ION HOMEOSTASIS AND VARIATION IN LOW TEMPERATURE PERFORMANCE IN THE FALL AND SPRING FIELD CRICKET (ORTHOPTERA: GRYLLIDAE)

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Abstract

Low temperature performance affects the geographical distribution of insects. The lower critical temperature limits of chill-susceptible insects are likely determined by failure of ion and water balance at low temperature. I used phenotypic plasticity in the cold tolerance of *Gryllus pennsylvanicus,* and the naturally higher cold tolerance of *Gryllus veletis* to test the hypothesis that variation in low temperature performance is accompanied by variation in ion and water homeostasis at low temperatures. Low temperature acclimation and cold adaptation enhanced performance at low temperatures. Groups with higher cold tolerance had an enhanced ability to prevent or mitigate the migration of hemolymph Na^+ and water into the digestive system, which ultimately resulted in smaller decreases in K^+ equilibrium potentials at the muscle tissue. The ability to maintain ion and water balance as a result of changes in gut membrane permeability increased performance at low temperatures, and reduced the onset of chilling injury.

Key Words

Gryllus pennsylvanicus, *Gryllus veletis*, Orthoptera, chilling injury, ion homeostasis, plasticity, CTmin, chill coma

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List of Abbreviations

CTmin: critical thermal minimum

CCR: chill coma recovery time

S.E.M: standard error of the mean

RCH: rapid cold hardening

SE: short-term exposure treatment

LE: long-term exposure treatment

AAS: atomic absorption spectrophotometer

E_K: Potassium muscle equilibrium potential

E_{Na}: Sodium muscle equilibrium potential

ATPase: adenosine triphosphate synthase

CHAPTER 1: INTRODUCTION

1.1 Insect Responses to Low Temperature

Temperature influences most physiological processes in insects (Chown and Nicolson, 2004). As ectotherms, body temperature of most insects closely matches the temperature of their surroundings. Consequently, exposure to low temperatures poses substantial challenges to insects. Low temperatures can decrease short-term performance, survival and reproductive success (Hutchinson and Bale, 1994). While some insects migrate to warmer areas to overwinter, thus avoiding stressful thermal conditions, the majority of insects overwinter in their local environment. Insects that overwinter in temperate and polar areas experience sub-zero temperatures for at least some part of the season, which increases the risk of their body fluids freezing. Insects have adopted two basic strategies to survive freezing temperatures (Lee et al., 2010a), 1) Freeze avoidance: insects that avoid freezing by maintaining their body fluids in a liquid state even at temperatures below their equilibrium freezing point or 2) freeze tolerance: insects can withstand formation of ice in the extracellular compartments of their body tissues (Sinclair and Renault, 2010). However, most insects are chill susceptible, meaning that they can only withstand moderately low temperatures above their freezing temperatures, and are injured or even killed by processes unrelated to freezing (Lee, 1991).

1.1.1 Chill coma Onset and Recovery

As a chill-susceptible insect cools, it reaches a critical thermal minimum (CTmin), a species-specific temperature at which the insect enters chill coma. Chill coma is a state characterized by a loss of muscle function and lack of response to external

stimuli (MacMillan and Sinclair, 2011; Mellanby, 1939). Exposure to a chill coma-inducing temperature for a brief period of time followed by rewarming usually results in recovery of locomotion and all other physiological functions of the insect (e.g. Gibert et al., 2001). The time taken for an individual to recover muscle function, chill coma recovery time (CCR), is often used as a proxy for the thermal tolerance of an insect (MacMillan and Sinclair, 2011; Gibert et al., 2001). However, a prolonged exposure to a chill coma-inducing temperature can result in irreversible physiological changes, and the accumulation of cellular and whole body damage (chilling injury), which may lead to sub-lethal fitness effects and death (Koštál et al., 2006).

1.1.2 Chilling Injury

Chilling injury in insects manifests itself as a departure from normal physiology or behaviour following prolonged exposure to chill coma-inducing temperatures. The short-term manifestation of chilling injury has been studied by assessing mortality or fitness proxies such as coordination, the ability of individuals to right themselves, and response to stimuli in individuals exposed to low temperatures in comparison to a control group (e.g. Koštál et al., 2004; Koštál et al., 2006; MacMillan and Sinclair, 2011). Koštál et al. (2006) identified defects in crawling, and uncoordinated movements as chilling injury in the tropical cockroach *Nauphoeta cinerea*. Cockroaches which showed rapid and coordinated crawling 48 h after exposure to 0 or 5 °C for up to 24 h were considered fit; cockroaches which moved in an uncoordinated manner were considered chilling-injured; and cockroaches which did not respond to any stimuli were considered dead. Other studies have focused on the long-term manifestation of chilling injury and its negative fitness impacts throughout the individual's lifetime and the carry-over effects

onto the next generation (e.g. Kayukawa and Ishikawa, 2005; Magiafoglou and Hoffmann, 2003; Yocum et al., 1994). For instance, Hutchinson and Bale (1994) found that increasing intensity of sublethal cold stress resulted in an increase in chilling injury that impaired development, reproductive capabilities and life expectancy of the aphid *Rhopalosiphum padi.*

Chilling injury in insects resulting from acute and chronic cold exposure can be classified as either direct or indirect (Lee, 1991; Sinclair and Roberts, 2005). Direct chilling injury is damage induced by short chilling episodes, often minutes to a few hours. Direct chilling injury is thought to result from cell membrane phase transitions from the liquid crystalline to the gel phase during rapid cooling which alter cell functioning (Chen et al., 1987; Drobnis et al., 1993; Quinn, 1985) and the induction of apoptosis (Yi et al., 2007). Indirect chilling injury occurs when exposure to low temperatures for longer periods of time, days to weeks, induces damage to the organism (Lee, 1991; Rojas and Leopold, 1996). Mismatching of metabolic pathways, oxidative stress and loss of ion homeostasis are proposed mechanisms of indirect chilling injury (Koštál et al., 2006; Rojas and Leopold, 1996; MacMillan and Sinclair, 2011a). The chilling injuries (whether direct or indirect), which affect the probability of an individual recovering, are dependent on the amount of time and exposure temperature. For example, in *Drosophila melanogaster* reared at 25 °C and exposed to sub-freezing temperatures of -2 °C, -3 °C, and -4 °C for up to 8 h, mortality increased as exposure time increased (MacDonald et al., 2004).

1.2 Plasticity of Cold Tolerance

1.2.1 Low Temperature Adaptation and Acclimatory Responses

Insects possess a diverse set of responses for dealing with thermal extremes, and these may act on different timescales, from long-term evolutionary adaptation to rapid phenotypic adjustment (Angilletta, 2009). On a long timescale, evolutionary diversification of cold tolerance has led to an increase in cold tolerance in temperate species or source populations in comparison to tropical ones (David et al. 2003). For example, in Australian fruit flies *Drosophila serrata* (Hallas et al., 2002) and *D. melanogaster* (Hoffmann et al., 2002b), chill-coma recovery time is inversely related to latitude. In some cases, a phenological shift without habitat or host change, has led to the establishment of different overwintering strategies in closely related species that overlap in distribution. For example, the fall and spring field crickets (*Gryllus pennsylvanicus* and *G. veletis*, respectively) are ecologically similar throughout their shared geographic range in Northeastern North America (Alexander and Bigelow, 1960). However, *G. pennsylvanicus* overwinters in the egg stage and matures in middle or late summer; late instar nymphs and adults are moderately chill susceptible. Conversely, *G. veletis* overwinters as late instar nymphs and matures in the early spring (Alexander and Bigelow, 1960).

On a shorter time scale than evolutionary adaptation, phenotypic plasticity is thought to facilitate the success of organisms in new environments and changing seasons (Ghalambor et al., 2007). Phenotypic plasticity can be defined as the ability of an organism with a given genotype to express different phenotypes in response to changes in environmental conditions (Garland and Kelly, 2006). Thermal acclimation is a form of phenotypic plasticity, where the response by an individual to a change in environmental

temperature leads to an alteration in its thermotolerance (Angilletta, 2009). Different forms of acclimatory responses exist, which can be rapid or gradual (e.g. RCH and seasonal acclimatization, respectively), and reversible (e.g. seasonal acclimatization) or irreversible (as in the case of developmental acclimation; Angilletta 2009; Chown and Nicolson 2004).

Developmental acclimation comprises irreversible changes in response to temperature conditions experienced during ontogeny. Organisms could respond to changes in temperature during one stage of their life cycle to enhance their fitness in a subsequent life stage, or parents could respond to changes in temperature experienced during their lifetime to enhance the performance of their offspring. In *Drosophila melanogaster,* parent flies reared and maintained at 29 °C produced offspring with higher average fitness (measured as per capita rate of population increase) than parents reared at 25 °C or 18 °C, independent of the thermal environment experienced by the offspring (Gilchrist and Huey, 2001). However, phenotypic plasticity may also have adverse effects on the fitness and survival of insects. For example, winter-acclimated prepupae of the emerald ash borer, *Agrilus planipennis,* reduce their cold tolerance in response to mid and late-winter warm periods. The loss of this winter acclimation response is not reversible and alters the ability of the insect to survive subsequent cold exposures likely to be experienced in nature (Sobek-Swant et al., 2012).

Reversible acclimation refers to responses induced by changing environmental temperatures that can be reversed given enough time. These responses can be induced in the laboratory, and also occur in the field in response to diel or seasonal changes (Angilletta, 2009). A brief exposure to extreme cold can often cause a change in

thermotolerance within hours (Sinclair and Roberts, 2005); a phenomenon referred to as rapid cold hardening (RCH). This change in thermotolerance is often lost within a matter of hours or days after the insect is removed from the temperature pre-treatment (Angilletta, 2009). Rapid cold hardening has been observed in several insect species from different orders such as Collembola (e.g., Worland and Convey, 2001), Coleoptera (e.g., Burks and Hagstrum, 1999), Diptera (e.g., et al., 1987, Czajka and Lee, 1990), Hemiptera (e.g., Powell and Bale, 2004), and Lepidoptera (e.g., Larsen and Lee, 1994). In *D. melanogaster,* RCH can impart increased tolerance to subsequent acute and chronic cold exposures, in spite of the different kinds of damage caused by these two stressors (Rajamohan and Sinclair, 2008). The physiological mechanisms underlying RCH are associated with the induction of the heat shock response (Colinet et al., 2009), and may be associated with the accumulation of a cryoprotectant such as glycerol and other low molecular weight polyhydric alcohols and sugars (Chen et al., 1987; Misener et al. 2001; Chen and Walker, 1994). Yi et al. (2007) have shown RCH may also be associated with a blocking of apoptosis-related pathways; this may explain why RCH can affect both acute and chronic cold tolerance (Rajamohan and Sinclair, 2008).

In contrast to RCH, long-term acclimation is a programmed set of responses to a prolonged, and often predictable, environmental cue (such as slowly declining temperatures or changes in photoperiod) that can trigger lasting effects in thermosensitivity (Angilletta, 2009). Acclimation of physiological capacities has been usually thought of as beneficial because it may compensate for a potentially negative influence of an environment change on a fitness-related performance trait. However, there has been considerable discussion in the literature about whether acclimation is

beneficial or not (see Chown and Nicolson, 2004 and Angilletta, 2009 for discussion). The importance of long-term acclimation is that the physiological changes can be established within days to weeks, which permits organisms to maintain fitness in environments that vary within generations (Tattersall et al., 2012). The physiological mechanisms underlying low temperature acclimation in insects include changes in sugar or polyol concentrations (Hendrix and Salvucci, 1998), as well as changes in membrane lipid composition (Hazel, 1995; Ohtsu et al. 1999).

1.3 Physiological processes underlying chill coma and chilling injury

1.3.1 Cellular Ion Homeostasis and Muscle Resting Potentials

Excitability of muscle cells and neurons at low temperatures, which dictates the thermal limits of activities such as foraging, mating, and predator avoidance, relies on the constant management of ion concentrations outside and inside cells (Koštál et al., 2004). At the cellular level, ion homeostasis is regulated through ATP-dependent ion pumps that work in synchrony with ion channels and secondary transporters to maintain intracellular and extracellular ion gradients within strict limits (Emery *et al*., 1988).

The lipid bilayer of biological membranes is largely impermeable to ions and other polar molecules, yet certain species of these molecules must cross these membranes in order for the cell to function properly. The bulk passive movement of ions down their concentration gradient through the membrane is facilitated by selective and non-selective ion channels. In insects, the resting membrane potential of muscle fibre membranes depends primarily on the concentration gradient and the relative permeability of the membrane to K^+ ions (Hoyle, 1953; Wood, 1962). In the resting state, the concentration of K^+ ions inside excitable cells is high and Na^+ concentration is low compared to the

extracellular space. Additionally, the permeability of the membrane to K^+ is relatively high (K^+ voltage-gated channels are open), while Na^+ permeablility is low (Na^+ voltage-gated channels are closed). The diffusion of K^+ ions across the membrane into the extracellular space (due to high membrane permeability) results in a local region of electronegativity on the interior side of the plasma membrane with respect to the exterior. This excess negative charge on the inside of the membrane creates an electrical force that tends to draw positive charges back into the cell. Thus, the driving force for the movement of ions across the membrane originates from the ion and electrical gradients themselves, which are originally established by the Na^+/K^+-ATPase pump (Moyes and Schulte, 2006).

The Na^+/K^+-ATPase pump actively transports Na^+ out of the cell and K^+ into the cell. The active transport of Na^+ and K^+ is of great physiological significance, as the Na^+/K^+ ion gradient controls cell volume, drives the secondary active transport of sugars and amino acids, and renders neurons and muscle cells electrically excitable (i.e. capable of generating and propagating nerve and muscle action potentials) (Hosler et al., 2000). Other ATPase pumps that are contributors to the establishment of ion gradients and the resting membrane potential are the Ca^{2+}-ATPase, which transports Ca^{2+} out of the cytoplasm and into the sarcoplasmic reticulum of muscle cells, and the vacuolar-type H^+-ATPase, which pumps H^+ into the gut lumen. A K^+/H^+ antiporter then uses the electrochemical proton gradient to drive secondary K^+ secretion (Wieczorek et al., 1991). The actual resting membrane potential then, is the result of passive and active forces working in synchrony: first and primarily, the diffusion of ions down their electrochemical gradient; second, the ATPase ion dependent pumps themselves.

1.3.2 Whole Body Ion Homeostasis

At the organismal level, ion homeostasis in most insects is managed by the interaction of the Malpighian tubules and the gut, particularly the hindgut (Zeiske, 1992; Djamgoz, 1987). The function of the hindgut is analogous to that of the vertebrate nephron and colon; it modifies the ion and water content of egesta. The Malpighian tubules, blind-ended, slender tube-like structures that extend from the midgut-hindgut junction and lie free in the hemolymph, are responsible for the excretion of waste and also aid in osmoregulation by removing excess ions and water from the insects' body (Harrison et al., 2012). Typically, the Malpighian tubules produce primary urine that is isosmotic with the hemolymph by actively transporting K^+, Cl^- and Na^+ transcellularly from the hemolymph into the lumen. This process maintains a gradient favouring the movement of water (through aquaporins), and harmful organic molecules (through selective transporters) into the lumen (Ramsay, 1954; Pannabecker, 1995; Spring et al., 2009). Most of the secreted fluid in the Malpighian tubule lumen moves posteriorly to the hindgut, particularly the rectum, were selective reabsorption of water, ions and nutrients back into the hemolymph takes place (MacMillan and Sinclair, 2011a; Harrison et al., 2012).

Advances in the understanding of transport function in insect Malpighian tubules (particularly in Orthoptera) have come from the locusts *Locusta migratoria* and *Schistocerca gregaria* (Phillips et al., 1981). A number of generalisations have been made about the mechanisms whereby the ionic content of the secreted fluid is regulated in Malpighian tubules. More recently, the chill susceptible fall field cricket, *Gryllus pennsylvanicus*, has been used as a model to characterize the loss of and return to osmotic

homeostasis following entry into and recovery from chill coma (see MacMillan and Sinclair, 2011a; MacMillan et al., 2012).

The extent to which insects depend on different ion pumps, selective and non-selective ion channels, symporters and antiporters, and the relative contribution of major cations to whole body ion homeostasis can vary greatly among taxa (Zeiske, 1992). For example, while some insects maintain high concentrations of Na^+ in their hemolymph in comparison to the lumen, other insects, such as the ribbed pine borer beetle *Rhagium inquisitor,* and the caterpillar of the moth *Manduca sexta,* have high hemolymph Mg^{2+} and K^+ concentrations instead, and ion regulation is driven by the activity of Mg^{2+} and H^+ ATPase pumps (Dow, 1986). In terrestrial plant feeding insects (particularly locusts), the primary active force that transports ions, and thus water, from the hindgut to the hemolymph is powered by the P-type Na^+/K^+ ATPase and an electrogenic Cl^- pump (Harrison et al., 2012). Yet in other species, particularly terrestrial blood feeding insects such as the yellow fever mosquito, *Aedes aegypti*, Na^+ and K^+ are thought to be excreted from the tubule cell via K^+/H^+ or Na^+/H^+ antiporter systems driven by the proton gradient generated by an electrogenic V- type H+ ATPase (Harvey and Wieczorek, 1997; Harvey et al., 1998). Hence, care and consideration should be taken when assessing the role and contribution of each ATP-dependent ion pump, ion channel, and the contribution of individual cations to insect whole body ion homeostasis.

