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# Cold Tolerance of each life stage of the sub-alpine willow leaf beetle, Chrysomela aeneicollis

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Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

# COLD TOLERANCE OF EACH LIFE STAGE OF THE SUB-ALPINE WILLOW LEAF BEETLE, *CHRYSOMELA AENEICOLLIS* (COLEOPTERA: CHRYSOMELIDAE)

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**Abstract**

To understand the potential effects of climate change on the sub-alpine willow leaf beetle, the response to cold and mechanisms involved in cold tolerance were investigated for all life stages. Microhabitat choice and temperatures within each microhabitat were related to median lethal temperature for each life stage. Summer active and winter quiescent adults are freeze tolerant, eggs and pupae are freeze avoidant and all larval stages are chill susceptible. Quiescent adults accumulated the highest concentration of glycerol (~24 mM) and haemolymph osmolality (875 mOsm). Haemolymph from pupae had angular crystals suggestive of antifreeze agent activity, but this was absent in the haemolymph of other life stages. Quiescent adults must move to a thermally buffered microhabitat in the soil to survive the winter in the Sierra Nevada Mountains in California. Low temperatures beyond the limits of the cold tolerance of the willow leaf beetle occur often at the mid-willow, but the quiescent adults are protected from these cold exposures in the soil beneath the willow. Overall, the willow leaf beetle uses both behavioural avoidance strategies and physiological mechanisms to survive cold exposures in its habitat.

**Keywords:**

Willow leaf beetle, *Chrysomela aeneicollis*, Coleoptera, Chrysomelidae, cold tolerance, cryoprotectants, freeze avoidance, supercooling, alpine, holometabolous, life stages, antifreeze agents, microhabitat temperature, quiescence

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**List of Abbreviations**

AFA: Antifreeze agent

BPC: Big Pine Creek

BC: Bishop Creek

GC: Gas chromatography

LLT: Lower lethal temperature

$LT_{50}$: Temperature at which 50% mortality is predicted

RCH: Rapid cold hardening

RC: Rock Creek

SCP: Supercooling Point

S.D.: Standard deviation

S.E.M.: Standard error of the mean

WLB: Willow leaf beetle

WMRS: White Mountain Research Station

Hsp: Heat shock protein

# CHAPTER 1. INTRODUCTION

## 1.1 Sub-alpine environments

Temperature is a factor limiting the survival and distribution of insect populations. Sub-alpine regions are ideal systems in which to study mechanisms by which organisms cope with a changing environment (Franklin and MacMahon, 2000). A sub-alpine environment is defined as a high elevation environment that is above the montane forest and below the tree line (Douglas, 1972). Globally, the elevation at which the tree line is found decreases with increasing latitude and can be altered over time by changes in climate (Körner, 1998). Sub-alpine and montane environments often have significant daily thermal fluctuations of tens of degrees centigrade (Sømme, 1989). These environments also tend to have drier climates due to decreased vapour pressures at high altitudes (Lazaridis, 2010). This decreased vapour pressure results in lower ambient temperatures and available water in relation to lowland environments (Lazaridis, 2010).

Higher elevation sites within sub-alpine areas typically have longer periods of snow cover and a longer winter season than lower elevation sites (Figure 1.1 ; Sømme, 1989) however, total annual snow cover has decreased by 10% since 1972 in the northern hemisphere (Serreze et al., 2000). Snow is an effective insulator against low temperatures, protects overwintering insects against abrasion by wind-blown ice crystals (Danks, 2004) and lowers the frequency of insect freeze-thaw cycles (Marshall and Sinclair, 2012; Sinclair, 2001b). Finding a protected microhabitat such as under the snow cover or under leaf litter is a common survival strategy among overwintering insects. An adequate layer of snow often prevents the temperature of the leaf litter and soil from falling much below 0 °C, providing an effective insulating buffer (Carcamo et al., 2009; Lam and Pedigo, 2000); under snow cover, daily temperatures only fluctuate by 1 - 2 °C (Bennett et al.,

2003). Increased daily thermal variations from reduced winter snow cover can increase the frequency at which insects are exposed to lethal cold events (Templer et al., 2012). Sub-alpine and montane environments are also subjected to night-time frosts during the summer months (Larcher et al., 2010), which could result in lethal cold exposures for local insect populations that would not normally be experienced in lowland areas.

Insects in montane environments have adaptations to survive in these harsh environments including: smaller body size, reduced flight capacity, increased melanisation, increased cold tolerance and life cycles synchronised with seasonal changes in climate to maximize resource availability (for review, see Sømme, 1989). Although most insect groups have montane representatives, the major terrestrial groups in the northern temperate alpine arthropod fauna are spiders (Lycosidae and Salticidae), springtails (Collembola), and beetles (Coleoptera) (Edwards, 1987). Many of these taxa rely on the native plant species for food and shelter to survive in the harsh climates of the alpine and sub-alpine zones. Several insect taxa are also important pollinators in montane environments including: beetles (Coleoptera), flies (Diptera), Butterflies (Lepidoptera) and sawflies (Hymenoptera) (Kevan and Baker, 1983).

Climate change models for montane taxa forecast net extinctions to increase at low elevations with migrations of populations to higher elevations (Parmesan, 1996). Montane ecosystems are increasingly recognized as vulnerable to climate change which is expected to threaten some species with a greater probability of extinction (IPCC 2007). Key factors influencing species vulnerability are potential changes in the frequency, intensity, and persistence of climate extremes (e.g. heat waves, heavy precipitation, and drought) and in climate variability (e.g., the El Niño Southern Oscillation) (IPCC 2007). Many alpine plant species are expected to go extinct with even small changes in the annual mean

temperature due to climate changes (Theurillat and Guisan, 2001) which could have detrimental effects on the available microhabitats for local insect species.

### 1.2 Microhabitats occupied by montane insects

Microhabitats are the small-scale physical areas inhabited by particular organisms or populations. Due to their small size, insects can avoid many annual thermal extremes by exploiting different microhabitats within their environment. Microhabitats include under shrubs (Tozetti and Martins, 2008), crevices under rocks (Sinclair et al., 2001), under the leaf litter (Werner, 1978) or under snow cover (Irwin and Lee, 2000; Irwin and Lee, 2003; Williams et al., 2012). Buffered temperatures in a protected microhabitat can potentially reduce the exposure to low temperatures and repeated freeze-thaw cycles for many insects (Coulson et al., 1995; Sinclair 1997; Ramløv 1999).

Snow cover can persist in alpine and sub-alpine regions for many months (Wipf and Rixen, 2010), and protect insects from low temperatures (Sømme, 1989). Due to the frequency of cold exposures in alpine and sub-alpine habitats both during the winter and summer months, there is increased cold tolerance in the high elevation insect populations over the lowland ones (Sømme, 1982). The harsh conditions of these habitats have resulted in a higher desiccation tolerance than lowland populations and a higher proportion of alpine insects using a freeze tolerant strategy (Sømme, 1989). Invertebrate microhabitats have been investigated in the context of overwintering (see Leather et al. 1993, for review), but few studies have addressed microhabitat selection in both summer and winter in alpine invertebrates (see Sinclair et al., 2001).

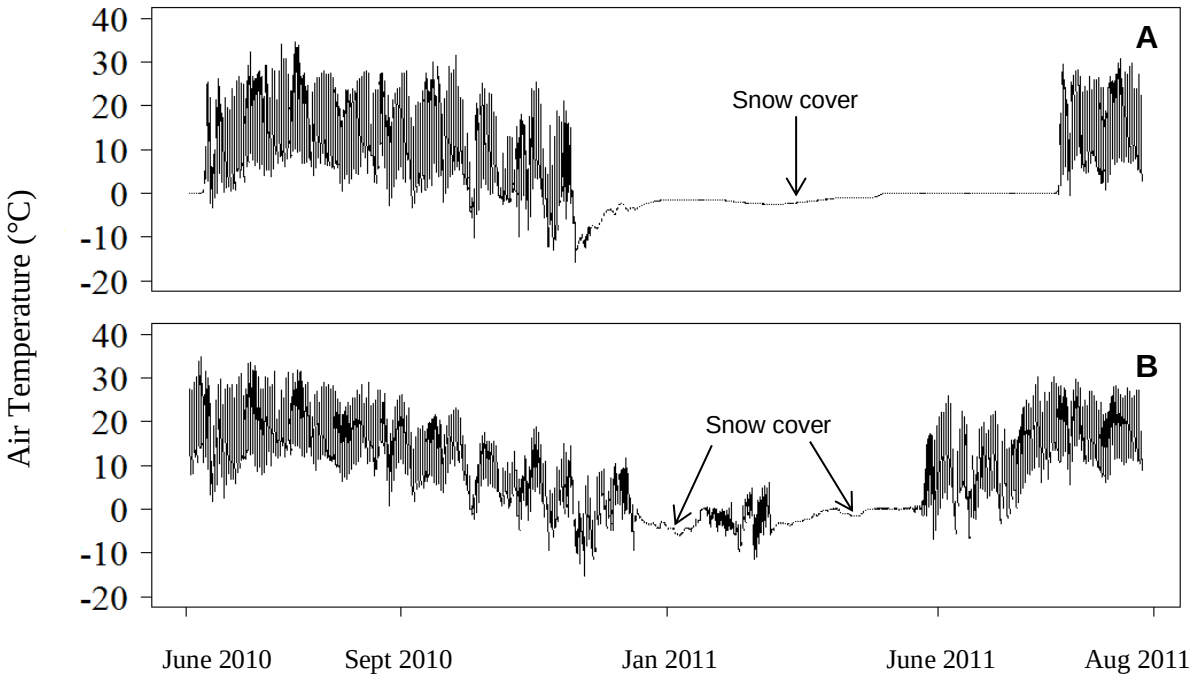


**Figure 1.1** Air temperatures recorded at approximately 1.2 m above the ground, inside the canopy of *Salix orestra*, from 2010-2011 at high and low elevation in the Big Pine Creek Drainage in the Sierra Nevada Mountains. Temperatures were recorded at 30 minute intervals and points that are very close in temperature and time appear almost black on the graph. **A.** Air temperatures at 3353 m. **B.** Air temperature at 2773 m. Periods of constant temperatures around 0 °C are indicative of snow cover.

### 1.3 Insect life history

A holometabolous insect's life cycle begins with development from an egg, through one or more larval stages and complete metamorphosis into its final adult form. A univoltine life cycle will have only one generation within each growing season. In temperate environments, one or more life stages must survive the winter months by developing increased cold tolerance if they are unable to migrate to a warmer habitat. Through the early life stages, growth and development in preparation for metamorphosis requires a constant influx of energy and may result in a trade-off of reduced cold tolerance to maintain high growth rates (Shiota and Kimura, 2007). The relationship between the phenology of the active life stages of an insect and its host plant can result in optimal resource availability for growth and to synchronize populations for events such as adult hatching (Danks, 1987; Denlinger, 2002). Temperature-driven phenology is the timing of natural events that is influenced by climate (Van Asch and Visser, 2007). Phenology research has focused on spring events such as first flight, flowering, or insect breeding dates (e.g. Thackeray et al., 2010). If insects shift their phenology and enter into diapause early, when autumn temperatures are still warm, they can suffer significant energy drain. This could result in life-history trade-offs such as decreases in adult size and fecundity that could lower the fitness of individuals and increase potential for population declines in temperate, univoltine, insects. (Williams et al., 2012).

The life cycle of many insects includes a specific life stage that enters into diapause which can last for many months. In most species, diapause is an endocrine-mediated period of developmental arrest that is often associated with times of unfavourable environmental conditions (e.g. cold, heat or lack of host plant resources) (Kostal 2006) and is accompanied by a cessation of growth and development (Tauber et

al., 1986). The onset of diapause for insects in temperate regions usually precedes seasonal drops in temperature and is often followed by a period of quiescence. Diapause prevents an insect from responding to environmental fluctuations that could cause the insect to resume active behaviours before their normal growing season begins (Danks, 2006). Whereas, quiescence is an environmentally-mediated period of dormancy due to low temperatures (Danks, 1987) that allows development in spring to resume when temperatures or photoperiod rise above a species-specific threshold (Koštál, 2006).

The life stage during which diapause occurs is species-specific. For example, the emerald ash borer (*Agrilus planipennis*) and the goldenrod gall fly (*Eurosta solidaginus*) both enter into diapause as prepupae, whereas the midge (*Chironomus riparius*) enters into diapause as a larva (Goddeeris et al., 2001) and the silkworm (*Bombyx mori*) enters into diapause as an egg (Zhao et al., 2012). Diapause can be either obligate or facultative depending on the insect species. Obligate diapause occurs at the same point in the life cycle for every generation regardless of the environmental conditions. For example, the ant, *Lepisiota semenovi*, enters into obligate diapause in response to signals from within the colony and temperature and photoperiod simply modify the timing of the diapause cue (Kipyatkov and Lopatina, 2009). Facultative diapause is initiated by changes in the environment experienced by individuals such as drops in temperature or a shorter photoperiod (Danks, 1987; Denlinger, 2002; Tauber et al., 1986). Some species of trichogrammatid wasps require both thermal and photoperiod cues to enter facultative diapause (Reznik, 2011).

When an insect is in diapause, its metabolic rate is depressed, all active behaviours, including mating and feeding, are stopped and its responses to external stimuli are greatly reduced via reduced thermal sensitivity (Koštál, 2006). The metabolic

rate of diapausing golden rod gall fly (*Eurosta solidaginis)* larvae in midwinter was just 35–40% of the early-autumn value at the same temperature (Irwin and Lee, 2002; Levin et al., 2003). Since all feeding behaviour is halted, insects in diapause must survive off their internal energy stores. By reducing metabolic rate, insects can extend the length of time they can wait out environmental stresses via the slow catabolism of stored body-fuel reserves (Hahn and Denlinger, 2007). Insects often halt the endocrine-mediated signalling pathway long before the unfavourable environmental conditions are actually lifted (Koštál, 2006). However, the insect remains in a state of quiescence until the environmental conditions improve (Koštál, 2006).

The onset of diapause often accompanies the development of cold hardening. Cold hardening is a change in the physiological make-up of a species that allows it to survive what would otherwise be lethal effects of low temperatures (Zachariassen, 1985). Functionally, the term 'hardening' refers to an increase in thermal tolerance that can happen on either a rapid or long-term acclimation time scale (Loeschcke and Sørensen, 2005). The flesh fly (*Sarcophaga crassipalpis*) requires the onset of diapause for the development of maximal cold hardiness (Lee and Denlinger, 1985; Michaud and Denlinger, 2007), while many other insect species develop cold hardiness independently of diapause (Hodkova and Hodek, 2004). Cold exposure and diapause can interact in upregulating aquaporins to permit water and glycerol transport in fat body tissues (Izumi et al., 2007) as well as the stimulation of cryoprotectant synthesis (Furusawa et al., 1982; Storey and Storey, 1991).

Chilling is the most common factor terminating winter diapause in the field (Hodek, 2002). However, chilling is not a prerequisite for the completion of diapause in all insects; there are multiple cues for diapause completion. For example, internal freezing

is a necessary factor for termination of prepupal diapause in the Japanese poplar sawfly *Trichiocampus populi* (Tanno, 1970). Whereas, photoperiod lengthening is a necessary factor in the termination of diapause for the predatory mite, *Euseius finlandicus* (Broufas et al., 2006).

**1.4 Insect responses to low temperatures**

Insects are ectotherms, meaning that their body temperature closely matches that of their environment. Therefore, the geographic distribution and fitness of many species of insects are limited, in part, by their tolerance to low temperatures (Chown, 2010; Crozier, 2004; Regniere et al., 2012). At low temperatures, insects are at risk of freezing if temperatures fall below their freezing point (Lee 2010a). The temperature at which the body fluids of an insect freezes is called the supercooling point (SCP) and is defined as the lowest temperature immediately preceding the exotherm from the latent heat of crystallization indicative of internal ice formation (Lee, 2010a) (Figure 1.2). The three most common responses to cold shown by insects are: chill susceptibility, freeze avoidance, and freeze tolerance (Lee, 2010a). These insect responses to cold can be divided into cold intolerant, or cold tolerant categories. Many insects in both of these categories can undergo cold hardening and increase their overall cold tolerance.

*1.4.1 Cold intolerant insects*

Chilling injuries cause many insects to die before they freeze (Lee, 2010a). Chill susceptible insects experience chilling injuries within their tissues that are unrelated to internal ice crystal formation (Koštál et al., 2004). For example, adult fruit flies (*Drosophila melanogaster*) are chill susceptible and die from exposure to -5 °C even though the SCP is ca. -20 °C (Czajka and Lee, 1990). The peach potato aphid (*Myzus persica*e) has a SCP of ca. -25 °C, but also dies after exposure to -5 °C (Bale et al., 1988).

Chilling injuries that cause this mortality can be the result of many biological processes being affected including: a loss of ion balance between the haemolymph and the gut tissues (MacMillan and Sinclair, 2011b), membrane phase transitions (Quinn, 1985), oxidative stress (Rojas and Leopold, 1996), mismatching of metabolic pathways (Koštál et al., 2004) and the activation of cellular apoptotic pathways (Yi et al., 2007).

*1.4.2 Cold tolerant insects*

Cold tolerant insects can broadly be categorised as either freeze avoidant or freeze tolerant. To determine the response to cold of an individual insect, two questions must be addressed: i) did the individual freeze upon exposure to low temperatures, and ii) did the individual die before freezing? Ice formation within the extracellular spaces can be survived by freeze tolerant insects while freeze avoidant insects will survive cold exposure until internal ice formation occurs. To increase their low temperature tolerance beyond the limits of cold hardening, cold tolerant insects employ mechanisms that are often absent in chill susceptible species.

One mechanism used by insects to increase their cold tolerance involves the upregulation of biochemical pathways involved in cryoprotectant synthesis which ultimately increases the concentration of cryoprotectants within the insect's haemolymph and tissues (Doucet et al., 2009). This accumulation of cryoprotectants results in an increase haemolymph osmolality (Zachariassen and Kristiansen, 2000) and a depression of the haemolymph melting point. A one osmole increase in haemolymph osmolality will depress the melting point by 1.86 °C (Zachariassen, 1985), which subsequently lowers the SCP and allows the insect to control when they freeze. Many cryoprotectants are common in insects that respond to cold with either a freeze avoidant or freeze tolerant strategy,

however, similar cryoprotectants can perform different functions in each of the responses to cold (Block, 1991).

