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Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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FREEZE TOLERANCE IN THE SPRING FIELD CRICKET, *GRYLLUS VELETIS*

(Thesis format: Monograph)

by

Alexander H McKinnon

Graduate Program in Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Masters in Biology

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Many insects are able to survive internal ice formation. However, the mechanisms underlying freeze tolerance are not well-understood, perhaps because of a lack of suitable model organisms. I found that the spring field cricket, *Gryllus veletis*, seasonally acquires freeze tolerance in the fall when kept outside in London, Ontario. Moreover, individuals acquired freeze tolerance in the laboratory in response to a simulated fall thermophotoperiod. Lab-acclimated *G. veletis* freeze at -6.1 ± 0.7 °C and the acquisition of freeze tolerance is accompanied by the accumulation of proline and trehalose. Crickets survived temperatures as low as -12 °C (1.5 h), and for one week at -8 °C. Lab-acclimated crickets were more cold-hardy than field-acclimatized crickets, with higher survival at both -12 °C and after one week at -8 °C. *Gryllus veletis* is a suitable candidate for further investigating freeze tolerance because it is easily reared and manipulated in a controlled laboratory environment.

Keywords

Spring field cricket, *Gryllus veletis*, Insecta, Orthoptera, Gryllidae, cold tolerance, freeze tolerance, overwintering, cryoprotectants, antifreeze proteins, ice nucleators, supercooling, model systems

Co-Authorship Statement

This work was conducted under the supervision of Dr. Brent J. Sinclair. All aspects of sampling design and analysis were planned in cooperation with Dr. Sinclair, and any publications subsequent to the completion of this work will be co-authored with him.

Acknowledgments

If I have seen further it is by standing on the shoulders of Giants in the Lab

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1 Introduction

Insects have colonized all terrestrial ecosystems, in which they experience a wide range of environments, exposing them to extreme high and low temperatures as well as to the risk of dehydration in many places. As ectotherms, insects are limited in their ability to regulate their internal temperature and are therefore at risk of injuries resulting from both high and low temperatures (Chown & Nicolson, 2004). At a global scale, absolute winter temperatures fluctuate more than summer temperatures and therefore may play a primary role in delineating latitudinal variation in insect processes (Williams *et al.*, 2015). Some insects, particularly those inhabiting alpine, polar and temperate regions, spend over half of their lives at risk to chilling and freezing injury and have evolved behavioural, physiological and biochemical mechanisms to cope with the stresses of low temperature (Williams *et al.*, 2015). Understanding how and why these adaptations are expressed in nature may provide explanations for current insect distributions and insight into predicting and controlling future distributions across changing climates and landscapes.

Cold-hardy insects survive sub-zero temperatures by either maintaining their internal fluids in a supercooled state below their melting point (freeze avoidance), or by surviving internal ice formation (freeze tolerance) (Denlinger & Lee, 2010; Zachariassen, 1985). Insect freeze tolerance was first described in the 18th century (Sømme, 2000), and has since received considerable scientific attention (Denlinger & Lee, 2010; Salt, 1961). Nevertheless, the underlying physiological mechanisms of freeze tolerance remain poorly understood, largely due to the lack of a tractable model organism, and few manipulative experiments (Hayward *et al.*, 2014; Sinclair & Renault, 2010). The overarching goal of my thesis is to assess the suitability of the spring field cricket, *Gryllus veletis*, as a new model organism that may be used in controlled, manipulative experiments to better understand the physiological mechanisms underlying insect freeze tolerance.

1.1 Insects at sub-zero temperatures

For overwintering insects, exposure to sub-zero temperatures may lead to freezing and potentially lethal cold injuries. In insects, internal freezing does not occur immediately upon cooling to 0 °C. Instead, insects are capable of cooling their hemolymph below their melting point without ice forming in a process called supercooling (Denlinger & Lee, 2010). The temperature at which an insect's body water spontaneously freezes is termed the supercooling point (SCP), which can be readily determined using a thermocouple to detect the exotherm associated with the release of heat as water forms an ice crystal (Denlinger & Lee, 2010). The majority of overwintering insects supercool extensively (Lee, 1989); therefore, determining the SCP of an insect provides insight into the relationship between exposure to low temperatures and the risk of internal ice formation.

Multiple insect lineages have independently evolved the ability to successfully overwinter in alpine, arctic and temperate regions where they will be exposed to sub-zero temperatures. Some insects that overwinter in these regions are able to avoid sub-zero temperatures by selecting warmer, buffered microhabitats, such as the acorn weevil, *Curculio glandium*, which burrows >5 cm into the soil while overwintering in Ontario, Canada (Udaka and Sinclair, 2014). However, the majority of insects that overwinter in alpine, arctic and temperate regions have enhanced cold tolerance through biochemical and physiological processes and are either freeze avoidant or freeze tolerant. Freeze tolerance may be advantageous in variable climates that have an unpredictable risk of ice nucleation, and in regions with very cold and long winters (Sinclair *et al.*, 2003). Insect freeze tolerance has repeatedly evolved across and within several lineages including Diptera, Coleoptera, Blattodea, Orthoptera, Hymenoptera and Lepidoptera (Chown & Sinclair, 2010). Further investigation into the precise mechanisms of freeze tolerance may provide insight into how and why these lineages evolved to become freeze tolerant. The remainder of this thesis will focus on insect freeze tolerance and elucidating these mechanisms.

1.2 Internal ice formation

For freeze-tolerant insects, the initiation of internal ice formation leads to multiple physiological stresses. To survive internal ice formation, freeze-tolerant insects must mitigate mechanical damage, cellular dehydration, anoxia and reduced metabolic activity, while maintaining necessary physiological processes (Lee, 2010). However, the mechanisms by which freeze-tolerant insects mitigate these freezing injuries as well as the processes of internal ice formation are not well understood (Sinclair & Renault, 2010). Throughout this thesis I follow with the general model of insect freeze tolerance in assuming that ice formation is restricted to extracellular spaces (Zachariassen, 1985). However it should be noted that intracellular ice formation has been observed in some freeze-tolerant insects, although its prevalence has not been fully explored (Sinclair & Renault, 2010).

The initiation of ice formation within an insect occurs either by homogeneous or heterogeneous nucleation (Zachariassen & Kristiansen, 2000). Homogeneous nucleation occurs at temperatures of -5 °C to -40 °C and requires a stochastic aggregation of supercooled water molecules that reach a critically large size (Zachariassen & Kristiansen, 2000). Heterogeneous nucleation, which appears to account for the majority of nucleation in insects, involves substances other than liquid water (Zachariassen *et al.*, 2004) (see section 1.1.2.1). These molecules are commonly referred to as ice nucleating agents (INAs), which configure and stabilize aggregations of water molecules, promoting ice formation at higher temperatures than homogenous nucleation (Tattersall *et al.*, 2012; Zachariassen & Kristiansen, 2000).

Freeze-tolerant insects may be able to influence the location and rate of internal ice formation through the production of endogenous INAs (see section 1.1.2.1) or the ingestion of exogenous INAs. Exogenous ice nucleators are likely retained in the gut of freeze-tolerant insects, as observed by Sinclair *et al.* (1999) in alpine and lowland weta, *Hemideina* spp. Some gut INAs are so efficient that they are produced commercially to induce ice formation at higher temperatures in skating rinks and ski hills, such as the ice-nucleating protein on the surface of bacteria (*Pseudomonas syringae*) present in the gut

of the lady beetle, *Hippodamia convergens* (Strong-Gunderson *et al.*, 1990). Species of the fungus *Fusarium* have also been identified as exogenous INAs in the gut of freeze-tolerant larvae of the rice stem borer, *Chilo suppressalis* (Tsumuki *et al.*, 1992; Hirai & Tsumuki, 1995).

During inoculative freezing, ice formation is initiated by the contact of external ice with internal fluids, and subsequent propagation within the insect. This mode of ice formation is necessary for some insects to survive freezing. For example, diapausing larvae of the dipteran, *Chymomyza costata*, can survive and develop after experiencing temperatures as low as -70 °C, but only if inoculative freezing occurs at -2 °C (Shimada & Riihimaa, 1988).

Once freezing begins, ice propagates rapidly at first, but the entire process may last for 1-2 days or until an equilibrium is reached (Lee, 1991). For example, when the freeze-tolerant larvae of the goldenrod gall fly, *Eurosta solidaginis*, are held at -23 °C, 10 % of the available water becomes ice within the first five minutes; however, it takes 6 h for 50 % of the water to become ice, and ice formation does not reach equilibrium (60 % ice) until the fly has been frozen for 40 h (Lee & Lewis, 1985). When freeze-tolerant Orthoptera, *Hemideina maori*, are exposed to -5 °C, ice formation progresses at a rate of 15 % of the total body water per hour for the first 3-4 hours and reaches equilibrium after being held frozen for approximately 9 h (Ramløv & Westh, 1993).

1.2.1 Freezing injury

Injury due to freezing may occur at the level of the cell, tissue, organ or whole organism. However, much of the theoretical framework for understanding the freezing injuries in insects is based on research directed toward cryopreservation of mammalian cells, particularly human red blood cells (Sinclair & Renault, 2010). Researchers should use care when extrapolating this framework to insect systems because human red blood cells are of an atypical nature (lacking nucleus and other cellular organelles), the fast cooling and warming rates ($\pm 10^4$ °C min⁻¹) used in cryopreservation research are not ecologically relevant, and the goal of cryopreservation is to prevent ice formation (vitrification) (Lee, 1991; Saragusty & Arav, 2011).

As ice forms in the extracellular spaces and the ice lattice grows, solutes become concentrated in the hemolymph. The resulting increase in osmotic pressure leads to the outflow of intracellular water down its osmotic gradient and ultimately causes cellular dehydration. In mammalian cells, a minimum cellular volume is required to maintain structural integrity (Meryman, 1974). If cells become too dehydrated, mechanical damage to the membrane via membrane fusions and phase transitions/separations of membrane components may cause cellular leakage (Quinn, 1985). At equilibrium, concentrations of intracellular solutes lower the melting point, which ensures that intracellular freezing does not occur at that temperature (Tattersall et al., 2012). These new osmotic concentrations may lead to other injuries caused by osmotic shock, pH shifts that alter protein structure and function, and elevated concentrations of signaling molecules (Lee, 1991; Meryman, 1974).

The rigid structure of ice crystals may also result in mechanical damage to the insect. As water turns to ice, it expands by approximately 10 %, potentially rupturing cellular membranes (Lee, 1991). During slow warming of frozen tissue or during prolonged freezing, larger ice crystals grow at the expense of smaller crystals in a process termed recrystallization, which can further distort surrounding cells and disrupt tissue organization (Tattersall *et al.*, 2012; Knight & Duman, 1986). Extracellular ice formation may also reduce the capacity of the insect's circulatory system, resulting in anoxic, acidotic, and high lactate conditions (Lee, 1991).

Freeze-tolerant insects not only have to mitigate freezing injuries but also survive the thawing process. As an insect is rewarmed the internal ice begins to melt, exposing cells to a relatively hypotonic extracellular solution. In mammalian cells, an influx of water into the cell can lead to swelling or lysis (McGrath, 1987). Simultaneously, reperfusion of tissues promotes oxidative stress, in which reactive oxygen species (ROS) are able to oxidize macromolecules, causing membrane damage via lipid peroxidation (Costanzo & Lee, 2013; Doelling *et al.*, 2014). In the context of cryopreservation, an overall increase in survival has been observed with increased warming rates (Nishino & Baust, 1989), possibly due to reduced growth and redistribution of ice crystals associated with recrystallization (Leopold, 1991).

Freeze-tolerant insects have lethal limits with respect to temperature, time spent frozen and number of freeze-thaw cycles. For example, in lowland weta (*Hemideina* spp.), *H. crassidens* survive freezing for 120 min at -5 °C but not at -7.5 °C, while *H. thoracica* can survive freezing at -5 °C for 150 min, but die after 210 min (Sinclair *et al.*, 1999). Independent of the duration of time spent frozen, Marshall and Sinclair (2011) observed a 30 % increase in mortality in woolly bear caterpillars, *Pyrrharctia isabella*, that were repeatedly frozen, which demonstrates that freeze-thaw cycles impose additional injuries to insects.

The proposed mechanisms of freezing injury in insects (mechanical, dehydration, oxidative stress) are neither mutually exclusive nor readily distinguishable. It is difficult to determine which of these freezing injuries are lethal, as current model systems for studying insect freeze tolerance are not easily manipulated in the lab. However, these proposed mechanisms, which are mostly based on correlative approaches or cryopreservation research, provide the foundation for the current model of how insects survive internal ice formation (Lee & Denlinger, 2010).

1.2.2 Biochemicals associated with freeze tolerance

The specific mechanisms of insect freeze tolerance are unknown, although multiple studies have characterized the physiological state of freeze-tolerant insects (e.g. Storey & Storey, 1983, Tsumaki *et al.*, 1992; Walters *et al.*, 2009). While strictly correlative, these studies have identified three main groups of biochemicals associated with freeze tolerance: ice nucleating agents, low-molecular-weight cryoprotectants, and thermal hysteresis agents.

1.2.2.1 Ice nucleating agents (INAs)

The ability to control heterogeneous nucleation is one of the few physiological differences between freeze-tolerant and freeze-avoidant insects (Tattersall *et al.*, 2012). Ice nucleating agents promote the transition of water from liquid to solid form by organizing water molecules into an ice crystal-like conformation (Zachariassen & Kristiansen, 2000). Freeze-tolerant insects potentially select for endogenous ice

nucleators, commonly in the form of protein ice nucleators, lipoprotein ice nucleators, and other inorganic molecules (Duman, 2001). For example, the freeze-tolerant orthopteran *H. maori*, produces a proteinaceous ice nucleating agent in its hemolymph (Wilson & Ramløv, 1995). Larvae of the freeze-tolerant crane-fly, *Tipula trivittata*, produce an ice-nucleating lipoprotein in their hemolymph that contains a phospholipid monolayer, which may be the active site for binding with water molecules (Duman *et al.*, 1985; Duman, 2001). Endogenous, inorganic ice nucleators are present in *E. solidaginis*, including inorganic ice nucleators such as calcium phosphate spherules in the Malpighian tubules, fat body cells, as well as potassium phosphate, potassium urate and sodium urate (Duman, 2001; Mugnano *et al.*, 1996).

1.2.2.2 Low-molecular-weight cryoprotectants

Low-molecular-weight molecules such as polyhydric alcohols (polyols), sugars, and amino acids are accumulated in both freeze-avoidant and freeze-tolerant insects. At high concentrations, low-molecular-weight cryoprotectants colligatively depress the melting point of an insect, with some freeze-avoidant insects accumulating over 3000 mM of polyols (Zachariassen, 1980). However, the relatively low concentration of these molecules in freeze-tolerant insects (~200-400 mM) compared to freeze-avoidant insects suggests that their primary cryoprotectant function may not be colligative.

Low-molecular-weight cryoprotectants, notably trehalose and proline, may play an important role in freeze tolerance by inhibiting damage to membranes and other macromolecules during the freezing process. Trehalose and proline decrease the packing density between membrane phospholipids, which may inhibit lateral-phase separations during freezing-dehydration by reducing the chance of van der Waals interactions between hydrocarbon chains (Crowe *et al.*, 1984). Košťál *et al.* (2011) suggest that proline may further enhance insect freeze tolerance by promoting the vitrification of unfrozen water in insect's tissues, as feeding a proline augmented diet to *Chymomyza costata* larvae resulted in the induction of freeze tolerance, as well as an increased propensity of water to undergo vitrification. Other low-molecular-weight molecules such as glycerol, sorbitol, ribitol, glucose and alanine accumulate in overwintering insects and

are correlated with insect freeze tolerance as well (Storey & Storey, 1983; Lee, 1991). However, no one molecule is common to all freeze-tolerant insects.

Accumulating cryoprotectants that are able to penetrate cellular membranes will increase the cell's non-aqueous volume, which may aid in maintaining the minimum cell volume (Meryman, 1974). These potential cryoprotectants are usually normal products of insect metabolism and can be readily transported through cellular membranes by aquaporin-like proteins (Tattersall *et al.*, 2012; Philip & Lee, 2010). In the freeze-tolerant beetle, *Pytho depressus*, glycerol is present in equal concentrations in intracellular and extracellular compartments (Zachariassen, 1979). Altogether, it is reasonable to expect that many other low-molecular-weight cryoprotectants would also be present in equal intra- and extra-cellular concentrations.

