between somatic maintenance and reproduction (Chapter 2). The results from this study indicate that, in the case of *E. solidaginis*, models that only incorporate the results from intensity and duration would overestimate fitness since stress is accumulating along the axes of frequency and period.

6.4.7   Conclusions

Species range boundaries are set by abiotic stresses acting on individuals. However, understanding the mechanisms that transduce this abiotic stress into fitness reductions remains elusive. Abiotic variables fluctuate to stressful levels within the timescale of individual lifetimes and individuals can respond to these fluctuations through plasticity on very short timescales. Therefore I suggest that frequency and period between stressful events, in addition to duration and intensity of the event, can also determine fitness. Here I present experimental evidence that frequency of low temperature stress is a more important determinant of fitness than intensity, duration, or period between exposures in the goldenrod gall fly *Eurosta solidaginis*. Extending the framework of the four axes of stress to other organisms and important fluctuating abiotic factors will provide better insights into the relationship between abiotic stress and fitness in individuals, allowing mechanistic scale-up to populations.

6.5   Acknowledgements

6.6   References

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Chapter 7

7   Discussion

Insects live in thermally-variable environments, and understanding the effects that temperature has on individuals in the field requires laboratory experiments that encompass ecologically-relevant temperature exposures. The studies I present in this thesis provide an overview of the effects of repeated cold exposure in insects with a broad range of life histories and responses to cold exposure. Despite these differences, repeated cold exposure was always more harmful to individuals than exposures matched for time and intensity. In the fruit fly *Drosophila melanogaster* (Diptera: Drosophilidae), repeated cold exposure caused less mortality than prolonged exposure, but also reduced the number of female offspring and therefore resulted in a decreased intrinsic rate of population increase (Chapter 3). In the woolly bear caterpillar *Pyrrharctia isabella* (Lepidoptera: Arctiidae), repeated freeze-thaw events increased damage to Malpighian tubules and hemocytes, which resulted in higher mortality than controls or individuals that received a prolonged freeze (Chapter 4). Similarly, in the freeze avoiding moth *Choristoneura fumiferana* (Lepidoptera: Tortricidae), repeated cold exposure increased glycerol concentration at a cost of glycogen reserves, and induced delayed mortality relative to individuals that received a prolonged exposure (Chapter 5).   This effect was independent of period between exposures, intensity, or number of exposures. Finally, in the freeze tolerant fly *Eurosta solidaginis* (Diptera: Tephritidae), repeated freeze-thaw cycles did not increase mortality, but they did decrease egg production relative to both control and individuals that received a prolonged freeze (Chapter 6). Like the spruce budworm, this effect was independent of the number of exposures, intensity of exposure, or period between exposures. Taken together, it is clear that the frequency of cold exposure is an important determinant of fitness in insects.

7.1   The impacts of repeated cold exposure on fitness measures

A central question in evolutionary physiology asks how phenotype, physiological performance, and fitness interact to determine population growth in the environment

(Feder *et al.*, 2000). The physiological mechanisms by which a stress encountered by an individual is translated into fitness are poorly understood, and there are few studies that simultaneously measure physiological traits and subsequent fitness (Kingsolver and Huey, 2003). The studies on repeated cold exposure contained in this thesis provide data on some of these links. To begin, it is clear that repeated cold exposure is a stress to insects: the consistent result found in all of the studies contained in this thesis is that repeated cold exposure results in lower fitness, even when intensity and duration of exposure are controlled.

In the two Lepidoptera I studied, *P. isabella* (Chapter 4) and *C. fumiferana* (Chapter 5), repeated cold or freezing reduced survival. This is in contrast to many studies on fluctuating thermal regimes (FTR), which show that damage accrued at low temperature can be repaired if individuals are given an opportunity to repair (Colinet *et al.*, 2006; Koštál *et al.*, 2007a; Renault *et al.*, 2004). The difference between my studies and FTR studies may be due to study design. In the *C. fumiferana* study, I followed individuals from the cold exposure through to eclosion as adults (a period of two months). I found that survival was not impacted immediately following cold exposure, but was affected later in development (Chapter 5). By contrast, many FTR studies only track individuals for a period of a few days (Renault, 2011). In addition, I matched the amount of time spent at low temperatures in my repeated cold exposure treatment, while FTR studies follow a "RCE *vs*. Cold" design (see discussion in Chapter 2). In the case of the freeze tolerant *P. isabella*, repeated freeze-thaw exposure caused increased tissue damage, which was reflected in whole-animal mortality. This result indicates that the freeze-thaw event itself is damaging, and this is similar to previous studies on the effects of repeated freezing in freeze tolerant insects (Brown *et al.*, 2004; Sinclair and Chown, 2005; Teets *et al.*, 2011). This decrease in survival was likely mediated through different mechanisms. In the case of *C. fumiferana*, it was either indirect chilling injury or by energetic depletion following repeated cold exposure. In the case of *P. isabella*, it was likely tissue damage that accrued over many freeze-thaw cycles. However, in both cases, this increased mortality following repeated low temperature exposure which indicates that repeated exposure increases the stress of low temperatures independently of intensity and duration of cold.

Repeated cold exposure did not reduce survival in all of the species investigated in my thesis. In the two dipteran species I studied, *D. melanogaster* (Chapter 3) and *E. solidaginis* (Chapter 6), repeated cold exposure resulted in no additional mortality relative to prolonged cold exposure (and even higher survival in the case of *D. melanogaster*). These species invested in additional cryoprotection at the cost of future fecundity. In the case of *D. melanogaster*, individuals synthesized glycerol at the expense of carbohydrate stores. While this result is only correlative, this investment is also accompanied by a decrease in the number of female offspring produced. Therefore I concluded there was a cost to repeated cold exposure. In *E. solidaginis*, there was increased investment into glycerol, sorbitol, and acetylated triacylglycerols (acTAGs) following repeated freezing. Similarly, this effect was accompanied by decreased egg production when compared with individuals that received the same duration and intensity of freezing. In both of these species, repeated low temperature exposure did not increase mortality; it rather induced a trade-off between somatic investment and fecundity.

I found that repeated cold exposure imposed significant stresses on all the insect species I studied. In addition, in both *E. solidaginis* and *C. fumiferana,* I was able to also investigate the effects of differing period, number, and intensity of low temperature exposure. In both of these studies I found that frequency of stress was more important than any of these factors for determining fitness effects. By contrast, the physiological effects of repeated cold exposure (as measured by cryoprotectant concentration and energy stores), were impacted by all of these factors and their interactions. My results represent the first experimental evidence that shows that the way that organisms experience abiotic stress is more reliant on frequency than intensity or duration. While all my studies were on the effects of low temperature exposure, it is possible that other abiotic stresses like high temperature, desiccation, or pH could induce similar effects since discontinuous physiological thresholds can occur in response to these stressors as well.

The low impact of differing period on survival and fecundity in *E. solidaginis* and *C. fumiferana* was unexpected. For example, for freeze tolerant syrphid *Syrphus ribesii* (Diptera: Syrphidae), increased period between freezing events reduced survival (Brown

et al., 2004). By contrast, in the freeze tolerant beetle *Hydromedion sparsutum* (Coleoptera: Promecheilidae), increased period between freezes increased survival (Bale *et al.*, 2001). These differences may be due to the ability of the individual to repair incurred damage between subsequent exposures, or they may be due to experimental design. In these previous studies, the total time over which the longest exposure period was performed was approximately a month, while in the studies presented in Chapters 5 and 6, the total experimental period was 100 days. Over this time, I found that cold hardiness correlates were plastic and so period of time between exposures is inevitably confounded with the time of year (and thus diapause intensity). Despite this, I found no significant differences due to period among measures of survival or fecundity in either species, suggesting either that 1) repair occurs on a timescale of less than a day, or that 2) repair does not occur.

7.2 Potential mechanisms of negative impacts of repeated cold exposure

Two fundamental mechanisms could account for the negative effects of repeated cold exposure on fitness that I observed (Figure 1.1). The first is direct damage: individuals accrued some cold or freeze- induced damage that could not be fully repaired, and this damage is more dependent on the transitions between relatively warm and cold temperatures rather than the total time spent at the low temperature. This damage can be at many different levels of biological organization, and is discussed in more detail below. The second mechanism of negative fitness impacts is more subtle, and relies on trade-offs in allocation between reproduction and survival induced by the stress of repeated cold exposure (Zera and Harshman, 2001). These trade-offs could be mediated by two separate processes: 1) the repair of damage induced by low temperature exposure and 2) the costs of preparing for subsequent cold exposures. Energetic resources that could have been used to fuel egg production could be shunted away for increased investment in cold hardiness or repair of cold and freeze-related damage, and this might be more likely in the nutrient-limiting overwintering life stage.

Cold- and freeze-induced damage to insects has been well investigated (Danks, 2005; Storey and Storey, 1988). This damage usually accumulates exponentially over time,

with increasing intensity, or with the interaction between the two (Nedvěd *et al.*, 1998). While the type of damage incurred cannot be directly inferred from my studies on *P. isabella* and *C. fumiferana*, there are several potential mechanisms for this damage that occur on multiple levels of biological organization (reviewed in Chapter 1). Direct effects of temperature occur within minutes, and would require repair immediately following temperature exposure. As a result, it is possible that these direct temperature effects could be responsible for the additional damage caused by repeated cold exposure.

Many insects increase the unsaturation of the fatty moieties in their phosopholipid membranes and triacylglycerols to increase fluidity at low temperatures (Michaud and Denlinger, 2006; Pruitt and Lu, 2008; van Dooremalen *et al.*, 2011). The double-bonds within these unsaturated moieties are particularly prone to oxidation, which can occur when reactive oxygen species are produced. For freeze tolerant species, repeated freeze-thaw with the reperfusion that accompanies each event could be an important cause of mortality (Joanisse and Storey, 1996). However, in freeze sensitive species, it is less clear that repeated cold exposure in particular would lead to additional cold injury since oxidative damage accrues slowly. In fact, it appears that recovery at warmer temperatures allows for the repair of oxidative damage incurred at low temperature (Lalouette *et al.*, 2011). In my *P. isabella* study (Chapter 4), the observed tissue damage could have been caused by reperfusion. While in the *C. fumiferana* study it was impossible to measure tissue damage due to the small size of the organisms (~100 μg), it is possible that oxidative damage to cell membranes may have caused the observed increased mortality following repeated cold exposure.

At the tissue level, it is clear that cold exposure damages insects. Freezing damages nervous tissue in *E. solidaginis* (Collins *et al.*, 1997), and similarly cold shock alters behaviour (and likely damages nervous system tissue) in the flesh fly *Sarcophaga crassipalpis* (Diptera: Sarcophagidae), (Yocum *et al.*, 1994). This type of damage may not be repairable (MacMillan and Sinclair, 2011b), and thus repeated exposures would be expected to cause increased damage with increased cold exposures. Tissue damage from low temperature exposure (whether due to induced apoptosis or necrosis) has been reported from several species, including *E. solidaginis* (Yi and Lee, 2003) and the

Antarctic midge *Belgica antarctica* (Diptera: Chironomidae), (Teets *et al.*, 2011). I found that repeated freezing induced tissue damage in *P. isabella*, but not in *E. solidaginis*, which suggests that accumulation of mechanical damage during repeated freeze-thaw may be important for species that are less low temperature tolerant.

7.2.1   The costs of repair

Whether the damage listed above accumulates following repeated cold exposures is dependent on the organism's ability to repair that damage. This repair can be costly, and, in a nutrient-limiting condition such as overwintering, can therefore induce trade-offs. For example, re-establishing ion homeostasis in the cricket *Gryllus pennsylvanicus* (Orthoptera: Gryllidae) causes an increase in CO_2 production indicating increased metabolic cost (MacMillan *et al.*, 2012). As cold exposure duration increases, the cost also increases, but in a saturating relationship with time (MacMillan *et al.,* 2012). Therefore it is likely that repair required by repeated low temperature exposures would induce additional metabolic costs that a single exposure would not.

Insects also have many biochemical mechanisms to repair damaged proteins following cold exposure. While I did not examine protein damage following repeated cold exposure, in a similar experiment in *D. melanogaster*, several genes related to actin and myosin were upregulated following repeated cold exposure, suggesting protein repair processes (Zhang *et al*., 2011). The protein ubiquitin binds to damaged proteins, and directs them towards the proteasome for degradation. In cold-exposed *D. melanogaster*, *Ubiquitin* is upregulated following cold exposure (Qin *et al.*, 2005). Similarly, heat shock proteins (Hsps) can aid restoration of protein conformation following denaturation (Hochachka and Somero, 2002). Heat shock proteins are frequently upregulated following cold exposure (Koštál and Tollarová-Borovanská, 2009; Qin *et al.*, 2005; Rinehart *et al.*, 2007). Similarly, *hsp70* is only upregulated in *B. antarctica* following repeated freezing, which suggests that it represents a repair response for additional freezing exposures (Teets *et al.*, 2011). In addition, cytoskeletal proteins may be damaged, and several genes related to cytoskeleton are significantly upregulated in *D. melanogaster* following repeated cold exposure (Zhang *et al.*, 2011). The costs of

synthesizing additional protein following each cold exposure are likely energetically expensive, and could induce the observed trade-off between survival of low temperature stress and reproductive output.

In the studies presented in this thesis I cannot distinguish with certainty between whether insects accumulated less damage with repeated cold stress, or whether they were actively repairing cold-induced damage. In *D. melanogaster*, the observed reduction in glycogen and triacylglycerol contents suggests that there may be energetic costs of repair (Chapter 3), and this suggestion is supported by gene expression data (Zhang *et al*., 2011). However the three other species I studied did not have reduced energetic stores following repeated cold exposures, suggesting that repair did not occur. The difference may be due to increased recovery temperature (*D. melanogaster* was allowed to recover at 22 ℃, while the other species recovered at 0 ℃), that *D. melanogaster* was not in winter dormancy during the experimental period, or *D. melanogaster* had access to food through the recovery period. These three explanations may not be mutually exclusive, but further investigation into repair processes following repeated low temperature exposure is a potentially fruitful avenue to dissecting costs of recovery from accumulation of damage.