Colligative cryoprotectants are usually simple sugars and polyols (e.g. glucose, glycerol and sorbitol) (Sømme, 1982) or other small molecules such as free proline (Nieminen et al., 2012) which act in a concentration-dependant manner (Storey, 1997). These compounds can accumulate to high concentrations after seasonal acclimatisation for the overwintering life stage of many insect species. For example, the concentration of haemolymph free proline can increase to 18 mM in the haemolymph of the fire bug, *Pyrrhocoris apterus*, (Koštál et al., 2011b) and 100 mM in the alpine weta, *Hemideina maori* (Ramløv, 1999). The concentration of glycerol can also be increased seasonally in many insects, such as 4 M in the freeze avoidant emerald ash borer, *Agrilus planipennis*, (Crosthwaite et al., 2011) and 600 mM in the freeze tolerant goldenrod gall fly, *Eurosta solidaginis* (Morrissey and Baust, 1976). The high concentrations of colligative cryoprotectants maintain cell volume during extracellular freezing and reduce the amount of extracellular ice by equalizing osmotic pressures inside and outside of the cell thereby preventing additional water from being drawn out of the intracellular compartment and increasing the minimum cell volume (Zachariassen, 1985).

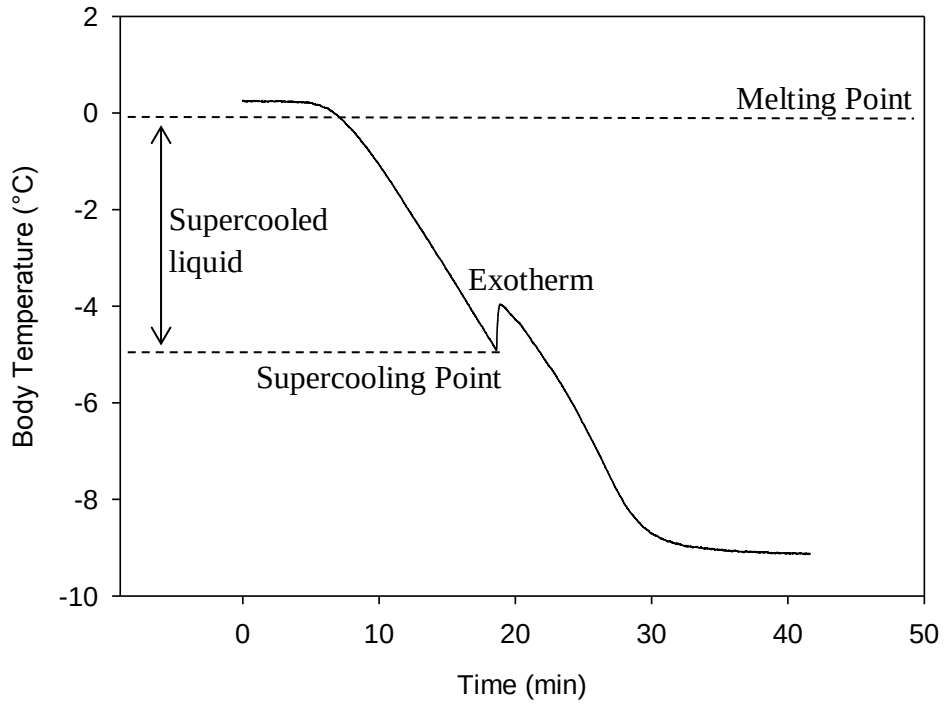


**Figure 1.2** Temperature trace of a cooling insect showing the supercooling point and an exotherm from the latent heat of crystallization of ice formation. The insect's body fluids remain liquid in a supercooled state as it cools below its melting point. Body fluids freeze at the supercooling point, defined as the lowest temperature before the exotherm. After Lee (1989).

Non-colligative cryoprotectants interact with ice and function either as antifreeze agents by preventing the growth and propagation of ice crystals or as recrystallisation inhibitors. Recrystallisation is the process of ice crystal growth in frozen solutions, as larger (lower energy-state) ice crystals accrete water molecules at the expense of smaller (higher energy-state) ice crystals (Lee, 2010a). Non-colligative cryoprotectants in insects include antifreeze proteins (Block, 1990) and glycolipids (Walters et al., 2011). Antifreeze proteins adsorb onto the surface of ice crystals at preferred growth sites thereby restricting growth. This process can be observed as a lowering of the thermal hysteresis freezing point (Raymond and Devries, 1977; Raymond et al., 1989).

Freeze tolerant insects initiate controlled ice crystal formation in their extracellular fluid at high sub-zero temperatures (Lee, 2010a). Some insects retain or produce ice nucleating agents to increase their SCP and initiate heterogeneous extracellular ice formation (Li, 2012) which protects their tissues against freezing injuries. Ice nucleating agents include food in the gut, ice nucleating proteins (Zachariassen and Hammel, 1976), crystalloid inorganic compounds such as calcium phosphate spherules (Mugnano et al., 1996) and ice-nucleating active bacteria (Kaneko et al., 1991). Frozen insects have lower metabolic rates which may conserve energy and water during winter (Irwin and Lee, 2002), however the risk of mechanical recrystallisation damage and osmotic stress on tissues and cells may increase (Storey and Storey, 1988). Antifreeze agents slow recrystallisation in the haemolymph by binding to the surface of ice crystals, and prevent uncontrolled growth around an ice nucleating agent (Barrett, 2001; Duman, 2001; Yu et al., 2010). Ice crystals incorporate water molecules to grow and reach their equilibrium content within the haemolymph over time (Chapsky and Rubinsky, 1997; Wang et al., 2012). Freezing in the extracellular compartment concentrates the

extracellular fluid and draws water out of the cells, down an osmotic gradient, which causes them to shrink (Zachariassen, 1985). The shrinking of the cells results in vapour pressure equilibrium between the intracellular and extracellular fluids, which prevents spontaneous ice formation inside the cells, regardless of their ice nucleating agent content (Zachariassen, 1985).

Freeze avoidant insects often have antifreeze proteins (Zachariassen and Kristiansen, 2000) and/or glycolipids (Walters et al., 2011), which stabilise supercooled haemolymph at low temperatures and prevent the growth of ice nuclei. High concentrations of low molecular weight cryoprotectants, such as glycerol, colligatively depress the SCP and stabilise protein structure against low temperature denaturation (Bale, 2002). Freeze avoidant insects must also eliminate or sequester ice nucleators in the gut and haemolymph to avoid ice formation, which further lowers their SCP (Lee, 2010a).

Inoculative freezing from external ice can be a major cause of mortality for freeze avoidant insects, but it can be avoided by secreting a waxy layer on the outside of the cuticle (Olsen et al., 1998; Crosthwaite et al., 2011). The emerald ash borer (*Agrilus planipennis*) is a freeze avoidant beetle that synthesises antifreeze agents, has a SCP below ca. -34 °C, a waxy cuticle and haemolymph glycerol concentrations of ca. 4 M in its overwintering prepupal stage (Crosthwaite et al., 2011).

Freeze avoidance and freeze tolerance represent alternative strategies for insect survival at low temperatures. Freeze avoidance is thought to be basal within the arthropod lineages and it seems that freeze tolerance has evolved convergently at least six times among insects (Sinclair et al., 2003). A freeze avoidant insect can benefit by remaining in a supercooled state for extended periods of time with minimal physiological stress (Sinclair, 1999; Voituron et al., 2002). However, if freeze avoidant insects experience

unexpected cold snaps in a summer-active state, with food present in the gut which as a source of ice nucleation, high rates of mortality may be observed. For example, summer cold snaps have been reported as a cause of death for freeze avoidant soil microarthropods that are otherwise cold hardy because the insects are unable to eliminate gut contents and undergo acclimatization as they would at the onset of winter conditions (Coulson et al., 1995; Sinclair et al., 2003).

According to Sinclair et al. (2003), freeze tolerance allows insects to take advantage of mild winter periods, which present opportunities for growth and development if the warming period were adequately long. During these periods, an individual must be able to make a rapid transition from a cold hardy to an active state, and survive the rapid onset of cold when the warm period ends. An insect that exhibits some freeze tolerance throughout the year could survive an unexpected freezing event without seasonal cold hardening, with food in the gut, especially if it was of a relatively short duration and temperatures did not cross lethal thresholds (Sinclair et al., 2003).

**1.5 Plasticity of cold tolerance in insects**

Some insects switch between responses to cold seasonally (Lee and Denlinger, 1985). For example, the gall fly (*Eurosta solidaginis*) can switch from freeze avoidance to freeze tolerance after it is acclimatised to the onset of winter conditions (Morrissey and Baust, 1976). Insects can also switch their responses to cold between years (Horwath and Duman, 1984). For example, the beetles, *Dendroides canadensis* and *Cucujus clavipes*, switched from freeze tolerant to freeze avoidant between 1980 and 1983 (Kukal and Duman, 1989).

All insects, cold tolerant and cold intolerant, can show remarkable plasticity in their cold tolerance within a single life stage. Plasticity can occur through acclimation

(>12 h) or through rapid cold hardening (minutes to hours) (Lee, 2010b). Some insects require cold hardening to survive exposure to the low temperatures experienced in their environment (Powell and Bale, 2006). Rapid cold hardening (RCH) is induced by severe low temperatures that could become lethal over a longer time period (Sinclair and Roberts, 2005). Through RCH and after recovery from the cold exposure, an insect can upregulate the production of heat shock proteins or low molecular weight cryoprotectants and increase their cold tolerance for future cold exposures (Lee et al., 1987; Lee, 2010b). For example, the sycamore lace bug (*Corythucha ciliata*) undergoes RCH after exposure to 0 °C for 4 h and after recovery, survival following exposure to -12 °C increased by 46% (Ju et al., 2011). Insect cold acclimation involves the accumulation of large quantities of low molecular weight cryoprotectants such as polyols, sugars or amino acids, including primarily, glycerol, sorbitol, trehalose, sucrose, proline and alanine (Lee, 2010a; Storey and Storey, 1988). Together, these cryoprotectants increase the osmolality of an insect's haemolymph and result in increased low temperature tolerance.

Longer term cold hardening processes, such as acclimation (in the laboratory) and seasonal acclimatisation (in the field), occur over a period of weeks or months (Bowler, 2005; Lagerspetz, 2006; Sinclair and Roberts, 2005).. Insects can adjust their thermal tolerance in response to daily or seasonal temperature changes, enhancing their activity and survival (Angiletta, 2009). Enzymes are key factors in this adjustment of thermal tolerance in insects after seasonal acclimatisation (for review, see Somero, 1995). Often, seasonal acclimatisation is anticipatory, and is a response to changes in photoperiod (Bradshaw, 2010). Without acclimatisation, temperature changes can disrupt an insect's metabolism by altering the thermal sensitivity of enzymes ($Q_{10}$ effects) (Hochachka and Somero, 1984) or through changes in enzyme kinetics and activity. To compensate for

this disruption, animals can increase enzyme concentrations in cell membranes, modify existing enzymes, increase ligand concentrations or synthesize different isozymes (Hochachka and Somero, 1984).

### 1.6 *Chrysomela aeneicollis*

The willow leaf beetle, *Chrysomela aeneicollis* (Coleoptera: Chrysomelidae), is a member of the family of leaf beetles, which is one of the largest families of beetles, with 35,000 described species worldwide (Riley et al., 2003). Willow leaf beetle populations can be found ranging from Lake Louise, Alberta at the northern range-edge to the Sierra Nevada Mountains in California at the southern range-edge (Brown, 1956). The willow leaf beetle is also found along a large elevation gradient within this distribution with populations in the mountains as well as along coastal areas (Rank, N.E., personal communication). The willow leaf beetle is a non-pest species living and feeding on willow leaves (*Salix spp*.). The willow leaf beetle has a holometabolous, univoltine life cycle meaning they progress through a life cycle with full metamorphosis once per year. Adults overwinter in diapause under the leaf litter (Brown, 1956), mate in the spring and lay eggs on the underside of willow leaves. After three larval instars, they pupate while hanging from the underside of the leaves. Adults eclose in the fall and have grey spots which turn red if the beetles feed on willow before entering diapause (Smiley and Rank, 1986).

Willow leaf beetle populations in the Sierra Nevada Mountains range from 2700 m to 3600 m above sea level (Dahlhoff et al., 2008). The field environmental conditions (temperature and snow cover) in the Sierra Nevada Mountains in California are driven by large scale climatic variability. The mid-latitude location of the range and its proximity to the moderating influence of the Pacific Ocean make the Sierra Nevada Mountains

vulnerable to climatic anomalies, such as the El Niño Southern Oscillation. These anomalies can produce large interannual differences in temperature in mountain ranges throughout the western Great Basin of North America (Melack et al., 1997). In general, the east slope of the Sierra Nevada Mountains is warmer than west slope at a comparable elevation, due to the primarily westerly wind over the mountain range, which often results in adiabatic warming as the air descends (Lundquist and Cayan, 2007).

There are large willow leaf beetle populations found along steep elevational gradients within three mountain drainages near the White Mountain Research Station (WMRS) on the eastern slope of the Sierra Nevada Mountains: Big Pine Creek (BPC), Bishop Creek (BC) and Rock Creek (RC). RC is the most northern drainage and BPC is the most southern drainage. The beetle populations within each of these drainages experience small scale environmental variation, RC is the coolest drainage and BPC is the warmest (Dahlhoff and Rank, 2000; Rank, 1992a) influenced by the slope aspect and gradient.

To date, research on *C. aeneicollis* has focused on summer heat tolerance and population dynamic trends correlated with genotype frequencies in summer-acclimated beetles. Willow leaf beetle populations differ in the frequency of phosphoglucose isomerase (PGI) genotypes. PGI enzymes catalyse the reversible conversion of glucose-6-phosphate to fructose-6-phosphate, and it is at the crux of both glycolysis and gluconeogenesis (Achari et al., 1981). In the willow leaf beetle populations, the distribution of the PGI 1 and PGI 4 alleles varies by drainage and this distribution affects several measures of performance. Summer adults with the PGI-1 allele ran faster up a wooden dowel after a single stressful exposure (hot or cold), whereas individuals with the PGI-4 allele ran faster after multiple stressful exposures; larval running speed was only

affected by single stressful exposures (Dahlhoff and Rank, 2007). Willow leaf beetle larvae upregulate the synthesis of heat-shock proteins (Hsps) after exposure to high summer temperatures (33 °C) more if they have the PGI-1 allele than if they have the PGI-4 allele (Dahlhoff and Rank, 2000).

PGI is one of very few enzymes to vary along with thermal tolerance, and it has been related to higher rates of survival after cold exposure in the willow leaf beetle (Neargarder et al., 2003) and in butterflies (Karl et al., 2008; Watt, 1977); however the reason for this is still unclear. The PGI 1 allele has been associated with upregulation of Hsps which results in higher cold tolerance for night-time low temperatures during the summer months (Dahlhoff and Rank, 2007; McMillan et al., 2005). The only cold tolerance data published to date shows the $LT_{50}$ for recently pupated summer adult beetles is -4.5 °C (Rank and Dahlhoff, 2002). Since mortality is caused by relatively mild low temperatures, these beetles may be chill susceptible.

The willow leaf beetle adults enter diapause shortly after emergence in late August, and they overwinter in the leaf litter beneath their host plants (Rank, 1992b). Nothing is known about the physiology of the adults during winter diapause, or the ability of each life stage to tolerate cold snaps in the spring, summer and fall. In the Sierra Nevada Mountains, the winter microclimate is changing much faster than the summer microclimate due to variation in snow cover between years (J. Smiley, personal communication).

**1.7 Objectives**

This thesis is part of a wider project on the willow leaf beetle aiming to understand the impacts of environmental thermal variation on fecundity and survival in this sub-alpine insect species in North America. The overall objective of this study is to

understand the cold tolerance of the willow leaf beetle in both the overwintering life stage as well as all of the summer life stages that have often been overlooked. Low temperatures can occur at any time of year in the Sierra Nevada Mountains, so in the context of the entire life cycle of the willow leaf beetle, there must be a balance between the demands for cold tolerance and the energetic demands of growth and development. My goal is to determine if there is a link between the cold tolerance correlates quantified in the lab and microhabitat temperatures.

My first objective is to determine the response to cold by each life stage of the willow leaf beetle, *Chrysomela aeneicollis.* I will distinguish between the response to cold (chill susceptibility, freeze avoidance or freeze tolerance) in each of the life stages. The life stages to be examined include: eggs, first instar larvae, second instar larvae, third instar larvae, pupae, summer adults and quiescent adults.

My second objective is to measure some common biochemical correlates of cold tolerance in all of the life stages of the willow leaf beetle. These biochemical correlates include glycerol content, thermal hysteresis activity, haemolymph osmolality and antifreeze agent activity. Differences in the amount of biochemical correlates of cold tolerance may account for observed variability in the mechanisms underlying differences in responses to cold between life stages.

My third objective is to use microhabitat temperature data to find the frequency, duration and extremes of cold exposures that fall below the median lethal temperatures for each life stage. Microhabitat temperature data collected using temperature data loggers placed at the mid-willow, base of the willow and soil beneath the willow will be compared for all analyses. I will link the microhabitat temperatures to changes in microhabitat choice for quiescent beetles. For one site within each of the three drainages

(RC, BC, BPC) at similar elevations, I will determine the differences between extreme temperatures and frequency of potentially lethal cold exposures experienced annually by each life stage. I will also compare extreme temperatures and frequency of potentially lethal cold exposures for each life stage at four sites along an elevation gradient within the Big Pine Creek drainage.

# CHAPTER 2: METHODS

## 2.1 Study animals

I collected *Chrysomela aeneicollis* of all life stages (eggs, larvae, pupae, non-diapausing and diapausing adults) from three drainages in the Sierra Nevada mountains in southern California: Big Pine Creek (BPC), Bishop Creek (BC) and Rock Creek (RC) (see Table 2.1). All beetles were collected at elevations between 3167 and 3215 m from all drainages in this study. Since the number of available individuals was not equal for each drainage, and initial analysis of supercooling point data did not show any difference among the drainages, the data were pooled. For all subsequent experiments, the beetles were randomly selected for use in each experiment. Eggs and first instar larvae were collected as full clutches by plucking the whole leaf on which the clutch was laid from the willow plant. For clutches of eggs and first instar larvae, no more than 2 individuals were used per clutch in any given experiment. All other life stages were collected as individuals and placed into a collection jar with several willow leaves. The collection jars (200 ml plastic jars) were insulated in my backpack while they were transported out of the mountain drainage to the White Mountain Research Station (WMRS). No more than 50 beetles, or 20 clutches were collected in each jar at one time, and they were kept in the jar for no more than 3 h. Upon returning to the WMRS in Bishop CA, USA, live beetles were maintained on a diet of fresh *Salix orestra* branches inside an incubator (Percival, Perry, IA, USA ) on a 12:12 L:D schedule that cycled from 20 °C during the day to 4 °C at night.Approximately 15 individuals per life stage per drainage were immediately placed in 0.6 mL microcentrifuge tubes and stored at -80 °C for later analysis of biochemical correlates of cold tolerance. Summer adults, eggs and

first instar larvae were collected and frozen for future analyses between July and August 2011. Second instar larvae, third instar larvae, pupae and newly eclosed adults were collected and frozen between July and August 2012. Quiescent adults were frozen for later analysis after being shipped to Western University in November 2011. Summer-active adult beetles (ca. 50 beetles/drainage) were personally brought back to Western University (London, Ontario, Canada) in a large cooler (25 cm x 60 cm x 25 cm; Cat. No. 2A20, Rubbermaid, Winchester, Va. USA) and reared in large plastic cages with mesh ventilation windows and ample branches of willow so that food was not limiting. Summer adult beetles were brought back in August 2011 and new adult beetles that had eclosed from their pupal casings were brought back in August 2012. Each plastic cage was approximately 40 cm x 30 cm x 40 cm and contained the entire population for a single drainage (up to 200 beetles). At Western, the beetles were provided with peachleaf willow branches (*Salix amygdaloides)* which was chosen for its bitter taste, indicative of a high salicylate content that is preferred by *C. aeneicollis* (Rank, 1992b). The plants were watered weekly, and liquid water sources were not provided for the beetles. These plastic cages were kept within a walk-in temperature-controlled growth chamber in the Biotron Institute for Experimental Climate Change Research (Western University). The beetles were acclimated to a 12 L:12 D photoperiod with the temperatures ramping from 20 °C during the day to 4 °C at night at a rate of ca. 0.1 °C/min; these conditions mimic non-stressful, mid-summer conditions.