1.2.2.3 Thermal hysteresis agents

Antifreeze proteins are a subset of proteinaceous thermal hysteresis agents, as they depress the freezing point of solutions further below the melting point than expected if they acted in a solely colligative manner (Zachariassen, 1985). However, the term recrystallization-inhibiting protein may better represent their primary function, as measurable thermal hysteresis is rarely observed in freeze-tolerant insects (Duman, 2001). These proteins are generally relatively small, threonine- and cysteine-rich in insects, and may function by binding to the surface of ice crystals, potentially inhibiting recrystallization at concentrations much lower than what is needed to produce thermal hysteresis (Duman, 2001). Thermal-hysteresis activity and recrystallization inhibition are enhanced by antifreezes forming complexes with other antifreezes or with other molecules such as glycerol and citric acid (Tattersall *et al.*, 2012). One explanation for the enhanced antifreeze activity of these complexes is that they are able to block a larger surface area of the ice crystal (Tattersall *et al.*, 2012). The presence of antifreeze proteins can increase low temperature survival in freeze-tolerant insects (Tursman & Duman, 1995). However, further studies are required to determine the mechanisms of cryoprotection and diversity of this class of proteins in freeze-tolerant insects.

1.2.3 Other molecules associated with insect freeze tolerance

There are other molecules associated with insect freeze tolerance that may mitigate damage accrued during freezing and thawing. Freeze-tolerant organisms must have the capacity to increase antioxidant capacity by upregulating antioxidant enzymes and substrates in anticipation or in the presence of ROS (Doelling *et al.* 2014; Silva *et al.*, 2013). Membrane-bound aquaporins and aquaglyceroporin-like proteins facilitate the rapid redistribution of water and other molecules associated with freezing under the assumption of extracellular ice formation. The upregulation of these transporters is correlated with the seasonal acquisition of freeze tolerance in *E. solidaginis* (Philip & Lee, 2010). MicroRNA expression is also differentially regulated during the acquisition of freeze tolerance in *E. solidaginis*, allowing for translational control of metabolism while the insect is in a frozen state (Courteau & Storey, 2012). Non-protein molecules such as xylomannan (fatty acids + saccharides) and acetylated triacylglycerols are also present in freeze-tolerant insects and may interact with ice to inhibit recrystallization and protect intracellular membranes (Walters *et al.*, 2009; Marshall *et al.*, 2014). It is important to note that the majority of these molecules, including the other cryoprotectant molecules mentioned above, were identified using correlative, seasonal approaches, and it is not yet known which molecules are specifically required for the induction of insect freeze tolerance.

1.2.4 The induction of insect freeze tolerance in the field and laboratory

Insects can increase their cold hardiness to adjust to seasonal temperature changes. These plastic responses are generally divided into two categories: Rapid cold-hardening and acclimatization. Rapid cold-hardening occurs over very short time-scales (30 min – 2 h) in response to a brief low temperature exposure, and increases the insects cold-hardiness in subsequent low temperature exposures (Lee & Denlinger, 2010). Acclimatization occurs over a longer period as the insect prepares for prolonged periods of stress (Rako & Hoffmann, 2006). Both of these plastic responses may rely on the previously described biochemical mechanisms to prepare an insect for exposure to sub-freezing temperatures.

Overwintering insects often rely on cues from their environment to initiate plastic responses in anticipation of low temperatures. Seasonal changes in temperature cycles (thermoperiod) and photoperiods, or when combined, thermophotoperiods, have a predictable relationship with changing seasons. As a result, these seasonal changes can induce acclimatization in the field, which can be simulated by acclimation in the laboratory (e.g. Rako & Hoffman, 2006). While many studies use constant temperature and photoperiods for acclimation, others use ecologically-relevant thermophotoperiods to simulate natural conditions (Kelty & Lee, 2001; Colinet *et al.*, 2015). In addition, the chemical composition of senescent plant tissues, which make up the diet and microhabitat of herbivorous overwintering insects, may initiate a transition to an insect's overwintering strategy (Baust & Nishino, 1991). Thus, manipulating temperature, photoperiod and diet may lead to developing a protocol for inducing insect freeze tolerance in a laboratory setting.

Manipulating an insect's diet in the laboratory can have significant effects on survival following internal ice formation. A proline-augmented diet enhances freeze tolerance in *Chymomyza costata*, following complete submergence in liquid nitrogen (Košťál *et al.*, 2011). Proline also facilitates freeze tolerance in the chill susceptible *Drosophila melanogaster*; however, flies can only tolerate freezing of half of their body water (Košťál *et al.*, 2012). While proline enhances freeze tolerance in both drosophilids, increased survival in chill-tolerant *C. costata* compared to chill-susceptible *D. melanogaster* suggests that complete freeze tolerance is achieved by insects through multiple mechanisms. Given the potential complexity of freeze tolerance, this phenomenon should also be examined in a way that better reflects the natural acquisition of insect freeze tolerance.

1.3 Model systems for studying insect freeze tolerance

The current understanding of processes describing insect freeze tolerance has relied heavily on mammalian cryoprotection research and relatively untested correlations associated with seasonal change. Consequently, the mechanisms of insect freeze tolerance remain undetermined (Hayward *et al.*, 2014). Correlative approaches make it

difficult to determine which molecules are both necessary and sufficient for insect freeze tolerance. For example, the low-molecular-weight cryoprotectant glycerol has been shown to accumulate in freeze-tolerant *E. solidaginis* and freeze-avoidant *E. scudderiana* prior to overwintering in a goldenrod host, despite opposite cold-tolerance strategies (Storey & Storey, 1983; Rickards *et al.*, 1987). One of the impediments to developing a greater understanding of the mechanisms underlying insect freeze tolerance using manipulative approaches has been the lack of a suitable model insect.

The August Krogh Principle states, “For many problems there is an animal on which it can be most conveniently studied,” (Krogh, 1929), and while the current study organisms have allowed us to build hypotheses of the mechanisms of freeze tolerance, they have inhibited our ability to rigorously test these hypotheses in a controlled laboratory setting. Insects that are currently used in freeze-tolerance research are not efficiently reared in the lab. For example, the long life span (e.g. *Hemideina* spp.) and seasonal constraints of field collections (e.g. *E. solidaginis*, *Belgica antarctica*, *P. isabella*) have often resulted in one-off characterizations of the insect’s physiological state. Furthermore, these traits make it difficult to account for variability in field conditions experienced by insects prior to bringing them into the lab, and may also limit sample sizes. Diapause is an endocrine-mediated dormancy that often coincides with insect cold hardening; however, freeze-tolerance can be achieved independent of diapause (Denlinger, 1991). Thus, diapause is a confounding variable for many species that can make it difficult to differentiate between diapause and freeze-tolerance responses (Lee, 1991). The size and body composition of current study organisms (e.g. *C. costata*, *E. solidaginis*) have made laboratory-based rearing and manipulations labour intensive or impossible (Brent J. Sinclair, personal communication). Additionally, with the exception of *B. antarctica*, none of these study organisms have thus far been amenable to genetic manipulation (Kelley *et al.*, 2014). Future investigations into the mechanisms of insect freeze tolerance require a study organism better suited than the current alternatives for laboratory-based manipulative approaches. I propose that the spring field cricket, *Gryllus veletis*, is a candidate model organism for studying insect freeze tolerance.

1.3.1 *Gryllus veletis* – a candidate model species for studying insect freeze tolerance

The spring field cricket, *Gryllus veletis* (Alexander & Bigelow) (Orthoptera: Gryllidae) overwinters as a late-instar nymph throughout temperate North America (Alexander & Bigelow, 1960). *G. veletis* nymphs overwinter in the soil where ambient temperatures are thermally buffered, and crickets become reproductively active in the late spring and early summer (Bégin & Roff, 2002; Alexander & Bigelow, 1960). A population of *G. veletis* originating from a wild population collected in Lethbridge, Alberta have been maintained and studied in the Biotron at Western University since 2010 (e.g. Coello Alvarado *et al.*, 2015). When reared under constant summer conditions (25 °C, 14 h Light: 10 h Dark, 70 % RH) the laboratory population produces a new cohort every two weeks. In a preliminary study (L.V. Ferguson personal communication, 2013), juvenile crickets from this population were acclimatized outdoors in London, Ontario from September 2012 through February 2013. Seven of these individuals were brought into the lab, cooled to -6.0 °C (0.25 °C min⁻¹) and held there for three hours. All seven crickets survived internal ice formation. However, non-acclimatized crickets from the laboratory-reared population were not able to survive freezing. Further investigation into the temperature, photoperiod and dietary conditions that lead to *G. veletis* freeze-tolerance in the field may provide insight into the acclimation conditions necessary for inducing freeze tolerance in the laboratory.

I propose that developing *G. veletis* as a new model system may overcome the limitations imposed by current model insects on the understanding of the underlying mechanisms of insect freeze tolerance. Access to a laboratory population removes the seasonal constraints of collecting insects from the field. Compared to other freeze-tolerant insects, the combined large size and easily discernable tissues of *G. veletis* better facilitate both *in vivo* and *in vitro* manipulations. Moreover, an assembled transcriptome for *G. veletis* provides the framework for gene-level investigations into the mechanisms underlying freeze tolerance through manipulative techniques such as RNA interference (A. H McKinnon, L. E. Des Marteaux, J. Toxopeus & B. J. Sinclair, unpublished data). My objective was to develop a protocol that induces *G. veletis* freeze tolerance in the

laboratory, thus facilitating future studies to test hypothesized mechanisms underlying freeze tolerance using manipulative approaches.

1.4 Goals and Objectives

The goal of my thesis was to assess the potential of *Gryllus veletis* as a new model organism for studying insect freeze tolerance. I accomplished this by addressing the following objectives:

1. **Assess freeze tolerance in field-acclimatized *G. veletis*.** I assessed freeze tolerance and used the field conditions experienced by the crickets to guide my laboratory acclimations.
2. **Develop a protocol that successfully induces *G. veletis* freeze tolerance in the laboratory.** I acclimated crickets under different diet, photoperiod and temperature conditions and tracked survival after internal ice formation. I also compared the lethal limits of laboratory-induced freeze tolerance to what I observed in field-acclimatized crickets.
3. **Characterize freeze tolerance in *G. veletis*.** I compared ice nucleation activity and cryoprotectant accumulation between freeze-tolerant crickets and freeze-intolerant (control) crickets to identify molecules that are associated with insects acquiring freeze tolerance.

2 Methods

2.1 Insect care and rearing

A laboratory colony of *Gryllus veletis* was collected from the University of Lethbridge campus in 2010 and maintained in the Biotron at Western University as described by Coello Alvarado *et al.* (2015). Starting in the fall of 2013, I housed crickets in 60 L plastic bins equipped with mesh lids and cardboard egg cartons for shelter, and provided them with water and food (Little Friends Rabbit Food, Martin Mills Inc., Elmira, ON, Canada) *ad libitum*. I reared the population under constant summer conditions (25 °C, 14:10 L:D, 70 % RH); and, to avoid overcrowding, the densities did not exceed 600 crickets/bin. Female *G. veletis* laid eggs into 500 mL plastic trays containing a mixture of 4:1 fine vermiculate to sand, which was replaced every two weeks. I placed trays containing eggs into new 60 L bins to develop at constant summer conditions for six to eight-weeks. I then isolated late instar nymphs from adults by the presence of wing pads that had yet to develop into adult wings and differentiated males from females by the lack of an ovipositor. I removed approximately 70 juvenile males from each cohort to use in my experiments.

2.2 Treatment groups

To determine the conditions that induce freeze-tolerance in *G. veletis*, I placed crickets either outside during the fall in London, Ontario or in one of several laboratory treatments. The purpose of the field-acclimatization treatment was to confirm that crickets can become freeze tolerant (L.V. Ferguson, unpublished observation, 2013) and to better understand the conditions that lead to freeze-tolerance so that I could simulate them in the laboratory treatments. In the laboratory, I exposed crickets to acclimation-treatments ranging from one- to eight-weeks, which manipulated diet, photoperiod and temperature. I also performed several short-term lab treatments that manipulated temperature and internal ice formation.

2.2.1 Field acclimatization

In early October 2013 and 2014, I acclimatized crickets outside prior to assessing and characterizing their freeze tolerance. Following a protocol that induced freeze tolerance in 2012 (L.V. Ferguson, personal communication), I placed late-instar male *G. veletis* along with soil, grass from a cultivated lawn, chicory (*Cichorium endivia*) leaves, black locust (*Robinia pseudoacacia*) leaves, and rabbit food into enclosed mesh cages (60 cm x 60 cm x 75 cm) in a shaded suburban garden in London, Ontario (42°59'N, 81°17'W, 251 m elevation) (Table 2.1). I placed a HOBO Pro v2 U23-003 data logger (Onset Computer Corporation, Bourne, MA, USA) in the garden with the probe situated on the top of the soil layer to record the temperature every 30 min throughout the acclimatization. Starting in December, I transferred acclimatized crickets to the lab and separated them into individual 180 mL plastic cups with mesh covering, a cardboard shelter, rabbit food and water, and placed them in an incubator (MIR153, Sanyo, Bensenville, IL) set to 6 °C with a short photoperiod (9:15 L:D). I allowed crickets to equilibrate in the incubator for 24 h prior to assessing their health and removed crickets that were unable to walk at this time. I assessed *G. veletis* freeze tolerance (see section 2.3) within 48 h of crickets being transferred to the lab and crickets used in other laboratory experiments (see section 2.4) were held in the incubator for a maximum of six days.

Table 2.1 - Field acclimatization treatments for juvenile male *Gryllus veletis*. Crickets were placed outdoors in London, Ontario, Canada. Air temperatures were recorded on the ground and day length for London was obtained from the National Research Council of Canada website (<http://www.nrc-cnrc.gc.ca/eng/services/sunrise/>) (Fig 3.1).

Treatment	Duration	Diet
F-2013a (Fig 3.1 A)	10 October 2013 – 3 December 2013	Grass, leaf litter, chicory, rabbit food
F-2013b (Fig 3.1 A)	10 October 2013 – 9 January 2014	Grass, leaf litter, chicory, rabbit food
F-2014 (Fig 3.1 B)	1 October 2014 – 5 December 2014	Grass, leaf litter, rabbit food

2.2.2 Temperature and photoperiod manipulations

To develop a laboratory protocol that induced freeze-tolerance, I exposed *G. veletis* to a range of laboratory manipulations and compared their subsequent ability to survive internal ice formation (Fig 1, Table 2.2). For these experiments, I separated late instar male *G. veletis* from the population reared at constant summer conditions in the Biotron into individual 180 mL plastic cups with mesh covering. The cups were equipped with a cardboard shelter and food and water were provided to the crickets *ad libitum*. These cages were placed in an incubator (MIR153, Sanyo, Bensenville, IL) in which I controlled temperature and photoperiod for multiple acclimation treatments.

I manipulated photoperiod, temperature, and food to generate several acclimation regimes (Table 2.2). I exposed crickets to long (14:10 L:D), intermediate (10:14 L:D) and short (9:15 L:D) photoperiods representative of summer, fall and winter, respectively. I also used a ramping treatment that decreased photoperiod by 18 min/week, starting from conditions in London, Ontario on October 5th, 2013 (11.5:12.5 L:D; National Research

Council Canada) (Table 2.2). Temperature regimes included: constant exposures to 25 °C, 4 °C and -2 °C (Fig. 2.1a); ramping treatments where temperatures decreased linearly from 25 °C to 4 °C (Fig. 2.1b); and treatments where temperatures fluctuated daily (Fig. 2.1c), with the daily high and low temperatures for each week based on the recorded upper- and lower-quartile values obtained from the data logger recordings in the 2013 field cage (Figure 3.1). Dietary manipulations included combinations of rabbit food, leaf litter and grass (Table 2.2). In all cases, the control group consisted of crickets fed rabbit food and held at summer rearing conditions in the Biotron (25 °C, 14:10 L:D, 70 % RH). Following each laboratory acclimation, I assessed the ability of crickets to survive freezing (see section 2.3).

To determine whether *G. veletis* become freeze-tolerant following an acute exposure to extreme temperatures, I exposed crickets to either 40 °C (S-HT, Table 2.2) or -2 °C (S-LT, Table 2.2) for 15 min. I placed crickets into 1.7 mL microcentrifuge tubes and inserted the tubes into an aluminum block with an ethylene glycol circulator (Model 1157P, VWR International, Mississauga, ON, Canada) set to either 40 °C or -2 °C. I then transferred the crickets to 180 mL plastic cups, and allowed them to recover at room temperature for 40 min prior to assessing their ability to survive freezing.

