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Mechanisms Underlying Variation in Insect Chill Tolerance: The Role of Ion and Water Transport

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Abstract

Water and ion homeostasis has emerged as an important factor limiting chill-susceptible insects at low temperatures; loss of this homeostasis in the cold likely contributes to chronic chilling injury, and reestablishment of homeostasis is required for recovery from chilling. Both plastic and interspecific variation in cold tolerance correlates with enhanced defense of water and ion homeostasis during cold exposure, however the mechanisms are poorly understood. Using *Gryllus* crickets, I generated and tested hypotheses about the mechanisms underlying this variation in transport function. I first related interspecific variation in cold tolerance to water and ion balance in early chill coma. A rapid influx of Na^+ to the hemolymph suggests that Na^+ first leaks from the tissues, and could drive migration of Na^+ and water to the gut. *Gryllus veletis* (a more cold-tolerant species) may avoid or slow this Na^+ leak by maintaining lower hemolymph Na^+ content and lower osmotic pressure between the gut and hemolymph, compared to *G. pennsylvanicus*. Plasticity in defense of water and ion homeostasis during cold exposure is thought to involve enhanced active transport function and/or decreased permeability of ionoregulatory tissues. Using *G. pennsylvanicus* I identified specific candidate mechanisms related to these transport function modifications by comparing the hindgut and Malpighian tubule transcriptomes of warm- and cold-acclimated individuals. Cold acclimation modified the expression of hindgut and Malpighian tubule ion transporters, and hindgut structural (cytoskeletal and cell junction) genes. Rectal macromorphology and rectal pad scalariform complex ultrastructure were unchanged (suggesting that modified permeability does not involve these structural elements), however cytoskeletal modifications do protect rectal pad actin stability during cold shock. Cold acclimation decreases excretion rate (i.e. active transport) across the Malpighian tubules, which may be driven by modified activity of Na^+-K^+ ATPase but not of V-ATPase. Increased expression of hindgut Na^+-K^+ ATPase did not alter the activity of this enzyme in the rectum. Overall I show that cold acclimation modifies active transport function in the Malpighian tubules and modifies rectal pad structure to enhance cytoskeletal stability during cold exposure.

Keywords

Cricket, *Gryllus,* cold tolerance, cold acclimation, ionoregulation, water balance, Malpighian tubules, hindgut, transcriptome.

Co-Authorship Statement

Chapter 2 was published in the *Journal of Insect Physiology* (see Appendix B for reprint permission). I am the first author, and co-author Brent J. Sinclair (BJS) contributed to experimental conception and design, and writing of the manuscript.

Chapter 3 is in revision at *BMC Genomics*. I am the first author, and Alexander H McKinnon (AHM), Jantina Toxopeus (JS), Hiroko Udaka (HU), and BJS were co-authors. AHM, JT, and HU helped with *de novo* transcriptome assembly and annotation. BJS contributed to experimental conception and design. I drafted the manuscript together with BJS and contributions from all other authors.

Chapter 4 is being prepared for publication. I will be the first author, with Joseph R. Stinziano (JRS) and BJS as co-authors. JRS helped with measurement and analysis of rectal pad ultrastructure and cytoskeletal stability. BJS contributed to experimental conception and design. I drafted the manuscript, with contributions from BJS and JRS.

Chapter 5 is being prepared for publication. I will be the first author, with Soheila Khazraeenia (SK), Natalia Li (NL), and BJS as co-authors. SK performed Ramsay assays and NL helped to adapt the NKA activity assay for cricket hindguts. BJS contributed to experimental conception and design. I drafted the manuscript together with BJS.

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"*Education is the path from cocky ignorance to miserable uncertainty*" – Mark Twain

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AP2	Adaptor-related protein complex 2 subunit α
ARP2/3	Actin-related proteins 2 and 3
bp	Base pair
CA1	Carbonic anhydrase isoform 1
CA9	Carbonic anhydrase isoform 9
CCRT	Chill coma recovery time
CT_{min}	Critical thermal minimum
EGFR/ErbB-1	Epidermal growth factor receptor
e-value	Expectation value
F-actin	Filamentous actin
hsp	Heat shock protein
MAP1A/1B	Microtubule-associated proteins 1A/1B
NHA	Na^+-H^+ exchanger
NKA	Na^+-K^+ ATPase
NKCC	Bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporter
NSF	N-ethylmaleimide-sensitive factor
PAR3	Partitioning defective 3
PKC-α	Protein kinase C α
PKC-i	Protein kinase C iota
RCH	Rapid cold-hardening
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SCP	Supercooling point
TGFβ2	Transforming growth factor β2
TUBA	Tubulin α
V-ATPase	Vacuolar-type H^+ ATPase
VE-PTP	Vascular endothelial protein tyrosine phosphatase

Chapter 1

1 General introduction

1.1 Importance of understanding insect cold physiology

Insects account for most of the eukaryotic ectotherm diversity and biomass in terrestrial and freshwater ecosystems (Costello et al., 2012). This group is important not only for their role in ecosystem function but also for their impacts on global health and economy. Some estimates place the annual global value of pollination services at over $500 billion (Breeze et al., 2016). Ecological services of unmanaged insect populations (e.g. pollination, facilitation of decomposition, pest control, and as a food source) have an estimated annual value exceeding $57 billion in the USA alone (Losey and Vaughan, 2006). Insects also cause great economic loss; they consume 10-16% of pre-harvest agricultural crops globally (Bradshaw et al., 2016) and can have devastating impacts on forest composition. For example, the invasive emerald ash borer has destroyed millions of North American ash trees, costing billions of dollars in management (Herms and McCullough, 2014). Insect disease vectors have large impacts on health, especially in developing countries; mosquito-vectored malaria alone killed nearly half a million people in 2015 (WHO, 2016).

Effective management and distribution forecasting of insect populations requires a firm understanding of their thermal physiology (Hawkins et al., 2007; Somero, 2010; Lehmann et al., 2015). The field of insect cold tolerance gained momentum with foundational studies by Reginald Salt in the mid-1900s (Salt, 1953, 1961) and has expanded rapidly since the 1980s (Zachariassen, 1985; Block et al., 1990; Lee, 1991; Bale, 1993; Sinclair et al., 2003b; Storey and Storey, 2012; Teets and Denlinger, 2013b). Although the literature has classically focused on insect freeze tolerance and freeze avoidance (Salt, 1961; Lee, 1991; Bale, 1993; Hochachka and Somero, 2002; Chown and Nicolson, 2004), recent mechanistic studies are addressing the means by which insects are limited by chilling (i.e. the physiological challenges of cold exposure unrelated to freezing; Baust and Rojas, 1985; Bale, 1987; Sinclair and Roberts, 2005).

As ectotherms, insect body temperatures typically reflect the ambient thermal environment (although some species are heterothermic; see, e.g., Heinrich, 1993). Because reaction rates in biological systems decrease with temperature, so too does the physiology of the insect as a whole (Hochachka and Somero, 2002; Tattersall et al., 2012; Sinclair, 2015). Temperature therefore indirectly regulates ecosystem function via direct effects on insect physiology and performance (Huey and Berrigan, 2001; Bale, 2002; Sunday et al., 2011). Insects in polar, temperate, and alpine zones spend over half of their lives overwintering and are thus challenged with prolonged and/or repeated cold exposures (Block et al., 1990; Hahn and Denlinger, 2007; Marshall and Sinclair, 2012). Cold exposures have variable consequences for insect energetics and homeostasis (Zachariassen, 1991; Lee, 2010; Hahn and Denlinger, 2011; MacMillan et al., 2015a), protein and membrane function (Hochachka and Somero, 2002), reproductive potential, and survival (Marshall and Sinclair, 2009; Williams et al., 2015). These overwintering challenges are further complicated by changing mean temperatures and increased climate variability (especially in the Northern Hemisphere; Hartmann et al., 2013; Scheffers et al., 2016), further necessitating adequate understanding of insect ecophysiology (Sinclair et al., 2003b; Kearney et al., 2009; Williams et al., 2015).

1.2 Strategies of insect cold tolerance

Insects have evolved a multitude of behavioural, morphological, and physiological means of surviving low temperatures. Behaviourally, insects can avoid the cold by migrating (Masters et al., 1988) or by selecting warmer microhabitats (e.g. remaining within soil or under bark; Willmer, 1982). Shivering and or basking (as exhibited by some flies, bees, butterflies, and moths) in addition to flight also help to raise thoracic temperature (Kukal et al., 1988; Masters et al., 1988; O'Neill et al., 1990; Heinrich, 1993; Van Dyck and Matthysen, 1998). Morphologically, darker pigmentation (e.g. in wood butterflies and ambush bugs) can facilitate active mate-searching at lower temperatures (Van Dyck and Matthysen, 1998; Punzalan et al., 2008).

Insects that do not avoid cold exposure (e.g. those overwintering at high latitudes or altitudes) exhibit a variety of physiological strategies to survive the cold. Some species

overwinter in diapause – an endocrine-controlled state of developmental arrest (Danks, 1987; Denlinger, 2002; Hahn and Denlinger, 2011). Although diapausing insects often exhibit enhanced cold tolerance, the mechanistic overlap between these states is unclear (Denlinger, 1991). Species considered most cold tolerant typically employ strategies of freeze tolerance or freeze avoidance (including cryoprotective dehydration; Lee, 1989; Bale, 1993; Ramløv, 2000; Sformo et al., 2010), and some species can switch between both strategies (Horwath and Duman, 1984; Sformo et al., 2009). Freeze avoidance appears to be more common than freeze tolerance, at least in the Northern Hemisphere (Bale, 1993; Sinclair et al., 2003a). Globally, however, a majority of insects are killed or injured at low but above-freezing temperatures and are considered chill-susceptible (Salt, 1961; Bale, 1996). I briefly introduce the physiology of insect freeze tolerance and freeze avoidance strategies before outlining the physiology of chill-susceptible species (which are the focus of this dissertation).

1.2.1 Freeze tolerance

Freeze tolerance has evolved independently in multiple lineages (Sinclair et al., 2003a), and internal freezing (and thawing) presents many physiological challenges for the insect (Lee, 2010). In the generally-accepted model of freeze tolerance, insects survive internal ice formation by confining freezing to the extracellular space (e.g. hemolymph) and controlling the rate and temperature at which that freezing occurs (Zachariassen, 1991; Ramløv, 2000; Chown and Nicolson, 2004). Some insects survive intracellular ice formation (at least in certain tissues; Worland et al., 2004; Sinclair and Renault, 2010; Wharton, 2011) and cells may freeze at the same temperature as the hemolymph (Toxopeus et al., 2016). Mechanisms underlying survival of intracellular freezing have received relatively little attention in the literature (Sinclair and Renault, 2010), but may involve control of ice crystal size (Raymond and Wharton, 2016).

As the extracellular fluid freezes, water is drawn from the cell. The consequent cytosolic dehydration concentrates solutes in the cytoplasm and lowers the supercooling point (SCP) such that intracellular freezing is generally prevented (Duman et al., 1991; Lee, 1991; Zachariassen, 1991; Storey, 1997; Danks, 2006). However, intracellular dehydration

causes osmotic stress, protein denaturation, concentration of toxins (Lee, 1991; Yi and Lee, 2003; Zachariassen et al., 2004), and can damage the membrane and cell structure (Ramløv, 2000). Recrystallization can also mechanically damage proteins and membranes (Storey and Storey, 1988). The temperature at which freezing is initiated matters; lower temperatures mean more rapid ice crystal growth and a larger proportion of body water freezes (causing further dehydration and associated challenges thereof; Duman et al., 1991; Zachariassen, 1991; Wharton, 2011). Damage also depends on the rates of cooling and rewarming; rapid cooling can lead to intracellular ice formation which is often lethal (Raymond and Wharton, 2016), while rapid rewarming can cause rehydration swelling and cell rupture (Gao and Critser, 2000; Dumont et al., 2004). To control the temperature at which freezing occurs, freeze-tolerant insects produce hemolymph ice-nucleating proteins to initiate extracellular freezing at relatively high sub-zero temperatures (e.g. -4°C in spring field cricket nymphs or -5°C in adult hornets; Duman et al., 1984; McKinnon, 2015), allowing ice to grow slowly (Duman et al., 1991; Lundheim and Zachariassen, 1993; Ramløv, 2000). Selection of moist microhabitats can also raise the freezing temperature via inoculation, as contact with external ice initiates internal freezing (Layne et al., 1990; Gehrken et al., 1991).

Water management is important for surviving dehydration stress during freezing; in goldenrod gall fly larvae, aquaporin function is crucial for protection of cell viability during both freezing and thawing (Philip et al., 2008). Insects can also accumulate high (molar) concentrations of sugars and polyols (e.g. glycerol and sorbitol) to limit structural and osmotic stresses of cellular dehydration. For example, high concentrations of hemolymph trehalose or glucose protect isolated Malpighian tubules of New Zealand alpine weta from freezing damage (Neufeld and Leader, 1998). Maintaining membrane function is also important; modification of phospholipid composition may prevent loss of membrane structure during freezing (Koštál et al., 2003), while production of polyols prevent protein denaturation and also stabilize the membrane (Storey and Storey, 1988; Block et al., 1990).

1.2.2 Freeze avoidance

Freeze-avoidant insects are killed by internal ice formation but survive sub-zero temperatures by depressing the SCP (Salt, 1961; Zachariassen, 1985). This strategy allows some insects to avoid freezing to extremely low temperatures (e.g. -54°C in a salpingid beetle, -63°C in a gall fly, or even -100°C in vitrified Arctic bark beetles; Miller, 1982; Ring, 1982; Lee, 1991; Addo-Bediako et al., 2000; Sformo et al., 2010). As in freeze-tolerant insects, survival of freeze-avoidant insects is time- and temperature-dependent; the chance of spontaneous freezing increases as temperatures decrease and cold exposure durations increase (Salt, 1961; Sømme, 1982).

To lower the SCP, freeze-avoiding insects typically increase their body osmolality by accumulating polyols and sugars (Zachariassen, 1991; Zachariassen et al., 2004), salts (Williams et al., 2014), or via dehydration (which effectively increases osmolality; Rickards et al., 1987; Lundheim and Zachariassen, 1993; Danks, 2000). For example, the emerald ash borer accumulates as much as 4 M glycerol in preparation for overwintering, which likely contributes to a 10°C reduction in SCP (Crosthwaite et al., 2011). Freeze-avoiding insects also mask or remove internal ice-nucleators (e.g. by clearing bacteria, fungi, and food particles in the gut; Salt, 1953; Sømme, 1982; Zachariassen, 1985; Lee et al., 1991; Tsumuki et al., 1992), and may select dry microhabitats to prevent inoculative freezing (Layne et al., 1990; Gehrken et al., 1991). Modification of cuticular waxes function to prevent internal freezing following contact with external nucleators (Olsen et al., 1998). In the event of spontaneous nucleation, accumulation of thermal hysteresis factors (e.g. antifreeze proteins) help freeze-avoidant insects to suppress ice crystal growth (DeVries, 1982; Zachariassen, 1991; Sinclair and Chown, 2002). Antifreeze proteins also help to lower the probability of freezing by stabilizing supercooled hemolymph (Zachariassen and Husby, 1982).

1.2.3 Chill susceptibility

We expect insects overwintering in exposed microhabitats or particularly cold regions to exhibit freeze avoidance and freeze tolerance strategies. However many species in such

regions overwinter in insulated habitats (under snow, soil, leaf litter, bark etc.; Willmer, 1982; Danks, 2006; Sformo et al., 2010; Udaka and Sinclair, 2014; Sinclair, 2015) or overwinter as embryos which have naturally low SCPs (due to their small size and impermeability; Sømme, 1964, 1982; Bale, 1993; Jing and Kang, 2003, Sinclair et al. 2003a). Many of these insects are chill susceptible, whereby injury or mortality from mild cold exposure is unrelated to ice formation or supercooling (Bale, 1993; Lee, 2010).

The activity of chill-susceptible insects is bounded at low temperatures by the critical thermal minimum (CT_{min}), below which insects enter chill coma, a reversible neuromuscular paralysis (Hosler et al., 2000; Gibert et al., 2001; Hazell and Bale, 2011; Findsen et al., 2014; Andersen et al., 2015). The time required to regain neuromuscular function upon rewarming (e.g. display correct posturing) is termed the chill coma recovery time (CCRT). During cold exposure, chill-susceptible insects can accumulate chilling injuries which manifest as loss of coordination (Koštál et al., 2006), behavioural changes (Yocum et al., 1994), disruption of development (Rojas and Leopold, 1996), and/or death (Chen et al., 1987; Koštál et al., 2004). The $CT_{min,}$ CCRT, and chilling injury are commonly-used cold tolerance metrics for chill-susceptible insect populations (MacMillan and Sinclair, 2011a; Kellermann et al., 2012; Andersen et al., 2014), and they appear to driven by interrelated but distinct underlying processes (Ransberry et al., 2011).

Insects in chill coma gradually lose ion and water homeostasis. Hemolymph Na^+ (as well as Ca^{2+} and Mg^{2+}) migrates to the gut lumen, driving a similar migration of water. This loss of hemolymph volume consequently raises hemolymph $[K^+]$ (Koštál et al., 2004; MacMillan and Sinclair, 2011b; Coello Alvarado, 2012; Findsen et al., 2014; MacMillan et al., 2014). High hemolymph $[K^+]$ during cold exposure was previously thought to explain muscular paralysis (MacMillan and Sinclair, 2011b), however chill coma onset – a relatively rapid process – precedes substantial imbalance of hemolymph K^+ (which increases gradually over hours to days; MacMillan and Sinclair, 2011b; Armstrong et al., 2012; MacMillan et al., 2014). Low temperature also directly inhibits muscle excitability (Wareham et al., 1974; MacMillan et al., 2014) and, in the migratory locust, accounts for a greater loss of muscle tetanic force than does increased extracellular $[K^+]$ (Findsen et al., 2014).

Why insects lose ion and water balance during cold exposure is not understood, but this loss of balance suggests a failure of transport function. Transporting epithelia rely on electrochemical gradients established by active ion transport enzymes (Shaw and Stobbart, 1963; Phillips et al., 1987). As temperatures decrease, so too do the reaction rates of these enzymes according to their temperature coefficient (Q_{10}; Hochachka and Somero, 2002; Nespolo et al., 2003), while passive leak may be relatively unaffected (Fig. 1.1A). We therefore expect ionic (and osmotic) gradients to be lost over time at low temperatures, assuming that the cold does not substantially alter epithelial permeability (but see Motais and Isaia, 1972; Dokladny et al., 2006; Ionenko et al., 2010). The CT_{min} thus appears to approximate the theoretical threshold below which an insect fails to maintain transport function (in addition to marking the temperature of neuromuscular silencing).

Recovery from chill coma corresponds with the re-establishment of water and ion homeostasis (MacMillan et al 2012). A low CT_{min} typically correlates with shorter CCRT (Ransberry et al., 2011), however the process of chill coma entry is somewhat complementary to that of chill coma recovery. Rewarming of the body to temperatures required for generation of muscular action potentials typically precedes an insect's CCRT (i.e. the actual regaining of neuromuscular function; Macdonald et al., 2004). As ion and water imbalance is progressive during cold exposure, increasing cold exposure durations are reflected by longer CCRTs (Macdonald et al., 2004; MacMillan et al., 2012). The CCRT therefore approximates the relative rate at which insects restore water and ion balance (Koštál et al., 2007; MacMillan et al., 2012). If cold exposure is deep or prolonged, insects may be unable to restore homeostasis and can accumulate chilling injuries (or fail to recover entirely; Lavy and Verhoef, 1998; Gibert et al., 2001; Macdonald et al., 2004; MacMillan, 2013).

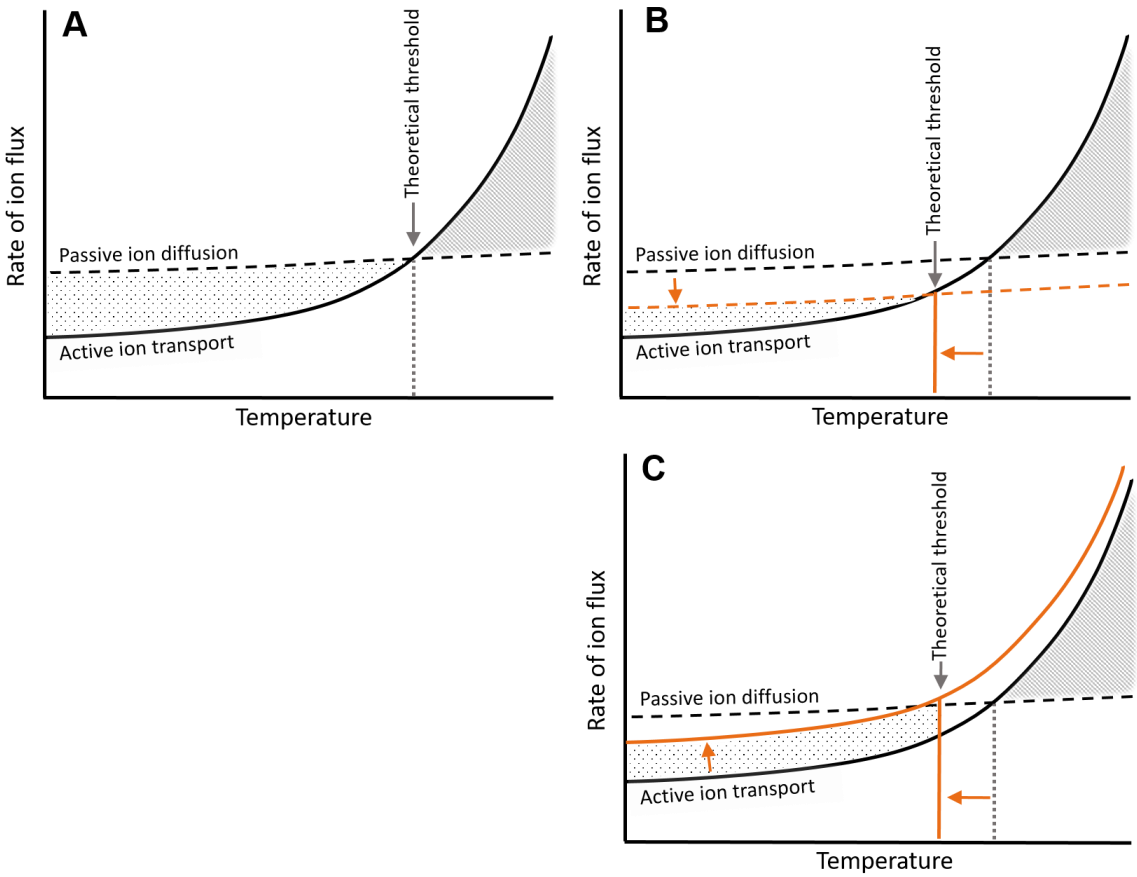

Figure 1.1. **Conceptual models for loss of homeostasis in chill-susceptible insects during cold exposure (A) and mechanisms by which cold acclimation may alter transport function to defend homeostasis (B, C).** The theoretical threshold temperature at which passive ion diffusion exceeds active ion transport could be lowered by reducing passive diffusion or by increasing active transport at lower temperatures (indicated in orange on panels B and C). Modified from MacMillan and Sinclair (2011a).

We understand relatively little about mechanisms underlying injuries unrelated to freezing (Sinclair and Roberts, 2005; MacMillan et al., 2015c), and the nature of such injuries also differs depending on the time and temperature of cold exposure. Brief cold shock causes acute (direct) injury while longer, milder cold exposures lead to chronic (indirect) injuries (Macdonald et al., 2004; Sinclair and Roberts, 2005). Potential mechanisms of acute injury include apoptosis, protein misfolding, and damage to DNA, the cell membrane, and

cytoskeleton (Ramløv, 2000; Yi et al., 2007; MacMillan et al., 2009; Teets et al., 2012; Yao and Somero, 2012; Štětina et al., 2015).

Chronic chilling injuries are accumulated gradually over days to months (Lee, 2010; Koštál et al., 2011; MacMillan, 2013), and proposed mechanisms include energy depletion and build-up of metabolic wastes (Koštál et al., 2011; Teets and Denlinger, 2013b). In addition to membrane damage and oxidative stress (Rojas and Leopold, 1996; Lalouette et al., 2011) osmotic stress (e.g. ion imbalance) during prolonged cold exposure is also thought to contribute to chronic chilling injury (Koštál et al., 2006; Lee, 2010; Findsen et al., 2014). Loss of ion balance leads to failure of trans-membrane potentials (e.g. neuromuscular impairment) and disruption of signaling pathways (which can initiate apoptosis; Lee, 1991; Heimlich et al., 2004; Michaud and Denlinger, 2004; Koštál et al., 2007; Teets et al., 2013; MacMillan et al., 2015c). Loss of transport function following enzyme misfolding and cytoskeletal failure (e.g. actin depolymerization) at low temperatures can further exacerbate loss of water and balance (Khurana, 2000; Kim et al., 2006; Kayukawa and Ishikawa, 2009). The gut – which is an important regulator of ion and water balance – may be particularly susceptible to damage from cold exposure (at least with freezing; Izumi et al., 2005; Teets et al., 2011). Re-establishment of ion and water balance is necessary for recovery, but is energetically costly (Koštál et al., 2007) and therefore has reproductive and fitness consequences for overwintering insects (Marshall and Sinclair, 2009; Arrese and Soulages, 2010).

1.3 Plasticity of cold tolerance

Insects can modify their cold tolerance in response to thermal conditions, and the nature and extent of this plasticity varies among lineages (Lee et al., 1987; Koštál et al., 2007; Findsen et al., 2013; Foray et al., 2013; Kvist et al., 2013; Jakobs et al., 2015; MacMillan et al., 2015d; Schoville et al., 2015). In some cases, plasticity can even account for a larger proportion of cold tolerance variation than do interspecific (adaptive) differences (Ayrinhac et al., 2004; Hoffmann et al., 2005; Koštál et al., 2012; McKinnon, 2015). This plasticity can be invoked by prior chilling at both short- and long-term scales (Chen et al.,

1987; Sinclair and Roberts, 2005; Somero, 2010). Mild cold exposures on the scale of hours (and in some cases in as little as ten minutes) can improve insect cold tolerance by the process of rapid cold-hardening (RCH; Lee et al., 1987; Chen et al., 1987). In the process of cold acclimation (akin to seasonal acclimatization), cold tolerance is enhanced by mild chilling over days, weeks, or months (Ding et al., 2003; Sinclair and Roberts, 2005; Rako and Hoffmann, 2006; Coello Alvarado et al., 2015). Insects can also be deacclimated by exposure to warmer conditions; simulated winter warm-snaps increase the SCP and lower hemolymph cryoprotectant content in Anise swallowtails and (irreversibly) in freeze-avoiding emerald ash borers (Sobek-Swant et al., 2012; Williams et al., 2014).

1.3.1 Rapid cold-hardening

RCH lowers the CT_{min} (Overgaard et al., 2005), can reduce the time required to re-establish homeostasis upon recovery (i.e. reduce the CCRT; Overgaard et al., 2005; Findsen et al., 2013), and improves survival of acute and chronic cold exposure (Chen et al., 1987; Lee et al., 1987; Yi et al., 2007). This swift plastic response likely prepares insects for the physiological challenges of daily temperature fluctuations or cold fronts (Gerken et al., 2015). The mechanisms of RCH remain poorly understood, however a number of candidate mechanisms are proposed (Teets et al., 2012; Teets and Denlinger, 2013b; Gerken et al., 2015). For example, RCH can increase hemolymph osmolality, glycerol content (Chen et al., 1987), and membrane fatty acid unsaturation (Overgaard et al., 2005; Michaud and Denlinger, 2007), which may account for a decreased SCP and protection against loss of membrane fluidity, respectively. However, these changes with RCH are not necessarily consistent across species (MacMillan et al., 2009). RCH can also alter or indirectly influence ion transport, signaling, apoptosis and autophagy, and the cytoskeleton (at least, in *Drosophila melanogaster*; Overgaard et al., 2005; Teets et al., 2012; Gerken et al., 2015) which may help to maintain homeostasis, tissue structure, and repair chilling injuries.

1.3.2 Cold acclimation

Cold acclimation regimes typically involve constant exposure to low temperatures (but may mimic fall-like conditions of decreasing or fluctuating temperatures and sometimes shorter photoperiods; Gibert and Huey, 2001; Koštál et al., 2006; Koštál et al., 2007; Lachenicht et al., 2010; Coello Alvarado et al., 2015; Jakobs et al., 2015; Sinclair et al., 2015; MacMillan et al., 2016), but may also include dietary manipulation (Koštál et al., 2012; McKinnon, 2015). These manipulations decrease the CT_{min} and CCRT, improve survival from cold exposure (Gibert and Huey, 2001; Ayrinhac et al., 2004; Sinclair and Roberts, 2005; MacMillan and Sinclair, 2011b; Coello Alvarado, 2012; MacMillan et al., 2015b). Enhancement of cold tolerance by acclimation can be more substantial than by RCH (at least for some metrics; Gibert and Huey, 2001; Ayrinhac et al., 2004; Sinclair and Roberts, 2005; Rako and Hoffmann, 2006; Ransberry et al., 2011). Cold acclimation can even confer freeze-tolerance to otherwise chill-susceptible *D. melanogaster* and spring field crickets (Koštál et al., 2012; McKinnon, 2015). RCH and cold acclimation can act synergistically (Gerken et al., 2015) or antagonistically (Rajamohan and Sinclair, 2009). For example, rearing temperature explains a greater variation in the CT_{min} and CCRT than does population latitude in *D. melanogaster* (Addo-Bediako et al., 2000; Gibert and Huey, 2001). The mechanistic overlap between cold acclimation and RCH is therefore not entirely clear (Sinclair and Roberts, 2005; Rako and Hoffmann, 2006; Teets and Denlinger, 2013b; Gerken et al., 2015).

Mechanisms underlying cold acclimation are not well-understood in general (Gerken et al., 2015), but this process should involve physiological modifications that either protect against chilling injury and/or facilitate repair of injuries upon rewarming. A number of studies have provided hints as to the potential mechanisms; in addition to changing membrane composition, cold acclimation alters expression of heat shock proteins (hsps), cryoprotectants, apoptosis and autophagy factors, cytoskeletal components, and ion transporters (Lee, 1991; Hazel, 1995; Chown and Nicolson, 2004; Loeschcke and Sørensen, 2005; Teets et al., 2012).

Modifications to maintain membrane fluidity in the cold (e.g. decreased fatty acid saturation) protect against acute chilling damage in cold-acclimated arctiid moths and flies *Chymomyza costata*, *Delia antiqua*, and *D. melanogaster* (Koštál and Simek, 1998; Koštál et al., 2003; Overgaard et al., 2005; Kayukawa et al., 2007). Maintenance of membrane fluidity at lower temperatures – which is important for water and ion transport function – also likely protects against chronic chilling injuries (Gonzalez-Mariscal et al., 1984; Khurana, 2000; section 1.4.3). In addition to their roles in freeze-tolerance and freeze-avoidance, cryoprotectants (e.g. sugars, polyols, and amino acids) accumulated during cold acclimation can also stabilize the membrane, enzymes, and other proteins to prevent chilling damage (Lee, 1991; Overgaard et al., 2007).

Expression of hsps can prevent chilling injuries not only by maintaining protein folding but also by stabilizing the cytoskeleton and preventing cell death (Huot et al., 1996; Russotti et al., 1997; Sonoda et al., 2006; Štětina et al., 2015). For example, suppression of *hsp70* and *hsp23* decreases cold survival in *Sarcophaga crassipalpis* flies (Rinehart et al., 2007), and *hsp70* among others inhibits stress-induced apoptosis (Yi et al., 2007). Hsps are also important for repair of damage; RNA-interference of *hsp70* in *Pyrrhocoris apterous* bugs hinders repair of chilling injuries (Koštál and Tollarová-Borovanská, 2009). Acclimation to cold or fluctuating temperatures increases expression of genes involved in repair of DNA and oxidative damage in *D. melanogaster* and alfalfa leaf cutter bees (Torson et al., 2015; MacMillan et al., 2016). Upregulation of genes promoting apoptosis and autophagy likely function to clear cold-damaged cells and cellular components, respectively (Teets and Denlinger, 2013a; Gerken et al., 2015). However, cold-hardening inhibits apoptotic cell death following cold shock in *D. melanogaster* (Yi et al., 2007).

As the cytoskeleton is involved in many of the aforementioned processes which are thought to underlie chilling injury, it is not surprising that acquired cold tolerance (i.e. RCH or acclimation) often correlates with changes in the expression of cytoskeletal genes (Teets et al., 2012; Gerken et al., 2015; Torson et al., 2015; MacMillan et al., 2016). These changes may act to protect cell volume and transport across the membrane (Cantiello, 1995; Khurana, 2000; Pedersen et al., 2001), tissue integrity and permeability (Madara et al.,

1986; Hartsock and Nelson, 2008), and regulate autophagy (Monastyrska et al., 2008; Monastyrska et al., 2009; Lee and Yao, 2010; Aguilera et al., 2012).

Recovery from cold exposure and avoidance of chilling injury are governed in part by water and ion homeostasis (see section 1.2.3). Cold-acclimated insects defend transport function to lower temperatures (Koštál et al., 2004; Koštál et al., 2006; MacMillan et al., 2015a) and recover water and ion balance faster upon rewarming, compared to warm-acclimated conspecifics (Rako and Hoffmann, 2006; Ransberry et al., 2011; Coello Alvarado et al., 2015). In this dissertation I focus on understanding the mechanisms underlying changes in transport function in cold-acclimated insects (and, to a lesser extent, the means by which cold acclimation defense against chilling injury). The mechanisms underlying modified transport function in cold-acclimated insects are introduced in more detail in section 1.4.3).

1.4 Regulation of ion and water balance

In insects, ion and water balance are regulated by the Malpighian tubules (which produce the primary urine) and the hindgut (where water and ions are selectively reabsorbed). Much of our basic understanding about these processes in terrestrial insects is derived from studies in locusts (Dow, 1981; Chamberlin and Phillips, 1982; Hanrahan and Phillips, 1982; Morgan and Mordue, 1985; Phillips et al., 1987; Phillips and Audsley, 1995; Findsen et al., 2014) and, as Orthoptera is the focus of this dissertation, the following sections pertain primarily to water and ion regulation in this group.

1.4.1 Primary urine production by the Malpighian tubules

The Malpighian tubules excrete water, ions, toxins, and metabolic wastes (the primary urine), and transport water and ions at some of the highest rates known among animals (e.g. the Malpighian tubules of blood-fed *Rhodnius prolixus* bugs turn over total cellular Cl^- and water volume every 5 and 15 seconds, respectively; Berridge, 1972; Beyenbach, 2003; O'Donnell and Simpson, 2008). Malpighian tubules may be simple or differentiated

into distal/main (secreting) or proximal (reabsorbing) regions (Irvine, 1969). Many endopterygotes including dipterans, hymenopterans, and lepidopterans possess two functionally-distinct tubule cell types: the numerous principal cells (responsible for water and cation transport, Fig. 1.2) and stellate cells (specialized for transcellular water transport and acting as Cl^- shunts; O'Donnell et al., 1996; Coast, 2012; Halberg et al., 2015). Evidence of stellate cells in Orthoptera, Hemiptera, and Coleoptera is limited (Halberg et al., 2015); transport of water and ions in these lineages may occur across an epithelium composed of a single (principal) cell type. Transport across the Malpighian tubules is also regulated by a suite of neuropeptides which differ depending on taxonomic group and target cell (principal or stellate; O'Donnell et al., 1996; Coast, 2007; Halberg et al., 2015).

The primary urine is isosmotic to the hemolymph (Ramsay, 1954), however excretion by the Malpighian tubules relies on local osmotic and electrochemical gradients established by facilitated, unidirectional cation transport at the folded apical border of principal cells (Berridge and Oschman, 1969; Pannabecker, 1995). First, basolateral Na^+-K^+ ATPase (NKA) generates a high extracellular (hemolymph) [Na^+], favoring secondary active transport of Na^+, K^+, and Cl^- from the hemolymph into the cell by Na^+-K^+-Cl^- (NKCC), K^+ channels, and K^+-Cl^- cotransporters (Ianowski and O'Donnell, 2004). Intracellular carbonic anhydrase (CA) catalyzes the hydration of CO_2 to produce carbonic acid (H_2CO_3), which spontaneously dissociates into a proton and bicarbonate (Edwards and Patton, 1967; Maddrell and O'Donnell, 1992; Wessing et al., 1997; Chintapalli et al., 2013). Bicarbonate is used to import hemolymph Cl^- via basolateral Cl^--HCO_3^- exchangers (Coast, 2012). Protons are exported to the lumen by apical H^+-ATPase, creating a lumen-positive transcellular voltage potential (Wessing et al., 1997; Coast, 2009; Harvey, 2009; Chintapalli et al., 2013). Lumenal protons are then exchanged for intracellular Na^+ or K^+ by cation-H^+ antiporters, resulting in a net cation secretion (Klein, 1992; Maddrell and O'Donnell, 1992; Harvey, 2009). Para- and transcellular Cl^- transport is passive and driven by the lumen-positive transcellular voltage potential (Nicolson, 1993; Coast, 1998; Ianowski and O'Donnell, 2006). Passive movement of water to the lumen by trans- or paracellular routes is favorited by transcellular osmotic gradients (O'Donnell and Maddrell, 1983; Collier and O'Donnell, 1997; Spring et al., 2009; Coast, 2012).

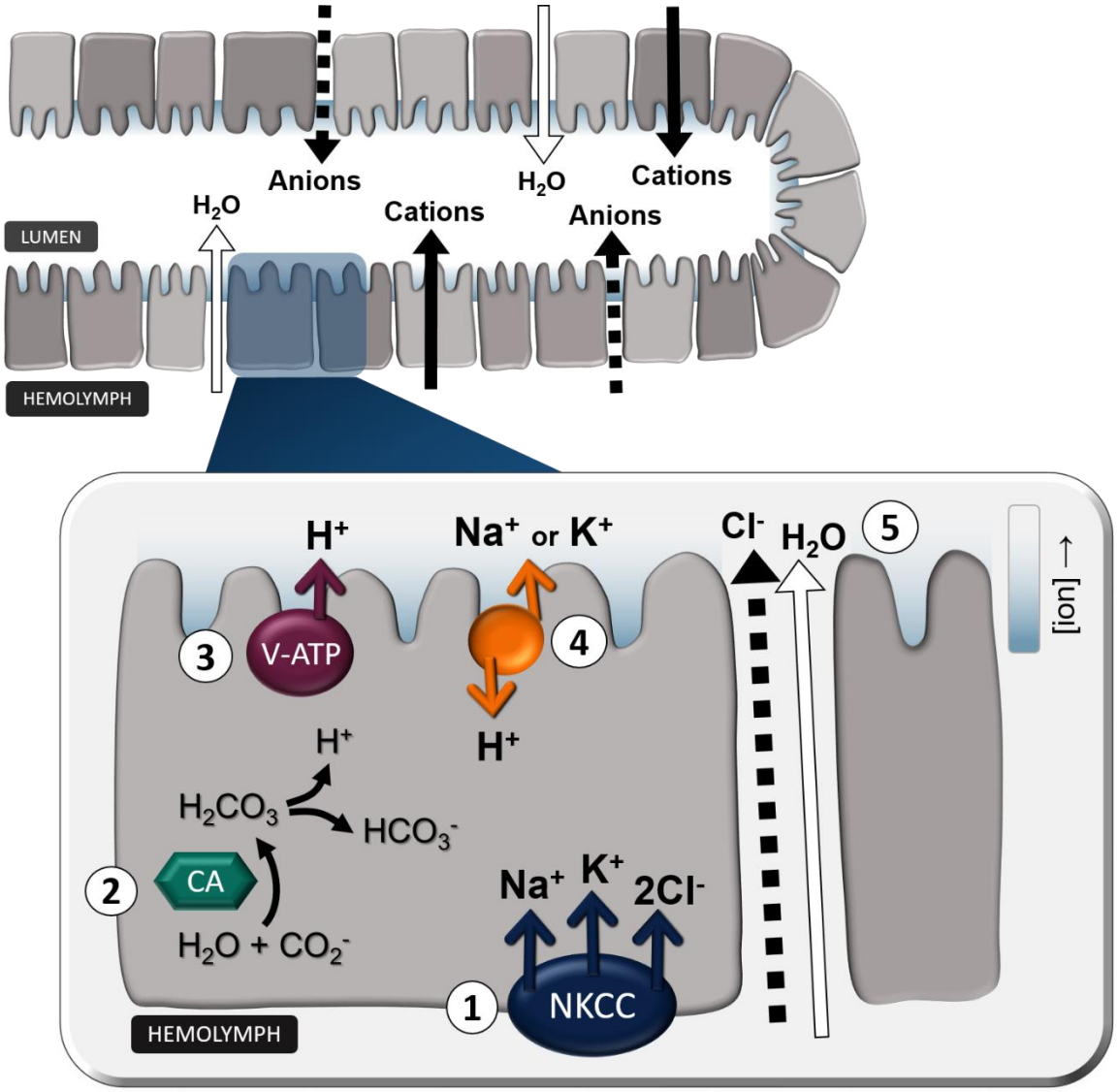

Figure 1.2. A simplified schematic of water and ion transport across the distal Malpighian tubules of an endopterygote insect. Na^+, K^+ and Cl^- enter the principal cell via basolateral Na^+- K^+-$2Cl^-$ cotransporter (NKCC) among other transporters (1). Carbonic anhydrase (CA) facilitates the production of protons which are then pumped into the lumen by apical V-ATPase (V-ATP) (2,3). Intracellular Na^+ and K^+ are transported to the lumen in exchange for protons (4). The lumen-positive voltage potential drives Cl^- (via paracellular shunt), while the osmotic gradient favors movement of water to the lumen (via paracellular routes as well as transcellularly via aquaporins) (5).