Quiescence was induced in newly-eclosed adult beetles brought to Western University from the field by low temperature acclimation starting in September in both 2011 and 2012. From September through March for both years, adult beetles were stored individually in 30 ml transluscent plastic cups (Solo, Cat. No. SOLO-PL1, Lake Forest,

IL, USA) with a small piece of moist paper towel in the dark at 0.5 °C. Adults that displayed a reduced speed in movement after prodding with blunted forceps at room temperature during this time period were classified as ‘quiescent’ adults. The willow branches and/or paper towels were changed and moistened at least once a week.

**Table 2.1** Summary of field sites in the Sierra Nevada Mountains. Beetles were collected from Upper Site, High Stream Crossing and Mosquito Flat for investigation of cold tolerance. Microhabitat temperature data for all sites were used for determination of differences between drainages, and differences along an elevational gradient within Big Pine Creek.

| Drainage | Field Site Name | Altitude | Coordinates |
|---|---|---|---|
| Big Pine Creek | Sam Mack Meadow | 3353 m | 37° 7'7.80"N 118°30'21.50"W |
| | Upper Site | 3215 m | 37° 7'27.50"N 118°30'8.40"W |
| | 40 Bog | 2905 m | 37° 7'53.90"N 118°28'22.90"W |
| | 26 Bog | 2773 m | 37° 8'12.50"N 118°27'49.00"W |
| Bishop Creek | High Stream Crossing | 3194 m | 37°10'37.60"N 118°33'4.50"W |
| Rock Creek | Mosquito Flat | 3167 m | 37°26'29.60"N 118°44'48.30"W |

**2.2 Determination of the responses to cold and median lethal temperature**

To determine the supercooling point (SCP) and the response to cold, groups of a minimum of eight individuals were used at a time for each life stage, up to the number of individuals outlined in Table 3.2. In the case of eggs and first instar larvae, no more than two individuals were tested from each clutch collected. SCP and median lethal temperature experiments were performed on field-caught beetles at the White Mountain Research Station for all life stages except for the quiescent adults which were acclimated to 0.5 ºC and tested at Western University. To determine SCP, individuals were placed in 0.6 ml microcentrifuge tubes in contact with 36 AWG (American wire gauge) type T (copper-constantan) thermocouples. For eggs and first instar larvae, the thermocouple was first dipped in silicone vacuum grease so that the individual would stick to the thermocouple throughout the experiment. The microcentrifuge tubes were inserted into an aluminum block cooled by methanol circulated by a Lauda Proline 3530 refrigerated circulator (Lauda, Wurzburg, Germany). The block was held at 0 °C for a 10 min equilibration period, then cooled to -30 °C at 0.5 °C/min. To monitor temperature, thermocouples were connected to a Picotech TC-08 thermocouple interface and PicoLog software for Windows (Pico Technology, Cambridge, UK). The SCP was taken as the lowest temperature recorded before the exotherm which is caused by internal ice formation (Figure 1.2).

To assess median lethal temperature ($LT_{50}$), groups of eight individuals were cooled at 0.1 °C/min to a given temperature (Table 2.2), held at that temperature for 1 h, and then re-warmed at 0.5 °C/min. Following cold exposure, the individuals were placed in individual wells of 6-well plates (Cat. No. 353224, Becton Dickinson, Franklin Lakes, NJ, USA) on moistened paper towel, then maintained in the refrigerator at 4 °C and

monitored for survival after 24 h. Control insects were placed in 6-well plates with moistened paper towel and put directly in the 4 °C refrigerator without any additional cold exposure. Survival was defined as successful hatching for eggs, and coordinated movement of at least one leg in all other life stages. Freeze-tolerant individuals are expected to survive exposure to internal ice formation. Freeze-avoidant individuals are expected to survive exposure to low temperatures as long as internal ice formation does not occur. Finally, chill-susceptible individuals will die after exposure to low temperatures well above their SCP.

**2.3 Survival after prolonged freeze exposure**

To determine the survival of the adult beetles after prolonged exposure to internal ice, four quiescent adults were exposed to -8 °C for 1, 4, 6, or 12 h. Individuals were cooled by 0.1 °C/min using the same protocol as noted above in the median lethal temperature determination. All individuals were cooled from 0 °C, and rewarmed to +4 °C, at 0.5 °C/min. After treatment, individuals were returned to their solo cups with moistened paper towel, and stored at 4 °C until survival was assessed 24 h post treatment.

**Table 2.2** Cold Exposure temperatures for determination of median lethal temperature ($LT_{50}$) for each life stage of the willow beetle.

| **Life Stage** | **Cold exposure temperatures (°C)** |
|---|---|
| Summer Adult | 0, -3, -6, -9, -12, -15 |
| Egg | 0, -5, -10, -15, -20, -40 |
| First Instar Larva | 0, -3, -6, -9 |
| Second Instar Larva | 0, -3, -6, -10 |
| Third Instar Larva | 0, -3, -6, -9, -12 |
| Pupa | 0, -3, -6, -12, -15, -20 |
| Quiescent Adult | 0, -6, -10, -20, -30 |

## 2.4 Whole animal carbohydrates

### *2.4.1 Gas chromatography coupled with flame ionisation detection*

Gas chromatography (GC) was used to identify carbohydrates present in homogenized whole beetles of each life stage. Carbohydrates were analysed as their alditol acetate derivatives, using methods derived from Blakeney et al. (1983) and described by Crosthwaite et al., (2011). Preparation and derivitisation of whole beetles consisted of four main steps: water extraction, hexane cleaning, reduction and acetylation.

*Water extraction*

Whole adult beetles and larvae whose mass exceeded 20 mg were immersed in 200 µL of milli-q water in a 1.7 mL microcentrifuge tube and ground up by hand using a blunted glass pestle. All other life stages were immersed in 100 µL of milli-q water and ground up using the same technique. The samples were then heated for 20 min in 100 °C water to denature enzymes. After this treatment, the samples were centrifuged at 25000 g for 10 min to help separate the remaining cuticle from the supernatant. Finally, a measured quantity (75-175 µL) of supernatant was removed with a glass syringe and transferred into a 4 mL glass screw-top vial for use in the remaining steps.

*Hexane cleaning*

To remove any remaining lipids and other non-polar substances from the samples, the samples were defatted prior to GC analysis to eliminate unwanted peaks. Sulphuric acid (0.1 M, 0.7 ml), then hexane (2 ml) was added to each of the 4 mL glass vials and the mixture was vortexed and allowed to settle into phases. The upper, non-polar, hexane layer included any lipids present. A 0.4 ml aliquot of the lower layer was removed to a new 4 ml glass vial. Ammonium hydroxide (8 M, 75 µL) along with xylitol standard (1

mg/mL in 1 M ammonia, 50 µL) was added to each sample. Finally, a 0.1 mL aliquot was removed and transferred to 10 mL screw top test tubes for use in the reduction step.

*Reduction*

A solution of sodium borohydride in DMSO was used to reduce monosaccarides to polyhydric alcohols. The solution was prepared by dissolving 2 g sodium borohydride in 100 ml anhydrous DMSO at 100 °C. Sodium borohydride solution (1 ml) was added to the 0.1 ml of sample and then heated at 40 °C for 90 minutes in a circulating water bath. After cooling to room temperature, 0.1 ml acetic acid (18 M) was added to decompose any excess sodium borohydride to borates.

*Acetylation*

Acetylation of the reduced sugars was done by adding 0.2 ml 1-methylimidazole (as a catalyst) followed by 2 ml acetic anhydride. After 10 minutes at room temperature (22 °C), 5 ml distilled, deionised water (milli-q) water was added to decompose excess acetic anhydride. To prepare for GC, 2 ml of dichloromethane was added, the solution mixed, then 1.5 ml of the dichloromethane layer removed to a 4mL glass vial, dried under nitrogen and re-suspended in 0.2 mL dichloromethane to concentrate the sample. The sample was then transferred to a 2 ml, septum-cap vial with a 200 µl insert (product no. 392611552, Varian, Inc., Palo Alto, CA, USA) for analysis. Samples not analysed immediately were stored at -20 °C.

*Gas chromatography*

To identify carbohydrates, derivatised samples were analysed by capillary gas chromatography with flame ionization detection (Varian Star CX3400, Varian, Inc., Palo Alto, USA). The column used was a CP-Sil 88, WCOT fused silica, 25 m x 0.25 mm i.d. (Varian). Nitrogen was used as the carrier gas at a flow rate of 2 mL/min. The injector

temperature was 250 °C and the FID temperature was 300 °C. The following temperature program was used to compare whole animal samples with standard sugar solutions: initial temperature 140 °C, hold for 5 minutes, ramp to 230 °C at 4 °C/min, hold for 15 minutes, ramp to 240 °C at 10 °C/min, hold for 6.5 minutes. Xylitol (product no. X3375-5G, Sigma-Aldrich, Inc.) was used as an internal standard for all beetle samples tested. A standard sugars mix measured for retention times included: glycerol, D-glucose, mannose, DL-arabinose, rhamnose, inositol, fructose, xylose, and galactose standards.

*2.4.2 Spectrophotometric glycerol assay*

Since glycerol was the predominant polyhydric alcohol found through gas chromatography in all life stages, glycerol content of whole animal extractions was measured using a spectrophotometric assay to allow higher throughput. Beetles were crushed manually using a glass pestle in 150 µl 0.05 % v/v Tween 20 if their mass was less than 15 mg, or 200 µl if their mass was greater than or equal to 15 mg. Samples (30 µl) were incubated in 96-well plates with 100 µl free glycerol reagent (product no. F6428-40ML, Sigma Aldrich, Inc., St Louis, MO, USA) at room temperature (22 °C) for five minutes. Following incubation, the plates were read at 540 nm on a microplate spectrophotometer (SpectraMax 340PC, Molecular Devices, Sunnyvale, CA, USA). Glycerol content was calculated by fitting a regression line through the absorbance values obtained from a standard curve on each plate. Using the equation of the regression line for each plate seperately, I calculated the amount of glycerol in each sample. The glycerol content (mg) was divided by the water content (%) for each life stage to get the glycerol concentration.

**2.5 Mass and water content**

To determine wet and dry mass gravimetrically, individuals from each life stage were stored at -80 °C for two weeks prior to weighing and drying. Individuals were weighed using a Mettler UMX-5 microbalance to determine wet mass, then dried at 70 °C for a minimum of 4 days, and reweighed to obtain dry mass. Dry mass was subtracted from wet mass to determine total water content.

**2.6 Antifreeze activity, thermal hysteresis and haemolymph osmolality**

Haemolymph osmolality and thermal hysteresis were measured using a nanolitre osmometer (Clifton Technical Physics, Hartford, NY, USA). As much haemolymph as possible was extracted from adult beetles (summer and quiescent) with minimal exposure to air using a pipette partially filled with type B immersion oil (cat. no. 16484, Cargille Laboratories, Cedar Grove, NJ, USA) and then emptied into a 0.6 ml microcentrifuge tube filled with 200 µL immersion oil to prevent oxidation of the haemolymph in air. The haemolymph was then snap-frozen in liquid nitrogen and stored at -80 °C until analysed. Whole animal samples from all other life stages were collected in the field, and stored at -80 °C until analysis. Haemolymph was extracted from these frozen whole animal samples immediately preceding osmometry. The haemolymph of three live quiescent beetles were tested by osmometry as a control for using frozen samples. There was no observed difference in ice crystal shape, thermal hysteresis or osmolality between frozen and live beetles.

To measure the melting point, small droplets of haemolymph (~20 nl) were suspended in small wells filled with type B immersion oil under a microscope and rapidly cooled until frozen. The droplets were then warmed slowly until the last crystal melted (the melting point). Melting point was converted to °C by dividing haemolymph melting

point (mOsm) by 1000 mOsm and multiplying by -1.86 °C (a 1000 mOsm solution has a melting point of -1.86 °C (Zachariassen, 1985)).

To detect thermal hysteresis, samples were refrozen following melting point determination, and warmed until only one or two ice crystals remain in the sample. The crystals were allowed to stabilize for one minute, and then slowly cooled until they were observed to grow. The temperature at which they began to grow was recorded as the freezing point. Thermal hysteresis is measured as the difference between melting point and freezing point. Both thermal hysteresis and angular ice crystal morphologies are indicative of antifreeze protein activity (Zachariassen and Kristiansen, 2000). Un-faceted and round growing ice crystals are indicative of no antifreeze agent activity, ice crystals that grow with one or more distinctly flat edges may be the result of antifreeze agent activity (Bar-Dolev et al., 2012).

**2.7 Microhabitat temperature**

Temperature data were collected at six sites throughout the three drainages in the Sierra Nevada Mountains using HOBO Pendant UA-002-08 data loggers (Onset Computer Corporation, Bourne, MA, USA). The data loggers were deployed in sets of three on the same willow plant: mid-willow (ca. 1.2 m above the ground), base of willow stem and in the buried ca. 3 cm in the soil next to the roots of the willow. The mid-willow data logger was placed inside a plastic cup with many small holes for air ventilation at ca. 1 m above the ground, and affixed to the willow with a plastic tie. The data loggers at the base of the willow and under the soil were affixed to the willow with plastic ties, but they were not placed in the plastic cups. Each data logger recorded the ambient temperature every 30 min. The data loggers were deployed in the mid-willow during the summer of

2005, whereas the base-of-the-willow and soil loggers were deployed in the summer of 2009 (Table 2.3).

Using these microhabitat temperature data and unpublished phenology data for each life stage (Rank, N.E., personal communication), I looked for cold events that crossed the $LT_{50}$ threshold for each life stage during the time of the year that they are present (methods from Sinclair, 2001a). I analysed for the frequency, duration and extreme low temperature from each microhabitat within each field site. These summary data were used for a comparison of microhabitat temperatures within each field site (mid-willow, base of willow, soil) as well as between field sites. Field sites were compared at similar elevations with one site within each drainage (RC, BC, BPC), and at four sites along an elevation gradient within the BPC drainage.

**2.8 Statistical analysis**

SCPs were compared among life stages using a one-way ANOVA followed by Tukey's *post hoc* tests in R (R Development Core Team 2010). Median lethal temperature was determined for each life stage by fitting a generalised linear model onto the survival after cold exposure data using R. Differences in the median lethal temperatures were determined by extending the range to include the predicted $LT_5$ through the $LT_{95}$ and looking for overlap between the ranges for each life stage. If the mortality range limits did not overlap between life stages, they were qualitatively determined to be significantly different.

Glycerol content measured in µg glycerol, water content, osmolality and thermal hysteresis were compared among life stages using an ANCOVA with body mass as a covariate and a Tukey's HSD *post-hoc* test for pairwise comparison. The volume of beetle water for glycerol content in mM was estimated by multiplying the mass of the

beetle by the mean % water content for each life stage. This was done because variation in the water content of the beetles would affect the glycerol concentration in the haemolymph. Glycerol concentration (in mM) was compared between life stages using an ANOVA and a Tukey's HSD *post-hoc* test to determine differences between mean glycerol concentration in each life stage.

**Table 2.3** Start and end dates for each temperature data set used in microhabitat temperature analysis of frequency of potentially lethal cold exposures for the willow leaf beetle. These data were collected by N.E. Rank and E.D. Dahlhoff.

| Drainage | Field Site | Temperature logger location | Start Date | End Date |
|---|---|---|---|---|
| Big Pine Creek | Sam Mack Meadow | mid-willow | 08/07/2005 | 07/06/2012 |
| | | base | 22/06/2009 | 07/06/2012 |
| | | soil | 22/06/2009 | 07/06/2012 |
| | Upper Site | mid-willow | 30/06/2005 | 06/08/2012 |
| | | base | 22/06/2009 | 26/06/2011 |
| | | soil | 22/06/2009 | 26/06/2011 |
| | 40 Bog | mid-willow | 18/08/2005 | 07/06/2012 |
| | | base | 22/06/2009 | 26/06/2011 |
| | | soil | 22/06/2009 | 07/06/2012 |
| | 26 Bog | mid-willow | 18/08/2005 | 07/06/2012 |
| | | base | 22/06/2009 | 07/06/2012 |
| | | soil | 22/06/2009 | 07/06/2012 |
| Bishop Creek | High Stream Crossing | mid-willow | 28/06/2005 | 01/06/2012 |
| | | base | 16/06/2009 | 31/05/2012 |
| | | soil | 16/06/2009 | 04/06/2012 |
| Rock Creek | Mosquito Flat | mid-willow | 09/07/2005 | 30/05/2012 |
| | | base | 14/06/2009 | 30/05/2012 |
| | | soil | 14/06/2009 | 30/05/2012 |

For microhabitat temperature analysis, each life stage was assigned a time frame which encompasses the first and last appearance of individuals of that life stage based on 1 h survey counts at each field site (Table 2.4; Rank and Dahlhoff unpublished data). The frequency and duration of potentially lethal cold exposures were extracted from the temperature data for each data logger (mid-willow, base of willow, soil underneath the willow) using R. Potentially lethal cold exposures were defined as temperatures that fell below the $LT_{50}$ for each life stage of the willow leaf beetle. Minimum temperature reached during the time frame for each life stage was compared qualitatively between temperature loggers for each site to determine differences between microhabitat temperatures.