1.4 The Onset of Chill-coma and loss of Ion Homeostasis

When insects are exposed to chill coma-inducing temperatures, postsynaptic potential increases in duration and decreases in amplitude (Esch, 1988; Goller and Esch, 1990), and muscle and nerve cells experience a progressive decline in resting membrane

potentials (Hosler et al., 1999; Heitler et al., 1977; Kivivouri et al., 1990; Xu and Robertson, 1994). Once the muscle depolarizes beyond a threshold potential, fibres seem unable to restore their resting membrane potential, rendering the cells unexcitable. After the initial generation of action potentials, no subsequent action potentials are generated, contraction is no longer possible and the insect is paralyzed (MacMillan and Sinclair, 2011a). In *Gryllus pennsylvanicus,* the progressive decline in muscle resting membrane potentials characteristic of chill coma on-set is due to the loss of whole-body ion homeostasis. The dissipation of membrane potentials and loss of muscle excitability is driven partly by a migration of the majority of hemolymph Na^+, Mg^{2+}, Ca^{2+} and water into the alimentary canal of the insect resulting from an inability to maintain whole body ion homeostasis while exposed to low temperature (MacMillan and Sinclair, 2011a). These movements of ions and water into the gut cause hemolymph Na^+ and Ca^{2+} concentrations to decrease and hemolymph K^+ concentration to increase. In turn, these changes in hemolymph ion concentrations drive decreases in K^+ and Ca^{2+} equilibrium potentials at the muscle tissue.

The onset of chill-coma is probably caused by electrophysiological failure of the neuromuscular system caused by the loss of ion homeostasis resulting from these changes in hemolymph ion concentrations (MacMillan and Sinclair, 2011b; Figure 1.1). Although the active transport of ions by ATPase ion pumps is an enzymatic process directly influenced by temperature (Koštál et al., 2006), passive diffusion of ions through the membrane is largely temperature-independent (Zachariassen et al. 2004). Hence, during exposure to low temperature, the activity of the ATPase ion pumps is expected to be reduced more so than the passive diffusion of ions through the membrane. The difference

in reduction of ATPase ion transport activity vs. the passive diffusion and leakage of ions through the gut epithelium in response to low temperature exposure likely plays a major role leading to the disruption water and ion homeostasis in an insect.

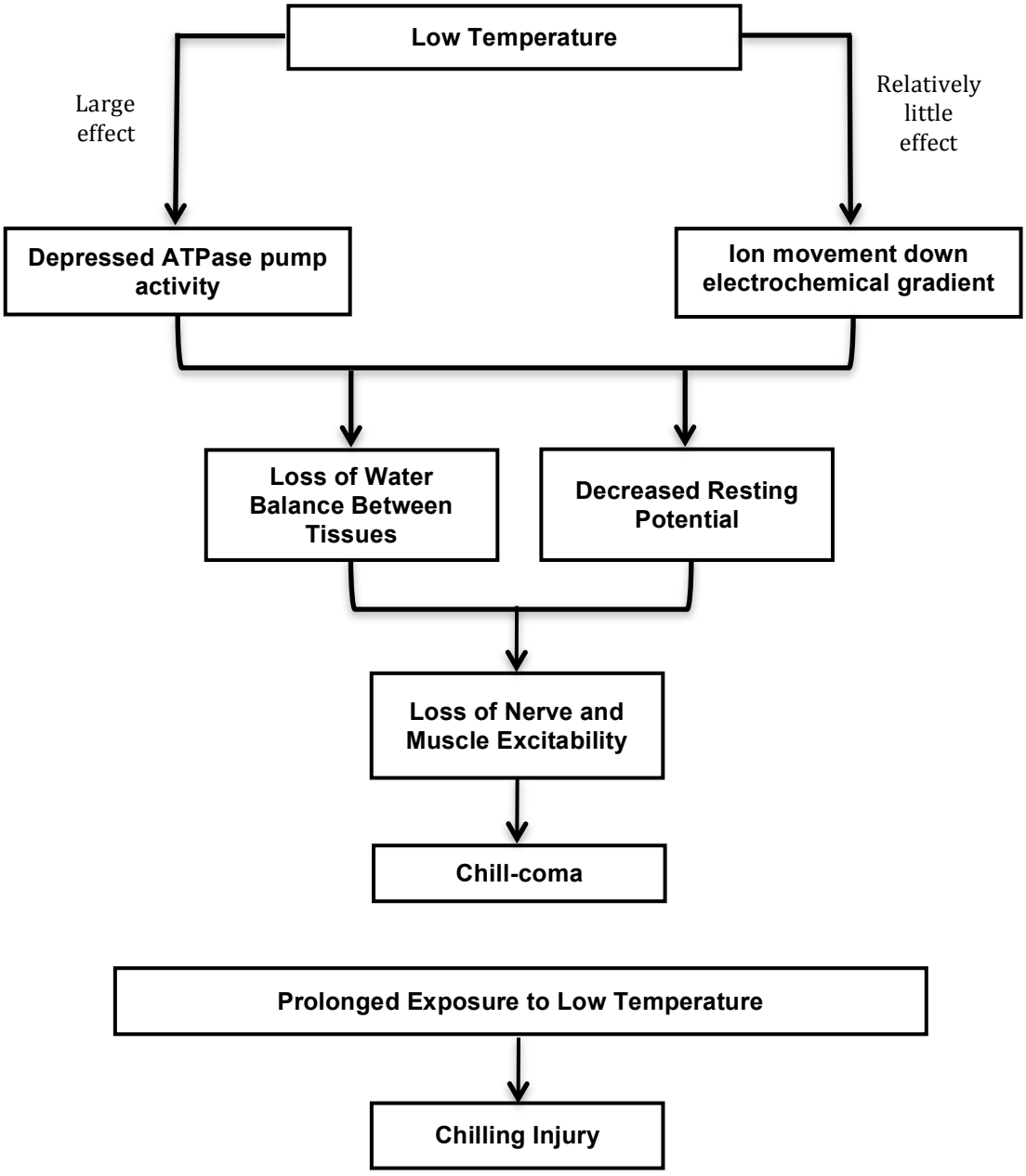

Fig 1.1 Schematic of physiological mechanisms though to underlie the neuromuscular transmission failure thought to underlie chill coma and the progression into chilling injury.

1.5 Chill coma Recovery and Chilling Injury

It is generally assumed that recovery from chill coma (CCR) is the reversal of the mechanisms resulting in the onset of chill coma (MacMillan and Sinclair, 2011b). However, biphasic patterns have been observed in chill-coma recovery time of *Gryllus pennsylvanicus* in response to increasing cold exposure (MacMillan et al., 2012). Similar biphasic patterns have been observed in CCR time of *Drosophila* exposed to varying temperatures (David et al., 2003; MacDonald et al., 2004). This biphasic response in CCR time suggests at least two underlying mechanisms are acting during chill coma recovery. It seems that following entrance into chill coma, a chill susceptible insect that is held at that chill coma-inducing temperature beyond a species-specific threshold will start to develop chilling injury.

When *Gryllus pennsylvanicus* recovers from chill coma, hemolymph Na^+ and K^+ concentrations return to control levels. Restoration of muscle K^+ equilibrium potential coincides with the average time required for crickets to recover movement. Throughout recovery, hemolymph volume and Na^+ content closely follows the decrease of gut water content, suggesting recovery of hemolymph involves the active movement of Na^+, and water, from the gut back into the hemocoel. The decline in hemolymph K^+ concentration observed during recovery is consistent with an increase in hemolymph volume while keeping a constant K^+ content. Thus, it appears that the re-establishment of K^+ homeostasis in *Gryllus pennsylvanicus* is largely driven by the redistribution of Na^+ and water from the gut lumen into the hemocoel (MacMillan et al., 2012).

1.5.1 Effect of Acclimation on Chill coma

Plasticity of chill coma, to some extent, has been demonstrated over a broad

2001; Chen and Walker, 1994). Provided the model of chill coma on-set and progression into chilling injury is correct (from Macmillan and Sinclair, 2011a; Figure 1.1), a shift in one trait and in its underlying mechanism, should influence all other traits as well. Hence, by looking at how ion and water homeostasis vary with variation in cold tolerance, I will determine the specific physiological changes result in enhanced cold tolerance. There are several possible ways an exposure to low temperature could affect the proposed mechanism of chill coma onset that would result in a more cold-hardy phenotype: 1) changes in the resting membrane potential Na^+ and K^+ concentrations necessary for ion homeostasis such that it takes a longer time to reach the threshold potential for loss of nerve and muscle function, 2) changes in membrane fluidity that affect the rate of cellular or whole body ion and water movement thus changing the rate of loss of ion homeostasis, or 3) changes in the rate of activity of ATPases that enhance their regulatory capacity and would change the threshold temperature at which loss of ion homeostasis begins. Any one or a combination of these changes could result in a phenotype that is less susceptible to the onset of chill coma and development of chilling injuries.

Objectives

In this study, short-term (SE) and long-term (LE) exposure to cold were used as treatments to induce plasticity (RCH and acclimation, respectively) in the cold tolerance of *Gryllus pennsylvanicus*, while the naturally higher cold tolerance of *Gryllus veletis* was used as additional comparative group. The different *G. pennsylvanicus* phenotypes resulting from these cold exposures, and *Gryllus veletis* were used to examine variation in low temperature performance and ion homeostasis. Given that CTmin, CCR, and survival after cold exposure are traits influenced by ion and water homeostasis, whose

mechanisms are thought to be partially coupled; a shift in one trait, which implies a shift in the mechanism underlying that trait, should influence all other traits as well. I will examine the following hypotheses and predictions:

1) **Low temperature acclimation and adaptation results in physiological changes that enhance performance at low temperatures**. I predict that a treatment that enhances cold tolerance should result in individuals with lower thermal limits, faster recovery times from chill coma, and higher rates of survival after cold exposures.

2) **Changes that increase performance at low temperature in response to low temperature acclimation and adaptation are associated with an enhanced ability to maintain ion homeostasis.** Consequently, I predict that treatment groups that perform better at low temperatures will be able to prevent or mitigate the migration of hemolymph Na^+ and water into their digestive system.

CHAPTER 2: METHODS

2.1 Rearing

I used a population of *Gryllus pennsylvanicus* derived from individuals collected from the University of Toronto Mississauga campus during 2004, and *Gryllus veletis,* derived from individuals collected from a wild population from the grounds around the campus of the University of Lethbridge, Alberta during late June of 2010 for my experiments. I reared crickets in 15L clear plastic bins with mesh covered lids until early into their last nymph instar stage, at which point I transferred them to 60L bins (600-1000 crickets/bin). This ensured there was no overcrowding, one of the main causes of death in cricket colonies (Kevin A. Judge personal communication). I maintained crickets under constant summer conditions (25 °C, 14L: 10D photoperiod, ~ 70% RH). I provided crickets with water and rabbit food pellets (Little Friends Rabbit Food, Martin Mills Inc., Elmira, ON, Canada) *ad libitum,* and 6-7 stacked cardboard egg cartons as shelter.

I gave ten- to twelve-week old adults access to 500 mL containers filled with a 4:1 mixture of fine vermiculite and sand for two weeks to lay eggs. Afterwards, I transferred the containers used to collect *Gryllus pennsylvanicus* eggs to 4 °C for three months to accommodate an obligate diapause and later returned the eggs to 25 °C to complete development. The containers used to collect *Gryllus veletis* eggs were left at 25 °C, as they do not require chilling to complete development. I used adult *Gryllus pennsylvanicus* males, approximately 3 weeks post final molt, and late instar *Gryllus veletis* juveniles, at least 180 mg in body mass, for all experiments. I differentiated adult *Gryllus pennsylvanicus* males from females and male juveniles by the lack of an ovipositor and the presence of wings respectively. I differentiated late instar *Gryllus*

veletis juveniles from females and male adults by the lack of an ovipositor and the presence of wing pads that had yet to develop into adult wings respectively.

2.2 Cold Exposure Treatments

Prior to being used in experiments, I transferred all *Gryllus pennsylvanicus* individuals from their rearing bin into individual 177 mL translucent cups (Polar Plastics, Summit Food Distributors Inc., London, ON, Canada) and provided food, water and a piece of egg carton as shelter to them. I covered each cup with mesh fabric and secured it with a rubber band, which allowed for ventilation. The individuals I subjected to a long-term exposure (LE) were cooled in an incubator (Sanyo MIR 154, Sanyo Scientific, Bensenville, Illinois) from 25 °C to 12 °C at 2.5 °C day^{-1} and kept at 12 °C with a 10L: 14D photoperiod for the subsequent three weeks. The individuals I subjected to a short-term exposure (SE) were kept under rearing conditions for a month. On the first day of experiments, I transferred individuals from 25 °C to 4 °C for four hours and gave them one hour to recover at 25 °C prior to using them in experiments. I kept *Gryllus veletis* and *G. pennsylvanicus* control crickets under summer rearing conditions for the duration of the acclimation period (Fig 2.1).

2.3 Cold Tolerance Measurements

2.3.1 Determination of Critical Thermal Minimum

I placed twelve crickets individually into covered 200 mL glass beakers jacketed in an insulated acrylic enclosure through which an ethylene glycol:water mix (1:1 v:v) was circulated from a programmable refrigerated bath (Model 1157P, VWR International, Mississauga, ON, Canada). I monitored the temperature inside each well with a type-T thermocouple connected to a computer *via* a Picotech TC-08 thermocouple

interface and PicoLog software (Pico Technology, Cambridge, UK). The chambers were cooled linearly from the rearing, recovery or acclimation treatment temperature at 0.25°C min^{-1} while I observed crickets continuously. The CTmin was the temperature at which the insect was no longer able to move, and failed to respond to being stimulated with a plastic probe.

2.3.2 Chill coma recovery time from an acute cold exposure

When I assessed chill coma recovery time, I placed twelve individuals into individual 14 mL polypropylene falcon tubes loosely covered with their lids (to allow air flow). I then cooled the crickets (Tenney ETCU Series Chamber, Thermal Product Solutions, White Deer, PA, USA) from their rearing, recovery or acclimation temperature to 0 °C at 0.25 °C min^{-1} and held at 0 °C (a temperature that would induce chill coma, but not ice formation) for 12 h. When I took the crickets out of the chamber, I laid them on their backs on a petri dish and recorded the time of 1) their first abdominal movement (an indication of the ability of nerves to generate action potentials or muscles to accomplish contractions) (MacMillan et al., 2012) and 2) the recovery of their righting response (a behaviour which requires coordinated movement; after David et al., 1998).

2.3.3 Survival assessment after chronic cold exposure

To assess survival, I placed crickets into individual fourteen mL falcon tubes, which were loosely covered (to allow air flow), were cooled from their rearing, recovery or acclimation temperature to 0 °C at 0.25 °C min^{-1} and held at 0 °C for up to 5 days. Every 12 h, I took a subset of crickets out of the chamber, transferred them back to a cup, gave them food and water, and left them to recover at 25 °C. I carried out the survival assessments 24 h after I took the individuals out of the cold. When I assessed survival, I

noted chilling injury. I categorized individuals as fit if they walked in a coordinated fashion and were able to perform a jump, injured if they walked in an uncoordinated fashion and were unable to perform a jump (regardless of the severity of injury), or dead if they failed to respond to any stimuli (After Koštál et al., 2006.) When I compared differences in survival among groups, I categorized individuals as alive (fit + injured) and dead.

2.4 Ion and Water Distribution in Gut and Muscle Tissues, and Hemolymph

2.4.1 Sampling Regime

I placed individuals into loosely-capped fourteen mL polypropylene falcon tubes, cooled them from their rearing, recovery or acclimation temperature and held them at 0 °C for up to 5 days. Every 12 to 24 h, I took a subset of individuals out of the cold to dissect their foregut, midgut, hindgut and muscle tissue, and extract hemolymph (Figure 2.1). With the exception of time 0 dissections, which I performed at room temperature (24 °C), I conducted all other dissections in a cold room at 4 °C.

2.4.2 Tissue Sampling

I made an incision at the joint of the hind leg and abdomen using microscissors and extracted as much hemolymph as possible using a 20 µL pipette. I removed the hind legs using microscissors and collected muscle tissue from both hind legs using fine forceps to gently squeeze and separate the tissue from the hind leg exoskeleton. I used microscissors, while being careful not to rupture any gut sections or the esophagus, to make a dorsal incision from the tip of the abdomen to the back of the head. I removed the foregut, midgut, and hindgut using fine forceps to clamp the ends of each gut section, thus ensuring there was no mixing of the gut content within the sections. I gently blotted

all tissues on a Kimwipe to remove residual hemolymph before storing samples at -20 °C in preweighed 200 µL PCR tubes.

2.4.3 Ion and water content analyses

I determined tissue wet mass by using a microbalance (Model MX-5, Mettler Toledo, Greifensee, Switzerland). Afterwards, I dried the tissues in a 70 °C oven for 48 h, and reweighed them to obtain their dry mass. I followed this with the addition of 200 µL of concentrated nitric acid to each PCR tube containing the dry tissue samples, and vortexed the tubes 2-3 times (to ensure the tissues were completely dissolved) during the subsequent 24 h, while leaving the samples at room temperature. I stored the tissues in a -20 °C freezer until used in analyses.

I diluted the tissue samples stored in concentrated nitric acid with double-distilled H_2O in 20 mL glass test tubes to bring them within the measurable range of the atomic absorption spectrometer (AAS; Model iCE 3300, Thermo Scientific, Waltham MA, USA). For K^+ measurements, I did a 1:50 dilution of sample stored in concentrated nitric acid to double-distilled water. I used a known volume of this 1:50 dilution to make an additional 1:250 dilution of sample stored in concentrated nitric acid to double-distilled water for the Na^+ measurements. These dilutions were derived from previous work on female *Gryllus pennsylvanicus* (Macmillan and Sinclair 2011a). I ran each of these Na^+ and K^+ diluted samples in triplicate through the AAS, measuring absorbance at 589.0 nm and 766.5 nm for Na^+ and K^+ respectively. The three absorption values obtained for each Na^+ and K^+ diluted sample were averaged and used to calculate the ion concentration and content for each individual.

I generated standard curves of Na^+ and K^+ from diluted standards containing nitric acid (after Macmillan and Sinclair 2011a). The inclusion of nitric acid in the standard curve dilutions accounted for any effects nitric acid might have had on the absorbance values. I used a 2% concentrated nitric acid solution to make my potassium standard curve and a 0.4% concentrated nitric acid solution to make my sodium standard curve. I accounted for any variation in absorbance measurements caused by instrument drift by multiplying the absorbance values obtained from the AAS by a correction factor. This correction factor was the mean absorbance value of a 1ppm of Na^+ and K^+ solution in my standard curve divided by the mean absorbance value of a 1 ppm Na^+ and K^+ solution run as quality control prior to every sample run. I used these corrected absorbance values to calculate Na^+ and K^+ ion concentration and content values for each ion tissue sample using Beer-Lambert's Law:

$$A = \varepsilon l c$$

where A is absorbance of the sample, ε is the molar absorption coefficient, l is the path length of the samples, and c is the concentration of the ion in solution. Given the volume of double-distilled H_2O the samples stored in concentrated nitric acid were diluted in and the water content of each tissue and hemolymph volume for each cricket was known, I was able to calculate the concentration and content of Na^+ and K^+ for each tissue for every individual.