7.2.2 The costs of preparation

Insects have a suite of physiological mechanisms for avoiding cold and freeze injury (see Chapter 1). While the seasonal acquisition of these mechanisms is well-studied (Bale, 2002; Danks, 2005; Danks, 2006a), it is also clear that insects can rapidly adjust their cold hardiness (Czajka and Lee, 1990; Rajamohan and Sinclair, 2009; Ransberry *et al.*, 2011; Teets *et al.*, 2011). These mechanisms can include synthesis of compounds such as cryoprotectants, Hsps, and antifreeze proteins. This increased cold hardiness may come at a cost—for example, in *E. solidaginis*, repeated freeze-thaw reduces available ATP supply which may be a result of additional cryoprotectant synthesis (Churchill and Storey, 1989). Again, in a nutrient-limiting environment, these short-term adjustments (if they occur following each cold exposure) can come at an energetic cost to insects and induce trade-offs after repeated exposures.

One common response to low temperature exposure is the production of additional cryoprotectant. Many species accumulate additional glycerol in response to a single short-term low temperature stress like that experienced in rapid cold hardening (RCH, Lee *et al.*, 1987). Glycerol is generally synthesized from glycogen stores, which is an ATP-demanding process (Storey and Storey, 1983), so investment in additional glycerol content could induce an energetic cost during repeated cold exposure. I found additional glycerol content in all four of my study species following repeated exposures, which may be driving the trade-offs between survival and reproductive output observed in *E. solidaginis* and *D. melanogaster*. While *E. solidaginis* also produces additional sorbitol following repeated cold exposures (Chapter 6, Churchill and Storey, 1989), production of this cryoprotectant is much more efficient in terms of carbon retained than the synthesis of glycerol (Storey and Storey, 1983). It is even possible that high cryoprotectant concentrations may incur costs due to direct toxicity (Fahy, 1986).

In addition to cryoprotection, there are several other preparatory mechanisms that could be costly. Similarly, there is evidence that cold exposure can induce an immune response in *D. melanogaster* (Le Bourg, 2011; Zhang *et al.*, 2011). In *P. isabella* I found repeated freezing events increased resistance to a fungal pathogen (Chapter 4), which indicates that immune function is upregulated to prepare for an additional stress. While it is unclear why insects might invest in immune function following cold exposure, it is possible that this represents preparation for subsequent exposures that may cause damage to the gut (MacMillan and Sinclair, 2011b), which could cause leakage of gut contents (including pathogens) into the hemocoel. Another potential candidate for energetic costs following cold shock is the expression of antifreeze proteins. While these do not appear to be induced in many species (Duman and Horwath, 1983), in a microarray experiment I found several antifreeze proteins were significantly upregulated following cold exposure in *C. fumiferana* (K. E. Marshall, unpublished observations). Finally, exposure to fluctuating thermal conditions can induce changes in membrane fluidity in the springtail *Orchesella cincta* (Collembola: Entomobryidae), (Van Dooremalen *et al.*, 2011). These changes again come with an energetic cost as enzymes to catalyze these reactions are synthesized, and particularly if the reactions themselves are ATP-dependent. In the

nutrient-limiting state of overwintering, every additional enzyme synthesized represents an energetic cost that may not be replaceable.

7.3   The relationship between number of exposures and costs

The timescale of insect responses to low temperature are usually split into two categories: long term seasonal acquisition of cold hardiness through acclimation, and short-term responses to cold shock (Bale, 2002; Rajamohan and Sinclair, 2009; Shintani and Ishikawa, 2007). In three of the four studies in this thesis (all but Chapter 3), the insects I studied were already seasonally acclimated or acclimatized. The short-term exposures I subjected the study species to were on the scale of RCH, yet I believe the responses I documented are distinct from RCH. I found that responses were either 1) entrained only after two or more low temperature exposures, or 2) increased with increasing numbers of exposures.

In my four studies, some responses were entrained after two or more temperature cycles. Mortality in *C. fumiferana* was unaffected by the number of cycles, but individuals that received three or more 12 h exposures had higher mortality than those that received one 120 h exposure (Chapter 5). That there was increased mortality (equally present at all temperatures) with this type of treatment seems inconsistent with the hypothesis of RCH response. In *E. solidaginis*, egg production was only impacted by frequency of exposure, but not intensity or duration of cold exposure. In addition, in *D. melanogaster*, glycerol production increased at the expense of glycogen in any flies that received two or more exposures (Chapter 3). These responses all indicated a saturating response with number of exposures, and demonstrated that frequency of low temperature stress was more important than duration.

In all four species, some physiological and fitness measures increased with increasing numbers of exposures. In *P. isabella*, increasing numbers of freeze-thaw cycles caused increasing accumulation of mortality (Chapter 4). Similarly, in *D. melanogaster*, increasing number of low temperature exposures reduced the intrinsic rate of population increase (Chapter 3). In *C. fumiferana* and *E. solidaginis*, increasing numbers of

exposures increased the change in physiological measures. In *C. fumiferana*, glycerol concentration significantly increased with increasing number of low temperature exposures at the expense of glycogen content (Chapter 5). In *E. solidaginis*, increasing number of freeze-thaws significantly increases sorbitol content only in pre-pupae exposed to a daily cycle (Chapter 6). The same occurs in *B. antarctica* — increased number of freeze-thaw cycles decreases survival and lipid and carbohydrate reserves (Teets *et al.*, 2011). These measures show that the accumulation of responses to repeated low temperature stress, and suggest that RCH (a single exposure) is not enough to induce the full response of these individuals.

Perhaps the strongest evidence for the independence of repeated cold exposures from RCH is from the *D. melanogaster* study (Chapter 3). There I directly compared the effects of repeated 2 h exposures to -0.5 ºC to a single 2 h cold exposure (which is equivalent to a RCH treatment in *D. melanogaster*, Czajka and Lee, 1990). I found that energetic reserves, cryoprotection, and fecundity were distinct between the repeated exposure treatment and the RCH treatment. In addition, following these treatments, gene expression patterns between the two treatments were highly divergent (Zhang *et al.*, 2011). Out of 85 genes differentially expressed following repeated low temperature exposures, only five were shared with the RCH treatment. Taken together, it is clear that even though repeated cold exposures increase cold hardiness, they do not constitute a "hardening" or "acclimation" response. Instead repeated cold exposure increases stress.

7.4 Life histories and responses to repeated cold exposures

A model of overwintering energetics suggests that, in addition to the costs of freeze-thaw cycles, the freeze tolerance strategy is favoured in life histories in which individuals begin winter with relatively high energetic reserves (Voituron *et al.*, 2002). This suggests that feeding strategy and the overwintering life stage are important predictors of response to low temperature exposure. The species I chose for my four studies all represent insects with differing life history traits and responses to cold exposure. Due to the confounding effect of phylogeny I cannot draw broad conclusions regarding the response to repeated cold exposures as a result of cold hardiness. However this diversity allows me to at least

explore potential effects of repeated cold exposure in relation to response to cold. I found that while all four insect species increased cold hardiness measures in response to repeated cold, that the responses of these species could be grouped into two categories. The first is those species in which repeated cold stress increased mortality (*P. isabella* and *C. fumiferana*), and the second is those that increased their investment in somatic maintenance at the cost of reproductive output (*E. solidaginis* and *D. melanogaster*).

I found that the relationship between life history traits, physiology, and response to repeated cold exposure is complex since there was no correspondence between cold hardiness strategy and response to repeated cold exposure. Nor was there a relationship between the ability to feed to recover energetic stores following overwintering and response to repeated cold. In my study, the freeze tolerance strategy was represented by *P. isabella* and *E. solidaginis*. Both overwinter in a relatively later life stage (final instar larva and pre-pupa, respectively, Irwin and Lee, 2003; Marshall and Sinclair, 2012). *Eurosta solidaginis* adults can feed on small amounts of nectar, but cannot replace resources gathered as larvae (Uhler, 1951), and while *P. isabella* caterpillars might be able to feed following overwintering, in my study they were not provisioned (and in a separate experiment, even if they were provisioned with pinto bean diet, they did not feed). By contrast *C. fumiferana* is freeze avoiding, overwinters on maternally-derived energetic reserves as a 2nd instar larva, and feeds exclusively following overwintering (Han and Bauce, 1993). Finally, while the ecology of *D. melanogaster* is not well-described, it is believed this species overwinters as a chill-susceptible adult with the opportunity to feed before subsequent reproduction (Hoffmann *et al.*, 2003; Izquierdo, 1991). The absolute cold hardiness of these species also did not predict response: *E. solidaginis* and *C. fumiferana* are both extremely cold hardy, both surviving exposure below -35 ºC (Bale *et al.*, 1989; Han and Bauce, 1995). By contrast, *D. melanogaster* can only survive brief exposures to -5 ºC (Czajka and Lee, 1990) and *P. isabella* does not survive below -20 ºC (Boardman *et al.*, 2011). In addition, overwintering microclimate is also not predictive: *C. fumiferana* overwinters in an exposed microclimate that closely tracks air temperature, while *P. isabella* overwinters in a buffered microclimate, yet both have reduced survival due to repeated low temperature exposure. Consequently, the

resources, life stage, overwintering microclimate, and basal response to cold are not predictive of response to repeated low temperature exposure.

In the case of *E. solidaginis*, it seems likely that the freeze treatments applied were insufficient to induce tissue damage. However, why the chill-susceptible *D. melanogaster* was able to repair chilling injury while the more cold-hardy *C. fumiferana* was unable is not immediately apparent. One potential explanation is that *C. fumiferana* was only able to feed at the end of the experiment (one month following the final low temperature exposure), while *D. melanogaster* was able to feed between each exposure. In addition, *D. melanogaster* recovered at substantially higher temperatures than *C. fumiferana* (22 ℃ *vs.* 0/2 ℃), which may have allowed for temperature-sensitive recovery processes between subsequent exposures—perhaps the induction of synthesis of actin and myosin (as observed by Zhang *et al.,* 2011).

In my studies, the fitness effects of repeated cold exposure cannot be generalized across cold hardiness response or life history. The only similarity among responses was within taxonomic groups—repeated low temperature exposure reduced survival in both lepidopterans, and both dipterans were able to trade-off future fecundity for investment in cryoprotection. Whether this taxonomic effect is broadly true or merely an artifact of the species chosen cannot be addressed with such a small number of species. This is generally a problem in the field of insect cold hardiness: it is unclear what effect particular life stages have on cold hardiness, independent of phylogeny. This problem could be addressed using a well-characterized radiation of species that overwinter in many different life stages, such as the mosquitoes of the Northeastern USA (Crans, 2004).

7.5 Interpreting responses to repeated cold exposure in the context of natural temperature variation

The temperature that an organism experiences in its environment is due to the additive effects of multiple, nesting scales of forcing (Lowry, 1997). The broadest scale is the climatological, which encompasses the impact of latitude on climate (Lowry, 1977). Nested within this are landscape effects on temperature, which may include topographical

features such as mountains and shoreline (Lowry, 1977). Finally, small-scale influences due to microclimate features such as vegetation and snow cover filter the influences of climate and landscape (Leather *et al.* 1995). Cold exposure depends on the influence of each of these scales, and can vary along many axes (intensity, duration, frequency, and period) simultaneously in natural environments, and in Chapters 5 and 6 I investigated the interactive impacts of differing duration, period, and intensity along with frequency of exposures. The results from these studies showed that frequency of low temperature exposure caused the greatest impacts on fitness, even though duration, period, intensity, and their interactions could affect immediate physiological response. However, natural temperature regimes vary in other ways as well: mid-winter warm spells, cooling rate, as well as seasonal variation. Overlain on this variability is also the influence of microclimate and climate change.

7.5.1 Microclimates

The influence of air temperature fluctuation on individual insects is filtered by microhabitat. Insects overwinter in a multitude of locations, each of which is differentially related to the air temperature, depending on the extent of buffering or additional heat gain available (Leather *et al.*, 1995). Some of these influences on microhabitat include urban heat island effects (Angilletta *et al.*, 2007), snow cover (Lawrence and Slater, 2009), thermal inertia, and aspect (Vermunt *et al.*, 2012b). Overwintering microhabitat can be an important determinant of spring fitness (Irwin and Lee, 2003; Marshall and Sinclair, 2012; Sinclair *et al.*, 2013). Understanding the influence of repeated cold exposure must be in the context of the microclimate experienced by the animal.

Snow cover has perhaps the best-explored influence on microhabitat. Many insect species (including *P. isabella*, occasionally *E. solidaginis*, and likely *D. melanogaster*) overwinter underneath the litter layer (Irwin and Lee, 2003; Izquierdo, 1991; Layne *et al.*, 1999; Leather *et al.*, 1995). Snow cover buffers microclimates from extreme cold, producing a net warming through the winter (Henry, 2008; Marshall and Sinclair, 2012). This warming can increase metabolic rate, thus increasing energetic drain through the winter either through direct effects of temperature on metabolism (Irwin and Lee, 2003),

or by reducing the amount of time spent frozen (Marshall and Sinclair, 2012). However, overwintering below the snow could also reduce injury from repeated freeze-thaw events (Chapter 4). Thus there may be a trade-off between the protection that snow cover affords and the resulting increased energy demand. Species like *P. isabella,* that overwinter under snow*,* may be generally poorly adapted to survive repeated freeze-thaw because it is not a common stress for them. These effects are further modulated by season: during fall and spring this buffering may be unavailable due to little or no snow cover.

In addition to snow cover, urban heat island effects, thermal inertia, and aspect of overwintering locale can combine to warm individual insects well above the air temperatures. For example, the emerald ash borer overwinters as a pre-pupa under the bark of ash trees that can occur in both rural and urban conditions (Crosthwaite *et al.*, 2011). This microclimate is significantly warmer than the surrounding air temperature due to the thermal inertia of the tree, and the differential is greater at the north-facing aspect, and in trees that occur in urban areas (Vermunt *et al.*, 2012a; Vermunt *et al.*, 2012b). These effects combine to reduce the intensity of low temperature exposure, while the frequency remains relatively unchanged. However, if repeated low temperature exposure is stressful in this species as it is for the species in this thesis, this thermal buffering may not be sufficient to protect individuals from thermal stress. Understanding the influence of microclimate on thermal regime is an essential part of predicting the impacts of repeated low temperature events on insects.