The ratio of K^+ to Na^+ in the primary urine may be modified depending on hemolymph composition and diet (e.g. K^+ is the dominant excreted ion in herbivorous insects, while some blood-feeders favor excretion of Na^+; Berridge and Oschman, 1969; Irvine, 1969; Maddrell and O'Donnell, 1992). In some species the primary urine is also partially modified by reabsorption of water and ions at the proximal Malpighian tubule (Beyenbach, 1995; Coast, 1998; Beyenbach, 2003). Urine mixes with the gut contents at the anterior hindgut, and this mixture is further modified by selective reabsorption across the ileum and rectum before voiding (Coast, 2007; O'Donnell and Simpson, 2008).

1.4.2 Reabsorption across the rectum

Conservation of water, ions, and other solutes is achieved by selective reabsorption across the hindgut. The final excreta may be hypo- or hyperosmotic to the hemolymph, as the proportion of gut water or ions recovered can range from 10 to 90% depending on the needs of the insect (Wall and Oschman, 1970; Phillips et al., 1987; O'Donnell and Simpson, 2008). In most insects the ileum reabsorbs some water and ions but is a major site of pH regulation (whereby protons, carried as NH_4^+, are exported in exchange for Na^+; Phillips et al., 1987; Phillips et al., 1988; O'Donnell and Simpson, 2008). The majority of water and ion reabsorption occurs across the rectal epithelium, which may be specialized as rectal papillae (e.g. in blowflies) or rectal pads (in cockroaches and orthopterans; Phillips, 1964; Oschman and Wall, 1969; Berridge, 1972; Phillips et al., 1987). Reabsorption across the rectum is regulated by a suite of diuretic and antidiuretic peptides (for more details, see Schooley et al., 2012).

The orthopteran rectum is comprised of six rectal pads composed of well-tracheated, thickened, pseudostratified columnar epithelium (Oschman and Wall, 1969; Berridge, 1972). The rectum is lined by thin, porous, unsclerotized cuticle that is permeable to water, amino acids, and monovalent ions (as well as Ca^{2+} and Mg^{2+}) but which excludes larger waste products (Phillips et al., 1987). Below the cuticle the apical plasma membrane is in-folded and rich in mitochondria (Oschman and Wall, 1969). In the most apical and basal cell regions, adjacent cells are closely-apposed by tight and septate (or adherens) junctions (Satir and Gilula, 1973; Phillips et al., 1987; Tepass et al., 2001; Matter and Balda, 2003).

In the mid-cell region, invaginated lateral cell borders form open, convoluted (stacked), intercellular channels (Wall and Oschman, 1970). These convoluted plasma membranes are packed with mitochondria, together forming the 'scalariform complex' (Hanrahan and Phillips, 1982; Chapman, 2013; Fig. 1.3). Basally, channels of the scalariform complex open into epithelial sinuses (Wall and Oschman, 1970; Phillips et al., 1987). Absorbate from the sinuses is further filtered by the basal lamina and must pass through valves in the underlying circular muscle to reach the hemolymph (Oschman and Wall, 1969).

Active transport of Na^+, K^+, and Cl^- establishes local osmotic gradients in the rectal pad, driving passive uptake of water from the lumen (despite an opposing osmotic gradient between the lumen and hemolymph; Wall and Oschman, 1970; Phillips et al., 1987). Chloride from the gut lumen is transported into the rectal epithelial cells by an apical Cl^- pump (the activity of which is regulated by neuropeptides acting via cAMP; Phillips et al., 1988; Coast, 2007; O'Donnell and Simpson, 2008). This negative intracellular voltage potential favors passive influx of Na^+ and K^+ through apical channels (Phillips et al., 1987; Phillips and Audsley, 1995). Influx of K^+ from the lumen is also favored by a steep pre-existing ionic gradient, as lumen $[K^+]$ is much higher than the hemolymph (Phillips et al., 1987). At the scalariform complex, NKA pumps intracellular Na^+ into the meandering paracellular channels in exchange for extracellular K^+ (Lechleitner and Phillips, 1988). This high $[Na^+]$ within the channels drives paracellular migration of water from the lumen to the hemolymph (Phillips et al., 1987). Solute reabsorption across the rectal pads is predominately transcellular (Phillips et al., 1987), however some ions and amino acids are swept to the hemolymph along with water (Berridge, 1972).

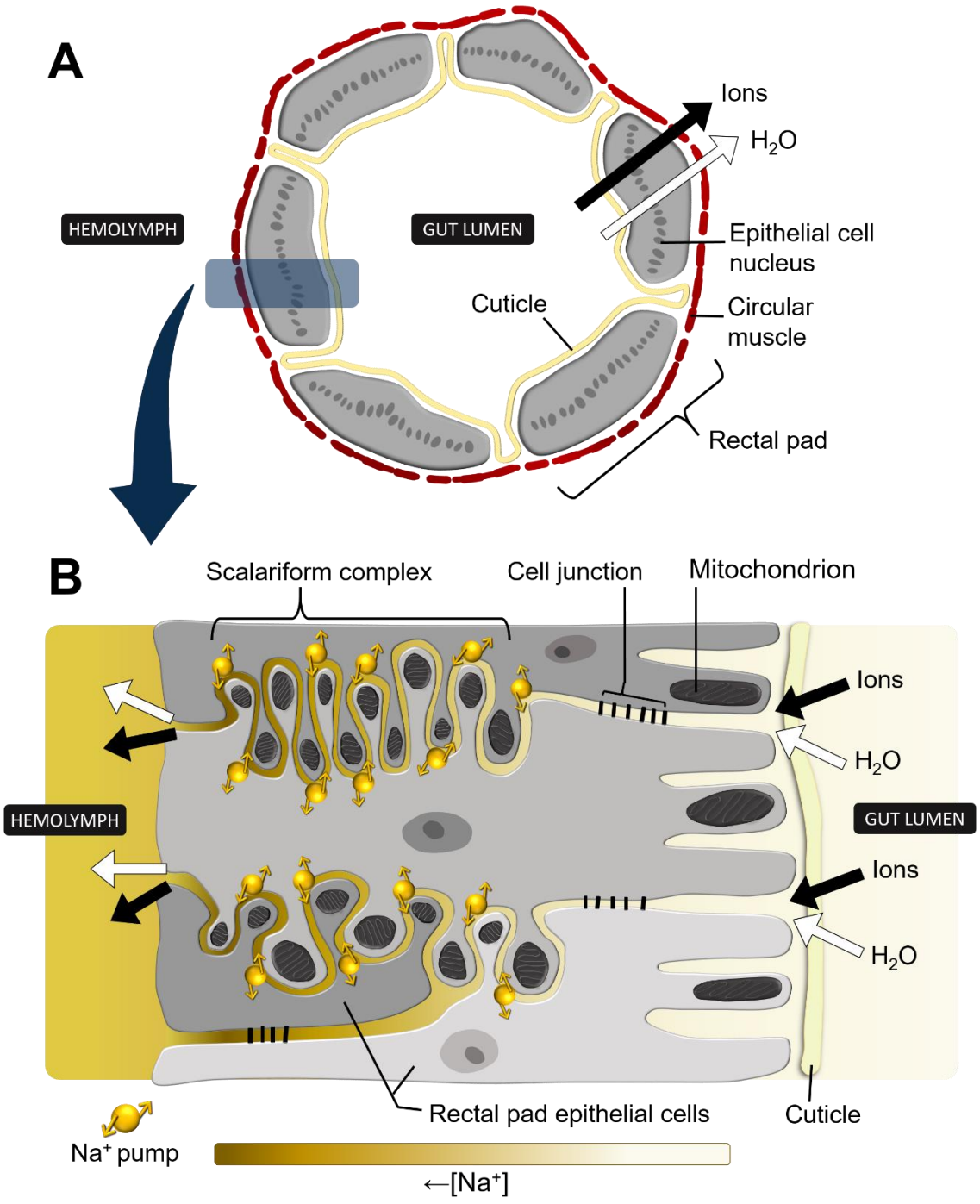
A
Ions
H_2O
HEMOLYMPH
GUT LUMEN
Epithelial cell nucleus
Cuticle
Circular muscle
Rectal pad
B
Scalariform complex
Cell junction
Mitochondrion
Ions
H_2O
HEMOLYMPH
GUT LUMEN
Ions
H_2O
Rectal pad epithelial cells
Cuticle
Na^+ pump
←[Na^+]

Figure 1.3. (above) A schematic of the rectum of an orthopteran insect (in cross section) (A) with a simplified schematic of water and ion transport across the rectal pad epithelium (B, detail). The basolateral borders of the epithelial cells are mitochondria-dense and form meandering paracellular channels lined with Na^+-K^+ ATPase (Na^+ pump), together comprising the scalariform complex. High [Na^+] within the channels (established by the Na^+ pump) favors migration of water from the lumen to the hemolymph (otherwise against osmotic and ionic gradients between the hemolymph and gut). Some extracellular Na^+ in the basolateral region is recycled into the cell. An apical semi-permeable cuticle filters the absorbate.

1.4.3 The role of cold acclimation in transport function

Because transport is temperature-sensitive, one might reasonably predict that cold acclimation modifies transport processes to maintain that function in the cold, but our current understanding of any such modifications is incomplete (Gibert and Huey, 2001; Ransberry et al., 2011; Findsen et al., 2013; MacMillan et al., 2015a). The severity of ion and water imbalance also impacts both chill coma recovery and chilling injury, and cold acclimation confers chill hardiness by improving defense of homeostasis at low temperatures and/or allowing faster re-establishment of homeostasis upon recovery. Terhzaz et al. (2015) recently showed that a neuropeptide (capa) which mediates water and ion balance is important for recovery from cold stress in *Drosophila*, however the role of the endocrine system during cold acclimation is generally not well explored and not addressed in this dissertation. I instead focus on the tissues regulating homeostasis (the rectum and Malpighian tubules), which are obvious targets for modification during the cold acclimation process.

Maintenance of transport function at low temperatures in the cold-acclimated hindgut and Malpighian tubules is likely achieved by either of two mechanisms: 1) by reduced rates of diffusion (Fig. 1.1B) or 2) by enhanced active transport to exceed passive diffusion (Fig. 1.1C). Here I address these hypotheses based on the following rationale: reduction in passive diffusion – which has received very little attention – likely involves modifications to epithelial structure that decrease ion and water permeability, e.g. via changes to cellular adhesion or the cytoskeleton (Gonzalez-Mariscal et al., 1984; Belous, 1992; Behrens et al., 1993; Turner et al., 1997; Kim et al., 2006). Because the primary urine is isosmotic to the

hemolymph, I do not expect Malpighian tubule permeability to change appreciably during cold acclimation. Instead, modification of transport function in cold-acclimated Malpighian tubules is more likely to reflect changes in active transport. Active transport rates could be maintained at low temperatures by increasing the expression, recruitment, and/or activity of active ion pumps (Koštál et al., 2007; McMullen and Storey, 2008; MacMillan et al., 2012; MacMillan et al., 2015a; MacMillan et al., 2015d). As the gut maintains ionic and osmotic gradients between the hemolymph and lumen, leak of water and Na^+ during cold exposure should occur by way of hindgut (or midgut) epithelium (Treherne, 1967). Epithelial permeability is therefore a likely target for modification during the cold acclimation process in the hindgut. Because reabsorption across the rectal pads relies on active transport, cold acclimation may also act to modify the function of ion transporters (e.g. NKA) at low temperatures.

1.5 Dissertation overview

In this dissertation I focused on generating and testing hypotheses about candidate mechanisms underlying a) the loss of ion and water homeostasis in chill-susceptible insects during cold exposure, and b) variation in cold tolerance as related to ion and water homeostasis. I also generate and test some candidate mechanisms of chilling injury. My approach was to exploit both plastic (intraspecific) and interspecific variation in insect cold tolerance and relate that variation to differences in transport function (active and passive; Fig. 1). For this work I used chill-susceptible adult female *Gryllus* crickets (Orthoptera: Gryllidae). To investigate interspecific variation (Chapter 2) I compared *Gryllus pennsylvanicus* (the fall field cricket) and *G. veletis* (the spring field cricket). These species are emerging models for studies of insect cold tolerance plasticity and homeostasis at low temperatures (MacMillan and Sinclair, 2011b; MacMillan et al., 2012; Coello Alvarado et al., 2015; McKinnon, 2015). *Gryllus pennsylvanicus* has an obligate embryonic diapause and is less cold-tolerant than *G. veletis* (which has a late-instar facultative diapause). I investigate cold tolerance plasticity by comparing *G. pennsylvanicus* acclimated to fall-like conditions (12°C and 10 h day length) to those acclimated to summer-like conditions

(25°C, 14 h day length; Chapters 3-5). Cold acclimation to fall-like conditions improves defense of ion and water homeostasis, lowers the CT_{min}, and improves survival following cold exposure (Coello Alvarado et al., 2015).

In Chapter 2 I explored ion and water balance in the early stages of cold exposure 1) to understand how rapidly homeostasis is lost, 2) to determine whether adaptive variation in cold tolerance corresponds with defense of homeostasis, and 3) to use the patterns of ion and water balance in *Gryllus* spp. to generate hypotheses about the mechanisms underlying loss of homeostasis and the means by which more cold-tolerant species defend homeostasis. I found that hemolymph Na^+ balance is lost rapidly during the first hour of cold exposure, and may result from leak of Na^+ from the tissues. Patterns of hemolymph [Na^+] differed from those in later (>12 h) stages of cold exposure, suggesting multiple processes underlying loss of ion and water balance. Patterns of ion and water balance during early cold exposure were similar for the two cricket species and therefore did not reflect interspecific variation in cold tolerance. However, ion and water gradients pre-cold exposure did differ between the two species.

In Chapter 3 I used a tissue-specific comparative transcriptomic approach to generate candidate mechanistic hypotheses about cold tolerance plasticity. Specifically, I compared the Malpighian tubule and hindgut transcriptomes of warm- and cold-acclimated *G. pennsylvanicus*. Differential gene expression profiles suggest that cold acclimation protects against a loss of hemolymph volume and Na^+ in the cold by three potential mechanisms: 1) by lowering primary urine production rates via reduced expression of V-ATPase (and perhaps CA) in the Malpighian tubules, 2) by increasing water and Na^+ uptake across the hindgut via increased expression of NKA, and 3) by restructuring of the epithelium to prevent ion and water leak. Cell or tissue restructuring and altered transport function could also protect from direct and indirect chilling injury, respectively. I used the candidate cold tolerance genes identified in this chapter to direct my approach for Chapters 4 and 5.

In Chapter 4 I aimed to address the hypothesis that cold acclimation alters ion and water diffusion (leak). In the context of tissue permeability (and chilling injury) I quantified the cell and tissue structural changes accompanying cold acclimation by comparing warm- and

cold-acclimated *G. pennsylvanicus* for hindgut structural differences at three levels: 1) macromorphology of the rectum (via brightfield microscopy), 2) ultrastructure of the rectal pad scalariform complexes (via transmission electron microscopy), and 3) stability of the actin cytoskeleton following a cold shock (via fluorescence confocal microscopy). I found no effect of cold acclimation on macromorphology or scalariform complex ultrastructure, however cold acclimation protected (and even enhanced) filamentous actin following cold shock. I discuss how protection of cytoskeletal structure at low temperatures can both maintain transport function and prevent chilling injury.

In Chapter 5 I test the hypothesis that cold acclimation alters active transport function. Using warm- and cold-acclimated *G. pennsylvanicus*, active transport function across the Malpighian tubules was first quantified by Ramsay assay. I found that cold acclimation decreased primary urine production rates across a broad range of temperatures, indicating a decrease in active ion pump activity and/or quantity. Although cold acclimation decreased V-ATPase expression in the Malpighian tubules (Chapter 3), the activity of this enzyme was not modified. NKA activity in the Malpighian tubules may be increased by cold acclimation, and this could drive reduced urine production, retain hemolymph Na^+, and rid excess hemolymph K^+. NKA activity in the rectum was not modified by cold acclimation (despite an increase in transcript abundance). Overall, shifts in active transport across the Malpighian tubules that slow primary urine production may allow cold-acclimated insects to retain hemolymph volume and ion balance during cold exposure.

In Chapter 6 I synthesize my findings into a conceptual framework about how cold acclimation alters transport function to prevent chilling injury and loss of homeostasis during cold exposure. My results support the hypothesis that altered active transport following cold acclimation help to maintain hemolymph volume and mitigate leak, but I find no evidence of a role for modification of passive diffusion based on rectal structural changes. I suggest future directions to strengthen our understanding of the cold acclimation process as related to homeostasis and chilling injury, and discuss some additional candidate mechanisms that warrant further exploration.

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Chapter 2

2 Ion and water balance in *Gryllus* crickets during the first twelve hours of cold exposure

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2.1 Introduction

Because insects are ectotherms, many of their physiological processes are directly influenced by ambient temperature. The mechanisms that underlie thermal physiology therefore determine how climate impacts insect performance and, consequently, ecosystem function (Sinclair et al., 2003; Chown and Terblanche, 2006; Somero, 2010; Williams et al., 2015). Insect performance is bounded at low temperatures by the critical thermal minimum (CT_{min}), below which insects enter a reversible paralysis termed chill coma. Insects lose ion and water homeostasis when in chill coma and regain homeostasis during recovery (Koštál et al., 2004; MacMillan et al., 2012). The ability to survive and maintain homeostasis in the cold is variable and plastic; cold-acclimated or -adapted insect populations sustain water and ion balance at lower temperatures than their warm-acclimated or -adapted counterparts (Gibert and Huey, 2001; Ayrinhac et al., 2004; Koštál et al., 2004; Koštál et al., 2006; Andersen et al., 2014; Coello Alvarado et al., 2015; MacMillan et al., 2015a).

In several insects (including crickets, locusts, and cockroaches), Na^+ and water migrate out of the hemolymph during chilling, while hemolymph $[K^+]$ increases (Koštál et al., 2006; MacMillan and Sinclair, 2011; Andersen et al., 2013; Findsen et al., 2014; Coello Alvarado et al., 2015). The migration of Na^+ is likely a result of reduced ion pumping (such that active ion transport no longer exceeds passive diffusion) and, as water balance is often tightly linked to Na^+ gradients, hemolymph water balance is lost as Na^+ balance is lost.

Increased hemolymph [K^+] is thought to result from decreased hemolymph volume (MacMillan and Sinclair, 2011). Chill coma onset occurs rapidly (within minutes of cold exposure) and appears to be mechanistically unrelated to processes underlying loss of water and ion homeostasis (Findsen et al., 2014; MacMillan et al., 2014; Andersen et al., 2015). In particular, previous authors have not observed a loss of water or ion homeostasis associated with chill coma paralysis within the first few minutes of cold exposure (Findsen et al., 2014; MacMillan et al., 2014; Andersen et al., 2015). However, loss of water and ion homeostasis during chilling is readily apparent at longer timescales (hours to days) in the context of studies of chill coma recovery time (CCRT) and chilling injury (e.g. Koštál et al., 2006; MacMillan and Sinclair, 2011; Findsen et al., 2013). In *Gryllus pennsylvanicus* crickets, the largest decrease in hemolymph [Na^+] and increase in hemolymph [K^+] occurs within the first 12 h of cold exposure (MacMillan and Sinclair, 2011), but we do not know how rapidly Na^+ or K^+ balance is lost, or whether the patterns of homeostasis in the initial cold exposure reflect those observed at longer timescales. Similarly, how ion and water imbalance during chilling relates to or predicts survival and chilling injury is not well understood (MacMillan et al., 2014).

Insects vary in their ability to maintain ion and water balance in the cold (Koštál et al., 2004; Koštál et al., 2007; Coello Alvarado et al., 2015; MacMillan et al., 2015a; MacMillan et al., 2015c). Our understanding about the mechanisms underlying this variation is incomplete (Gibert and Huey, 2001; Ransberry et al., 2011), but recent studies have revealed a potential role for modified Na^+ balance. Cold-acclimated *Drosophila melanogaster* maintain low hemolymph [Na^+] (and consequently low [K^+]) in both warm and cold conditions, and may also exhibit lower Na^+ transport capacity (MacMillan et al., 2015a; MacMillan et al., 2015c). *Gryllus veletis* (Alexander and Bigelow) nymphs maintain tissue and hemolymph Na^+ balance at 0°C, while *G. pennsylvanicus* adults (which are less chill tolerant) lose this Na^+ balance at 0°C unless they have undergone prior cold acclimation (Coello Alvarado et al., 2015).

Here I explored the patterns of water and ion balance at the organismal and tissue levels during the first 12 h of chilling with the aim of testing and generating mechanistic

hypotheses for why homeostasis is lost in the cold, and why chill-tolerant insects are better at maintaining homeostasis at low temperatures. I used two species of field cricket: *G. pennsylvanicus* (the species in which the initial model of loss of ion and water homeostasis in the cold was developed), and *G. veletis*, which are more chill-tolerant and maintain ion and water homeostasis to lower temperatures (Coello Alvarado et al., 2015).

2.2 Methods

Gryllus pennsylvanicus and *G. veletis* colonies originated from individuals collected from the University of Toronto at Mississauga campus, Ontario (2004) and the University of Lethbridge, Alberta (2010), respectively. I reared crickets under constant summer-like conditions (25°C, 14 light:10 dark photoperiod, 70% RH) at the University of Western Ontario Biotron Research Center, as described previously (MacMillan and Sinclair, 2011; Coello Alvarado et al., 2015). Crickets were housed in transparent plastic containers with stacked cardboard egg cartons for shelter and provided with tap water and *ad libitum* commercial rabbit food (Little Friends Original Rabbit Food, Martin Mills, Elmira, ON, Canada). I collected eggs in containers of moist vermiculite and sterile sand; *G. veletis* eggs hatched after two weeks, and I placed *G. pennsylvanicus* eggs at 4°C to accommodate an obligate three-month (minimum) diapause (Rakshpal, 1962) before returning them to 25°C to hatch. For all experiments I used adult virgin female *G. pennsylvanicus* and *G. veletis* (approximately 1 and 5 weeks post final molt, respectively). The difference in age reflected a longer development time for *G. veletis*. For one week prior to experiments, crickets were held individually in 180 mL transparent cups (Polar Plastics, Summit Food Distributors, London, ON, Canada) with mesh fabric lids and containing egg carton shelters, rabbit food, and water. Isolation prevented cannibalism and any associated changes in gut contents.

2.2.1 Measuring chill tolerance

I assessed low temperature performance of *G. pennsylvanicus* and *G. veletis* adult females by measuring the CT_{min}, CCRT, and survival following cold exposure. Measurement of the CT_{min} (n = 20 per species) was as described by (MacMillan and Sinclair, 2011). Briefly, I

placed crickets into individual 200 mL glass beakers within a Plexiglas® encasement containing a solution of equal parts ethylene glycol and water. The solution was circulated with a refrigerated bath (Model 1157P, VWR International, Mississauga ON). To monitor cricket body temperatures I placed a type T thermocouple in contact with each cricket. Thermocouples were connected to a computer by a Picotech TC-08 thermocouple interface and data were recording with PicoLog software (Pico Technology, Cambridge, UK). I cooled these crickets from room temperature at 0.25°C min^{-1} until the CT_{min} was reached. I defined the CT_{min} as the temperature at which physical stimulus with a metal probe elicited no muscular response. I defined CCRT as the time required for the righting response (a coordinated movement) after 48 h of cold exposure. To measure CCRT and survival of cold exposure I placed crickets (n = 24 per species) in 15 mL Falcon tubes immersed in an ice-water slurry at 0°C (a temperature that induced chill coma in both *G. pennsylvanicus* and *G. veletis* in preliminary experiments) for 48 h. This chilling duration should not cause mortality; *G. veletis* survive at least five days at 0°C, while approximately 20% of *G. pennsylvanicus* die after 108 h at 0°C (Coello Alvarado et al., 2015). After chilling I moved the crickets to room temperature, placed them on their dorsum in a 6-well plate, and video recorded their recovery for up to 9 h (Hazell et al., 2008). I extracted righting response times from the video. Crickets that did not exhibit signs of recovery within 9 h were not included in CCRT analyses. All crickets were then returned to 25°C in individual cups and provided with food, water, and shelter. After 24 h at 25°C, I assessed survival and injury (the latter defined as uncoordinated locomotion or inability to jump when stimulated with a probe; MacMillan and Sinclair, 2011).

2.2.2 Cold exposure and dissection

I held crickets at 25°C (control, 0 h) or exposed them to 0°C for a duration of 0.5, 1, 3, 6, or 12 h (n = 14-19 individuals per species per treatment). Size-matching of crickets ensured that mean wet mass did not differ among treatments within each species ($F_{5,83}$ = 0.30, P > 0.9 and $F_{5,89}$ = 0.32, P > 0.9 for *G. pennsylvanicus* and *G. veletis*, respectively). I placed cold-exposed crickets individually into loosely-capped 50 mL plastic tubes suspended in a bath of 50% methanol in water, pre-cooled to 0°C (Lauda Proline RP 3530, Würzburg,

Germany). I added a thermocouple in contact with one of the crickets to monitor its body temperature during cold exposure.

Immediately after removal from 0°C I dissected crickets on a Petri dish surrounded by ice within a large Styrofoam box. I punctured the pronotum with an insect pin and collected hemolymph (5-30 µl) with a micropipette, then opened the body cavity by a mid-dorsal incision and collected as much hemolymph from the body as possible by applying gentle pressure to the abdomen. I approximated hemolymph volume gravimetrically by weighing extracted hemolymph and assuming a density equal to water. This method of hemolymph extraction and approximation correlates linearly with inulin dilution estimates for hemolymph volume in *G. pennsylvanicus* (MacMillan et al., 2012). I pinned open the body cavity and removed the gut (from anterior foregut to rectum) into a pre-weighed microcentrifuge tube. I then severed the hind legs and used forceps to extract femur muscles into pre-weighed 0.2 mL microcentrifuge tubes.

To identify potential reservoirs of Na^+ (as I observed increased hemolymph Na^+ content during chilling), I measured Na^+ in the fat body, head, Malpighian tubules, and ovaries from an additional six control *G. pennsylvanicus* females. I calculated tissue water contents from the difference between the tissue fresh (wet) mass and mass after drying at 70°C for 24 h (muscle, Malpighian tubules, and fat body) or 48 h (gut, head, and ovaries).

2.2.3 Quantification of ion balance

I assessed ion homeostasis over 12 h of cold exposure by quantifying the concentration and content of Na^+ and K^+ in the hemolymph and tissues. I measured shifts in ion contents as they indicate bulk movement of Na^+ or K^+ between body compartments (which in turn affects bulk movement of water), and measured ion concentrations as they are important for neuromuscular function and as directional predictors of ion leak. I quantified ions as described by MacMillan and Sinclair (2011). Briefly, I digested hemolymph and dried tissues in nitric acid (70%) at room temperature for 24 h (hemolymph, muscle, fat body, and Malpighian tubules), 48 h (gut), or 72 h (head, ovaries). I quantified [Na^+] and [K^+] in

the dissolved, diluted hemolymph and tissue samples using an atomic absorption spectrometer (iCE 3300, Thermo Scientific, Waltham, MA, USA). From the measured absorbance, I calculated sample ion concentrations by comparison with standard curves generated from Na^+ and K^+ reference solutions containing 0.2% and 1% nitric acid, respectively. The water contents of each tissue (assumed to be intracellular water) or hemolymph (assumed to represent extracellular water) allowed me to calculate the ion concentration in the tissue or hemolymph. To determine sample ion content, I corrected ion concentrations for the volume or mass of hemolymph or tissue in the sample.

2.2.4 Data analyses

I expected that *G. veletis* would exhibit a lower CT_{min} and CCRT, and greater survival following cold exposure than *G. pennsylvanicus* (Coello Alvarado et al., 2015), therefore I made interspecies comparisons of the CT_{min}, CCRT, and survival following cold exposure using one-sided Welch's t-tests. I compared initial and endpoint (12 h) ion and water measurements as well as trajectories of ion and water balance during cold exposure among species, but I did not make point-by-point comparisons. To compare control ion or water measurements among species, I used two-sided Student's t-tests (if variance was equal) or Welch's t-tests (if variance was unequal). I quantified the relationship between cold exposure time and water or ion balance using generalized least squares models and linear regression, and compared discrete cold exposure time points by one-way ANOVA and Tukey's HSD. I log-transformed cold exposure times prior to analysis in cases when this transformation improved normality, and used exponential weighting for generalized nonlinear least squares models if variance was unequal across cold exposure times (Gałecki and Burzykowski, 2013). Tissue water and ion contents were positively correlated with tissue dry mass ($P < 0.05$, see Table A1) with the exception of muscle water ($P > 0.1$), therefore I corrected ion contents for tissue dry mass before quantifying the effect of cold exposure on water or ion content (i.e. cold exposure effects were modeled with the residuals of water or ion content regressed against tissue dry mass; MacMillan and Sinclair, 2011). Similarly, because hemolymph volume was positively related to cricket wet mass ($F_{1,85} = 61.89$, $P < 0.001$ and $F_{1,93} = 31.05$, $P < 0.001$ for *G. pennsylvanicus* and *G. veletis*,

respectively), I corrected hemolymph volume for cricket wet mass prior to quantifying the effect of cold exposure on hemolymph volume.

I calculated muscle Na^+ and K^+ equilibrium potentials at 23°C (control crickets) and at 0°C (cold-exposed crickets) as described by MacMillan and Sinclair (2011) using the Nernst equation (Nernst, 1888):

$$E = \left(\frac{RT}{zF}\right) \ln\left(\frac{[o]}{[i]}\right) \quad (1),$$

where R is the universal gas constant, T is the absolute temperature, z is the ionic charge (e.g. z for Na^+ or K^+ = 1), F is Faraday's constant, $[o]$ is the ion concentration outside of the muscle (i.e. the hemolymph), and $[i]$ is the ion concentration inside the muscle, i.e. the estimate from the tissue.

Descriptive values reported in the text, tables, and figures are given as mean ± s.e.m. Detailed statistics for regression models are included in supplementary material (Table A2). All statistical analyses were performed in R (v3.1.2, R Development Core Team, 2014).

2.3 Results

Gryllus veletis was more chill tolerant than *G. pennsylvanicus.* The CT_{min} of *G. veletis* (0.7 ± 0.2°C) was lower than that of *G. pennsylvanicus* (2.2 ± 0.13°C) ($t_{36.2}$ = 7.38, P < 0.001). Following exposure to 0°C for 48 h, *G. veletis* recovered 20-times faster than *G. pennsylvanicus* on average ($t_{8.02}$ = 4.75, P < 0.001). Sixteen of the 25 *G. pennsylvanicus* never regained righting ability within 9 hours of measuring CCRT (Fig. 2.1A), eight of which never recovered. Twenty-four hours after this cold exposure, 84% of *Gryllus pennsylvanicus* crickets were dead or injured, while only 20% of *G. veletis* crickets were injured and none were dead (Fig. 2.1B).

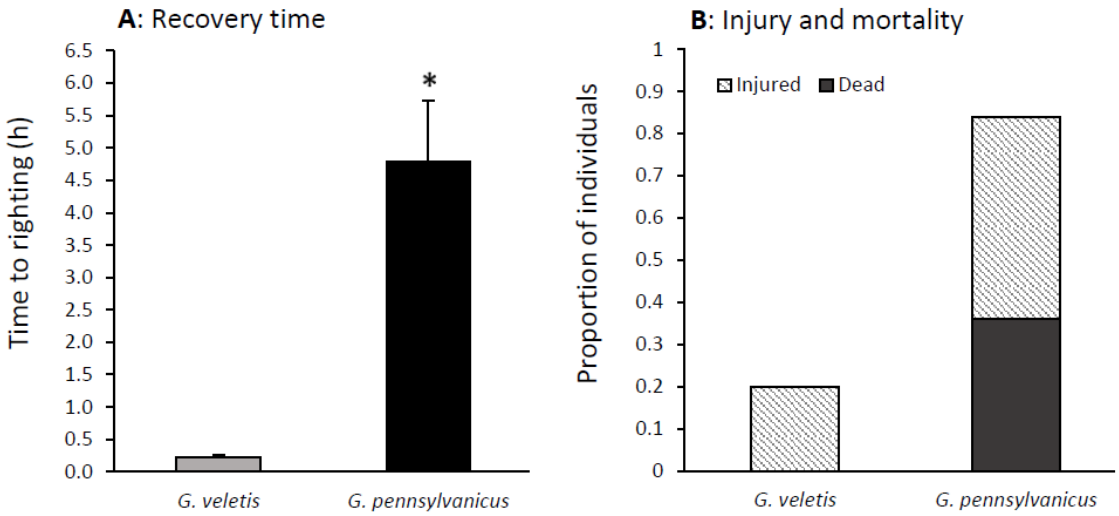

Figure 2.1. Recovery time (A) and injury and mortality (B) of *G. veletis* and *G. pennsylvanicus* after 24 h of recovery following 48 h in chill coma at 0°C. A. $n = 9$ and 24 for *G. pennsylvanicus* and *G. veletis*, respectively. B. $n = 25$ crickets per species.

2.3.1 Water balance

Under control conditions, hemolymph volume relative to gut water content was lower in *G. veletis* than in *G. pennsylvanicus* ($t_{31} = 2.49$, $P = 0.019$). The gut of *G. veletis* accounted for a slightly greater proportion of body fresh mass (11.5 ± 0.9%) compared to *G. pennsylvanicus* (8.2 ± 0.5%) ($t_{32} = 3.10$, $P = 0.004$). The volume of hemolymph relative to cricket fresh mass did not differ between species ($t_{32} = 1.59$, $P > 0.1$).

Gut water content increased over 12 h of cold exposure for both *G. pennsylvanicus* and *G. veletis* ($P = 0.032$ and $P = 0.004$, respectively; Appendix A, Fig. A1A). Hemolymph volume decreased by 25% for *G. veletis* during 12 h of cold exposure ($P = 0.001$). For *G. pennsylvanicus*, hemolymph volume first increased before decreasing slightly but I observed no significant change in hemolymph volume over the 12 h of cold exposure ($P = 0.091$; Appendix A, Fig. A1B). The water content of the hemolymph relative to the gut decreased linearly by 23% for *G. pennsylvanicus* and 38% for *G. veletis* ($P = 0.009$ and $P = 0.023$, respectively; Fig. 2.2A). Muscle water content was unchanged over 12 h of cold exposure for *G. pennsylvanicus* and *G. veletis* ($P > 0.3$ and $P > 0.2$).

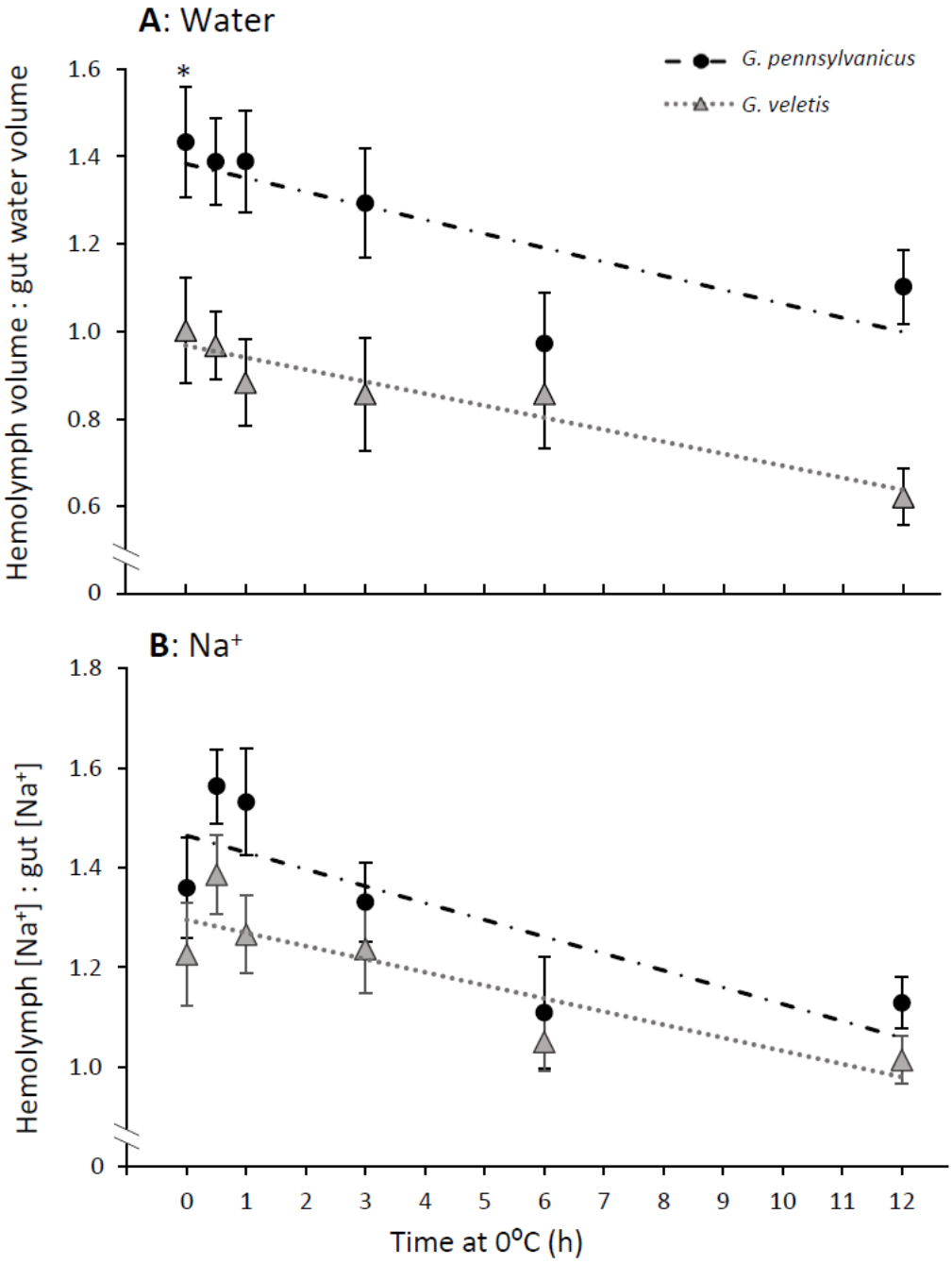

Figure 2.2. Ratio of hemolymph-to-gut water volume (A) and [Na^+] (B) in *G. pennsylvanicus* and *G. veletis* crickets exposed to 0°C for up to 12 h. Dashed lines indicate a significant linear relationship between water volume or [Na^+] ratio and cold exposure time. *n* = 11 to 18 crickets per species per time point; see Table A2 for statistics.