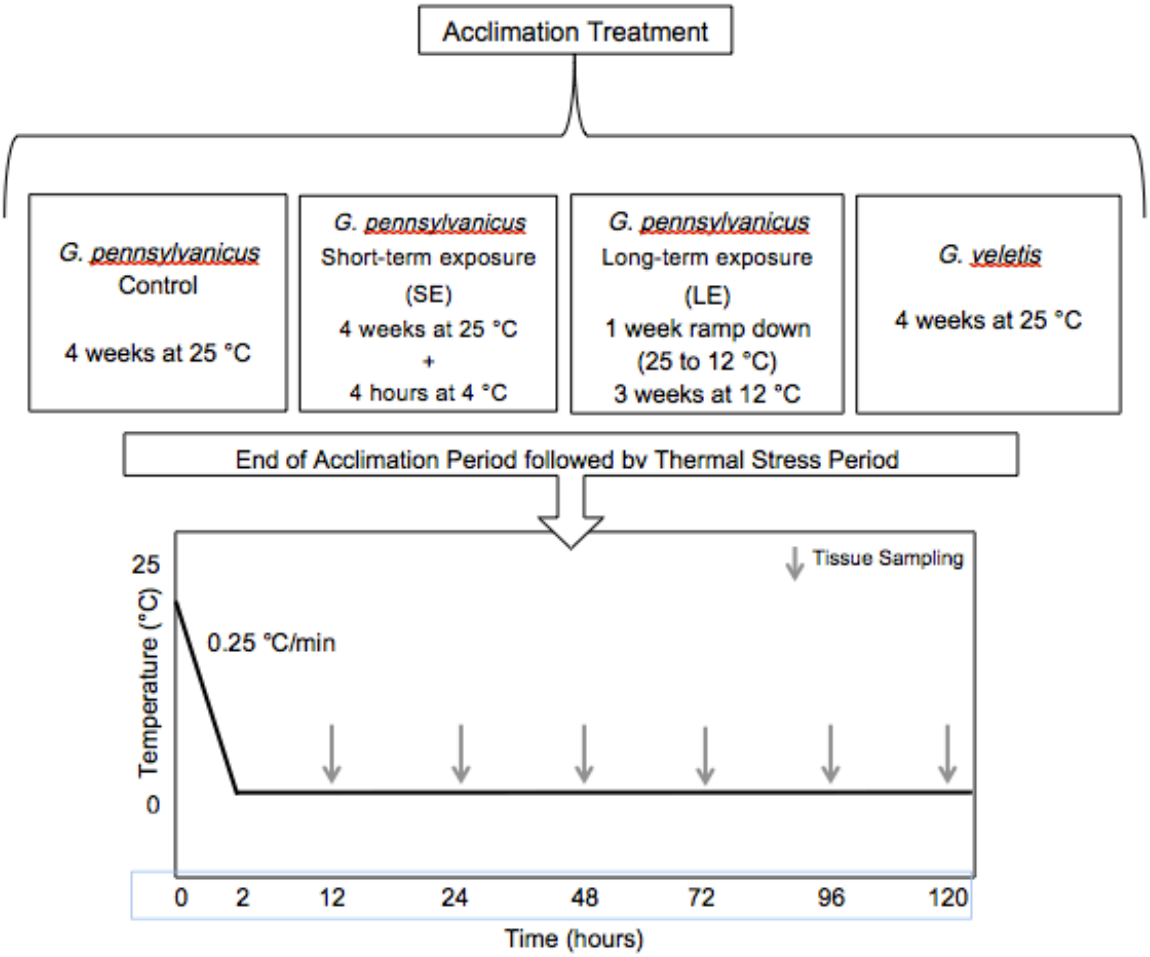

Fig 2.1 Experimental design for acclimation treatment and sampling regime used in the collection of survival, water content and ion distribution analyses. Individuals were acclimated for a four week period (including ramp down) prior to being exposed to a thermal stress at 0 °C for up to 120 h (5 days). Survival was assessed every 12 h. Hemolymph, muscle, and gut tissue samples were collected after 12, 24, 48, 72, 96, and 120 h of exposure to 0 °C.

2.5 Statistical Analyses

Critical thermal minima and chill coma recovery times were compared among species and treatments using Sigma Plot (v12.2, San Jose, CA, USA). There was no significant effect of body size on CTmin or CCR measurements among the treatment groups, so I compared CTmin and CCR among groups using a one-way ANOVA followed by a Tukey's *post hoc* test. Data are reported as mean ± S.E.M. Differences in chill coma recovery were analyzed in SPSS (v21.0. Armonk, NY: USA) using Log Rank survival curve analyses followed by a Holm-Sidak multiple comparison *post hoc* test. Survival data were analyzed in SPSS using a Binary Logistic Regression.

Simple linear regressions in R (R Development Core Team, 2010) were used to determine the effects of wet body mass and dry tissue mass on hemolymph, muscle and gut water content, and ion content. In all cases, body and dry tissue mass had a significant effect on water content and ion content that obscured differences among treatment groups. Hence, with the exception of hemolymph volume and ion concentrations, subsequent comparisons across time and among groups on the residuals of the linear regression (corrected for dry or body mass accordingly) of water content and ion content in muscle and gut tissue. Hemolymph volume was square-root transformed prior to analysis to improve normality. The equilibrium potentials (E) across the muscle cell membranes were calculated for each ion and each individual using the Nernst equation:

$$E = (RT/zF) \ln (Co/Ci)$$

where R is the gas constant, T is the absolute temperature, z is the charge of the ion, F is the Faraday constant, Co is the outer (hemolymph) and Ci is the inner (muscle)

concentration of the ion, respectively (Moyes and Schulte, 2006).

Generalized linear models built in R were used to determine the effect of time and acclimation treatment on residual water content, residual ion content and concentration in hemolymph, muscle and gut tissue between time point 0-12, and 12-120 h, and equilibrium potentials. This approach was taken because clear differences were observed in the direction and rate of change of most response variables between the first 12 h and the prolonged cold exposure. A model simplification approach was used, beginning with the saturated model including time, group and their interaction (Crawley, 2005). Model terms were retained on the basis of Akaike's Information Criterion until minimal adequate model was reached (Crawley, 2005). Tukey's HSD *post hoc* tests were used to identify statistically significant differences among the different treatment groups. Given *G. veletis* and *G. pennsylvanius* are different species, if a difference across time or groups was found between *G. pennsylvanicus* control and *G. veletis* individuals, the effect of time and treatment on *G. veletis* was evaluated separately.

CHAPTER 3: RESULTS

3.1 Critical Thermal Minimum and Chill coma Recovery Time

G*ryllus pennsylvanicus* LE individuals had the lowest CTmin, followed by the SE and control individuals (Figure 3.1). LE and SE individuals had a significantly lower CTmin than control individuals ($F_{3,48}$= 54.62, P< 0.001). However, CTmin of SE and LE individuals did not differ significantly (P= 0.149). *Gryllus veletis* had a significantly lower CTmin than all of the *G. pennsylvanicus* treatment groups (P<0.001).

Gryllus pennsylvanicus control individuals took significantly longer to recover from chill coma (12 hr exposure to 0 °C), in both the time taken to restore abdominal contractions (Log-rank statistic= 48.146, df=3 P<0.001; Figure 3.2) and time taken for individuals to right themselves, in comparison to SE, LE and *G. veletis* (Log-rank statistic=79.163, df=3 P< 0.001). The short-term exposure treatment and LE individuals took 6 to 4 fewer minutes to contract their abdomen after being transferred to room temperature (22-24 °C) than control individuals. Similarly, SE and LE individuals took 30-34 fewer minutes to right themselves from their back onto their abdomen than control individuals, which took close to an hour to recover. *Gryllus veletis* individuals were contracting their abdomen and moving their limbs while still in the falcon tubes at 0 °C. All *G. veletis* individuals righted themselves within 3 minutes of being removed from the cold.

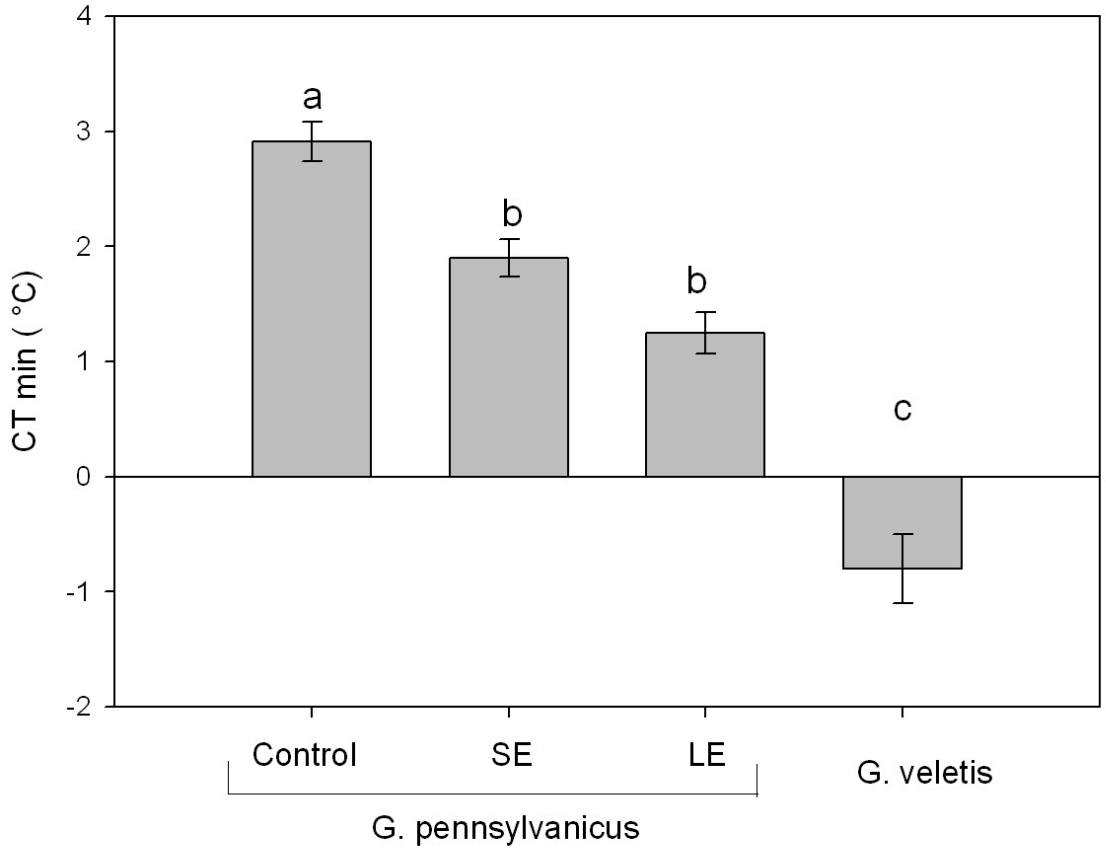

Figure 3.1 Mean ± S.E.M critical thermal minimum (CTmin) of *G. pennsylvanicus* treatment groups and *G. veletis*. N=12 for each treatment. Different letters denote a significant difference in CTmin among treatments (Tukey's HSD, $P<0.05$).

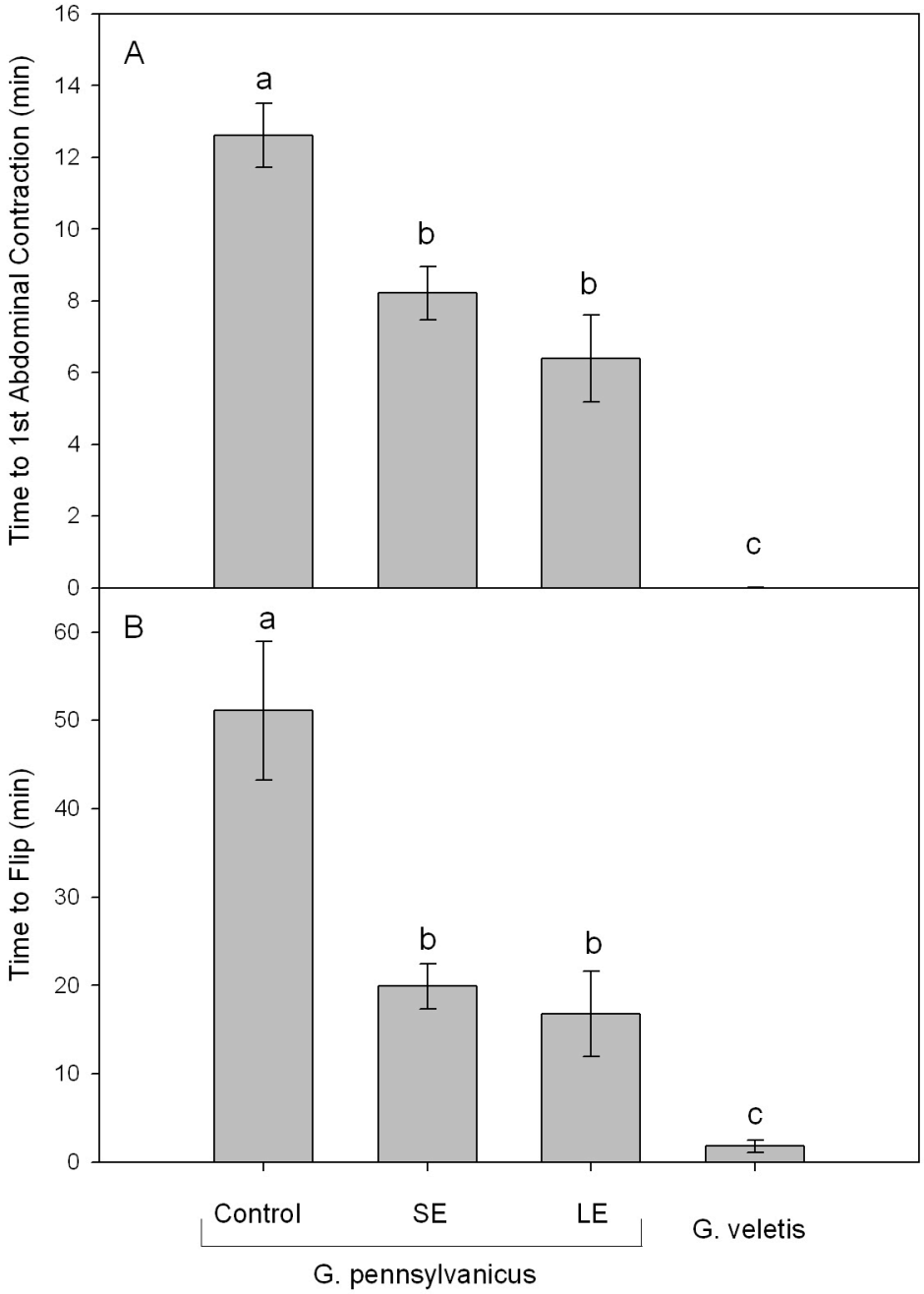

Figure 3.2 Mean ± S.E.M chill coma recovery time (CCR) of *G. pennsylvanicus* and *G. veletis* after a 12 hour exposure to 0 °C. CCR was measured as the time it took for individuals to A) be able to contract their abdomen and B) right themselves after being lip placed on their backs. N=12 for each treatment. Different letters denote a significant difference in CCR amongst treatments (Tukey's HSD, $P<0.05$).

3.2 Survival after chronic cold exposure

Exposure treatments and time exposed to cold had a significant effect on mortality (Wald χ^2: 18.882, df= 2, P<0.001 and Wald χ^2: 54.928, df= 1, P<0.001 respectively). Mortality was observed in *Gryllus pennsylvanicus* individuals that had been exposed to 0 °C for as little as 12 h (Figure 3.3). Control and SE crickets had similar proportions of dead individuals over time (P=0.340); mortality started around 36 - 48 h. This was followed by a slight increase in mortality between 36-48 h and 60 h, and a sharper increase in mortality between time points 72 and 96 h. After 96 h of exposure to 0 °C, control individuals experienced a steady increase in mortality, and only one control individual survived a 120 h exposure to 0 °C. Conversely, the proportion of SE individuals that experienced mortality decreased between time point 80 and 108 h. However, after a 120 h exposure to 0 °C, SE individuals also had a high proportion of dead individuals (7 out of 10). LE individuals started dying earlier than control and SE individuals, yet the proportion of dead LE individuals never rose above 0.5. Mortality of LE individuals fluctuated between 0.1 – 0.4 throughout the duration of the experiment; a significantly different trend than the SE and control individuals (P<0.001). After a 120 h of exposure to 0 °C, only 3 out of 10 LE individuals were dead. *Gryllus veletis* individuals did not exhibit chilling injury or mortality throughout the entire 120 hour exposure to 0 °C (Figure 3.3).