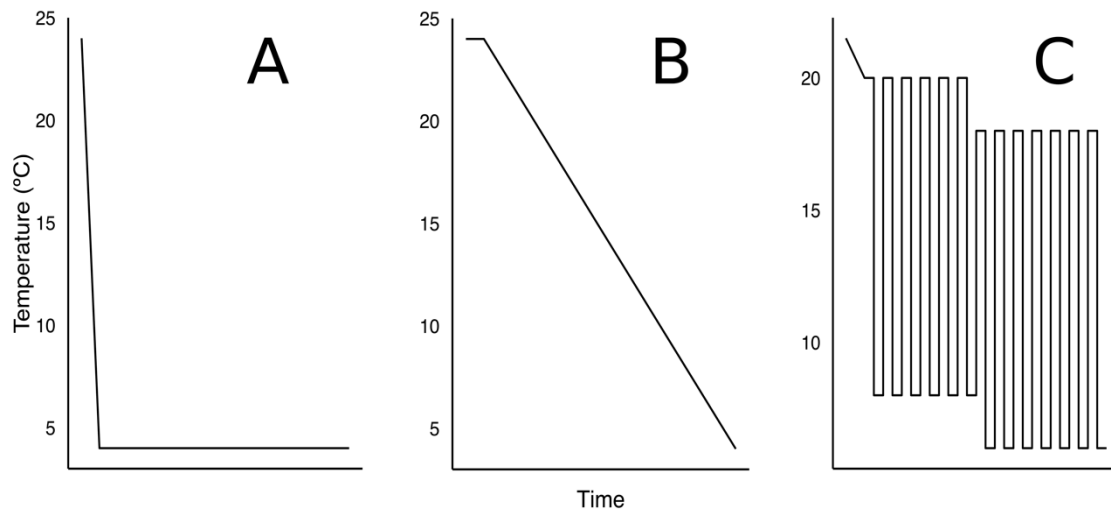


Figure 2.1 - Temperature ramping regimes used during lab-acclimation treatments. Juvenile male *Gryllus veletis* were exposed to: a rapid decrease to a constant temperature (A), a linear decrease in temperature (B), or daily thermal fluctuations (C). The duration of these exposures ranged from one to eight weeks.

Table 2.2 – Photoperiod and temperature manipulations applied to juvenile male *Gryllus veletis* in the laboratory. (OS = outdoor simulated; CP = constant photoperiod; CT = constant temperature; RP = ramping photoperiod; RT = ramping temperature; S-HT = short high temperature; S-LT = short low temperature; RF = rabbit food; CMB = combined [rabbit food, grass, leaf litter]).

Treatment	Duration	Diet	Photoperiod (L:D)	Temperature
Control-2	2 weeks	RF	14:10 (Constant)	25°C (Constant)
Control-4	4 weeks	RF	14:10 (Constant)	25°C (Constant)
Control-6	6 weeks	RF	14:10 (Constant)	25°C (Constant)
Control-8	8 weeks	RF	14:10 (Constant)	25°C (Constant)
OS-8	8 weeks	CMB	Ramping, Fig 2.2A	Fluctuating, Fig 2.2A
CPCT-1	1 week	RF	11:13 (Constant)	-2 °C (Constant)
CPCT-2	2 weeks	CMB	9:15 (Constant)	4°C (Constant)
CPCT-4	4 weeks	CMB	9:15 (Constant)	4°C (Constant)
RPCT-2	2 weeks	CMB	14:10 → 9:15 (Ramping)	4°C (Constant)
RPCT-4	4 weeks	CMB	14:10 → 9:15 (Ramping)	4°C (Constant)
CPRT-2	2 weeks	CMB	9:15 (Constant)	25°C → 4°C (Ramping)
CPRT-4	4 weeks	CMB	9:15 (Constant)	25°C → 4°C (Ramping)
S-HT	55 min	RF	n/a	15 min @ 40 °C → 40 min @ 22 °C
S-LT	55 min	RF	n/a	15 min @ -2 °C → 40 min @ 22 °C

Initial experiments indicated that *G. veletis* became freeze tolerant following an eight-week lab acclimation (Treatment OS-8, Table 2.2; 3.2) that mirrored the thermophotoperiod of London, Ontario from October 2013 through early December 2013 (Fig. 2.2 A). Thus, I exposed crickets to two-, four- or six-week segments of the OS-8 laboratory treatment (Table 2.3) to determine if exposure to these isolated components could induce freeze-tolerance in *G. veletis*.

Table 2.3 – Components of the OS-8 laboratory treatment applied to juvenile *Gryllus veletis*. (OS = outdoor simulated)

Treatment	Duration (Weeks)	Diet	Thermophotoperiod
OS-8.2	2	CMB	OS-8 conditions, removed at 2-weeks
OS-8.4	4	CMB	OS-8 conditions, removed at 4-weeks
OS-8.6	6	CMB	OS-8 conditions, removed at 6-weeks
OS-8₁₊₂	2	CMB	Weeks 1 + 2 of OS-8
OS-8₃₊₄	2	CMB	Weeks 3 + 4 of OS-8
OS-8₅₊₆	2	CMB	Weeks 5 + 6 of OS-8
OS-8₇₊₈	2	CMB	Weeks 7 + 8 of OS-8

To optimize a protocol for inducing freeze-tolerance in *G. veletis*, I refined the eight-week outdoor simulation (OS-8) to minimize acclimation time and eliminate any unnecessary conditions. I provided crickets with rabbit food and reduced the acclimation time by compressing the decrease in thermophotoperiod representative of London, Ontario from October 10th, 2013 through January 2nd, 2014 into a six-week acclimation (Treatment COS-6, Table 2.4, Fig 2.2B). The photoperiod began at 11.5:12.5 L:D and decreased by 36 min/week. The initial and final daily upper and lower temperature limits (16.5 °C, 11.8 °C) were set by calculating the weekly upper and lower quartile temperatures obtained from the data logger placed outside in London, Ontario from

October 10th, 2013 through October 17th, 2013 and December 27th, 2013 through January 2nd, 2014 respectively. The upper and lower temperature limits decreased by 1.58 °C and 1.26 °C respectively, every 3.5 days for six weeks.

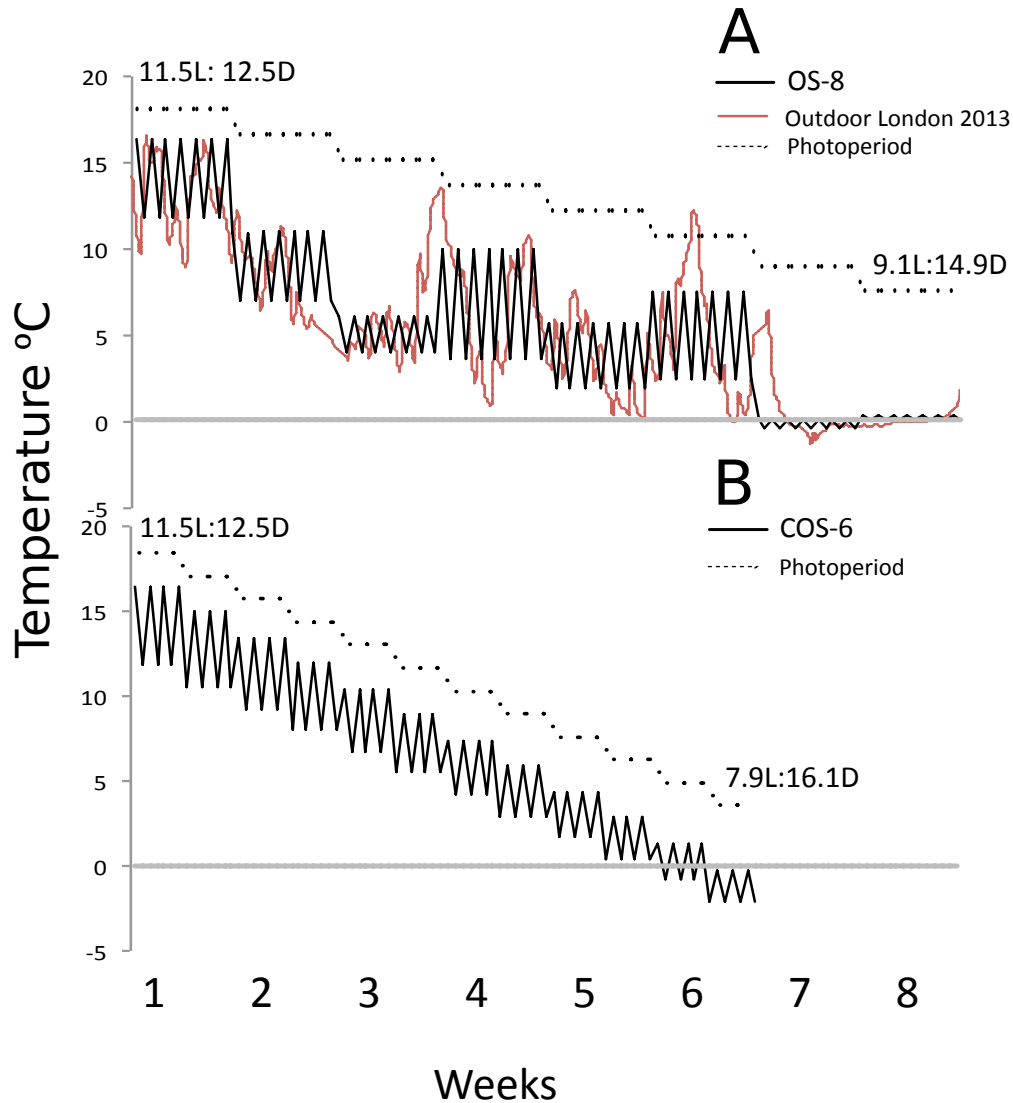


Figure 2.2 - Temperature and photoperiod regimes that induced *Gryllus veletis* freeze tolerance. The OS-8 (A) treatment group was exposed to a thermophotoperiod representative of London, Ontario from October 10th through December 5th, 2013. The COS-6 treatment group was exposed to a decreasing thermophotoperiod representative of October 10th, 2013 through January 2nd, 2014, but compressed into a six-week regime (B).

I divided late instar male nymphs into two treatment groups (n = 8) to examine the individual role of photoperiod and temperature in *G. veletis* freeze tolerance. I exposed one group (COS-6T, Table 2.4) to a six-week acclimation with the same thermoperiod regime as COS-6, but with a long photoperiod (14:10 L:D). I exposed the second group (COS-6P, Table 2.4) to the same six-week photoperiod regime as COS-6, however the temperature was set at a constant 25 °C.

Table 2.4 – Components of the six-week laboratory acclimations applied to juvenile *Gryllus veletis*. (RF = rabbit food).

Treatment	Duration (Weeks)	Diet	Photoperiod (L:D)	Temperature
COS-6	6	RF	Ramping, Fig 2.2B	Fluctuating, Fig. 2.2B
COS-6T	6	RF	14:10 (Constant)	COS-6 thermoperiod
COS-6P	6	RF	COS-6 photoperiod	25°C (Constant)

2.2.3 Ice nucleation manipulations

To determine if manipulating the initial site of ice nucleation could induce freeze tolerance, I divided juvenile male *G. veletis* into three treatment groups: inoculative freezing (INOC), gut nucleation ($Acclimation_{AgI}$) and hemolymph nucleation (HEM-AgI) (Table 2.5). In each treatment (n = 8 per treatment group), I used a known ice-nucleator, silver iodide (AgI), to initiate ice formation. To promote external ice formation, the INOC crickets were briefly submerged in a 25 mg/mL AgI in water slurry, prior to freezing. I manipulated ice nucleation in the gut of crickets by dusting rabbit food with AgI, throughout several laboratory acclimations. To manipulate ice-nucleating activity in the hemolymph, I injected 4 μ L of a 25 mg/mL AgI in water slurry underneath the pronotum, into the cricket's hemolymph using a 5 μ L Hamilton syringe and a 25-gauge needle (Hamilton Company, Reno, Nevada). I then placed the crickets in 180 mL plastic cups to recover at room temperature (22 °C) for 40 min. Following each manipulation, I assessed the ability of crickets to survive freezing (see section 2.3).

Table 2.5 – Manipulation of ice formation in juvenile male *Gryllus veletis*. (CP = constant photoperiod; CT = constant temperature; RP = ramping photoperiod; RT = ramping temperature; INOC = inoculation; HEM = hemolymph)

Treatment	Manipulation
INOC	Inoculation with external ice
CPCT-2 _{AgI}	CPCT-2 Photoperiod and temperature regime w/AgI in diet
RPCT-2 _{AgI}	RPCT-2 Photoperiod and temperature regime w/AgI in diet
CPRT-2 _{AgI}	CPRT-2 Photoperiod and temperature regime w/AgI in diet
CPCT-4 _{AgI}	CPCT-4 Photoperiod and temperature regime w/AgI in diet
RPCT-4 _{AgI}	RPCT-4 Photoperiod and temperature regime w/AgI in diet
CPRT-4 _{AgI}	CPRT-4 Photoperiod and temperature regime w/AgI in diet
HEM-AgI	Injection of silver iodide into hemolymph

2.3 Characterization of cold tolerance

I used groups of six to eight *G. veletis* to determine the supercooling point (SCP) and cold tolerance strategy for crickets from each treatment. First, I transferred crickets that had completed their field or lab treatment into individual 1.7 mL microcentrifuge tubes and placed a type T (copper-constantan) thermocouple next to their abdomen to monitor individual temperatures. I then placed the tubes into an aluminum block cooled by methanol from a programmable refrigerated circulator (Proline RP 855, Lauda, Wurzburg, Germany). The thermocouples were connected to a computer *via* a Picotech TC-08 thermocouple interface and the temperature was recorded every 500 ms using PicoLog software (Pico Technology, Cambridge, UK). To ensure that the crickets had similar starting temperatures, I held them at 6 °C for 15 min. I then cooled crickets at 0.25 °C min⁻¹ to sub-freezing temperatures, which were either -6 °C for field-acclimatized crickets or -8 °C for laboratory-acclimated crickets and held them at that temperature for

1.5 h. I recorded SCP as the lowest temperature before the exotherm associated with ice crystallization was detected. After freezing, the crickets were warmed to 15 °C at 0.25 °C min⁻¹. I then placed crickets into individual 180 mL plastic cups and allowed them to recover in an incubator before assessing survival (15 °C, 12:12 L:D). Due to not all crickets from the control group freezing at -8 °C, I performed a separate experiment to determine their SCPs in which crickets were cooled at 0.25 °C min⁻¹ until all crickets reached their SCP. Differences in SCPs among laboratory-acclimated groups were investigated using a one-way ANOVA, with planned contrasts between the control group and each treatment. Differences in SCPs among field-acclimatized groups were investigated using a one-way ANOVA.

I assessed the survival of *G. veletis* 48 h after removal from the freezing treatment. Crickets were given 15 min to acclimate to laboratory conditions (22 °C) and survival was defined as any movement in response to stimulus from the bristles of a small paintbrush. I considered treatments that resulted in greater than 75 % survival 48 h post-thaw to have successfully induced freeze tolerance.

2.3.1 Lethal limits

To determine both the lower lethal temperature (LLT) and the length of time that crickets can survive being frozen, late instar male crickets were acclimated for six weeks (COS-6) in the laboratory or placed outdoors in London from October until early December, 2014 (F-2014). Prior to determining these limits of freeze tolerance, a subset of each treatment (n = 8 per group) was assessed (see section 2.3, above) to ensure that freeze tolerance had been successfully induced.

To determine the LLT of freeze-tolerant crickets, I first cooled groups (n = 8 per treatment group) of laboratory-acclimated individuals (COS-6) in an aluminum block cooled by methanol to -6, -8, -10, -12 and -15 °C at 0.25 °C min⁻¹, held for 1.5 h, and warmed them to 15 °C at 0.25 °C min⁻¹. Due to a limited number of field-acclimatized crickets (F-2014), I only exposed groups of crickets (n=6 per treatment group) from this treatment to temperatures of -6, -9 and -12 °C. After warming to 15 °C, crickets were

placed into individual 180 mL plastic cups along with rabbit food and water to recover in an incubator (15 °C, 12:12 L:D). I assessed survival of individuals 24 and 48 h post-thaw.

To determine the influence of time frozen on survival of *G. veletis*, I cooled groups (n = 8 per treatment group) of lab-acclimated crickets (COS-6) in an aluminum block cooled by methanol to -8 °C at 0.25 °C min⁻¹ and held them at -8 °C for 3, 6, 12, 24, 48, 96, and 168 h. I then warmed them to 15 °C at 0.25 °C min⁻¹ and placed them into individual 180 mL plastic cups along with rabbit food and water to recover in an incubator (15 °C, 12:12 L:D). I assessed survival of individuals 24 and 48 h post-thaw. This experiment was repeated on field-acclimatized crickets (F-2014; n = 6 per treatment group), but only included 1.5, 24, 48 and 96 h exposures to -8 °C.