2.3.2 Ion balance

Na^+ gradients between the hemolymph and the gut did not differ between species under control conditions ($t_{33} = 0.927$, $P = 0.361$), however both species exhibited linear decreases in the hemolymph-to-gut [Na^+] ratio during 12 h of cold exposure ($P < 0.001$ and $P < 0.002$ for *G. pennsylvanicus* and *G. veletis*, respectively; Fig. 2.2B). Gut Na^+ content increased by approximately 21% during cold exposure for *G. veletis*, while a 29% increase in gut Na^+ content for *G. pennsylvanicus* was non-significant ($P = 0.032$ and $P = 0.073$, respectively; Fig. 2.3). Gut K^+ content did not change over cold exposure time in *G. pennsylvanicus* or *G. veletis* ($P > 0.8$) despite a decrease in gut [K^+] ($P = 0.036$ and $P = 0.005$, respectively).

In the hemolymph of *G. pennsylvanicus*, [Na^+] initially increased (from 110 mM to 130 mM within 0.5 h of cold exposure) before returning to control values by 6 h ($F_{5, 78} = 4.34$, $P < 0.002$) (Fig. 2.4A). A rise and fall of hemolymph [Na^+] also occurred in cold-exposed *G. veletis* but with a much smaller overall change (from 106 mM to 119 mM) ($F_{5,88} = 2.35$, $P = 0.048$), such that differences among time points were not identified using Tukey's HSD. General patterns of hemolymph [Na^+] during cold exposure in *G. pennsylvanicus* were mirrored by the hemolymph Na^+ content ($F_{5,77} = 2.42$, $P = 0.043$), however a similar trend observed for Na^+ content in the hemolymph of *G. veletis* was non-significant ($F_{5,88} = 2.25$, $P = 0.056$; Fig. 2.4C).

I observed a movement of Na^+ to the hemolymph in the first hour of exposure to 0°C, therefore I quantified [Na^+] and Na^+ content in the ovaries, fat body, head, and Malpighian tubules of *G. pennsylvanicus* under control conditions to identify potential reservoirs of Na^+. The [Na^+] in both the fat body and ovaries exceeded that of the hemolymph, while [Na^+] in the head and Malpighian tubules were lower than the hemolymph (Table 2.1). The ovaries –which accounted for 32 ± 1.7 % of the adult female body mass – held the largest reservoir of total Na^+. For both species, cold exposure caused linear increases in both hemolymph [K^+] ($P < 0.001$) and K^+ content ($P = 0.037$ and $P < 0.001$ for *G. veletis* and *G. pennsylvanicus*, respectively; Fig. 2.5A,C).

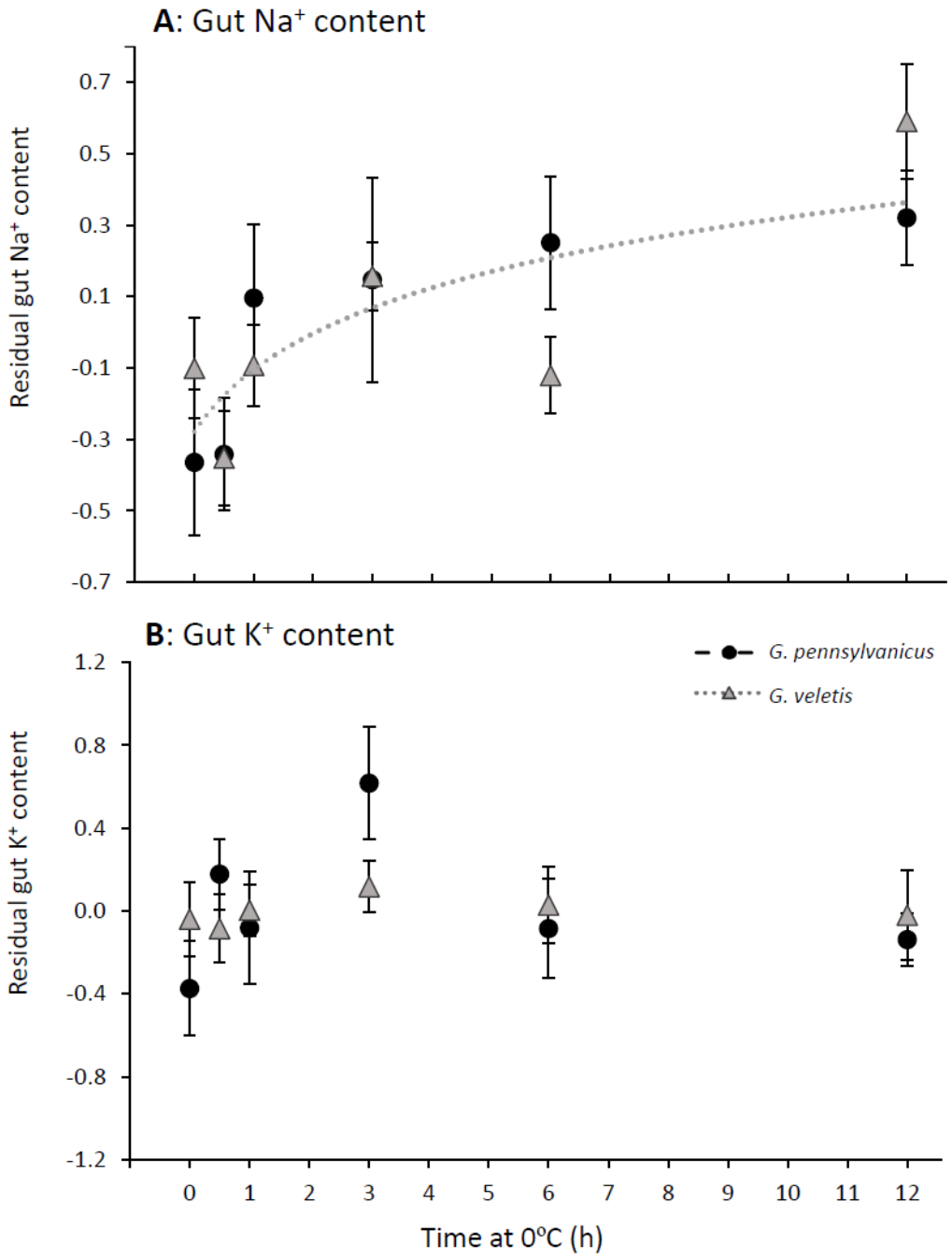

Figure 2.3. Content of gut Na^+ (A) and K^+ (B) in *G. pennsylvanicus* and *G. veletis* exposed to 0°C for up to 12 h. Ion contents are represented as the residuals of a regression of μmoles Na^+ or K^+ against gut dry mass and are expressed as mean residual ± s.e.m. The dashed line indicates a significant relationship between gut ion content and cold exposure time in *G. veletis*. *n* = 13 to 18 per species per time point; see Table A2 for statistics.

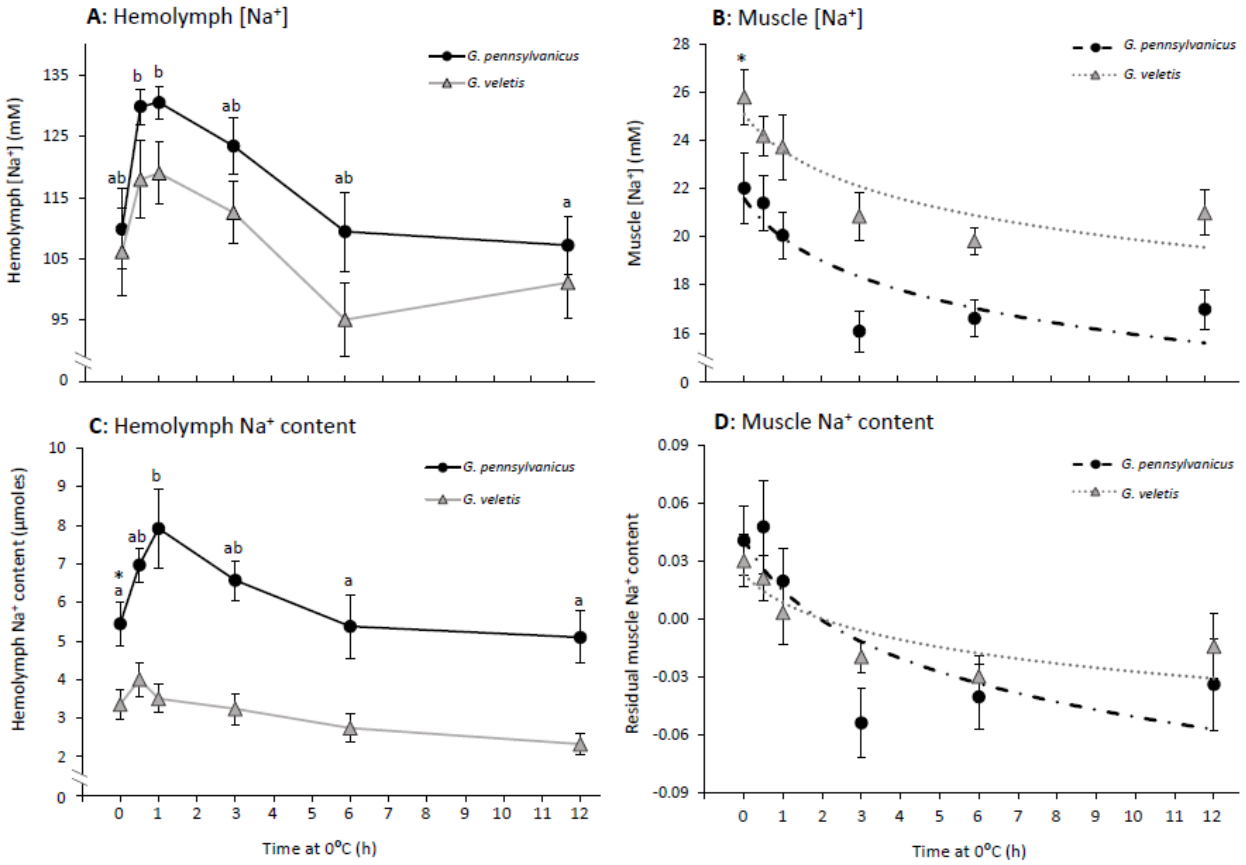

Figure 2.4. Balance of Na^+ in the hemolymph (A, C) and muscle (B, D) of *G. pennsylvanicus* and *G. veletis* crickets exposed to 0°C for up to 12 h. [Na^+] (A, B) is expressed in mM, while Na^+ content is expressed as total µmoles (C, D). Effects of cold on muscle Na^+ (B, D) were modeled using the residuals of a regression of total µmoles Na^+ against muscle dry mass. Dashed lines indicate significant relationships between muscle Na^+ and 0°C exposure time. Solid lines are used to illustrate trends in hemolymph Na^+ during cold exposure. Different letters indicate differences in mean hemolymph Na^+ of *G. pennsylvanicus* according to Tukey's HSD. Tukey's HSD failed to detect differences among mean for *G. veletis.* Asterisks denote significant differences in Na^+ between species at time = 0 h. n = 11 to 18 crickets per species per time point; see Table A2 for statistics.

Table 2.1. Content and concentration of Na^+ in the fat body, ovaries, head, Malpighian tubules, and hemolymph of adult *G. pennsylvanicus* crickets under control conditions (25°C, 14 h day length). n = 17 (hemolymph) or 6 (all other tissues).

Tissue	[Na^+] (mM)	Total Na^+ content (µmoles)
Malpighian tubules	65 ± 4.2	0.3 ± 0.03
Head	70 ± 3.8	2.2 ± 0.14
Hemolymph	110 ± 6.6	5.5 ± 0.57
Fat body	123 ± 5.3	0.5 ± 0.05
Ovaries	135 ± 6.0	11.5 ± 0.86

Gryllus pennsylvanicus had higher muscle [K^+] compared to *G. veletis* under control conditions ($t_{23.3}$ = 2.36, P = 0.027). I observed a slight increase in muscle [K^+] for *G. veletis* (P = 0.049) over 12 h, however cold exposure had no effect on muscle [K^+] in *G. pennsylvanicus* (P > 0.4). Muscle K^+ content was not affected by cold exposure in *G. pennsylvanicus* (P > 0.3) or *G. veletis* (P = 0.080, Fig. 2.5B,D). Muscle [Na^+] in *G. pennsylvanicus* was lower than in *G. veletis* under control conditions ($t_{30.5}$ = 2.04, P = 0.025). During 12 h of cold exposure, muscle [Na^+] decreased for both *G. pennsylvanicus* and *G. veletis* (P < 0.001) and this decrease reflected a loss of muscle Na^+ content (P < 0.002 and P = 0.007, respectively; Fig. 2.4B,D). *Gryllus veletis* appeared to lose muscle Na^+ more slowly than *G. pennsylvanicus*.

Control *G. pennsylvanicus* exhibited higher muscle Na^+ equilibrium potential (by c. 5.5 mV; t_{33} = 1.92, P = 0.032) and lower muscle K^+ equilibrium potential (by c. 11.5 mV; t_{23} = 2.38, P = 0.013) compared to *G. veletis* (Fig. 2.6). I did not observe significant changes in muscle Na^+ potential during 12 h of cold exposure for *G. pennsylvanicus* or *G. veletis* ($F_{5,80}$ = 1.20, P > 0.3 and $F_{5,85}$ = 0.79, P > 0.5, respectively). Muscle K^+ equilibrium potential depolarized from -75.4 mV (*G. pennsylvanicus*) and -63.9 mV (*G. veletis*) to approximately -40 mV in both species after 12 h at 0°C.

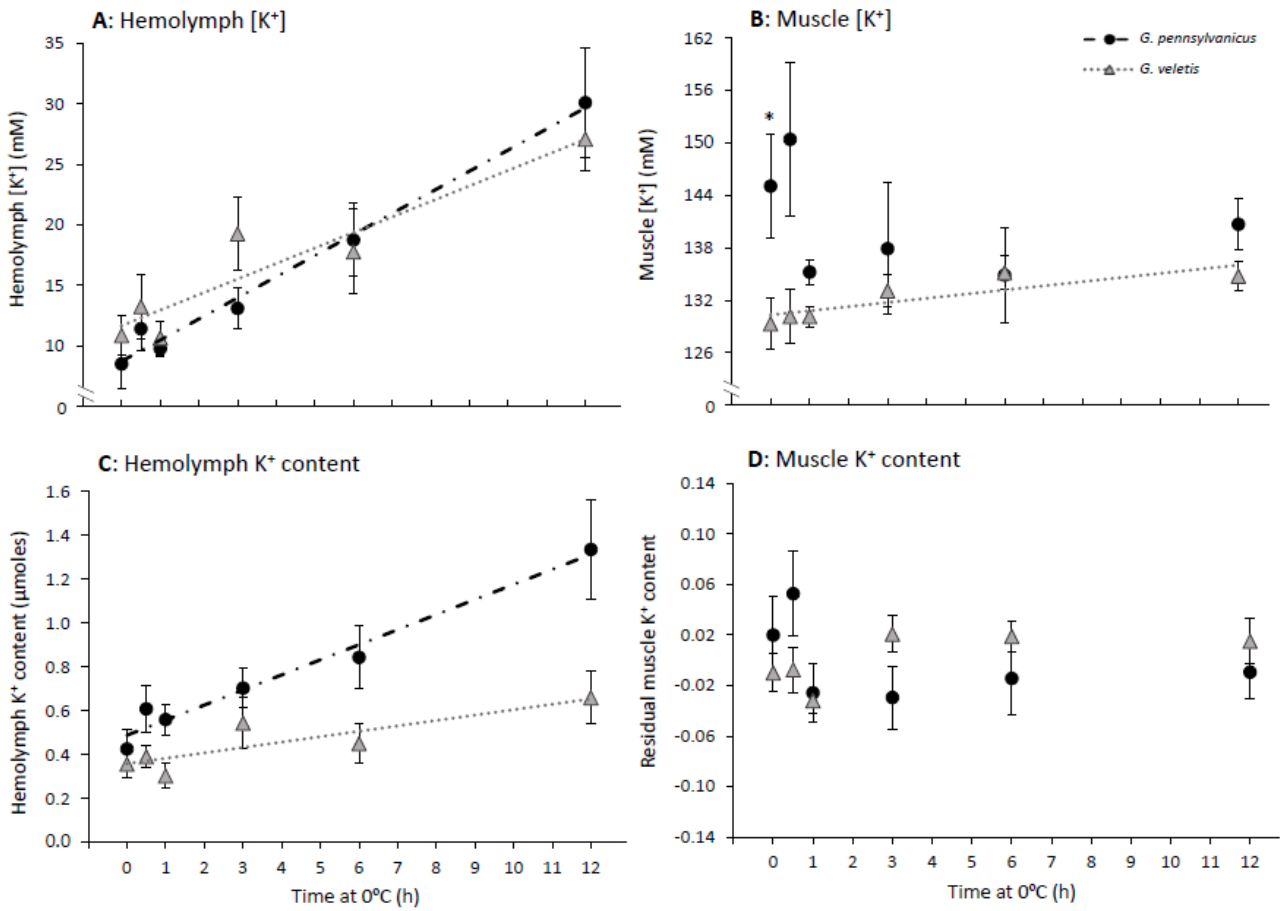

Figure 2.5. Balance of K^+ in the hemolymph (A, C) and muscle (B, D) of *G. pennsylvanicus* and *G. veletis* crickets exposed to 0°C for up to 12 h. Potassium concentration (A, B) is expressed in mM, while hemolymph K^+ content is expressed as total µmoles (C). Effects of cold on muscle K^+ content (D) was modeled as the residuals of a regression of total µmoles K^+ against muscle dry mass. Dashed lines indicate significant linear relationships between muscle or hemolymph K^+ and cold exposure time. Asterisks denote significant differences between species at time = 0 h (see Table A2 for statistics). *n* = 13 to 18 crickets per species per time point.

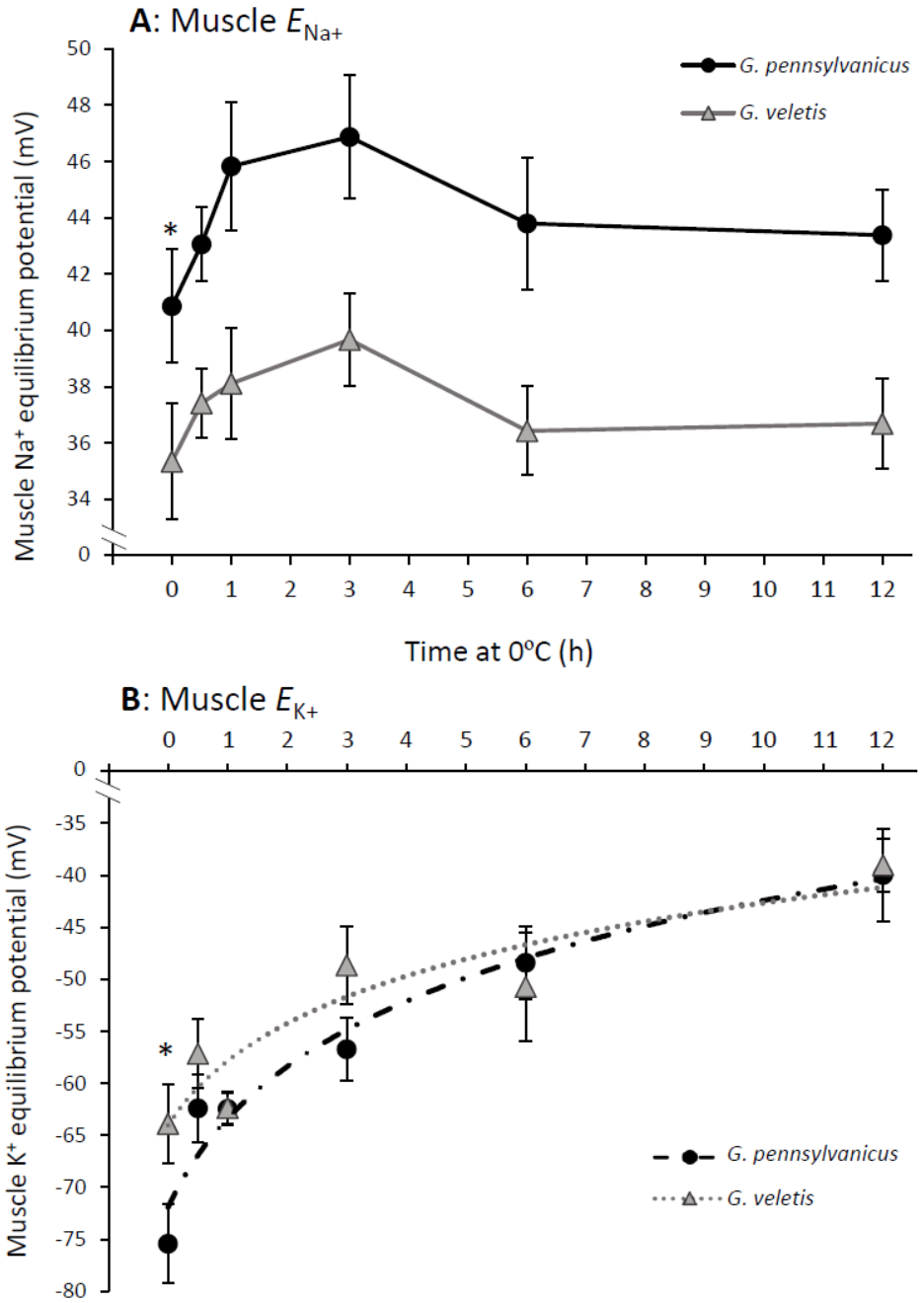

Figure 2.6. Na^+ (A) and K^+ (B) potentials (mV) across the muscle cell membrane in *G. pennsylvanicus* and *G. veletis* exposed to 0°C for up to 12 h. Solid lines are used to illustrate trends in muscle Na^+ potential, but muscle Na^+ potentials did not differ between cold exposure times for either species according to ANOVA. Dashed lines indicate significant relationships between muscle K^+ potential and cold exposure time. Asterisks denote significantly different potentials between species at exposure time = 0 according to a t-test. n = 12 to 18 per species per time point.

2.4 Discussion

The mechanisms underlying loss of ion and water balance at low temperatures and the means by which chill-tolerant insects avoid this loss are not fully understood. By quantifying the ion and water balance in crickets during the first 12 h of cold exposure I show that shifts in hemolymph Na^+ balance observed at later stages (days) of cold exposure do not reflect changes in these early stages. I hypothesize that loss of Na^+ balance during chill coma may be driven by a loss of Na^+ from the tissues. While neither species could defend water, $[Na^+]$, or $[K^+]$ balance during cold exposure, shifts in ion contents across the hemolymph and muscle were slower and/or less extensive in the more chill-tolerant cricket (*G. veletis*) compared to the less chill-tolerant cricket (*G. pennsylvanicus*). These findings support the hypothesis that chill tolerance (as assessed by the CT_{min}, CCRT, and survival of cold exposure) may be associated with a greater resistance of the tissues to ion leak in the cold (MacMillan et al., 2015a).

MacMillan and Sinclair (2011) report that hemolymph $[Na^+]$ of *G. pennsylvanicus* adults drops substantially by 12 h of cold exposure and decreases gradually thereafter over 120 h (MacMillan and Sinclair, 2011). However, within the first 12 h of cold exposure I instead observed a rapid increase in hemolymph $[Na^+]$, peaking at 1 h of exposure to 0°C and then returning to control values by 6 h such that there was no net change in $[Na^+]$ by 12 h. Some of this discrepancy could be explained by differences in hemolymph $[Na^+]$ of control crickets (a mean of 110 mM $[Na^+]$ was measured in the present study compared to an approximate 185 mM measured by MacMillan and Sinclair, 2011). Typical orthopteran hemolymph $[Na^+]$ is closer to 91 mM (Piek and Njio, 1979). Food and rearing conditions were identical between the present study and a previous study by MacMillan and Sinclair (2011), however I isolated crickets for one week prior to experiments to prevent cannibalism and any consequent effects on gut ion content. I also controlled for potential inconsistencies in mating status by ensuring that all females were virgin; gravid females used in the previous study likely exhibit some differences in ovary and/or fat body mass, and this could affect total available tissue Na^+. Finally, CO_2 used for cricket anesthesia in the previous study could affect hemolymph Na^+ balance (Stewart, 1978; Nilson et al., 2006;

Matthews and White, 2011). A higher hemolymph [Na^+], as measured by MacMillan and Sinclair (2011) would present a steeper gradient of Na^+ between the hemolymph and gut, favoring greater migration of Na^+ towards the gut (and perhaps this accounted for the rapid drop in hemolymph [Na^+] in the first 12 h).

In the present study, the peak of hemolymph [Na^+] in the first hour of cold exposure reflected a peak in hemolymph Na^+ content and also coincided with increases in gut Na^+ content (at least statistically for *G. veletis*). However, by 12 h in the cold I had observed no net change in hemolymph Na^+ content in either species. A net increase in gut Na^+ content without a net decrease in hemolymph Na^+ content was also observed by Coello Alvarado et al. (2015), and suggests that Na^+ may have entered the hemolymph from surrounding tissues before migrating to the gut where it remained. This hypothesis is supported by the loss of muscle Na^+ content observed during cold exposure, which agrees with previous observations for *G. pennsylvanicus* at 12 h in chill coma (MacMillan and Sinclair, 2011). Tissues other than the muscle could also lose Na^+ during cold exposure; the ovaries are a large potential reservoir of Na^+ and have a higher [Na^+] than the hemolymph. However, I did not measure changes in Na^+ balance within the ovaries during cold exposure. As male crickets lack ovaries, it is unclear whether they will exhibit a similar increase in hemolymph Na^+ content during early chill coma, or if the testes act as a potential source of this Na^+. Quantifying changes in Na^+ balance of non-muscle tissues (e.g. fat body, gonads, or ganglia) during chill coma would confirm whether a loss of homeostasis in the tissues manifests as imbalance in hemolymph Na^+ content.

Cold exposure caused a gradual redistribution of water between the hemolymph and gut, as observed during longer-term cold exposure (MacMillan and Sinclair, 2011; Coello Alvarado et al., 2015). However, gut water content in *G. pennsylvanicus* increased despite no measurable decrease in hemolymph volume. This phenomenon was also observed in *G. veletis* nymphs over longer cold exposures, and it is possible that dehydration of tissues accounted for the gain of gut water (Coello Alvarado et al., 2015). Cold-acclimated *Pyrrhocoris apterus* L. bugs lose water from the fat body during chill coma (Koštál et al., 2004), and while I did not observe changes in muscle water content in crickets during chill

coma, water could have been lost from the fat body or other tissues and followed Na^+ to the gut.

Cold exposure caused hemolymph [K^+] to increase steadily over 12 h in for both species, reflecting trends observed at longer durations of chilling (MacMillan and Sinclair, 2011; Coello Alvarado et al., 2015). Increased hemolymph [K^+] in the cold is thought to result from loss of hemolymph volume, rather than changes in hemolymph K^+ content (MacMillan and Sinclair, 2011). My observations support a gradual loss of hemolymph volume concurrent with a gradual increase in hemolymph [K^+], and without changes in gut K^+ content (similar trends were observed in crickets after a 120 h cold exposure; MacMillan and Sinclair, 2011; Coello Alvarado et al., 2015). However, I also observed an increase in hemolymph K^+ content during cold exposure. This K^+ was unlikely to be sourced from the muscle; unlike our observation of decreased muscle Na^+ content, muscle K^+ content did not change during cold exposure (similar to the findings of MacMillan and Sinclair, 2011). Potassium could enter the hemolymph from other tissues; *P. apterus* bugs lose K^+ from the fat body when exposed to -5°C (Koštál et al., 2004). Alternatively, the gut contents could act as a source of K^+; the gut lumen [K^+] is roughly 17-fold higher than the hemolymph and presents a steep gradient for K^+ favoring migration to the hemolymph. Leak of K^+ across the gut may be enhanced during cold exposure due to changes in the permeability of gut epithelium (Motais and Isaia, 1972; Dokladny et al., 2006; Ionenko et al., 2010). Although I did not observe a change in gut K^+ content during early chill coma, small amounts of K^+ lost from the gut could have large impacts on hemolymph K^+ content.

Increased hemolymph [K^+] during cold exposure (which disrupts muscle K^+ equilibrium potential) was initially proposed by MacMillan and Sinclair (2011) to explain chill coma paralysis by causing a loss of muscle resting potential. However recent studies of *Locusta migratoria* L. indicate that chill coma paralysis precedes hemolymph [K^+] imbalance and that low temperatures play a direct role in neuromuscular silencing (Koštál et al., 2006; Findsen et al., 2014; MacMillan et al., 2014; Andersen et al., 2015). It is therefore now generally accepted that chill coma onset and loss of ion homeostasis during cold exposure are mechanistically unrelated. I too show that loss of muscle E_{K^+} due to hemolymph [K^+]

imbalance does not account for a total loss of muscle resting potential during very early stages of cold exposure. The hypothesized muscle membrane potential threshold for chill coma is between -37 and -45 mV in *D. melanogaster* and *Apis mellifera* L. (Hosler et al., 2000), which is supported by Andersen *et al*. (2015) in locusts. Although chill coma onset is rapid, muscle potential based on $[K^+]$ balance in crickets did not reach -45 mV prior to 7 h in the cold.

2.4.1 Do chill-tolerant crickets maintain homeostasis better in the cold?

Gryllus veletis had better performance in the cold (faster CCRT, lower incidence of chilling injury, and increased survival) compared to *G. pennsylvanicus*, agreeing with Coello Alvarado et al. (2015) who compared chill tolerance of *G. pennsylvanicus* adults with *G. veletis* nymphs. However unlike *G. veletis* nymphs, *G. veletis* adults were not much better than *G. pennsylvanicus* at maintaining water balance and, in most cases, $[Na^+]$ and $[K^+]$ balance during 12 h of cold exposure were similar between the two species. It is not known whether sex or a 6-week age gap in *G. pennsylvanicus* adults accounted for differences in homeostasis observed by Coello Alvarado et al. (2015) and the present study. *Gryllus veletis* did, however, better-maintain hemolymph Na^+ and K^+ content and to some degree, muscle Na^+ content.

Under control conditions and during cold exposure, *G. veletis* exhibited less water in the hemolymph relative to the gut compared to *G. pennsylvanicus*. This difference was not due to a higher relative gut water content in *G. veletis*. Nevertheless, *G. veletis* did not avoid a loss of water balance over 12 h of cold exposure; the rate of water redistribution from hemolymph to gut was roughly parallel for the two species. This suggests that regulation of ion homeostasis may be more important than water balance for surviving cold exposure.

Hemolymph $[Na^+]$ was similar for both crickets under control conditions but changed less in *G. veletis* during 12 h of cold exposure due to lesser influx of Na^+ to the hemolymph. Coello Alvarado et al. (2015) also observed that *G. veletis* nymphs, and to some degree

cold-acclimated *G. pennsylvanicus* adults, avoid this Na^+ influx up to 120 h in the cold. Chill-tolerant insect tissues may therefore be more resistant to Na^+ leak in the cold; in support of this hypothesis, *G. veletis* appeared to lose muscle Na^+ content somewhat more slowly than *G. pennsylvanicus*. This prevention of ion leak could be achieved by a tightening paracellular junctions or other modification of epithelial ultrastructure. Additionally (but not necessarily alternatively), *G. veletis* could combat Na^+ leak by enhancing Na^+ pump activity in the cold (Galarza-Muñoz et al., 2011). However, chill tolerance in *D. melanogaster* is correlated with a decrease in whole-body Na^+-K^+ ATPase (NKA) activity (MacMillan et al., 2015c). As NKA maintains higher hemolymph [Na^+] relative to the gut, lower NKA activity suggests that chill-tolerant insects may reduce Na^+ gradients across the gut. Cold tolerance in *D. melanogaster* is correlated with a reduction in the [Na^+] gradient across the gut, and it is thought that this reduced gradient minimizes the driving force for bulk movement of Na^+ and water from the hemolymph to the gut during cold exposure (MacMillan et al., 2015a; MacMillan et al., 2015c). However, this hypothesis was not well-supported by my observations, as the mean hemolymph-to-gut [Na^+] ratio in *G. veletis* was not significantly lower than for *G. pennsylvanicus* under control conditions (nor did it appear lower throughout cold exposure). Neither species exhibited a net loss of hemolymph Na^+ content by 12 h of cold exposure, yet both species suffered a loss of hemolymph volume and a rise in hemolymph [K^+].

Increased hemolymph [K^+] during cold exposure may lead to chilling injury via signalling disruption and cell death (Rojas and Leopold, 1996; Koštál et al., 2006; MacMillan et al., 2015b), however the accumulation of chilling injuries in adult *Gryllus* crickets was not predicted by the ability to defend hemolymph [K^+] in the first 12 h of cold exposure. It is therefore unclear whether ion imbalance in the first 12 h of chill coma plays a role in chilling injury. *Gryllus veletis* did exhibit lesser increases in hemolymph K^+ content compared to *G. pennsylvanicus*, so perhaps the gut epithelium of *G. veletis* is more resistant to changes in ion permeability at low temperatures. This hypothesis could be tested by manipulating the [K^+] gradient between the hemolymph and gut prior to cold exposure by artificial diets, as was attempted in a previous study with *L. migratoria* (Andersen et al., 2013). Preventing leak of K^+ into the hemolymph could also explain shorter CCRT in *G.*

veletis, as recovery requires reestablishment of water balance in addition to the reversal of any bulk movement of ions that occurred during cold exposure (MacMillan et al., 2012).

Under control conditions, *G. veletis* exhibited a lower muscle Na^+ potential and higher muscle K^+ equilibrium potential compared to *G. pennsylvanicus*. Without direct measurements of muscle resting potential it is unclear whether these differences in Na^+ and K^+ potentials help *G. veletis* delay muscle depolarization in early chill coma or play some role in a more rapid CCRT compared to *G. pennsylvanicus* (MacMillan et al., 2014; Coello Alvarado et al., 2015). Nevertheless, both species entered chill coma well before muscle K^+ equilibrium potentials had reached the theoretical threshold for chill coma at 7 h of cold exposure.

2.4.2 Conclusions

After characterizing patterns of ion and water balance in the first 12 h of cold exposure, I propose some refinements to the current model of homeostasis in the cold. During cold exposure, Na^+ appears to be lost from tissues and enters the hemolymph before ultimately migrating to the gut along with water. Loss of hemolymph volume in addition to possible leak of K^+ from the gut to the hemolymph leads to an increase in hemolymph $[K^+]$. This K^+ imbalance does not cause paralysis in early stages of cold exposure, but may negatively affect CCRT. Chill tolerance based on avoidance of chilling injury was not associated with the ability to defend the water balance or ion concentrations, however chill-tolerant crickets (*G. veletis*) better defended the balance of Na^+ and K^+ contents compared to less chill-tolerant crickets (*G. pennsylvanicus*). I therefore hypothesize that in addition to the gut epithelium, other tissues (e.g. muscle or ovaries) in chill-tolerant insects have lower permeability to ions in the cold, such that Na^+ does not leak from tissues to the hemolymph and K^+ does not leak across the gut epithelium to the hemolymph. Thus, an important future direction is to quantify the effects of cold on tissue permeability and transport function, with special consideration of ultrastructure and ion pump activities (e.g. NKA or the proton pump) in the hindgut and Malpighian tubules, as these tissues are responsible for the bulk of ion and water transport.

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Chapter 3

3 Effects of cold acclimation on gene expression in the Fall field cricket (*Gryllus pennsylvanicus*) ionoregulatory tissues

A version of this chapter is in press at *BMC Genomics*.

3.1 Introduction

Most insects are chill-susceptible, such that their thermal performance and survival are limited in the cold at temperatures well above the freezing point (Bale, 1993). Although ice formation causes direct injury, cold injury not associated with ice formation is less well-understood. Direct cold shock probably causes immediate damage to cells, for example by causing membrane phase transition (Overgaard et al., 2005; Clark and Worland, 2008), disruption of the cytoskeleton (Belous, 1992; Örvar et al., 2000; Michaud and Denlinger, 2004; Kim et al., 2006; Kayukawa and Ishikawa, 2009), or induction of apoptosis (Yi et al., 2007). Indirect cold injury accumulates over time, most likely as a result of a loss of ion and water balance in the cold (MacMillan and Sinclair, 2011b; MacMillan et al., 2012; MacMillan et al., 2015a; MacMillan et al., 2015b; MacMillan et al., 2015c), although there is also evidence of roles for oxidative damage and disruption of signalling pathways (Michaud and Denlinger, 2004; Lalouette et al., 2011; Teets et al., 2013). Cold-acclimated insects better maintain homeostasis and avoid chilling injury and mortality at lower temperatures than warm-acclimated insects (Chen et al., 1987; Koštál et al., 2007; Armstrong et al., 2012; Coello Alvarado, 2012; Findsen et al., 2013; MacMillan et al., 2015d), but the underlying mechanisms are not completely understood.

During cold exposure, chill-susceptible insects lose water and Na^+ from the hemolymph to the gut lumen (Koštál et al., 2004; Koštál et al., 2006; MacMillan and Sinclair, 2011b; Findsen et al., 2014) and re-establish water and ion homeostasis during recovery (MacMillan et al., 2012). Ion and water homeostasis in insects is primarily regulated at the Malpighian tubules and hindgut (Phillips, 1981). The distal Malpighian tubules actively transport ions across a leaky epithelium to drive secretion of water, metabolic wastes, and

other ions into the tubule lumen. This primary urine – which is isosmotic to the hemolymph (Ramsay, 1954) – is partially modified at the proximal tubule (a tight epithelium) prior to entering the gut lumen (Beyenbach, 1995; Coast, 1998; Beyenbach, 2003). Water and ions are then selectively reabsorbed from the gut lumen by the hindgut, particularly across the rectum (Phillips et al., 1987). Na^+-K^+ ATPase (NKA) maintains high paracellular [Na^+] in the rectal epithelium, driving paracellular migration of water (and concurrent reabsorption of some Na^+ and Cl^-) from the gut lumen to the hemolymph. Secretion and reabsorption are regulated by diuretic and antidiuretic peptides (see Schooley et al., 2012), and these peptides are important for recovery from cold stress in *Drosophila* (Tehrzaz et al., 2015). Loss and recovery of ion and water balance in the cold is likely dependent upon processes at the Malpighian tubule and hindgut epithelia; specifically, changes in pumping rate at low temperatures could maintain ion balance for longer, and decreased epithelial permeability could reduce the rate of ion leakage.

Transport enzyme function is temperature-dependent (Wolfenden et al., 1999); thus cold exposure should limit ion pumping rates while the rate of passive leak should remain relatively unchanged (MacMillan and Sinclair, 2011a). To compensate, insects could increase transport capacity by expressing or mobilizing more transport enzymes (Storey and Storey, 1981; Fujiwara and Denlinger, 2007; Kayukawa et al., 2007; Clark and Worland, 2008). In *Drosophila melanogaster*, cold acclimation instead lowers hemolymph [Na^+] and whole-body NKA activity (MacMillan et al., 2015d), however it is unclear how NKA activity changes in ionoregulatory tissues specifically. An obvious hypothesis, then, is that expression or function of NKA and other transport enzymes in the Malpighian tubules and hindgut may be targeted for modification during cold acclimation.