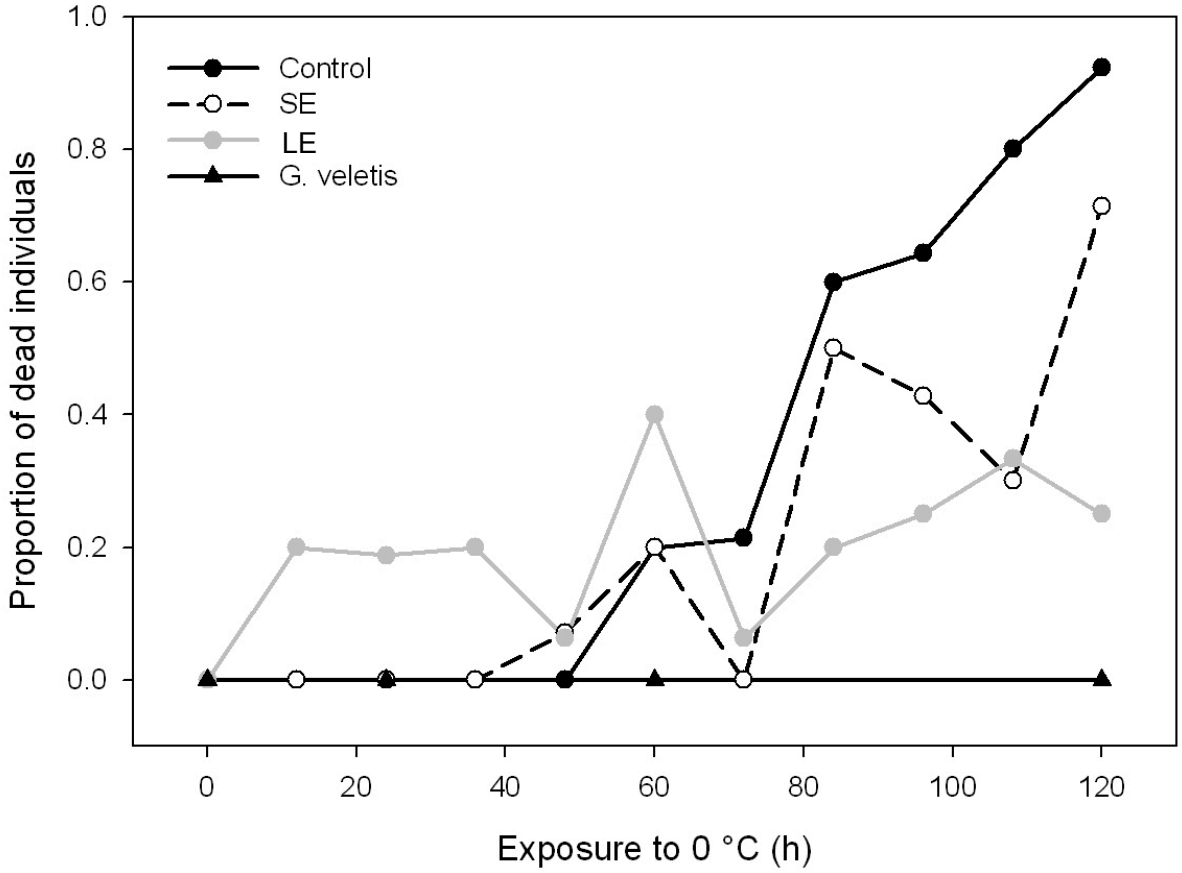

Figure 3.3 Mortality of *Gryllus pennsylvanicus* and *Gryllus veletis* 24 h after removal from exposure to 0 °C for up to 120 h. Survival data are presented as a proportion of dead individuals. N=10-14 crickets at each time point for *G. pennsylvanicus.* N=10 crickets at time point 0, 12, 24, 60 and 120h for *G. veletis*.

3.3 Hemolymph Volume and Tissue Water Content

3.3.1 Hemolymph Volume and Body Mass

Hemolymph volume decreased significantly with increasing time at 0 °C in all *G. pennsylvanicus* treatment groups ($F_{1,120}$=98.637, P<0.001; Figure 3.4). The decreases in hemolymph volume over the entire duration of cold exposure was smaller in LE individuals in comparison to control and SE individuals ($F_{2,120}$=13.713, P<0.001). *Gryllus veletis* individuals had significantly less hemolymph at the start of the cold exposure in comparison to *G. pennsylvanicus* control individuals ($F_{1,67}$=101.988, P<0.001), and their hemolymph volume did not significantly change with increasing time exposed to 0 °C ($F_{1,28}$=0.4751, P=0.496). There was a significant positive relationship between body mass and hemolymph volume in *G. pennsylvanicus* ($F_{1,120}$=19.408, P<0.001). Throughout a 120 h exposure at 0 °C, all *G. pennsylvanicus* treatments lost a similar amount (4%) of body mass ($F_{2,59}$=1.632, P=0.204). *Gryllus veletis* lost significantly less body mass (2%) than all *G. pennsylvanicus* treatments throughout the cold exposure ($F_{3,69}$=7.41, P<0.001). None of the *G. pennsylvanicus* treatments (t_{118}=1.32, P=0.126) or *G. veletis* (t_{18}=0.264, P=0.146) lost a significant amount of body mass with increasing time spent at 0 °C (t_{118}=1.32, P=0.126).

3.3.2 Gut Water Content

Gut water content significantly increased in all *G. pennsylvanicus* treatment groups during the first 12 h of cold exposure ($F_{1,31}$=13.262, P<0.001) and remained unchanged through the subsequent 108 h of cold exposure ($F_{1,118}$=0.0573, P=0.8112; Figure 3.5). Gut water content was similar among all *G. pennsylvanicus* treatments throughout the first 12 h of cold exposure ($F_{2,31}$= 0.456, P=0.638). However, LE

individuals had significantly less water in their gut throughout the last 108 h of cold exposure in comparison to SE and Control individuals ($F_{2,118}$=7.1362, P<0.005). Control and SE individuals accumulated similar amounts of water in their gut through the last 108 h of cold exposure (P=0.485). *Gryllus veletis* gut water content did not change during the first 12 h of exposure ($F_{1,10}$=0.406, P=0.5381) but rose significantly throughout the subsequent 108 h ($F_{1/20}$=12.45, P<0.01; Figure 3.5). Gut water content of *G. veletis* and *G. pennsylvanicus* control individuals was similar throughout the entire duration of exposure (Time point 0-12: $F_{1,20}$= 1.263, P>0.1; Time point 12-120: $F_{1,56}$=0.012, P>0.5).

3.3.3 Muscle Water Content

There was an increase in muscle water content in all *G. pennsylvanicus* treatment groups during the first 12 h of cold exposure ($F_{1,42}$=0.001, P<0.01; Figure 3.6), followed by a significant decrease in muscle water content throughout the subsequent 108 h of cold exposure ($F_{1,102}$=7.144, P<0.001). Muscle water content was similar among all *G. pennsylvanicus* treatments groups over the entire duration of exposure (Time point 0-12: $F_{3,42}$=1.256, P=0.302 ; Time point 12-120: $F_{2,102}$=0.116, P=0.891). Muscle water content was not significantly different between *G. pennsylvanicus* control individuals and *G. veletis* (Time point 0-12: $F_{1,21}$= 2.295, P= 0.1686 ; Time point 12-120: $F_{1,47}$=0.5043, P=0.481). However, unlike *G. pennsylvanicus* controls, increasing time of cold exposure did not significantly change muscle water content in *G. veletis* individuals (Time point 0-12: $t_{7.43}$=10.108, P=0.344; Time point 12-120: $F_{1,17}$=0.0451, P=0.834).

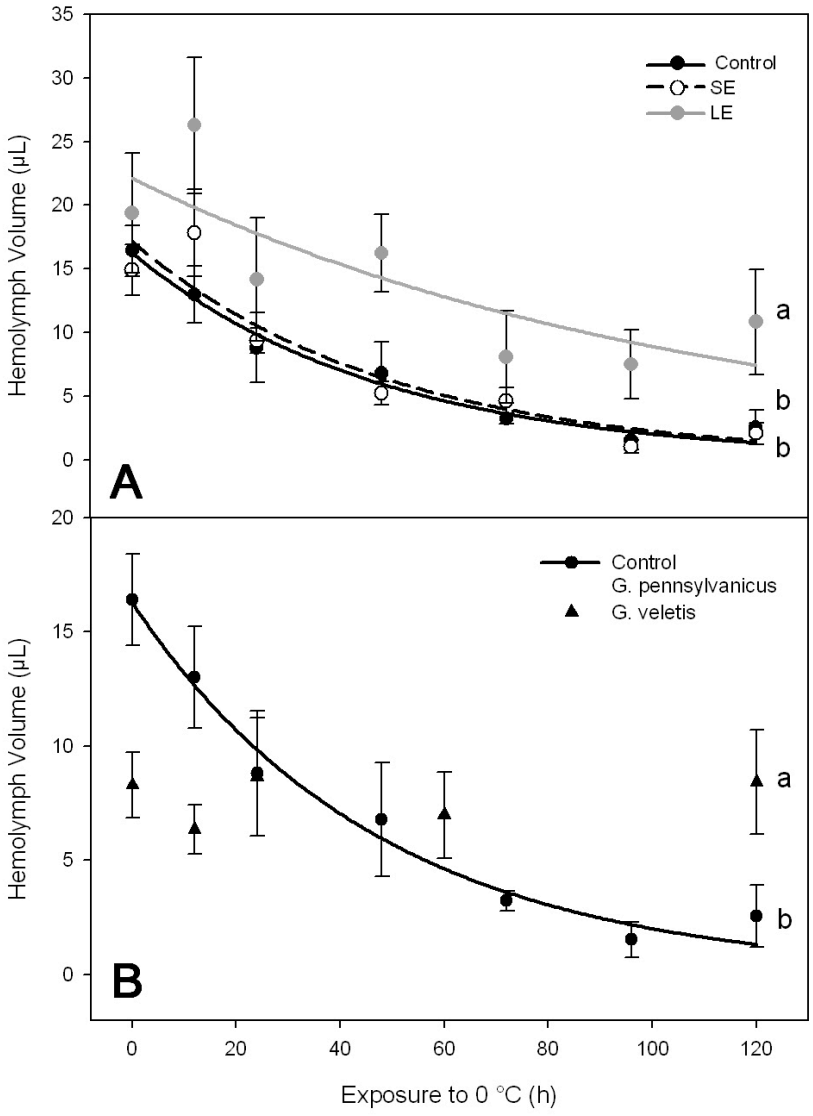

Figure 3.4 Mean ± S.E.M hemolymph volume of A) *Gryllus pennsylvanicus* treatment groups and B) *G. veletis* (with *G. pennsylvanicus* control for comparative purposes) exposed to 0 °C for up to 120 h. Lines denote a significant effect of cold exposure over time. Different letters at the end of each line denote a significant effect of treatment over the 120 h of cold exposure (Tukey's HSD, $P<0.05$). N=6 crickets per sampling period. Error bars that are not visible are obscured by the symbols.

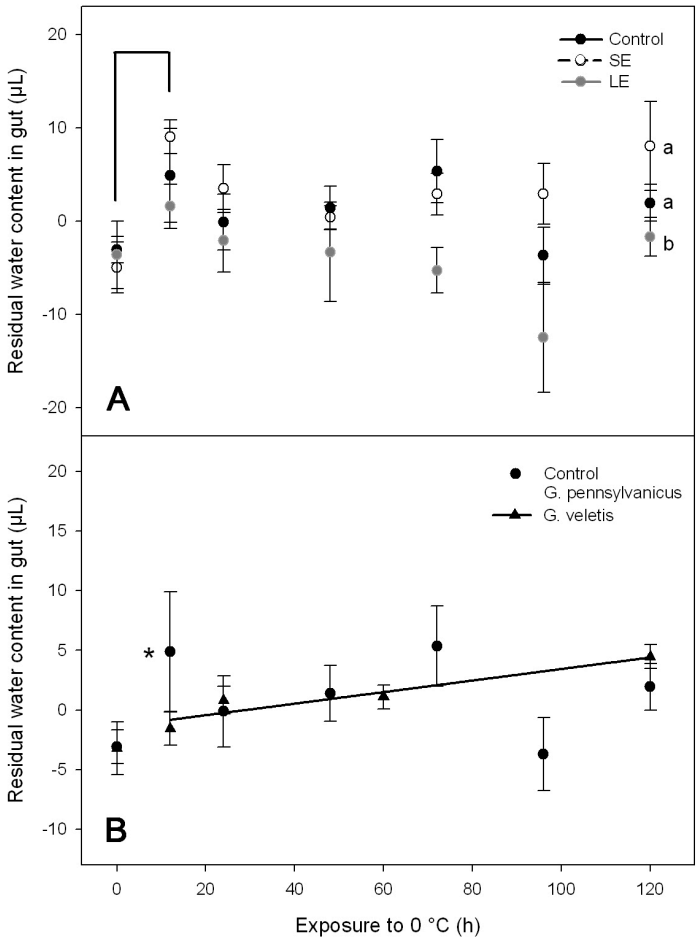

Figure 3.5 Mean ± S.E.M residuals of a linear regression of gut water content and dry mass of A) *Gryllus pennsylvanicus* treatment groups and B) *G. veletis* (with *G. pennsylvanicus* control for comparative purposes) exposed to 0 °C for up to 120 h. In panel A, bracket denotes a significant effect of initial cold exposure on all treatments. In panel B, asterisk denotes a significant effect of initial cold exposure only on the treatment * is adjacent to. Lines denote a significant effect of time exposed to 0 °C on gut water content throughout the last 108 h of cold exposure. Different letters at the end of each line denote a significant effect of treatment throughout the last 108 h of cold exposure (Tukey's HSD, $P<0.05$). N=6 crickets per sampling period.

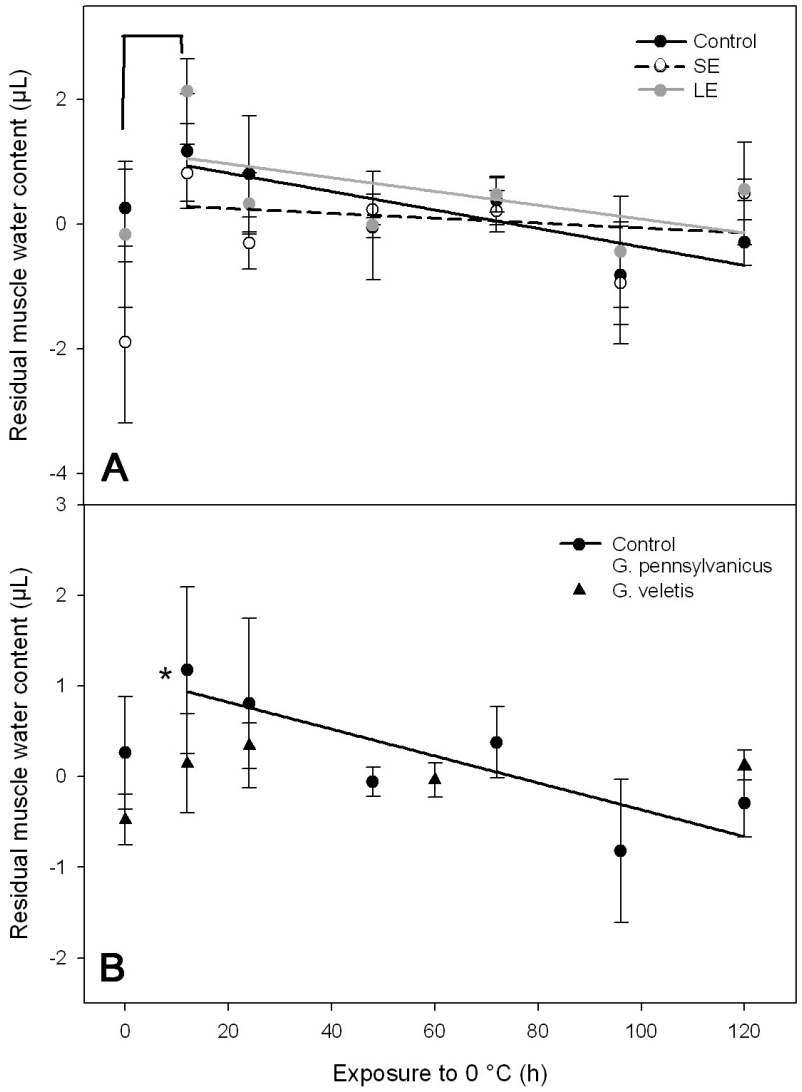

Figure 3.6 Mean ± S.E.M residuals of a linear regression of muscle water content and dry mass of A) *Gryllus pennsylvanicus* treatment groups and B) *G. veletis* (with *G. pennsylvanicus* control for comparative purposes) exposed to 0 °C for up to 120 h. In panel A, bracket denotes a significant effect of initial cold exposure on all treatments. In panel B, asterisk denotes a significant effect of initial cold exposure only on the treatment * is adjacent to. Lines denote a significant effect of cold exposure during the last 108 h. N=6 crickets per sampling period.

3.4 Tissue and hemolymph Ion Content

3.4.1 Hemolymph Sodium Content

Hemolymph Na^+ content did not significantly change in any *G. pennsylvanicus* treatment group during the first 12 h of cold exposure ($F_{1,29}$=1.062, P=0.311; Figure 3.7), and there were no significant differences in hemolymph Na^+ content among any of the *G. pennsylvanicus* treatment groups during this time period ($F_{2,29}$=0.879, P= 0.426). Hemolymph sodium content in all *G. pennsylvanicus* treatments significantly decreased over the subsequent 108 h of cold exposure ($F_{1,100}$=49.204, P<0.001). However, LE individuals' hemolymph Na^+ content was significantly higher in comparison to Control and SE individuals throughout the last 108 h of cold exposure (P<0.001). Hemolymph Na^+ content was similar between control and SE individuals throughout the last 108 h of cold exposure (P=0.985). *Gryllus veletis* Na^+ hemolymph ion content did not significantly change during the first 12 h ($F_{1,9}$=3.609, P>0.05) or the remainder of the cold exposure ($F_{1,20}$=1.047, P>0.1).

3.4.2 Hemolymph Potassium Content

Hemolymph potassium content was highly variable did not significantly change in any treatment group with increasing time spent at 0 °C for the entire duration of exposure (Time point 0-12h: $F_{1,39}$=0.155,P>0.5; Time point 12-120 h: $F_{1,121}$=0.008, P>0.5). Exposure treatment did not have a significant effect on hemolymph K^+ content throughout the entire duration of exposure (Timepoint 0-12h: $F_{3,39}$=0.548, P>0.5; Timepoint 12-120h: $F_{3,121}$= 0.073, P>0.5); K^+ hemolymph content was not significantly different between *G. veletis* and any *G. pennsylvanicus* acclimation groups (Figure 3.8).