I compared the lower lethal temperature and the length of time crickets can survive freezing between the two treatment-groups (COS-6 and F-2014) using a Cox proportional hazards regression model tested for differences between groups with Wald's statistic. I conducted this statistical analysis using the survival package in R (Therneau & Grambsch, 2000; R Development Core Team, 2008).

2.4 Identification of biochemicals associated with freeze tolerance

I compared groups of crickets that underwent a six-week laboratory acclimation that induces freeze tolerance by simulating outdoor conditions (COS-6) to groups of control crickets to identify potential mechanisms of freeze tolerance. I assessed ice nucleator activity in *G. veletis* by comparing SCPs of isolated tissues between groups of freeze-tolerant and control crickets. I measured the hemolymph osmolality in crickets and examined the hemolymph for the presence of thermal hysteresis and antifreeze activity. I then identified carbohydrates in the hemolymph using gas chromatography and measured hemolymph trehalose concentration using spectrophotometry. I also measured whole-body and hemolymph proline concentrations using spectrophotometry. I measured the

concentration of sodium and potassium in the hemolymph using atomic absorption spectroscopy.

2.4.1 Ice nucleation in isolated tissues

I removed crickets from their acclimation treatments and isolated tissues at room temperature (22 °C) following previously described methods (MacMillan & Sinclair, 2011a). With each cricket (n = 8 per treatment group), I made a small incision underneath the pronotum and used a pipette to collect 3 µL of hemolymph and place it in separate 0.2 mL microcentrifuge tubes along with 9 µL of 3 % ascorbic acid to prevent coagulation. I then made a longitudinal incision from the tip of the abdomen to the base of the head, and dissected out the gut, Malpighian tubules, and fat body. Each tissue was rinsed in ddH₂O and gently dabbed with a Kimwipe to remove hemolymph before being placed into a 0.2 mL microcentrifuge tube. 20 µL of 3 % ascorbic acid was added to gut, Malpighian tubules and fat body tissue so that their SCPs could be compared to hemolymph samples, which also contained ascorbic acid.

I compared the SCP of isolated *G. veletis* tissues between control and lab-acclimated crickets to determine potential sites of ice nucleation. I included 3 µL of insect Ringer's solution (110 mM Na⁺, 8.5 mM K⁺, 5.9 mM Mg²⁺, 7.1 mM Ca²⁺, 144.5 mM Cl⁻, 4 mM glucose, 5 mM HEPES, 80 mM proline; Coast *et al.*, 2007) in 9 µL of 3 % ascorbic acid and 20 µL of insect Ringer's solution as controls. A type-T thermocouple was attached to the outside of each 0.2 mL microcentrifuge tube, prior to being cooled to -35 °C at 0.25 °C min⁻¹. The SCPs were the lowest temperature recorded before the exotherm was detected. The SCP of each tissue was compared to the SCP of insect Ringer's solution in 3 % ascorbic acid using a one-way ANOVA with planned contrasts. I compared the SCPs of tissues between treatment groups using a one-tailed Welch's t-test with a Benjamini-Hochberg correction (Benjamini & Hochberg, 1995).

2.4.2 Antifreeze activity, thermal hysteresis and hemolymph osmolality

Following the protocol in Section 2.4.1, I collected 4 µL of hemolymph from control and freeze-tolerant crickets (n=8 per treatment group) and placed it into separate 0.2 mL

microcentrifuge tubes containing 12 μL of 3 % ascorbic acid to prevent coagulation. I then placed a layer of type B immersion oil (Cargille Laboratories, Cedar Grove, NJ, USA) on top of the sample to limit evaporation. I snap-froze the samples in liquid nitrogen vapour and stored them at $-80\text{ }^{\circ}\text{C}$ until analysis.

I measured osmolality and thermal hysteresis in *G. veletis* hemolymph using a nanolitre osmometer (Otago Osmometers, Dunedin, New Zealand) and following previously described methods (Crosthwaite *et al.*, 2011). I suspended small droplets (~ 20 nL) of my samples into small wells filled with type B immersion oil under a microscope and rapidly cooled the wells until the droplets froze. I then slowly warmed the droplets until the last ice crystal was stable (melting point). I used the melting point to determine osmolality, as there is a linear relationship between solute concentration and melting point depression: one mole of solutes depresses the melting point from $0\text{ }^{\circ}\text{C}$ to $-1.86\text{ }^{\circ}\text{C}$. I calculated the osmolality of each of my samples, accounting for the dilution in 3 % nitric acid.

To detect thermal hysteresis, I refroze my samples and warmed them to a temperature just below the melting point and only a single ice crystal was visible under the microscope. I then slowly cooled the sample until the ice crystal began to grow. Thermal hysteresis was measured as the difference between the melting point, and the temperature in which the ice grew. During this measurement, I also made note of ice-crystal morphology, as spicular and angular crystals can be representative of antifreeze activity (Scotter *et al.*, 2006). Differences in osmolality between treatments were investigated using a one-tailed Welch's t-test.

2.4.3 Hemolymph carbohydrates

2.4.3.1 Qualitative gas chromatography analysis

In a preliminary experiment, I qualitatively compared hemolymph carbohydrate composition between control (Control-6) and freeze-tolerant (COS-6) crickets ($n = 2$ per treatment group). Carbohydrates present in the hemolymph were analyzed as their alditol acetate derivatives, and were separated and identified with gas chromatography (GC)

using methods derived from Blakeney *et al.* (1983) and Crosthwaite *et al.* (2011). Sample preparation and derivatization was divided into four main steps: extraction, hydrolysis, reduction and acetylation. By comparing the hydrolyzed sample to its non-hydrolyzed equivalent and known carbohydrate standards, it is possible to detect the presence of both monosaccharides and oligosaccharides.

2.4.3.1.1 *Carbohydrate Extraction*

To extract carbohydrates, I collected 4 μL of hemolymph from each individual, and placed it in a separate 1.7 ml microcentrifuge tube prior to snap-freezing in liquid nitrogen vapour and storing at $-80\text{ }^{\circ}\text{C}$ until analysis. I transferred the hemolymph with 250 μL of sulphuric acid (0.1 M) into a 4 ml glass vial. I then added 2 mL of hexane to the hemolymph and vortexed the mixture. I allowed the sample to settle into two layers and removed two 100- μL aliquots from the lower, carbohydrate-containing aqueous layer and placed them into two separate 4 ml glass vials. One vial went on to hydrolysis, while the other bypassed hydrolysis, going directly to the reduction step.

I prepared a mixture of standards to be used in identifying carbohydrates by adding 25 μL of 20 mg/ml xylitol, glycerol, ribitol, mannitol, sorbitol, and trehalose to 975 μL of 1 M ammonia into a glass vial. I vortexed each vial and removed 100 μL to be used in the reduction step.

2.4.3.1.2 *Hydrolysis*

To concentrate samples for hydrolysis, I dried the 100 μL aliquots under nitrogen. I then added 62.5 μL of 13.5 M sulphuric acid under nitrogen gas and vortexed each sample. After 45 min at room temperature, I added 675 μL of distilled water under nitrogen and then heated the samples at $100\text{ }^{\circ}\text{C}$ for 3 h in an aluminum block connected to a VWR Signature heated/refrigerated circulator (model 1157, VWR International, West Chester, PA) containing 1:1 water:ethylene glycol. I removed the samples from the block and allowed them to cool to room temperature before adding 300 μL of 8 M ammonia. I then added 25 μL of 20 mg/ml xylitol as an internal standard. Finally, I vortexed each vial and removed 100 μL to be used in the reduction step.

2.4.3.1.3 *Reduction*

To reduce the carbohydrates, I added 1 ml of a 2 % (w/v) sodium borohydride (NaBH_4) in dimethyl sulfoxide solution to 100 μL of hydrolyzed and non-hydrolyzed samples. I heated the samples at 40 $^\circ\text{C}$ for 90 min in an aluminum block connected to a circulating bath (described in hydrolysis, above). I allowed the samples to cool to room temperature (22 $^\circ\text{C}$) and then added 100 μL of 18 M acetic acid to decompose excess NaBH_4 into borates prior to the acetylation step.

2.4.3.1.4 *Acetylation*

Directly following the reduction step I added 200 μL of 1-methylimidazole (catalyst) and 2 ml acetic anhydride to acetylate the polyhydric alcohols. After 10 min at room temperature (22 $^\circ\text{C}$), I added 5 ml of distilled water to decompose excess acetic anhydride. I then added 750 μL of dichloromethane (CH_2Cl_2), mixed the solution and transferred ~ 100 μL of the CH_2Cl_2 layer to a 2 ml, septum-cap vial with a 200 μL insert (product no. 392611552, Varian, Inc., Palo Alto, CA, USA). I stored the samples at -20 $^\circ\text{C}$ until GC analysis.

2.4.3.1.5 *Separation and identification of carbohydrates*

The acetylated samples were separated and analyzed by capillary GC with flame ionization detection (FID) on a Varian Star CX3400 GC (Varian, Inc., Palo Alto, CA, USA), equipped with a CP-Sil 88, WCOT fused silica, 25 m x 0.25 mm i.d. column (Varian) and an autosampler. One microlitre of sample was injected and eluted with the following temperature profile: 150 $^\circ\text{C}$ for 5 min, ramp to 230 $^\circ\text{C}$ at 4 $^\circ\text{C min}^{-1}$, hold for 15 min, ramp to 240 $^\circ\text{C}$ at 10 $^\circ\text{C min}^{-1}$ and hold for 9 min. The injector temperature was 250 $^\circ\text{C}$, the carrier gas was helium (1 ml min^{-1}). The FID temperature was 300 $^\circ\text{C}$ and its make-up gas was N_2 . I identified individual peaks by retention time and visually compared them to the known standards using Varian MS Workstation software version 6.9.2 (Varian, Inc., Palo Alto, CA, USA).

2.4.3.2 Trehalose quantification

Trehalose was the only carbohydrate that I identified using GC as being present in *G. veletis* hemolymph, furthermore it appeared to be differentially accumulating between control and freeze-tolerant crickets. Trehalose is a non-reducing disaccharide that can be hydrolyzed to two glucose molecules by trehalase. I quantified hemolymph trehalose using porcine trehalase and a glucose colorimetric assay (Tennesen *et al.*, 2014). I diluted 5 μ L of hemolymph from both control (Control-6) and freeze-tolerant (COS-6) crickets ($n = 5$ per treatment group) with 100 μ L of trehalase buffer (TB; 5 mM Tris, 137 mM NaCl, 2.7 mM KCl) in a 1.7 mL microcentrifuge tube. To deactivate hydrolases present in the hemolymph, I heated the samples for 10 min at 70 $^{\circ}$ C in an aluminum block connected to a circulating bath. I then centrifuged the samples at $21890 \times g$ for 3 min at 4 $^{\circ}$ C in a tabletop centrifuge and divided the supernatant into two 30 μ L aliquots. I added 30 μ L of trehalase stock (TS; 3 μ L of porcine trehalase (Sigma) per ml of TB) to one aliquot to convert free trehalose into glucose. I diluted the second aliquot with 30 μ L of TB; this sample was used to measure baseline levels of glucose in the hemolymph. I generated 0.01, 0.02, 0.04, 0.08 and 0.16 mg/mL standards for both glucose and trehalose. The glucose standards were diluted in TB and the trehalose standards were diluted using a 1:1 mix of TB and TS. I then incubated the samples and standards in a water bath set to 37 $^{\circ}$ C for 24 h to allow for breakdown of trehalose into free glucose.

Following incubation, I centrifuged the samples and standards at $21890 \times g$ for 3 min and transferred 10 μ L of each sample, in triplicate, to 96-well plates. I then added 90 μ L of liquid glucose reagent (Sigma-Aldrich, Inc., St Louis, MO, USA; GAHK20) to each well. I sealed the wells with parafilm to prevent evaporation and incubated the plate at room temperature (22 $^{\circ}$ C) for 15 min. Following incubation, absorbance was read at 340 nm on a microplate spectrophotometer (SpectraMax 340PC, Molecular Devices, Sunnyvale, CA, USA). I determined free glucose concentrations for samples not treated with trehalase based on the glucose standard curve. I then calculated trehalose concentrations for samples treated with trehalase based on the trehalose standard curve and subtracted the absorbance measured for free glucose in the untreated samples.

Differences in hemolymph trehalose concentrations between treatments were investigated using a one-tailed Welch's t-test.

2.4.4 Whole-body and hemolymph proline

I measured the proline concentration of whole crickets (excluding gut) and their hemolymph in groups ($n = 12$ per treatment group) of control (Control-6) and freeze-tolerant (COS-6; compressed outdoor simulation) *G. veletis* using a colorimetric assay (Carrillo & Gibon, 2011). First, I collected 4 μL of hemolymph and placed it in a separate 0.2 mL microcentrifuge tubes along with 50 μL of ice-cold 40 % ethanol. Hemolymph samples were then refrigerated at 4 °C for 24 h. Using the same crickets, I dissected out the gut (to remove dietary proline) and gently dabbed the remaining tissues with a Kimwipe to remove hemolymph. I then weighed the crickets, placed them into separate 1.7 mL microcentrifuge tubes and snap-froze the samples in liquid nitrogen vapour for 15 min. At room temperature (22 °C), I homogenized each sample with a pestle for 1 min, I then added 800 μL ice-cold 40 % ethanol and vortexed for 5 s. Tissue samples were then refrigerated at 4 °C for 24 h.

Following the 24 h incubation, hemolymph and tissue samples were centrifuged at $21890 \times g$ for 3 min at 4 °C. I removed the supernatant and further diluted the samples in ice-cold 40 % ethanol as follows: control hemolymph 1:1; control tissue 1:30; COS-6 hemolymph 1:10; and COS-6 tissue 1:80. I then pipetted 50 μL of sample into a new 1.7 mL tube along with 100 μL of ninhydrin reaction mix (1 % ninhydrin (w/v) in 60 % acetic acid (v/v) and 20 % ethanol (v/v)). I sealed the tubes, and heated at 95 °C for 20 min in an aluminum block connected to a VWR Signature heated/refrigerated circulator (model 1157, VWR International, West Chester, PA) containing 1:1 water:ethylene glycol. After cooling to room temperature (22 °C), I centrifuged the samples (1 min, $600 \times g$) and transferred 100 μL of the sample to 96-well plates. The absorbance of the samples were read at 340 nm on a microplate spectrophotometer (SpectraMax 340PC, Molecular Devices, Sunnyvale, CA, USA) and proline concentration was quantified with a standard curve of known concentrations of proline ranging from 4 mM to 0.015625 mM. I compared whole-body and hemolymph proline concentrations between control and

freeze-tolerant crickets and tested for differences using separate one-tailed Welch's t-tests. Differences in whole-body and hemolymph proline concentrations between control and freeze-tolerant crickets were investigated using separate one-tailed Welch's t-tests.

2.4.5 Hemolymph ion concentration

I measured the concentration of sodium (Na^+) and potassium (K^+) in the hemolymph of control (Control-6) and freeze-tolerant (COS-6) crickets following previously described methods (MacMillan & Sinclair, 2011a). I collected 4 μL of hemolymph from each cricket ($n=8$ per treatment group) and placed the hemolymph into separate 1.7 mL microcentrifuge tubes that contained 20 μL of 3 % nitric acid. I left the samples to digest in the acid for 24 h at room temperature (22 °C). I then centrifuged the samples (1 min, $600 \times g$) and diluted 20 μL of supernatant with 10 mL of double-distilled H_2O to bring the samples within the measurable range of the atomic absorption spectrometer (iCE 3300, Thermo Scientific, Waltham MA, USA). I compared the absorption values from my samples with standard curves of Na^+ and K^+ generated from diluted standards containing nitric acid. I compared hemolymph Na^+ and K^+ concentrations between control and freeze-tolerant crickets and tested for differences with separate two-tailed Welch's t-tests.

3 Results

3.1 Field acclimatization

I recorded the temperatures at the soil surface, which I assumed to be experienced by juvenile male *Gryllus veletis* in the field cages from 10 October 2013 through 9 January 2014 (Figure 3.1A) and 1 October 2014 through 5 December 2014 (Fig. 3.1B) with HOBO data loggers. In 2013-2014, 0 °C was first reached on 20 November, and the minimum temperatures experienced by F-2013a and F-2013b crickets were -1.2 °C on 25 November and -7.5 °C on 7 January, respectively. In the fall of 2014, 0 °C was first reached on 13 November, with a minimum temperature of -2.4 °C reached on 19 November. Overall, the F-2013a and F-2013b crickets experienced lower temperatures than F-2014 crickets.