Water and ion leak during cold exposure will depend on tissue permeability (MacMillan and Sinclair, 2011a, b; Coello Alvarado et al., 2015), particularly of paracellular pathways (e.g. by a loss of cell-to-cell junctional integrity; Armitage et al., 1994). Paracellular shunts are the primary pathway for water and anion movement across the Malpighian tubule and rectal epithelia (O'Donnell and Maddrell, 1983; Phillips et al., 1987; O'Donnell et al., 1996; Beyenbach, 2003). During cold exposure, loss of extracellular Ca^{2+} balance (MacMillan

and Sinclair, 2011b) could further alter epithelial permeability by altering junctional integrity (Martinez-Palomo et al., 1980; Dokladny et al., 2006), Malpighian tubule secretion rates (Morgan and Mordue, 1985), and/or cold-related cellular signalling (Teets et al., 2013). As paracellular shunts are plastic and temperature-sensitive (Gonzalez-Mariscal et al., 1984; Behrens et al., 1993; Turner et al., 1997), they could be modified during cold acclimation to reduce epithelial permeability.

Surprisingly few genes have been directly associated with insect cold tolerance (Clark and Worland, 2008; Storey and Storey, 2012), but transcriptomics approaches have revealed many candidates. For example, modified expression of cuticular genes underlie cold tolerance variation in New Zealand stick insects (Dunning et al., 2014; Dennis et al., 2015), and the gene *Frost* is associated with recovery from cold exposure in *Drosophila* (Sinclair et al., 2007; Colinet et al., 2010; Hoffmann et al., 2012), although its function remains elusive (Udaka et al., 2013). Cold shock recovery in *Sarcophaga bullata* flesh flies changes the expression of genes related to the membrane and cytoskeletal structure, apoptosis, protein folding, oxidative stress, and signaling (Teets et al., 2012), and many of these genes (in addition to those involving autophagy and ion transport) are also modified with cold acclimation and rapid cold-hardening in *D. melanogaster* (Gerken et al., 2015). Most transcriptomic studies have explored responses to acute cold exposure (e.g. Qin et al., 2005; Zhang et al., 2011), or compared natural variation among populations and species whose underlying differences may render the specific drivers of cold-related phenotypes difficult to detect (e.g. Dunning et al., 2014). Acclimation of a single population is therefore a useful approach to identify candidates associated with cold tolerance plasticity.

A few single population studies have identified transcriptomic changes associated with cold acclimation, e.g. MacMillan et al. (2016b) and Gerken et al. (2015) recently identified key pathways and 1000s of genes associated with cold acclimation in *D. melanogaster*. In these *Drosophila* datasets, modification of ion transport (particularly altered expression of Na^+ transporters) and cellular adhesion is consistent with our expectation that modulating epithelial transport is associated with cold tolerance plasticity. However, these and other related studies have examined the transcriptome of either entire animals (e.g. MacMillan

et al., 2016b), or mixed tissues (e.g. the entire excised heads of stick insects; Dunning et al., 2014; Dennis et al., 2015). Because the Malpighian tubules and hindgut effectively work antagonistically in insect ion and water balance, transcriptomic shifts in these epithelia are likely to be masked in whole-animal homogenates. Thus, a tissue-specific approach to transcriptomics is urgently needed to more precisely determine the cellular- and tissue-level changes underlying cold acclimation in chill-susceptible insects.

The fall field cricket, *Gryllus pennsylvanicus* (Orthoptera: Gryllidae) inhabits the Eastern North American temperate zone (Criddle, 1925). The species is univoltine and overwinters in diapause in the soil as an egg (Carrière et al., 1996). Adult *G. pennsylvanicus* are chill-susceptible; they develop chilling injuries in as little as 12 h at 0°C, and are killed by 3-5 d at this temperature (MacMillan and Sinclair, 2011b). *Gryllus pennsylvanicus* has also emerged as a model for understanding cold-induced loss of ion and water balance (MacMillan and Sinclair, 2011b; Coello Alvarado et al., 2015). Briefly, when these crickets are exposed to cold, Na^+, Ca^{2+}, Mg^{2+}, and water migrate from the hemolymph to the gut, hemolymph [K^+] rises, and muscle equilibrium potential is lost (MacMillan and Sinclair, 2011b; Coello Alvarado et al., 2015). Ion and water balance are actively re-established during recovery from cold (MacMillan et al., 2012). *Gryllus pennsylvanicus* exhibits plasticity in its cold tolerance whereby cold-acclimated individuals have improved defense of ion and water homeostasis in the cold, a lowered critical thermal minimum, faster chill coma recovery time, and suffer lower rates of injury and mortality following cold shock (Coello Alvarado et al., 2015).

Here I took a tissue-specific comparative gene expression approach to understand the processes of cold acclimation in the transporting epithelia of chill-susceptible insects. I assembled a transcriptome for *G. pennsylvanicus* and compared the expression of Malpighian tubule and hindgut genes between warm- and cold-acclimated adults (with a focus on genes involved in ion and water homeostasis and cellular and junctional integrity). I aimed to generate mechanistic hypotheses about specific molecular underpinnings of cold acclimation, and provide insights into the causes of water and ion disruption during cold exposure.

3.2 Methods

3.2.1 Insect rearing

A colony of *G. pennsylvanicus* originated from individuals collected in 2004 from the University of Toronto at Mississauga campus, Ontario and was reared under constant summer-like conditions (25ºC, 14 light:10 dark photoperiod, 70% RH), as described previously (MacMillan and Sinclair, 2011b; Coello Alvarado et al., 2015). At approximately 8 weeks post-hatch, and prior to sexual maturation, female crickets were separated from males to prevent mating. Adult females at approximately 11 weeks post-hatch were used for experiments.

3.2.2 Acclimation and dissection

Crickets were first isolated individually in common summer-like conditions in mesh-covered 180 mL transparent cups (Polar Plastics, Summit Food Distributors, London, ON, Canada) containing egg carton shelters, rabbit food, and water. Isolation prevented cannibalism and lasted one week. I then haphazardly assigned crickets into cold- and warm-acclimations (n = 30 per treatment). For warm acclimation, crickets remained in the rearing growth chamber under constant summer-like conditions. I cold-acclimated crickets in a Sanyo MIR 154 incubator (Sanyo Scientific, Bensenville, Illinois) at 10 light:14 dark with temperature decreasing from 25 to 12ºC over seven days followed by constant 12ºC for three weeks. This regime lowers the critical thermal minimum c. 2ºC, reduces chill coma recovery time by c. 65%, increases survival following 5 d at 0ºC by nearly 80%, and enhances maintenance of ion and water homeostasis in the cold (Coello Alvarado et al., 2015). While the two acclimation temperatures are likely to affect physiological ageing, the four weeks of acclimation represent approximately 20% of the adult cricket lifespan. Therefore I assumed that the effect of physiological age on gene expression would be less than the effect of acclimation temperature (Lai et al., 2007).

Cricket hindguts were dissected as described previously (Chapter 2) immediately following the four weeks of warm- or cold-acclimation. Under Ringer's solution (110 Na^+, 8.5 K^+, 6 Mg^{2+}, 7 Ca^{2+}, 144.5 Cl^-, pH 7.6, concentrations in mM, derived from measurements of *G.*

pennsylvanicus, Chapter 2) in a Petri dish the hindgut (rectum, colon, and ileum) was cut away from the gastrointestinal tract and flushed of fecal material with approximately 3 mL of Ringer's using a syringe (this procedure took < 3 min). Malpighian tubules were removed as a single bunch by detaching the ureter with forceps, rinsing briefly in Ringer's, and blotting on a tissue. Malpighian tubules and hindguts were flash-frozen in liquid nitrogen. The three biological replicates for the hindgut and Malpighian tubules were each comprised of tissue pooled from ten individuals. To maximize transcript coverage for the *de novo* assembly, warm- and cold-acclimated whole male and female adult crickets, eggs, and warm-acclimated juveniles were pooled and added to an additional 1.5 mL microcentrifuge tube and flash frozen in liquid nitrogen. All samples were stored at -80 ºC until RNA extraction.

3.2.3 RNA extraction & cDNA library preparation

I homogenized thawed tissues with a plastic micropestle (ThermoFisher Scientific, Ottawa ON, Canada) in 1.1 mL TRIzol (Invitrogen, Burlington ON, Canada), and extracted RNA according to manufacturer's instructions. I purified RNA extracts using the RNeasy Mini kit (Qiagen, Mississauga ON, Canada) according to manufacturer's instructions, measured absorbance at 260 nm to determine RNA concentrations, and checked for RNA quality with an Agilent Bioanalyzer. cDNA library production and sequencing were performed by the Donnelly Sequencing Center (Toronto ON, Canada). At 13 samples per lane, each cDNA library was sequenced twice using the Illumina HiSeq2500 platform (Illumina, San Diego, CA) with single-end, 50-bp reads.

3.2.4 *De novo* transcriptome assembly and annotation

I removed Illumina adapter sequences and discarded sequences shorter than 15 nucleotides or containing unknown bases using the Galaxy web service (Goecks et al., 2010). Sequenced libraries were then grouped and assembled *de novo* using Trinity release 2012-10-25 (Grabherr et al., 2011; Haas et al., 2013) on the SHARCNET computing cluster (https://www.sharcnet.ca), with 1 GB Jellyfish Memory and a minimum contig (a set of

overlapping sequence fragments representing a consensus regions of DNA) length criterion of 100 nucleotides. Transcriptome assembly "completeness" was compared to a database of arthropod Benchmark Universal Single Copy Orthologs (BUSCO) using BUSCO v1.22 (Simão et al., 2015). Contigs from the Trinity assembly were compared to the National Centre for Biotechnology Information (NCBI) non-redundant (nr) protein database (September 2013) by BLASTx (e-value threshold = 1×10^{-3}). Gene Ontology (GO) annotation (e-value threshold = 1×10^{-6}) was based on SwissProt BLAST matches using Blast2GO version 2.7.2 (Conesa et al., 2005). To filter out transcriptional artifacts, misassembled transcripts, and poorly-supported transcripts, the original cleaned sequence reads were mapped back onto the Trinity-assembly using Bowtie2 version 2.1.0 (Li et al., 2009; Langmead and Salzberg, 2012) and reassembled with the Cufflinks package version 2.1.1 (Trapnell et al., 2012). Blast2GO (Conesa et al., 2005) and the NCBI database was used to obtain putative identities and GO annotation for mapped transcripts. I accepted one hit for each transcript at an e-value threshold of 1×10^{-3}.

3.2.5 Gene expression analyses

I used normalized read counts of genes in warm- and cold-acclimated hindgut and Malpighian tubule libraries for differential gene expression analysis using the edgeR Bioconductor package (Robinson et al., 2010) in R (v3.2.2, R Development Core Team, 2015; Risso et al., 2014). Because each biological replicate was sequenced twice (two technical sequencing replicates), read counts from these technical replicate libraries were summed for each gene. For analyses I retained only those genes with at least 10 counts per million in three of the six libraries being compared (warm- vs cold-acclimated hindguts each had three biological replicates) (Robinson et al., 2010). Filtering yielded 11,140 and 11,066 contigs for differential gene expression analyses of hindgut and Malpighian tubules, respectively. I compared gene expression profiles within tissues (i.e. warm- vs cold-acclimated), and also compared the hindgut and Malpighian tubules for genes that were uniquely up- or downregulated between those tissues with cold acclimation. Individual genes were considered differentially expressed if the absolute fold change was ≥ 2 and if the P-value adjusted for false discovery rate (FDR) was < 0.05.

I used contigs that met the criteria for inclusion (fold change ≥ 2, FDR-adjusted P-value < 0.05) to identify the GO terms associated with the responses to cold acclimation in each tissue (note that I did not formally compare GO terms among tissues or treatments). Differentially-expressed pathways in warm- and cold-acclimated tissues were analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa and Goto, 2000). KEGG identities were assigned to contigs by the KEGG Automatic Annotation Server (Moriya et al., 2007), and differential expression analyses of pathway components were performed using the Generally Applicable Gene-set Enrichment (GAGE) and Pathview Bioconductor packages (Luo et al., 2009; Luo and Brouwer, 2013) in R. These packages identify coordinated differential expression in gene sets (pre-defined, functionally-related groups of genes; Luo et al., 2009). I accepted pathways as differentially-expressed if the FDR-adjusted P-value was < 0.1.

3.3 Results

Sequencing of 26 libraries yielded 286 million 50-bp reads, which were assembled into 70,037 contigs (Table 3.1). The transcriptome included 1808 (67.6%) complete and 415 (15.5%) fragmented of 2675 arthropod BUSCOs; which is similar to other recent arthropod transcriptome assemblies (e.g. Tassone et al., 2016; Theissinger et al., 2016), and comparable or better than the transcriptomes referred to by Simão et al. (2015). Approximately 44% of these contigs in the transcriptome had putative identities by BLAST (Supplementary material, Spreadsheet S1), and of these approximately 36% aligned to genes of the termite *Zootermopsis nevadensis*. Cold acclimation led to a two-fold or greater change in 1,493 genes in the hindgut and 2008 genes in the Malpighian tubules (Fig. 3.1). Within a given tissue, the number of genes up- and down-regulated were approximately equal. Approximately 52% of all upregulated genes and 60% of all downregulated genes exhibited unique differential expression across the two tissues. Eighty-one genes that appear to be important for cold acclimation (those with a 10-fold or greater change in expression) were unidentifiable by BLAST (Supplementary material, Spreadsheet S1). These represented 22 upregulated and 11 downregulated genes in the hindgut, and 26 upregulated and 22 downregulated genes in the Malpighian tubules.

Table 3.1. Summary of *G. pennsylvanicus* transcriptome *de novo* assembly.

Sequencing & Quality Control	
Libraries	26
50-bp reads (raw)	286 million
50-bp reads (trimmed/cleaned)	266 million
Trinity assembly	
Assembly length (bp)	92 million
Contigs	260,407
Mean contig length (bp)	352
Median contig length (bp)	156
N50	716
GC %	38
Cufflinks refined assembly	
Length (bp)	59 million
Contigs	70,037
Mean contig length (bp)	839
Median contig length (bp)	459
N50	1,524
GC %	39
Identification	
Contigs with BLAST hit	30,666
Contigs with GO description	5,292

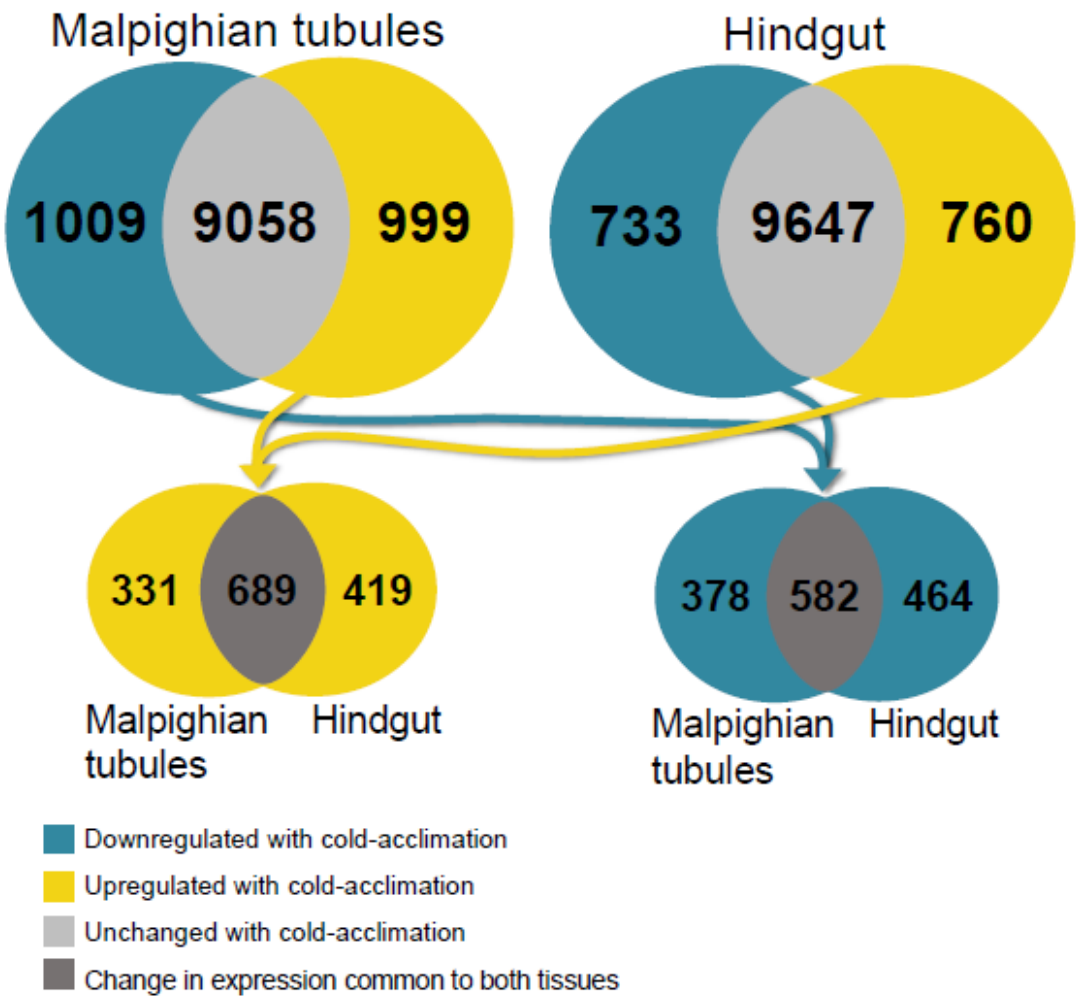

Figure 3.1. Number of genes upregulated, down-regulated, or unchanged in the hindgut and Malpighian tubules of *G. pennsylvanicus* crickets following cold acclimation. Differentially-expressed genes are those with an FDR alpha < 0.05 and a fold-change > 2. Note that due to some overlap in contigs the sum of genes up- or down-regulated across both tissues is less than the sum of genes up- or down-regulated in separate tissues (1,439 and 1,424 unique genes were up- and downregulated across both tissues, respectively).

The GO domain profiles that changed with cold acclimation were similar across the two tissues; of all up- or downregulated GO terms, just over half related to 'molecular function' (i.e. transport, binding, enzyme and receptor activities), over one third related to 'biological processes' (series of molecular events with a defined beginning and end), and roughly one tenth were 'cellular components' (i.e. specific locations of subcellular structures and

macromolecular complexes; Supplementary material, Spreadsheet S2). Within the molecular function GO domain, genes involved in nucleotide, protein, metal, and ion binding accounted for over 50% of the upregulated transcripts and approximately 30% of downregulated transcripts in both tissues. Most of the cellular components that were differentially-expressed with cold acclimation involved the cell membrane and extracellular region. In the Malpighian tubules, genes pertaining to the cytoskeleton and cellular junctions accounted for 5% of all upregulated cellular component transcripts, while V-ATPase accounted for 2% of the downregulated transcripts. Metabolic genes accounted for much of the downregulated biological process transcripts counts in both tissues. However, some unique differences in biological process profiles between the two tissues were apparent; approximately 17% of downregulated transcripts in the Malpighian tubules were transport-related (2% of which were ion transport-specific), compared to just over 8% of downregulated transcripts in the hindgut. Of the upregulated biological processes over 30% of transcripts in the hindgut involved stress response, protein folding, and repair, while over 10% of transcripts in the Malpighian tubules related to transport.

3.3.1 Hindgut

Cold-acclimated *G. pennsylvanicus* had altered expression of putative gene orthologs related to apoptosis, the cytoskeleton, detoxification and repair, ion transport and pH regulation, phosphorylation, protein folding, and signal transduction in the hindgut (Tables 3.2, 3.3). A complete list of differentially-expressed genes in the hindgut is provided in Supplemental material Spreadsheet S1. Upregulated genes involved in water and ion balance included those encoding atrial natriuretic peptide-converting enzyme, NKA α-subunit, and a Ca^{2+} release-activated Ca^{2+} channel protein, while downregulated genes included those putatively encoding bumetanide-sensitive Na^{+}-Cl^{+} channel (the Na^{+}-K^{+}-$2Cl^{-}$ cotransporter, or NKCC), carbonic anhydrase (CA) isozymes 1 and 9, and a mitochondrial Na^{+}-H^{+} exchanger (NHA). A gene encoding the putative microtubule-associated protein Jupiter increased during cold acclimation, while a putative enzyme involved in homeoviscous adaptation—Δ9 desaturase 1—was downregulated 3.9-fold. Among the genes most differentially-expressed in cold-acclimated crickets related to repair

and oxidative damage, including those encoding cytochrome P450 (26-fold increase) and a putative cytochrome P450 cyp44 (5.5-fold decrease), glutathione S-transferase (6.8-fold decrease), and vitellogenin (40-fold decrease). Cold acclimation altered expression of hindgut heat shock proteins (*hsp70* and *hsp90* were upregulated, while *hsp67B* and *hspβ11* were downregulated), and altered the expression of some apoptosis genes. Cold-acclimated hindguts exhibited upregulation of a number of protein kinases, phosphodiesterases, and adenylate cyclase. Circadian genes *per, clock,* and *nocturnin* were upregulated with cold acclimation, while *timeless* was downregulated.

More KEGG pathways in the hindgut were downregulated with cold acclimation than were upregulated (Fig. 3.2). Among 25 upregulated pathways, 'adherens junction' (Fig. 3.3) and 'gap junction' are likely to be directly relevant to ion and water balance. Cold acclimation shifted actin regulation within the 'adherens junction' pathway; some genes putatively encoding actin-associated proteins (FRG and α-actinin) were upregulated while others were downregulated (β/γ actin, vinculin, and α-catenin). The putative proteins vascular endothelial protein tyrosine phosphatase (VE-PTP), transforming growth factor β2 (TGFβ2), and partitioning defective protein 3 (PAR3) were also upregulated. Upregulation of the 'gap junction' pathway was driven by increased expression of a gene putatively encoding tubulin α (TUBA), and to some degree epidermal growth factor receptor (EGFR, or ErbB-1,1 listed as the receptor tyrosine kinase, RTK), while the gene encoding protein kinase C α (PKC-α) was downregulated. Many of the 47 downregulated KEGG pathways in the hindgut were related to metabolism, but also included 'cardiac muscle contraction' (Fig. 3.4) and 'synaptic vesicle cycle'. Downregulation of the 'cardiac muscle contraction pathway' was driven by a decrease in expression of the gene encoding cytochrome c reductase, however there were also significant increases in the expression of genes encoding the NKA α subunit, tropomyosin 1, and myosin heavy chain 6/7. Downregulation of the 'synaptic vesicle cycle' was driven by a reduction in the expression of the putative proton pump (V-ATPase), however the gene encoding the dynamin GTPase increased.

Table 2.2. Selected upregulated genes in the *G. pennsylvanicus* hindgut following cold acclimation whose putative function in relation to cold tolerance is discussed in the text. For a full list of the 760 upregulated hindgut genes, see Spreadsheet S1. P-values were adjusted for false discovery rate (FDR). For each gene, the species with the highest sequence similarity via BLAST is given. Species codes: *Aa (Aedes aegypti), Ap (Acyrthosiphon pisum), Gb (Gryllus bimaculatus), Gf (Gryllus firmus), Gm (Galleria mellonella), Ll (Lutzomyia longipalpis), Lm (Locusta migratoria), Md (Microplitis demolitor), Ms (Modicogryllus siamensis), Tc (Tribolium castaneum), Xt (Xenopus tropicalis), Zn (Zootermopsis nevadensis).*

Function	Description	Fold change	P-value	Species
Apoptosis	Caspase-6	4.0	$1.8E^{-31}$	*Gs*
	Caspase-8	4.0	$9.1E^{-39}$	*Zn*
Circadian	Clock	2.7	$1.7E^{-17}$	*Gb*
	Nocturnin	2.4	$1.8E^{-16}$	*Zn*
	Period	7.4	$1.6E^{-50}$	*Gb*
Cytoskeleton	Microtubule-Associated Protein Jupiter	4.2	$5.3E^{-31}$	*Zn*
Diuresis	Atrial Natriuretic Peptide-Converting Enzyme	3.5	$3.7E^{-15}$	*Zn*
Ion transport	Ca^{2+} Release-Activated Ca^{2+} Channel Protein 1	2.3	$4.3E^{-17}$	*Zn*
	Na^{+}-K^{+} ATPase Alpha Subunit	2.8	$2.7E^{-21}$	*Ll*
Neurotransmission	Na^{+}- and Cl^{-}-Dependent GABA Transporter Ine	2.1	$8.8E^{-10}$	*Md*
Phosphorylation	Dual Specificity Tyrosine-Phosphorylation-Regulated Kinase 2	2.7	$2.2E^{-04}$	*Zn*
	Serine Threonine-Protein Kinase Rio3	2.9	$3.7E^{-07}$	*Zn*
	Serine Threonine-Protein Kinase Sik3-Like Isoform X3	2.9	$8.7E^{-16}$	*Ap*
Protein folding	Heat Shock Protein 90	3.0	$1.7E^{-28}$	*Gf*
	Hsp70 Family Member	2.1	$8.0E^{-14}$	*Lm*
Repair/antioxidant	Cytochrome P450	26.0	$7.7E^{-108}$	*Aa*
	Cytochrome P450 4C1	2.0	$7.2E^{-05}$	*Zn*
	DNA Mismatch Repair Protein Mlh1	3.5	$1.2E^{-22}$	*Xt*
	DNA Repair Protein Complementing Xp-G Cells	2.7	$4.8E^{-20}$	*Zn*
	Glutathione S-Transferase D7	2.1	$2.6E^{-05}$	*Zn*
Signal transduction	cAMP-Specific 3',5'-Cyclic Phosphodiesterase, Isoform F Isoform X2	2.1	$3.3E^{-12}$	*Tc*
	Dual 3',5' Cyclic-AMP and -GMP Phosphodiesterase 11	2.5	$1.5E^{-17}$	*Zn*
	G Kinase-Anchoring Protein 1	3.8	$6.5E^{-34}$	*Zn*
	G-Protein Coupled Receptor Mth2-Like	2.2	$4.5E^{-14}$	*Ap*
	Protein Kinase C Iota (partial)	2.4	$1.5E^{-16}$	*Zn*
Signalling/gut contraction	Adenylate Cyclase Type (partial)	2.5	$1.2E^{-13}$	*Zn*

Table 3.3. Selected genes downregulated in the *G. pennsylvanicus* hindgut following cold acclimation whose putative function in relation to cold tolerance is discussed in the text. For a full list of the 733 downregulated hindgut genes, see Spreadsheet S1. P-values were adjusted for false discovery rate (FDR). For each gene, the species with the highest sequence similarity via BLAST is given. Species codes: *Ac (Acheta domesticus), Ap (Aphis gossypii), Ar (Athalia rosae), Bt (Bemisia tabaci), Dp (Diploptera punctata), Gb (Gryllus bimaculatus), Go (Gryllus orientalis), Lm (Locusta migratoria), Md (Microplitis demolitor), Mr (Megachile rotundata), Nv (Nasonia vitripennis), Ps (Plautia stali), Sg (Schistocerca gregaria), Tc (Tribolium castaneum), Zn (Zootermopsis nevadensis).*

Function	Description	Fold change	P-value	Species
Apoptosis	Apoptosis-Inducing Factor 3-Like	-4.4	$6.1E^{-29}$	*Nv*
Circadian	Timeless	-4.3	$2.3E^{-36}$	*Gb*
Ion transport	Bumetanide-Sensitive Na^+-Cl^- (partial) (NKCC)	-2.9	$6.9E^{-25}$	*Zn*
	Na^+-Independent Sulfate Anion Transporter-Like	-2.8	$1.9E^{-12}$	*Mr*
	Organic Cation Transporter Protein	-2.6	$4.2E^{-19}$	*Zn*
Ion transport/pH regulation	Carbonic Anhydrase 1	-4.6	$4.9E^{-49}$	*Tc*
	Carbonic Anhydrase 9	-3.9	$6.5E^{-40}$	*Zn*
	Mitochondrial Na^+-H^+ Exchanger NHA2	-2.9	$7.0E^{-18}$	*Zn*
Phospholipid biochemistry	Δ9 Desaturase 1	-3.9	$1.4E^{-10}$	*Ad*
Protein folding	Heat Shock Protein 67B2	-2.1	$3.3E^{-09}$	*Zn*
	Heat Shock Protein β-11	-4.5	$5.1E^{-29}$	*Zn*
Repair/antioxidant	Antioxidant Enzyme	-2.0	$1.6E^{-12}$	*Go*
	Cytochrome P450 4C1	-4.1	$4.0E^{-42}$	*Zn*
	Cytochrome P450 6A14	-3.8	$1.3E^{-12}$	*Zn*
	Cytochrome P450 9E1	-2.5	$9.5E^{-19}$	*Dp*
	Cytochrome P450 Cyp44	-5.5	$1.9E^{-43}$	*Zn*
	Epsilon Glutathione S-Transferase	-2.1	$7.8E^{-11}$	*Lm*
	Glutathione S-Transferase	-6.8	$1.2E^{-35}$	*Bt*
	Glutathione S-Transferase-Like	-2.5	$2.3E^{-15}$	*Md*
	Glutathione S-Transferase Sigma 1	-2.5	$2.1E^{-10}$	*Sg*
	Glutathione S-Transferase Sigma 7	-4.8	$1.5E^{-26}$	*Lm*
	Glutathione S-Transferase Theta 1	-2.8	$2.5E^{-21}$	*Lm*
	Peroxiredoxin	-2.6	$4.3E^{-17}$	*Go*
	Vitellogenin	-40.0	$1.7E^{-47}$	*Ar*
	Vitellogenin-2	-18.4	$1.2E^{-50}$	*Ps*

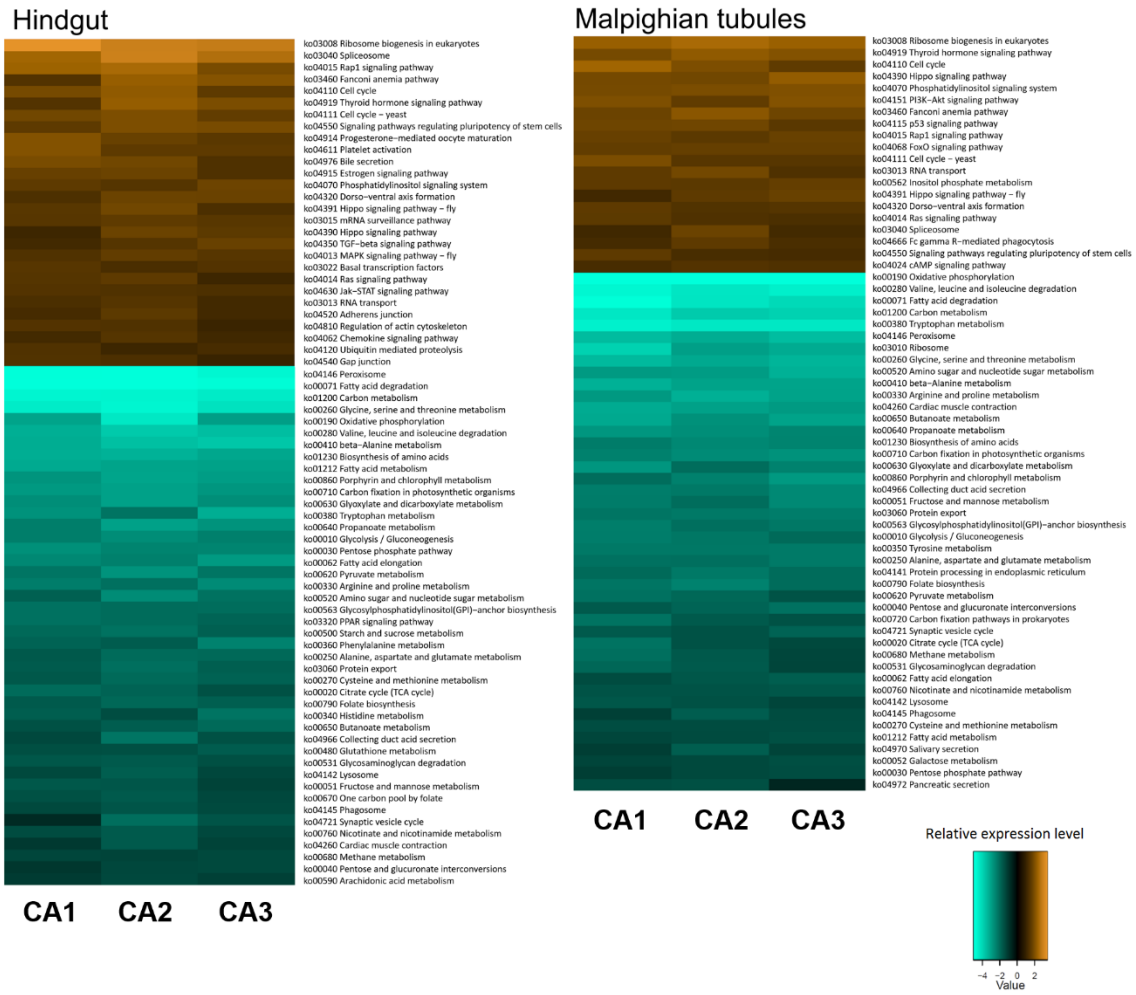

Figure 3.2. Heat map of differentially-expressed KEGG pathways in the hindgut and Malpighian tubules of *G. pennsylvanicus* crickets following cold acclimation. Upregulated pathways are given in orange and downregulated pathways are given in blue. Each heat map contains three column indicating three cold-acclimated biological replicates (CA 1-3) each compared to the mean expression among warm-acclimated replicates. For a complete description of each pathway, see the KEGG online resource (http://www.genome.jp/kegg/).

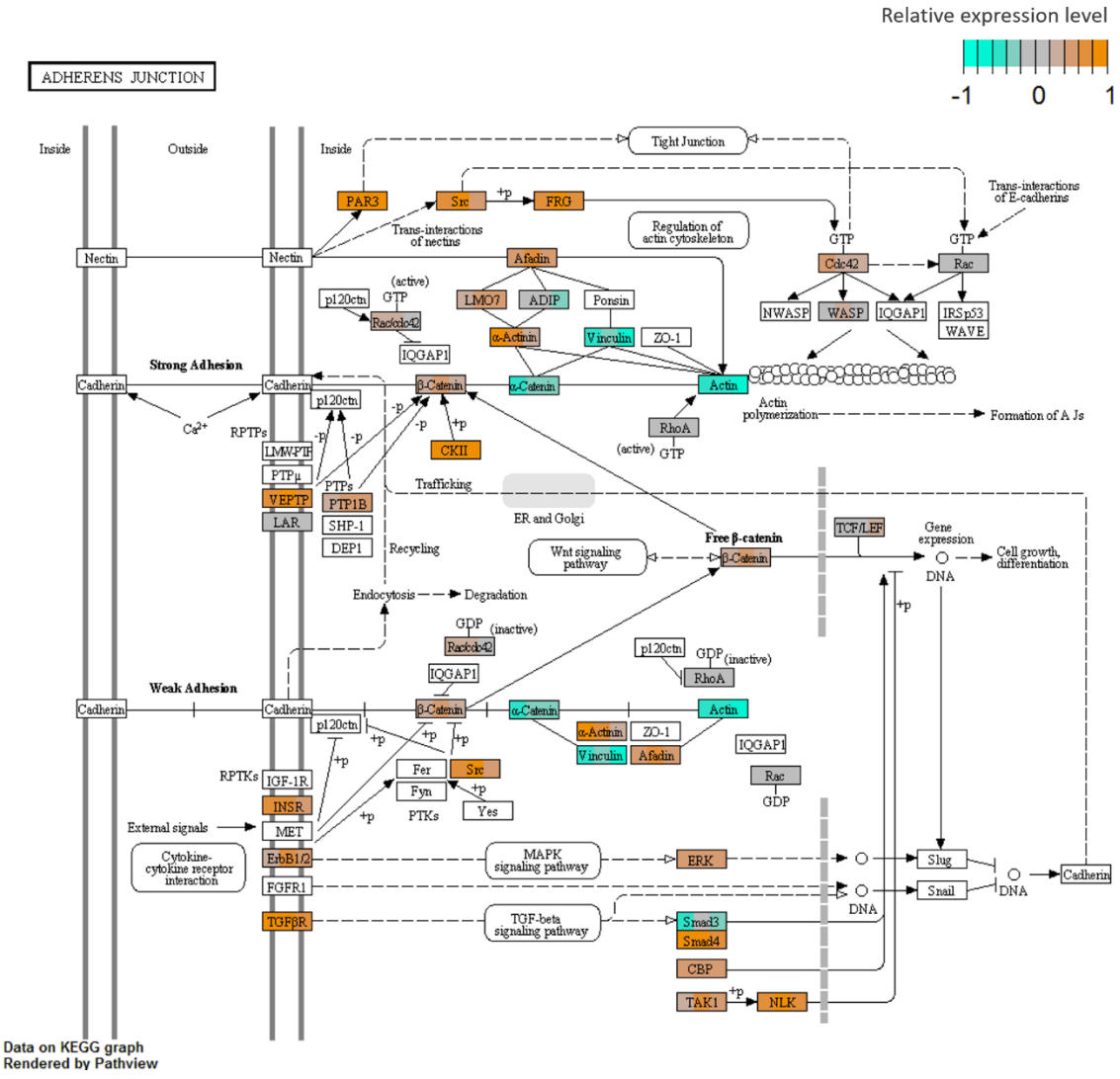

Figure 3.3. Shifts in the expression of 'adherens junction' KEGG pathway components in the *G. pennsylvanicus* hindgut following cold acclimation, as an example of a pathway that was significantly differentially regulated. Each pathway component contains three color bars indicating three cold-acclimated biological replicates each compared to the mean expression among warm-acclimated replicates. For cold-acclimated crickets relative to warm-acclimated crickets, shifts in expression are either upregulated (orange), downregulated (blue), or unchanged (grey). For a complete description of each pathway component, see the KEGG 'adherens junction' reference pathway (http://www.genome.jp/kegg-bin/show_pathway?ko04520).