3.4.3 Gut Sodium Content

Gut sodium content significantly increased in control and SE individuals during the first 12 h of cold exposure ($F_{1,26}$=9.231, P<0.001; Figure 3.9). Conversely, gut Na^+ content in LE individuals did not significantly change during the first 12 h of cold exposure (P=0.217). In the subsequent 108 h of cold exposure, there was a tendency for gut Na^+ content to decrease in all *G. pennsylvanicus* groups. However, this trend was marginally insignificant ($F_{1,105}$=3.660, P=0.059). Control and SE individuals had similar gut Na^+ content (P=0.795), while LE individuals had significantly less Na^+ in their gut in comparison to Control and SE groups ($F_{2,105}$= 32.417, P<0.001). *G. veletis* gut Na^+ content significantly increased during the first 12 h of cold ($F_{1,19}$=10.361, P<0.01; Figure 3.9) and remained unchanged throughout the subsequent 108 h of cold exposure ($F_{1,56}$=0.667, P>0.5). *G. veletis* gut Na^+ content was marginally non-significantly lower than *G. pennsylvanicus* control individuals Na^+ gut content throughout the last 108 h of cold exposure ($F_{1,56}$=3.923, P=0.0525).

3.4.4 Gut Potassium Content

Time exposed to cold ($F_{1,35}$=0.177, P>0.5) and treatment ($F_{3,35}$=1.210, P>0.1) did not affect the gut K^+ content of any *G. pennsylvanicus* exposure group and *G. veletis* during the first 12 h of cold exposure (Figure 3.10). Throughout the subsequent 108 h of exposure to 0 °C, gut K^+ content of *G. pennsylvanicus* treatment groups significantly decreased ($F_{1,126}$=8.358, P<0.005). This decrease in gut K^+ content was most evident in LE individuals in comparison to control and SE crickets ($F_{1,126}$=8.358, P<0.005), which had similar patterns of decease in gut K^+ content (P=0.977). In the last 108 h of cold exposure, there was a tendency for gut K^+ to increase in *G. veletis*, however this trend

was not significant ($F_{1,20}$=0.116, P=0.737) and the amount of K^+ in the gut of *G. veletis* and *G. pennsylvanicus* control individuals did not differ significantly throughout the last 108 of cold exposure ($F_{1,56}$=0.221, P=0.639).

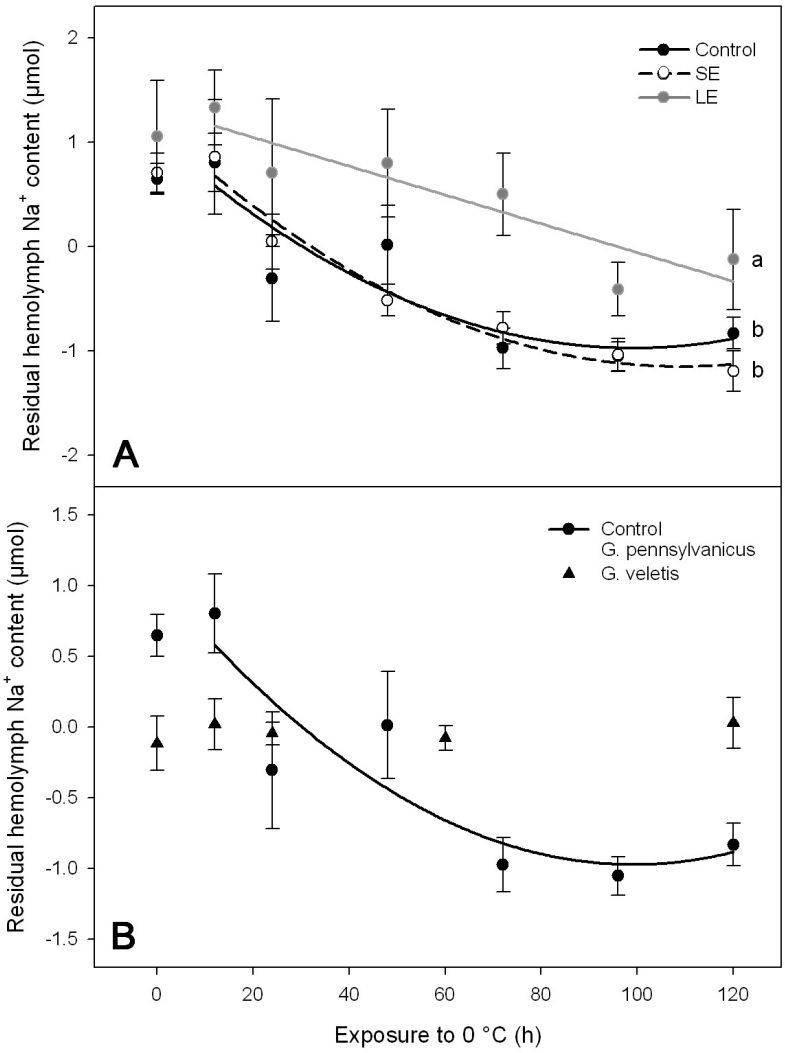

Figure 3.7 Mean ± S.E.M residuals of a linear regression of hemolymph Na^+ content and body mass of A) *Gryllus pennsylvanicus* treatment groups and B) *G. veletis* (with *G. pennsylvanicus* control for comparative purposes) exposed to 0 °C for up to 120 h. Lines denote a significant effect of cold exposure during the last 108 h. Different letters at the end of each line denote a significant effect of treatment during that same time period (Tukey's HSD, $P<0.05$).

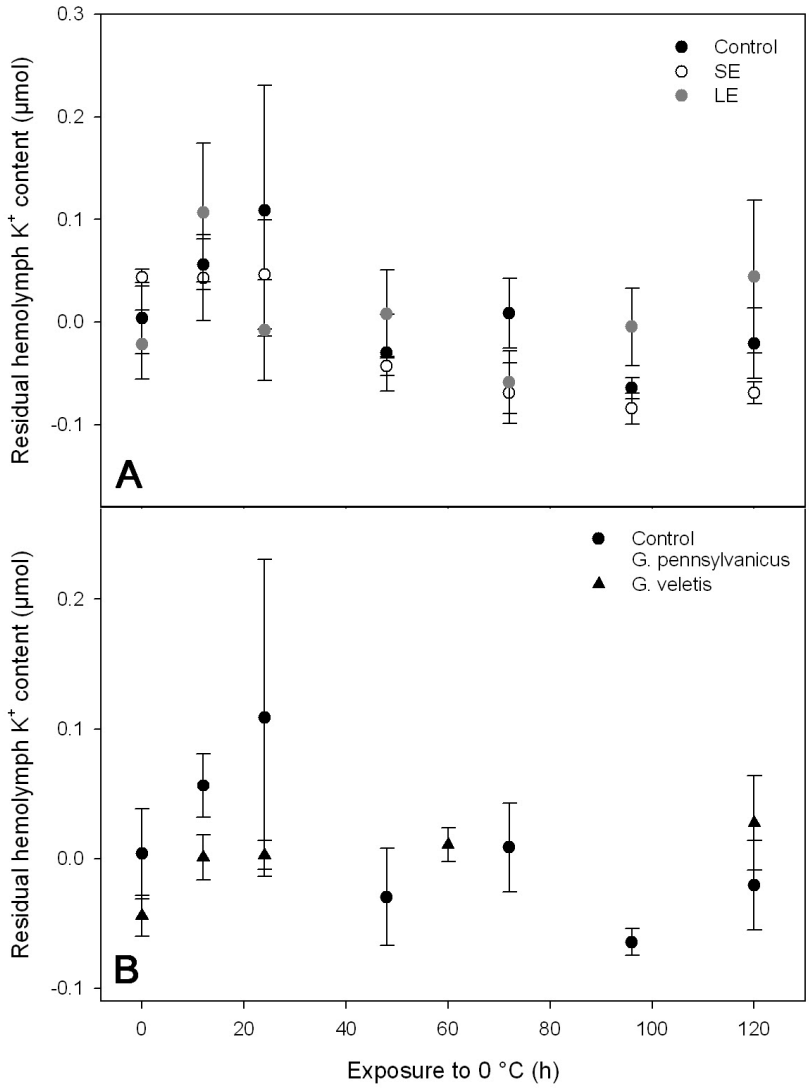

Figure 3.8 Mean ± S.E.M residuals of a linear regression of hemolymph K^+ content and body mass of A) *Gryllus pennsylvanicus* treatment groups and B) *G. veletis* (with *G. pennsylvanicus* control for comparative purposes) exposed to 0 °C for up to 120 h. Exposure to 0 °C did not significantly change hemolymph K^+ content of *G. pennsylvanicus* or *G. veletis* individuals. Hemolymph K^+ content was not significantly different between any *G. pennsylvanicus* treatment group and *G. veletis*. N=6 crickets per sampling period.

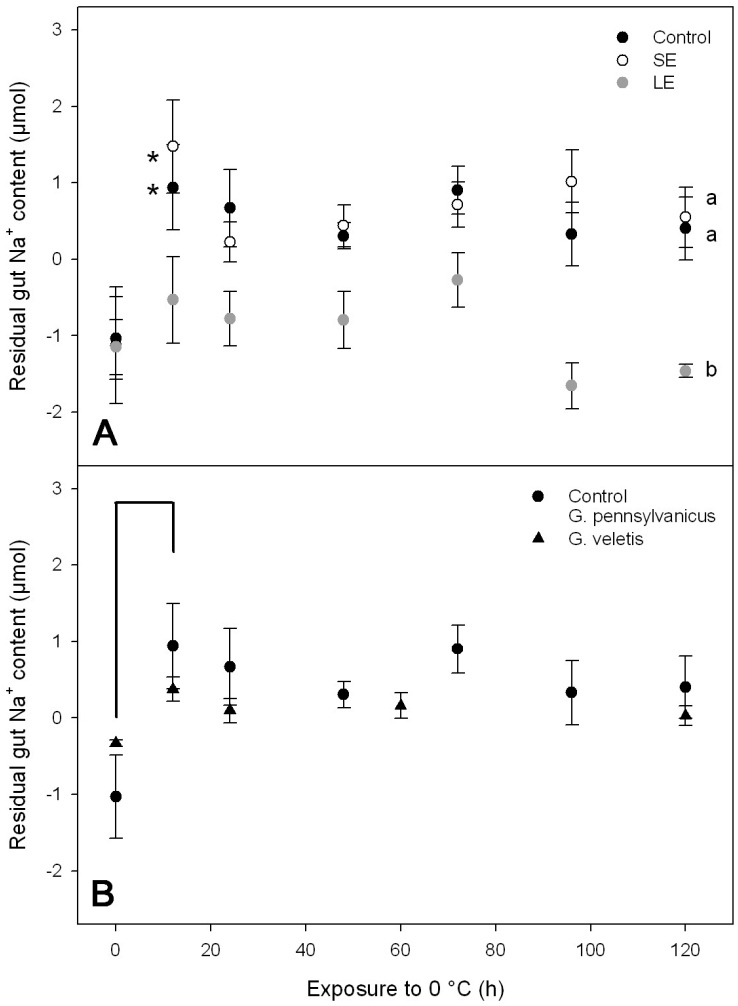

Figure 3.9 Mean ± S.E.M residuals of a linear regression of gut Na^+ content and dry mass of A) *Gryllus pennsylvanicus* treatment groups and B) *G. veletis* (with *G. pennsylvanicus* control for comparative purposes) exposed to 0 °C for up to 120 h. In panel A, asterisks denote a significant effect of initial cold exposure only on treatments * is adjacent to. In panel B, bracket denotes a significant effect of cold exposure on all treatments. Different letters at the end of each line denote a significant effect of treatment during the last 108 h of cold exposure (Tukey's HSD, $P<0.05$). N=6 crickets per sampling period.

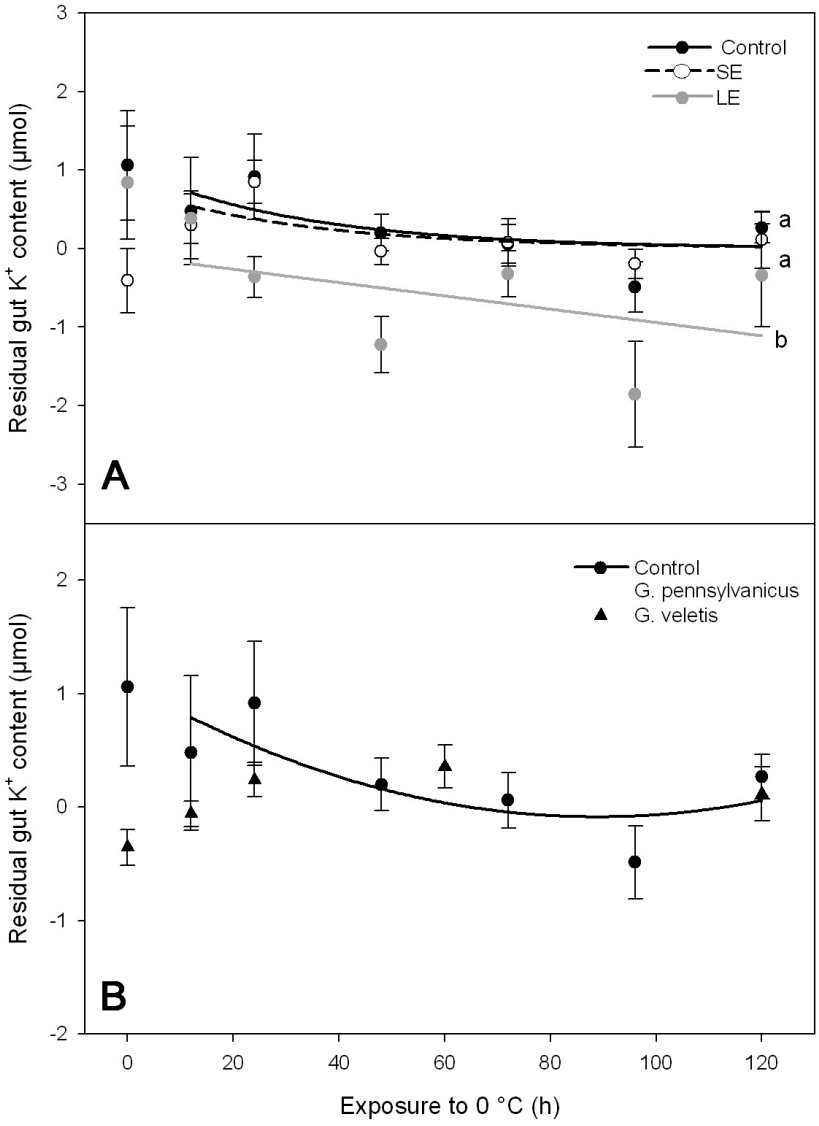

Figure 3.10 Mean ± S.E.M residuals of a linear regression of gut K^+ content and dry mass of A) *Gryllus pennsylvanicus* treatment groups and B) *G. veletis* (with *G. pennsylvanicus* control for comparative purposes) exposed to 0 °C for up to 120 h. Lines denote a significant effect of cold exposure during the last 108 h. Different letters at the end of each line denote a significant effect of treatment during that same time period (Tukey's HSD, $P<0.05$). N=6 crickets per sampling period.

3.5 Tissue Ion Concentration

3.5.1 Hemolymph Sodium Concentration

Time exposed to 0 °C did not significantly affect hemolymph Na^+ concentration in any *G. pennsylvanicus* treatment group during the entire duration of cold exposure (Timepoint 0-12: $F_{1,29}$=0.048, P=0.829; Time point 12-120: $F_{2,88}$=0.148, P=0.863; Figure 3.11). Hemolymph sodium concentrations were similar among all *G. pennsylvanicus* treatments groups throughout the 120 h exposure to 0 °C (Timepoint 0-12: $F_{2,29}$=0.652, P=0.529; Time point 12-120: $F_{2,88}$=0.148, P=0.863). Hemolymph sodium concentration in *G. veletis* significantly decreased during the first 12 h of cold exposure (t_{11}=11.8641, P<0.001), and significantly increased throughout the last 108 h of cold exposure ($F_{1,18}$=6.056, P<0.05). *Gryllus veletis* maintained significantly lower hemolymph [Na^+] in comparison to *G. pennsylvanicus* control individuals throughout the last 108 h of cold exposure ($F_{1,49}$=4.701, P<0.05).

3.5.2 Hemolymph Potassium Concentration

Hemolymph K^+ concentration in *G. pennsylvanicus* did not significantly change during the first 12 h of cold exposure ($F_{1,24}$= 0.186, P=0.670; Figure 3.12). There was a trend towards differences in hemolymph [K^+] among *G. pennsylvanicus* treatment groups during the first 12 h of cold exposure, but this trend was not statistically significant ($F_{2,24}$=15.728, P=0.059). The response of the LE individuals over these first 12 h of cold exposure was significantly different from control and SE crickets; hemolymph [K^+] in control and SE individuals tended to increase, while hemolymph [K^+] in LE individuals tended to decrease over these first 12 h of cold exposure ($F_{2,24}$=4.353, P<0.05). Throughout the subsequent 108 h of cold exposure, there was an increase in hemolymph

K^+ concentration in all *G. pennsylvanicus* treatment groups ($F_{1,84}$= 35.060, P<0.001). Hemolymph K^+ concentration was significantly higher in SE and control crickets in comparison to LE individuals throughout the last 108 h of cold exposure (P<0.05). Hemolymph [K^+] was comparable between control and SE individuals during this time period (P=0.436). Long-term exposure individuals had marginally lower hemolymph [K^+] than SE crickets (P=0.687), and a significantly lower hemolymph [K^+] concentration in comparison to control individuals ($F_{2,84}$= 6.151, P<0.001). *Gryllus veletis* hemolymph K^+ concentration was comparable to *G. pennsylvanicus* controls throughout the first 12 h of cold exposure ($F_{1,20}$=4.333, P>0.1). During the last 108 h of cold exposure, *G. veletis* hemolymph K^+ concentration did not significantly change ($F_{1,18}$=0.414, P=0.528).