The supercooling points (SCPs) of males that overwintered outdoors ranged from -2.4 °C to -5.5 °C. Mean SCP did not differ between groups of crickets that were placed outside at different time points during the late fall and early winter of 2013-14 or 2014 (Table 3.1; $F_{2,17} = 2.073$, $P = 0.156$). All crickets survived internal ice formation, indicating that *G. veletis* become freeze tolerant while overwintering outdoors in London, Ontario.

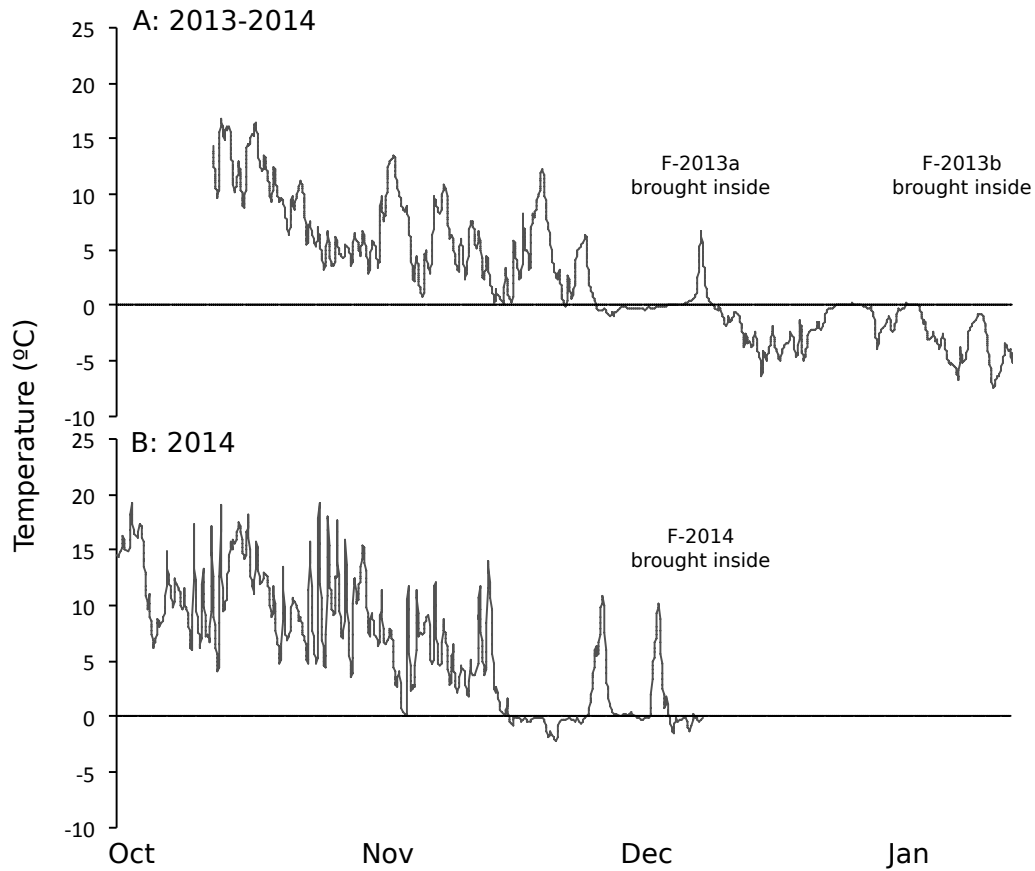


Figure 3.1 - Microclimate temperatures experienced by *Gryllus veletis* in London, Ontario. A. Temperature trace recorded in 2013-2014 within the cage of F-2013a and F-2013b acclimatization treatments. B. Temperature traces recorded in 2014-2015 with a logger recording ground temperature within the cage of the F-2014 acclimatization treatment. All temperatures were recorded with a HOBO data logger at ground level.

Table 3.1 – The effect of exposure to outdoor overwintering conditions on the survival of internal ice formation in juvenile male *Gryllus veletis*. Crickets were exposed to outdoor conditions in London, Ontario over the 2013-2014 and 2014-2015 winters (Table 2.1). Treatments that led to greater than 75 % survival at 48 h post-thaw were considered to have successfully induced freeze tolerance. Treatment did not have a significant effect on cricket SCP (see text for statistics).

Treatment	N	No. frozen	Survival 48 h post-thaw	Freeze tolerant?	Mean SCP (\pm SEM) ($^{\circ}$C)
F-2013a	6	6	6	Yes	-4.6 \pm 0.40
F-2013b	8	8	8	Yes	-3.8 \pm 0.34
F-2014	5	5	5	Yes	-3.6 \pm 0.28

3.2 Laboratory-acclimation

3.2.1 Survival of internal ice formation

To determine if juvenile male *G. veletis* become freeze-tolerant in the laboratory after acclimation treatments, I cooled crickets at $-0.25\text{ }^{\circ}\text{C min}^{-1}$, held them at $-8\text{ }^{\circ}\text{C}$ for 1.5 h, and assessed their survival at 24 and 48 h post-thaw. In all but two treatment groups, less than 75 % of crickets survived 48 h post-thaw (Table 3.2). The OS-8 (eight-week outdoor simulation) and COS-6 (compressed, six-week outdoor simulation) laboratory-acclimations successfully induced freeze tolerance in *G. veletis*, as the observed survival was 91 % and 92 %, respectively (Table 3.2). The survival of internal ice formation increased over the course of the OS-8 thermophotoperiod regime (Figure 3.2A), however, there was no difference in survival between groups of crickets exposed to isolated two-week components of this regime (Figure 3.2B). The combination of a fluctuating temperature regime and reduction in photoperiod was essential for the induction of freeze tolerance under COS-6 conditions as exposure to isolated temperature (COS-6T) and photoperiod (COS-6P) regimes did not induce freeze tolerance (Figure 3.3).

The SCPs of male *G. veletis* exposed to laboratory conditions ranged from $-3.3\text{ }^{\circ}\text{C}$ (S-AgI) to $-9.8\text{ }^{\circ}\text{C}$ (Control-SCP; Table 3.2). Not all control crickets reached their SCP during my assessment of survival at $-8\text{ }^{\circ}\text{C}$. However, the mean SCPs of control crickets that did freeze was not affected by the length of their treatment (Table 3.2; $F_{3,101} = 0.964$, $P = 0.413$). I determined the mean SCP of crickets exposed to control conditions for six-weeks (Control-SCP; $-7.7 \pm 0.10\text{ }^{\circ}\text{C}$) and used this value for comparisons to crickets exposed to other laboratory acclimations. Planned contrasts revealed that all laboratory-acclimation treatments resulted in higher mean SCPs than the Control-SCP group ($F_{28,299} = 10.82$, $P < 0.05$).

Table 3.2 – Survival of internal ice formation in juvenile male *Gryllus veletis* following various laboratory acclimations. Treatments that led to greater than 75 % survival at 48 h post-thaw were considered to have successfully induced freeze tolerance. Asterisks indicate significant difference in mean SCPs between the treatment group and Control-SCP ($P < 0.05$). See tables 2.2 - 2.5 for treatment conditions.

Treatment	N	No. Frozen	Survival 48 h post-thaw	Freeze tolerant?	Mean SCP (\pm SEM) ($^{\circ}$ C)
Control-2	24	16	0	No	-7.6 \pm 0.10
Control-4	24	22	0	No	-7.4 \pm 0.15
Control-6	48	43	0	No	-7.3 \pm 0.10
Control-8	36	30	0	No	-7.2 \pm 0.14
Control-SCP	64	64	n/a	No	-7.7 \pm 0.10
S-HT	8	8	0	No	-6.36 \pm 0.21*
S-LT	8	8	0	No	-5.6 \pm 0.18 *
CPCT-1	8	8	0	No	-6.03 \pm 0.21*
CPCT-2	8	8	0	No	-6.9 \pm 0.31*
RPCT-2	8	8	0	No	-6.5 \pm 0.26*
CPRT-2	8	8	0	No	-6.8 \pm 0.21*
CPCT-4	8	8	0	No	-6.4 \pm 0.25*
RPCT-4	8	8	0	No	-6.4 \pm 0.25*
CPRT-4	8	8	1	No	-6.5 \pm 0.25*
OS-8	32	32	29	Yes	-6.7 \pm 0.15*
OS-8.2	8	8	0	No	-6.7 \pm 0.22*
OS-8.4	8	8	1	No	-6.2 \pm 0.25*

Continued on next page

Table 3.2 – Continued.

Treatment	N	No. Frozen	Survival 48 h post-thaw	Freeze tolerant?	Mean SCP (\pm SEM) ($^{\circ}$C)
OS-8.6	8	8	5	4	-6.5 \pm 0.31*
OS-8 ₁₊₂	8	8	0	No	-6.7 \pm 0.22*
OS-8 ₃₊₄	8	8	1	No	-6.7 \pm 0.31*
OS-8 ₅₊₆	8	8	2	No	-6.9 \pm 0.24*
OS-8 ₇₊₈	8	8	1	No	-6.6 \pm 0.24*
COS-6	24	24	22	Yes	-6.1 \pm 0.15 *
COS-6P	8	8	0	No	-6.9 \pm 0.28*
COS-6T	8	8	0	No	-7.0 \pm 0.29*
INOC	8	8	0	No	-5.0 \pm 0.38 *
CPCT-2 _{Agl}	8	8	0	No	-5.8 \pm 0.21*
RPCT-2 _{Agl}	8	8	0	No	-5.8 \pm 0.27*
CPRT-2 _{Agl}	8	8	0	No	-5.9 \pm 0.33*
CPCT-4 _{Agl}	8	8	0	No	-5.8 \pm 0.21*
RPCT-4 _{Agl}	8	8	0	No	-6.2 \pm 0.25*
CPRT-4 _{Agl}	8	8	0	No	-5.8 \pm 0.29*
INOC	8	8	0	No	-3.6 \pm 0.36 *

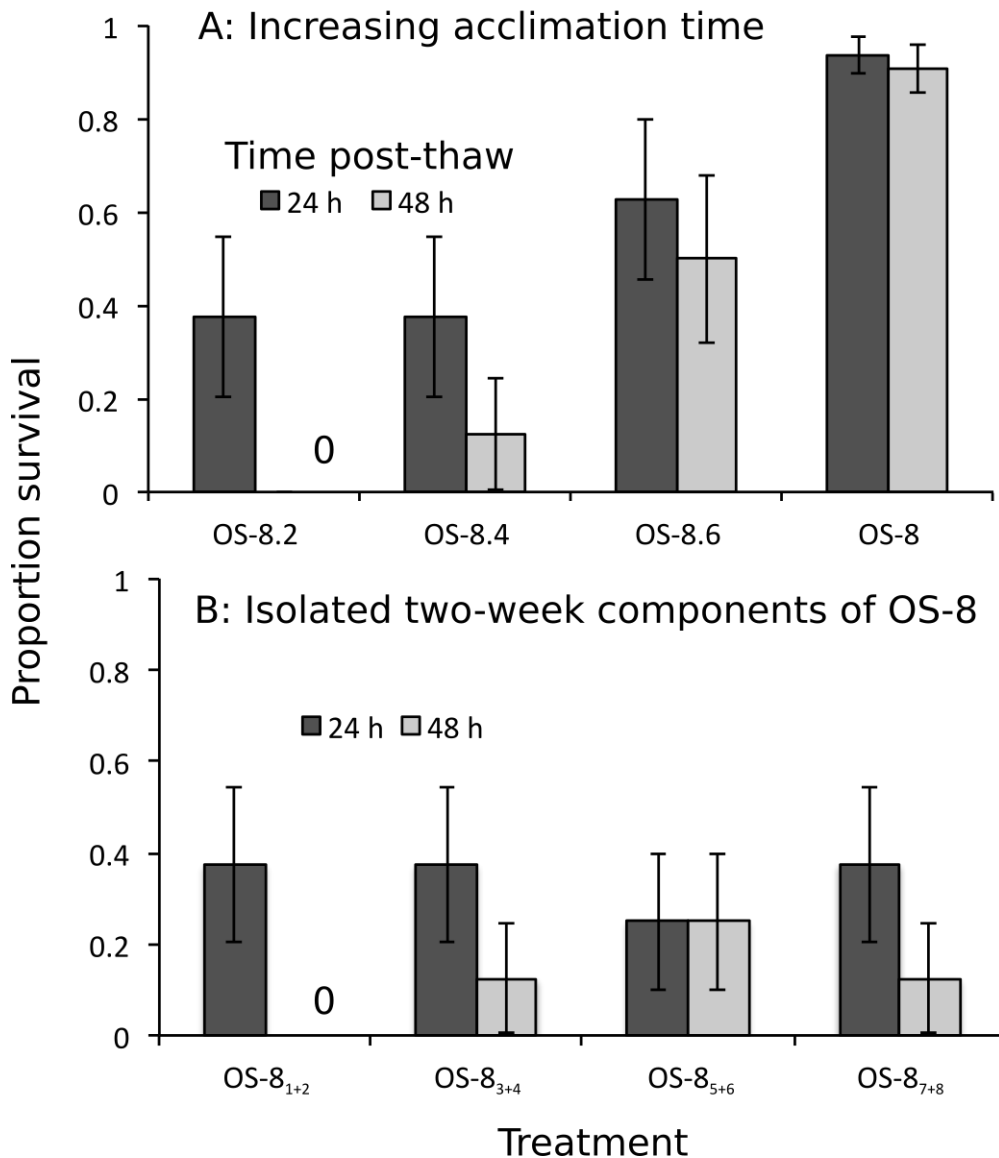


Figure 3.2 - Survival of internal ice formation in juvenile male *Gryllus veletis* exposed to components of the OS-8 laboratory-acclimation. Crickets were acclimated in the laboratory (Table 2.2 and 2.3), prior to being held at -8 °C for 1.5 h and the proportion survival (\pm Standard error of proportion; SEP) was assessed at 24 and 48 h post-thaw. A. Survival of freezing in crickets exposed to two, four, six and eight weeks of the OS-8 laboratory-acclimation. B. Survival of freezing in crickets exposed to isolated two-week components of the OS-8 laboratory-acclimation. N = 32 for OS-8; N = 8 for all other treatments.

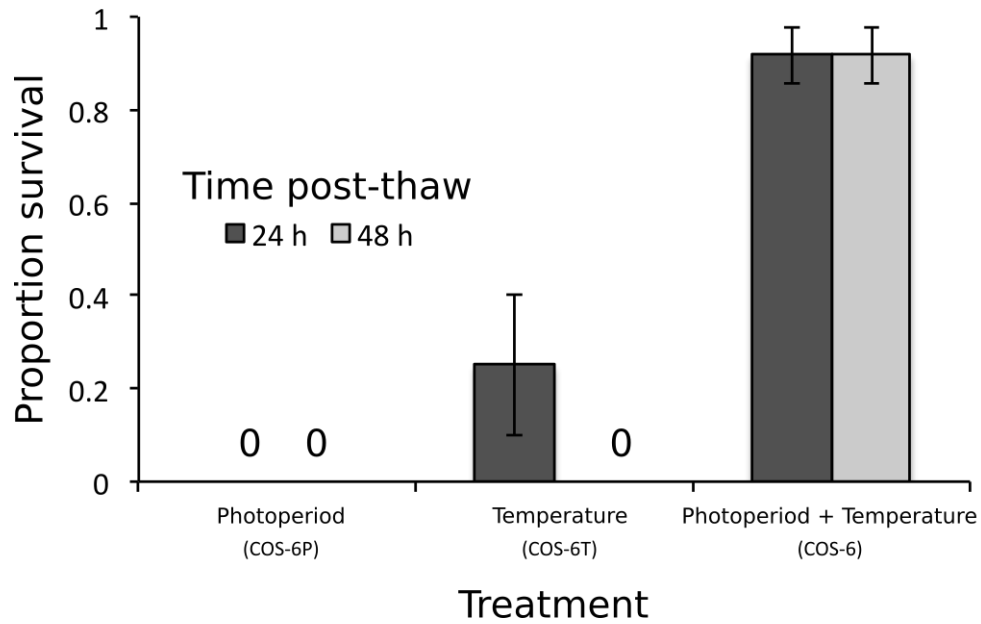


Figure 3.3 - Survival of internal ice formation in juvenile male *Gryllus veletis* exposed to isolated temperature and photoperiod regimes of the COS-6 laboratory-acclimation. Crickets acclimated in the laboratory (Table 2.4), prior to being held at -8 °C for 1.5 h. Proportion survival (\pm SEP) was assessed at 24 and 48 h post-thaw. N = 24 for COS-6; N = 8 for COS-6P and COS-6T.