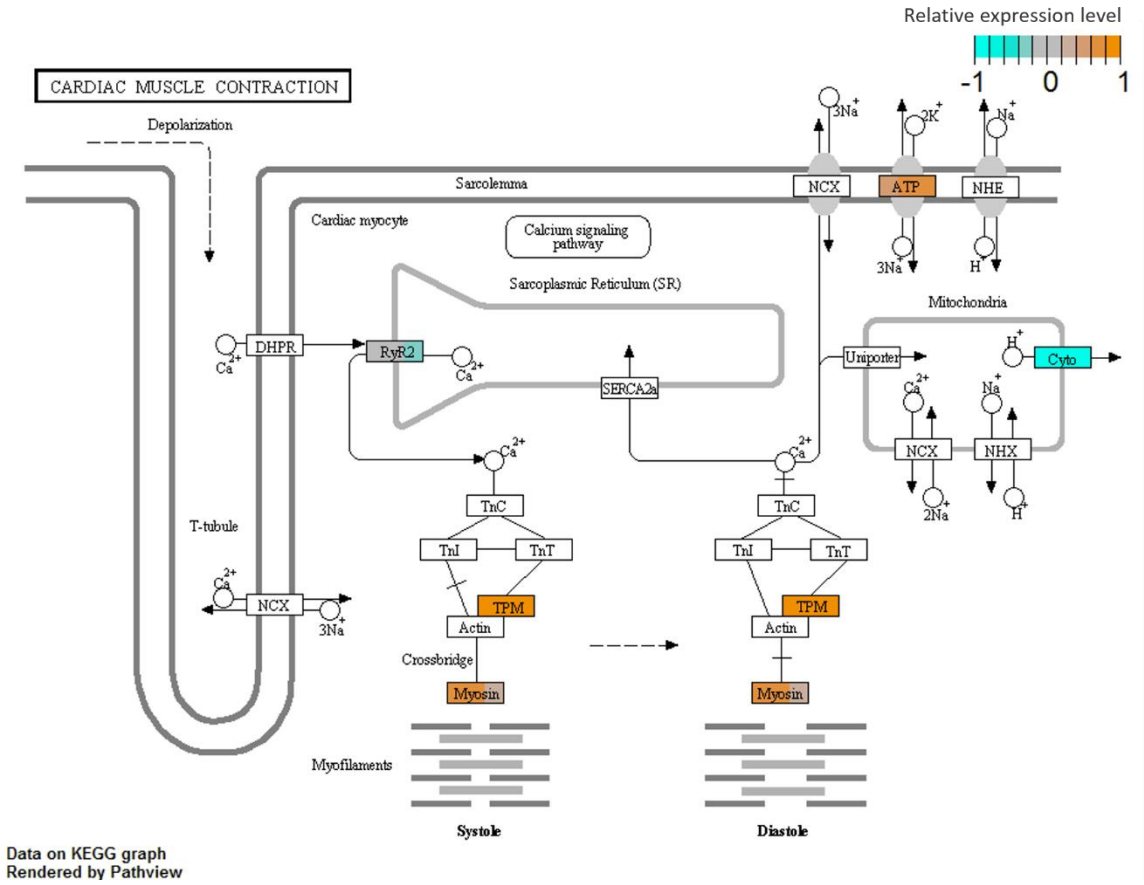

Figure 3.4. Shifts in expression of the 'cardiac muscle contraction' KEGG pathway components in the *G. pennsylvanicus* hindgut following cold acclimation, as an example of a pathway that was significantly differentially regulated. Cardiac muscle is analogous to insect striated muscle. Each pathway component contains three color bars indicating three cold-acclimated biological replicates each compared to the mean expression among warm-acclimated replicates. For cold-acclimated crickets relative to warm-acclimated crickets, shifts in expression are either upregulated (orange), downregulated (blue), or unchanged (grey). ATP - Na^+-K^+ ATPase α subunit, Cyto - cytochrome c reductase iron-sulfur subunit, TPM - tropomyosin 1, Myosin - myosin heavy chain 6/7. For a complete description of each pathway component, see the KEGG 'adherens junction' reference pathway (http://www.genome.jp/kegg-bin/show_pathway?ko04520).

3.3.2 Malpighian tubules

Cold acclimation shifted the expression of Malpighian tubule genes related to apoptosis and autophagy, the cytoskeleton, detoxification and repair, ion transport, pH regulation, phosphorylation and signal transduction, and protein folding (Tables 3.4, 3.5; see Supplemental material, Spreadsheet S1A for a complete list of differentially-expressed genes). Ion transporters included a putative V-ATPase (downregulated 2-fold), Ca^{2+} and anion transporters (upregulated over 2-fold), and both CA 1 and 9 (downregulated 2- and 4-fold, respectively). Cold acclimation led to variable expression of cytoskeletal genes, increased the expression of two apoptosis genes, and decreased expression of one gene involved in autophagosome formation. Similar to the hindgut, cold-acclimated Malpighian tubules also exhibited increased expression of *hsp70* and downregulation of *hsp67B*, and both up- and downregulation of multiple repair and antioxidant genes (e.g. cytochrome P450s and glutathione S-transferases). Multiple kinase genes were upregulated in cold-acclimated Malpighian tubules while inositol monophosphatase expression decreased (6.9-fold). Altered expression of circadian genes following cold acclimation were also similar to that of the hindgut, and juvenile hormone expression was reduced nearly 11-fold.

Similar to patterns in the hindgut, more KEGG pathways were downregulated than were upregulated in cold-acclimated Malpighian tubules (Fig. 3.2). Many of the 20 upregulated pathways were involved in signaling, and most of the 47 downregulated pathways related to metabolism. The ‘cardiac muscle contraction’ pathway (analogous to insect striated muscle; Piek and Njio, 1979) was downregulated based on reduced expression of a cytochrome c reductase gene. Unlike in the hindgut, NKA, tropomyosin, or myosin heavy chain components of this pathway were not upregulated in Malpighian tubules. The ‘synaptic vesicle cycle’ pathway exhibited downregulation overall (driven by downregulation of V-ATPase), however a number of genes involved in endocytosis and vesicle-membrane fusion were upregulated. These upregulated genes include those encoding putative N-ethylmaleimide-sensitive factor (NSF, an ATPase), dynamin, AP2 complex α (a protein associated with endocytosis of clathrin-coated vesicles), and syntaxin 1A (involved in vesicle fusion for exocytosis).

Table 3.4. Selected upregulated genes in *G. pennsylvanicus* Malpighian tubules following cold acclimation whose putative function in relation to cold tolerance is discussed in the text. For a full list of the 999 upregulated Malpighian tubule genes, see Spreadsheet S1. P-values were adjusted for false discovery rate (FDR). For each gene, the species with the highest sequence similarity via BLAST is given. Species codes: *Aa (Aedes aegypti), Gb (Gryllus bimaculatus), Hs (Harpegnathos saltator), Lm (Locusta migratoria), Ms (Modicogryllus siamensis), Nv (Nasonia vitripennis), Phc (Pediculus humanus corporis), Tc (Tribolium castaneum), Xt (Xenopus tropicalis), Zn (Zootermopsis nevadensis).*

Function	Description	Fold change	P-value	Species
Apoptosis	Caspase-8	2.1	$1.7E^{-5}$	*Zn*
	Programmed Cell Death Protein 2	2.4	$7.0E^{-10}$	*Nv*
Circadian	Clock	2.5	$6.4E^{-9}$	*Gb*
	Nocturnin	3.5	$1.2E^{-23}$	*Zn*
	Period	7.6	$2.5E^{-63}$	*Ms*
Cytoskeleton	Gamma-Tubulin Complex Component 6	2.2	$8.6E^{-10}$	*Zn*
	Kinesin-Like Protein Costa	2.8	$2.6E^{-9}$	*Zn*
	Protein Shroom	2.5	$1.6E^{-9}$	*Zn*
	Microtubule-Associated Protein Jupiter	2.2	$1.8E^{-8}$	*Zn*
Ion transport	Ca^{2+}-Transporting ATPase Type 2C Member 1	2.2	$1.1E^{-8}$	*Zn*
	Solute Carrier Organic Anion Transporter Family Member 5a1	2.4	$3.0E^{-11}$	*Hs*
Phosphorylation	Inositol Polyphosphate Multikinase	2.2	$2.6E^{-15}$	*Zn*
	Serine Threonine-Protein Kinase rio3	3.4	$5.4E^{-35}$	*Zn*
	Serine Threonine-Protein Kinase pctaire-2	2.6	$3.9E^{-14}$	*Zn*
	Serine Threonine-Protein Kinase Tousled-Like 2	2.9	$2.7E^{-11}$	*Zn*
	Tyrosine-Protein Kinase Transmembrane Receptor ror1	2.5	$6.6E^{-10}$	*Phc*
	Tyrosine-Protein Phosphatase Non-Receptor Type 23	2.5	$1.6E^{-15}$	*Zn*
Protein folding	Hsp 70 Family Member	2.0	$1.0E^{-10}$	*Lm*
	Hsp 70-binding Protein 1	3.3	$2.7E^{-22}$	*Zn*
Repair/antioxidant	Alkylated DNA Repair Protein Alkb-Like Protein	2.6	$1.4E^{-12}$	*Zn*
	Cytochrome P450	14.6	$3.7E^{-58}$	*Aa*
	Cytochrome P450 2j2	3.4	$4.6E^{-7}$	*Zn*
	Cytochrome P450 partial	6.1	$9.1E^{-11}$	*Zn*
	DNA Mismatch Repair Protein mlh1	4.2	$3.9E^{-24}$	*Xt*
	Glutathione S-Transferase Sigma 7	2.3	$9.5E^{-9}$	*Zn*
	Peroxiredoxin-6	2.1	$4.0E^{-9}$	*Lm*
Signal transduction	cAMP-Specific 3',5'-Cyclic Isoform F Isoform X2	3.2	$2.4E^{-30}$	*Tc*
	G Kinase-Anchoring Protein 1	3.7	$1.2E^{-28}$	*Zn*

Table 3.5. Selected downregulated genes in *G. pennsylvanicus* Malpighian tubules following cold acclimation whose putative function in relation to cold tolerance is discussed in the text. For a full list of the 1009 downregulated Malpighian tubule genes, see Spreadsheet S1. P-values were adjusted for false discovery rate (FDR). For each gene, the species with the highest sequence similarity via BLAST is given. Species codes: *Aa (Aedes aegypti), Bt (Bemisia tabaci), Dp (Diplotera punctata), Gb (Gryllus bimaculatus), Go (Gryllotalpa orientalis), Lm (Locusta migratoria), Md (Microplitis demolitor), Tc (Tribolium castaneum), Zn (Zootermopsis nevadensis).*

Function	Description	Fold change	P-value	Species
Autophagy	Autophagy-Related Protein 2-Like Protein B	-2.1	$1.5E^{-7}$	*Zn*
Circadian	Timeless	-3.0	$3.3E^{-13}$	*Gb*
Cytoskeleton	Microtubule-Associated Proteins 1A 1B Light Chain 3C	-4.9	$8.5E^{-23}$	*Zn*
	Epidermal Growth Factor Receptor Kinase Substrate 8-Like Isoform X1	-2.4	$1.9E^{-9}$	*Md*
	Gamma-Tubulin Complex Component 3	-2.0	$4.7E^{-9}$	*Zn*
Development	Juvenile Hormone-Inducible	-10.6	$3.8E^{-50}$	*Aa*
Ion transport/pH regulation	Carbonic Anhydrase 9	-4.2	$2.1E^{-33}$	*Zn*
	Carbonic Anhydrase 1	-2.2	$5.9E^{-15}$	*Tc*
	V-ATPase Subunit D	-2.1	$1.2E^{-14}$	*Lm*
Phosphorylation	Inositol Monophosphatase	-6.9	$1.1E^{-58}$	*Zn*
	Serine Threonine-Protein Phosphatase 2B Catalytic Subunit 2-Like Isoform X2	-2.1	$9.0E^{-14}$	*Tc*
Protein folding	Heat Shock Protein 67B2	-2.8	$1.6E^{-16}$	*Zn*
Repair/antioxidant	Antioxidant Enzyme	-2.9	$3.7E^{-23}$	*Go*
	Cytochrome P450	-2.9	$1.4E^{-11}$	*Bt*
	Cytochrome P450 4C1	-2.7	$3.2E^{-22}$	*Zn*
	Cytochrome P450 6A14	-2.5	$3.2E^{-13}$	*Zn*
	Cytochrome P450 9E2	-2.3	$1.4E^{-11}$	*Zn*
	Cytochrome P450 (partial)	-2.3	$3.6E^{-12}$	*Zn*
	Cytochrome P450 9E1	-2.2	$3.3E^{-14}$	*Dp*
	Glutathione S-Transferase	-3.2	$1.8E^{-23}$	*Bt*
	Glutathione S-Transferase Theta 1	-3.1	$1.9E^{-28}$	*Lm*

3.4 Discussion

Cold acclimation altered the expression of genes associated with water and ion homeostasis and cellular junctions in addition to those involved in prevention and/or repair of chilling injury (e.g. those associated with apoptosis, cell structure, detoxification and repair, and protein folding). Similar functional changes occur during both cold acclimation and rapid cold-hardening in *Drosophila* (Gerken et al., 2015; MacMillan et al., 2016a). In crickets, stress response, protein folding, and repair appear to be particularly important aspects of cold acclimation in the hindgut, while cold acclimation in the Malpighian tubules appears to involve shifts in transport function. In both tissues, cold acclimation reduced metabolic gene expression and altered the expression of many genes encoding components of the membrane and extracellular space.

3.4.1 Water balance

Only one gene with known function in insect water homeostasis – that encoding atrial natriuretic peptide-converting enzyme – was upregulated in the hindgut following cold acclimation. Homologs of this enzyme in mosquitos stimulate primary urine production by the Malpighian tubules by increasing secretion of Na^+ and Cl^- (Petzel et al., 1985; Schooley et al., 2012). Precisely how shifts in the expression of this peptide act on the insect hindgut is less certain, but stimulation of Na^+ and Cl^- transport would suggest enhanced water reabsorption by the rectum (Phillips and Audsley, 1995), and this could aid in the maintenance of hemolymph volume during cold exposure. The effect of cold acclimation on the expression of other known diuretic peptides in the nervous system tissues should be characterized in future (cf. Paluzzi et al., 2008). Although some aquaporins are associated with insect desiccation and freeze-tolerance (Philip et al., 2008; Philip and Lee, 2010; Goto et al., 2011), their role in cold acclimation among chill-susceptible insects remains to be investigated. None of the six known water-transporting insect aquaporins were differentially-expressed genes in the hindgut or Malpighian tubules.

3.4.2 Ion transport

Cold acclimation altered the expression of putative NKA, NKCC, CA, NHA, and V-ATPase, which are typically enriched in insect transporting epithelia (Chintapalli et al., 2013). While all of these transport enzymes contribute to primary urine production by the Malpighian tubules (Ianowski and O'Donnell, 2004), most of these gene expression changes were observed in the hindgut; cold acclimation in the Malpighian tubules only downregulated genes encoding V-ATPase and CAs 1 and 9.

CA catalyzes the hydration of CO_2 to produce H_2CO_3, a source of protons for export by apical V-ATPase by the Malpighian tubules (Maddrell and O'Donnell, 1992; Wessing et al., 1997; Chintapalli et al., 2013). These lumenal protons are then exchanged for Na^+ or K^+ by NHA and K^+-H^+ antiporters (Ianowski and O'Donnell, 2006; Chintapalli et al., 2013), driving passive excretion of water and anions (Nicolson, 1993; Beyenbach, 2003). Downregulation of CA9 (membrane-bound), CA1 (cytosolic), and V-ATPase in the Malpighian tubules during cold acclimation could therefore have an antidiuretic effect, perhaps defending hemolymph volume in the cold. Indeed, cold-acclimated *G. pennsylvanicus* Malpighian tubules produce primary urine more slowly (Chapter 5).

Paracellular Na^+ gradients across the rectal pads drives passive reabsorption of water against osmotic gradients (Hanrahan and Phillips, 1982; Phillips et al., 1987; Chapman, 2013). Failure of NKA to maintain these Na^+ gradients during cold exposure could account for leak of Na^+, and consequently water, to the gut. We might therefore expect cold acclimation to increase NKA protein abundance as compensation for slower enzyme activity at low temperatures. In support of this prediction, expression of the α (catalytic) subunit of NKA increased nearly 3-fold in the cricket hindgut after cold acclimation (and despite overall metabolic downregulation in that tissue). However, cold acclimation in *D. melanogaster* lowers whole-body NKA activity (MacMillan et al., 2015d), suggesting a decoupling of Na^+ gradients across the gut rather than compensation for slowed NKA activity in the cold. This discrepancy in the apparent strategies of maintaining Na^+ balance stresses the importance of tissue-specific analysis. Alternately, it is possible that Diptera

and Orthoptera use different acclimation strategies with regards to Na^+ balance; a possibility that merits additional exploration. Hindgut NKCC (which imports Na^+, K^+, Cl^- basally and drives apical ion exchange; Zeuthen and MacAulay, 2012; Chintapalli et al., 2013) was downregulated, however it is difficult to predict how this might influence homeostasis as the role of NKCC in insect hindgut transport has received relatively little attention.

3.4.3 Cell junctions and structure

Reduced rectal pad permeability could enhance cold tolerance by minimizing Na^+ and water leak in the cold (MacMillan and Sinclair, 2011b). Cold acclimation did indeed change the expression of many tissue structural genes, which could indicate modified rectal pad epithelium. Upregulated hindgut genes involved in cell growth, differentiation, and adhesion included endothelial growth factor (EGFR), PAR3, VE-PTP, and TGFβ2 (Boyer et al., 1999; Ebnet et al., 2003; Broermann et al., 2011), and I observed both upregulation of apoptotic promoters (caspases 6 and 8) and down-regulation of a mitochondrial apoptosis-inducing factor. CA9 may reduce cellular adhesion (via interaction with β-catenin; Švastová et al., 2003) and also regulates proliferation, and differentiation (at least in mammals) (Gut et al., 2002), therefore a downregulation of CA9 expression in and hindgut and Malpighian tubules during cold acclimation may increase cellular adhesion and epithelial tightness (but see also comments above about the role of CA9 in providing protons to V-ATPase; the precise role of this enzyme in the rectum is uncertain). Tissue-specific post-translational modification of CA9 is an important means of regulating expression of this gene (Hilvo et al., 2004), however I could not identify such modifications in the present study.

Candidates for modification of epithelial permeability include septate and/or adherens junctions (comprising the bulk of rectal pad paracellular connections; Satir and Gilula, 1973; Phillips et al., 1987; Tepass et al., 2001; Matter and Balda, 2003; Adam, 2015). Cold acclimation changed the expression of hindgut adherens junction components (and potentially septate junctions, which share many components with adherens junctions; Matter and Balda, 2003). In addition to promoting actin stabilization (discussed below), I

observed both up- and down-regulation of genes encoding actin-membrane anchors with cold acclimation, which could influence cell junction characteristics or cell shape. During cold exposure, migration of water distends the gut (MacMillan and Sinclair, 2011b) and osmotic stress or changes in membrane viscosity could alter both cell shape and volume. Thus, reduced actin-membrane anchoring could minimize tension, shearing damage, or stretch-activation of membrane-bound ion channels (Örvar et al., 2000; Lecuit and Lenne, 2007). Cold acclimation also altered some gap and tight (septate) junction components, which are important for electrochemical coupling and ion and water recycling between the cytoplasm and paracellular channels (Gupta et al., 1980; Haraguchi et al., 2006), electrical insulation, shunt pathways, and selectivity of absorption (Reuss, 2001; Hartsock and Nelson, 2008), respectively. Upregulation of PAR3 (Ebnet et al., 2003) and downregulation of PKC-α (Rosson et al., 1997) suggests increased septate junction formation and therefore increased tightness of the hindgut epithelium during acclimation. Enhanced epithelial tightness could mitigate ion and water leak, however changes in septate junction morphology in the rectal pads following acclimation should be confirmed by transmission electron microscopy or immunostaining (Berridge, 1972; Dokladny et al., 2006). Leak of water and ions across the Malpighian tubules may also be altered with cold acclimation, as I observed shifts in the expression of multiple genes involving the cytoskeleton and cell junctions (e.g. protein shroom) (Hildebrand and Soriano, 1999; Hildebrand, 2005). Whether these structural changes actually affect ion and water balance requires some assessment of Malpighian tubule permeability following cold acclimation.

3.4.4 Chilling injury

Cold-attributed oxidative stress, disruption of homeostasis and signaling, protein mis-folding, and loss of membrane and cytoskeletal integrity may all contribute to chilling injury and mortality in chill-susceptible insects (Rojas and Leopold, 1996; Yu et al., 2001; Heimlich et al., 2004; Kim and Denlinger, 2009; Teets et al., 2013; MacMillan et al., 2015c; Štětina et al., 2015). Cold hardening must therefore induce physiological changes that prevent cold damage and/or repair damage upon rewarming. Upregulation of putative apoptosis genes (e.g. those encoding caspase 6 - an apoptosis initiator and caspase 8 - an apoptosis effector; Elmore, 2007) during cold acclimation might contribute to rectal pad or

Malpighian tubule epithelial restructuring (discussed above) and/or repair of damage. Similarly, shifts in autophagy-related gene expression (e.g. upregulation of Ras and ubiquitin signaling KEGG pathways) indicates that clearing of cell components (potentially following cold damage) is an important aspect of cold tolerance (Denton et al., 2012; Pérez et al., 2015). Polymorphisms or shifts in the expression of genes associated with both apoptosis and autophagy appear to be common to the rapid cold-hardening processes (Gerken et al., 2015), as well as dehydration (Teets and Denlinger, 2013; Lee and Denlinger, 2014).

Cytoskeletal components such as actin and tubulin depolymerize at low temperatures in fish, mammals, and insects (Madara et al., 1986; Belous, 1992; Kayukawa and Ishikawa, 2009; Kim and Denlinger, 2009). This depolymerization could cause chilling injury by modifying ion transporter recruitment to the membrane (e.g. lack of V-ATPase recruitment could lead to ion and pH imbalance; Breton et al., 2000), or by disrupting signaling (e.g. via Ca^{2+} influx; Örvar et al., 2000). Water loss (which occurs during cold exposure in chill-susceptible species) appears to drive shifts in cytoskeletal gene expression in other insects (Lee and Denlinger, 2014). Not surprisingly, cold acclimation enhanced expression of cytoskeletal branching and stabilizing proteins, similar to observations in cold-acclimated *Culex pipiens* (Kim et al., 2006), *Delia antiqua* (Kayukawa and Ishikawa, 2009), alfalfa (*Medicago sativa*) (Örvar et al., 2000), and *Drosophila melanogaster* (Cottam et al., 2006; MacMillan et al., 2016a). Specifically, α-catenin – which suppresses actin polymerization by binding with cadherin-β-catenin (Knudsen et al., 1995; Drees et al., 2005) – was downregulated, while ARP2/3 – which serves as a nucleation site for actin polymerization (Drees et al., 2005) – was upregulated. Tropomyosin (one of whose functions is to stabilize actin) was also upregulated in the cold-acclimated hindgut, as were microtubule protein Jupiter and MAP1A/B which promote microtubule polymerization and stabilization, respectively (Karpova et al., 2006; Riederer, 2007).

Cold exposure appears to cause oxidative stress (Huot et al., 1996; Lalouette et al., 2011), which may be exacerbated by decreased activity of antioxidant enzymes. Cold acclimation increases antioxidant activity and/or expression in a number of insects (Lalouette et al.,

2011; Storey and Storey, 2012), and this was true for crickets; I observed substantial increases in the expression of putative cytochrome P450s in both the hindgut and Malpighian tubules, in addition to some increases in DNA repair genes and a glutathione S-transferase. These may represent only a subset of cold tolerance genes as antioxidant expression can also be upregulated upon rewarming (Joanisse and Storey, 1996; Lalouette et al., 2011; Storey and Storey, 2012). It should be noted that cold acclimation also reduced the expression of multiple cytochrome P450 and glutathione S-transferase genes. This downregulation could be a consequence of lower metabolic rates during cold acclimation (i.e. lower oxygen radical stress; Lalouette et al., 2011; Niehaus et al., 2012).

Cold acclimation increased the expression of *hsp70* in both tissues (as well as hindgut *hsp90* expression). In addition to its role as a protein chaperone, hsp70 also acts to suppress apoptosis, promote DNA repair, and enhance cell survival (Moskalev et al., 2008). Hsps 70 and 90 may also protect against thermal disruption of tight junctions (Dokladny et al., 2006), which could help to prevent epithelial ion leak in the cold. Downregulated hsps included the less-characterized *hspβ11* (involved in the vertebrate heat stress response; Quinn et al., 2011) and *hsp67B2* or *hsp67Bb* (which may protect against heat and oxidative stress in *Drosophila*; Pauli et al., 1988; Moskalev et al., 2008).

3.4.5 Other differentially-expressed genes

Membrane remodeling is likely an important aspect of cold acclimation (Clark and Worland, 2008; Lopez-Martinez et al., 2009; MacMillan et al., 2009) which may help to defend transport function in addition to membrane viscosity at low temperatures. Indeed, a large proportion of differentially-expressed GO cellular components in the cold-acclimated Malpighian tubules and hindgut were membrane-associated. In the hindgut, a gene encoding Δ9 desaturase – which is implicated in homeoviscous adaptation – was downregulated with acclimation. Cold acclimation in carp and two flies (*Belgica antarctica* and *Delia radicum*) has been associated with upregulation of Δ9 desaturase (Tiku et al., 1996; Kayukawa et al., 2007; Zerai et al., 2010). We do not know whether cold acclimation causes changes in membrane fluidity in *G. pennsylvanicus*, but this would require acyl

chain desaturation or choline intercalation, which could also be achieved by other enzymes or by post-translational modification of desaturases.

Loss of synaptic transmission and/or signal transduction in the cold is a proposed mechanism of chill coma, and recent evidence suggests that paralysis during cold exposure results from direct inhibitory effects of low temperature on neuromuscular function (Koštál et al., 2006; Findsen et al., 2014; MacMillan et al., 2014; Andersen et al., 2015, Chapter 2). Cold acclimation altered the expression of some signal transduction proteins (e.g. cAMP, G-proteins, and PKC) and upregulated some neurotransmission proteins (a GABA transporter and Ca^{2+} channel), however it is difficult to predict if and how these changes enhance cold tolerance by lowering the critical thermal minimum. Perhaps of greater interest were upregulated vesicle localization and fusion genes such as dynamin (which mediates membrane-vesicle fusion), NSF (a vesicle-fusing ATPase), AP2 (involved in vesicle endocytosis), and syntaxin 1A (which promotes vesicle-membrane docking). Vesicle-membrane localization is important for both neurotransmission and recruitment of ion transporters (the latter of which could directly affect ion homeostasis in the cold; Bezzerides et al., 2004). Neurotransmitters are accumulated in synaptic vesicles by the action of V-ATPase (Moriyama et al., 1992) which was downregulated with cold acclimation.

Cold acclimation affected the expression of some genes associated with circadian rhythm, storage and metabolism, development, and phosphorylation. Differential expression of circadian genes was likely a result of the cold (e.g. low temperatures decrease the expression of *timeless* and increase the expression of *per* in flesh flies; Goto and Denlinger, 2002). Cold acclimation drastically lowered the expression of hindgut genes encoding vitellogenin – a yolk protein precursor. I suspect that vitellogenin mRNA came from traces of fat body remaining on the gut during dissections (Bownes, 1986). Adenylate cyclase was upregulated with cold acclimation, and may increase hindgut muscular contraction, which in turn could reduce the depth of unstirred boundary layers to aid in ion transport (Wright et al., 1986; Collier and O'Donnell, 1997).

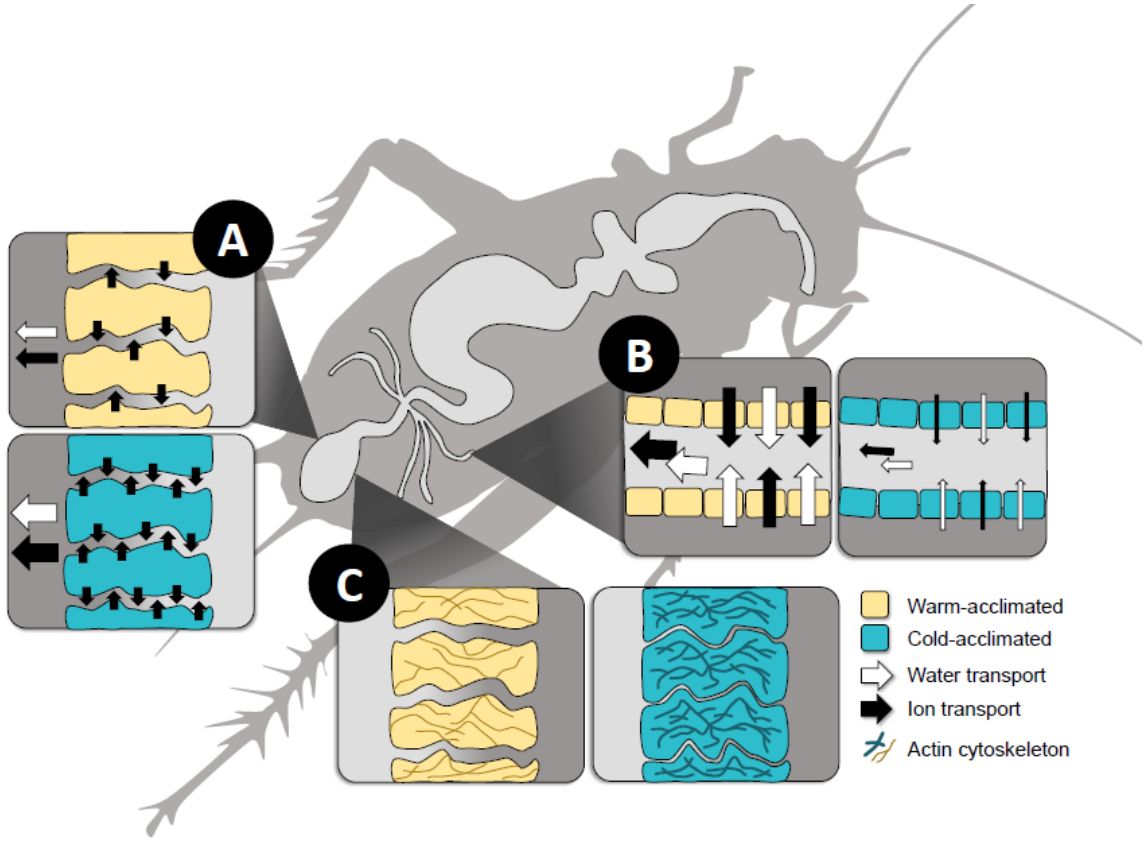

Figure 3.5. Candidate mechanisms of cold acclimation in *G. pennsylvanicus*. A) Increased abundance of NKA in rectal pad epithelia should increase Na^+ and water reabsorption; this may counteract leak of water and ions and aid in chill coma recovery). B) Downregulation of CA and V-ATPase expression in the Malpighian tubules should slow primary urine production, thereby retaining hemolymph volume. C) Cytoskeletal and junctional remodeling of the hindgut may mitigate water and ion leak during cold exposure.

3.4.6 Conclusions

I have sequenced and assembled the first transcriptome of *Gryllus pennsylvanicus* and my tissue-specific comparisons suggest that cold acclimation involves modification of both ion transport function and cellular/junctional integrity (summarized in Fig. 3.5, above). I have generated three precise mechanistic hypotheses about the cold acclimation process. 1) Ion transport modifications defend hemolymph volume in the cold; decreased Malpighian tubule V-ATPase and CA expression should slow primary urine production, while upregulation of hindgut NKA should increase Na^+ and water reabsorption and may account for faster chill coma recovery in cold-acclimated insects. 2) Remodeling of the

cytoskeleton and adherens junctions (and potentially tight junctions) may mitigate paracellular leak of water and ions in both the hindgut and Malpighian tubules. 3) Cold-acclimated crickets may prevent direct chilling injury by stabilizing the actin cytoskeleton and by changing the way in which actin anchors to the membrane, while upregulation of antioxidant, DNA repair, apoptosis, autophagy, and chaperone genes may aid in repair of chilling injuries.

3.5 References

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Chapter 4

4 Effects of cold acclimation on rectal macromorphology, ultrastructure, and cytoskeletal stability

I have prepared this chapter for submission to the *Journal of Experimental Biology*.

4.1 Introduction

Chill-susceptible insects lose ion and water homeostasis at low temperatures and may accumulate chilling injuries if the cold exposure is deep or prolonged (Koštál et al., 2004; Koštál et al., 2006; MacMillan and Sinclair, 2011b; Findsen et al., 2014; Coello Alvarado et al., 2015). This loss of homeostasis is thought to result from the failure of active transport to combat Na^+ leak down concentration gradients (MacMillan and Sinclair, 2011a), leading to bulk migration of Na^+ and water from the hemolymph to the gut (MacMillan and Sinclair, 2011b; Coello Alvarado et al., 2015). To recover from cold exposure, insects must re-establish ion and water balance and repair chilling injuries (MacMillan et al., 2012; Findsen et al., 2013; Findsen et al., 2014). Currently, our understanding of the mechanisms underlying loss of homeostasis in the cold, chilling injury, and how these two process are related, is incomplete (Rojas and Leopold, 1996; Yu et al., 2001; Teets et al., 2013; MacMillan et al., 2015c; Štětina et al., 2015). Even less is understood about the mechanisms by which cold-adapted or -acclimated insect populations sustain water and ion balance at lower temperatures and avoid chilling injury (Chen et al., 1987; Gibert and Huey, 2001; Ayrinhac et al., 2004; Koštál et al., 2004; Koštál et al., 2006; Findsen et al., 2013; Andersen et al., 2014; Coello Alvarado et al., 2015; MacMillan et al., 2015b).

Water and ion homeostasis is largely maintained by the Malpighian tubules (which excrete primary urine) and the rectum (where water and ions from the gut lumen are selectively reabsorbed). In orthopterans, reabsorption occurs across specialized epithelia of the rectal pads. The lateral borders of rectal pad cells form meandering channels that are intimately-associated with mitochondria, collectively termed the scalariform complex (Wall and Oschman, 1970; Noirot and Noirot-Timothée, 1976; Noirot-Timothée and Noirot, 1980). Sodium-Potassium ATPase (NKA) in the lateral cell membrane generates a high $[Na^+]$ in

the paracellular channels, driving water para- and transcellularly from the lumen across the rectal pads (Wall et al., 1970; Phillips et al., 1987). Absorbate enters the hemolymph via one-way valves in the muscle underlying the rectal pads (Oschman and Wall, 1969). Unlike in the Malpighian tubules, where the lumen is isosmotic to the hemolymph (Ramsay, 1954), the rectal pads establish steep osmotic and ionic gradients between the gut lumen and hemolymph (Dow, 1981; MacMillan and Sinclair, 2011b; Chapter 2).

Cold-acclimated insects defend water and ion balance to lower temperatures than warm-acclimated insects, and this is likely achieved by enhanced active transport function in the cold and/or reduction in epithelial permeability to minimize water and ion leak (Koštál et al., 2004; MacMillan and Sinclair, 2011a, b; Coello Alvarado et al., 2015). Some of these active transport modifications have been explored recently; seasonally-acquired cold tolerance has been related to whole-body NKA activity in the flies *Drosophila melanogaster* and *Eurosta solidaginis* (Koštál et al., 2007; McMullen and Storey, 2008; MacMillan et al., 2015a; MacMillan et al., 2015c), and variation in cold tolerance among *Drosophila* spp. has been correlated with selectivity of cation excretion by the Malpighian tubules (MacMillan et al., 2015a). However, the means by which epithelial permeability may be altered via tissue structural modifications during cold acclimation has been relatively unexplored. The insect gut is damaged by cold exposure (Yi and Lee, 2003; Izumi et al., 2005; Sinclair and Chown, 2005; Yi et al., 2007; Philip et al., 2008), and this is likely to exacerbate epithelial leak across the rectum. I hypothesize that cold acclimation reduces epithelial permeability (i.e. reduces water and ion diffusion) by modifying rectal tissue structure. For example, thickening of the rectal pads and associated tissues (e.g. muscle or inner cuticle) would increase water and ion diffusion distance, while narrowing and/or lengthening of the scalariform complex channels would reduce paracellular permeability across the rectum.

Multimeric cytoskeletal components (e.g. actin and tubulin) depolymerize during cold exposure in plants, mammals, and insects (Job et al., 1982; Belous, 1992; Russotti et al., 1997; Örvar et al., 2000; Pokorna et al., 2004; Cottam et al., 2006; Kim et al., 2006), and depolymerization is likely to impair gut transport function (Cantiello, 1995a; Tilly et al.,

1996; Khurana, 2000). Cytoskeletal failure may also cause a loss of cell junction integrity and exacerbate paracellular leak of water and ions (Gonzalez-Mariscal et al., 1984; Belous, 1992; Behrens et al., 1993; Turner et al., 1997), while loss of epithelial rigidity could lead to cell swelling or collapse as water traverses the rectum (Berridge, 1972). Actin is particularly important for the regulation of ion transport (e.g. for localizing enzymes to the membrane and maintaining membrane fluidity; Cantiello, 1995a; Hilgemann, 1997; Khurana, 2000), and membrane (transcellular) permeability (O'Donnell and Maddrell, 1983). Depolymerization of actin filaments activates Na^+ channels in amphibian renal cell lines (Cantiello, 1995b) and, in rats, unpolymerized actin stimulates renal NKA activity by increasing affinity for Na^+ (Cantiello, 1995a). As NKA activity is crucial for establishing osmotic gradients within the scalariform complex, actin failure in the cold could directly hinder water and Na^+ reabsorption. Actin depolymerization is associated with membrane damage in cold-exposed *Delia antiqua* onion maggots (Kayukawa and Ishikawa, 2009). Actin failure could also impede repair of chilling injuries (as epithelial wound closure requires localization of actin to the cell membrane; Rodriguez et al., 2003; Fernandez-Gonzalez et al., 2009; Fernandez-Gonzalez and Zallen, 2013).

If the actin cytoskeleton is damaged by cold, then cold acclimation should protect insects from chilling injury and loss of transport function by stabilizing actin at low temperatures (Khurana, 2000; Yi et al., 2007; Kayukawa and Ishikawa, 2009; Gerken et al., 2015). Acquired cold tolerance appears to involve modification of genes associated with the actin cytoskeleton (Teets et al., 2012; Gerken et al., 2015; Torson et al., 2015; MacMillan et al., 2016), which corroborates this hypothesis. Both cryoprotective dehydration and rehydration in the freeze-avoidant Antarctic midge also cause shifts in actin gene expression (Lopez-Martinez et al., 2009). In the dipterans *Culex pipiens* and *D. antiqua*, acquired cold tolerance is associated with defense of F-actin polymerization during cold exposure; Kim et al., 2006; Kayukawa and Ishikawa, 2009). In *C. pipiens*, improved actin filament stability appears to be driven by upregulation of *actin* genes (Kim et al., 2006). In Chapter 3 I showed that cold acclimation modifies the expression of multiple actin-associated genes in the cricket hindgut, but how these modifications affect cold tolerance or transport function is unknown.

The fall field cricket, *Gryllus pennsylvanicus* (Burmeister) (Orthoptera: Gryllidae) is a chill-susceptible species that has emerged as a model for understanding cold-induced loss of ion and water balance (MacMillan and Sinclair, 2011b; Coello Alvarado et al., 2015; Des Marteaux and Sinclair, 2016). When exposed to 0°C, *G. pennsylvanicus* exhibits chilling injury in as little as 12 h and mortality at 3-5 d (MacMillan and Sinclair, 2011b), however cold tolerance in this species is plastic; prior cold-acclimation lowers the critical thermal minimum, chill coma recovery time, incidence of injury and mortality following cold shock, and improve defense of ion and water homeostasis in the cold (Coello Alvarado et al., 2015). Cold acclimation in this species also causes differential expression of multiple cytoskeletal genes in the hindgut, many of which are actin-associated (e.g. actin-stabilizing and actin-to-membrane anchoring proteins; Fig. 4.1 and Chapter 3).

Here I aimed to test two hypotheses: 1) that cold acclimation alters the structure of the rectum and/or rectal pad scalariform complex, and 2) that cold acclimation protects cytoskeletal integrity at low temperatures. Using warm- and cold-acclimated *G. pennsylvanicus* I measured the macromorphological characteristics of the rectum (via brightfield microscopy), ultrastructure of the scalariform complex (via transmission electron microscopy), and rectal pad actin polymerization before and after cold shock (via fluorescence confocal microscopy).

4.2 Methods

Gryllus pennsylvanicus were reared as described by Des Marteaux and Sinclair (2016) (Chapter 2). Briefly, I reared crickets under constant summer-like conditions (25°C, 14 light:10 dark photoperiod, 70% RH) in transparent 60 L plastic containers with stacked cardboard egg cartons for shelter, tap water, and *ad libitum* commercial rabbit food (Little Friends Original Rabbit Food, Martin Mills, Elmira, ON, Canada). I collected eggs in containers of moist vermiculite and sterile sand and placed them at 4°C to accommodate an obligate three-month diapause (Rakshpal, 1962) before returning them to 25°C to hatch. I used adult female crickets at approximately three months post-hatch for all experiments.