3.5.3 Muscle Sodium Concentration

Exposure to cold did not significantly change Na^+ concentration in the muscle tissue of *G. pennsylvanicus* or *G. veletis* during the first 12 h ($F_{1,26}$=0.157, P=0.695) or the remainder of the cold exposure ($F_{1,116}$=1.149, P=0.286; Figure 3.13). Muscle [Na^+] was comparable among all *G. pennsylvanicus* treatment groups and *G. veletis* throughout the first 12 h of cold exposure ($F_{2,26}$=1.964, P=0.161). Throughout the last 108 h of cold exposure, *G. pennsylvanicus* SE individuals had a significantly lower concentration of Na^+ in their muscle tissue in comparison to LE and statistically marginally lower (P=0.044) to *G. veletis* individuals ($F_{3,116}$=6.367, P<0.001). All other treatment groups had comparable muscle Na^+ concentrations throughout the last 108 h of cold exposure (P>0.05).

3.5.4 Muscle Potassium Concentration

Muscle K^+ concentration did not significantly differ ($F_{3,35}$=2.090, P=0.1136) or change among any *G. pennsylvanicus* treatment group and *G. veletis* over the first 12 h of cold exposure ($F_{1,35}$=2.419, P=0.129; Figure 3.14). Potassium concentration in the muscle tissue of all *G. pennsylvanicus* treatments and *G. veletis* increased over the subsequent 108 h of cold exposure ($F_{1,116}$=10.335, P<0.01), and this change was similar among all the treatment groups and between *G. veletis and G. pennsylvanicus* ($F_{3,116}$=0.202, P=0.895).

3.5.5 Muscle Equilibrium Potentials

There were no significant differences or changes in Na^+ muscle equilibrium potentials among any of the *G. pennsylvanicus* treatment groups and *G. veletis* throughout the 120 h exposure to 0 °C (Time: $F_{1,121}$=0.102, P=0.750; Group: $F_{1,121}$=0.104, P=0.957; Figure 3.15). Potassium muscle equilibrium potentials significantly depolarized during the first 12 h of exposure ($F_{1,27}$=8.501, P<0.001) and continued to depolarize throughout the subsequent 108 h of cold exposure in all *G. pennsylvanicus* treatment groups ($F_{1,87}$=30.018, P<0.001). During the first 12 h of cold exposure, the amount of depolarization for muscle E_{K+} was comparable among all *G. pennsylvanicus* groups (F2,27= 0.575, P= 0.569). Throughout the remaining 108 h of cold exposure, the depolarization of K^+ equilibrium potentials was significantly smaller in LE individuals in comparison to control and SE individuals ($F_{1,87}$=8.109, P<0.05). *Gryllus veletis* K^+ equilibrium potentials also depolarized during the first 12 h of cold exposure ($F_{1,20}$=8.109, P<0.001); the amount of depolarization was similar to that of *G. pennsylvanicus* controls throughout these first 12 h of cold exposure (P=0.857).

Throughout the subsequent 108 h, unlike *G. pennsylvanicus* control K^+ equilibrium potentials, which continued to decline over time, *G. veletis* K^+ equilibrium potentials plateaued around -54 mV±2.09 ($F_{1,48}$=12.942, P<0.001; Figure 3.15).

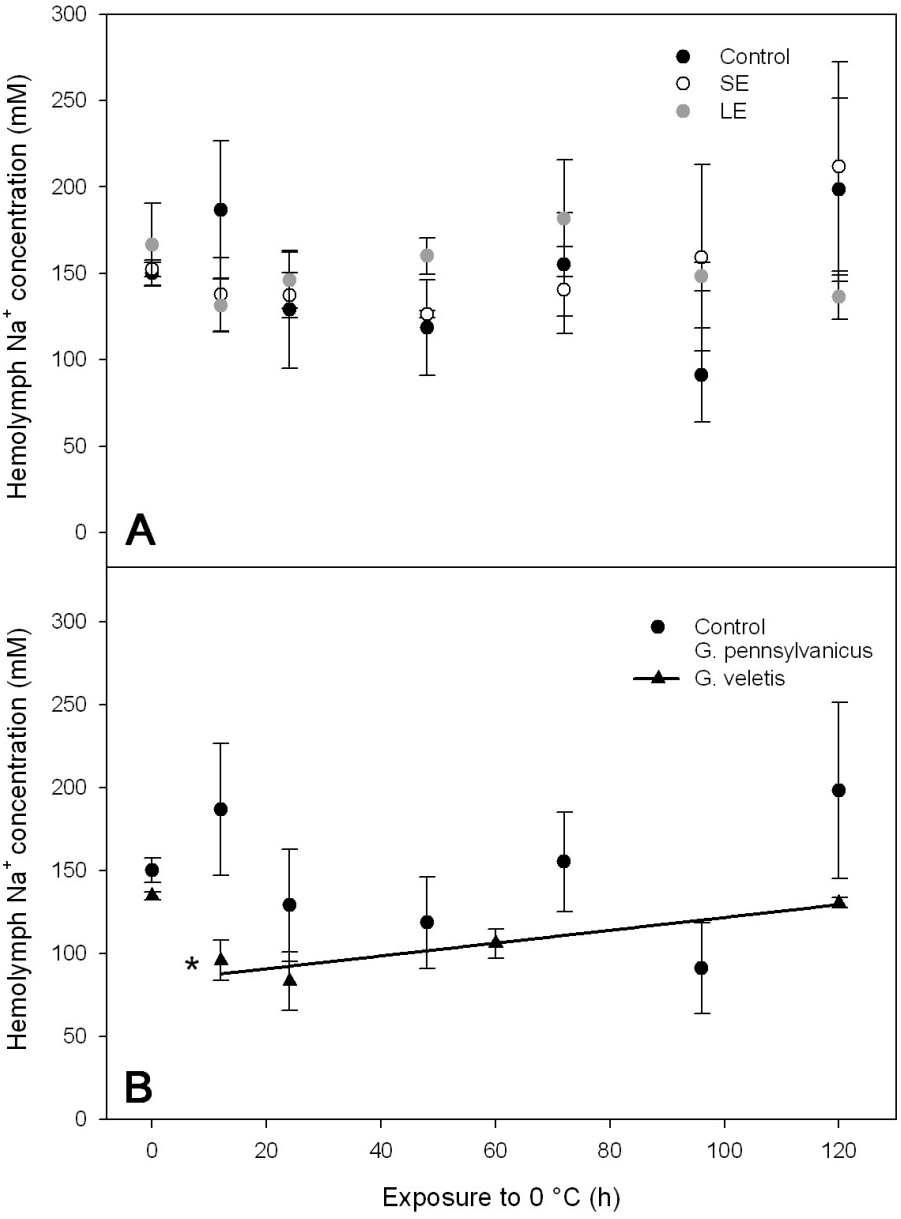

Figure 3.11 Mean ± S.E.M hemolymph [Na^+] of A) *Gryllus pennsylvanicus* treatment groups and B) *G. veletis* exposed to 0 °C for up to 120 h. Lines denote a significant effect of increasing time of exposure to 0 °C on hemolymph [Na^+] during the last 108 h. In panel B, asterisk denotes a significant effect of initial cold exposure only on treatment * is adjacent to. N=6 crickets per sampling period.

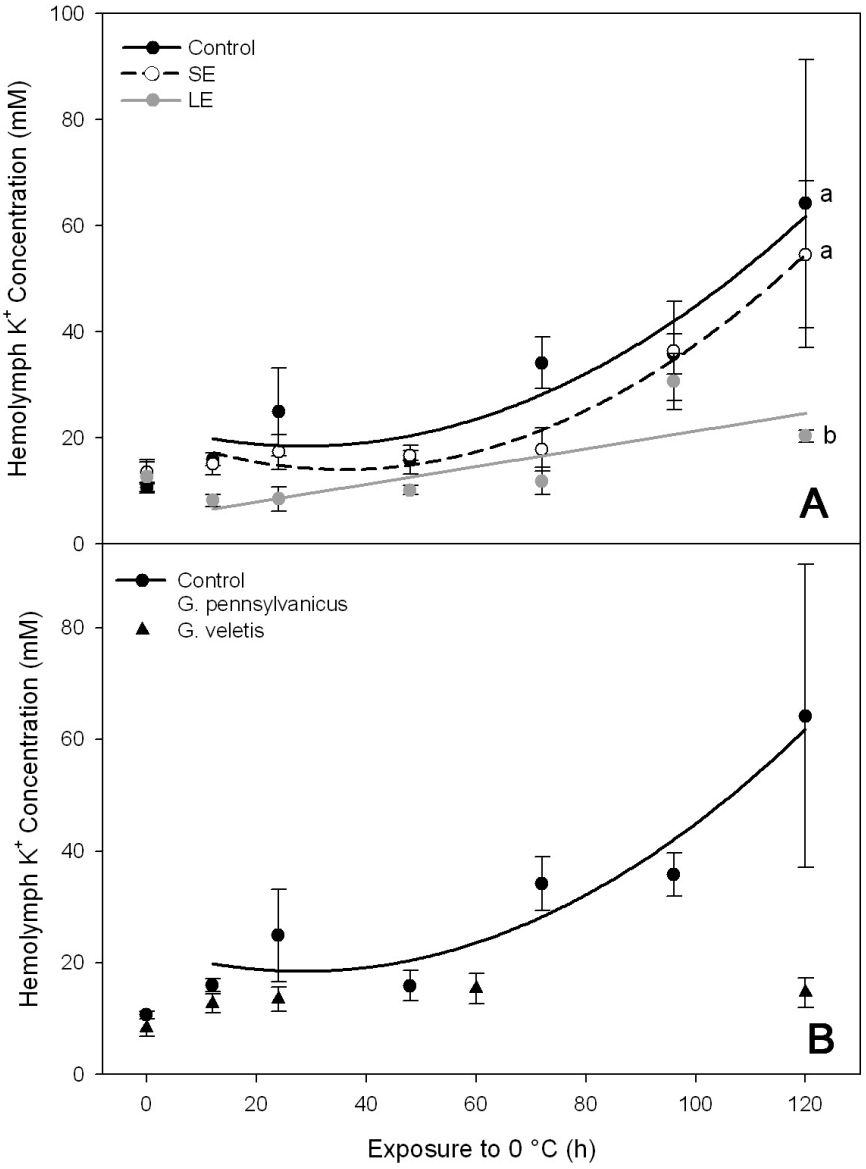

Figure 3.12 Mean ± S.E.M hemolymph [K^+] of A) *Gryllus pennsylvanicus* treatment groups and B) *G. veletis* (with *G. pennsylvanicus* control for comparative purposes) exposed to 0 °C for up to 120 h. Lines denote a significant effect of increasing time exposure to 0 °C on hemolymph [K^+] during the last 108 h. Different letters at the end of each line denote a significant effect of treatment during that same time period. N=6 crickets per sampling period.

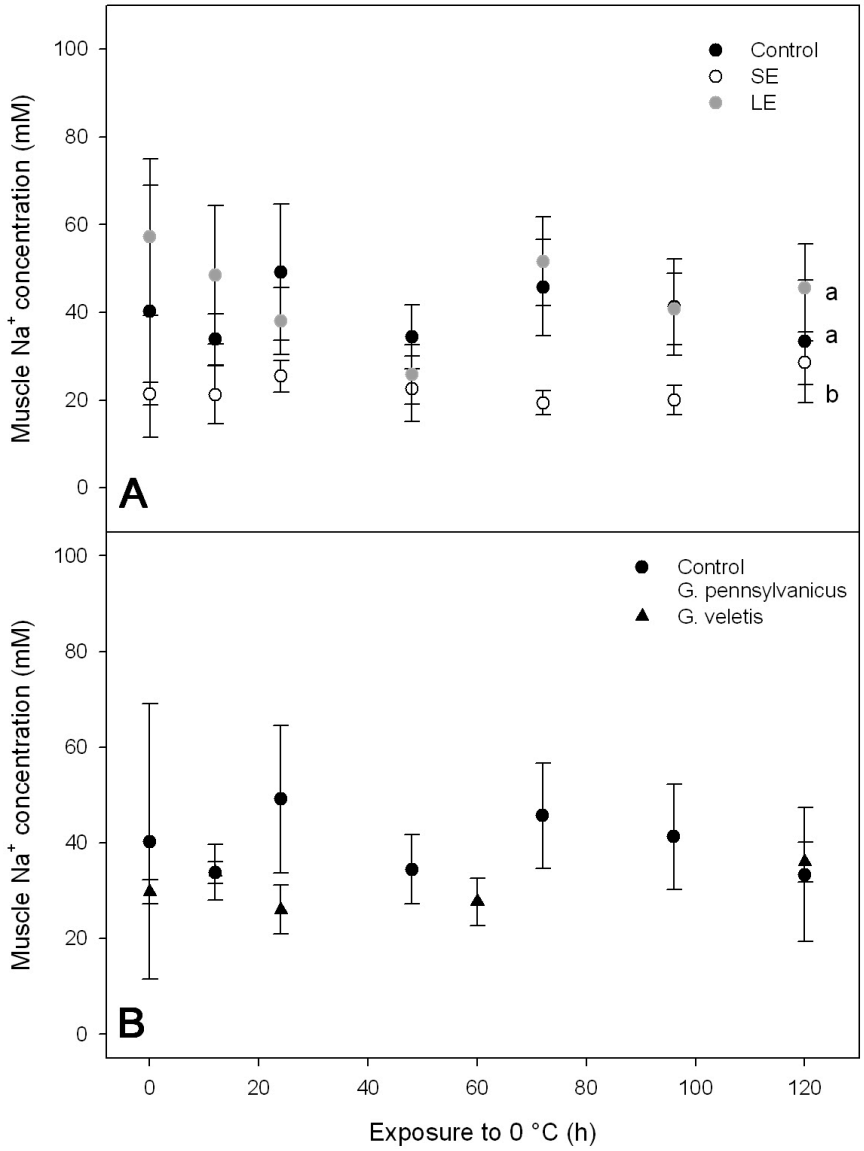

Figure 3.13 Mean ± S.E.M muscle [Na^+] of A) *Gryllus pennsylvanicus* treatment groups and B) *G. veletis* (with *G. pennsylvanicus* control for comparative purposes) exposed to 0 °C for up to 120 h. Increasing exposure to 0 °C did not significantly change muscle [Na^+] of *G. pennsylvanicus* or *G. veletis*. Different letters at the far right side denote a significant effect of treatment during the last 108 h of cold exposure (Tukey's HSD, $P<0.05$). N=6 crickets per sampling period.

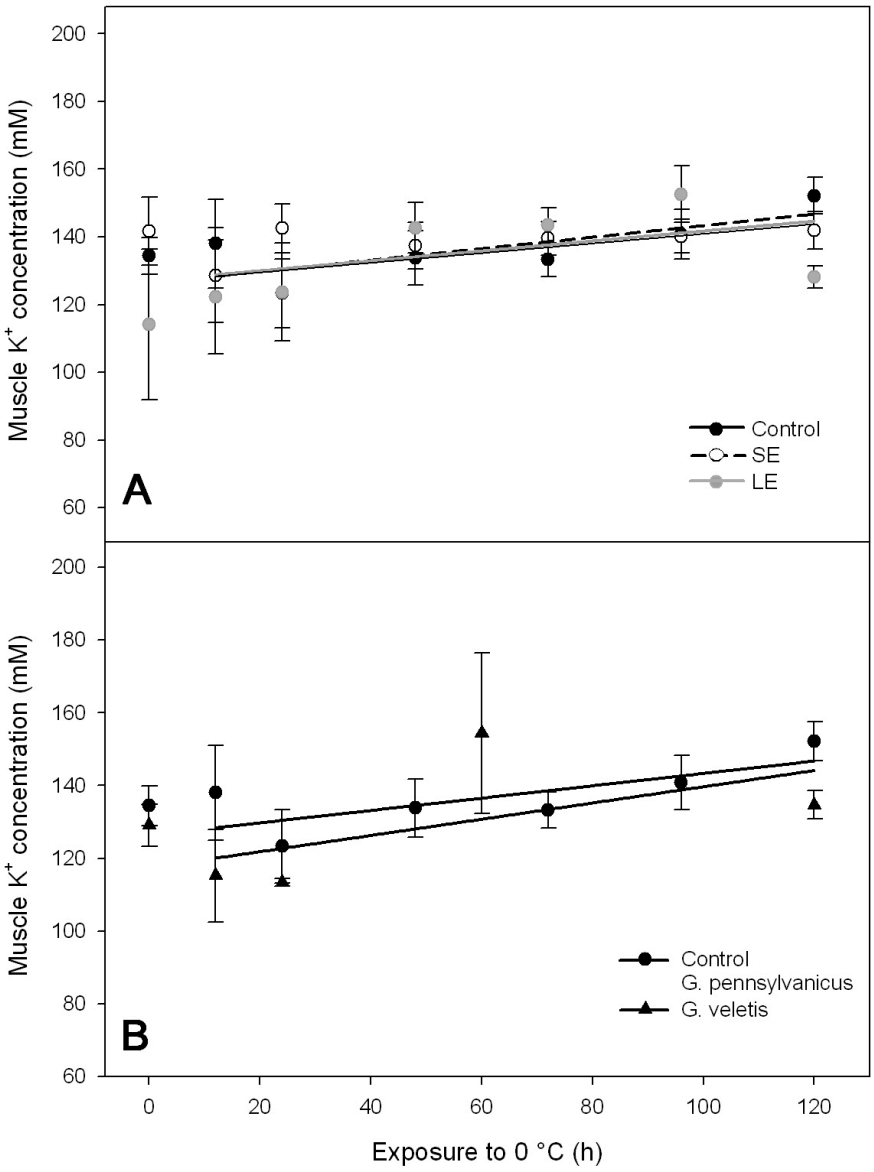

Figure 3.14 Mean ± S.E.M muscle [K^+] of A) *Gryllus pennsylvanicus* treatment groups and B) *G. veletis* (with *G. pennsylvanicus* control for comparative purposes) exposed to 0 °C for up to 120 h. Lines denote a significant effect of increasing time exposed to 0 °C on muscle [K^+] during the last 108 h. There were no significant differences in muscle K^+ concentration among any treatment group throughout the duration of the cold exposure. N=6 crickets per sampling period.