3.2.2 Lethal limits

To assess the lower lethal temperature of freeze-tolerant juvenile male *Gryllus veletis*, laboratory-acclimated (COS-6) and field-acclimatized (F-2014) crickets were exposed to a series of low temperatures (Figure 3.4). The survival of groups of field and laboratory crickets did not differ at decreasing temperatures (Wald's statistic = 0.96 on one df, $P = 0.326$) and crickets from both treatments were capable of surviving $-12\text{ }^{\circ}\text{C}$ for 1.5 h. There was a sharp decline in survival at temperatures below $-9\text{ }^{\circ}\text{C}$ (F-2014) and $-10\text{ }^{\circ}\text{C}$ (COS-6), and laboratory-acclimated crickets did not survive exposure to $-15\text{ }^{\circ}\text{C}$ for 1.5 h.

To assess the influence of time frozen on survival, groups of COS-6 and F-2014 were exposed to $-8\text{ }^{\circ}\text{C}$ for durations of 1.5 to 196 h (Figure 3.5). Laboratory-acclimated crickets survived internal ice formation for a longer duration (37 % survival after 168 h) than field-acclimatized crickets (0 % survival after 96 h) (Wald's statistic = 7.88 on one df, $P < 0.05$).

I compared the distribution of SCPs of groups of laboratory-acclimated (COS-6), field-acclimatized (F-2014), and control crickets (Figure 3.6). The mean SCPs differed between all treatments and ice nucleation occurred at significantly higher temperatures in freeze-tolerant crickets compared to control crickets (Tukey's HSD, $P < 0.05$).

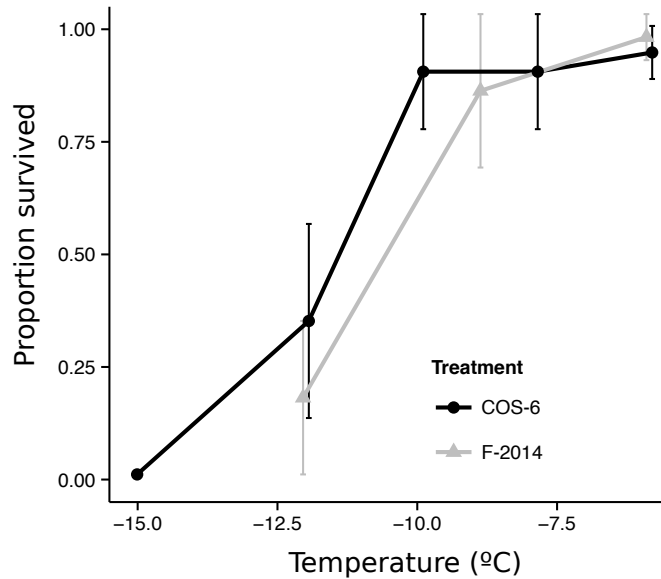


Figure 3.4 - Survival after acute low temperature exposure in laboratory-acclimated and field-acclimatized juvenile male *Gryllus veletis*. Acclimated (COS-6) and acclimatized (F-2014) crickets were held frozen for 1.5 h and survival was assessed 24 h post-thaw. Each point represents the proportion (\pm SEP) of crickets that survived the cold exposure. There was no difference in survival at decreasing temperatures between the two treatments (see text for statistics). N = 6 for F-2014; N = 24 for the -8 °C COS-6 exposure; and N = 8 for all other COS-6 cold exposures.

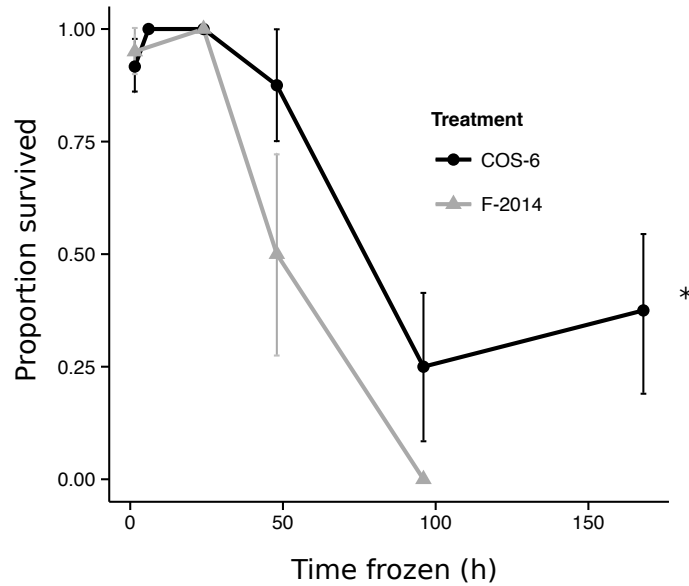


Figure 3.5 - Survival of juvenile male *Gryllus veletis* following extended freezing events. Lab-acclimated (COS-6) and field-acclimatized (F-2014) crickets were cooled at $0.5\text{ }^{\circ}\text{C min}^{-1}$ and held at $-8\text{ }^{\circ}\text{C}$ for 1.5 to 168 h, survival was assessed 24 h post-thaw. Each point represents the proportion (\pm SEP) of crickets that survived freezing. Lab-acclimated crickets survived significantly longer exposures to sub-freezing conditions (see text for statistics). $N = 6$ for F-2014; $N = 24$ for the 1.5 h COS-6 exposure; and $N = 8$ for all other COS-6 cold exposures.

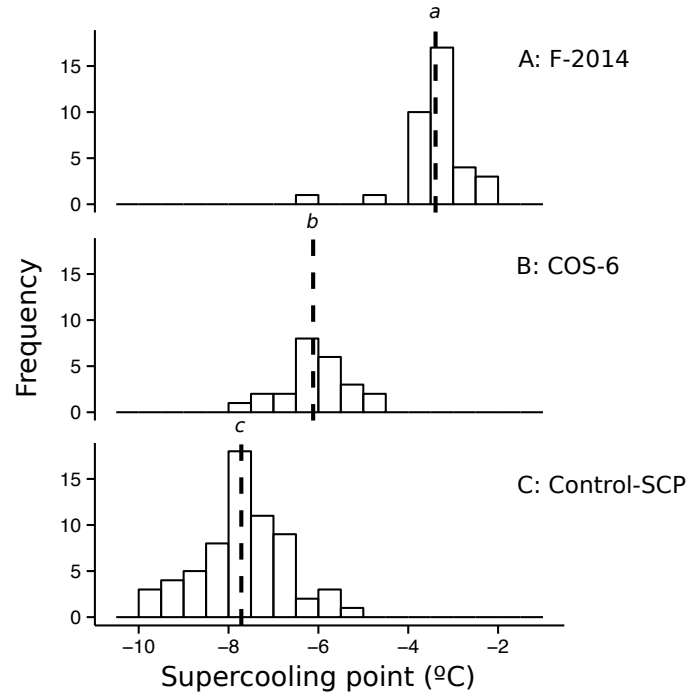


Figure 3.6 - Distribution of supercooling points (SCPs) of field-acclimatized (A), laboratory-acclimated (B), and control (C) juvenile male *Gryllus veletis*. The dashed line indicates the mean SCP for each treatment. Lower-case letters indicate SCPs that significantly differ between treatments (Tukey's HSD, $P < 0.05$). $N = 24$ for COS-6; $N = 36$ for F-2014; $N = 64$ for Control-SCP.

3.3 Physiological changes associated with freeze tolerance

To determine the potential physiological changes associated with becoming freeze tolerant, I characterized and compared control crickets (Control-6) to lab-acclimated, freeze-tolerant crickets (compressed, six-week outdoor simulation; COS-6).

3.3.1 Site of ice nucleation

To determine potential sites of ice nucleation in freeze-tolerant crickets, I compared the mean SCPs of tissues isolated from freeze-tolerant crickets with those from control crickets (Figure 3.7). The highest SCP recorded for a tissue was $-4.6\text{ }^{\circ}\text{C}$ in the gut isolated from a freeze-tolerant (COS-6) cricket, and the lowest SCP recorded was $-18.5\text{ }^{\circ}\text{C}$ in hemolymph from a control cricket. Planned contrasts revealed that all cricket tissues had higher SCPs than that of insect Ringer's solution in 3 % ascorbic acid ($F_{9,70} = 27.18$, $P < 0.001$, all contrasts $P < 0.05$). The SCPs of the hemolymph and gut were significantly higher in freeze-tolerant crickets than in controls ($P < 0.05$) and there was no difference in the SCPs of fat bodies or Malpighian tubules between treatments ($P > 0.05$).

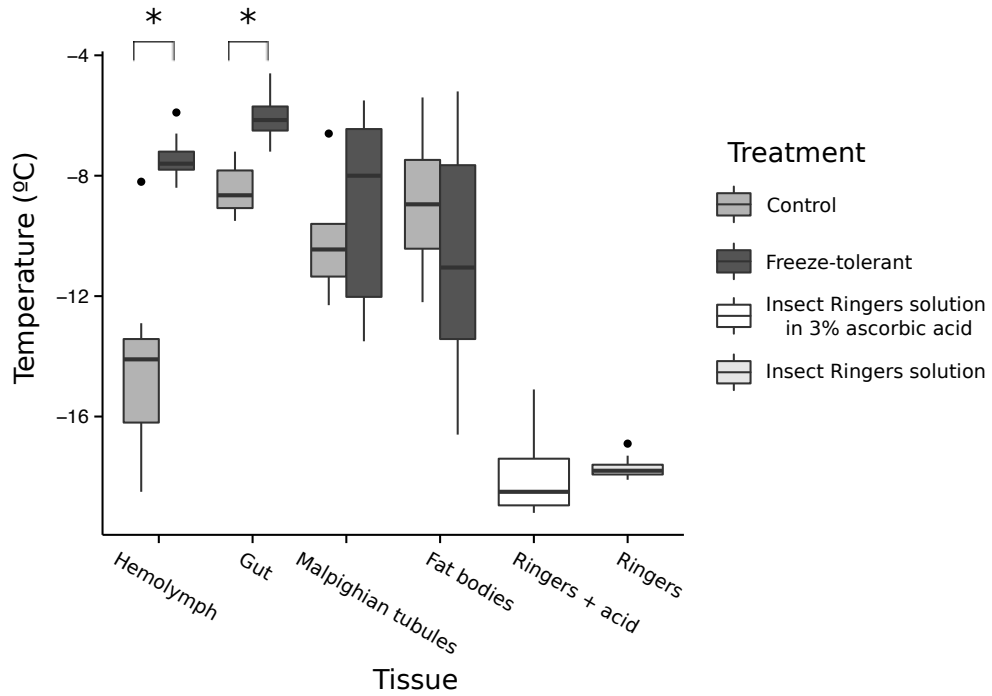


Figure 3.7 - Supercooling points (SCPs) of tissues isolated from juvenile male *Gryllus veletis* exposed to a six-week laboratory-acclimation (COS-6) and control conditions (Control-6). The top and bottom of each box represents the upper and lower quartile, respectively, the horizontal line represents the median, the vertical lines extend to the minimum and maximum values within 1.5 times the inter-quartile range and black dots indicate outliers. Mean tissue SCPs that are significantly different between treatments are denoted with an asterisk ($P < 0.05$). The mean SCPs of all tissues differed from insect Ringer's solution in 3 % ascorbic acid ($P < 0.05$). $N = 8$ for all treatments.

3.3.2 Biochemical composition of freeze-tolerant crickets

I compared hemolymph composition between freeze-tolerant (COS-6) and control (Control-6) crickets, specifically comparing carbohydrates, free proline, sodium, potassium, and total hemolymph osmolality (Figure 3.8). Freeze-tolerant crickets had a significantly higher hemolymph osmolality than control crickets (Figure 3.9; $F_{1,14} = 31.79$, $P < 0.001$). I did not observe thermal hysteresis in the hemolymph of crickets from either group.

I determined through qualitative gas chromatography that trehalose was the only carbohydrate present at detectable concentrations in *G. veletis* hemolymph. Unhydrolyzed hemolymph produced several peaks with retention times that did not correspond with retention times for xylitol, glycerol, ribitol, mannitol or sorbitol standards (Figure 3.10 A & C; Table 3.3). However, the peak with a retention time of 30.5 min corresponded to that of my trehalose standard. Cricket hemolymph that had been hydrolyzed produced a single non-artifact peak with a retention time of 27.1 min, which corresponded with my sorbitol standard (Figure 3.10 B & D; Table 3.3). I attributed the appearance of the sorbitol peak in the hydrolyzed sample to the hydrolysis of trehalose into two glucose molecules and the subsequent reduction of glucose into sorbitol.

I compared the concentrations of trehalose and sorbitol between control and freeze-tolerant crickets. Hemolymph isolated from freeze-tolerant crickets had greater area under the curve for their trehalose and sorbitol peaks compared to hemolymph isolated from control crickets (Figure 3.10). This suggested that freeze-tolerant crickets may have higher trehalose concentrations in their hemolymph than control crickets, however this needed to be confirmed with a trehalose assay.

I confirmed the presence of trehalose in *G. veletis* and measured its concentrations in the hemolymph of control and freeze-tolerant crickets using a

spectrophotometric assay. Freeze-tolerant crickets had 318% more trehalose in their hemolymph than control crickets ($P < 0.001$; Figure 3.9B).

I compared the whole-body and hemolymph concentration of proline between control and freeze-tolerant crickets. Freeze-tolerant crickets had >300 % higher concentrations of proline in both their whole-body ($P < 0.001$) and hemolymph ($P < 0.001$) than control crickets (Figure 3.9C,D). The accumulation of free proline in freeze-tolerant crickets accounts for at least part of the difference in hemolymph osmolality between the two treatments (Figure 3.8).

I compared the concentrations of sodium and potassium in the hemolymph of freeze-tolerant and control *G. veletis* and found no difference between treatments in their concentrations ($P > 0.05$; Figure 3.9 E & F). The cumulative concentration of sodium and potassium accounts for approximately 44 % of total hemolymph osmolality in control crickets and 28 % in freeze-tolerant crickets.

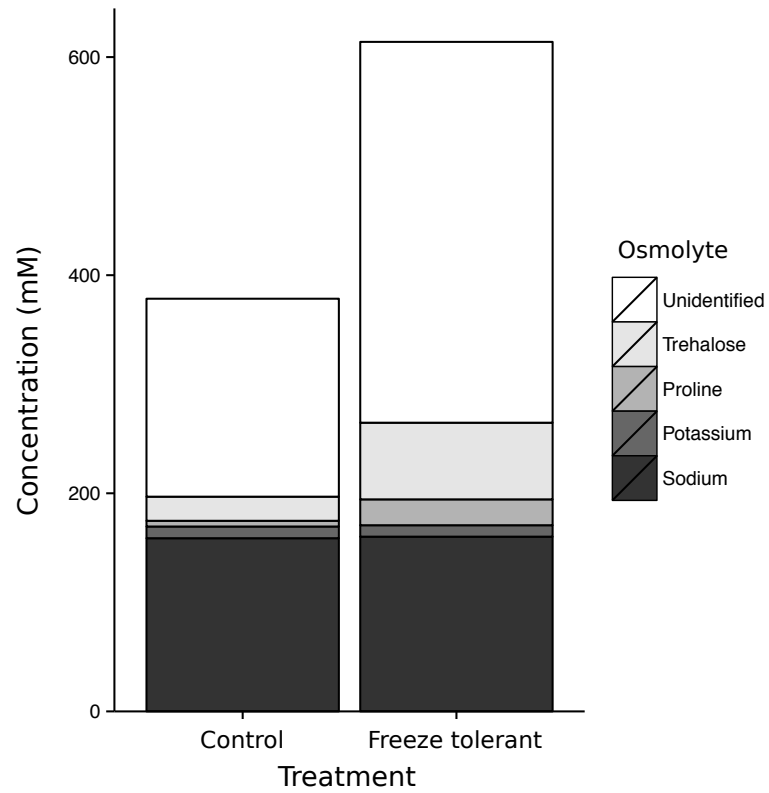


Figure 3.8 – Hemolymph composition in freeze-tolerant *Gryllus veletis*. Hemolymph extracted from control and freeze-tolerant crickets were compared for differences in osmolality, trehalose, proline, potassium, and sodium concentrations.