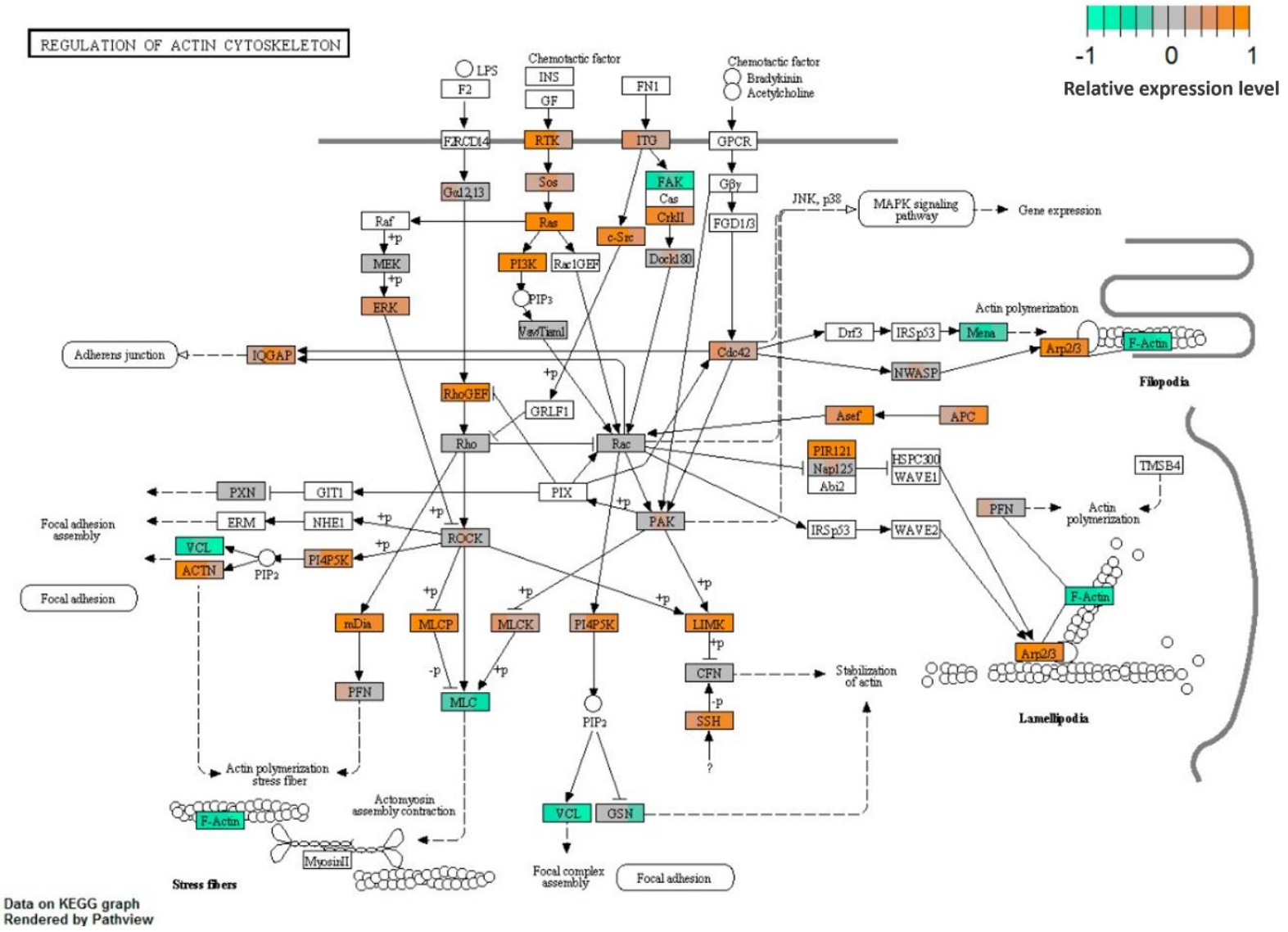
REGULATION OF ACTIN CYTOSKELETON
-1
0
1
Relative expression level
LPS
F2
Chemotactic factor
INS
GF
FN1
Chemotactic factor
Bradykinin
Acetylcholine
FERCD14
RTK
ITG
GPCR
Gα12,13
Sos
FAK
Cas
CrkII
Gβγ
FGD1/3
JNK, p38
MAPK signaling pathway
Gene expression
Raf
Ras
c-Src
Dock180
PI3K
Rac1GEF
PIP3
Vav/Tiam1
MEK
ERK
Actin polymerization
Adherens junction
IQGAP
Cdc42
Drf3
IRSp53
Mena
Arp2/3
F-Actin
NWASP
Filopodia
RhoGEF
GRLF1
Asef
APC
Rho
Rac
PIR121
Nap125
Abi2
HSPC300
WAVE1
TMSB4
PXN
GIT1
PIX
PAK
PFN
Actin polymerization
Focal adhesion assembly
ERM
NHE1
IRSp53
WAVE2
VCL
ACTN
PIP2
PI4P5K
ROCK
F-Actin
Focal adhesion
mDia
MLCP
MLCK
PI4P5K
LIMK
Arp2/3
CFN
Stabilization of actin
PFN
MLC
PIP2
SSH
Lamellipodia
Actin polymerization stress fiber
Actomyosin assembly contraction
F-Actin
MyosinII
VCL
GSN
Stress fibers
Focal complex assembly
Focal adhesion
Data on KEGG graph
Rendered by Pathview

Figure 4.1. (above) Shifts in the expression of the 'regulation of actin cytoskeleton' KEGG pathway in cold-acclimated *G. pennsylvanicus* cricket hindguts. Each pathway component contains three color bars (right to left) indicating three biological replicates comparing warm- and cold-acclimation. For cold-acclimated crickets relative to warm-acclimated crickets, shifts in expression are either upregulated (orange), downregulated (blue), or unchanged (grey). For a complete description of each pathway component, see the reference pathway (http://www.genome.jp/kegg-bin/show_pathway?ko04810). F-actin - filamentous actin, ACTN - α-actinin, Arp2/3 - actin related proteins 2 and 3, VCL - vinculin (Chapter 3).

4.2.1 Cold acclimation and cold shock

During acclimation, crickets were isolated in 180 mL transparent cups (Polar Plastics, Summit Food Distributors, London, ON, Canada) with mesh fabric lids, containing egg carton shelters, rabbit food, and water. Warm-acclimated crickets remained in summer-like conditions (25°C, 14 light:10 dark photoperiod) for one week, while cold-acclimated crickets were housed in a Sanyo MIR 154 incubator (Sanyo Scientific, Bensenville, Illinois) at 12°C, 10 light:14 dark photoperiod for one week. I cold-shocked crickets for 1 h at -4°C in loosely-capped 50 mL plastic tubes suspended in a pre-cooled bath of 50:50 methanol:water (Lauda Proline RP 3530, Würzburg, Germany). Eight crickets (four warm- and four cold-acclimated) were cold-shocked for cytoskeletal stability measurements (described below). To assess whether cold shock affects survival, 40 crickets (20 warm-acclimated, 20 cold-acclimated) were cold-shocked, then returned to cups with food and water at 25°C. I assessed survival 48 h later.

4.2.2 Rectal macromorphology and ultrastructure

Warm-acclimated crickets (mean ± s.e.m. mass: 491 ± 17 mg) and cold-acclimated crickets (506 ± 36 mg) were size-matched for measurements ($t_{4.3}$ = 0.36, P = 0.73). Warm- and cold-acclimated crickets were secured to Sylgard-lined Petri dish by a pin through the pronotum and dissected. I opened the body cavity by a mid-dorsal incision and pinned the body open to remove the rectum with microscissors. The severed rectum was placed in a droplet of Ringer's solution (110 Na^+, 8.5 K^+, 6 Mg^{2+}, 7 Ca^{2+}, 144.5 Cl^-, pH 7.6,

concentrations in mM, derived from measurements of *G. pennsylvanicus* in Chapter 2) and the fecal material flushed out with a Ringer's-filled 5 mL syringe.

I used brightfield microscopy to visualize rectal macrostructure in cross-section. Recta were fixed in 10% formalin and paraffin-embedded, mounted, cross-sectioned, and stained with Movat's pentachrome stain (Movat, 1955) at the Robarts Research Institute (Molecular Pathology Facility, London, ON, Canada). I captured images of the cross-sections with an AxioImager Z1 Microscope (Carl Zeiss Microscopy GmbH, Jena, Germany), and used Image-Pro Premier software (Media Cybernetics Inc, Rockville, MD) to measure macrostructural features. I counted the number of nuclei (as a proxy for epithelial cell density) and measured the length of all six rectal pads for each cricket (Fig. 4.2C). Nuclei count per rectal pad was averaged across the six rectal pads for each cricket. For each rectal pad I made five to seven width measurements (at regular intervals). For the entire cross-section I made sequential clockwise measurements of outer circular muscle width (at least 75 measurements per section) and cuticle width (at least 30 measurements per section) at regular intervals. I used the grand mean (± s.e.m.) for each metric for each cricket to compare warm- and cold-acclimated individuals (n = 4 per treatment) with Welch's t-tests in R (v3.2.2, R Development Core Team, 2015).

I used transmission electron microscopy (TEM) to visualize the ultrastructure of the mid-cell scalariform complexes in rectal pad epithelia. Recta were fixed (2.5% glutaraldehyde, 0.1 M cacodylate, pH 7.4) and then stained with 1% (v/v) osmium tetroxide (pH 7.4), with three washes in 0.1 M cacodylate buffer (pH 7.4). Recta were then serially dehydrated in acetone and embedded in Epon-Araldite resin (Electron Microscopy Sciences, Fort Washington, PA, USA), which was polymerized at 60°C for 48 hr. I cut 0.5 μm sections and stained with 2% uranyl acetate (20 min) followed by Reynold's lead citrate (1 min) (Graham and Orenstein, 2007). I imaged sections with a Philips CM10 Transmission Microscope (Philips Electron Optics, Eindhoven, The Netherlands) and AMT Advantage digital imaging system with Hamamatsu Orca 2 MPx HRL Camera (Advanced Microscopy Techniques, Woburn, MA). I measured scalariform complex tortuosity (n = 3 cold-acclimated and 4 warm-acclimated crickets, 1-9 different scalariform complexes measured

per cricket) and channel widths (n = 4 crickets per treatment, 2-8 channels measured per cricket) using ImageJ software (Schindelin et al., 2015). Tortuosity was quantified as the length of scalariform complex channel relative to the length of a straight trajectory between each end of the channel. I compared measurements from warm- and cold-acclimated crickets using Welch's t-tests in R. Reported values are means (± s.e.m.).

4.2.3 Cytoskeletal stability

To determine whether cold acclimation protects cytoskeletal stability at low temperatures, I quantified actin polymerization in recta of warm- and cold-acclimated crickets with and without cold shock (described above, n = 4 crickets per treatment combination). Recta were dissected from each cricket and flushed with insect Ringer's (as above). I fixed recta in 4% paraformaldehyde overnight at 4°C and then embedded, cross-sectioned, and mounted the tissues on slides. Tissues were deparaffinized in xylene and progressively rehydrated to 70% ethanol before rinsing in water and PBS. To stain for filamentous actin (F-actin), I first permeabilized rectal sections for 5 min using 0.1% (v/v) Triton X-100 in PBS and I applied Background Sniper (Biocare Medical, Concord, CA) for 5 min to reduce background florescence. Sections were stained in PBS with 2.5 % Phalloidin (Alexa-Fluor 488, Thermo Scientific, Mississauga, ON) for F-actin and 0.6 μM DAPI for nuclei. I used PermaFluor mountant (Thermo Scientific, Mississauga, ON) to reduce fading and stored slides at 4°C until imaging.

I imaged rectal pad F-actin with a Zeiss LSM 5 Duo Vario confocal microscope and ZEN Pro software (Carl Zeiss Microscopy GmbH, Jena, Germany). F-actin fluorescence was converted to grayscale for analysis using ImageJ. Because F-actin density was consistently higher in the basal region of the rectal pads (generally between the nuclei and basal lamina) compared to the apical region (between the nuclei and apical border), apical and basal F-actin intensities were measured separately. Approximately five rectal pads (but ranging from one to six) were measured for each cricket. For each rectal pad measured, four to five 80 μm^2 regions in each of the apical and basal areas were haphazardly selected to quantify grey pixel intensity. The grey pixel density of the background (regions where no tissue was present) were measured in two 80 μm^2 regions per section and subtracted this value from

rectal pad measurements. Grey pixel intensity was averaged first for each section, then averaged for the whole individual prior to analysis. Grey pixel intensity measurements were natural log-transformed prior to analysis to meet the assumption of normality, and these measurements were compared for warm- and cold-acclimated crickets using a three-way ANOVA in R. The standardized effect size for acclimation was calculated as the difference in grey pixel intensity between warm and cold acclimated rectal pads divided by their pooled standard deviation. The standardized effect size for cold shock was calculated as the difference in grey pixel intensity of cold shocked and non-cold shocked rectal pads divided by their pooled standard deviation.

4.3 Results

4.3.1 Rectal macromorphology and ultrastructure

Cold acclimation had no discernible effects on rectal pad length ($t_{4.3}$ = 1.47, P = 0.21) or width ($t_{4.5}$ = 0.70, P = 0.52; Figs. 4.2, 4.3A), nor did it alter the thickness of the cuticle ($t_{5.9}$ = 0.11, P = 0.92) or outer circular muscle ($t_{4.7}$ = 0.01, P = 0.99; Figs. 4.2,4.3B). Mean nuclear density of cold-acclimated rectal pad cross sections (55 ± 5 nuclei per pad) did not differ from that of warm-acclimated rectal pads (57 ± 4 nuclei per pad; $t_{3.3}$ = 0.26, P = 0.81). Paracellular channel structure differed between apical (Fig. 4.4) and mid-cell regions (Fig. 4.5) of the rectal pads. Tortuosity of the mid-cell scalariform complexes did not differ between warm-acclimated crickets (5.3 ± 0.4 μm/μm) and cold-acclimated crickets (5.2 ± 1.5 μm/μm) ($t_{2.3}$ = 0.105, P = 0.925). Similarly, cold acclimation did not alter the mid-cell scalariform channel width (26.4 ± 1.5 nm in warm-acclimated crickets, 29.3 ± 1.7 nm in cold-acclimated crickets) ($t_{4.8}$ = 0.89, P = 0.42; Fig 4.5).

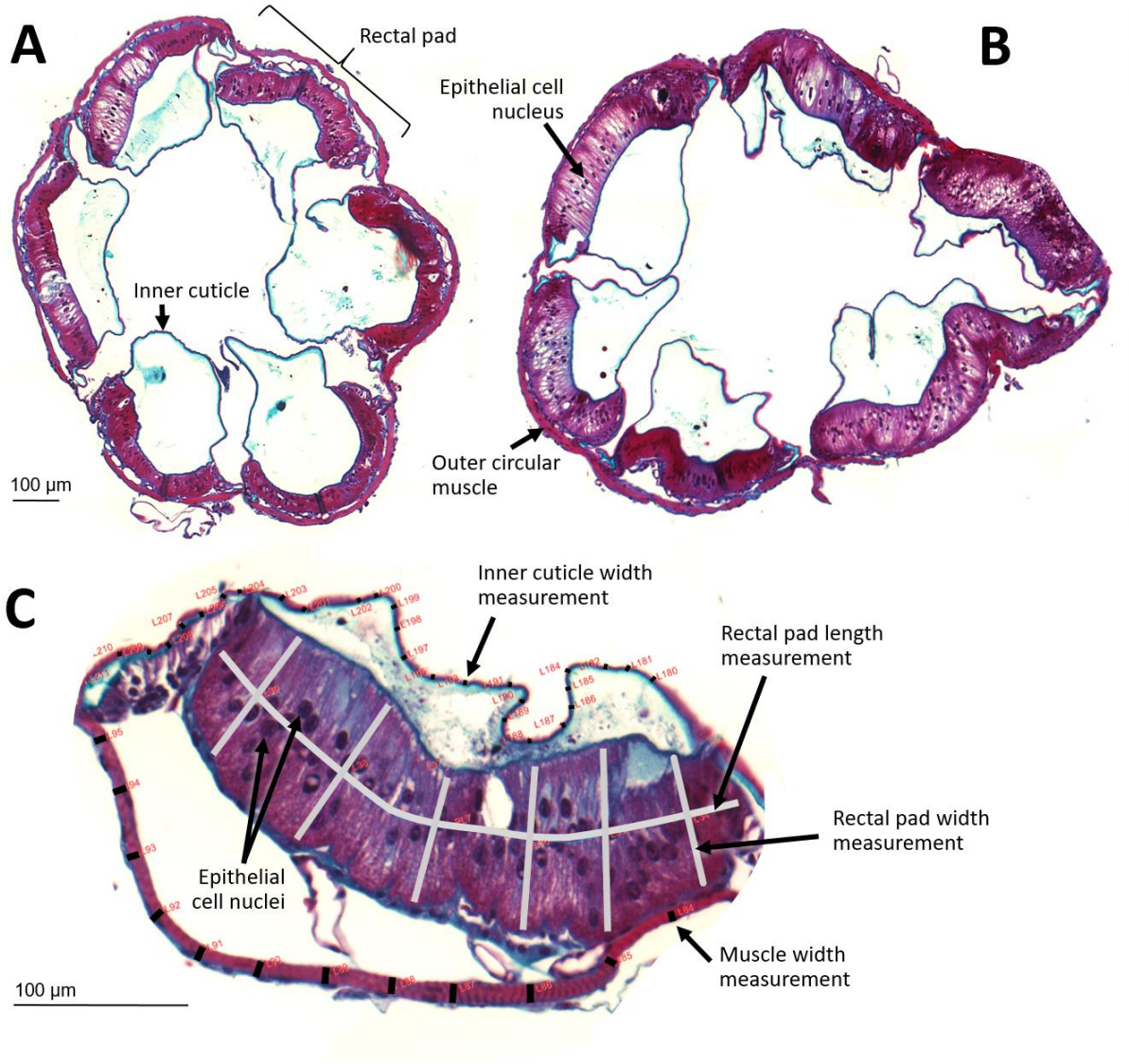

Figure 4.2. Representative rectal cross sections from warm-acclimated (A) and cold-acclimated (B) adult *G. pennsylvanicus*. I compared the rectal macromorphology (C) of crickets that were cold-acclimated (12°C, 10 light:14 dark photoperiod for one week) with those that were warm-acclimated (25°C, 14 light:10 dark photoperiod for one week). Sections were stained with Movat's stain: nuclei/elastin (black), ground substance/mucin (blue), cytoplasm/muscle (red).

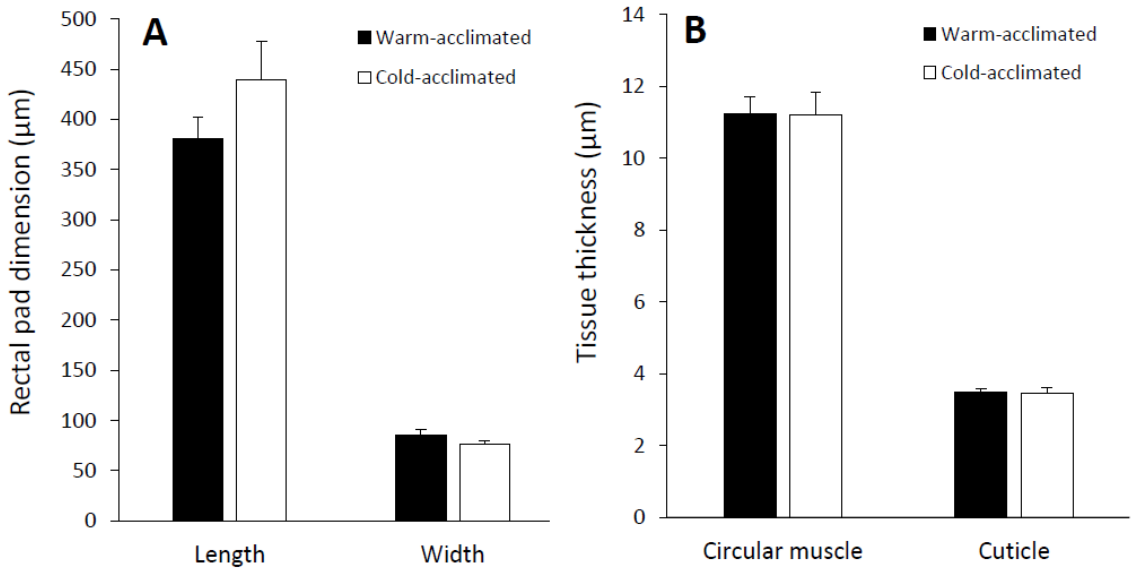

Figure 4.3. Effect of cold acclimation on the macromorphology of *G. pennsylvanicus* rectal pads, as measured by brightfield microscopy of stained rectal cross sections. Neither the mean length or width of rectal pads (A) nor the mean thickness of the rectal cuticle and circular muscles (B) differed between warm- and cold-acclimated crickets.

4.3.2 Cytoskeletal stability

The density of F-actin was higher in the cytoplasm basal to nuclei compared to the cytoplasm apical to the nuclei (Fig. 4.6). These differences may reflect greater density of organelles (e.g. mitochondria) in the scalariform complexes of the basal cell regions (Khurana, 2000). Cold shock alone did not reduce F-actin density (P = 0.098, standardized effect size = -0.53; Fig. 4.7). Cold acclimation enhanced F-actin in the basal cell region ($F_{1,14}$ = 10.7, P = 0.006) but not in the apical region ($F_{1,14}$ = 0.93, P = 0.35).

F-actin density in the basal region following cold shock was higher in cold-acclimated crickets compared to warm-acclimated crickets ($F_{1,14}$ = 11.4, P = 0.004; Tukey's HSD P < 0.001, standardized effect size = 0.71). Although I found a significant interaction between acclimation and cold shock in the apical region ($F_{1,14}$ = 5.1, P = 0.04), I could not determine the driver of this interaction using Tukey's HSD. All crickets survived cold shock, regardless of acclimation.

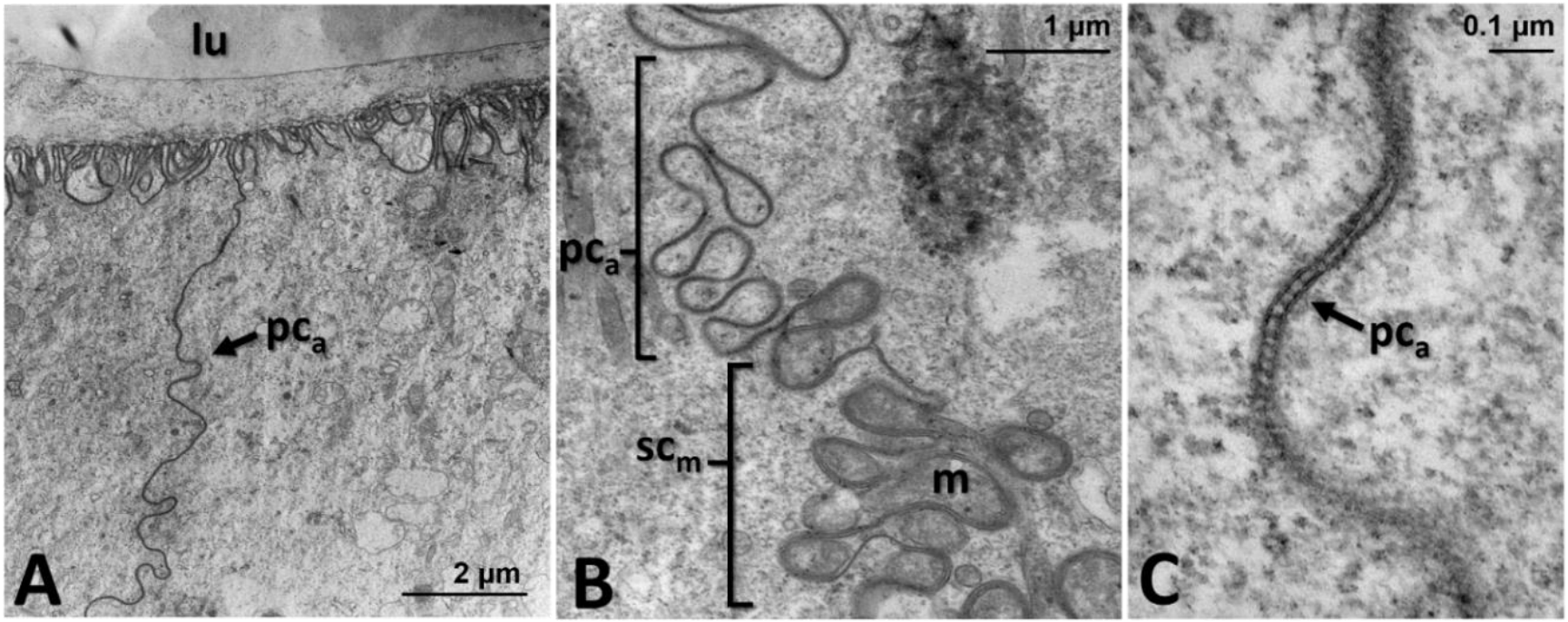

Figure 4.4. Representative cross sections of *G. pennsylvanicus* rectal pads imaged by TEM. A) a meandering apical paracellular channel (pc_a) separates two rectal pad epithelial cells. lu - lumen. B) the apical and mid-cell intersection is marked by a shift in lateral cell border characteristics; the paracellular channel in close association with mitochondria (m) forms the scalariform complex (sc_m) in the mid-cell region. C) ladder-like structure of the tightly-opposed apical paracellular channel. Sections A and C belonged to warm-acclimated crickets, while section B belonged to a cold-acclimated cricket.

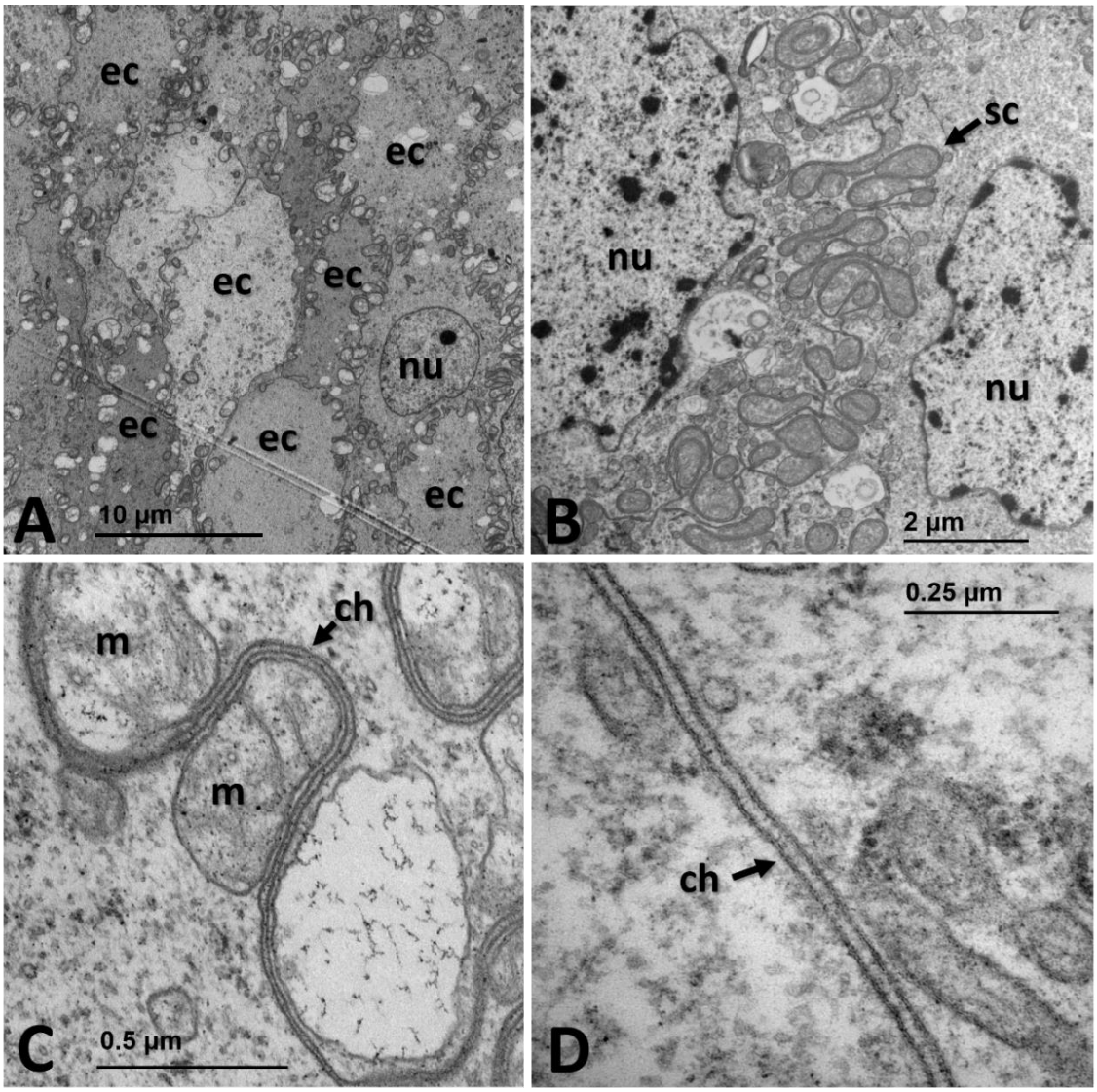

Figure 4.5. Example cross-sections of *G. pennsylvanicus* rectal pads for ultrastructure measurements. Sections represent the mid-cell region (in the vicinity of the nuclei) and were imaged by TEM at increasing magnification from A to D. A) epithelial cells (ec) are each bordered by meandering scalariform complexes. One nucleus (nu) is visible. B) scalariform complex (sc) between the nuclei of two epithelial cells. This approximate magnification was used for measurements of scalariform tortuosity. C) close association of mitochondria (m) with the paracellular channel (ch) of the scalariform complex. D) the paracellular channel of a scalariform complex. This magnification was used to measure channel width. Sections A and C belonged to warm-acclimated crickets, while sections B and D belonged to cold-acclimated crickets.

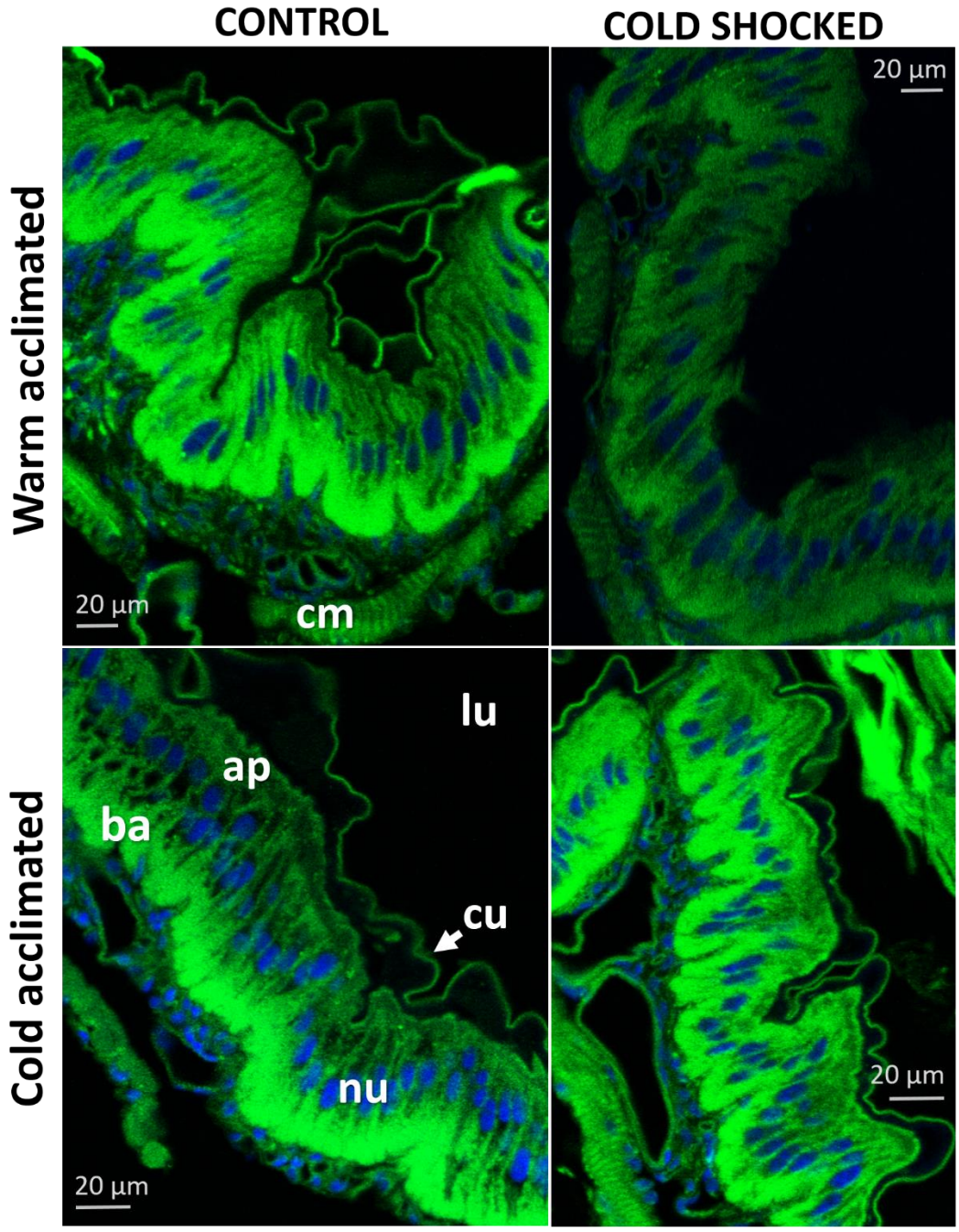

Figure 4.6. Effect of cold acclimation and cold shock on the density of filamentous actin in the rectal pads of adult *G. pennsylvanicus* crickets. Warm- and cold-acclimated crickets were either not cold shocked (control) or cold shocked at -4°C for 1 h. Images represent one of four crickets from each treatment combination. F-actin was stained with phalloidin (green). ap - apical region, ba - basal region, cm - circular muscle, cu - cuticle, lu - gut lumen, nu - nuclei (blue).

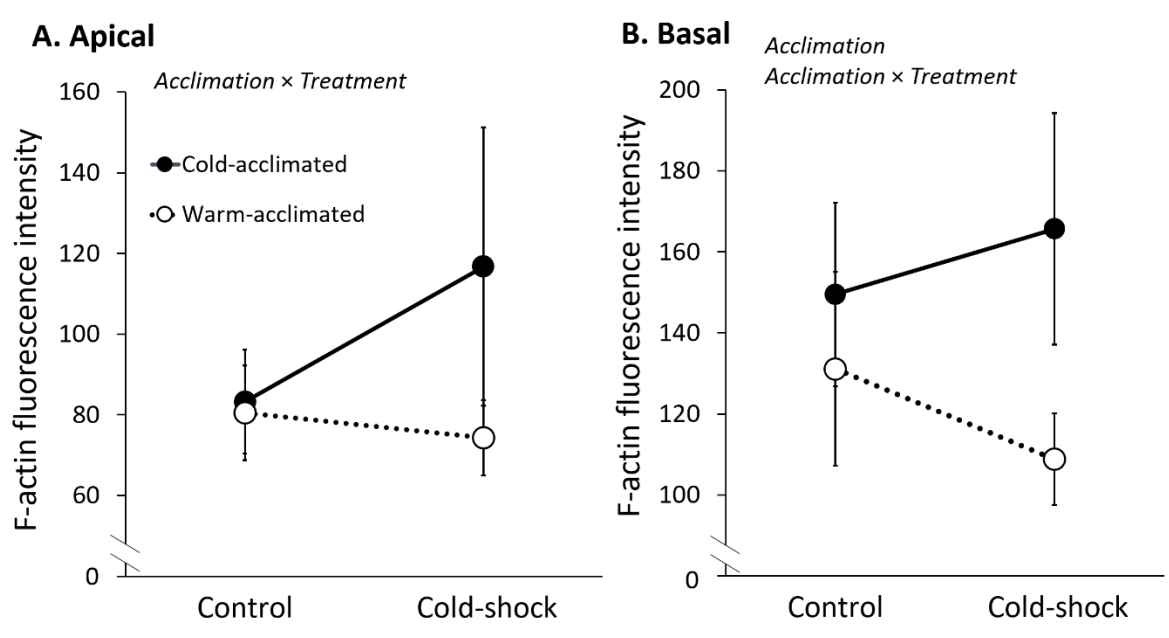

Figure 4.7. Effect of cold acclimation and acute cold exposure on the polymerization state of actin in *G. pennsylvanicus* cricket rectal pads. Actin polymerization was measured by fluorescence of F-actin. Both warm- and cold-acclimated crickets were either cold shocked (exposed to -4°C for 1 h) or not cold shocked (control). Significant effects of acclimation, cold shock (treatment), or their interaction are indicated above each figure in italics.

4.4 Discussion

Cold acclimation may allow crickets to defend water and ion homeostasis during cold exposure by restructuring the ionoregulatory tissues to reduce permeability (MacMillan and Sinclair, 2011a). Structural modifications that enhance cytoskeletal stability in the cold should also protect ionoregulatory tissues chilling injury and loss of transport function (Kim et al., 2006; Kayukawa and Ishikawa, 2009; Teets et al., 2012; Torson et al., 2015). By comparing rectal tissue structure of warm- and cold-acclimated crickets I show that cold acclimation does not modify rectal macromorphology or the structure of the rectal pad scalariform complex. Cold acclimation does modify the cytoskeleton such that actin polymerization is protected (and even enhanced) following cold shock.

4.4.1 Rectal macromorphology and scalariform complex ultrastructure are not targets of cold acclimation

I hypothesized that cold acclimation could reduce hindgut permeability (thereby minimizing leak during cold exposure) by thickening the rectal pads, inner cuticle, and/or rectal musculature to increase diffusion distance. Cold acclimation could also narrow the scalariform complex channels and/or increase the tortuosity of those channels. The benefit of the former to maintain water and ion homeostasis would be two-fold; narrowed channels could prevent leak of Na^+ and water from the hemolymph, while decreased channel volume would reduce the NKA activity required to achieve a given [Na^+] within the channels (thereby mitigating effects of reduced enzyme activity in the cold). In the latter scenario, increased channel tortuosity would increase diffusion (leak) distance. However, I observed none of these predicted structural changes, indicating that if cold acclimation modifies tissue permeability this modification involves other aspects of tissue structure.

I observed altered tissue structural and cytoskeletal gene expression in *G. pennsylvanicus* hindguts following a four-week cold acclimation regime (Chapter 3), and crickets in the present study were acclimated for only one week. However, one week of cold acclimation is as effective at enhancing cold tolerance as a four-week acclimation (based on similar or greater reduction the CT_{min} and CCRT, and improved survival of chronic cold exposure, Chapter 5). Therefore I assume that any tissue structural modifications involved in prevention of chilling injury or re-establishment of water and ion homeostasis during recovery from cold exposure should be apparent within one week of cold acclimation.