**Table 2.4** Span of observations during which each life stage can be observed in the Sierra Nevada Mountains. These dates are based on the earliest and last date when each life stage can be found during field surveys (from Rank, N.E., Personal communication). The median lethal temperatures ($LT_{50}$) reported here were used to determine the frequency of potentially lethal cold exposures in microhabitat temperature analysis.

| **life stage** | **Start Date** | **End Date** | **$LT_{50}$** |
|---|---|---|---|
| Summer adult | May-30 | Jun-30 | -9.2 |
| Egg | Jun-15 | Jul-15 | -20.1 |
| First instar | Jul-01 | Jul-31 | -3.3 |
| Second instar | Jul-15 | Aug-15 | -6.2 |
| Third instar | Aug-01 | Aug-30 | -13.3 |
| Pupa | Aug-15 | Sep-15 | -11.2 |
| Quiescent adult | Sep-01 | May-30 | -15.0 |

# CHAPTER 3: RESULTS

## 3.1 Cold tolerance

Both summer-active and quiescent adults survived internal ice formation indicating that adult beetles are freeze tolerant. All other life stages did not survive internal ice formation, indicating they are freeze intolerant. Eggs and pupae survived exposure to low temperatures as long as internal ice formation did not occur for that individual, indicating that these life stages are freeze avoidant. All larval stages died after exposure to low temperatures above their supercooling point (SCP) indicating they are chill susceptible (Table 3.1).

### *3.1.1 Supercooling point*

There was no significant difference between the mean SCPs of the summer active and winter quiescent adults. The SCPs of all larval instars were not significantly different from each other, but were all lower than the adult beetles. The eggs had a mean SCP that was significantly lower than all other life stages (Table 3.2, One-way ANOVA, $F_{8, 265}=413.39$, $p<0.001$).

Some pupae showed a single exotherm (6 of 24 pupae) indicative of internal ice formation, while others showed two exotherms (18 of 24 pupae) that were separated by an average of 3.9 °C (Figure 3.1, Table 3.2). The SCP of the single exotherm (-11.1 ± 2.8 °C) was not significantly different from the first of the paired exotherms (-13.6 ± 3.2 °C) (Table 3.2, One-way ANOVA, $F_{8, 265}=413.39$, $p<0.001$). Several pupae showing either one exotherm or two exotherms were dissected to determine differences in morphology and stages of pupal development (Figure 3.2). The pupae that showed two exotherms all had tissue from the third instar larva that had not become fully desiccated. The pupae that showed one exotherm had only the fully desiccated cuticle from the third instar remaining.

*3.1.2 Median lethal temperature for cold exposure ($LT_{50}$)*

The median lethal temperature for each life stage was compared for significant differences by comparing the $LT_5 – LT_{95}$ range for overlap between them (Table 3.3). The quiescent adults had a significantly lower $LT_{50}$ (ca. -15.0 °C) compared to all summer life stages, except for the eggs and pupae. Eggs had the lowest $LT_{50}$ (ca. -20 °C) of all life stages. The $LT_{50}$ of all juvenile life stages (larvae and pupae) were not significantly different from each other. The pupae had the greatest variance in $LT_{50}$ of any life stage, with a $LT_5 – LT_{95}$ range spanning almost 7 °C.

*3.1.3 Long term freeze exposure*

Quiescent adult beetles exposed to -8 °C were all confirmed to have internal ice formation using the same methods as SCP determination. There was 100% survival for all individuals that remained frozen for one hour. There was 75% survival after four hours exposure to internal ice, 25% survival after six hours and no beetles survived after being frozen for 12 h. Control beetles exposed to -4 °C (higher temperature than the SCP for all individuals) did not freeze after 12 h, and had 100% survival.

**Table 3.1** Summary of the response to cold for each life stage of the willow leaf beetle. Data are presented as the percent of individuals killed for each life stage either before or by freezing. The responses to cold were determined by assessing survival in individuals before and after exposure to internal ice formation.

| **Life Stage** | **Killed before freezing?** | **Killed by Freezing?** | **Response to cold** |
|---|---|---|---|
| Summer Adult | 0/264 | 3/264 | Freeze Tolerant |
| Egg | 0/72 | 72/72 | Freeze Avoidant |
| First Instar Larva | 44/44 | 44/44 | Chill Susceptible |
| Second Instar Larva | 40/40 | 40/40 | Chill Susceptible |
| Third Instar Larva | 57/57 | 57/57 | Chill Susceptible |
| Pupa | 0/66 | 61/66 | Freeze Avoidant |
| Quiescent Adult | 0/30 | 0/30 | Freeze Tolerant |

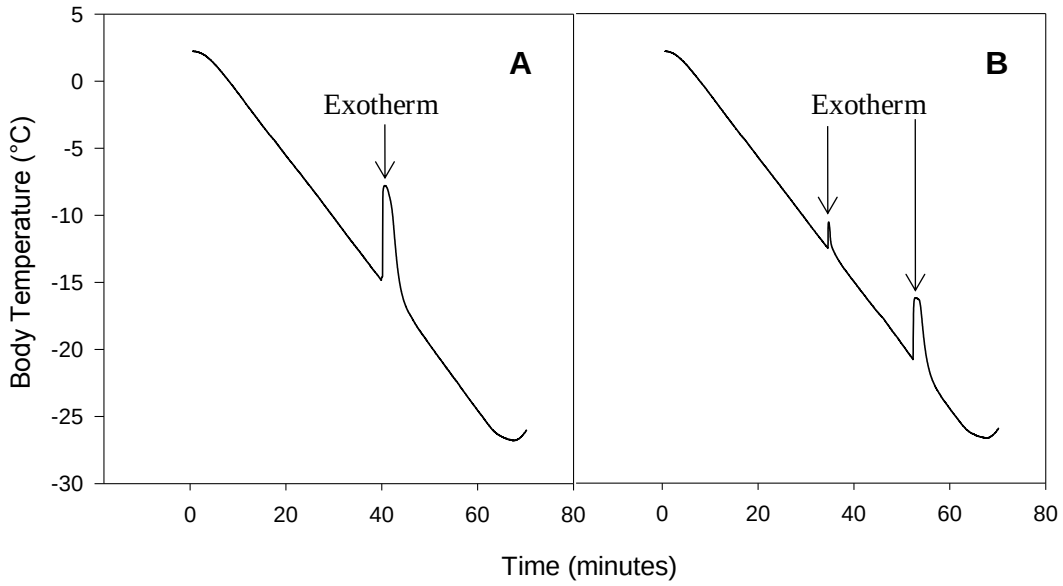


**Figure 3.1** Representative single and double exotherms observed in the pupae of the willow leaf beetle. **A.** Single exotherm observed in 6 of 24 pupae. **B.** Paired exotherms observed in 18 of 24 pupae.

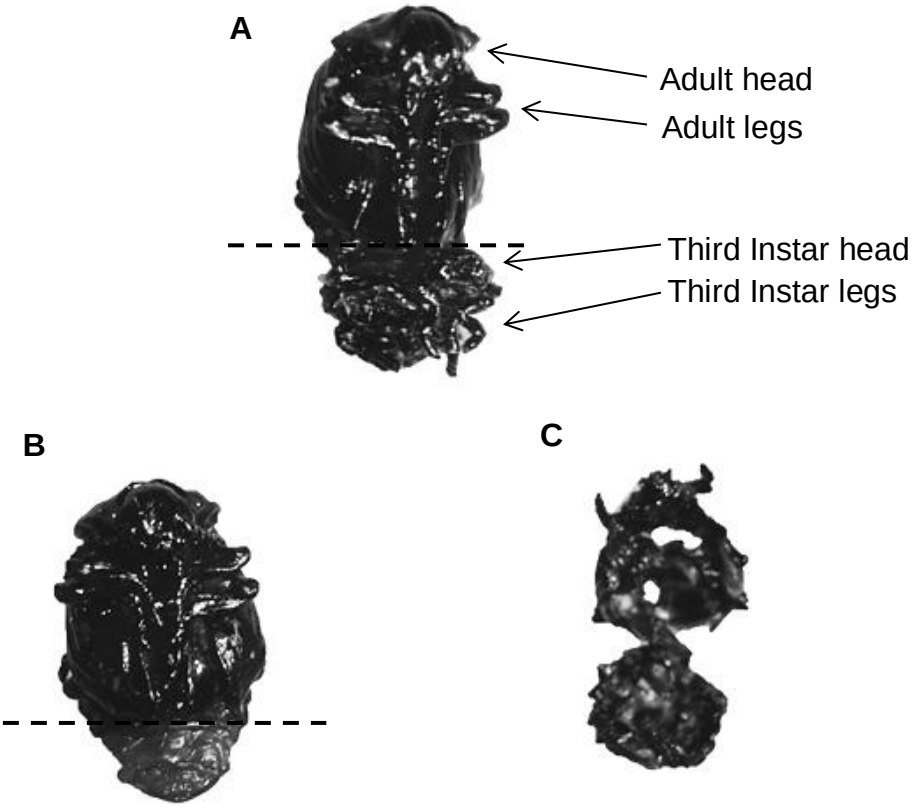


**Figure 3.2** Representative pupa showing tissue development of the willow leaf beetle. **A.** Intact pupa showing the adult head and legs as well as third instar head and legs. **B.** Pupa with third instar tissue removed. **C.** Fully desiccated third instar larval tissue removed from the pupa in panel B. The areas below the dashed lines indicate the location of the third instar larva tissue. Photo credit: Jordan Sayre.

**Table 3.2** Mean supercooling point for each life stage of the willow leaf beetle. The data are presented as mean ± SEM. Similar superscript letters indicate no significant difference between the supercooling points ($p<0.001$).

| **Life stage** | **n** | **Supercooling point (°C)** |
|---|---|---|
| Summer Adult | 95 | -4.9 ± 0.05 $^{A}$ |
| Egg | 47 | -23.4 ± 0.18 $^{F}$ |
| First Instar Larva | 22 | -10.6 ± 0.47 $^{B}$ |
| Second Instar Larva | 16 | -10.8 ± 0.68 $^{B}$ |
| Third Instar Larva | 32 | -8.6 ± 0.62 $^{B}$ |
| Pupa - Single Exotherm | 6 | -11.1 ± 1.14 $^{BC}$ |
| Pupa - First Exotherm | 18 | -13.6 ± 0.05 $^{CD}$ |
| Pupa - Second Exotherm | 18 | -17.5 ± 0.75 $^{E}$ |
| Quiescent Adult | 28 | -5.3 ± 0.11 $^{A}$ |

**Table 3.3** Median lethal temperature for each life stage of the willow leaf beetle. The median lethal temperature data were determined by fitting a generalised linear model onto survival data. They are represented as the $LT_{50} \pm LT_5 - LT_{95}$ range. Similar letters indicate an overlap in the $LT_5 - LT_{95}$ range.

| **Life Stage** | **Median Lethal Temperature (°C)** |
|---|---|
| Summer Adult | -9.17 ± 0.43 [a] |
| Egg | -20.09 ± 0.75 [d] |
| First Instar Larva | -3.26 ± 3.53 [b] |
| Second Instar Larva | -6.21 ± 4.77 [ab] |
| Third Instar Larva | -8.7 ± 0.37 [a] |
| Pupa | -11.4 ± 6.79 [abc] |
| Quiescent Adult | -14.98 ± 0.60 [c] |

*3.1.4 Thermal hysteresis, antifreeze agent activity, osmolality and water content*

Ice crystal morphology indicative of low antifreeze agent activity was observed in first and second instar larvae and in pupae. The larvae showed half circle-shaped ice crystal growth with a single distinctly flat edge. The pupae showed either rectangular or trapezoidal shaped ice crystal growth indicating antifreeze agent activity. All other life stages showed uniform, round ice crystal growth. Thermal hysteresis did not differ significantly between the life stages (Table 3.4, One-way ANOVA $F_{6, 23}$=18.932, $p<0.001$). All life stages showed some thermal hysteresis activity, values ranged from 0.25 °C for quiescent adult beetles to 0.05 °C for first instar larvae. Osmolality differed significantly between the life stages (Table 3.4, One-way ANOVA $F_{6, 23}$=31.569, $p<0.001$). Osmolality ranged from 380 ± 7 mOsm for summer adults to 875 ± 13 mOsm for quiescent adults (Table 3.4). Water content ranged from 92 ± 8% of wet mass in pupae to 67 ± 5% of the wet mass in the summer adults. Total water content was highest in pupae, lowest in the adult beetles and intermediate for all other life stages (Figure 3.3, one-way ANOVA, $F_{6, 49}$=13.193, $p<0.05$).

**Table 3.4** Changes through ontogeny in haemolymph properties of the willow leaf beetle. Using a Clifton nanoliter osmometer, haemolymph from each life stage was assayed for melting point, freezing point, thermal hysteresis osmolality and ice crystal growth shape. Data are shown as mean ± SD. Similar letters indicate no significant difference, $p<0.05$.

| Sample | n | Ice Crystal Shape | Melting Point (°C) | Freezing Point (°C) | Thermal Hysteresis (°C) | Osmolality (mOsm) |
|---|---|---|---|---|---|---|
| Summer Adult | 5 | Round | -0.71 ± 0.01 | -0.79 ± 0.01 | 0.08 ± 0.01 $^{a}$ | 380 ± 7 $^{A}$ |
| Egg | 5 | Round | -1.39 ± 0.01 | -1.58 ± 0.03 | 0.19 ± 0.04 $^{bc}$ | 746 ± 16 $^{B}$ |
| 1$^{st}$ instar larva | 5 | Half Circle | 1.28 ± 0.02 | -1.33 ± 0.06 | 0.05 ± 0.08 $^{a}$ | 687 ± 30 $^{BC}$ |
| 2$^{nd}$ instar larva | 5 | Half Circle | -0.83 ± 0.03 | -0.92 ±0.03 | 0.10 ± 0.03 $^{ab}$ | 443 ± 15 $^{AD}$ |
| 3$^{rd}$ instar larva | 5 | Round | -0.86 ± 0.03 | -0.95 ± 0.02 | 0.09 ± 0.03 $^{a}$ | 463 ± 11 $^{AD}$ |
| Pupa | 5 | Rectangular | -1.06 ± 0.05 | -1.15 ± 0.05 | 0.09 ± 0.02 $^{a}$ | 570 ± 29 $^{CD}$ |
| Quiescent Adult | 5 | Round | -1.63 ± 0.03 | -1.88 ± 0.03 | 0.25 ± 0.04 $^{c}$ | 875 ± 13 $^{B}$ |

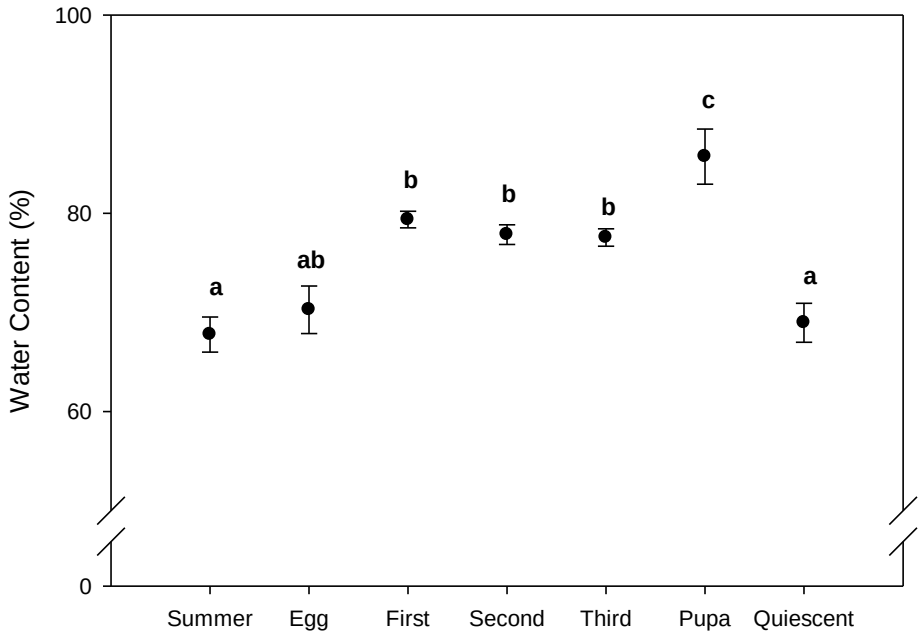


**Figure 3.3** Total water content determined gravimetrically for each life stage of the willow leaf beetle. Data are presented as percent of wet mass shown as mean ± SD, n = 8 for each life stage. Different letters indicate significant difference between life stages ($p<0.05$).

*3.1.5 Whole animal carbohydrates and polyhydric alcohols*

Gas chromatography of the standard sugars mix produced a single major peak for each sugar tested as well as a large reagent peak that eluted separately in all chromatograms. The reagent peak eluted at the same time in all standard sugar samples (mixed and individually tested). The reagent peak is likely an artifact from the reduction and acetylation process. Peaks eluted that were consistent with glycerol and either rhamnose or fructose in all life stages (Figures 3.4, 3.5, 3.6). The quiescent adults had a sugar that eluted consistent with glucose (Figure 3.4). There was no evidence of inositol, mannose, arabinose or galactose in any life stage of the WLB.

Using enzymatic analysis, quiescent adult beetles had a significantly higher content of glycerol than all other life stages (1.3 ± 0.45 µg glycerol/mg beetle tissue) (Figure 3.7; ANCOVA, $F_{7, 47}$=11.49, $p<0.001$). The concentration of glycerol estimated using the total water content was also significantly higher for quiescent adults (23.9 ± 12.3 mM; One-way ANOVA, $F_{7, 48}$=5.32, $p<0.001$). There was no significant difference between any other life stages (Tukey's HSD, $p<0.05$).