7.5.2 Timescales and types of temperature variation

In natural environments, mid-winter warming could affect overwintering individuals. A warm temperature exposure mid-winter could initiate deacclimation and reduce cold hardiness of insects. For example, in the freeze avoidant emerald ash borer *Agrilus planipennis* (Coleoptera: Buprestidae), as little as two days at 10 ºC can significantly increase the supercooling point and reduce hemolymph osmolality, glycerol content, and thermal hysteresis (Sobek-Swant *et al.*, 2011). This reduction in cold hardiness measures is irreversible; despite a week of reacclimation to -10 ºC, *A. planipennis* pre-pupae did not recover their acclimation, so a second cold exposure could have significant

deleterious consequences in this insect. By contrast, fluctuating thermal regimes (FTR) exposures offer the opportunity for chill susceptible insects to repair chilling injury and prepare for subsequent exposures at the higher temperature exposure. The recovery temperatures in FTR experiments are usually quite warm compared with the recoveries in my experiments; 20 ℃ is a common recovery temperature. Some observed effects included reduced oxidative stress (Lalouette *et al.*, 2011), increased chaperone synthesis (Colinet *et al.*, 2007), maintenance of reproductive output (Renault, 2011), and increased cryoprotectant synthesis (Koštál *et al.*, 2012). However, these studies do not control for the total amount of time spent at the low temperature, and it is therefore difficult to ascertain whether some of these effects are at least partially due to lesser accumulation of chilling injury (Chapter 2). Only my *D. melanogaster* study (Chapter 3) allowed recovery at a warm temperature (22 ℃), similar to FTR exposures. While my temperature regimes were modeled after naturally-occurring mid-winter variation in southern Ontario conditions, including warm spells could allow for investigation of recovery processes following repeated cold exposures.

Cooling rate can also vary in natural systems. In laboratory studies, increased cooling rates can significantly increase mortality in overwintering insects. For example, in *E. solidaginis,* cooling rates of 1 ℃/min or greater to -40 ℃ increases mortality with increasing rate (with only 7 % survival after cooling at 10 ℃/min, Bale *et al.*, 1989). By contrast, cooling at 0.1 ℃/min to -40 ℃ causes no additional mortality compared to controls. Similarly, cooling the grain aphid *Sitobion avenae* (Hemiptera: Aphididae) at slower rates (0.1 or 0.05 ℃/min) confers resistance to a subsequent cold shock more than cooling aphids at 1 ℃/min. In addition, cooling more slowly (0.1 or 0.05 ℃/min *vs*. 1 or 0.5 ℃/min) lowers the temperature at which *D. melanogaster* loses muscle function by 2.5 ℃ (Kelty and Lee, 1999). In nature, cooling rates are frequently much slower than rates used in the laboratory. In one study of cooling rates in the Alpine zone in New Zealand, rates varied between 0.062 and 0.001 ℃/min (Sinclair, 2001a), and I found in southern Ontario cooling rates similarly are very slow (Chapter 6). While I kept all rates in Chapters 5 and 6 constant at 0.1 ℃/min, investigations into the interaction between cooling rate and frequency of low temperature exposure, in the context of natural cooling rates, could determine whether rate was an important parameter.

In temperate systems, the predominant long-term cycle is the progression of seasons. In the studies in this thesis, I applied all treatments to individuals following a common summer and fall, and following overwintering exposed individuals to identical spring conditions. However this assumption does not necessarily hold in natural systems. During late summer and autumn, many insects are already diapausing and cannot feed, therefore the warm temperatures during this period are important determinants of overwintering energetic stores (Hahn and Denlinger, 2011; Han and Bauce, 1998; Williams *et al.*, 2012). In addition, spring conditions can also cause substantial mortality if deacclimated individuals are exposed to sudden low temperature snaps (Gray, 2008; Han and Bauce, 1995). In species that feed following winter, like *C. fumiferana*, spring conditions may be especially important as they feed to replenish depleted energy reserves. *Eurosta solidaginis*, as a species that feeds only on a single host plant, may be especially vulnerable to the effects of warm fall seasons depleting energetic reserves. Seasonal changes in cold hardiness affect the ability of individuals to respond to low temperature exposures, which I observed in my *C. fumiferana* study (Chapter 5). In particular, I found that the inducible changes in cold hardiness are strongly affected by time of year in this species, which has important implications for interpreting the impacts of repeated low temperature exposure.

7.5.3   Effects of climate change

Overlain on the dynamic cycles of temperature variability and the influence of microclimate is anthropogenic climate change (IPCC, 2007). Climate change encompasses more than the simple warming of the Earth's surface: it also includes changing frequency of extreme temperature events (Easterling *et al.*, 2000) and reorganization of precipitation regimes (IPCC, 2007; Lawrence and Slater, 2009). Many of these changes are expected to be particularly concentrated in the winter season, and changing thermal and precipitation patterns will influence the frequency, duration, number, and period of low temperature exposures (IPCC, 2007). There is little information on change in frequency of low temperature exposure outside of changes to the number of frost days (Henry, 2008; Kodra *et al.*, 2011). There are suggestions that the number of extreme low temperature events has significantly decreased over the past

30 years (Caprio *et al.*, 2008; Easterling *et al.*, 2000), these effects can be reversed on the regional scale, with increased numbers of low temperature events in some regions (Shabbar and Bonsal, 2003). In addition, extreme cold events are predicted to persist well into the future (Kodra *et al.*, 2011). While extreme low temperature events cause significant mortality in insects, the studies in this thesis predict that number of exposures is also an important variable.

An additional consideration is that warming winter temperatures may increase the number of freeze-thaw cycles a freeze tolerant organism experiences, depending on the local climate regime and the supercooling and melting point of the organism. Supercooling and melting points of freeze tolerant (and indeed all) insects tend to be separated by at least several degrees C. For example, *P. isabella* freezes between -11 and -7 °C (Chapter 4), but does not melt until -3.4 °C (based on glycerol concentration, Marshall and Sinclair, 2012). Air temperatures in temperate environments frequently average between -11 and -4 °C between these points, meaning that there are frequent freeze-thaw cycles for these organisms (see example data in Han and Bauce, 1998; Irwin and Lee, 2003; Marshall and Sinclair, 2012; Sinclair *et al.*, 2013; Vermunt *et al.*, 2012b). Warming may increase the probability that the melting temperature is reached, shortening the length of freeze-thaw cycles, but increasing their frequency. Knowing that increased freeze-thaw cycles reduce fitness independently of duration means that this increased frequency could have serious effects for these species.

Climate change is also predicted to influence the duration and extent of snow cover, which will alter subnivean microclimates. Modelling of Northern Hemisphere snow cover suggests that while snow precipitation will increase, warming temperatures will decrease the duration of snow cover (Lawrence and Slater, 2009). Changing snow cover regimes have increased the number of soil freeze-thaw cycles across Canada (Henry, 2008), and decreased the extent of snow covered area in high latitude regions (Serreze *et al.*, 2000). In addition, spring snow cover duration across the Canadian Arctic has decreased significantly over the past decades (Derksen *et al.*, 2012). These changes will alter the frequency of repeated cold exposure events in those species that overwinter beneath the snow. Increased frequency may increase the amount of damage accrued by

individuals and depress survival (Chapter 4), but loss of snow cover may lower overwintering metabolism thus increasing spring fitness (Marshall and Sinclair, 2012).

7.6 Implications for future studies in thermal biology

Thermal biology is concerned with the patterns and processes of organismal response to temperature (Angilletta *et al.*, 2006). An underlying assumption in this field is that negative fitness impacts accumulate with increasing intensity and duration of low temperature exposure (MacMillan and Sinclair, 2011a; Nedvĕd *et al.*, 1998). Through the four studies presented in this thesis, I demonstrate that intensity and duration of low temperature stress are not sufficient for predicting survival or fecundity of insects. In addition, every species I studied is capable of increasing measures of cold hardiness in response to repeated low temperature stress. These results suggest that changes to the design of both laboratory and modelling experiments are necessary if studies are to better reflect the impacts of natural temperature conditions on insects.

7.6.1 Laboratory experiments

Studies of response to a single cold shock or freeze-thaw (Chen and Walker, 1994; Joplin *et al.*, 1990; MacMillan *et al.*, 2012; Storey and Storey, 1985; Teets *et al.*, 2012b) have elucidated the broad range of these mechanisms in response to short-term cold exposure. Similarly, studies of seasonal acclimation (Crosthwaite *et al.*, 2011; Rinehart *et al.*, 2007; Storey and Storey, 1986) have defined the role of these mechanisms in the context of survival of long term adverse conditions. In this thesis I document several responses to repeated low temperature exposure that suggest that there are additional mechanisms that can be induced, and several other studies have additionally found unique responses to repeated low temperature exposure (Teets *et al.,* 2011, Zhang *et al.,* 2011). Given that insects live in thermally-fluctuating environments, these novel responses may be more ecologically-relevant than the processes found during studies of the effects of a single cold shock.

First, I found that glycerol is produced in *D. melanogaster* only following repeated low temperature exposures (Chapter 3). Glycerol is not generally believed to be used by *D. melanogaster* following cold shock (Kelty and Lee, 1999), yet I found that with

increasing number of low temperature exposures there was increasing glycerol production. This increase was not sufficient to cause any appreciable effects on supercooling point, but this could be a method to protect protein function or cellular membranes at low temperatures (Koštál *et al.*, 2007b). In addition, repeated cold exposures in this species produce patterns of gene expression that are completely unique to those produced by single cold exposures, even when intensity and duration are matched (Zhang *et al.*, 2011). In *P. isabella*, repeated freezing resulted in significantly increased immune function (Chapter 4), which was not found in individuals that received a single freezing event. If the goal of a laboratory study is to predict how individual insects may respond to low temperature exposure in natural conditions, the impacts of repeated cold exposure should be considered. Repeated cold exposure elicits unexpected physiological responses that may form an important part of insect's response to cold in natural conditions.

I also found that two species (*E. solidaginis* and *D. melanogaster*) traded-off additional cryoprotection in exchange for lowered reproductive output. I hypothesized this link is energetic, but there could be additional signaling pathways that mediate this trade-off— for example insulin-signalling mediates fitness trade-offs due to starvation (Chippindale *et al.*, 1996). This trade-off suggests that fitness estimates derived in the laboratory following a single low temperature exposure are inadequate for elucidating the complexity of low temperature responses in the field. Laboratory studies should incorporate long-term tracking of individuals when possible to account for the potential for delayed mortality (as in Chapter 5) or these trade-offs.

7.6.2 Modelling species range limits

An important goal of thermal biology is extrapolating thermal tolerance measured in the laboratory to effects on populations through modelling. These models are important in a many applied fields, including forensic entomology (Ames and Turner, 2003), agricultural and forest pest management (Gray, 2008; Knight, 2007), as well as predicting the potential effects of climate change on species ranges (Régnière *et al.*, 2012). There are several types of models that seek to predict species population dynamics, including correlating species range extent to climate, bioclimatic envelope models, and mechanistic

models. These models are an integral part of making thermal biology relevant to real-world conditions, and all could benefit from incorporating the effects of repeated cold exposure that I document here.

The simplest models are those that correlate thermal tolerance of animal species with environmental conditions that individuals experience. The broadest predictor of thermal tolerance is latitude, which does not allow dissection among the effects of temperature means and extremes (Addo-Bediako *et al.*, 2000; Sunday *et al.*, 2011). Other studies test multiple measures of temperature against thermal limits (Bozinovic *et al.*, 2011; Gray, 2008; Somero, 2005). There have already been several calls to move beyond mean temperatures (Gaines and Denny, 1993) and into higher temporal resolution data (Kearney *et al.*, 2012) for predicting the extent of species ranges. In addition, the minimum environmental temperature has been shown in several studies to be significantly correlated with northern range limits in several species (Calosi *et al.*, 2010; Gray, 2008). Considering that both my prolonged and repeated low temperature treatments would be treated identically in these models (both had the same minimum temperature), I believe that number of low temperature exposures should be explored as a possible predictor of species ranges. Extracting this type of information is relatively straight-forward, and code in both Excel and R is available to do so (Marshall and Sinclair, 2012; Sinclair, 2001b). This would be a relatively simple first step to understanding broad-scale impacts of repeated cold exposure on ectotherm populations.

Bioclimatic envelope models (reviewed in Chapter 1) correlate climate variables to species range extent to predict range shifts in future climates. In the context of repeated cold exposure, these models cannot incorporate information about fitness reductions that are not captured in mean or minimum temperature data, nor do they account for the physiological responses to repeated cold exposures. By contrast, mechanistic or ecophysiological models (reviewed in Chapter 1) use physiological data to predict species ranges in relation to climate data (Buckley *et al.*, 2010). However these models also generally assume that physiology is relatively inflexible, and incorporating effects of repeated cold exposure could improve model prediction, particularly if the range studies I

recommend above indicate a significant impact of repeated cold exposure on species distribution.

7.7 Limitations and future directions

In the studies presented in this thesis I have demonstrated that repeated low temperature exposure is stressful to insects and that this stress can be detected through changes in survival or trade-offs with reproduction. I have also shown that insects respond to repeated low temperature stress by synthesizing additional cryoprotection. In addition, I have shown that these results occur in insects from both cold hardiness strategies, as well as chill-susceptible insects. These results suggest stress from low temperature accrues along axes other than intensity and duration.

Two limitations of my studies (cooling rate and the reliance on time of year for the effects of period) have been discussed in more detail above, but I will mention them briefly here. I did not investigate cooling rate in my studies, but it is possible that rate also interacts with intensity, duration, frequency, and period. I studied four different species from two orders, and found that responses to repeated low temperature split along taxonomic lines rather than response to cold hardiness or life history. With this small number of species I did not have power to disentangle the effects of phylogeny from cold hardiness, but additional investigations into whether cold stress can induce life history trade-offs in lepidopterans would be a logical next step.

7.7.1 Time course following exposure

In Chapter 1, I review how individual insects repair damage and prepare for subsequent exposures while recovering from low temperature events. This dynamic response to low temperature suggests that by a second low temperature event, the individual is physiologically altered, and may experience a second event differently. This suggests that the physiological events between low temperature stresses are critical for determining the effects of subsequent stressors. However, due to the complex experimental design, I was forced to limit the sampling time-points of physiological measures following cold exposures, despite the potential for individuals to adjust physiology along a time course after my sampling.