Cold acclimation may cause other hindgut structural modifications that were not apparent by the methods I employed. Cell junctions (e.g. tight and occluding junctions) are temperature-sensitive (Gonzalez-Mariscal et al., 1984; Behrens et al., 1993; Turner et al., 1997) and their failure may contribute to paracellular water and ion leak during cold exposure. Narrowing or reducing the thermal sensitivity of cell junctions in the apical (or basal) rectal pad regions (Fig. 4.3) could therefore reduce paracellular permeability. Indeed, the expression of multiple genes encoding components of tight and adherens (septate) junctions (e.g. *vinculin, partitioning defective protein 3*, *protein shroom*, *α-*

actinin, and *casein kinase II*) are altered by cold acclimation in crickets (Chapter 3). In flies, acquired cold tolerance is also accompanied by shifts in the expression cellular adhesion genes (Teets et al., 2012; MacMillan et al., 2016). To reduce transcellular permeability, cold acclimation could reduce the abundance or membrane-localization of aquaporins and/or ion channels. Shifts in the type or abundance of aquaporins appear to be important for acquired freeze tolerance in dipterans (Philip et al., 2008; Philip and Lee, 2010), however I found no evidence of altered aquaporin transcript abundance following cold acclimation in crickets (Chapter 3). Cold acclimation does reduce the expression of Na^+-K^+-$2Cl^-$ cotransporter and organic anion/cation transporters in cricket hindguts. Post-translational modifications of aquaporins, ion transporters, and cytoskeletal components could also affect transport function (Seo and Lee, 2004; Teets and Denlinger, 2016). Whether transcriptional or post-translational shifts correlate with a reduction in ion channel abundance and hindgut permeability requires verification.

4.4.2 Cold acclimation protects cytoskeletal stability from cold shock

Both cold acclimation and seasonally-acquired cold tolerance correlate with shifts in cytoskeletal gene expression, and actin stability appears to be particularly important for survival of cold exposure (Kim et al., 2006; Kayukawa and Ishikawa, 2009; Teets et al., 2012; Gerken et al., 2015; Torson et al., 2015). In cold-acclimated crickets, genes promoting actin stability are upregulated in the hindgut specifically (Chapter 3). Here I demonstrated that these transcriptional changes protect F-actin against depolymerization in the rectal pads during cold shock. By quantifying F-actin polymerization before and after cold shock, I also showed that cold shock can either enhance or reduce F-actin polymerization depending on whether or not crickets were cold acclimated. For example, cold shock increased F-actin density in cold-acclimated rectal pads compared to warm-acclimated rectal pads. Increased polymerization and distribution of actin in the midguts of cold-exposed *C. pipiens* mosquitoes is more pronounced in diapausing, rather than non-diapausing individuals (Kim et al., 2006). It is unclear how cytoskeletal modifications allow for cold exposure to directly enhance actin polymerization.

In addition to cold-acclimated crickets and diapausing *C. pipiens*, both diapausing and cold-acclimated *D. antiqua* onion maggots also defend actin polymerization in the cold (Kayukawa and Ishikawa, 2009). Therefore cytoskeletal modification appears to be a shared mechanism among diapause and cold acclimation processes. Which molecules actually promote actin stability is unclear; defense of actin polymerization in the cold correlates with *actin* expression in *C. pipiens*, *hsp60* expression in *D. antiqua*, and a plethora of cytoskeleton-associated genes in *G. pennsylvanicus* (Chapter 3). The cytoskeleton includes multiple accessory proteins in addition to actin and tubulin, and transcriptional changes in one of these components may not necessarily alter overall cytoskeletal stability. To identify which proteins enhance cytoskeletal stability in the cold, cold tolerance and actin polymerization could be compared for insects with or without knockdown or mutation of candidate cytoskeletal components.

Although crickets were not injured by the cold shock I applied, loss of cytoskeletal structure likely contributes to transport failure, chronic chilling injury, and/or failure to clear or repair damaged cellular components (Koštál et al., 2006; Kayukawa and Ishikawa, 2009; Monastyrska et al., 2009; Lee, 2010; Fernandez-Gonzalez and Zallen, 2013; Findsen et al., 2014). In the rectum, defense of cytoskeletal structure during cold chronic exposure should therefore help cold-acclimated insects to maintain transport function, thereby directly or indirectly preventing chilling injuries. Changes in actin should be linked to improved cell and tissue survival following chronic cold exposure.

4.4.3 Conclusions

I aimed to demonstrate the functional significance of modified hindgut tissue and cell structural gene expression following cold acclimation. I hypothesized that structural changes to reduce rectal epithelial permeability should prevent water and ion leak during cold exposure, however these permeability changes do not appear to involve modification of rectal macromorphology or rectal pad scalariform complex ultrastructure. Cold acclimation does protect actin from depolymerization at low temperatures, suggesting that cytoskeletal modification plays a role in preventing cellular chilling injury and maintaining transport function in the insect rectum.

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Chapter 5

5 Effect of cold acclimation on active ion transport in insect ionoregulatory tissues

I have prepared this chapter for submission to *Comparative Biochemistry and Physiology A.*

5.1 Introduction

Chill-susceptible insects lose ion and water homeostasis at temperatures below their critical thermal minimum (the CT_{min}). This loss of homeostasis progresses over hours to days and appears to be driven by gradual migration of Na^+ down a concentration gradient from the hemolymph to the gut lumen (MacMillan and Sinclair, 2011b; Coello Alvarado et al., 2015). Water follows the migration of Na^+, leading to decreased hemolymph volume and consequent increase in the concentration of hemolymph K^+ (in addition to Mg^{2+} and Ca^{2+}) (Koštál et al., 2006; MacMillan and Sinclair, 2011b; Coello Alvarado et al., 2015; MacMillan et al., 2015a; Chapter 2). This ionic imbalance increases the time required for insects to recover from chill coma (Koštál et al., 2007; MacMillan et al., 2012; Findsen et al., 2013; MacMillan et al., 2014) and likely contributes to the accumulation of chronic chilling injuries (Koštál et al., 2006; Lee, 2010; Findsen et al., 2014; MacMillan et al., 2015b). Defense of water and ion homeostasis during cold exposure is plastic (i.e. improved with prior mild chilling; Koštál et al., 2006; Coello Alvarado et al., 2015; MacMillan et al., 2015a), but the mechanisms underlying transport plasticity are not well understood.

Insects maintain water and ion balance via the Malpighian tubules (which excrete primary urine) and hindgut (across which selective reabsorption of water and ions occurs; Phillips et al., 1988; O'Donnell and Simpson, 2008). Although the primary urine is isosmotic to the hemolymph, excretion by the Malpighian tubules is dependent on ionic gradients established at the apical cell membrane by active cation transport (Beyenbach, 2003). These transporters include the Na^+-K^+-$2Cl^-$ cotransporter (NKCC, which imports Na^+, K^+, and Cl^- into the basal cell), carbonic anhydrase (CA, which provides cytosolic protons),

and V-ATPase (which pumps protons to the lumen for future exchange with intracellular cations; Coast, 2012; Chintapalli et al., 2013; Halberg et al., 2015). Within the rectal pads, highly convoluted, mitochondria-dense paracellular channels form the scalariform complex, in which membrane-bound Na^+-K^+ ATPase (NKA) establishes a high extracellular [Na^+] (Phillips et al., 1988; O'Donnell and Simpson, 2008). This Na^+ concentration gradient drives migration of water from the rectal lumen to the hemolymph against an osmotic gradient.

During cold exposure, active transport of ions across ionoregulatory epithelia is thought to be exceeded by passive leak of ions down their concentration gradients (MacMillan and Sinclair, 2011a). Cold-acclimated insects are therefore expected to defend water and ion homeostasis by reducing epithelial permeability (to minimize water and ion leak) and/or by enhancing active ion transport at lower temperatures (MacMillan and Sinclair, 2011a). The latter hypothesis is supported by shifts the transcription of ion pumps driving transport across both the Malpighian tubules and hindguts of cold-acclimated fall field crickets [*Gryllus pennsylvanicus* (Burmeister), Orthoptera: Gryllidae, Chapter 3]. However, V-ATPase expression in the Malpighian tubules was actually downregulated with cold acclimation, while hindgut NKA expression increased. These transcriptional changes suggest that cold acclimation reduces active transport across the Malpighian tubules while enhancing active transport across the rectum.

I hypothesized that cold acclimation: 1) reduces excretion rates by decreasing Malpighian tubule V-ATPase activity, and 2) increases NKA activity in the rectum (which would likely enhance reabsorption of Na^+ and water). To test these hypotheses I compared Malpighian tubule excretion rates (a proxy for active transport) of warm- and cold-acclimated insects, and related transcriptional changes in ion pumps (NKA and/or V-ATPase) accompanying cold acclimation (Chapter 3) to functional changes in tissue transport via enzyme activity assays in homogenized Malpighian tubules and recta. For this work I used warm- and cold-acclimated *G. pennsylvanicus*; an emerging model system for the study of cold tolerance plasticity and its relation to water and ion homeostasis (MacMillan and Sinclair, 2011b; MacMillan et al., 2012; Coello Alvarado et al., 2015; Des Marteaux and Sinclair, 2016).

5.2 Methods

5.2.1 Insect rearing

I reared *G. pennsylvanicus* crickets as described in Chapter 2. Briefly, crickets were housed in transparent 60 L plastic containers with stacked cardboard egg cartons for shelter, tap water, and *ad libitum* commercial rabbit food (Little Friends Original Rabbit Food, Martin Mills, Elmira, ON, Canada) and developed under constant summer-like conditions (25°C, 14 light:10 dark photoperiod, 70% RH). Crickets laid eggs in containers of moist vermiculite and sterile sand which were placed at 4°C to accommodate an obligate three-month diapause (Rakshpal, 1962) before being returned to 25°C to hatch. I used adult female crickets at approximately three months post-hatch for all experiments.

5.2.2 Cold acclimation

Crickets were isolated in 180 mL transparent cups (Polar Plastics, Summit Food Distributors, London, ON, Canada) with mesh fabric lids, containing egg carton shelters, rabbit food, and water. Warm-acclimated crickets remained in summer-like conditions (25°C, 14 light:10 dark photoperiod) for the week, while cold-acclimated crickets were placed in a Sanyo MIR 154 incubator (Sanyo Scientific, Bensenville, Illinois) at 12°C, 10 light:14 dark photoperiod for one week.

5.2.3 Cold tolerance measurements

The CT_{min} was quantified as described by MacMillan and Sinclair (2011b) and defined as the temperature at which physical stimulus with a metal probe elicited no movement. Crickets were cooled from room temperature to the CT_{min} at 0.25°C min^{-1} (n = 10 per acclimation). To measure chill coma recovery time (CCRT) I placed crickets in 15 mL Falcon tubes immersed in an ice-water slurry at 0ºC (n = 9 crickets per acclimation). After 12 h, crickets were returned to room temperature, placed on their dorsum in wells of flat-bottom six-well cell culture plates, and I measured the time taken for crickets for right themselves (the chill coma recovery time, CCRT). To assess survival of chronic cold exposure, warm- and cold-acclimated crickets (n = 12 and 10, respectively) were placed in

15 mL Falcon tubes immersed in an ice-water slurry at 0ºC for 72 h. Crickets were then returned to 25°C in transparent cups containing food, water, and shelter, and I assessed mortality and injury (uncoordinated locomotion or the inability to jump when prodded) 24 h later.

5.2.4 Dissections

Crickets were affixed to a Petri dish by a pin through the pronotum and the body cavity was opened by mid-dorsal incision. The Malpighian tubules were removed as a bundle by detaching the ureter from the gut with forceps. The rectum was severed from the rest of the gut with microscissors. Both tissues were immediately placed in droplets of simple Ringer's solution specific to *G. pennsylvanicus* hemolymph: (in mM) 110 Na^+, 8.5 K^+, 6 Mg^{2+}, 7 Ca^{2+}, 144.5 Cl^-, pH 7.6 (Chapter 2). Any adhering fat body or tracheae were removed from tissues. For Ramsay assays, individual Malpighian tubules were detached from the bundle by severing with forceps as close as possible to the ampulla (where multiple tubules coalesce towards the ureter; Wall et al., 1975).

For enzyme activity assays, entire Malpighian tubule bundles were blotted on tissue paper, flash frozen in liquid nitrogen, and stored at -80°C until use. Recta were cut open with microscissors to empty the lumen of fecal material, blotted on a tissue, and stored on ice for enzyme activity assays performed on the same day. Each replicate for enzyme activity assays in the Malpighian tubules was comprised of entire Malpighian tubule bundles pooled from five crickets. For enzyme activity assays in the recta, each replicate was comprised of 8-11 pooled cricket recta.

5.2.5 Active transport across the Malpighian tubules (Ramsay assay)

The rate of primary urine excretion (a proxy for active transport function) was quantified by Ramsay assay (Ramsay, 1954) using methodology modified from Rheault and O'Donnell (2004). Assays were carried out using a custom acrylic enclosure. The top surface of the enclosure contained four, flat-bottomed wells (3.5 cm diameter, 2.5 cm

depth) which were filled with paraffin oil. Well temperature was monitored with type-T thermocouples connected to Picotech TC-08 interface and processed by PicoLog software (Pico Technology, Cambridge, UK). The enclosure was connected to a refrigerated circulator (Model 1157P, VWR International, Mississauga, ON, Canada) filled with an equal ratio mixture of ethylene glycol and water.

Four Sylgard® blocks (5 x 2.5 mm) were affixed to the bottom of each well in the enclosure, and a shallow incision was made by razorblade medially on the top edge of each block. A 10 μL droplet of Ringer's (with 4 mM glucose and 15 mM HEPES, buffered to pH 7.6) was added 3 mm from each block and one Malpighian tubule was placed individually into each droplet. The distal end of each tubule was pulled from the droplet through the paraffin oil and 'cleated' into the incision on the edge of a block. The region of tubule between the droplet and block was gently punctured using fine forceps to produce an initial droplet of primary urine. This first droplet was discarded after 15 min. Each tubule was then allowed to excrete through this puncture for 2 h and the diameter of all droplets were measured using a microscope with an ocular micrometer. Droplet diameters were used to calculate droplet volume ($\pi d^3/6$) excreted per hour, and divided by the length of tubule within the droplet (measured by ocular micrometer). Malpighian tubule excretion rate was measured at 24, 16, 12, 8, and 4°C (n = 4, 5, 5, 6, and 2 crickets per treatment, respectively). The excretion rate for each cricket was taken as the mean of the excretion rates measured from six individual Malpighian tubules.

5.2.6 Malpighian tubule NKA and V-ATPase activity

I measured NKA and V-ATPase activity in homogenized Malpighian tubules of warm- and cold-acclimated crickets using an NADH-linked activity assay. Pooled tissues were diluted in 400 μL in SEID buffer (in mM: 150 sucrose, 10 EDTA, 50 imidazole, and 2.5 Na^+-deoxycholate, pH 7.3) and homogenized on ice for 10 s with a 7 mm attachment on a Polytron PT 10-35 homogenizer (Kinetica, USA). Homogenates were centrifuged at 10000 × g for 10 min at 4°C and the supernatant was collected. Supernatants were diluted 5-fold further with SEID for use in activity assays. A reaction buffer was comprised (in mM) of 47 NaCl, 2.6 $MgCl_2$, 10.5 KCl, 50 imidazole, 0.27 NADH, 2.6 ATP, and 2.1

phosphoenolpyruvate, with 3 $U.mL^{-1}$ lactate dehydrogenase (E.C. 1.1.1.27) and 3.75 $U.mL^{-1}$ pyruvate kinase (E.C. 2.7.1.40), pH 7.5.

Duplicate wells on a 96-well plate each received 10 μL of dilute supernatant and 200 μL of either assay buffer, assay buffer with 5 mM ouabain (to inhibit NKA), or assay buffer with 10 mM bafilomycin A1 (to inhibit V-ATPase). NADH absorbance (at 340 nm) of the reaction at 21°C (n = 6 biological replicates per acclimation) was then measured each minute for 30 min in a Multiskan® Spectrum spectrophotometer and SkanIt Software (v2.2) (Thermo Scientific, Wilmington, DE, USA), simultaneously for all samples. Total protein concentrations of dilute sample supernatants were quantified by Bradford assay against albumin standards (Kruger, 1994). Enzyme activities were calculated as the difference in rates between reactions with and without enzyme inhibitors, corrected for total protein abundance.

5.2.7 Hindgut NKA activity

I quantified the activity of NKA in homogenized recta from warm- and cold-acclimated crickets using assays modified from MacMillan et al. (2015c) (see 5.2.3). Briefly, recta were diluted in 14 volumes of homogenization buffer (25 mM imidazole, 10 mM β-mercaptoethanol, 0.2% w/v Na^+-deoxycholate, pH 7.5), homogenized with a Polytron PT 10-35, and sonicated with a Virsonic 100 (VirTis, Gardiner, NY, USA). Tissues were homogenized and sonicated each in four, 10 s bursts followed by 20 s on ice. Homogenates were then centrifuged at 7000 × *g* for 5 min at 4°C and the supernatant was collected. 300 μL aliquots of supernatant were filtered through a size-exclusion column (a 3 mL syringe barrel plugged with glass wool, containing 3 mL of Sephadex G50, and equilibrated with homogenization buffer) by centrifuging at 500 × *g* for 1 min. The total protein concentrations of filtered supernatants were quantified by Bradford assay against albumin standards.

I added 10 μL of filtered sample to each of four ultra-micro cuvettes; one pair of cuvettes then received 350 μL of reaction buffer (30 mM KCl, 156 mM NaCl, 7.8 mM $MgCl_2$, 74 mM imidazole, pH 7.5), while a second pair of cuvettes received 350 μL of reaction buffer

also containing 1.0 mM ouabain. I then added phosphoenolpyruvate, NADH, lactate dehydrogenase, and pyruvate kinase (final reaction concentrations of 4 mM, 300 mM, 20 $U.mL^{-1}$, and 20 $U.mL^{-1}$, respectively). Reactions were initiated by adding 40 µL of 50 mM ATP in reaction buffer.

NADH absorbance of each reaction was recorded five times per min for 20 min at 21°C or 6°C (n = 6 biological replicates per acclimation per temperature) in a Cary 100 Bio spectrophotometer (Varian, Palo Alto, CA, USA) using WinUV Thermal Application software (v3.0, Agilent Technologies). Temperature was maintained with a Cary Temperature Controller (Varian, Palo Alto, CA, USA). To monitor temperature, a type-T thermocouple connected to a TC-08 interface was placed in a microvolume cuvette containing 400 µL of water. Enzyme activities were calculated as the difference in rates between reactions with and without ouabain, corrected for total protein abundance.

5.2.8 Data analyses

I compared the CT_{min}, CCRT, and Malpighian tubule enzyme activities from warm- and cold-acclimated crickets using Welch's t-tests. I used two-way ANOVAs to compare the Malpighian tubule excretion rates and rectal NKA activity of warm- and cold-acclimated crickets. Values reported in the text are means ± s.e.m. All statistical analyses were performed in R (v3.2.2, R Development Core Team, 2015).

5.3 Results

5.3.1 Effect of a one-week cold acclimation on cold tolerance

One week of cold acclimation enhanced multiple aspects of cold tolerance. In cold-acclimated cricket the CT_{min} was approximately 1.7°C lower ($t_{13.2}$ = 7.9, P < 0.001), and chill coma recovery was over 3.5-fold faster compared to warm-acclimated crickets ($t_{8.9}$ = 7.0, P < 0.001; Fig. 5.1). Chronic cold exposure injured only 30% of cold-acclimated crickets, but injured and killed 50 and 33% of warm-acclimated crickets, respectively.

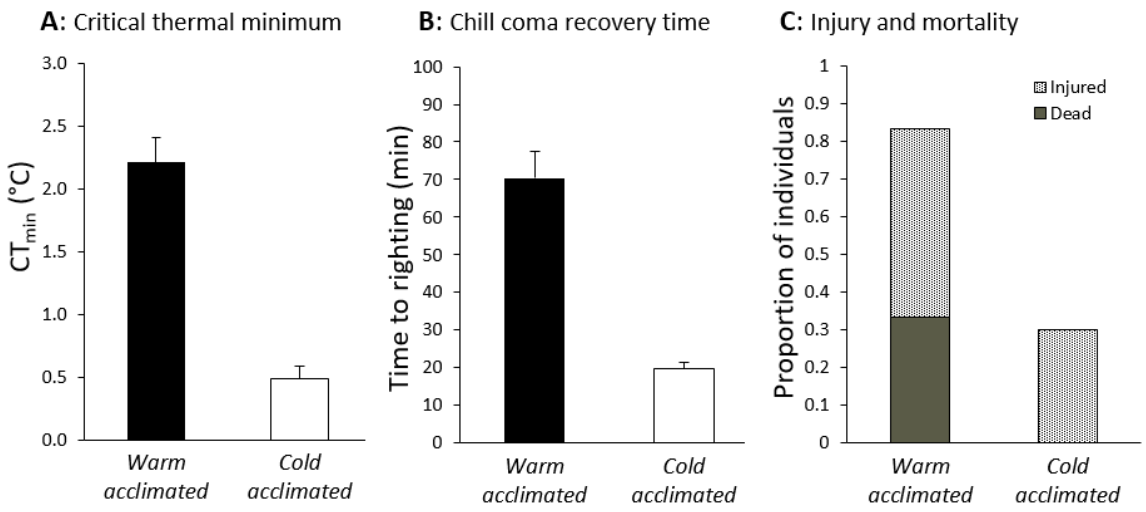

Figure 5.1. One week of cold acclimation improves cold tolerance of adult *G. pennsylvanicus* crickets. Cold acclimation decreased the CT_{min} (A), CCRT following 12 h at 0°C (B), and mortality following three days at 0°C (C) (see text for statistics).

5.3.2 Active transport across the Malpighian tubules

The rate of primary urine production by the Malpighian tubules decreased with temperature ($F_{1,40}$ = 102, P < 0.001). The Q_{10s} of secretion rate for warm- and cold-acclimated tubules were 2.2 and 1.9, respectively (calculated between 15.4°C and 24.8°C). Primary urine production by cold-acclimated crickets was approximately 35% slower at all temperatures compared to warm-acclimated crickets ($F_{1,40}$ = 20.5, P < 0.001; Fig. 5.2). I found no interaction between temperature and acclimation ($F_{1,40}$ = 0.046, P > 0.8).

5.3.3 NKA and V-ATPase activities in the Malpighian tubules

NKA activity in the Malpighian tubules of cold-acclimated crickets was higher than for warm-acclimated crickets, and this was nearly significant (t_{10} = 2.17, P = 0.055). A power analysis based on the observed standardized effect size (the difference in mean enzyme activities divided by the pooled variance) indicated that a sample size of eight would be sufficient to detect this difference; this was close to my current sample size of six. I did not observe a decrease in V-ATPase activity (a prediction based on previous observations of

decreased transcript abundance; $t_{6.5}$ = 1.54, P = 0.92; Fig. 5.3). Total protein abundance did not differ between warm- and cold-acclimated Malpighian tubules ($t_{6.1}$ = 0.73, P = 0.49).

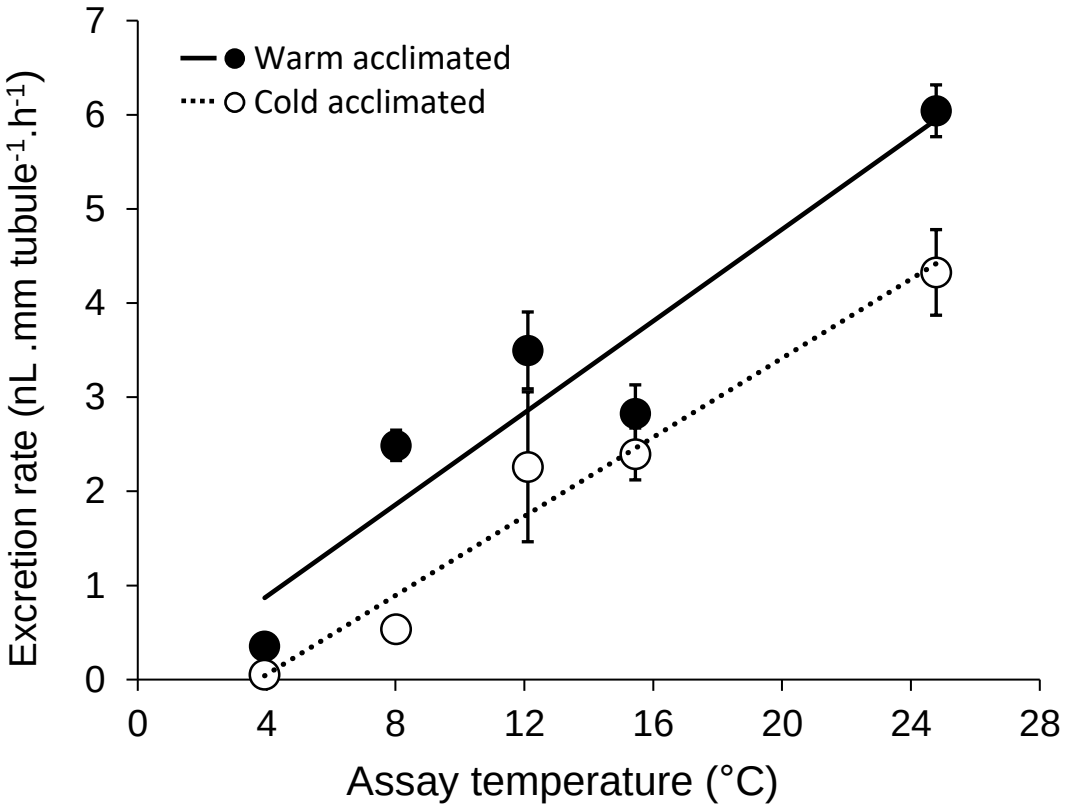

Figure 5.2. Effect of cold acclimation on primary urine excretion rate (a proxy for active ion transport) by the Malpighian tubules in adult *G. pennsylvanicus* crickets. Primary urine production was measured on isolated tubules using the Ramsay assay (n = 12 to 36 tubules per temperature-acclimation combination). The effect of temperature and acclimation on excretion rates were compared by two-way ANOVA (see text for statistics), and displayed trend lines represent linear models for each acclimation treatment.

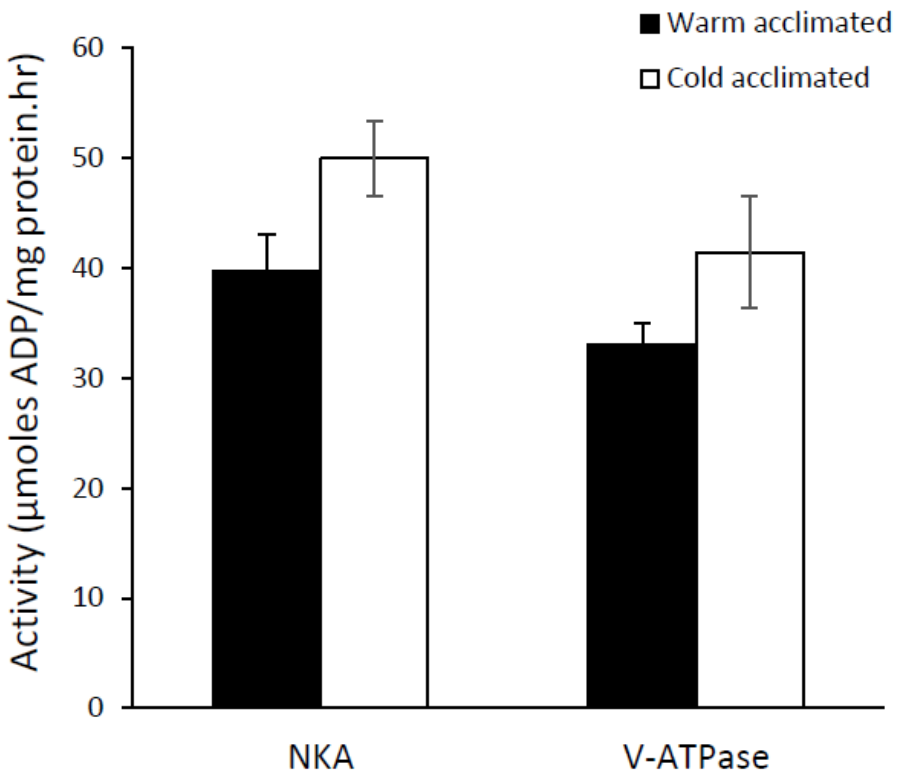

Figure 5.3. Effect of cold acclimation on the activity of Na^+-K^+ ATPase (NKA) and V-ATPase in Malpighian tubules of *G. pennsylvanicus* crickets. Activity rates were measured at 21°C via NADH-linked assays, and given as moles of ADP converted per hour (corrected for protein concentration in homogenates). Differences in enzyme activities between warm- and cold-acclimated crickets were statistically non-significant, however an increase in NKA activity with cold acclimation was nearly-significant.

5.3.4 NKA activity in the rectum

NKA activity in homogenized recta decreased with temperature ($F_{1,20}$ = 16.6, P < 0.001), but was unaffected by acclimation ($F_{1,20}$ = 2.5, P = 0.13). The interaction between acclimation and assay temperature was also non-significant ($F_{1,20}$ = 2.4, P = 0.14; Fig. 5.4). Although NKA activity at 21°C was higher in the recta of cold-acclimated crickets, a power analysis based on the observed standardized effect size indicated that a sample size of 15 would be required to detect this difference; the current sample size was six.

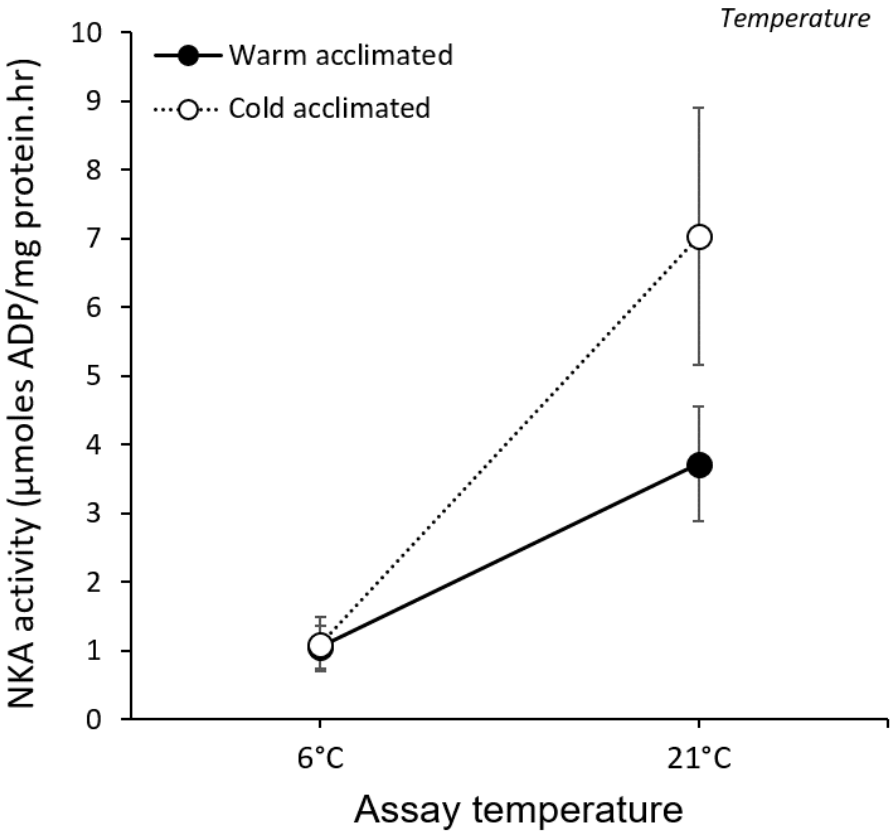

Figure 5.4. Effect of cold acclimation on the activity of Na^+-K^+ ATPase (NKA) activity in adult *G. pennsylvanicus* cricket recta. NKA activity was measured in homogenized recta by NADH-linked activity assay. Significant effects of acclimation, assay temperature, or their interaction is indicated at the top right of the figure in italics (see text for statistics).

5.4 Discussion

I hypothesized that cold-acclimated insects defend hemolymph volume by slowing primary urine excretion rates, and that this is driven by a reduction in V-ATPase activity. Cold acclimation did modify active transport across the Malpighian tubules, manifesting as a reduction in primary urine excretion rate at both low and optimal temperatures. However, slower primary urine was not related to V-ATPase activity, rather it was coincident with increased NKA activity. Although I expected cold acclimation to increase rectal NKA activity (thereby enhancing water and ion reabsorption), I did not observe this increase in activity at either 6°C or 21°C.

5.4.1 Cold acclimation modifies active transport across the Malpighian tubules

Primary urine excretion by the Malpighian tubules is driven by active ion transporters, most of which are temperature-sensitive (Dietz et al., 2001; Somero, 2004; O'Donnell and Simpson, 2008; Galarza-Muñoz et al., 2011). MacMillan and Sinclair (2011a) hypothesized that cold acclimation modifies active ion transport such that ion pumping rates are maintained to lower temperatures compared to warm-acclimated insects, however I show that the Malpighian tubules of cold-acclimated crickets excrete urine more slowly across a range of temperatures. Seasonal acclimatization between September and December also corresponds with a reduction in the rate of Malpighian tubule transport in *Eurosta solidaginis* larvae (Yi and Lee, 2005). By reducing active transport across the Malpighian tubules, cold-acclimated insects may retain hemolymph volume (i.e. mitigate leak of water) during cold exposure. However this mechanism may not be conserved within or among insect lineages; in *D. melanogaster*, knockdown of diuretic capa peptides instead slows chill coma recovery (Tehrzaz et al. 2015).

Proton pumping drives net cation transport across the Malpighian tubules, and V-ATPase is central to this process (Klein, 1992; Chintapalli et al., 2013). Although V-ATPase mRNA abundance is reduced in the Malpighian tubules of cold-acclimated crickets (Chapter 3), cold acclimation did not cause a reduction in the activity of this enzyme overall. Decreased primary urine production should therefore involve modification of other enzymes (e.g. CA or NKA). CA in the Malpighian tubules provides protons for transport by V-ATPase and provides the counterions (H^+ and HCO_3^-) for import of hemolymph Na^+, K^+, and Cl^- (Henry, 1984; Phillips et al., 1987; Wessing et al., 1997; del Pilar Corena et al., 2005). A decrease in CA activity could therefore drive decreased primary urine excretion in cold-acclimated crickets. Because CA is a thermally-insensitive enzyme (Feller and Gerday, 1997), cold exposure alone would not be expected to reduce activity. A reduction in CA protein abundance, membrane localization, or other post-translational modification may be involved (see section 5.4.3). CA expression is indeed reduced in the Malpighian tubules of cold-acclimated crickets (Chapter 3), but whether this correlates with reduced enzyme activity requires verification (e.g. by colorimetric assay; Wilbur and Anderson, 1948).

Cold acclimation may increase Malpighian tubule NKA activity (based on a trend in cold-acclimated crickets at 21°C), and this could have multiple effects on water and ion balance in the hemolymph. In *Rhodnius* spp., inhibition of NKA stimulates diuresis (Grieco and Lopes, 1997; Caruso-Neves and Lopes, 2000), therefore increased NKA activity in the Malpighian tubules could account in part for the decreased primary urine production rate observed in cold-acclimated crickets. NKA activity in the Malpighian tubules also regulates selectivity of excreted cations. For example, inhibition of NKA by ouabain increases the Na^+:K^+ ratio of the primary urine in *Acheta domesticus* crickets (Coast, 2012). Therefore, an increase in Malpighian tubule NKA activity under optimal temperatures could hasten to the removal of K^+ to re-establish hemolymph [K^+] during recovery from cold exposure (Beyenbach, 2003). Chill-tolerant *Drosophila* spp. indeed excrete primary urine with lower Na^+:K^+ ratios compared to chill-susceptible species (MacMillan et al., 2015a). Although I did not measure Malpighian tubule enzyme activities at low temperatures, enhanced NKA activity during cold exposure could prevent or delay imbalance of hemolymph Na^+, water, and K^+ during chill coma (both reducing the CCRT and the energetic costs of re-establishing ionic and osmotic gradients; MacMillan et al., 2012).

5.4.2 Rectal NKA activity is unchanged by cold acclimation

I hypothesized that cold acclimation increases hindgut NKA activity. As NKA partially drives reabsorption across the rectal pads (Tolman and Steele, 1976; Phillips et al., 1988), increasing rectal NKA activity at low temperatures could allow cold-acclimated insects to maintain transport function during cold exposure. Increased rectal NKA activity during rewarming should also increase Na^+ and water reabsorption rates (i.e. reduce CCRT). However I found no evidence of this increase in NKA activity at 6°C, and higher NKA activity in cold-acclimated recta at 21°C was non-significant. Thus NKA does not appear to be a target of modification by cold acclimation. The significance of increased hindgut NKA transcript abundance in cold-acclimated *G. pennsylvanicus* (Chapter 3) therefore remains in question (although mRNA this does not necessarily reflect increased enzyme abundance; Gygi et al., 1999). It should be determined if and how cold acclimation

modifies active transport function across the rectum, either at low or optimal temperatures (e.g. with an Ussing chamber; Ussing and Zerahn, 1951; Clarke, 2009).

Many other hindgut enzymes could be modified by cold acclimation, however multiple enzymes controlling reabsorption across the rectum remain unidentified (O'Donnell and Simpson, 2008; Chintapalli et al., 2013). This poses a challenge for predicting how modification of hindgut water or ion transporters may affect transport in the cold. For example, cold acclimation decreases the expression of hindgut NKCC and two CAs (Chapter 3), however, unlike in the Malpighian tubules, the precise roles of these enzymes in rectal reabsorption have not been demonstrated. Similarly, very little is known about a cAMP-stimulated apical Cl^- pump thought to drive rectal transport (Phillips et al., 1987). To determine how cold acclimation modifies transport function we therefore require a better understanding about the fundamentals of hindgut transport (and this has not been revisited appreciably since the 1990s). The specific enzymatic targets of cold acclimation (and their relative contribution to altered transport function) could be determined by comparing active transport rates across the rectum *ex vivo* with and without selective enzyme inhibitors (Hanrahan et al., 1984; Bertram et al., 1991; Clarke, 2009).

5.4.3 A global role for NKA in cold acclimation

Acquired cold tolerance is associated with a reduction in whole-body NKA activity in *Drosophila melanogaster* (MacMillan et al., 2015c) and goldenrod gall fly larvae (*Eurosta solidaginis*; McMullen and Storey, 2008). However, the functional significance of modified active transport should depend on the specific enzyme and tissue in which that modification occurs. In the Malpighian tubules of cold-acclimated *G. pennsylvanicus* I instead observed potentially increased NKA activity, and this should prevent loss of hemolymph volume during cold exposure. It is possible that cold acclimation in dipterans modifies transport function differently than in orthopterans. However, as NKA is ubiquitously expressed, comparisons of whole-body NKA activity are not informative for predicting how cold acclimation affects transport function in ionoregulatory tissues specifically.

Changes in total rectal protein abundance could not explain a potential increase in Malpighian tubule NKA activity, however it is possible that the abundance of NKA increases proportionally with decreased abundance of other enzymes (e.g. V-ATPase), and I did not measure NKA abundance specifically. Cold-acclimated crickets could instead express NKA isozymes with different activities or thermal sensitivities (Blanco, 2005; Galarza-Muñoz et al., 2011), and post-transcriptional modification (via RNA editing) can also affect NKA activity (Colina et al., 2010).

Cold acclimation may also regulate active transport by post-translational modifications (e.g. phosphorylation or dephosphorylation) of NKA or other enzymes (Seo and Lee, 2004; McMullen and Storey, 2008; Poulsen et al., 2010). Kinase-mediated phosphorylation is proposed to reduce NKA activity in overwintering goldenrod gall flies (McMullen and Storey, 2008). Transcriptional upregulation of protein kinases could reduce NKA activity in cold-acclimated *G. pennsylvanicus* hindgut and Malpighian tubules (Chapter 3), however this requires experimental verification. Membrane localization affects the function of membrane-bound enzymes (Khurana, 2000; Lai and Jan, 2006), and genes regulating endocytosis and vesicle-membrane fusion were upregulated in the Malpighian tubules cold-acclimated crickets. Membrane fluidity also affects enzyme activity (Lam et al., 2004), therefore cold acclimation could regulate NKA activity by modifying membrane composition. The expression of multiple membrane-associated genes indeed changed with cold acclimation in crickets.