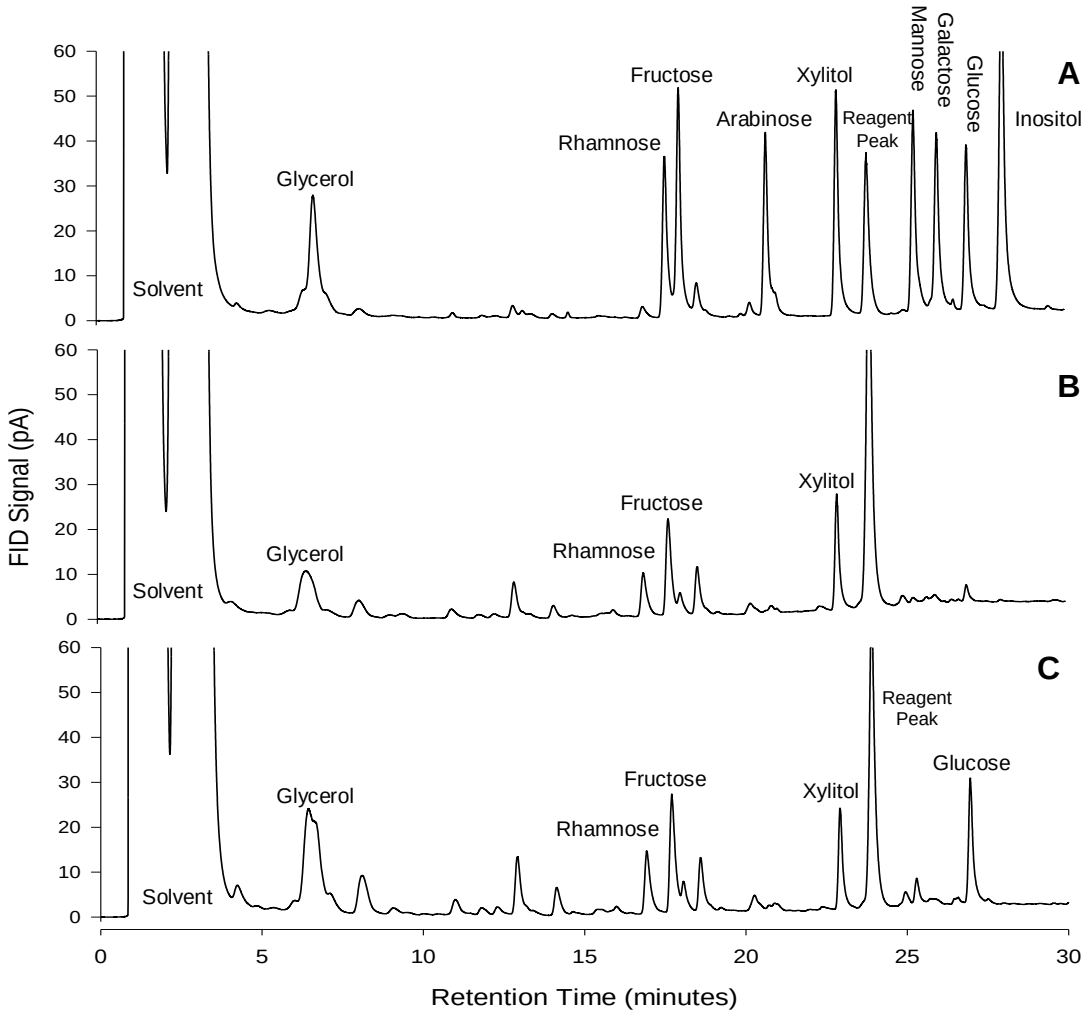


**Figure 3.4** Representative chromatograms from gas chromatography comparing a mix of standard sugars to whole animal homogenates of willow leaf beetle adults. **A**. Profile of polyhydric alcohol standards. **B**. Profile of acetylated summer adult homogenate. **C**. Profile of acetylated Quiescent Adult homogenate. Xylitol was used as an internal standard.

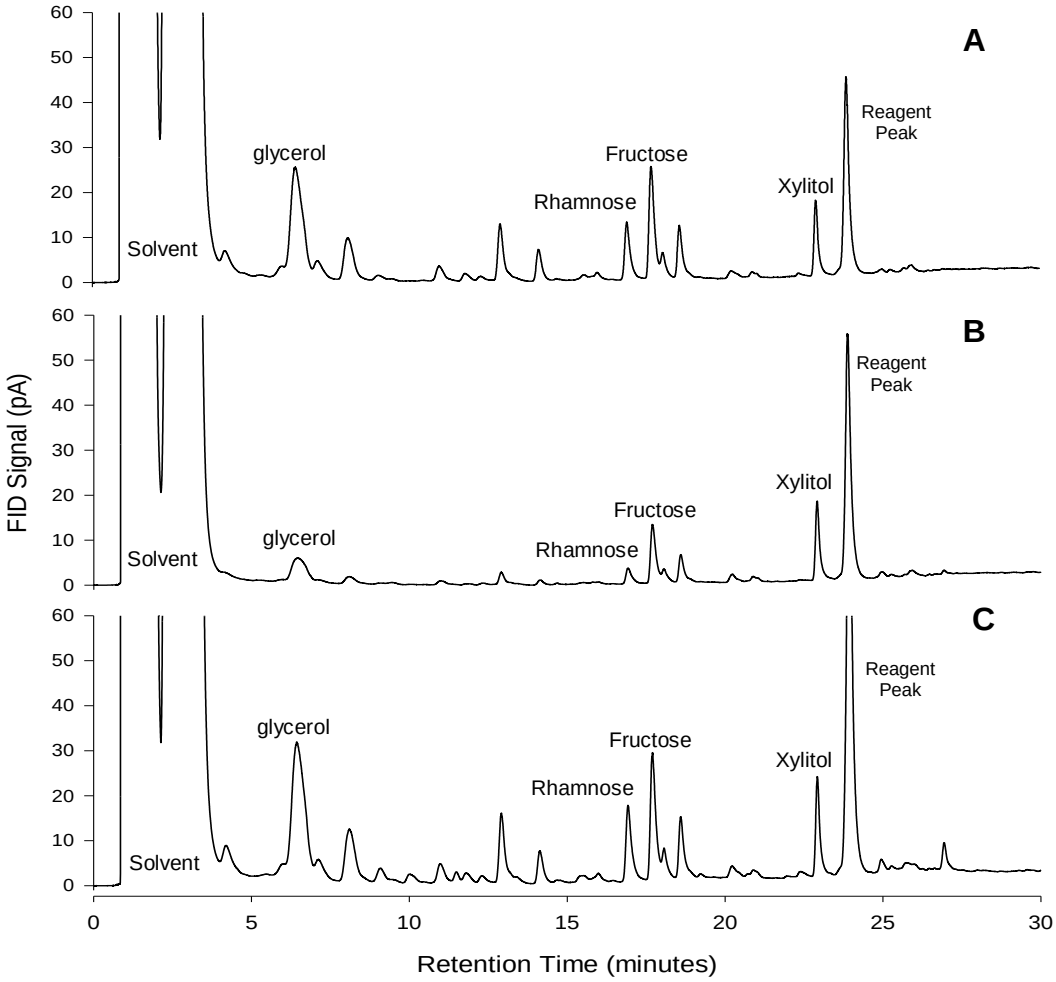


**Figure 3.5** Representative chromatograms from gas chromatography comparing whole animal homogenates of willow leaf beetle larvae. **A**. Profile of acetylated first instar larva homogenate. **B**. Profile of acetylated second instar larva homogenate. **C**. Profile of acetylated third instar larva homogenate. Xylitol was used as an internal standard.

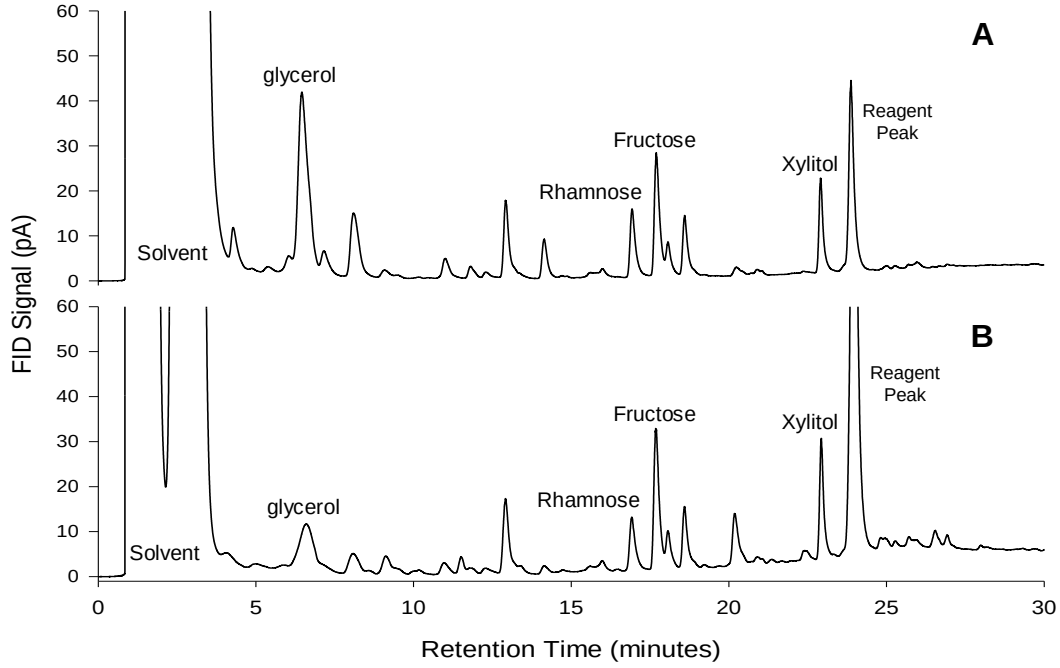


**Figure 3.6** Representative chromatograms from gas chromatography comparing whole animal homogenates of the willow leaf beetle pupa and egg. **A**. Profile of acetylated pupa homogenate. **B**. Profile of acetylated egg homogenate. Xylitol was used as an internal standard.

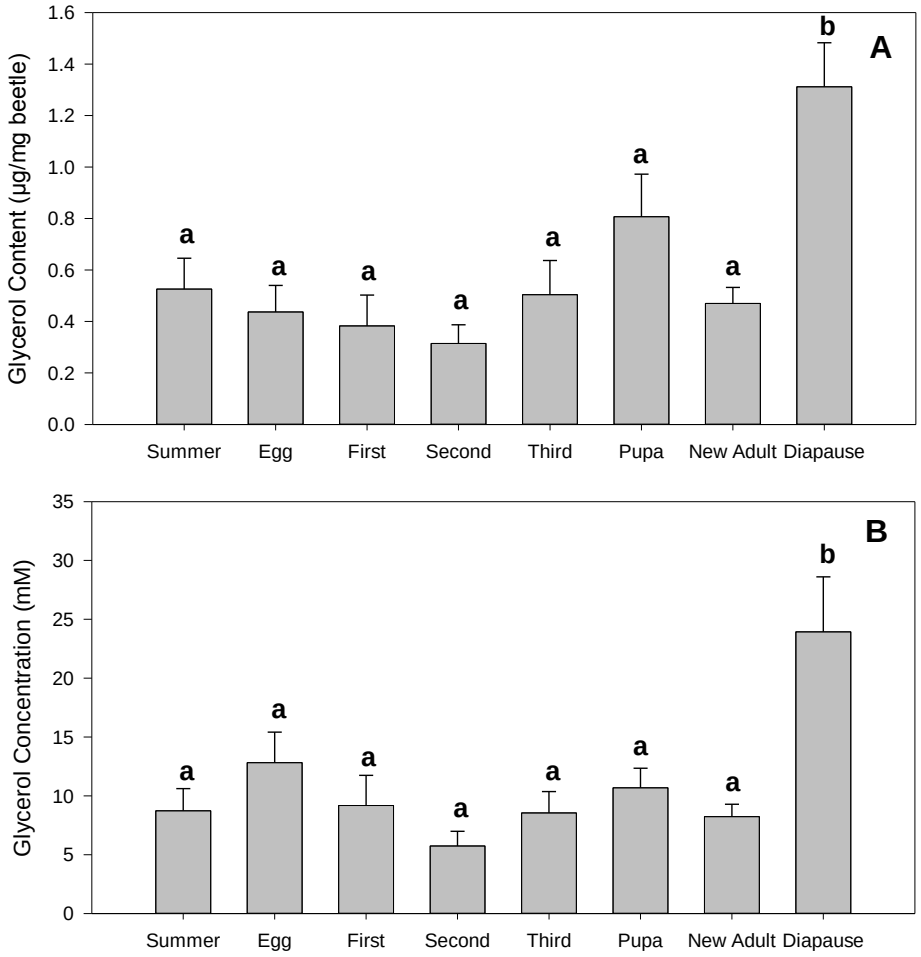


**Figure 3.7** Glycerol content determined enzymatically in each life stage of the willow leaf beetle. **A.** Glycerol content represented as ug glycerol/mg beetle tissue. Different letters indicate a significant difference in glycerol mass, $p<0.001$. **B.** Glycerol concentration (mM) calculated from average % water content for each life stage, Different letters indicate a significant difference in glycerol concentration, $p<0.01$. Data are presented as mean ± SEM, n=7.

### 3.2 Microhabitat temperatures

Field sites were chosen at similar elevations between the three drainages, and four field sites along an elevational gradient within the BPC drainage (Table 2.1). The mid-willow temperature loggers at all six field sites recorded the lowest minimum temperatures, ranging from -19.6 °C to -30.6 °C (Table 3.5). The dates at which the minimum temperatures occurred were not recorded during analysis. The mid-willow temperature logger in RC had the lowest minimum temperature recorded of -30.6 °C. The base of the willow loggers consistently had median minimum temperatures recorded, ranging from -9.1 °C to -22.7 °C. The soil loggers consistently recorded the mildest minimum temperatures ranging from -2.4 °C to -8.2 °C. In BPC, there was a 2.1 °C difference in the mean mid-willow temperature between 3353 m and 2773 m, indicating a 0.35 °C decrease in mean temperature per 100 m elevation gain (Table 3.5). The period of buffered temperatures recorded by both the mid-willow and the soil logger in winter that indicates snow cover increased in length with increasing elevation. At 3353 m, snow cover lasted from early October to mid-June and at 2775 m snow cover lasted from late October to early May (Figure 1.1). At the mid-willow temperature logger, 2007 was the coldest year for all field sites because there was no evidence of a thermally buffered period around 0 °C indicative of snow cover. The minimum temperature recorded at 3353 m was mild compared to lower altitude sites due to temperature logger failure for 2007. Temperature logger failure caused the coldest year (2007) to be missing from the data set for the site at 3353 m.

The minimum temperatures recorded at the mid-willow were lower than the $LT_{50}$ temperature for quiescent adults at all sites along an elevation gradient (Figure 3.8) as well as between drainages at similar elevations (Figure 3.9). The minimum temperatures also crossed the $LT_{50}$ threshold for the summer adults on very few occasions, but no other life stages experienced potentially lethal events during the time periods outlined for each life stage in Table 2.4. The minimum temperatures at the base of the willow as well as in the soil along an elevational gradient within BPC were consistently milder than the mid-willow, with soil temperatures being the warmest (Figure 3.8). The minimum temperature at the base of the willow fell below the $LT_{50}$ of the quiescent adults in RC in 2011, but for all other years, there was a trend of increasing minimum temperatures going from the mid-willow to the soil (Figure 3.9).

**Table 3.5** Summary of microhabitat temperature data from the Sierra Nevada Mountains in California. Mean temperatures are shown as ± SD for the entire data set.

| Field Site | Logger | Minimum Temperature (°C) | Maximum Temperature (°C) | Mean Temperature (°C) |
|---|---|---|---|---|
| Big Pine Creek – 3353 m | mid-willow | -19.6 | 41.5 | 2.8 ± 7.7 |
| | base | -13.3 | 36.2 | 3.0 ± 7.1 |
| | soil | -2.4 | 33.7 | 2.9 ± 4.0 |
| 3215 m | mid-willow | -28.0 | 38.7 | 1.9 ± 8.0 |
| | base | -11.2 | 43.1 | 1.9 ± 6.1 |
| | soil | -7.3 | 26.1 | 1.3 ± 3.9 |
| 2905 m | mid-willow | -24.8 | 38.5 | 3.3 ± 8.0 |
| | base | -9.1 | 33.9 | 4.1 ± 8.8 |
| | soil | -3.4 | 40.2 | 4.0 ± 5.5 |
| 2773 m | mid-willow | -22.5 | 34.7 | 4.9 ± 8.9 |
| | base | -10.8 | 41.7 | 5.2 ± 7.9 |
| | soil | -5.9 | 29.0 | 4.7 ± 5.6 |
| Bishop Creek – 3194 m | mid-willow | -26.9 | 36.1 | 2.5 ± 9.1 |
| | base | -13.9 | 28.7 | 0.4 ± 5.9 |
| | soil | -8.2 | 33.2 | 2.7 ± 5.1 |
| Rock Creek – 3167 m | mid-willow | -30.6 | 39.6 | 2.5 ± 9.3 |
| | base | -22.7 | 46.6 | 3.8 ± 8.7 |
| | soil | -7.8 | 35.9 | 2.6 ± 5.2 |

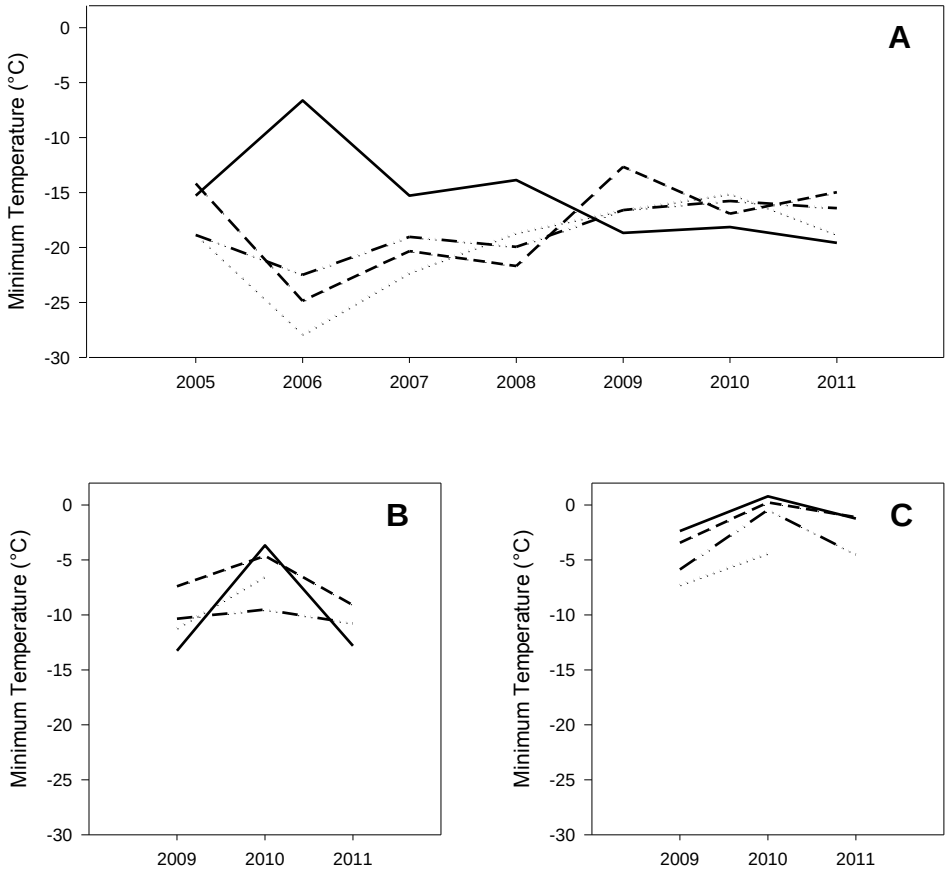


**Figure 3.8** Minimum temperature reached in each year for the time period when quiescent adult beetles are the dominant life stage in the Big Pine Creek Drainage. **A.** Temperatures at the mid-willow **B.** base of willow **C.** Temperatures in the soil under willow. Temperatures are compared along an elevational gradient. 3353 m(——), 3215 m(·····), 2905 m(– – –), 2773 m(— · ·).