In Chapters 4-6, I sampled *P. isabella*, *C. fumiferana*, and *E. solidaginis* 24 h following the last exposure. The reasoning behind this time-point was that this would allow sufficient time for biochemical adjustments following cold exposure, while not providing so much time that the signal of cold exposure was lost. In Chapter 3, I sampled *D. melanogaster* on a three-day time course following exposure, which showed that glycogen concentration remained similar regardless of the time sampled, although triacylglycerol and glycerol concentrations were impacted only immediately following the exposure. Ideally sampling on a time course could have been repeated for Chapters 4-6, although the number of individual insects required would have been prohibitive. While this short time scale fluctuation may be the case in *D. melanogaster*, in *E. solidaginis* and the freeze avoidant moth *Epiblema scudderiana*, most metabolites remain stable for at least 24 h following repeated cold exposure (Churchill and Storey, 1989). In a follow-up experiment on *C. fumiferana* I used microarrays to study gene expression on a 36 h time scale following a 120 h exposure to -15 ℃. I found that all changes to gene expression occurred by 4 h following low temperature exposure and remained stable for the following 32 h after exposure (K.E. Marshall, unpublished observation). This suggests that the 24 h time point was appropriate for measuring physiological changes as a result of cold exposure. It is also unclear whether the time course of repair and preparatory events remains stable following repeated *vs*. prolonged cold exposure. In addition, since both *C. fumiferana* and *E. solidaginis* showed long-term fitness effects due to repeated cold exposure, ideally metabolic fuels would have been either during or immediately prior to pupation to investigate the cause of these long-term effects.

7.7.2   Other stressors

In this thesis I have focused on the effects of fluctuating temperatures, but in the natural world many abiotic stressors are similarly variable and can potentially interact. The conclusions I draw about the importance of frequency of stress can be extended to other abiotic stressors such as pH, water availability, and anoxia. The effects of repeating these stresses likely depend on relationships such as the speed of organismal response to the stress, the mechanisms that increased intensity or duration increases stress response, and the costs of physiological mechanisms for responding to that stress. For there to be an

effect of repeated stress exposure, the following conditions must be met: 1) the environmental factor (humidity, pH, etc.) must fluctuate on short time-scales, 2) these environmental factors must be detected by the animal in some fashion (whether through direct effects on physiology by the factor or through receptors, Chown and Terblanche, 2007), 3) and the insect must respond to that factor through pathways of damage, repair, or preparation for subsequent exposures. Similarly, the effects of the four axes of stress will depend on whether there are direct effects of the stress on physiology (which influences the effect of intensity), and the timescale of response.

The rapid responses of insects to desiccation stress are beginning to be explored (Chown and Terblanche, 2007). In *D. melanogaster*, a "rapid desiccation hardening" effect has been described, whereby an exposure to desiccation stress increases resistance to subsequent desiccation stresses (Bazinet *et al.*, 2010). Similarly, in *B. antarctica,* repeated exposure to desiccation stress significantly depletes glycogen stores and decreases survival relative to a single stress (Teets *et al.*, 2012a). It is less clear how period between exposures would influence response to repeated desiccation. The speed of animal response relies on water loss rates which are influenced by surface area to volume ratio, metabolic rate, and cuticular lipids (Benoit *et al.*, 2007), or the ability for individuals to rehydrate (whether through diffusion, drinking, or catabolism of energetic resources, Bazinet *et al*., 2010). Because there are no direct effects of humidity on insects (other than increased water loss rate), the effects of intensity and period may be less important than duration in this type of stress, while frequency might be much more important.

Other natural stresses that vary include wave forces (Gracey *et al.*, 2008), wind speed (Gaines and Denny, 1993), and oxygen availability (López-Martinez and Hahn, 2012). In marine systems, pH is also highly variable and can fluctuate as much as 0.3 units on a daily basis (Hofmann *et al.*, 2011). Oxygen availability and pH have direct effects on animal physiology, while the effects of wave action and wind speed do not, so repeated exposure is likely more important in the former two stressors than the latter. In addition, the study of the interactions between these stressors could benefit from the framework that I utilized in this thesis.

7.8   Conclusions

Insects respond physiologically to fluctuations in their environment that occur on multiple temporal scales. In this thesis, I show that there are two types of response to repeated low temperature stress. The first, observed in *P. isabella* and *C. fumiferana*, is the accumulation of unrepaired damage with each cold exposure that leads to increased mortality. The second, observed in *E. solidaginis* and *D. melanogaster*, is that repeated low temperature stress induces additional investment into repair of damage or preparation for subsequent exposures. The lesson in this response is that there is no such thing as a free lunch—particularly in overwintering insects. Any investment into repair of damage or preparation comes from finite energetic resources.

A secondary conclusion from this thesis is that stress from cold exposure accumulates along axes other than intensity and duration. Frequency of low temperature stress can induce additional stress in itself. This translates not only into understanding of the importance of damage and repair processes, but also has important implications for predicting the effects of climate change on insect population density and distribution, and the fine-scale thermal history of an insect matters.

Finally, this thesis demonstrates that temporal and spatial resolution of temperature data matters. Absolute temperature is not as important as frequency of low temperature exposure, and this result holds for every species I studied. As a result, increased monitoring of temperature and incorporation of finer scale resolution of temperature data into models predicting species responses to temperature is an important next step.

7.9   References

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Appendix A: Chapter 2 Supplementary Material

Table A.1 The 26 studies of repeated cold exposure (RCE) on insects reported in this review. In Duration of exposures/Recovery time, the period of recovery is only noted if not immediately apparent from frequency of exposure. See "Design of repeated cold exposures experiments" for details on experiment types.

Taxonomic group	Species	Temperature of exposure/recovery (°C)	Frequency of exposure	Duration of exposures/Recovery time	Number of exposures	Freezing?	Cooling rate	Reference
Compared between months of collection								
Diptera: Tephritidae	*Eurosta solidaginis*	0/20	Weekly	4 days	4	No	Immediate transfer	Pio and Baust 1988
Compared two frequencies of exposure								
Collembola: Entomobryidae	*Orchesella cincta*	10/20	Daily or weekly	1 or 7 days each temperature	21 or 3	No	Immediate transfer	Driessen *et al.*, 2011
RCE *vs*. Cold								
Coleoptera: Curculionidae	*Rhynchaenus fagi*	10/-5, 2/-5, or 2/-15	Daily or weekly	24 h/24 h	14, 28, 42, 56, 70, or 84 days	No	Immediate transfer	Coulson and Bale, 1996
Coleoptera: Chrysomelidae	*Leptinotarsa decemlineata*	-5/5 or -10/5	Daily	12 h/12 h	1, 2, or 3	No	Immediate transfer	Yocum 2001
Collembola: Entomobryidae	*Orchesella cincta*	5/10	Every 2 days	2 days/2 days	5	No	Immediate transfer	Van Dooremalen *et al.*, 2011
Lepidoptera: Nymphalidae	*Aglais urticae, Inachi io*	-5/10	Daily for 5 days, two days rest	8 h/16 h	4	No	Immediate transfer	Pullin and Bale, 1989
Orthoptera: Acridiidae	*Locusta migratoria*	5/10/15, 0/5/10, -5/0/5, -4/20	Every 4 hours or daily	8 h/8 h/8 h, or 2 h/2 h/2 h, or 2h	2, 3, 8, 10, 20, or 40	No	Immediate transfer or 12h cooling	Wang *et al.* 2006
Soil microarthropods	Microarthropods (Collembolans and Acari)	-2/2	Every 5 days	3 days/2 days	1, 4, 8, or 16	Probably no	Unknown	Sjursen *et al.*, 2005

Table A.1 continued

Taxonomic group	Species	Temperature of exposure/recovery (°C)	Frequency of exposure	Duration of exposures/Recovery time	Number of exposures	Freezing?	Cooling rate	Reference
RCE *vs*. Matched Cold								
Diptera: Anthomyiidae	*Delia radicum*	-10.2/2 or -14.8/2	Two week interruption between exposures	18, 24, or 30 days in -10.2 ℃ experiment, 2, 5, 6, or 7 days in -14.8 ℃ experiment	2	No	Immediate transfer	Turnock *et al.*, 1985
Diptera: Chironimidae	*Belgica Antarctica*	-5/4	Daily	12 h/12 h	1, 2, 3, 4, or 5	Yes/No	Immediate transfer	Teets *et al.* 2011
Diptera: Drosophilidae	*Drosophila melanogaster*	-0.5/22	Daily	2 h/22 h	5	No	Immediate transfer	Marshall and Sinclair 2010
Diptera: Drosophilidae	*Drosophila melanogaster*	-0.5/22	Daily	2 h/22 h	5	No	Immediate transfer	Zhang *et al.*, 2011
Lepidoptera: Arctiidae	*Pyrrharctia Isabella*	-12/0 or -14/0	Every 5 days	7 h/113 h	5	Yes	0.5 °C/min	Chapter 4
RCE *vs*. Warm								
Coleoptera: Carabidae, Coleoptera: Staphylinidae	*Bembidion lampros, Tachyporus hypnorum*	-2/6	Daily or biweekly	8h/16h or 1 week/1 week	28, 32, 56, 63, or 70 (daily), or 10, 14 (weekly)	No	Immediate transfer	Petersen *et al.*, 1996
Coleoptera: Perimylopidae	*Hydromedion sparsutum*	-6.5/5 or-2.3*/5	Every 4 days, daily for 10 days, or every 30 minutes	15 min	10	Yes	0.5 °C/min	Bale *et al.*, 2001

*Indicates that individuals were exposed to their individual supercooling points. Value given is the mean exposure temperature.

Table A.1 continued

Taxonomic group	Species	Temperature of exposure/recovery (°C)	Frequency of exposure	Duration of exposures/Recovery time	Number of exposures	Freezing?	Cooling rate	Reference
RCE *vs*. Warm								
Diptera: Drosophilidae	*Drosophila melanogaster*	0/25	Daily for 5 days, 2 days warm, daily for 5 days	15, 30 or 60 min	5 or 10	No	Immediate transfer	Le Bourg, 2007
Diptera: Drosophilidae	*Drosophila melanogaster*	0/25	Daily for 5 days, 2 days warm, daily for 5 days	60 min	10	No	Immediate transfer	Le Bourg *et al.*, 2009
Diptera: Drosophilidae	*Drosophila melanogaster*	0/25	Daily for 5 days, 2 days warm, daily for 5 days	60 min	5 or 10	No	Immediate transfer	Le Bourg, 2010
Diptera: Drosophilidae	*Drosophila melanogaster*	0/25	Daily for 5 days, 2 days warm, daily for 5 days	60 min	5 or 10	No	Immediate transfer	Le Bourg, 2011
Diptera: Drosophilidae	*Drosophila melanogaster*	9/23	Daily	2 h/22 h	6	No	1 °C/h	Kelty and Lee, 2001
Diptera: Drosophilidae	*Drosophila melanogaster*	5/15, 11/21, 16/26, 21/31, 9/21, 21/33, 7/29, 10/32, 4/26, 9/33, or 21/34	Daily	12 h/12 h	Through development	No	Immediate transfer	Petavy *et al.*, 2001

Table A.1 continued

Taxonomic group	Species	Temperature of exposure/recovery (°C)	Frequency of exposure	Duration of exposures/Recovery time	Number of exposures	Freezing?	Cooling rate	Reference
RCE *vs*. Warm								
Hemiptera: Reduviidae	*Panstrongylus megistus*	0/28 or 5/28	8, 18, 24, or 72 hours after first shock	12 h	2	No	Immediate transfer	Garcia *et al.*, 2002
Lepidoptera: Noctuidae	*Sodoptera exigua*	0/20, 5/20, or 10/20	Daily	12 h/12 h	Through development	No	Immediate transfer	Kim *et al.*, 2000
Lepidoptera: Olethreutidae, Diptera: Tephritidae	*Epiblema scudderiana, Eurosta solidaginis*	-16/3	Every other day	24 h/24 h	12	Yes/No	Unknown	Churchill and Storey, 1989
Lepidoptera: Tinidae	*Pringleophaga marioni*	-5/5	Daily	2 h/22 h	5	Yes and no	0.1 °C/min	Sinclair and Chown, 2005
Diptera: Syrphidae	*Syrphus ribesii*	-10/2 or -7.5*/2	Hourly, daily, or weekly	15 min	5	Yes	0.5 °C/min	Brown *et al.*, 2004

*Indicates that individuals were exposed to their individual supercooling points. Value given is the mean exposure temperature.

Table A.2 Effects of repeated cold exposure (RCE) on insects. Effects are relative to all other experimental groups in study, unless noted otherwise.

Effects studied	Impacts	Reference
Compared between months of collection		
Glycerol and sorbitol production	Increased glycerol only in January and February larvae	Pio and Baust 1988
Compared two frequencies of exposure		
Mass change	No difference	Driessen *et al.* 2011
Survival	No difference	Driessen *et al.* 2011
Starvation resistance	No difference	Driessen *et al.* 2011
RCE *vs*. Cold		
Mass	Less mass loss relative to sustained cold	Coulson and Bale 1996
	Greater mass loss relative to sustained cold	Pullin and Bale 1989
	Less mass loss than sustained warm	Pullin and Bale 1989
Supercooling point	No difference	Coulson and Bale 1996
	Decreased relative to control at 30 C only	Wang *et al.* 2006
	Lowered compared to all other groups	Pullin and Bale 1989
Survival	Increased relative to sustained cold	Coulson and Bale 1996, Wang *et al.* 2006
	Increased relative to sustained warm	Pullin and Bale 1989
	No difference	Turnock *et al.* 1985
Hsp transcript expression	Increased	Yocum 2001, Wang *et al.* 2006
Cryoprotectant concentrations	Increased mannitol relative to sustained warm and cold	Wang *et al.* 2006
	Increased sorbitol relative to sustained warm and cold	Wang *et al.* 2006
	Increased myo-inositol relative to sustained warm and cold	Wang *et al.* 2006
	Increased trehalose relative to sustained warm and cold	Wang *et al.* 2006

Table A.2 continued

Effects studied	Impacts	Reference
RCE *vs*. Cold		
Fatty acid composition	Increased unsaturation relative to sustained cold	van Dooremalen *et al.* 2011
Heat knock down temperature	Increased survival relative to sustained cold	van Dooremalen *et al.* 2011
Chill coma recovery time	Increased survival relative to sustained warm	van Dooremalen *et al.* 2011
Microarthropod community composition	No change in Collembolans, increased numbers of Acaridida relative to both sustained warm and cold	Sjursen *et al.* 2005
Sex ratio	No difference	Turnock *et al.* 1985
RCE *vs*. Matched Cold		
Cell damage	Increased	Teets *et al.* 2011
Glycogen	Decreased	Chapter 3
Other	Increased total energy content	Teets *et al.* 2011 (in supercooling treatments only)
	Differences between sustained warm and cold in whole genome gene expression	Zhang *et al.* 2011
Survival	Increased relative to sustained cold	Chapter 3
	Unchanged	Teets *et al.* 2011(in supercooling treatments only)
Cryoprotectant concentrations	Increased trehalose relative to sustained cold	Teets *et al.* 2011 (in supercooling treatments only)
Sex ratio	Male-biased	Chapter 3
Lipid concentration	No difference	Teets *et al.* 2011 (in supercooling treatments only), Chapter 3
RCE *vs*. Warm		
Survival	Increased relative to sustained warm	Petersen *et al.* 1996
	Decreased egg viability	Petavy *et al.* 2001
Mass	Decreased	Petersen *et al.* 1996,
Supercooling point	Decreased	Kim and Song 2000