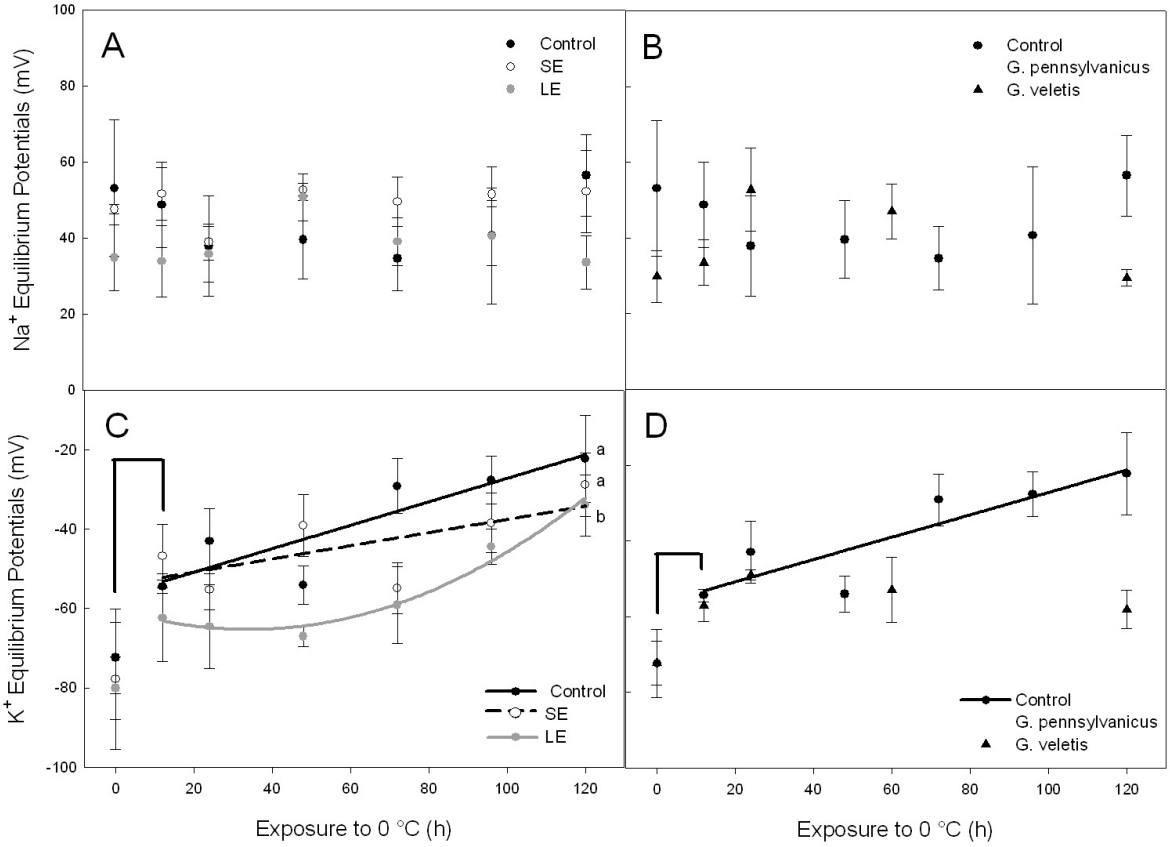

Figure 3.15 Mean ± S.E.M Na^+ and K^+ muscle equilibrium potentials muscle equilibrium potentials of *Gryllus pennsylvanicus* treatment groups (A and C) and *G. pennsylvanicus* controls and *G. veletis* (B and D) exposed to 0 °C for up to 120 h. Exposure to 0 °C did not significantly change Na^+ equilibrium potentials of *G. pennsylvanicus* treatment groups and *G. veletis*. Bracket denotes a significant effect of cold exposure on all treatments during the first 12 h of exposure to 0 °C. Lines denote a significant effect of cold exposure during the last 108 h of cold exposure. Different letters at the end of each line denote a significant effect of treatment during that same time period (Tukey's HSD, $P<0.05$). N=6 crickets per sampling period.

CHAPTER 4: DISCUSSION

In this study, I used cold exposure treatments that induced plasticity in the cold tolerance of *Gryllus pennsylvanicus,* and the naturally higher cold tolerance of *Gryllus veletis* to study variation in low temperature performance and ion homeostasis. Given CTmin, CCR, and survival after cold exposure are related cold tolerance traits thought to be driven by aspects of ion homeostasis, and their mechanisms are presumed to partially overlap; a shift in one trait, which implies a shift in the mechanism underlying that trait, should influence all other traits. By looking at how variation in ion homeostasis correlated with variation in cold tolerance, I was able to determine specific physiological changes that result in enhanced cold tolerance in *Gryllus pennsylvanicus*. I used a short-term (SE) and long-term exposure treatment (LE) to elicit plasticity in the cold tolerance of *G. pennsylvanicus*.

I first examined whether the exposure treatments and cold adaptation (*G. veletis*) resulted in physiological changes that enhanced performance at low temperatures. The long-term exposure treatment (LE) induced plasticity in the cold tolerance of *G. pennsylvanicus* and created a more cold-hardy phenotype. The short-term exposure treatment (SE) induced plasticity in some cold tolerance traits, such as CTmin and CCR, but did not induce changes in the mechanisms associated with indirect chilling injury. Thus, the SE treatment resulted in a more cold-hardy phenotype than control crickets, but not as cold-hardy as the LE phenotype. The changes induced in cold tolerance of *G. pennsylvanicus* by a long-term exposure were not as profound as those induced by cold adaptation in *G. veletis.*

I then examined whether the shifts in cold tolerance that resulted from acute and prolonged low temperature exposure treatments or cold adaptation were accompanied by a shift in the ability to prevent or mitigate the migration of hemolymph Na^+ and water into the digestive system of crickets. Comparison of the physiology of these phenotypes with different cold tolerance levels can be used to examine which mechanisms underlie plasticity in cold tolerance. Some of the mechanisms regulating ion and water movement between compartments during stressful cold exposure (0 °C for 120 h) and the means by which insects modulate their cold tolerance through changes in gut permeability to ions and water are plastic. The shift in cold tolerance as a result of the long-term exposure was accompanied by an improvement in the ability to mitigate the migration of hemolymph Na^+ and water into the digestive system in comparison to SE and controls.

Gryllus veletis did not experience a loss of hemolymph water into the gut, or a large movement of Na^+ from the hemolymph to the gut. *Gryllus pennsylvanicus* control and SE treatments had similar patterns of water and ion redistribution, suggesting SE does not induce lasting changes or have a lasting effect in the mechanisms associated with indirect chilling injury in *G. pennsylvanicus*. Aside from the lack of movement of water and Na^+ into the gut, which mitigates an increase in hemolymph K^+ concentration and decrease in E_K potentials; all other patterns of movement of ions and water were similar among the *G. pennsylvanicus* control and *G. veletis*. This confirms the ability to prevent the drift of water and Na^+ into the gut is associated with being able to mitigate chilling injury and enhanced cold hardiness, which results in crickets being able to survive a 120 h exposure to 0 °C.

4.1 Plasticity of Cold Tolerance

The long-term exposure treatment (LE) and cold adaptation resulted in individuals that had lower thermal limits, faster recovery times from chill coma, and higher survival after cold exposures in comparison to SE and control individuals. Despite being reared at the same temperature as *G. pennsylvanicus* control individuals, *G. veletis* had a lower CTmin than *G. pennsylvanicus* LE crickets; *G. veletis* did not go into chill coma after 12 h at 0 °C and had 0 % mortality after 120 h at 0 °C. This indicates adaptation to low temperature has resulted in much more profound physiological changes in the cold tolerance traits measured in this study than phenotypic plasticity induced by the short or long-term cold exposure treatments I applied to *G. pennsylvanicus*.

All exposure treatments induced plasticity in the CTmin of *G. pennsylvanicus;* SE and LE individuals had lower CTmins than control individuals (Figure 3.1) while *G. veletis* nymphs had the lowest CTmin out of all treatments. Although not a significant difference, LE individuals had a lower CTmin than SE individuals, suggesting the exposure treatments may a) affect the mechanisms underlying chill coma onset in *G. pennsylvanicus* in different ways, or b) the SE treatment changed some, yet not all the underlying mechanisms of chill coma onset the LE treatment changed. CTmin is plastic in a broad range of taxa in response to different rearing temperatures, acclimation and hardening treatments that vary in length of exposure, and thermal stress periods (Ransberry et al. 2012). A prolonged acclimation period can have a more profound effect on measures of chill coma than hardening treatments (e.g., Rako and Hoffmann 2006; Ransberry et al. 2012), which could explain why (although not significant) LE individuals had a lower CTmin than SE crickets. Control male *G. pennsylvanicus*

individuals had a similar CTmin to female *G. pennsylvanicus* crickets (MacMillan and Sinclair 2011a)

Low temperature exposure treatments also induced plasticity of the chill coma recovery time (CCR) of *G. pennsylvanicus*; both SE and LE individuals recovered faster than control individuals (Figure 3.2). However, there were no significant differences in CCR between LE and SE individuals. Control male *G. pennsylvanicus* crickets and female *G. pennsylvanicus* crickets had similar chill coma recovery times (MacMillan et al., 2012). By contrast, *Gryllus pennsylvanicus* nymphs used in a study by Niehaus et al. (2012), reared at 25 °C and cooled gradually to 12 °C over the course of 30 days (same temperature used in the present study), showed no plasticity in CCR. While I took my cold tolerance measurements after crickets were exposed to a prolonged static period of exposure at 12 °C, Niehaus et al. (2012) took their cold tolerance measurements immediately after the ramp down from 25 °C to 12 °C was completed. There may be a threshold time-temperature interaction that induces plasticity in CCR of *G. pennsylvanicus*. Induction of plasticity in CCR can happen after being exposed to a low temperature for a short period of time (as seen in SE treatment), yet these physiological changes may take longer to occur at a milder low temperature (LE treatment). This would explain why I saw plasticity in CCR of *G. penssylvanicus* LE and SE crickets that was not seen in Niehaus et al. (2012) study. Niehaus et al. (2012) could test whether in fact a longer static period of exposure to a mild temperature results in plasticity of CCR by exposing *G. pennsylvanicus* nymphs to a longer exposure period at 12 °C prior to taking CCR measurements.

G. veletis individuals reared under summer conditions did not enter chill coma after being exposed to 0 °C for 12 h; an indication this species of cricket is inherently more cold tolerant in their nymph stage than the adult fall field cricket. This higher basal cold tolerance is likely the product of overwintering as nymphs (Alexander and Bigelow, 1960); cold adaptation likely resulted in a lower or broader thermal range of performance for *G. veletis* nymphs in comparison to *G. pennsylvanicus* adults. In nature, *G. pennsylvanicus* is a nymph during summer months, and then molts into adulthood in the early fall. These adult individuals lay eggs throughout the fall and die during the early winter months (Niehaus et al., 2012). Thus, *G. pennsylvanicus* overwinters in its egg stage. Given the adults are already at the end of their life stage in the beginning of winter, there is likely very little selection pressure to elicit plasticity in cold tolerance as most of their energy is likely allotted to reproduction efforts.

Control and SE *G. pennsylvanicus* showed similar mortality with increasing exposure to 0 °C, and the majority of crickets in both treatments were killed after 120 h. Conversely, most of the crickets which underwent the LE treatment were still alive after a 120 h exposure to 0 °C (Figure 3.3). This lower mortality after cold exposure in LE crickets in comparison to the control treatment, in addition to the CTmin and CCR findings, indicates that a prolonged exposure to 12 °C resulted in an acclimation response in *G. pennsylvanicus*. Interestingly, LE crickets showed mortality before control and SE treatment individuals (after 12 hrs). There may be costs incurred in acclimating to a mild low temperature (12 °C) from the summer conditions the crickets were reared in that detrimentally affect survival at a more extreme low temperature (0 °C). The number of LE individuals categorized as dead at each time that point varied among assessment time

points, suggesting roughly 30% of the crickets did not undergo low temperature acclimation. This is another indication there may be costs associated with low temperature acclimation to 12 °C that lead to tradeoffs in fitness at other temperatures, such that only 70% of the individuals underwent the process.

Evidence for trade-offs has been found in cold-acclimated *D. melanogaster* larvae and adults, where being acclimated to low temperatures decreased the ability of flies to find food at high temperatures in comparison to control flies (Kristensen et al., 2008). In the adult ladybird *Adalia bipunctata,* acclimation to cold temperatures reduced pupae-to-adult survival (Sørensen, 2012). Rapid cold hardening and cold-shock can also reduce adult longevity in the housefly, *Musca domestica* (Coulson and Bale, 1992). The energetic costs of the cellular response involved in physiological acclimation are difficult to quantify and have yet to be properly investigated, but qualitative assessments suggest the costs are substantial (DeWitt et al., 1998; Angilletta, 2009). Future studies should attempt to quantify the costs and tradeoffs of acclimation responses to low temperature in insects. This would aid in understanding plasticity in cold tolerance and its underlying mechanisms, as well as understanding population variation in survival, and heritability of cold tolerance traits. Given plasticity in cold tolerance can be elicited in *G. pennsylvanicus* and some of the underlying mechanisms of chill coma onset and recovery (MacMillan et al., 2012) have been described in this organism, *G. pennsylvanicus* is a great model to continue to use in the study of plasticity in insect cold tolerance.

The overall similarity between SE and control mortality with increasing time spent at 0 °C indicates *G. pennsylvanicus* does not show a rapid cold hardening response after exposure to 0 °C for four hours. A lack of RCH response has also been seen in the

adult Karoo beetle *Afrinus* sp., where a 2 h pre-exposure to -2 °C induces RCH, while pre-exposure to 0 °C for 2 h does not (Sinclair and Chown, 2006). It may be the case that *G. pennsylvanicus* can exhibit RCH, but a 4 h exposure to 0 °C does not induce this response. The ability of *G. pennsylvanicus* to rapidly cold harden could be tested by subjecting individuals to more extreme pre-exposure temperatures. Alternatively, SE could enhance acute cold tolerance (tolerance to intense short bouts of cold) rather than prolonged cold tolerance (tolerance to mild extended bouts of cold), which was not tested in the present study). Future studies should examine more closely whether the SE treatment improves survival during cold shock. Despite having a CTmin that is only a few degrees lower than *G. pennsylvanicus*, all *G. veletis* nymphs fully recovered from a 120 h exposure to 0 °C. This relatively small difference in CTmin between *G. veletis* and *G. pennsylvanicus* is accompanied by a large difference in survival after a prolonged cold exposure, suggesting the mechanisms involved in chill coma onset are not the same as those involved in chilling injury and recovery from injury.

When I performed the survival assays, I categorized crickets as fit, injured or dead, where individuals were classified as injured regardless of the severity of the injury. In several cases I noticed that injury for some individuals was so severe it was likely the cricket would not recover (responsive, but unable to walk or jump). In other individuals, injury was minor (slightly impaired gait); representing a decrease in short-term fitness that may have little to no effect on an individuals' mating capacity and longevity. Future studies examining chilling injury should follow the survival assay procedures used in the present study, with the addition of a numeric scale of injury. Tracking the severity of the

injury and variation in severity of chilling injury at each time point could help explain some of the variation in the physiological parameters measured.

4.2 Hemolymph volume and tissue water content

Hemolymph volume decreased with increasing time of cold exposure for all *G. pennsylvanicus* groups; however, the rate of decrease was slower in LE individuals (Figure 3.4). This suggests that low temperature acclimation results in changes that make crickets more resistant to leakage of water into their gut (i.e. changes in membrane permeability) or an enhanced ability to remove water from their gut back into the hemocoel. Conversely, *G. veletis* had no changes in hemolymph volume over the 120 h exposure to 0 °C, which confirms that increased cold tolerance involves the ability to maintain a constant hemolymph volume.

Gut water content significantly increased in all *G. pennsylvanicus* treatment groups over the first 12 h of cold exposure (Figure 3.5). MacMillan and Sinclair (2011a) observed a similar response in female *G. pennsylvanicus*, where gut water content increased in an exponential manner throughout the first 72 h of exposure to 0 °C. In the present study, after the increase in gut water over the first 12 h of cold exposure, gut water content remained unchanged during the last 108 h of cold exposure. Throughout this time period, the amount of water in the gut of LE individuals was consistently lower than that of Control and SE individuals. This suggests low temperature acclimation results in changes that make male crickets more resistant to the redistribution of water between the gut and hemolymph.

Overall, I found a weak correlation between the loss of water from the hemolymph and an increase of water in the gut. In contrast, MacMillan and Sinclair

(2011a) found a strong correlation between the loss of water from the hemolymph and an increase of water in the gut of female *G. pennsylvanicus*; total water accumulation in the gut accounted for 86% of lost hemolymph volume. The discrepancy in hemolymph and gut water redistribution found in the present study suggests water could have been distributed to other tissues that were not sampled. The increase in muscle water content observed during the first 12 h of cold exposure in all *G. pennsylvanicus* groups suggests water could also have been distributed to the intracellular space of cells.

A 120 h exposure to 0 °C likely resulted in whole-animal dehydration of *G. pennsylvanicus*. This could account for the majority of the 4% decrease in body mass observed over the 120 h at 0 °C and the lack of concordance between hemolymph volume loss and gut volume increase seen in male *G. pennsylvanicus*. When *Drosophila melanogaster* undergo dehydration, flies preferentially lose water from the hemolymph (Folk et al., 2001). If *G. pennsylvanicus* also preferentially loses water from the hemolymph, resulting in a higher ion concentration in the hemolymph, desiccation may create a gradient that facilitates the diffusion of some water from the gut back into the hemocoel. This may have caused the decreasing trend in residual water content throughout the last 108 h of cold exposure after the initial increase during the first 12 h of cold exposure. Female *G. pennsylvanicus* individuals used by MacMillan and Sinclair (2011a), which came from the same colony as the male *G. pennsylvanicus* crickets used in the present study and weigh 500-700 mg (H. A. MacMillan, personal communication), lost ~2.5% of body mass over the 120 h of exposure to 0 °C (MacMillan and Sinclair, 2011a). Female *G. pennsylvanicus* from this colony are on average twice the size of the adult males; the higher surface area to body mass ratio of males likely makes them more

prone to dehydration than females (Renault and Coray, 2004). To assess how much body water male *G. pennsylvanicus* lose throughout a 120 h cold exposure, the difference in body mass prior to exposure and dry mass of crickets that were cold exposed could be compared to individuals that did not receive the cold exposure. This information would give an indication of what percentage of the hemolymph loss seen in male *G. pennsylvanicus* could be attributed to whole body dehydration effects.