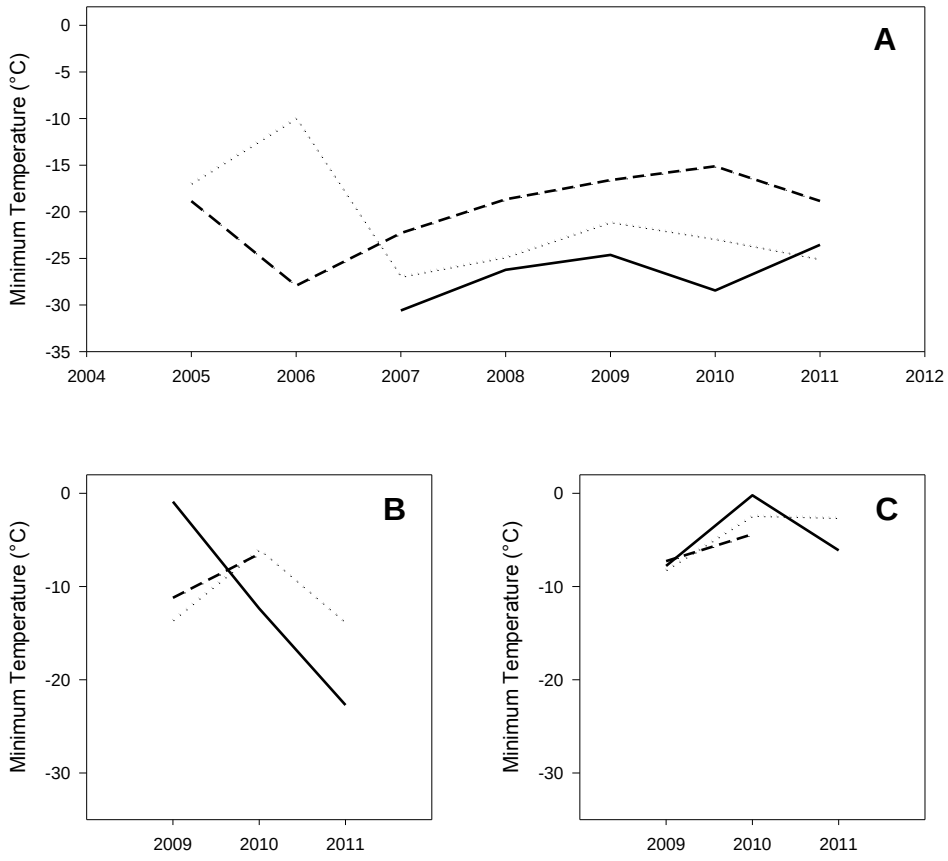


**Figure 3.9** Minimum temperature reached in each year for the time period when quiescent adult beetles are the dominant life stage. **A.** Temperatures at the mid-willow **B.** base of willow **C.** Temperatures in the soil under willow. Temperatures are compared between drainages at similar elevation. Rock Creek (3167 m, ——**),** Bishop Creek (3194 m, ·····), Big Pine Creek (3215 m, **– –** ).

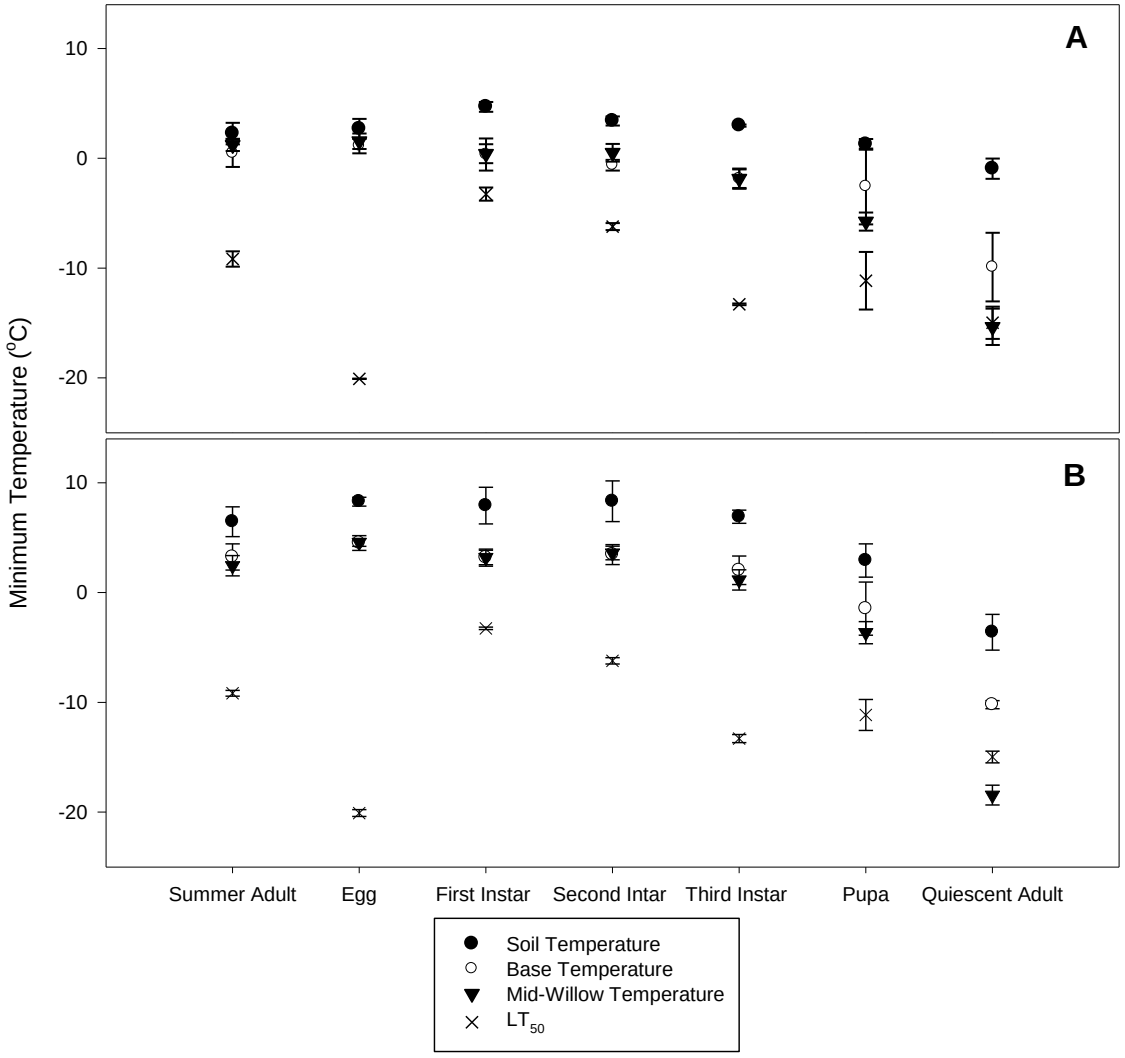


**Figure 3.10** Mean minimum temperature for all years at the mid-willow, willow base and soil under the willow compared to the median lethal temperature ($LT_{50}$) for each life stage of the willow leaf beetle. **A**. Willow minimum temperatures at 3353 m in Big Pine Creek. **B.** Willow minimum temperatures at 2770 m in Big Pine Creek. Minimum temperature data are presented as mean ± SEM. Median lethal temperature data are presented as $LT_{50}$ ± $LT_5$ – $LT_{95}$ range.

# CHAPTER 4: DISCUSSION

The overall objective of my thesis was to frame the cold tolerance of each life stage of the willow leaf beetle in the context of the field environmental conditions they experienced and life cycle adaptations as a whole. Specifically, my goal was to determine if there was a link between the mechanisms underlying cold tolerance quantified in the lab (glycerol, thermal hysteresis and osmolality) and survival through behaviour and life cycle timing for each life stage in field conditions (microhabitat choice, minimum temperature, life cycle timing).

The results of this study indicated the cold tolerance of the willow leaf beetle (WLB) is sufficient to prevent mortality from low air temperature exposures in all of the life stages within their seasonal microhabitats (Figure 3.10). The quiescent adult WLB uses behavioural avoidance strategies to survive winter cold exposures by moving into the soil. This behavioural strategy is especially important at low elevations where the persistence of snow cover and the range of daily temperatures (Figure 1.1) fluctuate between years.

## 4.1 Temperature variability and snow cover in the drainages of the Sierra Nevada Mountains

The three drainages in this study, Rock Creek (RC), Bishop Creek (BC) and Big Pine Creek (BPC) are all on the eastern slope of the Sierra Nevada Mountains with similar vegetation. There were no observed differences in the range of temperatures recorded at similar elevations (Table 3.5) in the three drainages. Similar climates in the drainages may be the result of large scale air currents rather than local variations in topography (Lundquist and Cayan, 2007). There is less variability in the recorded extreme temperatures than was previously thought. Previous studies have only considered summer

temperatures at the mid-willow logger. They found a trend of increasing minimum temperatures moving south through the drainages. Specifically, RC (most northern drainage) has the lowest minimum temperatures, and BPC (most southern drainage) has the highest minimum temperatures (Dahlhoff and Rank, 2000; McMillan et al., 2005; Rank and Dahlhoff, 2002). In this study, fall and winter temperatures were added to the data set, resulting in a loss of the trend between minimum temperatures and the drainages (Table 3.5). RC still showed the lowest minimum mid-willow temperature, BPC was intermediate and BC has the warmest minimum temperatures.

The three drainages are not homogeneous in slope, aspect and sun exposure which affects temperatures experienced by the beetles within their microhabitats and along their elevational gradients. Big Pine Creek (BPC) was the focus of a comparison between microhabitat temperatures at four different elevations within the drainage. In BPC, there was a 2.1 °C difference in the mean temperature between 3353 m and 2773 m, indicating a 0.35 °C decrease in mean temperature per 100 m elevation gain. In montane environments of the US Pacific northwest, mean air temperature decreases by between 0.4 °C (moist air) - 0.98 °C (dry air) per 100 m gain in elevation (Dodson and Marks, 1997; Rolland, 2003). At the mid-willow temperature logger in 2010-2011, there was a distinctly thermally buffered snow cover period at all high elevation sites (2905 m to 3353 m), but there was no evidence of snow cover at the lowest elevation site (2773 m). There was a distinctly thermally buffered period of snow cover in 2010-2011 at the soil for all elevations. Elevation is a more important factor in determining the cold exposures of the willow leaf beetle than the drainage in the Sierra Nevada Mountains. Increased diurnal temperature fluctuations and reduced persistence of snow cover at low elevation can result in more extreme cold events throughout the year.

## 4.2 Summer and quiescent adult willow leaf beetle

### *4.2.1 Cold tolerance*

The summer active and quiescent adult willow leaf beetles (WLB) are freeze tolerant. For both of these life stages, mortality was not observed until temperatures dropped below their freezing point in the lab. Summer and quiescent adult beetles had high SCPs, which is typical for freeze tolerant insects (Ohyama and Asahina, 1972). A high SCP allows freeze tolerant insects to control ice crystal formation and protect tissues against freezing injury (Zachariassen, 1985). Freeze tolerance has been described in nine families within the order Coleoptera, including Chrysomelidae, with SCPs ranging from -3 °C to -54 °C (Sinclair, 1999). The $LT_{50}$ for quiescent adults (ca. -15 °C) was significantly lower than summer adults (ca. -9 °C), which indicates increased cold tolerance. SCP and $LT_{50}$ can be altered, in part, by an increase in haemolymph osmolality (Zachariassen, 1985).

Quiescent adult beetles had higher osmolality and thermal hysteresis (875 mOsm) than summer adults (380 mOsm; Table 3.4). The increase in osmolality after seasonal acclimatization in the WLB is low in comparison to other freeze tolerant beetles. For example, the spruce bark beetle, *Ips typographus*, increases its osmolality from 339 mOsm in the summer to 1359 mOsm in the winter (Koštál et al., 2011a). The freeze tolerant beetle, *Phyllodecta laticollis*, increased its osmolality from 500 mOsm to 2500 mOsm after winter acclimatization (Van der Laak, 1982). For both of these freeze tolerant beetles, glycerol is a major driver of the increase in osmolality.

Quiescent adult beetles had significantly higher amounts of glycerol (24 mM) in whole body analyses compared to summer adults (9 mM; Figure 3.7). However, an increase in concentration of over 500 mM of glycerol would be expected if glycerol was a

major driver of the increase in osmolality of the quiescent adults compared to the summer adult beetles. Glycerol is a very commonly found cryoprotectant in insects, but it can be accumulated at much higher quantities than found in the WLB. For example, the freeze tolerant spruce bark beetle, *Ips typographus*, had a glycerol content of 11.2 µg/mg beetle in the winter (Koštál et al., 2011a) and the quiescent WLB accumulated only 1.3 µg/mg beetle tissue (Figure 3.7).

Other carbohydrates may have also contributed to the increase in osmolality of the quiescent adult willow leaf beetles along with the accumulation of glycerol. Elution peaks were consistent with glycerol, rhamnose and fructose in both summer and quiescent adult beetles (Figure 3.4). However, only the quiescent adult beetles showed a peak consistent with glucose. Glycerol and fructose are accumulated after cold exposure in some insects as cryoprotectants. Rhamnose was probably present in the gut contents since whole beetles were used for GC analysis. The presence of rhamnose could be the product of cellulose breakdown by yeast in the WLB beetle gut. Several yeast strains capable of digesting rhamnose were isolated from the digestive tracts of leaf beetles (Coleoptera: Chrysomelidae) (Nguyen et al., 2006). Glucose may be present in the quiescent beetle as another colligative cryoprotectant. The spruce bark beetle, *Ips typographus*, accumulated high levels of glucose as a cryoprotectant after seasonal acclimatization to winter conditions (Koštál et al., 2011a). In low concentrations, glucose may be the result of the breakdown of glycogen reserves from fat body cells (Hahn and Denlinger, 2011) rather than as a cryoprotectant. Some overwintering insects switch from lipids to glucose as a primary energy source during diapause and/or quiescence when lipid stores become depleted or during exposure to hypoxia (Hahn and Denlinger, 2011). Future studies may quantify changes in lipids and sugars over the course of the winter, during diapause and

quiescence of the willow leaf beetle, to determine if the glucose found in this study plays a role in cryoprotection, energy metabolism, or both.

Quiescent adult beetles had higher thermal hysteresis activity (0.25 °C) than summer adults (0.08 °C; Table 3.4). While the level of thermal hysteresis detected in the WLB haemolymph was quite low, this is not uncommon in insects. Modest levels of TH typically 0.2 – 0.5 °C or less (Duman et al., 2004), do not appear to prevent the formation of ice. Low thermal hysteresis activity in freeze tolerant insects may be used to promote controlled freezing at high sub-zero temperatures using ice nucleating agents (Zachariassen and Hammel, 1976). AFAs are thought to have a primary function of preventing recrystallisation. Antifreeze proteins and glycoproteins produce thermal hysteresis (Devries, 1986). Insects may not exhibit measureable TH in spite of producing large molecular weight antifreezes, and instead have only pronounced hexagonal crystal growth and/or recrystallisation inhibition (Tursman et al., 1994).

There was no evidence of faceting during ice crystal growth in any of the adult WLBs, all ice crystals were round (Table 3.4). Recrystallisation can cause ice crystals to continue to grow over time which could result in piercing of insect tissues. Since there is no detectable antifreeze agent activity in the adults, recrystallisation could be causing the mortality in quiescent adults after 4.5 h exposure to internal ice. Recrystallisation inhibitors with no detectable thermal hysteresis activity may play an important role in membrane stabilization, and preventing inoculative freezing in freeze tolerant insects if they are associated with the cuticle or the gut (e.g. Wharton et al., 2005).

*4.2.2 Avoidance behaviour and life cycle timing*

The summer and quiescent adult willow leaf beetles have limited cold tolerance in comparison to other freeze tolerant insects. Species with limited cold tolerance may

compensate with a behavioural strategy in which adults migrate to the soil to overwinter. Quiescent WLB moved from the mid-willow to the soil beneath the willow for their winter hibernaculum (J. Smiley, personal communication). The minimum temperature recorded at the mid-willow was consistently lower than the soil temperature with a difference of over 16 °C. Cold exposures beyond the limits of an insect's cold tolerance may be avoided by remaining in the winter hibernaculum (Wagner et al., 2012). The quiescent adult willow leaf beetles would be exposed to potentially lethal cold events at the mid-willow at all sites along an elevation gradient (Figure 3.8) and among the drainages (Figure 3.9).

The summer and the quiescent adult WLB were the only life stages to experience potentially lethal low temperatures ($LT_{50}$) at the mid-willow. During the summer adult time period (see Table 2.4), the minimum temperature fell below $LT_{50}$ at the mid-willow only five times between all field sites and lasted between 0.5 h to 2 h. The transition between a cold-hardy quiescent adult, to a summer adult that has acclimatised to summer temperatures likely does not occur in a step-wise fashion, but gradually over time. The potentially lethal cold exposure temperatures recorded for the summer adults in all years ranged from -9.6 °C to -14 °C which are all lower than their $LT_{50}$ (ca. -9 °C). These minimum temperatures are not low enough to fall below the $LT_{50}$ for quiescent adults (ca. -15 °C). If these potentially lethal cold events occurred very early in the spring when extreme diurnal temperature fluctuations are common, their cold tolerance may have been higher than the levels indicated in this study. The summer adult beetles during early spring may have retained some overwintering cold tolerance correlates accumulated during quiescence, or they may have undergone cold hardening in response to low temperatures experienced above their $LT_{50}$.

Insects in variable climates like the arctic or in the alpine regions may be more likely to retain freeze tolerance throughout the year as well as a faster cold hardening response to unexpected cold snaps than insects in less variable climates (Danks et al., 1994). The quiescent adults were only able to survive in a frozen state for 4.5 h, indicating low freeze tolerance, however this may be sufficient to survive unexpected overnight cold snaps before moving to a protected microhabitat. The willow leaf beetle adults are able to survive mild cold exposures without showing a loss of coordinated limb movements indicative of chilling injuries (MacMillan and Sinclair, 2011a). Future studies may look at the transition from quiescent levels of cold hardiness to summer levels in order to understand the impacts of early snow melts and shifts in phenology on survival of adult WLB.

**4.3 Cold tolerance of the willow leaf beetle eggs and larvae**

During the summer (June – August), minimum air temperatures at the mid-willow never reached the $LT_{50}$ for any of the juvenile life stages (egg, first, second and third instar larva). Willow leaf beetle eggs progressed to hatching as long as internal ice formation did not occur indicating they are likely to be freeze avoidant. All three larval instars had an $LT_{50}$ that was above their SCP indicating they are all chill susceptible.

Eggs have a lower supercooling point (ca. -20 °C) when compared to other life stages, likely because of their composition and small size. Insect eggs are comprised of proteins, fats, amino acids and sugars (Giron and Casas, 2003) and often have low SCPs. For example, eggs of the multicoloured asian lady beetle (*Harmonia axyridis*) have a SCP as low as -27 °C (Koch et al., 2004). The SCPs were not significantly different between the three larval instars of the willow leaf beetle (ca. -8 to -11 °C; Table 3.1). There was a

trend towards a decrease in the $LT_{50}$ temperatures from the first instar larvae (-3.3 °C) through the third instar larvae (-8.7 °C; Table 3.3).