Table A.2 continued

Effects studied	Impacts	Reference
RCE *vs*. Warm		
Survival after cold shock	Increased	LeBourg 2007, Kelty *et al.* 2001, Kim and Song 2000
Longevity	Increased in males	LeBourg 2007, LeBourg *et al.* 2009, LeBourg 2010, LeBourg 2011
Survival after heat shock	Increased	LeBourg 2007, LeBourg *et al.* 2009, LeBourg 2010, LeBourg 2011, Kelty *et al.* 2001
Starvation resistance	Decreased	LeBourg 2007
Climbing ability	Preserved in old age	LeBourg 2007, LeBourg 2010
Learning ability	Increased	LeBourg 2007
Immune function	Increased in response to fungal pathogen	LeBourg *et al.* 2009, LeBourg 2010, LeBourg 2011
Critical thermal minimum	Lowered	Kelty *et al.* 2001
Hsp concentration	No difference	Kelty *et al.* 2001
Cryoprotectant concentrations	No difference	Kelty *et al.* 2001, Churchill and Storey 1989
Development time	Decreased at low temperatures	Petavy *et al.* 2001
Sex ratio	Female-biased	Petavy *et al.* 2001
Nuclear phenotype	Increase in necrosis with advancing time since shock	Garcia *et al.* 2002
	Decrease in giant nuclei	Garcia *et al.* 2002
	Decrease in apoptosis and suspicion of apoptosis	Garcia *et al.* 2002

Table A.3 Effects of repeated freeze-thaw (RFT) on insects. Effects are relative to all other experimental groups in study, unless noted otherwise.

Effects studied	Impacts	Reference
RFT *vs*. Matched Cold		
Cell damage	Increased	Teets *et al.* 2011, Chapter 4
Glycogen	Decreased relative to sustained freeze	Teets *et al.* 2011
	No difference	Chapter 4
Total energy content	Decreased total energy content	Teets *et al.* 2011
Survival	Increased relative to sustained freeze	Teets *et al.* 2011
	Decreased relative to sustained freeze	Chapter 4
Hsp transcript expression	Increased relative to sustained freeze	Teets *et al.* 2011
Cryoprotectant concentrations	Increased glucose	Teets *et al.* 2011
	No difference in glycerol	Teets *et al.* 2011
	Increase in glycerol	Chapter 4
	Decreased trehalose relative to sustained freeze	Teets *et al.* 2011
Lipid concentration	No difference	Chapter 4
	Decreased relative to control only	Teets *et al.* 2011
Supercooling point	No difference	Chapter 4
Metabolic rate	No difference	Chapter 4
Immune function	Increased in response to fungal pathogen	Chapter 4
RFT *vs*. Warm		
Survival	Increased with greater time allowed for recovery, decreased relative to sustained warm	Bale *et al.* 2001, Brown *et al.* 2004
	No difference	Sinclair and Chown 2005
Mass	Decreased	Sinclair and Chown 2005
Supercooling point	Decreased	Bale *et al.* 2001, Brown *et al.* 2004
	No difference	Sinclair and Chown 2005
Survival after cold shock	Increased	Bale *et al.* 2001

Table A.3 continued

Effects studied	Impacts	Reference
RFT *vs*. Warm		
Critical thermal minimum	No difference	Sinclair and Chown 2005
Cryoprotectant concentrations	Increased sorbitol (in freeze tolerant species only)	Churchill and Storey 1989
Energy charge	Decreased (in freeze tolerant species only)	Churchill and Storey 1989
Glycogen	Decreased (in freeze tolerant species only)	Churchill and Storey 1989
Gut mass	Decreased	Sinclair and Chown 2005
Gut water content	Increased	Sinclair and Chown 2005

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Appendix B: Chapter 5 Supplementary Material
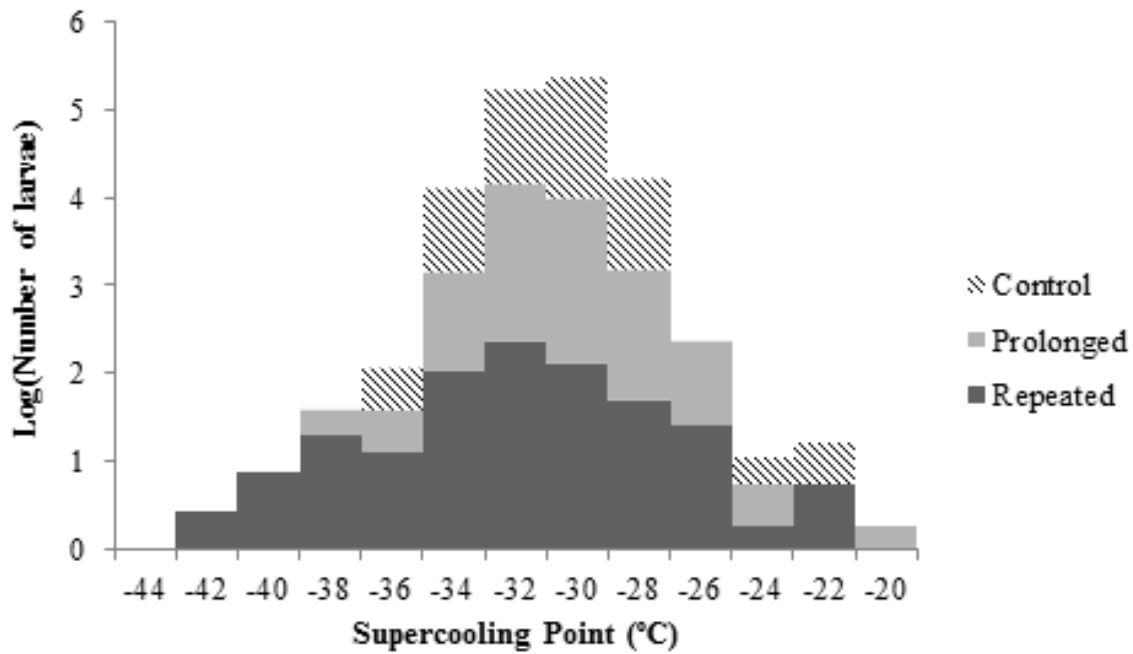
Figure B.1 The distribution of supercooling points of 2nd instar Choristoneura fumiferana larvae. Larvae were either given 1 × 120 h exposure ("Prolonged"), or repeated 12 h exposures to low temperature. Number of larvae were log-transformed to normalize sample sizes among groups.
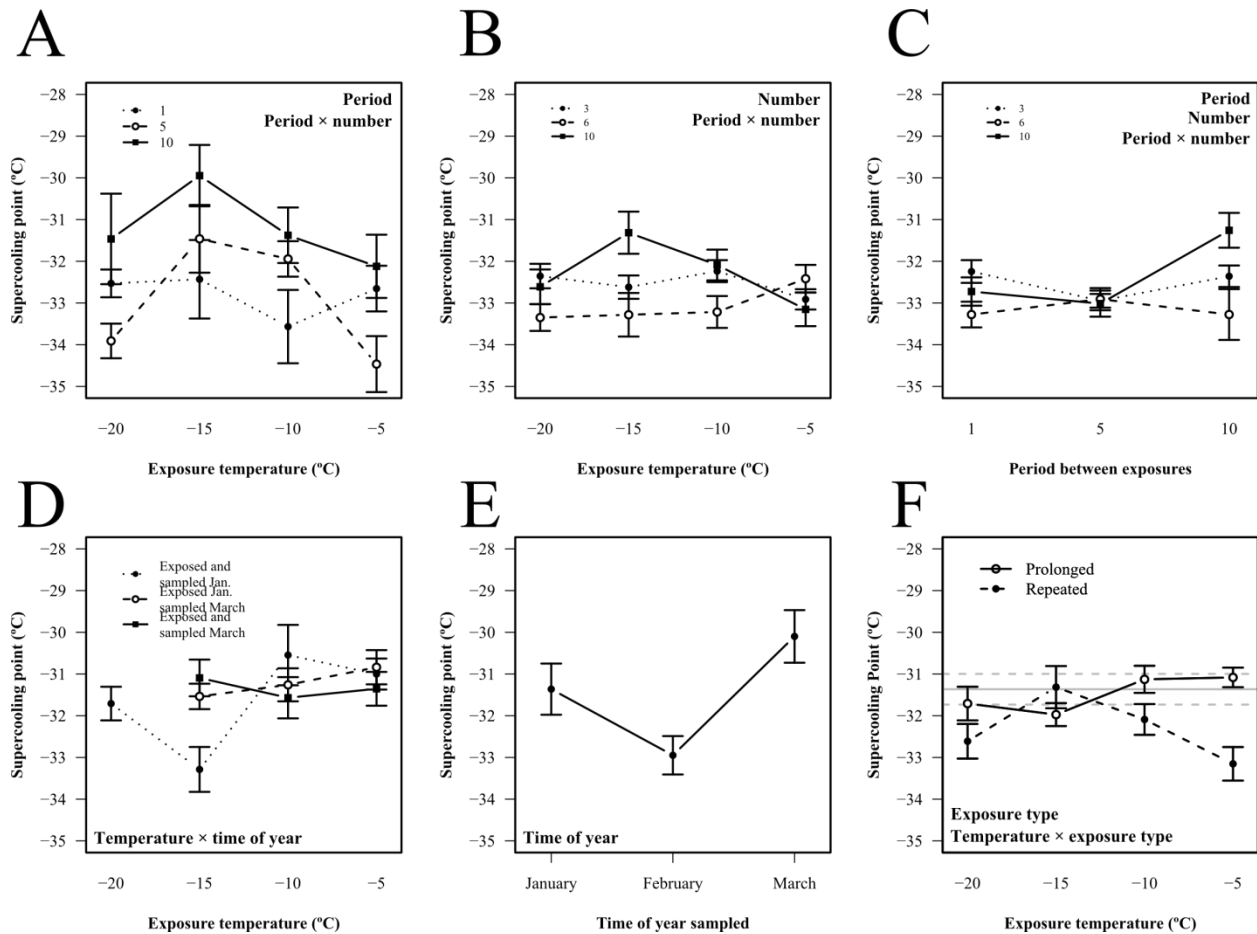
Figure B.2 The effects of cold exposure on the supercooling point of 2nd instar *Choristoneura fumiferana* **larvae.** All significant effects are indicated by the bold terms in each figure, and accompanying statistics are found in Tables 5-8. "Period" = days between cold exposure (daily, every five days, or every 10 days), "Intensity" = temperature of cold exposure, "Number" = number of cold exposures (three, six, or 10), "Time of Year" = time of year exposed or sampled. "Exposure Type" = either "Repeated" 12 h exposures, or a single "Prolonged" 120 h cold exposure. A) Larvae that received 10 twelve hour exposures. B-C) Larvae that received repeated 12 h cold exposures as 2nd instar larvae. D) Larvae that received a single 120 h cold exposure, exposed in either early January or late March, and sampled either early January or late March E) Control larvae, sampled in early January, mid-February, or late March. F) All exposure types together. Solid and dotted horizontal grey lines represent mean ± SE of control moths, respectively.

Figure B.3 The effects of cold exposure on glucose content of 2nd instar *Choristoneura fumiferana* larvae. All significant effects are indicated by the bold terms in each figure, and accompanying statistics are found in Tables 5-8. "Period" = days between cold exposure (daily, every five days, or every 10 days), "Temperature" = intensity of cold exposure, "Number" = number of cold exposures (three, six, or 10), "Time of Year" = time of year exposed or sampled. "Exposure Type" = either "Repeated" 12 h exposures, or a single "Prolonged" 120 h cold exposure. A) Larvae that received 10 twelve hour exposures. B-C) Larvae that received repeated 12 h cold exposures as 2nd instar larvae. D) Larvae that received a single 120 h cold exposure, exposed in either early January or late March, and sampled either early January or late March E) Control larvae, sampled in early January, mid-February, or late March. F) All exposure types together. Solid and dotted grey lines represent mean ± SE of control moths, respectively.

Table B.1 The effects of repeated 12 h cold exposures during the 2nd instar on adult male characteristics of *Choristoneura fumiferana*. Values are ANOVA statistics comparing adult characteristics (body mass and development time) among male *Choristoneura fumiferana* moths as a result of exposure temperature (either -5, -10, -15, or -20 °C), period between exposures (daily, every five days, or every 10 days), and number of exposures (either three, six, or 10 exposures) as 2nd instar caterpillars. Retained terms with significant p-values ($p < 0.05$) are in bold typeface.

Y	Initial model	Minimal adequate model	Terms from minimal adequate model	F	df	P
Male body mass	wing length + exposure temperature × period between exposures × number of exposures	wing length + exposure temperature + period between exposures+ exposure temperature × period between exposures	**Wing length** **Temperature** **Period** **Temperature × Period**	**104.6** **8** **4.36** **3.58** **2.70**	**1, 246** **3, 246** **2, 246** **6, 246**	**<0.001** **0.005** **0.029** **0.015**
Male development time	exposure temperature × period between exposures × number of exposures	exposure temperature × period between exposures × number of exposures	Temperature **Period** Number Temperature × Period **Temperature × Number** Period × Number **Temperature × Period × Number**	1.58 **3.96** 0.28 1.70 **2.90** 0.56 **4.55**	3, 235 **2, 235** 1, 235 6, 235 **3, 235** 2, 235 **6, 235**	0.196 **0.020** 0.597 0.121 **0.036** 0.575 **<0.001**

Table B.2 The effects of a 120 h cold exposure during the 2nd instar on adult male characteristics of *Choristoneura fumiferana*. Values are ANOVA statistics comparing adult characteristics (body mass, survival, sex ratio, and development time) among male *Choristoneura fumiferana* moths as a result of exposure temperature (either -5, -10, -15, or -20 °C) or time of year exposed (January or March) after 120 hours of low temperature exposure while 2nd instar caterpillars. Retained terms with significant p-values ($p < 0.05$) are in bold typeface

Y	Initial model	Minimal adequate model	Terms from minimal adequate model	F	df	P
Male body mass	wing length + exposure temperature × time of year exposed	wing length + exposure temperature +	**Wing length** **Temperature**	**34.98** **4.88**	**1, 81** **3, 81**	**< 0.001** **0.003**
Male development time	exposure temperature × time of year exposed	time of year exposed	**Time of year**	**13.54**	**1, 84**	**<0.001**

Table B.3 The effects of low temperature exposure during the 2nd instar on adult male characteristics of *Choristoneura fumiferana*. Values are ANOVA statistics comparing adult characteristics (body mass, survival, sex ratio, and development time) among *Choristoneura fumiferana* moths as a result of low temperature exposure type (ten 12 hour exposures or one 120 hour exposure) as 2nd instar caterpillars. Retained terms with significant p-values (p < 0.05) are in bold typeface.