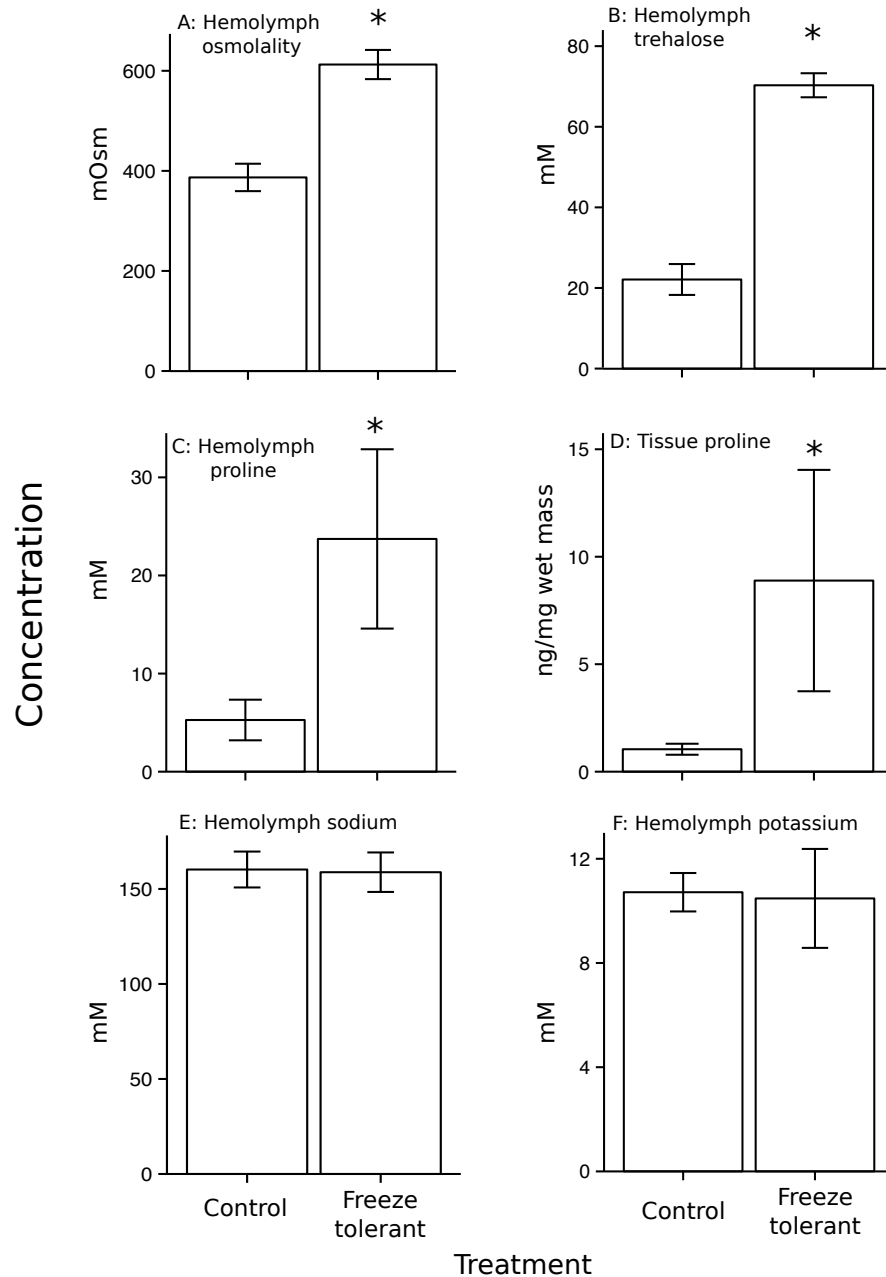


Figure 3.9 – Concentrations of biochemicals associated with freeze-tolerance in *Gryllus veletis*. Control and freeze-tolerant crickets were compared with respect to: (A) hemolymph osmolality; the concentration of trehalose (B), proline (C), sodium (E), and potassium (F) in the hemolymph; and the concentration of proline in the tissues (D). Asterisk indicates significant difference in mean concentrations between control and freeze-tolerant crickets ($P < 0.05$).

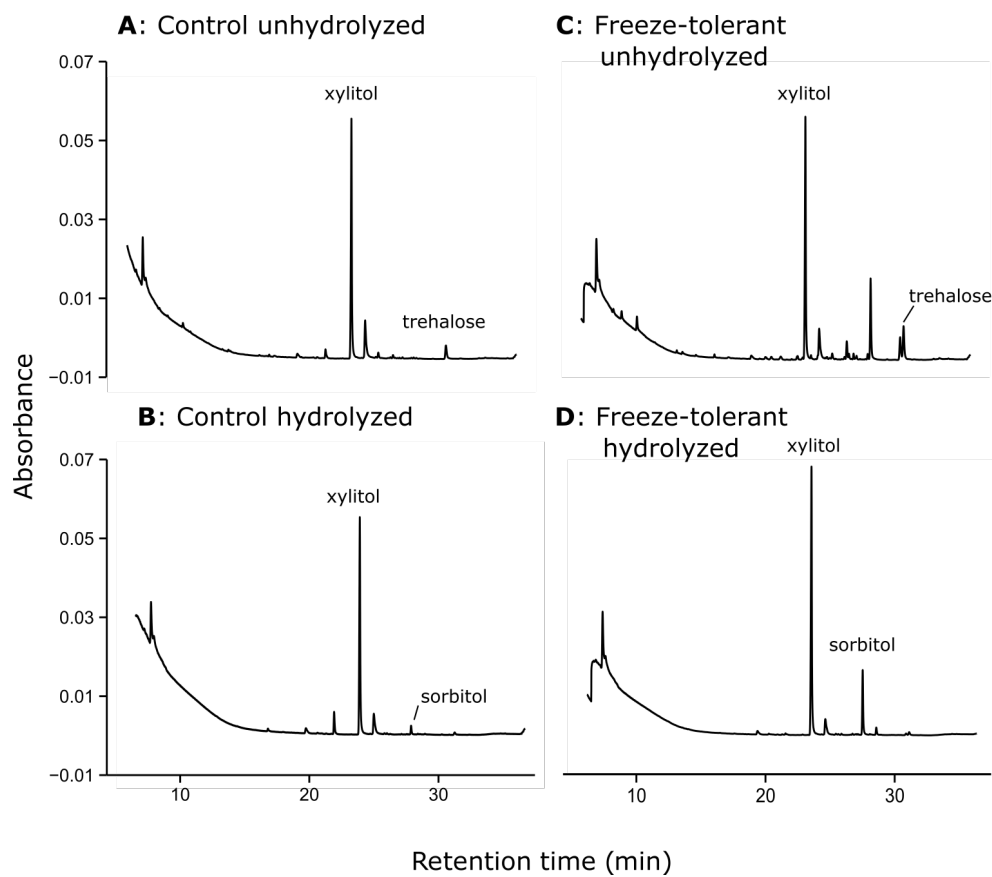


Figure 3.10 – Carbohydrates identified in freeze-tolerant and control *Gryllus veletis*, using gas chromatography (GC). Chromatograms profiling reduced and acetylated hemolymph from control (A) and freeze-tolerant (C) crickets, and their respective hydrolyzed samples (B & D). Xylitol was used as an internal standard and carbohydrates were identified by comparing retention time of peaks with known standards (Table 3.3).

Table 3.3 – Retention time of known standards separated using gas chromatography and their presence (X) in hemolymph isolated from control and freeze-tolerant crickets.

Carbohydrate	Retention time (min)	Freeze-tolerant		Control	
		Unhydrolyzed	Hydrolyzed	Unhydrolyzed	Hydrolyzed
Glycerol	6.8				
Ribitol	20.6				
Xylitol (Internal std.)	23.2	X	X	X	X
Mannitol	25.5				
Sorbitol	27.1		X		X
Trehalose	30.5	X		X	

4 Discussion

Insects inhabit thermally variable environments where they can be exposed to sub-zero temperatures and are at risk of freezing. At these temperatures, freeze-intolerant insects are killed if internal ice formation occurs, while freeze-tolerant insects are not (Denlinger & Lee, 2010). Currently, the physiological mechanisms underpinning insect freeze tolerance are not fully understood, a result of limited manipulative experiments and the lack of a tractable model organism (Sinclair & Renault, 2010; Hayward *et al.*, 2014). I found that juvenile male *G. veletis* can survive internal ice formation following a reduction in thermophotoperiod fluctuations in the field or laboratory. Freeze tolerance in *G. veletis* was accompanied by an accumulation of proline and trehalose, as well as evidence suggesting ice nucleation is initiated in the gut and hemolymph. My protocol for inducing freeze tolerance in *G. veletis* now allows for a direct comparison of crickets from the same population that are and are not freeze tolerant. With this, *G. veletis* may be used in future manipulative studies to test hypotheses and determine the mechanisms underlying insect freeze tolerance.

4.1 The induction of freeze-tolerance in *Gryllus veletis*

When reared exclusively at constant summer conditions in the laboratory, *G. veletis* did not survive internal ice formation. However, crickets became freeze tolerant after an eight-week field acclimatization in London, Ontario from October through January. Thus, I conclude that freeze-tolerance is a seasonally acquired plastic trait in *G. veletis*, similar to other freeze-tolerant insects such as *Eurosta solidaginis* and *Pyrrharctia isabella* (Morrissey & Baust, 1976; Layne *et al.*, 1999). I obtained the photoperiod and temperature conditions experienced by overwintering crickets, and used these conditions to guide my attempt to induce freeze tolerance in the laboratory.

I was able to induce freeze tolerance in over 90 % of crickets exposed to eight-week fluctuating thermophotoperiod regimes that simulate outdoor conditions (OS-8). I refined this acclimation into a six-week protocol (COS-6), which reduced acclimation

time by 25 % and allowed for a higher throughput of freeze-tolerant crickets. The proportion of crickets surviving internal ice formation increased with acclimation time, displaying a pattern of freeze-tolerance acquisition over time similar to that of *E. solidaginis* (Bennett & Lee, 2002). A minimum acclimation time may be required for insects to undergo the necessary physiological changes associated with freeze-tolerance such as the accumulation of cryoprotectants (Lee & Denlinger, 1991) and modifications of the lipid membranes to increase fluidity at low temperatures (Overgaard *et al.*, 2006). In future studies, cryoprotectants could be measured at time points throughout the acclimation to determine if a relationship exists between their accumulation and survival of internal ice formation. Investigating this relationship may identify critical components of the acclimation process relevant to refining the laboratory-acclimation protocol.

I found that a greater proportion of crickets became freeze tolerant following exposure to a fluctuating thermal regime compared to crickets exposed to constant-temperature and linear ramping thermal regimes. Exposure to fluctuating thermal regimes during acclimation increased cold tolerance of chill-susceptible *Drosophila melanogaster* (Overgaard *et al.*, 2011) and helped reduce chilling-injuries in the freeze-intolerant Orthoptera, *Locusta migratoria* (Jing *et al.*, 2005). Compared to constant and linear ramping thermal regimes, fluctuating thermal regimes may better simulate the natural cues that are more likely to induce phenotypic plasticity (Colinet *et al.*, 2015). Alternatively, intermittent periods of higher temperatures may allow insects to undergo physiological changes (e.g. cryoprotectant accumulation, tissue repair, membrane modifications) that would not occur during constant low temperatures (Colinet *et al.*, 2015). This hypothesis could be tested by measuring and comparing response variables associated with insect freeze tolerance (e.g. cryoprotectant concentrations) at several time points throughout a fluctuating thermal regime to determine if cryoprotectant accumulation occurs more readily during periods of exposure to high-temperatures compared to low-temperatures.

Photoperiod and temperature are two of the most common cues that trigger seasonal cold hardening in insects (Block *et al.*, 1990). The interaction of physiological responses (e.g. cryoprotectant accumulation, supercooling capacity) to temperature and

photoperiod cues can lead to increased cold tolerance in insects, as observed by Kim and Song (2000) in the beet armyworm, *Spodoptera exigua*. While I did not determine the extent to which reduced photoperiod and fluctuating temperature are required for freeze tolerance, I found that their combination was necessary to induce freeze-tolerance in *G. veletis*. In order to further assess the relative contribution of temperature, photoperiod, and their interaction to insect freeze tolerance, physiological changes associated with freeze tolerance should be compared between groups of *G. veletis* exposed to isolated and combined thermophotoperiod regimes.

The majority of my laboratory treatments did not induce freeze tolerance in *G. veletis*. No crickets became freeze-tolerant following two weeks of constant low temperatures, daily reduction in temperatures, constant short day length, daily reduction in day length or diet manipulations. The multiple laboratory manipulations that did not result in freeze tolerance underscore the complexity of the cues and responses required to induce insect freeze tolerance. In *E. solidaginis*, exposure to a reduced photoperiod can produce responses in the nervous and endocrine systems, which induce overwintering diapause but does not directly trigger polyol synthesis, which is regulated by temperature and development stage (Storey & Storey, 1991). Teets *et al.* (2008) demonstrated that an insect's response to low temperature exposure is a cellular response in freeze-tolerant Antarctic midge, *Belgica antarctica*. *G. veletis* may require an interaction of similar cellular and organismal level responses to survive internal ice formation.

While freeze tolerance has been observed in other Orthoptera (Table 4.1), these species are freeze-tolerant year-round. The seasonally acquired freeze tolerance that I observed in *G. veletis* reflects an ecological pattern described by Sinclair *et al.* (2003), who suggest that environmental predictability (or lack thereof) leads to two scenarios in which alternate freeze tolerance strategies would be advantageous for insects. The first is an unpredictable environment (common to the southern hemisphere) in which moderate, year-round freeze tolerance allows insects to survive summer cold snaps and take advantage of mild winters without the expense of seasonal cold hardening. Conversely, in more predictable environments like those inhabited by *G. veletis*, freeze-tolerant insects can withstand very low sub-zero temperatures for long periods but generally only become

freeze-tolerant prior to overwintering. Teets & Denlinger (2013) suggest that exploring limits of insect cold tolerance will provide insight into how different strategies evolved, and is essential for predicting future insect ranges in a changing climate.

4.2 Lethal limits of freeze tolerance in *Gryllus veletis*

I determined the lower lethal temperature (LLT) and the length of time that *G. veletis* could survive freezing, and compared these measurements between groups of freeze-tolerant crickets that were field-acclimatized or laboratory-acclimated. There was no difference in survival after acute low temperature exposure (90 min; LLT), but laboratory-acclimated crickets survived longer being held frozen at -8 °C (168 h) than field-acclimatized crickets. Although specific mechanisms for this difference cannot be inferred from my study, I speculate that this is because of the laboratory-acclimated crickets having easier access to food and water, which might allow them to better accumulate energy stores to fuel the physiological responses of acclimation.

Manipulating diet and access to water for *G. veletis* throughout acclimation and tracking survival could be used to test this hypothesis. Additionally, the acclimatized crickets experienced repeated exposures to more extreme absolute temperatures, which can result in the accumulation of chilling and freezing injuries (Marshall & Sinclair, 2012a), which may have limited their capability of surviving prolonged exposure to sub-zero temperatures. Nevertheless, I can conclude that crickets acclimated in the laboratory are equally, if not more, cold-hardy than crickets exposed to outdoor conditions.

While I cannot draw definitive conclusions about specific mechanisms of freezing damage in *G. veletis*, the decline in survival after exposure to decreasing sub-freezing temperatures and increased time spent frozen suggests that mortality from freezing in *G. veletis* was both temperature- and time-dependent. The LLTs for freeze-tolerant insects range from -4 °C to -196 °C (Sinclair, 1999) with the LLT for *G. veletis* falling between -12 and -15 °C, which is comparable to other freeze-tolerant Orthoptera (Table 4.1). However, the steep decline in survival at temperatures below -10 °C suggests that freezing damage begins to accumulate at temperatures slightly warmer than the LLT. Laboratory-acclimated *G. veletis* demonstrate greater than 85 % survival after being

frozen for 48 h, but survival decreases to 38 % after being frozen for one week. The relationship that I observed in *G. veletis* between mortality, temperature and time frozen could be a result of the quantity of ice in the cricket that surpasses a critical quantity, leading to lethal accumulation of cellular damages (Sinclair *et al.*, 1999). Measuring ice content in *G. veletis* over time and at different temperatures using a calorimeter and tracking survival could test this hypothesis. If no correlation between ice content and mortality is observed, then it is likely that mortality results from mechanisms other than cellular dehydration and high solute concentration.