Eggs and larvae have similar low glycerol content (Figure 3.7). Glycerol can be accumulated after cold exposure as a cryoprotectant, such as in locust eggs (*Locusta migratoria*) (Wang et al., 2010). Eggs and larvae also showed evidence of fructose and rhamnose through GC analysis (Figures 3.6 and 3.5 respectively) which may contribute to their increased osmolality. Eggs had relatively high thermal hysteresis (0.19 °C) and osmolality (746 mOsm) compared to the other life stages of the WLB. Eggs of the stonefly, (*Arcynopteryx compacta*) have a thermal hysteresis of up to 1.8 °C in summer (Gehrken and Sømme, 1987). All three larval instars of the WLB had haemolymph osmolality of ca. 443 – 687 mOsm, and low thermal hysteresis (0.05 – 0.10°C; Table 3.4). It is unlikely that WLB eggs have AFAs based on the round ice crystal growth shape and lack of thermal hysteresis observed in this study. First and second instar larvae showed mild AFA activity since their ice crystals had a distinctly flat edge on an otherwise round ice crystal; the third instar larva showed no AFA activity (Table 3.4). Since, the willow leaf beetle eggs and larvae are present in the mid-willow between June and August when air temperatures are warm, there is no need for extensive cold hardening.

**4.4 Cold tolerance of the pupa of the willow leaf beetle**

Willow leaf beetle pupae were all killed by low temperature exposures that caused freezing, suggesting that WLB pupae are freeze avoidant. One third of the WLB pupae showed a single exotherm and two thirds of the pupae showed two exotherms (Figure 3.1). The single exotherm and the first of the paired exotherms were not significantly different from each other (Table 3.2). The pupal body consists mostly of forming adult tissues. The similar SCPs could be the result of freezing events in the

extracellular spaces of these forming adult tissues. The SCP of the second exotherm begins at (-17.5 ℃) and is likely the result of a freezing event in the dehydrating third instar larval tissues (Figure 3.2). When the third instar tissue fully dehydrates, it can no longer freeze, which could explain the absence of a second (lower) exotherm in some pupae.

The total water content of pupae is higher than that of the other life stages (Figure 3.3) as well as other insect pupae. For example, pupae of Hessian fly, *Mayetiola destructor*, have 63-65% water content (Benoit et al., 2010) whereas the WLB pupae have ca. 83% water content (Figure 3.3). Pupal development stage was not controlled for water content determination, so a higher water content may be a consequence of tissue restructuring associated with metamorphosis (Rjordan, 1970). Not controlling for development stage also resulted in higher variance in water content measured in the pupae (Figure 3.3). Since no pupae survived internal ice formation, the onset of the freeze tolerant strategy in adults must happen after eclosion, but in the absence of acclimation (Chen et al., 1987).

In the fall of 2011, there was a night-time frost in the Big Pine Creek drainage while many pupae were on the leaves in the mid-willow. This frost was observed to be on the cuticle of the pupae, and caused all of the pupae to die. The new adult beetles that had already eclosed were also observed to be in contact with frost and did not show signs of mortality (J. Smiley personal communication). The minimum air temperature recorded at the mid-willow for the pupae in 2011 did not reach the $LT_{50}$ determined for this life stage in the lab. This observed mortality may have been the result of inoculative freezing from contact with ice crystals after the frost. Since the eclosed adult beetles showed no mortality, they may be resistant to inoculative freezing and freeze tolerant.

WLB pupae had low haemolymph osmolality (570 mOsm) and thermal hysteresis (0.9 °C) which was similar to that of the larvae (Table 3.4). The pupae showed the most AFA activity out of any of the life stages. The ice crystals had at least 2 flat edges, and some were distinctly square in shape (Table 3.4). Typically, moderate to high AFA activity causes ice crystals to become angular, spicular, or bipyramidal (Bar-Dolev et al., 2012). The weak faceting of the ice crystals observed in the WLB can be attributed to either weak AFA activity, or a low concentration of AFA (DeLuca et al., 1996) in the haemolymph of the pupae.

In some insects, AFAs are associated with the cuticle, where they function to prevent inoculative freezing from external ice (Olsen et al., 1998). For example, the cockroach (*Celatoblatta quinquemaculata*) demonstrates antifreeze activity and high levels of thermal hysteresis in the gut tissue that are absent in the haemolymph (Wharton et al., 2009). Future studies could look for tissue specific AFA in the pupae of the WLB to determine if they play a role in protection against inoculative freezing during over-night frosts.

**4.5 Conclusions and future directions**

The willow leaf beetle uses different cold tolerance strategies throughout its life cycle. Eggs are freeze avoidant, larvae are chill susceptible, pupae are freeze avoidant and all adults are freeze tolerant. Quiescent adults overwinter during the lowest temperatures of the year in the Sierra Nevada Mountains. The quiescent adults are the most cold tolerant life stage with the highest level of cold tolerance correlates including glycerol and haemolymph osmolality. The behavioural strategy of moving into the soil protects quiescent adults from cold exposures beyond the limits of their cold tolerance, indicating that behavioural avoidance strategies can be just as important as cold tolerance. Freeze

tolerance helps both summer and winter adult beetles survive overnight cold snaps. Juvenile life stages were not subjected to lethal cold temperatures based on summer air temperatures, however field observations showed that inoculative freezing from frosts caused mortality in pupae.

Future studies should focus on the cold tolerance of the willow leaf beetle during the spring and fall in the Sierra Nevada Mountains. The spring and fall are highly variable on a daily as well as an interannual time scale due to the persistence and the timing of snow cover. The summer adults and the pupae are the life stages that are most vulnerable to lethal cold events due to this variability in the spring and fall. In the fall, studies could focus on the mechanisms needed for resistance to inoculative freezing and the limits to cold tolerance plasticity in the pupae. With the variable temperatures of the fall when frosts frequently occur, tissue-specific antifreeze agents may reduce the mortality suffered by pupae due to overnight frosts. After eclosion from the pupa, significant cold hardening is needed before the beetles reach the quiescent adult levels of cold tolerance. The survival of the beetle during this transition period may require significant plasticity and rapid cold hardening which remains to be investigated.

Willow leaf beetle quiescent and summer adults are both freeze tolerant. The quiescent adults have greater cold tolerance than the summer adults which have lost their accumulated cryoprotectants. In the early spring, the transition from quiescent adult to summer adult and the loss of cryoprotectants could begin after emergence from their protected winter hibernaculum. Future studies on the plasticity of cold tolerance in the adult willow leaf beetle through acclimatisation during this transition period are needed. The rate of change between the quiescent to the summer adult levels of cold tolerance depends on the air temperatures experienced after emergence. The daily and seasonal

temperature variability may also require plasticity in cold tolerance to survive unpredictable cold events caused by local weather patterns. The mechanisms underlying plasticity in cold tolerance as well as the persistence of freeze tolerance after the loss of cryoprotectants remains to be investigated.

## CHAPTER 5: REFERENCES

Achari, A., Marshall, S. E., Muirhead, H., Palmieri, R. H. and Noltmann, E. A. (1981). Glucose-6-phosphate isomerase. Philosophical Transactions of the Royal Society of London B 293, 145-157.

Angiletta, M. J. (2009). Thermal adaptation: Atheoretical and empirical synthesis. New York, New York: Oxford University Press.

Bale, J. S. (2002). Insects and low temperatures: From molecular biology to distributions and abundance. Philosophical Transactions of the Royal Society of London B 357, 849-861.

Bale, J. S., Harrington, R. and Clough, M. S. (1988). Low-temperature mortality of the peach-potato aphid *Myzus persicae*. Ecological Entomology 13, 121-129.

Bar-Dolev, M., Celik, Y., Wettlaufer, J. S., Davies, P. L. and Braslavsky, I. (2012). New insights into ice growth and melting modifications by antifreeze proteins. Journal of the Royal Society Interface (In Press).

Barrett, J. (2001). Thermal hysteresis proteins. International Journal of Biochemistry & Cell Biology 33, 105-117.

Bennett, V. A., Lee, R. E., Nauman, J. S. and Kukal, O. (2003). Selection of overwintering microhabitats used by the arctic woollybear caterpillar, *Gynaephora groenlandica.* Cryoletters 24, 191-200.

Benoit, J. B., Morton, P. K., Cambron, S. E., Patrick, K. R. and Schemerhorn, B. J**.** (2010). Aestivation and diapause syndromes reduce the water balance requirements for pupae of the hessian fly, *Mayetiola destructor*. Entomologia Experimentalis Et Applicata 136, 89-96.

Block, W. (1990). Cold tolerance of insects and other arthropods. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 326, 613-&.

Block, W. (1991). To freeze or not to freeze - invertebrate survival of subzero temperatures. Functional Ecology 5**,** 284-290.

Bowler, K. (2005). Acclimation, heat shock and hardening. Journal of Thermal Biology 30, 125-130.

Bradshaw, W. E., Holzapfel, C. M. (2010). Insects at not so low temperature: Climate change in the temperate zone and its biotic consequences. In Low temperature biology of insects, (ed. D. L. Denlinger, Lee, R. E.), pp. 242-275: Cambridge University Press.

Broufas, G. D., Pappas, M. L. and Koveos, D. S. (2006). Effect of cold exposure and photoperiod on diapause termination of the predatory mite *Euseius finlandicus* (Acari : Phytoseiidae). Environmental Entomology 35, 1216-1221.

Brown, W. J. (1956). The new world species of *Chrysomela* l. (Coleoptera: Chrysomelidae). Canadian Entomologist 88 Supp. 3, 3-54.

Chapsky, L. and Rubinsky, B. (1997). Kinetics of antifreeze protein-induced ice growth inhibition. Febs Letters 412, 241-244.

Chen, C. P., Denlinger, D. L. and Lee, R. E. (1987). Cold-shock injury and rapid cold hardening in the flesh fly *Sarcophaga crassipalpis*. Physiological Zoology 60, 297-304.

Chown, S. L., Sinclair, B. J. (2010). The macrophysiology of insect cold-hardiness. In Low temperature biology of insects, (ed. D. L. Denlinger, Lee, R. E.), pp. 191-222: Cambridge University Press.

Coulson, S. J., Hodkinson, I. D., Block, W., Webb, N. R. and Worland, M. R. (1995). Low summer temperatures - a potential mortality factor for high arctic soil microarthropods. Journal of Insect Physiology 41, 783-792.

Crosthwaite, J. C., Sobek, S., Lyons, D. B., Bernards, M. A. and Sinclair, B. J. (2011). The overwintering physiology of the emerald ash borer, *Agrilus planipennis fairmaire* (Coleoptera: Buprestidae). Journal of Insect Physiology 57, 166-173.

Crozier, L. (2004). Warmer winters drive butterfly range expansion by increasing survivorship. Ecology 85, 231-241.

Czajka, M. C. and Lee, R. E. (1990). A rapid cold-hardening response protecting against cold shock injury in *Drosophila melanogaster*. Journal of Experimental Biology 148, 245-254.

Dahlhoff, E. P., Fearnley, S. L., Bruce, D. A., Gibbs, A. G., Stoneking, R., Mcmillan, D. M., Deiner, K., Smiley, J. T. and Rank, N. E. (2008). Effects of temperature on physiology and reproductive success of a montane leaf beetle: Implications for persistence of native populations enduring climate change. Physiological and Biochemical Zoology 81, 718-32.

Dahlhoff, E. P. and Rank, N. E. (2000). Functional and physiological consequences of genetic variation at phosphoglucose isomerase: Heat shock protein expression is related to enzyme genotype in a montane beetle. Proceedings of the National Academy of Sciences of the United States of America 97, 10056-10061.

Dahlhoff, E. P. and Rank, N. E. (2007). The role of stress proteins in responses of a montane willow leaf beetle to environmental temperature variation. Journal of Biosciences 32, 477-488.

Danks, H. V. (1987). Insect dormancy: An ecological perspective. Ottawa: Biological Survey of Canada Press.

Danks, H. V. (2004). Seasonal adaptations in Arctic insects. Integrative and Comparative Biology 44, 85-94.

Danks, H. V. (2006). Short life cycles in insects and mites. Canadian Entomologist 138, 407-463.

Danks, H. V., Kukal, O. and Ring, R. A. (1994). Insect cold-hardiness - insights from the arctic. Arctic 47, 391-404.

Deluca, C. I., Chao, H., Sonnichsen, F. D., Sykes, B. D. and Davies, P. L. (1996). Effect of type iii antifreeze protein dilution and mutation on the growth inhibition of ice. Biophysical Journal 71, 2346-2355.

Denlinger, D. L. (2002). Regulation of diapause. Annual Review of Entomology 47, 93-122.

Dodson, R. and Marks, D. (1997). Daily air temperature interpolated at high spatial resolution over a large mountainous region. Climate Research 8, 1-20.

Doucet, D., Walker, V. K. and Qin, W. (2009). The bugs that came in from the cold: Molecular adaptations to low temperatures in insects. Cellular and Molecular Life Sciences 66, 1404-1418.

Douglas, G. W. (1972). Subalpine plant communities of the western north Cascades, Washington. Arctic and Alpine Research 4, 147-166.

Duman, J. G. (2001). Antifreeze and ice nucleator proteins in terrestrial arthropods. Annual Review of Physiology 63, 327-357.

Duman, J. G., Bennett, V., Sformo, T., Hochstrasser, R. and Barnes, B. M. (2004). Antifreeze proteins in Alaskan insects and spiders. Journal of Insect Physiology 50, 259-266.

Edwards, J. S. (1987). Arthropods of alpine aeolian ecosystems. Annual Review of Entomology 32, 163-179.

Franklin, J. F. and Macmahon, J. A. (2000). Ecology - messages from a mountain. Science 288, 1183-1185.

Furusawa, T., Shikata, M. and Yamashita, O. (1982). Temperature-dependent sorbitol utilization in diapause eggs of the silkworm, *Bombyx mori*. Journal of Comparative Physiology 147, 21-26.

Gehrken, U. and Sømme, L. (1987). Increased cold hardiness in eggs of *Arcynopteryx compacta* (Plecoptera) by dehydration. Journal of Insect Physiology 33, 987-991.

Giron, D. and Casas, J. (2003). Mothers reduce egg provisioning with age. Ecology Letters 6, 273-277.

Goddeeris, B. R., Vermeulen, A. C., De Geest, E., Jacobs, H., Baert, B. and Ollevier, F. (2001). Diapause induction in the third and fourth instar of *Chironomus riparius* (Diptera) from Belgian lowland brooks. Archiv Fur Hydrobiologie 150, 307-327.

Hahn, D. A. and Denlinger, D. L. (2007). Meeting the energetic demands of insect diapause: Nutrient storage and utilization. Journal of Insect Physiology 53, 760-773.

Hahn, D. A. and Denlinger, D. L**.** (2011). Energetics of insect diapause. In Annual review of Entomology, vol. 56 eds. M. R. Berenbaum R. T. Carde and G. E. Robinson), pp. 103-121. Palo Alto: Annual Reviews.

Hochachka, P. W. and Somero, G. N. (1984). Biochemical adaptation: Mechanism and process in physiological evolution. New York: Oxford University Press.

Hodkova, M. and Hodek, I. (2004). Photoperiod, diapause and cold-hardiness. European Journal of Entomology 101, 445-458.

Horwath, K. L. and Duman, J. G. (1984). Yearly variations in the overwintering mechanisms of the cold-hardy beetle *Dendroides canadensis*. Physiological Zoology 57, 40-45.

Irwin, J. T. and Lee, R. E. (2002). Energy and water conservation in frozen vs. supercooled larvae of the goldenrod gall fly, *Eurosta solidaginis* (fitch) (Diptera : Tephritidae). Journal of Experimental Zoology 292, 345-350.

Irwin, J. T. and Lee, R. E., Jr. (2000). Mild winter temperatures reduce survival and potential fecundity of the goldenrod gall fly, *Eurosta solidaginis* (Diptera: Tephritidae). Journal of Insect Physiology 46, 655-661.

Irwin, J. T. and Lee, R. E., Jr. (2003). Cold winter microenvironments conserve energy and improve overwintering survival and potential fecundity of the goldenrod gall fly, *Eurosta solidaginis*. Oikos 100, 71-78.

Izumi, Y., Sonoda, S. and Tsumuki, H. (2007). Effects of diapause and cold-acclimation on the avoidance of freezing injury in fat body tissue of the rice stem borer, *Chilo suppressalis*. Journal of Insect Physiology 53, 685-690.

Ju, R. T., Xiao, Y. Y. and Li, B. (2011). Rapid cold hardening increases cold and chilling tolerances more than acclimation in the adults of the sycamore lace bug, *Corythucha ciliata* (Hemiptera: Tingidae). Journal of Insect Physiology 57, 1577-1582.

Kaneko, J., Kita, K. and Tanno, K. (1991). INA bacteria isolated from diamondback moth, *Plutella xylostella* l pupae (Lepidoptera, Yponomeutidae). Japanese Journal of Applied Entomology and Zoology 35, 7-11.

Karl, I., Schmitt, T. and Fischer, K. (2008). Phosphoglucose isomerase genotype affects life-history traits and cold stress resistance in a copper butterfly. Functional Ecology 22, 887-894.

Kevan, P. G. and Baker, H. G. (1983). Insects as flower visitors and pollinators. Annual Review of Entomology 28, 407-453.

Kipyatkov, V. E. and Lopatina, E. B. (2009). Temperature and photoperiodic control of diapase induction in the ant *Lepisiota semenovi* (Hymenoptera, Formicidae) from Turkmenistan. Journal of Evolutionary Biochemistry and Physiology 45, 238-245.

Koch, R. L., Carrillo, M. A., Venette, R. C., Cannon, C. A. and Hutchison, W. D. (2004). Cold hardiness of the multicolored asian lady beetle (Coleoptera : Coccinellidae). Environmental Entomology 33, 815-822.

Körner, C. (1998). A re-assessment of high elevation treeline positions and their explanation. Oecologia 115, 445-459.

Koštál, V. (2006). Eco-physiological phases of insect diapause. Journal of Insect Physiology 52, 113-127.

Koštál, V., Dolezal, P., Rozsypal, J., Moravcova, M., Zahradnickova, H. and Simek, P. (2011a). Physiological and biochemical analysis of overwintering and cold tolerance in two central European populations of the spruce bark beetle, *Ips typographus*. Journal of Insect Physiology 57, 1136-1146.

Koštál, V., Renault, D. and Rozsypal, J. (2011b). Seasonal changes of free amino acids and thermal hysteresis in overwintering heteropteran insect, *Pyrrhocoris apterus*. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 160, 245-251.