Groups tested	Y	Initial model	Minimal adequate model	Terms from minimal adequate model	F	Df	P
Repeated: period **Prolonged**: pooled	Male body mass	wing length + exposure temperature × exposure type	wing length + exposure temperature × exposure type	**Wing length**	**59.24**	**1, 156**	**<0.001**
				Temperature	**6.83**	**3, 156**	**<0.001**
				Exposure type	2.10	3, 156	0.103
				Temperature × Type	**2.14**	**9, 156**	**0.029**
Repeated: pooled **Prolonged**: time of year exposed	Male development time	exposure temperature × exposure type	exposure type	**Exposure type**	**6.95**	**2, 170**	**0.001**

Appendix C: Chapter 6 Supplementary Material Results

Results

Male development time

There were significant interactions between number of exposures and temperature of exposure, period between exposure and temperature of exposure, as well as between number of exposures and period between exposure on development time of male flies (Table C.3). Flies that received 10 exposures every 10 days had significantly longer development times when exposed to -20 °C, while other relationships were less clear. In male flies that received a single prolonged exposure, there was no impact of time of year exposed on development time, but there was an impact of exposure temperature (Table C.4). Flies that received lower temperature exposures developed in less time than flies that received higher temperature exposures. Finally, when all flies were compared together, there was a significant interaction between exposure temperature and exposure type, which *post-hoc* testing showed was generally driven by the long development time of male flies exposed at -20 °C every 10 days.

Adult male mass

In male flies, there was a significant three-way interaction between exposure temperature, number of exposures, and period between exposures (Table C.3) on mass. Flies that received daily exposures had greater mass than flies that received exposures every five days or every 10 days, but this effect was more pronounced at lower temperatures. Meanwhile, flies that received 10 exposures had higher mass than flies with fewer exposures at lower temperatures, while at higher temperatures, male flies that received more exposures were smaller as adults. In flies that received prolonged exposures, there was a significant interaction between exposure temperature and time of year exposed (Table C.4), however *post-hoc* testing showed no significant differences between temperatures and time of year exposed. Finally, when all exposure types were compared, there was a significant effect of exposure type on male adult mass (Table C.5), yet again, there was no significant differences when tested *post-hoc*.

Metabolites

Glycogen mass

Neither temperature, period between exposures, or number of exposures impacted glycogen mass in flies that received repeated low temperature exposures (Table C.16, Figure C.3). There was no impact of time of year of exposure on glycogen content in flies that received prolonged exposures, but as temperature decreased, amount of glycogen also decreased significantly (Table C.17, Figure C.3). Therefore temperature of exposure was retained in the model comparing prolonged and repeated cold exposures. In control flies, glycogen content dropped significantly through the experiment (Table C.18, Figure C.3). Finally, when all flies from all exposure groups were compared, there were no significant differences in glycogen content (Table C.19, Figure C.3).
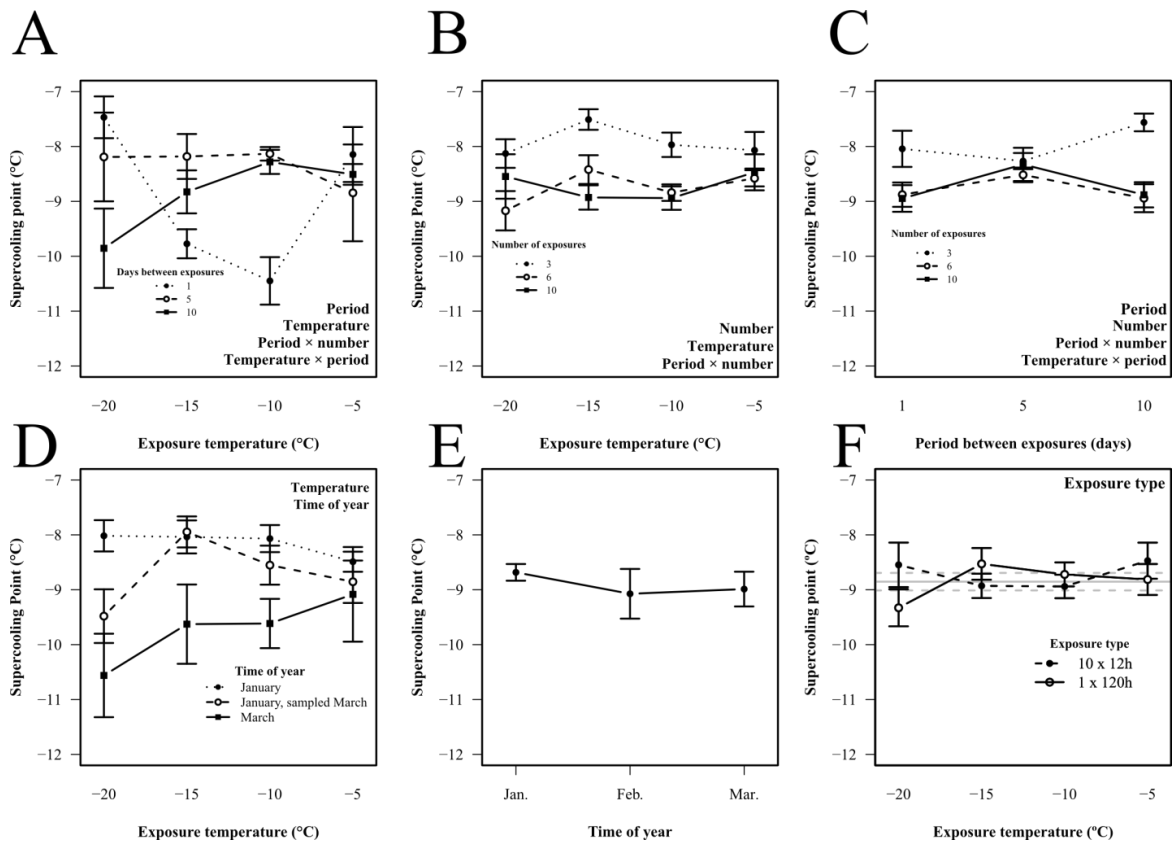
Figure C.1.   The effects of low temperature exposure on supercooling point of *Eurosta solidaginis* **pre-pupae.** All significant effects are indicated by the bold terms in each figure, and accompanying statistics are found in Appendix C Tables C.6-7. "Period" = time between cold exposures (one, five, or ten days), "Intensity" = temperature of cold exposure, "Number" = number of cold exposures (three, six, or ten days). A) Flies that received ten 12 h exposures as pre-pupae. B-C) Flies that received repeated 12 h exposures as pre-pupae. D) Flies that received a single 120 h cold exposure in January, received a bout in January but were sampled in March, or received a single 120 h bout in March and were sampled immediately. E) Control flies maintained at 0/2 °C all winter and sampled throughout. F) Comparison of flies that received repeated 12 h *vs*. a single 120 h cold exposure.   Solid and dotted grey lines represent mean and standard error of control flies, respectively.
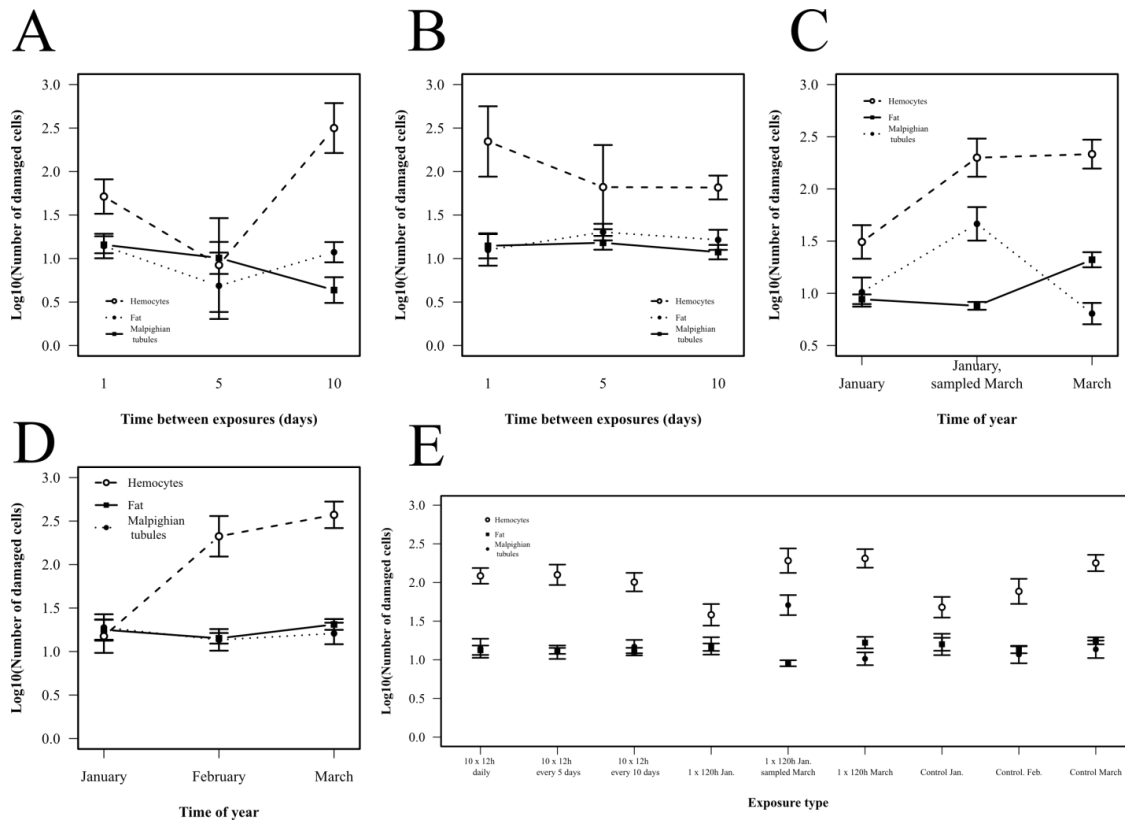
Figure C.2 The effects of low temperature exposure on cell damage in *Eurosta solidaginis.* Accompanying statistics are presented in Appendix C Tables C.8-10. A) Pre-pupae that received three 12 h exposures to -20 ℃ at different time intervals. B) Pre-pupae that received ten 12 h exposures to -20 ℃ at different time intervals. C) Pre-pupae that received one 120 h exposure to -20 ℃ in either January or March. D) Flies kept in control conditions at 0/2 ℃. E) All experimental treatments compared against each other. There were no significant effects in any comparison.
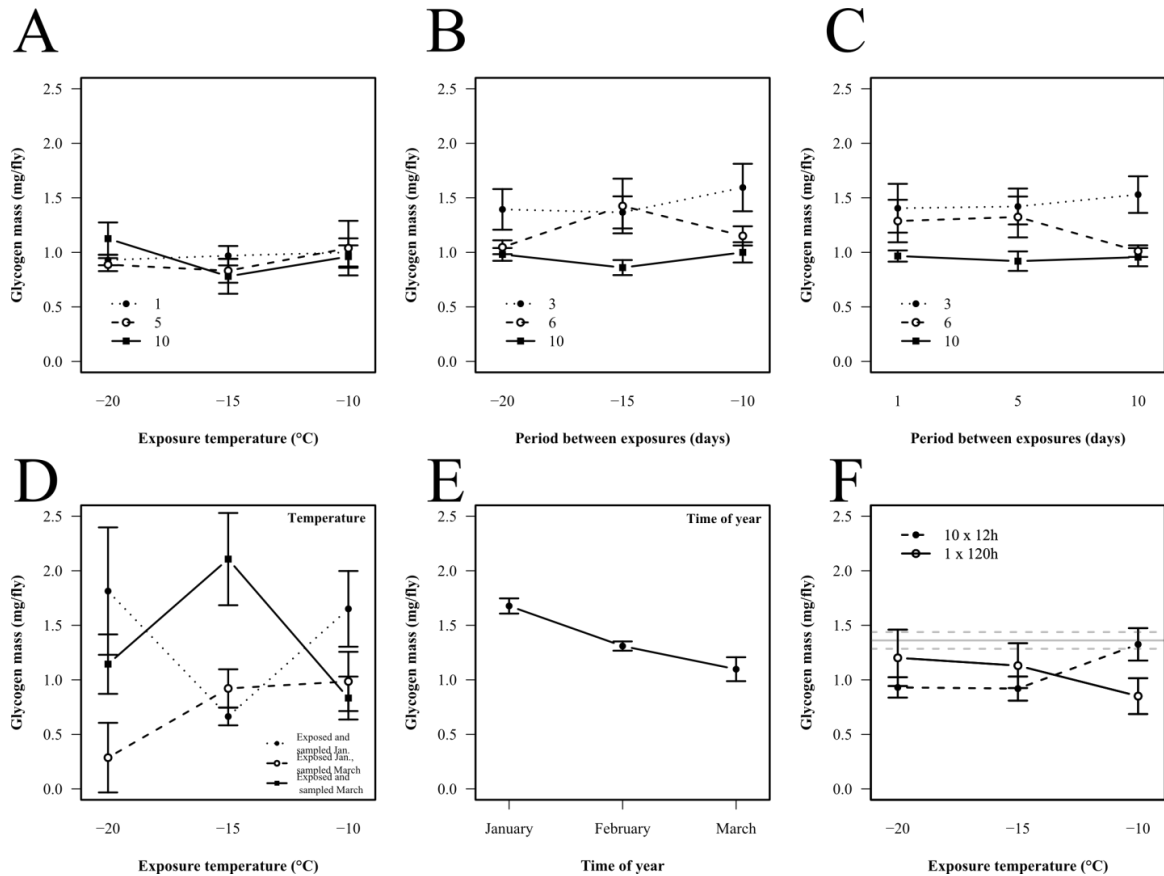
Figure C.3 The effects of low temperature exposure on glycogen mass in *Eurosta solidaginis*. All significant effects are indicated by the bold terms in each figure, and accompanying statistics are found in Appendix C Tables C.16-19. "Period" = time between cold exposures (one, five, or ten days), "Intensity" = temperature of cold exposure, "Number" = number of cold exposures (three, six, or ten days). Solid and dotted horizontal grey lines represent mean and standard error of control flies, respectively. A) Flies that received ten 12 h exposures as pre-pupae. B-C) Flies that received repeated 12 h exposures as pre-pupae. D) Flies that received a single 120 h cold exposure in January, received a bout in January but were sampled in March, or received a single 120 h bout in March and were sampled immediately. E) Control flies maintained at 0/2 °C all winter and sampled throughout. F) Comparison of flies that received repeated 12 h *vs*. a single 120 h cold exposure.