G. veletis had a consistent increase in gut water content over the last 108 h of cold exposure that cannot easily be explained given there was no significant loss of hemolymph volume over this same time period. *G. veletis* had similar amounts of water in the gut, yet less hemolymph than control *G. pennsylvanicus* individuals, even prior to the cold exposure. Unlike *G. pennsylvanicus*, water content in the gut of *G. veletis* did not change during the first 12 h of cold exposure. Given that *G. veletis* is not in chill coma after 12 h of exposure to 0 °C, the lack of increase in the gut water content in the first 12 h may be an indication that, unlike *G. pennsylvanicus*, ion balance is unaffected by cold throughout this time period. I have observed that *G. veletis* is not in chill coma even after a 120 h exposure to 0 °C. Hence, the steady increase in gut water content observed during the last 108 h of cold exposure could be a product of the ongoing metabolic processes *G. veletis* is able to maintain at low temperatures. All crickets were kept unfed and without access to water throughout the cold exposure; *G. veletis* may absorb more water from the food content in its alimentary canal in order to avoid chilling injury or desiccation. This could be tested by increasing the humidity in the chamber when exposing *G. veletis* to 0 °C. Additionally, future studies should assess whether the patterns of redistribution in hemolymph and gut water content of *G. veletis* when exposed to a temperature that

causes the level of chilling injury characteristic in adult male *G. pennsylvanicus* when exposed to 0 °C are similar to the patterns observed in *G. pennsylvanicus*.

4.3 Hemolymph and Gut Ion Content

Total hemolymph Na^+ content declined with increased time spent at 0 °C in all *G. pennsylvanicus* treatment groups. However, LE individuals' decrease in hemolymph Na^+ content was slower in comparison to Control and SE individuals throughout the last 108 h of cold exposure (Figure 3.7). The decrease in hemolymph Na^+ content seen in control and SE individuals was accompanied by an increase in Na^+ content in the gut throughout the first 12 h of exposure in these same treatments (Figure 3.9). These results are consistent with the observations made in *G. pennsylvanicus* females; Na^+ ion content also decreased in the hemolymph and increased in the gut with increasing time spent at 0 °C (MacMillan and Sinclair, 2011a). The slower decrease in hemolymph Na^+ content and lack of changes in Na^+ content in the gut of LE individuals in comparison to control and SE crickets suggests that the gut of LE individuals became less permeable to Na^+ as a result of low temperature acclimation.

The lack of significant changes in gut Na^+ content across time in LE individuals groups despite a significant decrease in hemolymph Na^+ content also suggests sodium is being transported to other tissues or compartments. Given that water passively follows the movement of Na^+, the increase in muscle water content observed during the first 12 h of cold exposure in all *G. pennsylvanicus* groups suggests Na^+ might have been sequestered in the muscle cells. This contrasts with MacMillan and Sinclair (2011a, who found no changes in muscle water or ion content over the duration of the cold exposure in female *G. pennsylvanicus*. Future studies examining the role of Na^+ in ion homeostasis of

Gryllus pennsylvanicus when exposed to low temperature could measure ion content in the muscle and other tissues to confirm this. Hemolymph sodium content in *G. veletis* did not significantly change with increasing time exposed to 0 °C, yet there was a significant increase in gut Na^+ content during the first 12 h of cold exposure. This increase in gut Na^+ content cannot be easily explained, and suggests Na^+ is being transported from some other tissues or compartment not measured in this study into the gut. Overall, long-term exposure and *G. veletis* individuals had the smallest change to no change, respectively, in hemolymph and gut Na^+ content in comparison to SE and control individuals. This suggests a link between enhanced cold tolerance and the ability of an individual to mitigate or prevent the movement of Na^+ (and thus water) from the hemolymph into the gut. To test this, the Na^+ content in the hemolymph and gut tissues of more *Gryllus* species with different basal cold tolerance levels should be incorporated in future studies of variation in ion homeostasis and low temperature performance.

Hemolymph K^+ content did not change over time or differ among the *G. pennsylvanicus* treatment groups and *G. veletis* (Figure 3.8). Gut K^+ content did not change over time in *G. veletis* and only marginally for *G. pennsylvanicus* control and SE individuals (Figure 3.10). However, there was a significant decline in gut K^+ content of LE individuals throughout the last 108 h of cold exposure. The movement of K^+ from the gut into the hemocoel does not seem like an adaptive response. This movement of ions, in addition to the movement of Na^+ and water into the gut, would only exacerbate the increase in hemolymph K^+ concentration which leads to the loss of muscle equilibrium potential (MacMillan and Sinclair, 2011a). In larval Lepidoptera, a V-H^+ATPase and K^+-$2H^+$ antiport combination actively transports K^+ from the hemolymph to the midgut

(Wieczorek et al., 1991). To my knowledge, such a mechanism has not been described in Orthoptera, but if low temperature acclimation resulted in K^+ being actively transported out of the midgut of *G. pennsylvanicus*, it could explain the drop in K^+ in the gut of LE individuals during exposure to 0 °C.

Low temperature acclimation may involve changes that increase membrane permeability to K^+ or Cl^-. The electrogenic co-transport of Cl^- with K^+ across the hindgut plays an important role in ion balance in the dessert locust, *Schistocerca gregaria.* In the hindgut, the production of feces that have a low water content is driven by cycling of ions (Na^+, K^+ and Cl^-) that drive absorption of water from the hindgut against the prevailing osmotic gradient (Phillips et al., 1986). Perhaps Cl^- plays a more important role than previously thought in maintenance of water and ion homeostasis in *Gryllus pennsylvanicus*. Additionally, if low temperature acclimation resulted in the electrogenic co-transport of Cl- with K+ across the hindgut undertaking a more prevalent role in maintaining ion balance in *G. pennsylvanicus* at low temperatures, it could explain the larger decrease in K^+ gut content seen over time in LE individuals. Future studies should attempt to examine whether K^+ is cotransported with Cl^- out of the gut and into the hemolymph and the importance of Cl^- transport in ion homeostasis in *Gryllus pennsylvancus.* If Cl^- transport is found to have an important role in ion balance, subsequent studies should examine the effects of low temperature on Cl- transport in *Gryllus pennsylvanicus.*

4.4 Hemolymph and Muscle Ion Concentration

Hemolymph Na^+ concentration remained unchanged throughout the cold exposure in all *G. pennsylvanicus* treatment groups (Figure 3.11). The lack of change in

hemolymph [Na^+] in male *G. pennsylvanicus* individuals contrasts with the observations made in female crickets; where [Na^+] hemolymph declined throughout the 120 h the crickets were exposed to 0 °C (MacMillan and Sinclair, 2011a). MacMillan and Sinclair (2011a) concluded the decline in [Na^+] hemolymph seen in female crickets was due to a greater loss of Na^+ than water from the hemolymph into the gut. The lack of change in hemolymph Na^+ concentration despite seeing a decrease in hemolymph Na^+ content suggests that equal amounts of Na^+ and water are moving into the gut of male *G. pennsylvanicus*, such that hemolymph Na^+ concentration appears unchanged. *Gryllus veletis* hemolymph [Na^+] significantly decreased during the first 12 h of cold exposure and returned to control levels throughout the remainder of the cold exposure. These changes in hemolymph sodium concentration in *G. veletis* could have been driven by small fluctuations in hemolymph volume (despite not being statistically significant).

Throughout the last 108 h of cold exposure, there was an increase in hemolymph [K^+] in all *G. pennsylvanicus* treatment groups (Figure 3.12). This increase in hemolymph [K^+] was similar between control and SE individuals, and significantly smaller by comparison in LE individuals. This increase in [K^+] hemolmyph is consistent with the findings of MacMillan and Sinclair (2011a) in female *G. pennsylvanicus* crickets, where hemolymph [K^+] also increased despite seeing no significant changes in hemolymph K^+ content. Thus, in the case of both male and female *G. pennsylvanicus,* it appears decreases in hemolymph volume, and not increases in the amount of extracellular K^+ lead to an overall increase in hemolymph [K^+]. This increase in hemolymph [K^+] likely drives decreases in K^+ equilibrium potentials (E_K) at the muscle, which is thought to lead to chill coma in insects. There were no significant changes in [K^+] in the

hemolymph of *G. veletis* throughout the 120 h of exposure to 0 °C, which corresponds with the lack of changes in hemolymph volume in these same individuals.

Increasing time spent at 0 °C did not significantly affect $[Na^+]$ in the muscle tissue of any *G. pennsylvanicus* treatment groups or *G. veletis* (Figure 3.13). Muscle $[K^+]$ increased throughout the last 108 h of cold exposure for all *G. pennsylvanicus* treatment groups. These patterns of muscle $[Na^+]$ and $[K^+]$ over time are different from the observations made on female *G. pennsylvanicus* (MacMillan and Sinclair, 2011a), where muscle $[K^+]$ did not change with increasing time spent at 0 °C. The increase in muscle $[K^+]$ concentration observed in male *G. pennsylvanicus* was likely caused by the decrease in muscle water content seen over the last 108 of cold exposure.

4.5 Equilibrium Potentials

There were no significant changes with cold exposure, and no significant differences among treatments in Na^+ muscle equilibrium potentials (E_{Na}) in *G. pennsylvanicus* treatments and *G. veletis* during the 120 h exposure to 0 °C (Figure 3.15). My findings are consistent with MacMillan and Sinclair (2011a), where cold exposure did not significantly affect muscle E_{Na} in female *G. pennsylvanicus* crickets. However, not all chill-susceptible insects respond to prolonged cold exposure the same way; in the tropical cockroach *Nauphoeta cinerea,* E_{Na} declines with duration of cold exposure (Koštál et al., 2006).

Muscle E_K significantly depolarized in all *G. pennsylvanicus* treatment groups following exposure to 0 °C for 120 h; female *G. pennsylvanicus* reared under control conditions had a similar response to cold exposure (MacMillan and Sinclair 2011a). The decline observed in E_K would be sufficient to dissipate muscle membrane potential, as

membrane potential closely follows E_K in non-lepidopteran insects (Hoyle, 1953; Wood, 1957). The similar pattern of depolarization between control and SE crickets indicates the short-term exposure treatment did not induce physiological changes in *G. pennsylvanicus* that made individuals more resistant to a decrease in muscle E_K. Potassium equilibrium muscle potentials of LE individuals also depolarized, but the depolarization occurred at a slower rate in comparison to the control individuals. The slower increase in hemolymph $[K^+]$ seen in LE individuals likely mitigates or prolongs the onset of muscle E_K depolarization during a 120 h exposure to 0 °C. This is consistent with LE individuals having lower mortality after cold exposure in comparison to controls, yet still being in chill coma after as little as 12 h of being exposed to 0 °C.

The muscle E_K of *Gryllus veletis* also depolarized during the first 12 h of cold exposure, but subsequently reached a plateau around -54 mV. Overall, the muscle E_K of *G. veletis* did not depolarize as extensively as any of the other *G. pennsylvanicus* treatment groups, which indicates adaptation to cold has resulted in better maintenance of homeostasis that mitigate the decrease in E_K muscle potentials. This ability to prevent the depolarization of muscle E_K is consistent with an increased performance at low temperatures; *G. veletis* is not in chill coma after 120 h of being exposed to 0 °C. The initial depolarization of muscle E_K during the first 12 h of cold exposure, despite *G. veletis* not being in chill coma, suggests there are other mechanisms at play in chill coma onset.

4.6 The role of ion and water balance in cold tolerance

A prolonged exposure to 0 °C caused a migration of the majority of hemolymph and Na^+, and the accumulation of some volume of water into the gut of *G.*

pennsylvanicus. These patterns of ion and water movement caused an increase in K^+ hemolymph concentration, driving a decrease in K^+ equilibrium potentials (E_K) at the muscle tissue. *G. pennsylvanicus* control and SE treatments had similar patterns of water and ion redistribution. This indicates our short-term exposure treatment can induce plasticity in some cold tolerance traits, such as CTmin and CCR, but is unable to induce lasting changes or have a lasting effect in the mechanisms associated with indirect chilling injury.

While LE individuals were not able to completely prevent the movement of water from the hemolymph into the gut, these patterns of movement happened at a slower rate in comparison to SE and control individuals. Thus, there was still an increase in hemolymph K^+ concentration and a decrease in E_K muscle potentials, yet in both cases these changes were smaller in comparison to the control and SE. This suggests that long-term exposure to 12 °C induced changes in ion and water gut permeability that mitigate the detrimental effects of a prolonged exposure to 0 °C.

G. veletis did not experience a loss of hemolymph water into the gut, or a large movement of Na^+ from the hemolymph to the gut. Therefore, the concentration of K^+ in the hemolymph did not increase enough to decrease E_K in the muscle tissue to the same extent observed in *G. pennsylvanicus*. Aside from the lack of movement of water and Na^+ into the gut, which mitigates an increase in hemolymph K^+ concentration and decrease in E_K potentials; all other patterns of movement of ions and water were similar among the *G. pennsylvanicus* control and *G. veletis*. This confirms that the ability to prevent the drift of water and Na^+ into the gut is associated with being able to mitigate chilling injury and enhanced cold hardiness, which results in being able to survive and be fit after a 120 h

exposure to 0 °C. The full recovery of *G. veletis* after a 120 h exposure to 0 °C while still observing a decrease in muscle E_K suggest there are other mechanisms (not tested in the present study) that play a role in chill coma onset and preventing indirect chilling injury in *G. veletis*.

The different responses of ion and water redistribution between SE, LE, and *G. veletis* indicates the short-term and long-term exposure treatments, and adaptation to cold affect the mechanisms underlying chill coma onset and chilling injury in different ways; in the present study, adaptation to cold had the most profound effect. *Gryllus veletis* individual's ability to withstand entry into chill coma after being exposed to 0 °C for 12 h, and to fully recover from a 120 h exposure to 0 °C reinforces this idea. While LE individuals had less mortality in comparison to SE and control individuals, the majority of the LE individuals were injured as opposed to fit after being subjected to a 120 h cold exposure. This indicates the long-term treatment is able to induce physiological changes in *G. pennsylvanicus* that somewhat mitigate chilling injury, but not to the same extent cold adaptation does. Basal cold tolerance may limit acclimation capacity in *G. pennsylvanicus;* the potential costs of the biochemical and physiological changes needed to enhance performance at mild low temperatures may result in tradeoffs in performance at lower temperatures.

4.7 Conclusions and Future Directions

In control *G. pennsylvanicus,* exposure to 0 °C caused a migration of hemolymph, Na^+, and water into the gut of male *G. pennsylvanicus*, which caused an increase in K^+ hemolymph concentration, driving the depolarization of muscle K^+ membrane potentials (E_K) at the muscle tissue. My findings show some of the mechanisms regulating ion and

water movement between compartments during cold exposures and the means by which insects modulate their cold tolerance through changes in gut permeability to ions and water are plastic. Using a long-term exposure to 12 °C, I induced plasticity in the cold tolerance of *G. pennsylvanicus* and created a more cold-hardy phenotype; the short-term exposure treatment induced plasticity in some cold tolerance traits, such as CTmin and CCR, but was unable to induce lasting changes in the mechanisms associated with indirect chilling injury. The shift in cold tolerance as a result of the long-term exposure treatment was accompanied by a shift in the ability to prevent or mitigate the migration of hemolymph Na^+ and water into the digestive system. The changes in cold tolerance of *G. pennsylvanicus* induced by a long-term exposure were not as profound as those induced by cold adaptation in *G. veletis,* a sister species that is evolutionarily more cold-tolerant, suggesting there are considerable costs to low temperature acclimation. However, both low temperature acclimation and cold adaptation resulted in changes in the redistribution of water and ions that enhanced performance at low temperatures; individuals had lower thermal limits, faster recovery times from chill coma inducing temperatures, and higher rates of survival after cold exposures in comparison to control. This confirms there is a link between ion and water balance and the symptoms of indirect chilling injury in the chill susceptible insect *G. pennsylvanicus*.

Future directions for understanding the mechanisms underlying chilling injury should aim to identify some key markers of loss of ion homeostasis that can be measured non-lethally (e.g hemolymph $[K^+]$), and see if they can be correlated with the severity of chilling injury. Future survival assays should aim for a maximum time exposure period that results in high severe injury rates in controls, yet high fitness rates in the acclimated

individuals (much like the discriminating temperatures used in many RCH studies). In the present study, after 120 h at 0 °C most SE and control individuals were dead, while the majority of LE individuals were injured (as opposed to fit). There could be mechanisms involved in ion homeostasis that immediately stop functioning upon death of the individual and obscure trends or patterns of water and ion redistribution. Moreover, future studies for understanding the mechanisms of chilling injury in *G. pennsylvanicus* should endeavour to reduce the effects of desiccation caused by a prolonged exposure to low temperature. This would reduce variance in ion concentration measurements and help clarify some of the discrepancies in hemolymph volume and gut water content seen in this study.

To predict the effects of changing climates on insect distribution and abundance, a clear understanding of the physiological mechanisms underlying thermal thresholds and performance at low temperature, as well as how these thresholds are modified is required. In this study, the induction of plasticity through cold exposure treatments in the cold tolerance of *Gryllus pennsylvanicus*, and the naturally higher cold tolerance of *Gryllus veletis* were used to examine variation in low temperature performance and ion homeostasis. My study shows the ability to maintain ion and water balance as a result of changes in gut membrane permeability enhance cold tolerance, thus increasing performance at low temperatures and reducing the onset of chilling injury.

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