Koštál, V., Vambera, J. and Bastl, J. (2004). On the nature of pre-freeze mortality in insects: Water balance, ion homeostasis and energy charge in the adults of P*yrrhocoris apterus*. Journal of Experimental Biology 207, 1509-1521.

Kukal, O. and Duman, J. G. (1989). Switch in the overwintering strategy of two insect species and latitudinal differences in cold hardiness. Canadian Journal of Zoology 67, 825-827.

Lagerspetz, K. Y. H. (2006). What is thermal acclimation? Journal of Thermal Biology 31, 332-336.

Larcher, W., Kainmuller, C. and Wagner, J. (2010). Survival types of high mountain plants under extreme temperatures. Flora 205, 3-18.

Lazaridis, M. (2010). First principles of meteorology and air pollution. New York, Heidlberg, London: Springer Dordrecht.

Lee, R. E. (2010a). A primer on insect cold-tolerance. In low temperature biology of insects, (ed. D. L. Denlinger, Lee, R. E.), pp. 3-34: Cambridge University Press.

Lee, R. E. (1989). Insect cold-hardiness - to freeze or not to freeze - how insects survive low temperatures. Bioscience 39, 308-313.

Lee, R. E., Chen, C. P. and Denlinger, D. L. (1987). A rapid cold-hardening process in insects. Science 238, 1415-1417.

Lee, R. E. and Denlinger, D. L. (1985). Cold tolerance in diapausing and non-diapausing stages of the flesh fly, *Sarcophaga crassipalpis*. Physiological Entomology 10, 309-315.

Lee, R. E., Denlinger, D. L. (2010b). Rapid cold-hardening: Ecological significance and underpinning mechanisms. In low temperature biology of insects, (ed. D. L. Denlinger, Lee, R. E.), pp. 35-58: Cambridge University Press.

Levin, D. B., Danks, H. V. and Barber, S. A. (2003). Variations in mitochondrial DNA and gene transcription in freezing-tolerant larvae of *Eurosta solidaginis* (Diptera : Tephritidae) and *Gynaephora groenlandica* (Lepidoptera : Lymantriidae). Insect Molecular Biology 12, 281-289.

Li, N. G. (2012). Relationships between cold hardiness, and ice nucleating activity, glycerol and protein contents in the hemolymph of caterpillars, *Aporia crataegi*. Cryoletters 33, 135-143.

Loeschcke, V. and Sørensen, J. G. (2005). Acclimation, heat shock and hardening - a response from evolutionary biology. Journal of Thermal Biology 30, 255-257.

Lundquist, J. D. and Cayan, D. R. (2007). Surface temperature patterns in complex terrain: Daily variations and long-term change in the central Sierra Nevada, California. Journal of Geophysical Research-Atmospheres 112.

Macmillan, H. A. and Sinclair, B. J. (2011a). Mechanisms underlying insect chill-coma. Journal of Insect Physiology **5**7, 12-20.

Macmillan, H. A. and Sinclair, B. J. (2011b). The role of the gut in insect chilling injury: Cold-induced disruption of osmoregulation in the fall field cricket, *Gryllus pennsylvanicus*. Journal of Experimental Biology 214, 726-734.

Marshall, K. E. and Sinclair, B. J. (2012). Threshold temperatures mediate the impact of reduced snow cover on overwintering freeze tolerant caterpillars. Naturwissenschaften 99, 165-166.

Mcmillan, D. M., Fearnley, S. L., Rank, N. E. and Dahlhoff, E. P. (2005). Natural temperature variation affects larval survival, development and Hsp70 expression in a leaf beetle. Functional Ecology 19, 844-852.

Melack, J. M., Dozier, J., Goldman, C. R., Greenland, D., Milner, A. M. and Naiman, R. J. (1997). Effects of climate change on inland waters of the Pacific coastal mountains and western great basin of North America. Hydrological Processes 11, 971-992.

Michaud, M. R. and Denlinger, D. L. (2007). Shifts in the carbohydrate, polyol, and amino acid pools during rapid cold-hardening and diapause-associated cold-hardening in flesh flies (*Sarcophaga crassipalpis*): A metabolomic comparison. Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology 177, 753-763.

Morrissey, R. E. and Baust, J. G. (1976). Ontogeny of cold tolerance in gall fly, *Eurosta solidagensis*. Journal of Insect Physiology 22, 431-437.

Mugnano, J. A., Lee, R. E. and Taylor, R. T**.** (1996). Fat body cells and calcium phosphate spherules induce ice nucleation in the freeze-tolerant larvae of the gall fly *Eurosta solidaginis* (Diptera, Tephritidae). Journal of Experimental Biology 199, 465-471.

Neargarder, G., Dahlhoff, E. P. and Rank, N. E. (2003). Variation in thermal tolerance is linked to phosphoglucose isomerase genotype in a montane leaf beetle. Functional Ecology 17, 213-221.

Nguyen, N. H., Suh, S. O., Erbil, C. K. and Blackwell, M. (2006). *Metschnikowia noctiluminum* sp nov., *Metschnikowia corniflorae* sp nov., and *Candida chrysomelidarum* sp nov., isolated from green lacewings and beetles. Mycological Research 110, 346-356.

Nieminen, P., Paakkonen, T., Eerila, H., Puukka, K., Riikonen, J., Lehto, V. P. and Mustonen, A. M. (2012). Freezing tolerance and low molecular weight cryoprotectants in an invasive parasitic fly, the deer ked (*Lipoptena cervi*). Journal of Experimental Zoology Part a-Ecological Genetics and Physiology 317A, 1-8.

Ohyama, Y. and Asahina, E. (1972). Frost resistance in adult insects. Journal of Insect Physiology 18, 267.

Olsen, T. M., Sass, S. J., Li, N. and Duman, J. G. (1998). Factors contributing to seasonal increases in inoculative freezing resistance in overwintering fire-colored beetle larvae *Dendroides canadensis* (Pyrochroidae). Journal of Experimental Biology 201, 1585-1594.

Parmesan, C. (1996). Climate and species' range. Nature 382, 765-766.

Powell, S. J. and Bale, J. S. (2006). Effect of long-term and rapid cold hardening on the cold torpor temperature of an aphid. Physiological Entomology 31, 348-352.

Quinn, P. J. (1985). A lipid-phase separation model of low-temperature damage to biological membranes. Cryobiology 22, 128-146.

Ramløv, H. (1999). Microclimate and variations in haemolymph composition in the freezing-tolerant new zealand alpine weta *hemideina maori* Hutton (orthoptera : Stenopelmatidae). Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology 169, 224-235.

Rank, N. E. (1992a). A hierarchical analysis of genetic differentiation in a montane leaf beetle *Chrysomela aeneicollis* (Coleoptera, Chrysomelidae). Evolution 46, 1097-1111.

Rank, N. E**.** (1992b). Host plant preference based on salicylate chemistry in a willow leaf beetle *Chrysomela aeneicollis*. Oecologia 90, 95-101.

Rank, N. E. and Dahlhoff, E. P**.** (2002). Allele frequency shifts in response to climate change and physiological consequences of allozyme variation in a montane insect. Evolution 56, 2278-2289.

Raymond, J. A. and Devries, A. L. (1977). Adsorption inhibition as a mechanism of freezing resistance in polar fishes. Proceedings of the National Academy of Sciences of the United States of America 74, 2589-2593.

Raymond, J. A., Wilson, P. and Devries, A. L. (1989). Inhibition of growth of nonbasal planes in ice by fish antifreezes. Proceedings of the National Academy of Sciences of the United States of America 86, 881-885.

Regniere, J., St-Amant, R. and Duval, P. (2012). Predicting insect distributions under climate change from physiological responses: Spruce budworm as an example. Biological Invasions 14, 1571-1586.

Reznik, S. Y. (2011). Ecological and evolutionary aspects of photothermal regulation of diapause in Trichogrammatidae. Journal of Evolutionary Biochemistry and Physiology 47, 512-523.

Rickards, J., Kelleher, M. J. and Storey, K. B. (1987). Strategies of freeze avoidance in larvae of the goldenrod gall moth, *Epiblema scudderiana* - winter profiles of a natural-population. Journal of Insect Physiology 33, 443-450.

Riley, E., Clark, S. and Seeno, T. (2003). Catalog of leaf beetles of North America and Mexico: Coleopterists Society.

Rjordan, K. (1970). Development of *Glossina pal palis* r.D. Inside the puparium. Entomologist, 171-181.

Rojas, R. R. and Leopold, R. A. (1996). Chilling injury in the housefly: Evidence for the role of oxidative stress between pupariation and emergence. Cryobiology 33, 447-458.

Rolland, C. (2003). Spatial and seasonal variations of air temperature lapse rates in alpine regions. Journal of Climate 16, 1032-1046.

Serreze, M. C., Walsh, J. E., Chapin, F. S., Osterkamp, T., Dyurgerov, M., Romanovsky, V., Oechel, W. C., Morison, J., Zhang, T. and Barry, R. G. (2000). Observational evidence of recent change in the northern high-latitude environment. Climatic Change 46, 159-207.

Sformo, T., Walters, K., Jeannet, K., Wowk, B., Fahy, G. M., Barnes, B. M. and Duman, J. G. (2010). Deep supercooling, vitrification and limited survival to -100 degrees C in the Alaskan beetle *Cucujus clavipes puniceus* (Coleoptera: Cucujidae) larvae. Journal of Experimental Biology 213, 502-509.

Shiota, H. and Kimura, M. T. (2007). Evolutionary trade-offs between thermal tolerance and locomotor and developmental performance in drosophilid flies. Biological Journal of the Linnean Society 90, 375-380.

Sinclair, B. J. (1999). Insect cold tolerance: How many kinds of frozen? European Journal of Entomology 96, 157-164.

Sinclair, B. J. (2001). Field ecology of freeze tolerance: Interannual variation in cooling rates, freeze-thaw and thermal stress in the microhabitat of the alpine cockroach *Celatoblatta quinquemaculata*. Oikos 93, 286-293.

Sinclair, B. J., Addo-Bediako, A. and Chown, S. L. (2003). Climatic variability and the evolution of insect freeze tolerance. Biological Reviews 78, 181-195.

Sinclair, B. J., Lord, J. M. and Thompson, C. M. (2001). Microhabitat selection and seasonality of alpine invertebrates. Pedobiologia 45, 107-120.

Sinclair, B. J. and Roberts, S. P. (2005). Acclimation, shock and hardening in the cold. Journal of Thermal Biology 30, 557-562.

Smiley, J. T. and Rank, N. E. (1986). Predator protection versus rapid growth in a montane leaf beetle. Oecologia 70, 106-112.

Somero, G. N. (1995). Proteins and temperature. Annual Review of Physiology 57, 43-68.

Sømme, L. (1982). Supercooling and winter survival in terrestrial arthropods. Comparative Biochemistry and Physiology A-Physiology 73, 519-543.

Sømme, L. (1989). Adaptations of terrestrial arthropods to the alpine environment. Biological Reviews of the Cambridge Philosophical Society 64, 367-407.

Storey, K. B. and Storey, J. M. (1988). Freeze tolerance in animals. Physiological Reviews 68**,** 27-84.

Storey, K. B. and Storey, J. M. (1991). Biochemistry of cryoprotectants. In insects at low temperature, eds. D. L. Denlinger and R. E. Lee), pp. 64-93. New York: Chapman and Hall.

Tanno, K. (1970). Frost injury resistance in the poplar sawfly, *Trichiocampus populi okamoto*. Contributions from the Institute of Low Temperature Science Hokkaido University B 16, 1-41.

Tauber, M. J., Tauber, C. A. and Masaki, S. (1986). Seasonal adaptations of insects. New York: Oxford University Press.

Templer, P. H., Schiller, A. F., Fuller, N. W., Socci, A. M., Campbell, J. L., Drake, J. E. and Kunz, T. H. (2012). Impact of a reduced winter snowpack on litter arthropod abundance and diversity in a northern hardwood forest ecosystem. Biology and Fertility of Soils 48, 413-424.

Thackeray, S. J., Sparks, T. H., Frederiksen, M., Burthe, S., Bacon, P. J., Bell, J. R., Botham, M. S., Brereton, T. M., Bright, P. W., Carvalho, L. et al. (2010). Trophic level asynchrony in rates of phenological change for marine, freshwater and terrestrial environments. Global Change Biology 16, 3304-3313.

Theurillat, J. P. and Guisan, A. (2001). Potential impact of climate change on vegetation in the European alps: a review. Climatic Change 50, 77-109.

Tozetti, A. M. and Martins, M. (2008). Habitat use by the South-American rattlesnake (*Crotalus durissus*) in south-eastern Brazil. Journal of Natural History 42, 1435-1444.

Tursman, D., Duman, J. G. and Knight, C. A. (1994). Freeze tolerance adaptations in the centipede, *Lithobius forficatus*. Journal of Experimental Zoology 268, 347-353.

Van Asch, M. and Visser, M. E. (2007). Phenology of forest caterpillars and their host trees: The importance of synchrony. In Annual review of Entomology, vol. 52, pp. 37-55. Palo Alto: Annual Reviews.

Van Der Laak, S. (1982). Physiological adaptations to low-temperature in freezing-tolerant *Phyllodecta laticollis* beetles. Comparative Biochemistry and Physiology a-Physiology 73, 613-620.

Voituron, Y., Mouquet, N., De Mazancourt, C. and Clobert, J. (2002). To freeze or not to freeze? An evolutionary perspective on the cold-hardiness strategies of overwintering ectotherms. American Naturalist 160, 255-270.

Wagner, D., Doak, P., Sformo, T., Steiner, P. M. and Carlson, B. (2012). Overwintering physiology and microhabitat use of *Phyllocnistis populiella* (Lepidoptera: Gracilliariidae) in interior Alaska. Environmental Entomology 41, 180-187.

Walters, K. R., Serianni, A. S., Voituron, Y., Sformo, T., Barnes, B. M. and Duman, J. G. (2011). A thermal hysteresis-producing xylomannan glycolipid antifreeze associated with cold tolerance is found in diverse taxa. Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology 181, 631-640.

Wang, S., Amornwittawat, N. and Wen, X. (2012). Thermodynamic analysis of thermal hysteresis: Mechanistic insights into biological antifreezes. Journal of Chemical Thermodynamics 53, 125-130.

Wang, X. H., Qi, X. L. and Kang, L. (2010). Geographic differences on accumulation of sugars and polyols in locust eggs in response to cold acclimation. Journal of Insect Physiology 56, 966-970.

Watt, W. B. (1977). Adaptation at specific loci .1. Natural-selection on phosphoglucose isomerase of Colias butterflies - biochemical and population aspects. Genetics 87, 177-194.

Werner, R. A. (1978). Overwinter survival of spear-marked black moth, *Rheumaptera hastata* (Lepidoptera geometridae), pupae in interior Alaska. Canadian Entomologist 110, 877-882.

Wharton, D. A., Barrett, J., Goodall, G., Marshall, C. J. and Ramlov, H. (2005). Ice-active proteins from the Antarctic nematode *panagrolaimus davidi*. Cryobiology 51**,** 198-207.

Wharton, D. A., Pow, B., Kristensen, M., Ramlov, H. and Marshall, C. J. (2009). Ice-active proteins and cryoprotectants from the New Zealand alpine cockroach, *Celatoblatta quinquemaculata*. Journal of Insect Physiology 55, 27-31.

Williams, C. M., Marshall, K. E., Macmillan, H. A., Dzurisin, J. D. K., Hellmann, J. J. and Sinclair, B. J. (2012). Thermal variability increases the impact of autumnal warming and drives metabolic depression in an overwintering butterfly. Plos One 7, e34470.

Wipf, S. and Rixen, C. (2010). A review of snow manipulation experiments in arctic and alpine tundra ecosystems. Polar Research 29**,** 95-109.

Yi, S. X., Moore, C. W. and Lee, R. E**.** (2007). Rapid cold-hardening protects *Drosophila melanogaster* from cold-induced apoptosis. Apoptosis 12, 1183-1193.

Yu, S. O., Brown, A., Middleton, A. J., Tomczak, M. M., Walker, V. K. and Davies, P. L. (2010). Ice restructuring inhibition activities in antifreeze proteins with distinct differences in thermal hysteresis. Cryobiology 61, 327-334.

Zachariassen, K. E. (1985). Physiology of cold tolerance in insects. Physiological Reviews 65, 799-832.

Zachariassen, K. E. and Hammel, H. T. (1976). Nucleating agents in hemolymph of insects tolerant to freezing. Nature 262, 285-287.

Zachariassen, K. E. and Husby, J. A. (1982). Stabilization of highly supercooled insects by thermal hysteresis antifreeze agents. Cryoletters 3, 316-316.

Zachariassen, K. E. and Kristiansen, E. (2000). Ice nucleation and anti-nucleation in nature. Cryobiology 41, 257-279.

Zhao, A. C., Long, D. P., Ma, S. Y., Xu, L. X., Zhang, M. R., Dai, F. Y., Xia, Q. Y., Lu, C. and Xiang, Z. H. (2012). Efficient strategies for changing the diapause character of silkworm eggs and for the germline transformation of diapause silkworm strains. Insect Science 19, 172-182.

## APPENDIX 1: CURRICULUM VITAE

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| **Name:** | Evelyn Boychuk |
| **Post-secondary education and degrees:** | University of Calgary<br>Calgary, AB, Canada<br>B.Sc. Zoology |
| **Awards:** | Research Contributions Scholarship, 2012<br>Graduate Thesis Research Award, 2012<br>White Mountain Research Station Minigrant, 2011<br>Jason Lang Scholarship, 2010 |
| **Work Experience:** | Graduate Teaching Assistant<br>Western University<br>2010-2012 |
| **Peer-reviewed Publications:** | Shartau, R.B., S. Harris, **E.C. Boychuk** and J.I Goldberg. 2010. Rotational behaviour of encapsulated pond snail embryos in diverse natural environments. *Journal of Experimental Biology*. **213**, 2086-2093. |
| **Conference Oral Presentations:** | Cold tolerance of the willow leaf beetle (*chrysomela aeneicollis*) is life-stage specific. Canadian Society of Zoologists 2012, Sackville, New Brunswick.<br><br>Behavioural, neural and ciliary responses to environmental stressors in embryos of the pond snail *Helisoma trivolvis*. Canadian Society of Zoologists 2010, Vancouver, British Columbia.<br><br>Characterization of neuronal activity using embryonic rotational behaviour in the pond snail *Helisoma trivolvis.* Annual Biological Sciences Student Conference, University of Calgary, 2009 |
| **Conference Poster Presentations:** | Cold tolerance of all life stages and overwintering physiology of the willow leaf beetle (*Chrysomela aeneicollis*). Canadian Society of Zoologists 2011, Ottawa, Ontario.<br><br>Characterization of embryonic rotational behaviour reveals neuronal response to hypoxia in the pond snail *Helisoma trivolvis*. Canadian Society of Zoologists 2009, Scarborough, Ontario. |