Table C.1 Coefficients from the MANCOVA described in Table 6.1. The term with the largest absolute value of coefficient is bolded in each column. Terms with significant effects are also in bold.

Term	Body Mass	Number of Eggs	Development time
Intercept	3.39	**75.92**	27.95
Thorax width	3.14	**26.41**	0.003
Period (every 10 days)	-2.94	**-23.55**	-1.58
Period (every 5 days)	2.10	**-5.64**	0.79
Number (3 exposures)	-0.87	**-9.8**	-0.61
Number (6 exposures)	1.85	**16.66**	-1.00
Temperature × Period (every 10 days)	-0.20	**-1.05**	-0.18
Temperature × Period (every 5 days)	0.19	**-0.58**	0.12
Period (every 10 days) × Number (3 exposures)	0.37	**-3.96**	-0.76
Period (every 5 days) × Number (3 exposures)	2.29	**-4.44**	1.45
Period (every 10 days) × Number (6 exposures)	-0.63	**6.30**	-0.35
Period (every 5 days) × Number (6 exposures)	-3.45	**-48.36**	3.20

Table C.2 Coefficients from the MANCOVA described in Table 6.2. The term with the largest absolute value of coefficient is bolded in each column.

Term	Body Mass	Number of Eggs	Development time
Intercept	-4.37	**63.05**	25.30
Thorax width	3.20	**26.72**	-0.14
Exposure type: Repeated	**6.90**	-2.43	1.86
Exposure type: Prolonged	4.59	**4.95**	0.38

Table C.3. ANCOVA statistics comparing adult characteristics among *Eurosta solidaginis* flies as a result of exposure temperature (either -5, -10, -15, or -20 °C), period between exposures (daily, every 5 days, or every 10 days), and number of exposures (either 3, 6, or 10 exposures) as pre-pupae. All pre-pupae were given repeated 12 hour low temperature exposures. Retained terms with significant p-values (p < 0.05) are bolded. In the case of number of eggs, a generalized linear model was fit with a quasipoisson distribution and the statistic given is the coefficient for the term. Similarly, in the case of survival, a generalized linear model was fit with a binomial distribution, and the statistic given is the coefficient for term.

Measure	Initial model	Minimal adequate model	Term	F	df	P
Female body mass	Y = thorax width + exposure temperature × period between exposures × number of exposures	Y = thorax width + temperature + period + number + temperature × period + period × number	**Thorax width**	**54.802**	**1, 195**	**< 0.001**
			Temperature	0.001	1, 195	0.971
			Period	0.230	2, 195	0.795
			Number	0.450	2, 195	0.639
			Temperature × Period	**4.889**	**2, 195**	**0.008**
			Period × Number	**14.836**	**2, 195**	**0.027**
Eggs	Y = body mass + exposure temperature × period between exposures × number of exposures	Y = body mass	**Body mass**	**0.038**	**207**	**<0.001**
Female development time	Y = exposure temperature × period between exposures × number of exposures	Y = exposure temperature × period between exposures × number of exposures	Temperature	1.972	1, 116	0.163
			Period	**3.385**	**2, 116**	**0.037**
			Number	**2.530**	**2, 116**	**0.048**
			Temperature × Period	**10.095**	**2, 116**	**<0.001**
			Temperature × Number	**5.752**	**2, 116**	**0.004**
			Period × Number	1.693	4, 116	0.156
			Temperature × Period × Number	**5.491**	**4, 116**	**<0.001**
Survival	Y = exposure temperature × period between exposures × number of exposures	Y = exposure temperature + period between exposures + temperature × period	Temperature	1.238	1, 33	0.266
			Period	3.183	2, 31	0.204
			Temperature × Period	**11.367**	**2, 29**	**0.003**
Male mass	Y = thorax width + exposure temperature × period between exposures × number of exposures	Y = exposure temperature × period between exposures × number of exposures	Temperature	0.375	1, 211	0.541
			Period	**11.889**	**2, 211**	**<0.001**
			Number	0.164	2, 211	0.849
			Temperature × period	1.623	2, 211	0.200
			Temperature × number	**3.635**	**2, 211**	**0.028**

			Period × number	0.738	4, 211	0.567
			Temperature × period × number	**3.216**	**4, 211**	**0.014**
Male development time	Y = exposure temperature × period between exposures × number of exposures	Y = temperature + period + number + temperature × period + temperature × number + period × number	Temperature	0.245	1, 215	0.621
			Period	1.501	2, 215	0.225
			Number	0.103	2, 215	0.903
			Temperature × period	**10.078**	**2, 215**	**<0.001**
			Temperature × number	**6.302**	**2, 215**	**0.002**
			Period × number	**2.453**	**5, 215**	**0.047**

Table C.4. ANCOVA statistics comparing adult characteristics (body mass, number of eggs, and development time) among *Eurosta solidaginis* **flies as a result of exposure temperature** (either -5, -10, -15, or -20 °C) or time of year exposed (January or March) after 120 hours of low temperature exposure. Retained terms with significant p-values (p < 0.05) are bolded. In the case of number of eggs, a generalized linear model was fit with a quasipoisson distribution and the statistic given is the coefficient for the term. Similarly, in the case of survival, a generalized linear model was fit with a binomial distribution, and the statistic given is the coefficient for term.

Measure	Initial model	Minimal adequate model	Term	F	Df	P
Female body mass	Y = thorax width + exposure temperature × time of year exposed	Y = thorax width	**Thorax width**	**16.941**	**1, 52**	**< 0.001**
Eggs	Y = body mass + exposure temperature × time of year exposed	Y = body mass	**Body mass**	**0.044**	**53**	**<0.001**
Female development time	Y = exposure temperature × time of year exposed	Y = null				
Survival	Y = exposure temperature × time of year exposed	Y = time of year exposed	**Time of year**	**12.242**	**1, 3**	**<0.001**
Male mass	Y = thorax width + exposure temperature × time of year exposed	Y = thorax width + exposure temperature × time of year exposed	Thorax width	1.267	1, 43	0.267
			Temperature	**4.814**	**1, 43**	**0.034**
			Time of year	0.125	1, 43	0.725
			Temperature × time of year	**5.753**	**1, 43**	**0.021**
Male development time	Y = exposure temperature × time of year exposed	Y = exposure temperature	**Temperature**	**5.719**	**1, 46**	**0.021**

Table C.5. ANCOVA statistics comparing adult characteristics (body mass, number of eggs, and development time) among *Eurosta solidaginis* **flies as a result of exposure type** (ten 12 hour exposures, one 120 hour exposure, or control). Retained terms with significant p-values (p < 0.05) are bolded. In the case of number of eggs, a generalized linear model was fit with a quasipoisson distribution and the statistic given is the coefficient for the term. Similarly, in the case of survival, a generalized linear model was fit with a binomial distribution, and the statistic given is the coefficient for term.

Measure	Exposure groups	Initial model	Minimal adequate model	Term	F	Df	P
Female body mass	**Repeated**: all periods separate **Prolonged**: pooled	Y = thorax width + exposure temperature × exposure type	Y = thorax width + exposure temperature × exposure type	**Thorax width** Temperature Type **Temperature × type**	**81.20** 0.60 0.15 **2.13**	**1, 245** 1, 245 3, 245 **9, 245**	**< 0.001** 0.616 0.927 **0.023**
Eggs		Y = body mass + exposure type	Y = body mass + exposure type	**Body mass** **Exposure type (repeated)** Exposure type (prolonged)	**0.039** **-0.221** -0.149	**262** **262** 262	**<0.001** **0.032** 0.157
Female development time		Y = exposure temperature × exposure type	Y = exposure temperature × exposure type	Temperature **Exposure type** **Temperature × exposure type**	0.211 **4.826** **7.236**	1, 116 **3, 116** **3, 116**	0.647 **0.003** **<0.001**
Survival		Y = exposure type	Y = exposure type	**Exposure type**	**24.097**	**5, 35**	**<0.001**
Male mass		Y = thorax width + exposure type	Y = exposure type	**Exposure type**	**3.097**	**4, 235**	**0.0181**
Male development time		Y = exposure temperature × exposure type	Y = exposure temperature × exposure type	**Temperature** **Type** **Temperature × type**	**0.223** **7.925** **9.917**	**1, 116** **3, 116** **3, 116**	**0.638** **<0.001** **<0.001**

Table C.6. ANOVA statistics comparing supercooling points of *Eurosta solidaginis* pre-pupae exposed to repeated (12 hour exposures) and prolonged exposures (120 hour exposures) to -5, -10, -15, or -20 °C. Flies that were repeatedly exposed were exposed either 3, 6, or 10 times either daily, every 5 days or every 10 days. Retained terms with significant p-values (p < 0.05) are bolded. Control flies were maintained at 0 °C through the experiment.

Exposure group	Initial model	Minimal adequate model	Term	F	df	P
Repeated	Y = exposure temperature × period between exposures × number of exposures	Y = temperature + period between exposures + number of exposures + temperature × period + period × number of exposures	Temperature	0.119	1, 743	0.730
			Period	1.114	2, 743	0.329
			Number	**13.519**	**2, 743**	**<0.001**
			Temperature ×	**5.743**	**2, 743**	**0.003**
			period	**2.852**	**4, 743**	**0.023**
			Period × number			
Prolonged	Y = exposure temperature × date exposed and sampled	Y = temperature + date exposed and sampled	Temperature	1.062	1, 247	0.304
			Date of exposure and assay	**10.179**	**2, 247**	**<0.001**
Control	Y = time of year	Null model				

Table C.7. ANOVA statistics comparing supercooling points of *Eurosta solidaginis* pre-pupae exposed to repeated (12 hour exposures) and prolonged exposures (120 hour exposures) to -5, -10, -15, or -20 °C. Flies that were repeatedly exposed were exposed 10 times either daily, every 5 days or every 10 days. Retained terms with significant p-values (p < 0.05) are bolded.

Exposure groups	Initial model	Minimal adequate model	Term	F	Df	P
Prolonged: time of year **Repeated:** period between exposures	Y = temperature of exposure × exposure group	Y = exposure group	**Exposure group**	**4.757**	**5, 479**	**<0.001**

Table C.8. ANOVA statistics comparing log number of damaged cells of *Eurosta solidaginis* pre-pupae exposed to repeated (12 hour exposures) exposures to -20 °C. Flies that were repeatedly exposed were exposed either 3, 6, or 10 times to either daily, every 5 days or every 10 days. Retained terms with significant p-values (p < 0.05) are bolded.

Tissue	Initial model	Minimal adequate model	Term	F	Df	P
Fat	Y = total cells + period between exposures × number of exposures	Y == total cells + period + number	Total cells	2.621	1, 24	0.119
			Period	2.368	2, 24	0.115
			Number	3.768	1, 24	0.064
Hemocytes	Y = total cells + period between exposures × number of exposures	Y = period + number + period × number	Period	2.413	2, 23	0.111
			Number	0.778	1, 23	0.387
			Period × number	2.802	2, 23	0.082
Malpighian tubules	Y = total cells + period between exposures × number of exposures	Y = null model				

Table C.9. ANOVA statistics comparing log number of damaged cells of *Eurosta solidaginis* pre-pupae exposed to prolonged (120 h) exposures to -20 °C in either January or March, as well as flies exposed in January then sampled in March. Retained terms with significant p-values (p < 0.05) are bolded.

Tissue	Initial model	Minimal adequate model	Term	F	df	P
Fat	Y = total cells + time of year exposed and sampled	Y = total cells + time of year exposed and sampled	Total cells	4.789	1, 11	0.051
			Time of year	3.407	2, 11	0.071
Hemocytes	Y = total cells + time of year exposed and sampled	Y = total cells	**Total cells**	**10.108**	**1, 13**	**0.007**
Malpighian tubules	Y = total cells + time of year exposed and sampled	Y = total cells	**Total cells**	**41.323**	**1, 13**	**<0.001**

Table C.10. ANOVA statistics comparing log number of damaged cells of control *Eurosta solidaginis* pre-pupae maintained at 0 °C for three months, and sampled at three different points (beginning of January, middle of February, and end of March). Retained terms with significant p-values (p < 0.05) are bolded.

Tissue	Initial model	Minimal adequate model	Term	F	df	P
Fat	Y = total cells + time of year	Y = total cells	**Total cells**	**14.94**	**1, 13**	**0.003**
Hemocytes	Y = total cells + time of year	Y = total cells + time of year	**Total cells**	**9.482**	**1, 11**	**0.015**
			Time of year	3.443	2, 11	0.069
Malpighian tubules	Y = total cells + time of year	Y = total cells	**Total cells**	**19.952**	**1, 13**	**<0.001**

Table C.11. ANOVA statistics comparing log number of damaged cells in *Eurosta solidaginis* pre-pupae exposed to repeated (12 hour exposures) and prolonged exposures (120 hour exposures) to -20 °C. Flies that were repeatedly exposed were exposed either 3, 6, or 10 times either daily, every 5 days or every 10 days. Retained terms with significant p-values (p < 0.05) are bolded. Control flies were maintained at 0 °C through the experiment.