Table 4.1 Characteristics of freeze-tolerant orthopterans. (NZ = New Zealand, NA = North America, SA = South America)

Family	Species	Life stage	Habitat	SCP (°C)	LLT (°C)	Length of freeze survival	Cryoprotectant				Reference
							Trehalose	Proline	Ice nucleating activity	Antifreeze activity	
Prophalgopsidae	<i>Cyphoderris monstrosa</i>	Nymph	Montane, NA	-6	-12	> 12 h	n/a	n/a	n/a	n/a	McKinnon, unpubl.
Acrididae	<i>Arphia conspersa</i>	Nymph	Alpine, NA	-9.2	-19	n/a	n/a	n/a	n/a	n/a	Alexander, 1967
	<i>Meridacris subaptera</i>	Adult	Alpine, SA	-4	-10	> 8 h	n/a	n/a	hemolymph	n.a	Sømme, 1986
	<i>Sigaüs australis</i>	Adult	Alpine, NZ	-4	-11	> 48 h	n/a	n/a	endogenous	n/a	Sinclair, 2001; Hawes, 2014
	<i>Xanthippus corallipes</i>	Nymph	Alpine, NA	-9.4	-19	n/a	n/a	n/a	n/a	n/a	Alexander, 1967
Stenopelmatidae	<i>Deinacrida connectens</i>	Adult	Alpine, NZ	-5	-12	n/a	n/a	n/a	n/a	n/a	Sinclair, 1999
	<i>D. parva</i>	Adult	Montane, NZ	-5	-5	n/a	n/a	n/a	n/a	n/a	Sinclair, 1999
	<i>Hemideina maori</i>	Adult	Alpine, NZ	-5	-10	> 5 days	>300 mM	41 mM	hemolymph protein & gut	None	Ramløv <i>et al.</i> , 1992; Sinclair <i>et al.</i> , 1999; Neufeld & Leader, 1998
	<i>H. thoracica</i>	Adult	Lowland, NZ	-5	-5	230 min	n/a	n/a	hemolymph & gut	None	Sinclair, 1999; Sinclair <i>et al.</i> , 1999
	<i>H. crassidens</i>	Adult	Lowland, NZ	-5	-6.5	165 min	n/a	n/a	hemolymph & gut content	n/a	Sinclair <i>et al.</i> , 1999

4.3 Physiological changes associated with freeze tolerance in

Gryllus veletis

I compared SCPs, hemolymph composition, and tissue concentration of proline between control and freeze-tolerant crickets to identify physiological changes associated with becoming freeze-tolerant. I found that freeze-tolerant crickets initiate ice formation at higher temperatures in the hemolymph and gut tissue. Additionally I found freeze-tolerant crickets have higher hemolymph osmolality due, in part, to higher trehalose and proline concentrations, as well higher whole-body proline concentrations. While I do not provide direct evidence that these physiological changes are necessary or sufficient for the survival of internal ice formation, they can provide a starting point for future manipulative studies that will help determine the underlying mechanisms of insect freeze tolerance.

4.3.1 Ice nucleating activity

In freeze-tolerant insects the transition to the cold-hardy state is accompanied by an increase in nucleating activity and subsequent increase in supercooling point (Zachariassen & Kristiansen, 2000). I found that freeze-tolerant crickets have higher and more tightly distributed SCPs than control crickets, suggesting some form of control over ice nucleation. Similar to other Orthoptera, this control of ice formation is likely initiated in the hemolymph and gut, as these appear to be the first tissues that freeze, and SCPs of these tissues were significantly higher in freeze-tolerant crickets (Table 4.1; Sinclair *et al.*, 1999). Control and freeze-tolerant crickets received the same diet and it is unlikely that exogenous ice nucleators were introduced to freeze-tolerant crickets in another form. Thus, I hypothesize that like other Orthoptera, *G. veletis* are producing an endogenous ice nucleator (Table 4.1). An exogenous ice nucleator, such as food or bacteria may have been introduced to the acclimatized crickets which would account for increase in SCP compared to the freeze-tolerant crickets acclimated in the lab. However, this increase in SCP did not enhance survival of internal ice formation.

Zachariassen and Hammel (1976) suggest that ice-nucleating agents in freeze-tolerant insects ensure that ice formation begins in the extracellular space, which leads to the cryoprotective dehydration of insect's cells. This is a potential mechanism by which ice nucleators present in the hemolymph and gut of *G. veletis* may aid in surviving internal ice formation. However, the manipulation of SCP through the introduction of an exogenous ice nucleator, silver iodide, did not induce freeze tolerance. This may be a result of ice-nucleators needing to be present in both the hemolymph and the gut simultaneously and silver iodide not being transferred from the gut to the hemolymph and vice versa. Alternatively, the control of ice-nucleation may not be sufficient for freeze tolerance in *G. veletis*.

The prevalence of intracellular ice formation in freeze-tolerant insects is not fully understood. However, of the freeze-tolerant insects studied, many appear to survive intracellular freezing in at least one cell type, usually fat body or gut cells (Sinclair & Renault, 2010). Sinclair and Renault (2010) speculated that intracellular freezing may be advantageous to an insect by reducing cellular and organismal dehydration. The real-time tracking of ice formation *in vivo*, using microscopy and other visualization techniques (X-ray, magnetic resonance) is needed to further explore the process of ice formation in *G. veletis*, particularly in the gut.

4.3.2 Hemolymph composition

I compared the hemolymph composition of freeze-tolerant and control crickets to identify potential cryoprotectants that would contribute to an increase in freeze-tolerance. I expect that the majority of cryoprotectants are present in the hemolymph if they are interacting with extracellular ice and protecting the outside of cell membranes. Additionally, many insects possess aquaporins, aquaglyceroporins and aquaglyceroporin-like proteins and other transporters that allow cryoprotectants to readily cross cellular-membranes to the extent that it is reasonable to assume that their intra- and extracellular concentrations would be similar (Philip & Lee, 2010). Thus, the cryoprotectants that I identified in *G. veletis* hemolymph are likely present in the tissues as well.

Hemolymph osmolality increased by 63 % in freeze-tolerant compared to control crickets. Approximately 25 % and 10 % of this difference in hemolymph osmolality is accounted for by the accumulation of trehalose and proline, respectively, in the hemolymph of freeze-tolerant crickets. I found no difference in sodium or potassium concentrations, which account for 30-40 % of the hemolymph solutes in *G. veletis*. I predict that large portions (~50-80 %) of the unidentified osmolytes are chloride and other anions that allow for a net ionic balance. Altogether, hemolymph extracted from control and freeze-tolerant *G. veletis* mirrors the composition and concentrations of hemolymph extracted from summer and overwintering *H. maori*, respectively (Neufeld & Leader, 1998; Ramløv *et al.*, 1991). I did not measure protein or amino acid content (other than proline), but these solutes may also account for a portion of the remaining unidentified osmolytes.

The ~200 mM increase in hemolymph osmolality that I observed in freeze-tolerant crickets may function to reduce the quantity of extracellular ice. Layne and Blakely (2002) found that a 200-300 mM increase in glycerol significantly reduced the amount of freezable water in *P. isabella* by approximately 12.5 %. In addition to colligative cryoprotection, some of these osmolytes, particularly trehalose and proline, may provide non-colligative cryoprotection by stabilizing membranes and other macromolecules in *G. veletis*.

Similar to other freeze-tolerant Orthoptera (Table 4.1), trehalose was the only major carbohydrate present in *G. veletis* hemolymph, and its accumulation corresponded with the induction of freeze tolerance. It is possible that carbohydrates other than trehalose are present in *G. veletis*; however, the concentrations of these carbohydrates were too low to detect using gas chromatography and likely too low to contribute to overall cryoprotection, in comparison to trehalose. Proline, like trehalose, has been identified as a potential non-colligative cryoprotectant that accumulates over winter in freeze-tolerant Orthoptera (Ramløv, 1999). Consistent with this, I found increased hemolymph and whole-body concentrations of proline in freeze-tolerant crickets compared to controls.

Under the assumption of extracellular freezing, ice does not penetrate into cells unless membranes are broken from ice or other low-temperature stresses (Storey & Storey, 2004). Trehalose and proline are both known anhydroprotectants and are often associated with membrane protection during dehydration stress in insects (Storey, 1997). Both compounds are believed to interact directly with the polar head groups of membrane lipids to stabilize the bilayer structure and prevent destructive events such as fusion, phase transitions and elevation of permeability (Crowe *et al.*, 1987). However, other membrane components (e.g. aquaporins, ion channels, trehalose transporters, cholesterol, etc.) should be examined in *G. veletis* as they may also play a role in insect cold tolerance (MacMillan & Sinclair, 2011b).

The concentrations of sodium and potassium in the hemolymph were the same for groups of control and freeze-tolerant *G. veletis*. Inorganic ions, such as sodium and potassium, perform a wide range of necessary functions within an insect (e.g. signaling, co-factors, electrochemical gradient), and a disruption in ion homeostasis is a primary cause of injury to insects during cold exposure (Zachariassen *et al.*, 2004; MacMillan & Sinclair, 2011a). Compared to the less cold-hardy field cricket, *Gryllus pennsylvanicus*, *G. veletis* better maintains ion homeostasis during exposure to low temperatures (Coello Alvarado *et al.*, 2015). The ability to maintain ion concentrations throughout acclimation and at low temperatures may be a beneficial trait in *G. veletis* as ion balance plays an important role in an insect's recovery from low-temperature exposure and is likely driven by ion-motive ATPases, ion channel gating mechanisms, and/or the lipid membrane composition (MacMillan & Sinclair, 2011b). Zachariassen *et al.* (2004) suggest that freeze-tolerant insects specifically, have large ATP stores that facilitate active transport of sodium and are able to rapidly restore sodium gradients upon thawing.

Hayward *et al.* (2014) suggest that the adoption of single gene ablations will provide direct and compelling evidence for the role of specific genes, and thus mechanisms, underlying insect freeze tolerance. Access to a *G. veletis* transcriptome along with other molecular techniques such as RNA-interference, which has been successfully performed on other Gryllidae (Takekata *et al.*, 2012), could allow for future manipulations of cryoprotectant synthesis and transport. Inhibiting cryoprotectant

synthesis and transport in acclimated *G. veletis* would help determine which molecules are necessary. However, because both trehalose and proline are involved in other physiological processes, most importantly energy metabolism, their reduction may be lethal regardless of freezing. Thus, experiments that increase cryoprotectants in control crickets prior to exposure to sub-freezing may be required to determine which molecules are sufficient for the survival of internal ice formation. Košťál *et al.* (2012) were able to induce freeze tolerance in *Drosophila melanogaster* by augmenting the insect's diet with proline. Similarly, cryoprotectant concentrations could be manipulated in *G. veletis* by diet manipulations and as well as direct injections into the hemolymph.

4.4 Future directions

This study is a step forward in our ability to determine the underlying mechanisms of insect freeze-tolerance. In *G. veletis*, we have a tractable model system to manipulate the potential mechanisms of insect freeze tolerance in a controlled, laboratory setting. While I have identified three potential cryoprotective molecules in *G. veletis* (trehalose, proline and ice nucleators in the gut and hemolymph), these results are strictly correlative. Further studies are needed to test the hypothesis stemming from my results as well as to determine how and where freezing damage is accruing, to evaluate the advantages of becoming freeze-tolerant, and to build and test hypotheses for the mechanisms underlying insect freeze tolerance.

I propose that proline and trehalose work concurrently with ice nucleating agents in freeze-tolerant *G. veletis* to limit intracellular damage during extracellular freezing. Multicomponent cryoprotectant systems are present in other freeze tolerant insects, such as *E. solidaginis* (Baust, 1986). This multicomponent hypothesis could be examined in a manipulative experiment, similar to that done on *H. maori* by Sinclair and Wharton (1997). In this experiment, tissues from *G. veletis* would be isolated and frozen in a medium with or without ice-nucleating agents and varying concentrations of proline and trehalose, with cellular survival measured post-thaw. This would help determine the role of each proposed cryoprotectant and the potential necessity of multiple cryoprotectant mechanisms. However, *in vivo* manipulations (RNA-interference, augmenting diets,

hemolymph injections) are also required to determine which biochemicals are necessary and sufficient for insect freeze tolerance.

I suggest that initial studies could focus on determining where lethal cryoinjury is occurring in freeze-intolerant *G. veletis*, as this would provide insight into the mechanisms of freezing injury and where cryoprotection is likely provided in freeze-tolerant crickets. This study could utilize live/dead staining (see Marshall & Sinclair, 2011) to compare cellular survival between crickets that die or survive exposure to sub-freezing temperatures. For this experiment, I suggest that crickets should first be exposed to one of the several laboratory-acclimations that resulted in approximately 50 % survival of internal ice formation. Therefore, differences in cellular survival between groups that die or survive freezing, can be attributed to the freezing event rather than differences in the acclimation conditions. Furthermore, the relationship between mechanisms for chilling and freezing damage is not fully understood as it these injuries can be difficult to tease apart (Hayward *et al.*, 2014; Teets & Denlinger, 2013). Cooling *G. veletis* to their mean SCP would lead to freezing in approximately half of the individuals, cellular damage could then be compared to differentiate between chilling and freezing damages.

The advantages of adopting a freeze-tolerance rather than a freeze-avoidance strategy remain unclear (Sinclair *et al.*, 2003). While the goal of my study was to assess the suitability of *G. veletis* as a model for studying the underlying mechanisms of insect freeze-tolerance, my protocol for laboratory-induced freeze-tolerance in *G. veletis* could also be used to understand why an insect would evolve freeze tolerance. With no phylogenetic patterns to explain for the distribution of freeze-tolerance within insects (Block, 1991), energetic models are likely to provide insight on the evolution of insect freeze tolerance. A current model, developed by Voituron *et al.* (2002) assumes a fitness cost associated with freezing and thawing. Although this assumption does not hold true for all freeze-tolerant insects (Marshall & Sinclair, 2012b), we can use *G. veletis* as a model to further understand the fitness (e.g. survival, reproduction) and energetic (e.g. cryoprotectant synthesis) costs associated with insect freeze tolerance, while controlling for factors such as diet, genetics and life history. Additionally, we can begin to examine benefits of freeze tolerance including the potential for protection against pathogens and

parasites. By determining the costs and benefits associated with freezing using empirical data from model insects such as *G. veletis*, we can begin to explain the use of freeze tolerance as a strategy to survive cold.

Gryllus veletis can become freeze-tolerant during a six-week laboratory acclimation and likely utilize several mechanisms to survive internal ice formation. The osmolytes I measured in freeze-tolerant *G. veletis* account for less than half of the increase in total osmolality during the acclimation treatment. Thus, it seems likely that unidentified osmolytes could also play an important role in *G. veletis* freeze tolerance. Using a metabolomics approach, Michaud & Denlinger (2007) identified 62 metabolites including amino acids, polyols, carbohydrates and metabolic intermediates in overwintering *Sarcophaga crassipalpis*. A similar metabolomics profile that utilizes gas chromatography-mass spectroscopy, liquid chromatography-mass spectroscopy, or nuclear magnetic resonance would identify other carbohydrates, polyols and amino acids that are potentially relevant to freeze tolerance. Additionally, other ions, specifically chloride should be measured to confirm that the majority of unidentified osmolytes in *G. veletis* are anions.

Hypothesis generating “-omics” approaches would be an ideal way to identify the mechanisms underlying insect freeze tolerance during acclimation. One could combine transcriptomic and metabolomic profiles of freeze-tolerant *G. veletis* to provide a holistic view of the physiological processes within freeze-tolerant insects (Teets & Denlinger, 2013). I have recently helped to assemble and annotate the transcriptome of control *G. veletis*, which will expedite the pipeline for differential gene expression comparing freeze-tolerant and freeze-intolerant (control) crickets. Furthermore, these “-omics” approaches could be used to connect gene expression to changes in phenotype (Teets and Denlinger, 2013). Together, these comparisons would help identify potential mechanisms of freeze tolerance in *G. veletis*, which as Hayward *et al.* (2014) suggest, can now be tested using manipulative experiments, similar to experiments that I have outlined above. Knocking down cryoprotectant synthesis and transport pathways using RNA-interference as well as artificially enhancing cryoprotectant concentrations via diet-augmentation and

hemolymph injections will help to determine what molecules are sufficient and necessary for insect freeze tolerance.

4.5 Conclusions

Freeze tolerance in *G. veletis* is a phenotypically-plastic trait acquired during exposure to a fluctuating reduction in thermophotoperiod. The acquisition of freeze tolerance is accompanied by increased control of ice formation in the hemolymph and gut tissue, as well as an accumulation of trehalose and proline that account for a portion of increased hemolymph osmolality. These patterns of cryoprotectant accumulation in *G. veletis* are similar to what is observed in other freeze-tolerant orthopterans. This study is one of the first to induce insect freeze tolerance in the laboratory and may provide researchers with an effective tool to determine what mechanisms are necessary and sufficient for surviving internal ice formation. Altogether, *G. veletis* has the necessary attributes to be further developed as a new model system for studying insect freeze tolerance.

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