Exposure groups	Tissue	Initial model	Minimal adequate model	Term	F	df	P
Repeated: Period Prolonged: time of year exposed and sampled Control: time of year sampled	Fat	Y = total cells + exposure group	Y = total cells	**Total cells**	**17.00**	**1, 43**	**<0.001**
Repeated: Period Prolonged: time of year exposed and sampled Control: time of year sampled	Hemocytes	Y = total cells + exposure group	Y = total cells	**Total cells**	**11.07**	**1, 43**	**0.002**
Repeated: Period Prolonged: time of year exposed and sampled Control: time of year sampled	Malpighian tubules	Y = total cells + exposure group	Y = total cells	**Total cells**	**49.66**	**1, 43**	**<0.001**

Table C.12. Coefficients from the MANCOVA described in Table 6.4. The term with the largest absolute value of coefficient is bolded in each column.

Exposure group	Term	lcTAG	FFA	acTAG
Repeated	Intercept	3.44	3.58	**4.70**
	Temperature	0.07	0.06	**0.09**
	Period (every 5 days)	-0.56	-0.47	**-1.03**
	Period (every 10 days)	-1.51	-1.24	**-2.46**
	Number (3 exposures)	**0.34**	0.20	0.33
	Number (6 exposures)	**0.16**	0.02	0.09
	Temperature × Period (every 5 days)	-0.04	-0.04	**-0.09**
	Temperature × Period (every 10 days)	-0.09	-0.07	**-0.16**
Prolonged	Intercept	2.78	3.12	**3.68**
	Exposed Jan., sampled March	-0.27	**-0.65**	-0.36
	Exposed March, sampled March	-0.47	-0.56	**-0.71**

Table C.13. Coefficients from a MANCOVA comparing lipid components among exposure types (either ten 12 hour exposures, one 120 hour exposure, or control conditions) in *Eurosta solidaginis* pre-pupae. The term with the largest absolute value of coefficient is bolded in each column.

Term	lcTAG	FFA	acTAG
Intercept	2.32	2.62	**3.08**
Control	-0.25	-0.20	**-0.51**
Prolonged	0.04	0.10	**0.26**

Table C.14. Coefficients from the MANCOVA described in Table 6.5. The term with the largest absolute value of coefficient is bolded in each column.

Exposure group	Term	Glucose	Glycogen	Glycerol	Sorbitol
Repeated	**Intercept**	1.06	-0.55	**1.75**	0.64
	Protein mass	0.27	**0.47**	-0.04	0.01
	Temperature	-0.03	-0.01	0.01	-0.01
	Period (daily)	0.14	**-0.36**	0.26	0.30
	Period (every 10 days)	**0.69**	0.21	0.32	-0.03
	Number (3 exposures)	**-1.80**	-0.13	-0.04	-0.32
	Number (6 exposures)	0.39	0.21	**-0.34**	-0.06
	Temperature × Period (daily)	0.01	**-0.02**	0.01	0.01
	Temperature × Period (every 10 days)	**0.05**	0.01	0.03	0.01
	Temperature × number (3 exposures)	**-0.11**	-0.02	-0.01	-0.02
	Temperature × number (6 exposures)	0.01	0.02	**-0.03**	-0.01
	Period (daily) × number (3 exposures)	**-1.67**	-0.02	-0.92	-0.60
	Period (every 10 days) × number (3 exposures)	**1.29**	0.83	0.56	0.63
	Period (daily) × number (6 exposures)	**0.96**	0.72	0.33	0.28
	Period (every 10 days) × number (6 exposures)	-0.38	**-0.59**	-0.37	-0.37
	Temperature × Period (daily) × number (3 exposures)	**-0.11**	<0.01	-0.06	-0.04
	Temperature × Period (every 10 days) × number (3 exposures)	**0.06**	0.04	0.02	0.03
	Temperature × Period (daily) × number (6 exposures)	**0.05**	0.03	0.02	0.03
	Temperature × Period (every 10) × number (6 exposures)	-0.02	**-0.03**	-0.03	-0.03
Prolonged	**Intercept**	**2.20**	-0.23	1.57	0.90
	Protein mass	0.01	**0.85**	0.03	0.01
	Temperature	-0.02	**0.09**	0.03	0.02
	Exposed Jan.	0.47	0.77	0.57	**0.79**
	Exposed Jan., sampled March	-0.61	**-1.11**	-0.65	-0.66
	Temperature × exposed Jan.	-0.04	**0.06**	0.02	0.04
	Temperature × exposed Jan., sampled March	-0.04	**-0.08**	-0.04	-0.05

Table C.15. Coefficients from a MANCOVA comparing carbohydrate components among exposure types (either ten 12 hour exposures, one 120 hour exposure, or control conditions) in *Eurosta solidaginis* pre-pupae. The term with the largest absolute value of coefficient is bolded in each column.

Term	Glucose	Glycogen	Glycerol	Sorbitol
Intercept	**2.21**	-1.17	1.12	0.59
Protein mass	0.08	**0.69**	0.01	-0.01
Control	**0.25**	-0.06	-0.24	-0.24
Repeated	**-0.26**	0.07	0.24	0.21

Table C.16. ANCOVA statistics comparing amount of glucose and glycogen among *Eurosta solidaginis* flies as a result of exposure temperature(either -5, -10, or -15 °C) , period between exposures (daily, every 5 days, or every 10 days), and number of exposures (either 3, 6, or 10 exposures) as pre-pupae. All pre-pupae were given repeated 12 hour low temperature exposures. Retained terms with significant p-values (p < 0.05) are bolded.

Measure	Initial model	Minimal adequate model	Term	F	df	P
Glucose	Y = protein mass + exposure temperature × period between exposures × number of exposures	Y = protein mass + exposure temperature × period between exposures × number of exposures	**Protein**	**22.288**	**1, 116**	**< 0.001**
			Temperature	2.561	1, 116	0.112
			Period	0.302	2, 116	0.740
			Number	**4.242**	**2, 116**	**0.017**
			Temperature × Period	**2.973**	**2, 116**	**0.055**
			Temperature × Number	**14.836**	**2, 116**	**<0.001**
			Period × Number	0.025	4, 116	0.999
			Temperature × Period × Number	**3.577**	**4, 116**	**0.009**
Glycogen	Y = protein mass + exposure temperature × period between exposures × number of exposures	Y = protein mass	**Protein**	**79.200**	**1, 133**	**<0.001**
Glycerol	Y = protein mass + exposure temperature × period between exposures × number of exposures	Y = protein mass + exposure temperature × period between exposures × number of exposures	**Protein**	**6.971**	**1, 116**	**0.009**
			Temperature	1.972	1, 116	0.163
			Period	**3.385**	**2, 116**	**0.037**
			Number	**2.530**	**2, 116**	**0.048**
			Temperature × Period	**10.095**	**2, 116**	**<0.001**
			Temperature × Number	**5.752**	**2, 116**	**0.004**
			Period × Number	1.693	4, 116	0.156
			Temperature × Period × Number	**5.491**	**4, 116**	**<0.001**
Sorbitol	Y = protein mass + exposure temperature ×	Y = exposure temperature × period	Temperature	1.224	1, 117	0.271
			Period	**21.755**	**2, 117**	**<0.001**

	period between exposures × number of exposures	between exposures × number of exposures	Number	0.019	2, 117	0.981
			Temperature × Period	1.722	2, 117	0.183
			Temperature × Number	**5.080**	**2, 117**	**0.008**
			Period × Number	**8.571**	**4, 117**	**<0.001**
			Temperature × Period × Number	**3.443**	**4, 117**	**0.011**
acTAG	Y = total lipid mass + exposure temperature × period between exposures × number of exposures	Y = total lipid mass + exposure temperature	**Total lipid mass**	**2287.28**	**1, 132**	**< 0.001**
			Temperature	**6.10**	**1, 132**	**0.0148**
lcTAG	Y = total lipid mass + exposure temperature × period between exposures × number of exposures	Y = total lipid mass + temperature + number of exposures	**Total lipid mass**	**2287.28**	**1, 13**	**< 0.001**
			Temperature	**4.57**	**1, 130**	**0.0344**
			Number	2.98	2, 130	0.054

Table C.17. ANCOVA statistics comparing amount of glucose and glycogen among *Eurosta solidaginis* flies as a result of exposure temperature (either -5, -10, or -15 °C) or time of year exposed (January or March) after 120 hours of low temperature exposure. Retained terms with significant p-values (p < 0.05) are bolded.

Measure	Initial model	Minimal adequate model	Term	F	Df	P
Glucose	Y = protein mass + exposure temperature × time of year exposed and sampled	Y = null model				
Glycogen	Y = protein mass + exposure temperature × time of year exposed and sampled	Y = protein mass + temperature	**Protein** **Temperature**	**40.050** **6.638**	**1, 42** **1, 42**	**<0.001** **0.014**
Glycerol	Y = protein mass + exposure temperature × time of year exposed and sampled	Y = protein mass + exposure temperature × time of year exposed and sampled	Protein **Temperature** **Time of year** Temperature × Time of year	0.272 **5.700** **7.713** 2.836	1, 38 **1, 38** **2, 38** 2, 38	0.605 **0.022** **0.002** 0.071
Sorbitol	Y = protein mass + exposure temperature × time of year exposed and sampled	Y = exposure temperature × time of year exposed and sampled	**Temperature** **Time of year** **Temperature × time of year**	**6.428** **10.651** **8.352**	**1, 39** **2, 39** **2, 39**	**0.015** **<0.001** **<0.001**
acTAG	Y = total lipid mass + exposure temperature × time of year exposed and sampled	Y = total lipid mass	**Total lipid mass**	**768.90**	**1, 43**	**<0.001**
lcTAG	Y = total lipid mass + exposure temperature × time	Y = total lipid mass + temperature × time of year	**Total lipid mass**	**643.40** 1.04	**1, 38** 1, 38	**<0.001** 0.315

of year exposed and sampled	Temperature	1.16	2, 38	0.324
	Time of year	**3.79**	**2, 38**	**0.032**
	Temp × time of year			

Table C.18. ANCOVA statistics comparing glucose and glycogen mass among *Eurosta solidaginis* pre-pupae during control conditions. Flies were maintained at 0 °C for three months, and sampled either early January, in the middle of February, or the end of March. Retained terms with significant p-values (p < 0.05) are bolded.

Measure	Initial model	Minimal adequate model	Term	F	Df	P
Glucose	Y = protein mass + time of year sampled	Y = protein mass	**Protein**	**4.701**	**1, 13**	**0.049**
Glycogen	Y = protein mass + time of year sampled	Y = protein mass + time of year sampled	**Protein** **Time of year**	**7.016** **6.641**	**1, 11** **2, 11**	**0.023** **0.013**
Glycerol	Y = protein mass + time of year sampled	Y = null model				
Sorbitol	Y = protein mass + time of year sampled	Y = time of year	**Time of year**	**4.716**	**2, 12**	**0.031**
acTAG	Y = total lipid mass + time of year sampled	Y = total lipid mass	**Total lipid mass**	**242.50**	**1, 13**	**<0.001**
lcTAG	Y = total lipid mass + time of year sampled	Y = total lipid mass	**Total lipid mass**	**242.50**	**1, 13**	**<0.001**

Table C.19. ANCOVA statistics for glucose and glycogen in overwintering *Eurosta solidaginis* pre-pupae as a result of exposure type. Repeatedly-exposed flies were exposed to either -10, -15, or -20 °C, with periods between exposures of either daily, every 5 days, or every 10 days, and received 10 exposures as pre-pupae. Flies that received prolonged exposures either received their exposure at -10, -15, or -20 °C in January (sampled either immediately or in March) or March (sampled immediately). Control flies were maintained at 0 °C all winter and were sampled in either January, February, or March (time of year sampled was retained only for sorbitol, see Table 27). Retained terms with significant p-values (p < 0.05) are bolded.

Measure	Initial model	Minimal adequate model	Term	F	Df	P
Glucose	Y = protein mass + exposure group	Y = null model				
Glycogen	Y = protein mass + exposure group	Y = protein mass	**Protein**	**120.89**	**1, 193**	**<0.001**
Glycerol	Y = protein mass + exposure group	Y = protein mass + exposure group	Protein **Exposure group**	0.228 **5.930**	1, 85 **18, 85**	0.634 **<0.001**
Sorbitol	Y = protein mass + exposure group	Y = exposure group	**Exposure group**	**8.814**	**20, 84**	**<0.001**
acTAG	Y = total.lipid.content + exposure group	Y = total.lipid.content + exposure group	**Total lipid content Exposure group**	**2132.58** **5.90**	**1, 144** **4, 144**	**<0.001** **<0.001**
lcTAG	Y = total.lipid.content + exposure group	Y = total.lipid.content + exposure group	**Total lipid content Exposure group**	**1189.46** **2.78**	**1, 91** **12, 91**	**<0.001** **0.003**

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Sue Chamberlain

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Sent: 09 November 2012 19:12
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Marshall, KE & Sinclair, BJ. (2011). The sub-lethal effects of repeated freezing in the woolly bear caterpillar *Pyrrharctia isabella*. *JEB* 214:1205-1212.
 Marshall, KE & Sinclair, BJ. (2012). The impacts of repeated cold exposure on insects. *JEB* 215, 1607-1613.

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Jennifer

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From: Katie Marshall

Sent: 09 November 2012 19:22
To: proceedingsb
Subject: Right to publish paper in thesis

Dear editor,

I'm a graduate student at the University of Western Ontario currently in the process of preparing my PhD thesis.

An important chapter in my thesis was published in your journal (Marshall, KE and Sinclair BJ, 2010, 277: 963-969). I've followed the link you recommended for obtaining permission to publish this paper I wrote in my thesis, however it is currently requiring me to pay for this service.

Is there a way I can obtain permission that does not require pay?

Cheers and thanks,
Katie

Curriculum Vitae

Name:	Katie Elizabeth Marshall
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Publications:

A total of 12 publications in peer-reviewed journals.

Sinclair, B.J., Stinziano, J.R., Williams, C. M., MacMillan, H.A., **Marshall, K.E.,** and Storey, K.B. (2013). Real-time measurement of metabolic rate during freezing and thawing of the wood frog, *Rana sylvatica*: Implications for overwinter energy use. *Journal of Experimental Biology.* 216, 292-302.

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Bazinet, A., **Marshall, K.E.**, MacMillan, H.A., Williams, C.M., Sinclair, B.J. (2010) Rapid changes in desiccation resistance in *Drosophila melanogaster* are facilitated by changes in cuticular permeability. *Journal of Insect Physiology.* 56, 2006-2012.

Marshall, K.E. & Sinclair, B.J. (2010) Repeated cold exposure results in a survival-reproduction trade-off in *Drosophila melanogaster*. *Proceedings of the Royal Society B.* 277, 963-969.