5.4.4 Conclusions

Cold acclimation modifies active transport function across the insect Malpighian tubules and possibly the rectum, and these modifications could explain why cold-acclimated insects defend hemolymph ion and water balance to lower temperatures and/or recover that balance more rapidly upon rewarming. Cold-acclimated crickets retain hemolymph volume at both low and optimal temperatures by reducing primary urine production (driven by modification of enzymes other than V-ATPase). Upon rewarming, enhanced Malpighian tubule NKA activity may allow cold-acclimated insects to retain hemolymph Na^+ content and counteract high hemolymph [K^+] by excreting more K^+.

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Chapter 6

6 General discussion

6.1 Thesis summary

Chill-susceptible insects lose water and ion homeostasis during cold exposure, and variation in insect cold tolerance is intimately linked with defense of this homeostasis during cold exposure, however the mechanisms are poorly understood. To generate hypotheses about why homeostasis is lost in the cold what mechanisms may explain interspecific cold tolerance variation I first compared patterns of water and ion balance in *Gryllus veletis* and *G. pennsylvanicus* crickets during early stages cold exposure. Hemolymph Na^+ balance was lost rapidly during cold exposure (preceding a gradual loss of water balance), and the pattern of Na^+ balance in early chill coma was unlike that observed at later stages (12 h or longer; Chapter 2). A bulk influx of Na^+ to the hemolymph within the first hour of cold exposure may indicate that leak of Na^+ from the tissues precedes (and may partially drive) migration of Na^+ and water to the gut. *Gryllus veletis* may avoid or slow or reduce Na^+ and water migration during early chill coma by maintaining a lower hemolymph Na^+ content and lower osmotic pressure between the hemolymph water compared to the less cold-tolerant *G. pennsylvanicus*.

I then generated and tested hypotheses about the mechanisms underlying cold tolerance plasticity by quantifying the effects of cold acclimation on transport-related gene expression and transport function in *G. pennsylvanicus* crickets. To generate hypotheses I performed tissue-specific transcriptome comparisons of warm- and cold-acclimated crickets (Chapter 3). Differential gene expression analyses revealed multiple candidate mechanisms related to both transport function and chilling injury. Changes in the expression of hindgut cytoskeletal and cell junction components suggested tissue restructuring, which could act to modify epithelial permeability. I tested this hypothesis by comparing the hindgut macromorphology and scalariform complex ultrastructure of warm- and cold-acclimated crickets, however I observed none of these structural changes (Chapter 4). Modification of water and ion permeability across the rectum by cold acclimation would

therefore involve other structural aspects. Cytoskeletal modifications did protect and (even enhance) F-actin polymerization during cold shock, which may protect the rectal pads from chilling injury and loss of transport function.

Cold acclimation decreased the expression of Malpighian tubule V-ATPase and increased expression of hindgut NKA, suggesting a reduction in primary urine production and an increase in reabsorption across the rectum. Ramsay assays confirmed that cold-acclimated Malpighian tubules indeed produce primary urine more slowly across a range of temperatures, however this was not explained by lower V-ATPase activity (Chapter 5). Increased NKA activity in the Malpighian tubules could partially account for reduced excretion and may also decrease Na^+:K^+ ratios in the excreta. NKA in the rectum activity was not modified by cold acclimation, therefore if cold acclimation modifies rectal transport function, those modifications must target other enzymes.

Overall, my work helps to revise the conceptual model of homeostasis during chill coma and supports the hypothesis that modified active transport function underlies plasticity in cold tolerance (by both preventing loss of ion and water balance in the cold and by aiding in recovery of that balance). While tissue structural modifications following cold acclimation have an as yet to be determined influence on passive leak, I demonstrate their functional significance with regards to protection against chilling injury. I suggest future approaches to test other mechanistic hypotheses about cold acclimation that I generated in this dissertation.

6.2 Revisiting loss of water and ion homeostasis during chill coma

The critical thermal minimum (CT_{min}) appears to align with the threshold temperature below which enzyme pumping rates are exceeded by passive diffusion. At or below the CT_{min}, hemolymph Na^+ and water gradually leak to the gut and hemolymph volume decreases. Consequently, hemolymph [K^+] increases and this high extracellular [K^+] was

proposed to explain the onset of chill coma paralysis via loss of muscle resting potential (MacMillan and Sinclair, 2011b). This conceptual model was based on water and ion imbalance apparent after 12 h of chill coma (Koštál et al., 2004; Koštál et al., 2006; MacMillan and Sinclair, 2011). However, some questions remained: how soon is water and ion balance perturbed upon entry into chill coma, and do patterns of water and ion balance in the early stages of chill coma reflect those in later stages? Furthermore, can a gradual loss of water and ion balance explain the onset of chill coma paralysis (which occurs within minutes of cold exposure)?

I show that Na^+ balance is lost rapidly during chill coma, and that both the content and concentration of hemolymph Na^+ increase (rather than decrease) within the first hour. This transient increase in hemolymph Na^+ had not been captured by previous studies, and introduces another step in the process of ion and water disruption during chill coma. It is likely that Na^+ first leaks to the hemolymph from surrounding tissues before migrating to the gut lumen. Although the hindgut maintains high paracellular [Na^+], a relatively stable gut Na^+ content during the first hour of chill coma (and increase thereafter) eliminates this tissue as a contributor to the initial hemolymph Na^+ influx. Femur muscle did lose Na^+ content rapidly, which is supported by decreased muscle [Na^+] without changes in muscle water content observed in a similar study (MacMillan and Sinclair, 2011b). Leak of Na^+ from other tissues may also contribute to the hemolymph Na^+ influx during cold exposure. For instance, the egg masses may have provided a large reservoir of Na^+ (and egg [Na^+] favors leak of this ion towards the hemolymph). It is not known whether males exhibit a similar spike in hemolymph Na^+. Determining the origin of the hemolymph Na^+ influx therefore requires analyses of Na^+ content in other tissues of both male and female insects during early chill coma.

Water and [K^+] imbalance proceed gradually during the first 12 h of chill coma (as predicted by progressive imbalance during later chill coma), but bulk movement of K^+ is not included in the conceptual model of homeostasis during chill coma (hemolymph K^+ content in *G. pennsylvanicus* does not change appreciably over days of cold exposure; MacMillan and Sinclair, 2011b; Coello Alvarado et al., 2015). However, I observed

hemolymph K^+ content to increase during the first 12 h of chill coma; this K^+ may have leaked from the gut lumen down a concentration gradient. However, hemolymph [K^+] imbalance in crickets does not account for muscle depolarization prior to 12 h of cold exposure (MacMillan and Sinclair, 2011b; Coello Alvarado et al., 2015; Chapter 2). This is also true for locusts (MacMillan et al., 2014) and tropical cockroaches (Koštál et al., 2006). It is now clear that while loss of homeostasis (in particular K^+ balance) can explain muscle depolarization in later chill coma, it does not explain the onset of chill coma paralysis. Rather, paralysis likely results from direct effects of low temperatures on muscle or nervous system excitability (Goller and Esch, 1990; Hosler et al., 2000; Armstrong et al., 2012; Findsen et al., 2014; MacMillan et al., 2014; Andersen et al., 2015). Unlike in the hemolymph, cold exposure causes a rapid surge in extracellular [K^+] in the nervous tissues and neural depolarization (Robertson, 2004; Armstrong et al., 2012). However, this K^+ disruption may not actually cause paralysis; rapid cold-hardening (RCH) lowers the temperature of chill coma onset but does not prevent or mitigate the surge of K^+. To my knowledge the effect of cold acclimation on defense of ion homeostasis in the nervous tissues has not yet been explored.

6.2.1 Interspecific variation in cold tolerance

Although both cricket species progressively lost water and ion balance, this loss was slower and less-severe in the more cold-tolerant species (*G. veletis*). It is possible that *G. veletis* tissues are more resistant to passive ion leak during cold exposure (discussed in more detail in section 6.3.1). However, *G. veletis* also maintained lower hemolymph Na^+ content (and, perhaps consequently) lower osmotic pressure between the hemolymph and gut even prior to cold exposure. Passive leak could thus be minimized by reducing transepithelial Na^+ and osmotic gradients, although manipulation of osmotic gradient alone (via high dietary xylitol) does not appear to affect cold tolerance (Lebenzon et al., 2017; Yerushalmi et al., 2016). That lower Na^+ gradients between hemolymph and gut explain interspecific differences in cold tolerance is supported by recent work in *D. melanogaster* (MacMillan et al., 2015b). However, dietary manipulation to reduce this Na^+ gradient in *D. melanogaster* and *G. pennsylvanicus* improved CCR and defense of hemolymph salt balance without improving survival of chronic cold stress (in the latter species, survival

actually decreased; Lebenzon et al., 2017; Yerushalmi et al., 2016). These findings therefore somewhat contradict the hypothesis that loss of ion balance during chronic cold exposure is a mechanism underlying chilling injury and mortality (Koštál et al., 2006; Lee, 2010; Findsen et al., 2014). To determine whether hemolymph ion imbalance damages the tissues independent of the cold, cell death in the hindgut or other tissues could be quantified after repeated salt injection (i.e. chronic hemolymph ionic stress; Weidler, 1977).

6.3 Mechanisms of cold acclimation

Cold-acclimated insects maintain water and ion homeostasis to lower temperatures (and/or recover homeostasis faster upon rewarming) compared to warm-acclimated conspecifics (Sinclair and Roberts, 2005; Koštál et al., 2006; Findsen et al., 2013; Coello Alvarado et al., 2015), suggesting that cold acclimation modifies transport function. The current conceptual model presents two overarching means by which cold acclimation could protect transport function: 1) by lowering tissue permeability to reduce ion and water leak, and/or 2) by maintaining rates of active transport to lower temperatures (Fig. 1; MacMillan and Sinclair, 2011a). The mechanisms underlying these modifications have been the main focus of my dissertation.

6.3.1 Modification of diffusion

MacMillan and Sinclair (2011) hypothesized that cold acclimation reduces ion and water diffusion during cold exposure by reducing epithelial permeability to water and/or ions (Fig 1.1B). Changes to tissue permeability are likely to be structural, and structural modifications are indeed emerging as a potential mechanism of acquired cold tolerance (Chapter 3; Teets et al., 2012; Gerken et al., 2015; Torson et al., 2015; MacMillan et al., 2016). Water and ions are likely to leak across the hindgut (and possibly the midgut) during cold exposure, and I find transcriptional but not histological evidence of reduced hindgut permeability in cold-acclimated *G. pennsylvanicus*. Changes to gut permeability following cold acclimation could be determined *ex vivo* by measuring electrophysiological resistance (e.g. by Ussing chamber; Ussing and Zerahn, 1951; Clarke, 2009; Brun et al., 2014), or by

tracking the movement of marker or labeled solutes across the tissue (Ordin and Bonner, 1956; Dow, 1981; Peters and Wiese, 1986). Although I have attempted Ussing chamber work with *G. pennsylvanicus* hindguts, loss of tissue viability was too rapid to measure steady resistance. Transport across the ileum and rectum in locusts has been measured successfully in Ussing chambers, therefore locusts may be better-suited orthopteran models for this technique (Hanrahan and Phillips, 1984; Thomson et al., 1988; Audsley et al., 1992).

6.3.1.1 Paracellular permeability

Paracellular permeability is particularly important for reabsorption of water and Na^+ across the hindgut (O'Donnell and Simpson, 2008), therefore cell junctions are a likely target for cold acclimation. In cold-acclimated crickets, differential expression of PAR3 and PCK-α suggest increased tight junction assembly and enhanced tightening of those junctions (Rosson et al., 1997; Ebnet et al., 2003). I also observed transcriptional modification of several genes involved in cell structure and adherens junctions. However, tightening of adherens junctions in the scalariform complex was not apparent in histological comparisons of warm- and cold-acclimated crickets (Chapter 4). Structural modifications to increase diffusion distance (e.g. scalariform complex tortuosity or epithelial cell thickening) or to increase absorptive surface area (e.g. by rectal pad cell proliferation) were also not apparent. I did not measure apical or basal paracellular channel widths, nor did I characterize the structure or localization of junction-related components, however these features could also be modified to reduce permeability. The extent to which water and ions leak via paracellular routes during cold exposure and how cold acclimation modifies paracellular leak should be first determined, e.g. by quantifying mannitol migration across warm- and cold-acclimated guts at different temperatures (Barthe et al., 1998).

The cytoskeleton also regulates cell-to-cell adhesion (Knudsen et al., 1995; Drees et al., 2005; Hartsock and Nelson, 2008), therefore cold acclimation could reduce tissue paracellular permeability by modifying cytoskeletal components involved in cell junction structure. For example, hindgut α-actinin was upregulated with cold acclimation (Chapter 3) which may indicate enhanced cell-to-cell adhesion (Knudsen et al., 1995). The

contribution of specific cytoskeletal and cell-junction components to enhanced cold tolerance and modified water or ion leak could be investigated in *D. melanogaster* by loss-of-function assays (e.g. by mutation or knockdown via the GAL4/UAS system; Roulier et al., 1992; Bellen et al., 2004; Ni et al., 2011), or gain-of-function assays for those components (e.g. using CRISPR; Sander and Joung 2014).

6.3.1.2 Transcellular permeability

Cold acclimation could mitigate transcellular leak of water and ions by reducing membrane recruitment or abundance of aquaporins and ion channels (Köttgen et al., 2005; Spring et al., 2009). Modified aquaporin expression does indeed correlate with acquired cold tolerance in the goldenrod gall fly (Philip et al., 2008; Philip and Lee, 2010). However, I did not identify any putative aquaporins in the *G. pennsylvanicus* transcriptome (potentially due to a lack of an annotated genome for this species), and few ion channels were transcriptionally modified by cold acclimation in the hindgut. The relative contribution of transcellular leak to water and ion imbalance during cold exposure should first be determined, e.g. by measuring transcellular resistance in an Ussing chamber-impedance spectroscopy technique (Krug et al., 2009). The effect of cold acclimation on transcellular permeability could then be quantified, and related to modification of aquaporins and ion channels by chemical blocking or knockdown of those proteins (Böhme et al., 1992; Philip et al., 2008; Neubauer et al., 2013; Drake et al., 2015).

6.3.2 Modification of active transport

Reduced enzyme activity at low temperatures likely explains loss of water and ion balance during chill coma (Nespolo et al., 2003; MacMillan and Sinclair, 2011a), and cold-acclimated insects are better able to defend this balance in the cold (Koštál et al., 2004; Coello Alvarado et al., 2015). Modifying ion pumps to maintain transport function at lower temperatures is therefore proposed as a mechanism of cold acclimation (Fig. 1.1C). Enhanced active transport function at higher temperatures should also speed the recovery of water and ion balance upon rewarming, and cold-acclimated insects do exhibit shorter CCRTs (Ayrinhac et al., 2004; Rako and Hoffmann, 2006). I show that cold acclimation

modifies active transport function in the Malpighian tubules at both optimal and low temperatures, and this modification may explain improved defense of hemolymph volume, Na^+ balance, and K^+ balance.

6.3.2.1 Malpighian tubule transport

Cold acclimation may defend hemolymph water and ion homeostasis by enhancing active transport at low temperatures (MacMillan and Sinclair, 2011a). Cold acclimation indeed modifies active transport across the Malpighian tubules such that excretion is reduced across a range of temperatures, and this may allow cold-acclimated insects to retain hemolymph volume during cold exposure. However a reduction in excretion rate implies that active ion transporter activities in the Malpighian tubules are generally reduced by cold acclimation, rather than enhanced. The effect of cold acclimation on active transport function therefore depends on the tissue in question, illustrating the importance of tissue-specific comparisons. Although V-ATPase expression was downregulated in cold-acclimated Malpighian tubules, lack of altered V-ATPase activity suggests that modification of other enzymes accounts for reduced excretion. CA expression was also reduced following cold acclimation, however the functional significance of this transcriptional shift requires a comparison of CA activity in warm- and cold-acclimated Malpighian tubules.

Cold acclimation does appear to enhance the activity of NKA in the Malpighian tubules (at least at 21°C), and this was not caused by changes in total protein or NKA transcript abundance. It is therefore likely that cold acclimation regulates Malpighian tubule NKA activity by post-translational modifications (see section 6.3.2.4). Increased pumping of NKA in the Malpighian tubules should reduce excretion rates (Grieco and Lopes, 1997; Caruso-Neves and Lopes, 2000) and decrease the primary urine Na^+:K^+ ratio (Coast, 2012). Loss of hemolymph water and Na^+, and increased hemolymph K^+ during cold exposure could therefore be mitigated if cold acclimation also enhances Malpighian tubule NKA activity at low temperatures. At least upon rewarming, increased NKA activity may explain shorter CCRT by re-establishing Na^+ and K^+ balance more rapidly (MacMillan et al., 2015a).

6.3.2.2 Hindgut transport

To maintain water and ion homeostasis during cold exposure and restore that homeostasis faster upon rewarming (i.e. to shorten the CCRT), cold acclimation should increase rates of reabsorption across the rectum at both low and optimal temperatures. Enhanced activity of rectal NKA would drive this reabsorption, however despite an increase in hindgut NKA transcript abundance following cold acclimation NKA activity in the rectum was unchanged at either 6°C or 21°C. First, a more general approach should be taken to determine whether cold acclimation indeed modifies rectal reabsorption rate. For example, active transport across the recta of warm- and cold-acclimated insects should be compared at both low and optimal temperatures (e.g. by Ussing chamber or the scanning ion-selective electrode technique; Ussing and Zerahn, 1951; Clarke, 2009; Nguyen and Donini, 2010). The contribution of other enzymes to altered rectal transport could then be quantified by selective inhibition (Hanrahan et al., 1984; Bertram et al., 1991; Clarke, 2009).

Although transport mechanisms of the Malpighian tubules have been relatively well-studied in the last 60 years, our understanding of hindgut transport mechanisms is lagging (Chintapalli et al., 2013). As such I can only speculate about the functional significance of downregulated NKCC, CA, V-ATPase, and a Na^+-H^+ exchanger in cold-acclimated hindguts. For example, NKCC is important for unidirectional transport of ions across Malpighian tubule principal cells (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2004), but its specific roles in the hindgut have not been demonstrated (Phillips et al., 1987; Chintapalli et al., 2013). The activity of CAs in the gut is coupled with V-ATPase and NHA for proton exchange (Zeiske, 1992; Chintapalli et al., 2013). Downregulation for these enzymes in the hindgut could potentially reduce apical Na^+ export at the expense of pH regulation (O'Donnell and Simpson, 2008; Chintapalli et al., 2013). Cricket hindgut transcriptomes did not reveal changes in the expression of any putative apical Cl^- pump (thought to be an important driver of reabsorption across the rectum; Phillips et al., 1988; Coast, 2007; O'Donnell and Simpson, 2008). However, neuropeptide stimulation of active Cl^- transport acts through cAMP (Phillips et al., 1988; O'Donnell and Simpson, 2008) which was upregulated in the hindgut following cold acclimation (see section 6.4.2).

6.3.2.3 The diverse roles of NKA in cold acclimation

Insect cold tolerance should be improved if water and ion homeostasis is defended in the cold (or at least re-established more rapidly upon recovery). This could be achieved by increasing NKA activity in the Malpighian tubules (reducing diuresis) and rectum (enhancing reabsorption), and I provide some evidence in support of this in crickets. However, interspecific and acquired cold tolerance has been associated previously with decreased whole-animal NKA activity, at least in dipterans (McMullen and Storey, 2008; MacMillan et al., 2015c). MacMillan et al. (2015c) suggest that a reduction in Na^+ pumping may reduce Na^+ gradients across insect epithelia, thereby minimizing the leak of Na^+ (and therefore water) during cold exposure (see section 6.2.3). It is possible that these disparate findings simply illustrate variation in cold tolerance mechanisms among insect lineages (see section 6.4.1). However, the effect of NKA on Na^+ transport differs among tissues (NKA activity prevents net Na^+ transport across the Malpighian tubules but drives Na^+ transport across the rectum). Therefore it is difficult to make specific predictions about how overall transport and ion balance may be modified based on whole-body NKA activity comparisons. The role of NKA in interspecific and plastic cold tolerance variation likely differs among lineages according to the dominant hemolymph cation (whether Na^+ or K^+). For example, I would predict that acquired cold tolerance should correlate with increased NKA activity in ionoregulatory tissues if Na^+ is the dominant hemolymph cation. To determine the ubiquity of NKA modification for maintaining transport function in the cold, variation in cold tolerance within and among lineages should be linked to NKA activity in specific ionoregulatory tissues.

6.3.2.4 Other mechanisms underlying modified active transport

In this dissertation I have not endeavored to identify active transport modifications unrelated to altered transcript or total protein abundance. Expression of isozymes with lower thermal sensitivities could allow cold-acclimated insects to maintain transport function at lower temperatures (Angilletta et al., 2003; Blanco, 2005). Isozymes are difficult to distinguish in *de novo* transcriptome assemblies (as fine sequence variation is not apparent after alignment), but their expression could be investigated by amplification

and sequencing for ion pumps of interest. Post-translational modifications (e.g. phosphorylation, oxidation of protein motifs, complexation with other molecules, and membrane recruitment) could also underlie plasticity in water and ion transport function in the cold (Merzendorfer et al., 1997; Khurana, 2000; Wieczorek et al., 2000; Seo and Lee, 2004; Hilvo et al., 2008; Galarza-Muñoz et al., 2011; Zeuthen and MacAulay, 2012). For example, suppression of NKA activity in overwintering *Eurosta solidaginis* gall flies is proposed to occur by kinase-mediated phosphorylation (McMullen and Storey, 2008). Multiple protein kinases in *G. pennsylvanicus* ionoregulatory tissues were upregulated by cold acclimation (Chapter 3), however should reduce NKA activity, in contrast to my observations (Chapter 5). The role of these protein kinases in transport modification of NKA or other enzymes could be quantified by comparing the phosphorylation states of ion pumps in the Malpighian tubules and recta of warm- and cold-acclimated insects. Similarly, we could measure the effect of cold acclimation on recruitment of ion pumps to the cell membrane (Hundal et al., 1992; Martens et al., 2004; Misonou et al., 2004), and relate those differences to modified expression of vesicle transport genes.

Transport function of ion pumps and channels can also be modified indirectly via changes to membrane fluidity at low temperatures (Lam et al., 2004; Galarza-Muñoz et al., 2011), and many membrane-associated genes in the hindgut and Malpighian tubules were transcriptionally altered with cold acclimation. Similarly, modification of the actin cytoskeleton (which was also apparent in cold-acclimated tissue transcriptomes) can also regulate enzymatic pump activity (Cantiello, 1995; Hilgemann, 1997; Khurana, 2000). The activity of enzymes such as V-ATPase are hormonally-regulated in insects (Phillips and Audsley, 1995; O'Donnell et al., 1996; Phillips et al., 1998; Harrison, 2001; Coast, 2012; Paluzzi, 2012), but the role of hormones in cold acclimation is almost completely unexplored (see section 6.4.3).

6.3.3 How does cold acclimation protect against chilling injury?

Although many mechanisms have been proposed to explain insect chilling injury, empirical evidence for these mechanisms is currently lacking. Part of the problem is that we have yet to identify the cells and tissues most susceptible to chilling (both acute and chronic), and the extent to which cell death is necrotic or apoptotic. The midgut and fat body appear to be particularly susceptible to freezing damage (Izumi et al., 2005; Philip et al., 2008), and the gut may be damaged by cold exposure itself (Sinclair and Chown, 2005). Acute cold shock causes apoptosis in *D. melanogaster* flight muscle, and this cell death is reduced by RCH (potentially via decreased abundance of apoptosis initiator and executioner caspases; Yi et al., 2007). However, I observed an upregulation of apoptosis-promoting caspases in cold-acclimated crickets (Chapter 3). It is possible that apoptosis is required for tissue restructuring during cold acclimation (as evinced by transcriptomic comparisons, see section 6.3.1). Tissues exhibiting apoptotic cell death with chilling could be confirmed by TUNEL assay (Gavrieli et al., 1992; Yi et al., 2007; Vasudevan and Ryoo, 2016).

My attempts to identify chilling-attributed cell death in *G. pennsylvanicus* ionoregulatory tissues by live-dead staining have been unsuccessful, but *Drosophila melanogaster* or *Sarcophaga crassipalpis* appear to be suitable models for this technique (personal observations; Yi and Lee, 2004). Cellular components with non-repairable damages must be cleared, and regulation of autophagy is also emerging as an important aspect of acquired cold tolerance (Teets and Denlinger, 2013a; Gerken et al., 2015). Crickets exhibited shifts in the expression of genes related to autophagy following cold acclimation (Chapter 3), however their role in repair of chilling injuries requires further investigation. For example, we may compare survival of injury-inducing cold exposure for insects with and without knockdown of specific autophagy-regulating genes or proteins (Juhász et al., 2003; Pattingre and Levine, 2006).

Water and ion imbalance does not appear to cause immediate damage; *G. pennsylvanicus* survival is relatively high despite substantial loss of hemolymph water and Na^+ during the first day of chilling at 0°C (MacMillan and Sinclair, 2011b; Coello Alvarado et al., 2015). This imbalance during prolonged cold exposure is instead proposed to cause chronic

chilling injury (Koštál et al., 2006; Lee, 2010; Findsen et al., 2014), but the precise mechanisms remain unknown. We should first correlate the onset of lethal chilling injury with the extent of water and ion imbalance. For example, a steep increase in mortality for *G. pennsylvanicus* occurs at two to three days of chilling at 0°C (MacMillan and Sinclair, 2011b; Coello Alvarado et al., 2015). If hemolymph ionic imbalance accounts for this mortality, then mortality or injury should be induced by manipulating cricket extracellular ion concentrations (via injection of salts) to reflect hemolymph conditions after two to three days of chilling. Damage to specific tissues could then be assessed by live-dead stain.

As cold exposure affects membrane structure and fluidity, ion and water imbalance is likely exacerbated by chilling damage to the cell membrane (O'Donnell and Maddrell, 1983; Ramløv, 2000; Lam et al., 2009). Cold tolerance is thus commonly associated with membrane modification (Hazel, 1989; Hazel, 1995; Gerken et al., 2015). In crickets, a relatively large proportion of genes encoding membrane-associated cellular components were differentially-expressed following cold acclimation, however I did not further investigate the relationship of these genes to modified membrane fluidity. Enhanced cold tolerance corresponds with increased unsaturation of the phospholipid bilayer in dipterans and moths (Koštál and Simek, 1998; Koštál et al., 2003; Overgaard et al., 2005; Shreve et al., 2007), however to my knowledge a link between these membrane modifications and enhanced transport function in the cold has not been demonstrated.

Chilling injury could result from cytoskeletal failure at low temperatures (Madara et al., 1986; Belous, 1992; Khurana, 2000; Pedersen et al., 2001; Kim and Denlinger, 2009; Monastyrska et al., 2009), and modified expression of actin- and tubulin-related genes is emerging as an important aspect of the cold-hardening process (RCH, cold acclimation, and even diapause; Cottam et al., 2006; Kim et al., 2006; Kayukawa and Ishikawa, 2009; Teets et al., 2012; Gerken et al., 2015; MacMillan et al., 2016). Actin appears to be a central target for modification, and genes that promote actin branching and stabilization were upregulated in crickets following cold acclimation (Chapter 3). I show in Chapter 4 that these transcriptional shifts correlate with both the protection (and even enhancement) of F-actin polymerization following cold shock. The effect of cold-hardening on the

microtubules is somewhat less clear; diapause entry and cold exposure reduces *beta-tubulin* expression and microtubule abundance in *C. pipiens* (at least in flight muscles; Kim and Denlinger, 2009), while cold acclimation in *G. pennsylvanicus* transport tissues increases the expression of at least two genes promoting microtubule stability and polymerization. Altered expression of microtubule-associated proteins 1A/1B light chain 3A in crickets may also modify autophagic processes (Pankiv et al., 2007). Phosphorylation of cytoskeletal components could also affect cell structure in the cold; and this appears to at least partially underlie rapid cold hardening in *Sarcophaga bullata* (Teets and Denlinger, 2016). We now need to show that these cytoskeletal modifications improve cell survival and/or transport function following cold exposure (see section 6.3.1).

To better understand how cold acclimation enhances survival of chilling, candidate cold tolerance mechanisms should be linked with cellular, tissue, and whole-organism performance in the cold. Ideally, the expression or titres of specific candidate molecules (e.g. antioxidants, cytoskeletal stabilizers, and those involved in apoptosis or autophagy) would be manipulated via RNA interference or gene editing using CRISPR and correlated with survival of cold exposure (Huvenne and Smagghe, 2010; Ni et al., 2011; Sander and Joung, 2014; Dong et al., 2015). A time-course analysis of gene expression prior to and following cold exposure could also help to define which molecules are involved in prevention of chilling injury, repair, or both. Transcriptome comparisons of chilling-susceptible tissues (such as the fat body and midgut) after acute and chronic chilling could provide hints about the nature of each type of injury.

6.4 Other aspects of cold tolerance and transport function

Regulation of transport function involves multiple molecules and processes, many of which appear to be modified by cold acclimation in the ionoregulatory tissues of *G. pennsylvanicus* (Chapter 3). In the following sections I briefly discuss some of these processes and their potential relationship to modified transport function and survival of cold exposure.

6.4.1 Neuropeptides and secondary messengers

Excretion and reabsorption of water and ions are controlled by multiple neuropeptide families acting on the Malpighian tubules and hindgut (Phillips and Audsley, 1995; Audsley et al., 2013; Halberg et al., 2015). Terhzaz et al. (2015) recently demonstrated a role for the diuretic capa peptides in insect cold tolerance, whereby knockdown of the *capa* gene increased CCRT in *D. melanogaster*. Chilling of *D. melanogaster* at 0°C increased *capa* mRNA abundance, and release of the peptide from neuroendocrine cells occurred primarily during recovery from cold stress (Terhzaz et al., 2015). This transcriptional shift indicates that modified endocrine function is one mechanism of cold acclimation (with regards to chill coma recovery).

Cold acclimation upregulated atrial natriuretic peptide converting enzyme (which stimulates Malpighian tubule secretion in mosquitoes; Petzel et al., 1985), however the role of this enzyme in hindgut reabsorption is not known. I also did not observe shifts in the expression of excretory neuropeptide receptors in the hindgut or Malpighian tubules of cold-acclimated insects. Whether or not cold acclimation alters neuropeptide expression in crickets and other insects may be best determined by comparing the nervous tissue transcriptomes or hemolymph proteomes of warm- and cold-acclimated individuals. The functional significance of specific neuropeptides (e.g. antidiuretic hormone, ion transport peptide, or capa) could also be determined by comparing the cold tolerance of insects with and without peptide injection (Phillips and Audsley, 1995; Paluzzi, 2012; Halberg et al., 2015; Terhzaz et al., 2015).

Most insect neuropeptides stimulate excretion and reabsorption via secondary messengers such as cAMP and cGMP, inositol triphosphate (IP3), and adenylate cyclase (Schooley et al., 2012). A number of these secondary messengers were transcriptionally-altered by cold acclimation, however the functional significance of their alteration for defense of hemolymph volume and ion balance is not entirely clear. For example, ion transport peptide stimulates ileal Na^+ reabsorption by elevating cytosolic cAMP and cGMP (Audsley et al., 2013). Increased expression of hindgut adenylate cyclase (which produces cAMP) could

thereby enhance ion reabsorption across the ileum in cold-acclimated *G. pennsylvanicus* (Schooley et al., 2012). However, upregulation of hindgut cAMP/cGMP phosphodiesterases might depress reabsorption rates by reducing cytosolic cAMP or cGMP (Schooley et al., 2012). Upregulation of a putative cAMP phosphodiesterase and downregulation of inositol monophosphatase (which is required for IP3 recycling) in the Malpighian tubules of cold-acclimated crickets could contribute to reduced excretion rates (Coyle and Duman, 2003). If these transcriptional shifts reflect altered secondary messenger abundance, we could perhaps quantify their contribution to altered transport function by selective inhibition in the hindgut and Malpighian tubules *ex vivo* (Sharma et al., 1975; Genain et al., 1995; Sarkar et al., 2005).

6.4.2 Ca^{2+} imbalance and signaling

Literature on the mechanistic roles of Ca^{2+} balance in cellular chilling injury, cold-sensing, and both interspecific and plastic variation in cold tolerance for plants is extensive (Minorsky, 1985; Knight et al., 1996; Jian et al., 1999; Thomashow, 1999; Nayyar et al., 2005; Lukatkin et al., 2012). However, relatively little is known about the role of Ca^{2+} balance in insects during cold exposure. Hemolymph Ca^{2+} in crickets was unchanged within the first 12 h of cold exposure (personal observations), but both the content and concentration of hemolymph Ca^{2+} appear to decrease gradually on the order of days (MacMillan and Sinclair, 2011b). Decreased extracellular Ca^{2+} can reduce rates of primary urine production by the Malpighian tubules (Morgan and Mordue, 1985), but this does not appear to defend hemolymph volume during cold exposure (MacMillan and Sinclair, 2011b). Ca^{2+} imbalance itself appears to be important for insect cold sensing and RCH (Teets et al., 2008; Teets et al., 2013).

Intracellular [Ca^{2+}] in goldenrod gall fly tracheal cells nearly doubles with chilling at 0°C (Teets et al., 2013). Although total muscle [Ca^{2+}] was unchanged over five days at 0°C in *G. pennsylvanicus* (MacMillan and Sinclair, 2011b), this does not necessarily reflect cytosolic (active) [Ca^{2+}]. Muscle depolarization resulting from hemolymph ion imbalance during chill coma in general could initiate a voltage-dependent release of Ca^{2+} to the cytosol (Koštál et al., 2006), and even minute shifts in free intracellular [Ca^{2+}] can have

drastic impacts on signaling cascades and other cellular processes (Orrenius et al., 1989). For example, sustained increased cytosolic [Ca^{2+}] can cause membrane degradation, cytoskeletal disruption, oxidative stress, and damage to DNA and cellular components (Hochachka, 1986; Orrenius et al., 1989; Wahlström et al., 2006; Lukatkin et al., 2012). Export or sequestration of cytosolic Ca^{2+} to reduce [Ca^{2+}] is likely to be hindered at low temperatures by slowed Ca^{2+}-ATPase activity. Cold-acclimated insects may defend cytosolic [Ca^{2+}] by modifying Ca^{2+} transport, and this hypothesis is supported by an increase in the expression of Malpighian tubule Ca^{2+}-ATPase and hindgut Ca^{2+}-release-activated Ca^{2+} channels for cold-acclimated *G. pennsylvanicus* (Chapter 3). These transcriptional modifications should now be linked to enhanced abundance and/or activity of these enzymes in the ionoregulatory tissues.

6.4.3 Unidentified molecules associated with cold acclimation

Our understanding of water and ion transport processes in the Malpighian tubules and hindgut within and among insect lineages is incomplete. For example, neuroendocrine control and partitioning of anion and cation transport across Malpighian tubule cells appears to vary considerably among insects but only a few taxonomic orders have been studied in this regard (Halberg et al., 2015). Comparative studies on hindgut transport function are even fewer, and the hindgut is enriched in many genes with unknown function (Chintapalli et al., 2013). In both the hindgut and Malpighian tubules of *G. pennsylvanicus*, many of the genes most altered by cold acclimation had no putative identity via BLAST (these included contigs downregulated by 37-fold in both tissues, and genes upregulated 59- and 100-fold in the hindgut and Malpighian tubules, respectively; Chapter 3). Identification of these genes would be invaluable not only for understanding about hindgut and Malpighian tubule transport function in Orthoptera but how those processes relate to cold acclimation and defense of homeostasis at low temperatures. These genes may be identifiable as genomic databases become more robust (especially for non-model species such as *G. pennsylvanicus*).

6.5 Dissecting plastic and interspecific mechanisms of cold tolerance

Many questions about acquired cold tolerance still remain: what are the limits of cold tolerance plasticity and what are the associated costs or trade-offs? How conserved are the mechanisms of acquired cold tolerance within and among taxa? How rapid is the cold acclimation process, and what distinguishes it from RCH? Cold tolerance plasticity appears to involve multiple physiological systems and various timescales. For example, cold acclimation regimes in the literature span from two days to four months (Ding et al., 2003; Rako and Hoffmann, 2006), and the duration of acclimation is likely to influence conclusions about the underlying mechanisms. At least in adult *G. pennsylvanicus*, a one-week cold acclimation regime improves cold tolerance as effectively as a four-week cold acclimation under similar conditions (Coello Alvarado et al., 2015). One week may therefore be sufficient for steady-state alteration of cold tolerance phenotype, but do the physiological mechanisms driving this phenotype differ between one and four weeks of cold acclimation? The energetic costs of enhanced cold tolerance may change over time, and could reflect different mechanisms (e.g. short-term mechanisms may involve active transport modifications while longer-term mechanisms may involve structural modifications).

Cold acclimation and RCH appear to share some targets for modification (e.g. the membrane, cytoskeleton, ion transport, apoptosis, and autophagy; Teets et al., 2012; Gerken et al., 2015; Koštál and Simek, 1998; Overgaard et al., 2005; Findsen et al., 2013). Distinguishing the mechanistic overlap of RCH and cold acclimation processes is therefore an ongoing area of investigation (Colinet and Hoffmann, 2012; Teets and Denlinger, 2013b; Gerken et al., 2015). Although acclimation is commonly associated with gradual, transcription-driven modifications (Clark and Worland, 2008), RCH can also alter gene expression and induce alternative splicing (Overgaard et al., 2005; Qin et al., 2005; Teets et al., 2012; Gerken et al., 2015). It is suggested that RCH (as well as cold acclimation) can act via post-translational modifications (Misener et al., 2001; Storey and Storey, 2012; Colinet et al., 2013; MacMillan et al., 2015c), and RCH has recently been shown to modify signaling cascades that alter protein phosphorylation (Teets and Denlinger, 2016).

6.6 Applications in insect management

Plasticity can enhance cold tolerance beyond adaptive differences (e.g. based on phylogeny; Ayrinhac et al., 2004; Ransberry et al., 2011; McKinnon, 2015). However in an increasingly variable climate this plasticity may pose a problem with regard to insect pests, especially if species' invasive potential is associated with a greater capacity for cold tolerance plasticity (Lehmann et al., 2015). Uncovering common themes of interspecific and plastic cold tolerance could help to identify widely-applicable physiological targets for manipulating insect cold tolerance (Huvenne and Smagghe, 2010; Andreadis and Athanassiou, 2017). For example, cold tolerance within and among *Drosophila* spp. is correlated with a reduction in hemolymph [Na^+] (MacMillan et al., 2015b; MacMillan et al., 2015c), and this strategy could be common to other dipterans or other lineages. Manipulation of insect water and ion homeostasis is a promising means of population management (Cohen, 2013), therefore understanding the link between water or ion homeostasis and insect cold tolerance would add to the utility of these manipulations for controlling pests or beneficial species at low temperatures.

6.7 Concluding remarks

In addition to understanding the evolution of insect cold tolerance, uncovering the means by which insects modify their cold tolerance has substantial practical importance for mass-rearing or population management of disease vectors, pest, and beneficial insects. Insects lose hemolymph water and ion balance rapidly upon exposure to the cold, and this process may involve leak of ions from surrounding tissues. Although loss of homeostasis does not account for paralysis in early stages chill coma, progressive water and ion imbalance hinders chill coma recovery and may lead to the accumulation chilling injuries by as yet to be determined mechanisms. Cold acclimation appears to involve both a modification of both Malpighian tubule active transport function and rectal cytoskeletal structure, and these changes may explain improved chilling survival and enhanced defense or recovery of water and ion homeostasis. Many questions still remain about the mechanisms underlying both plastic and interspecific variation in cold tolerance for chill-susceptible insects. My work

has yielded specific hypotheses about these mechanisms with regard to transport function, which should help to move the insect thermal physiology field from correlative to manipulative, causative approaches to address candidate mechanisms of cold tolerance.

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Appendix A: Chapter 2 supplementary material

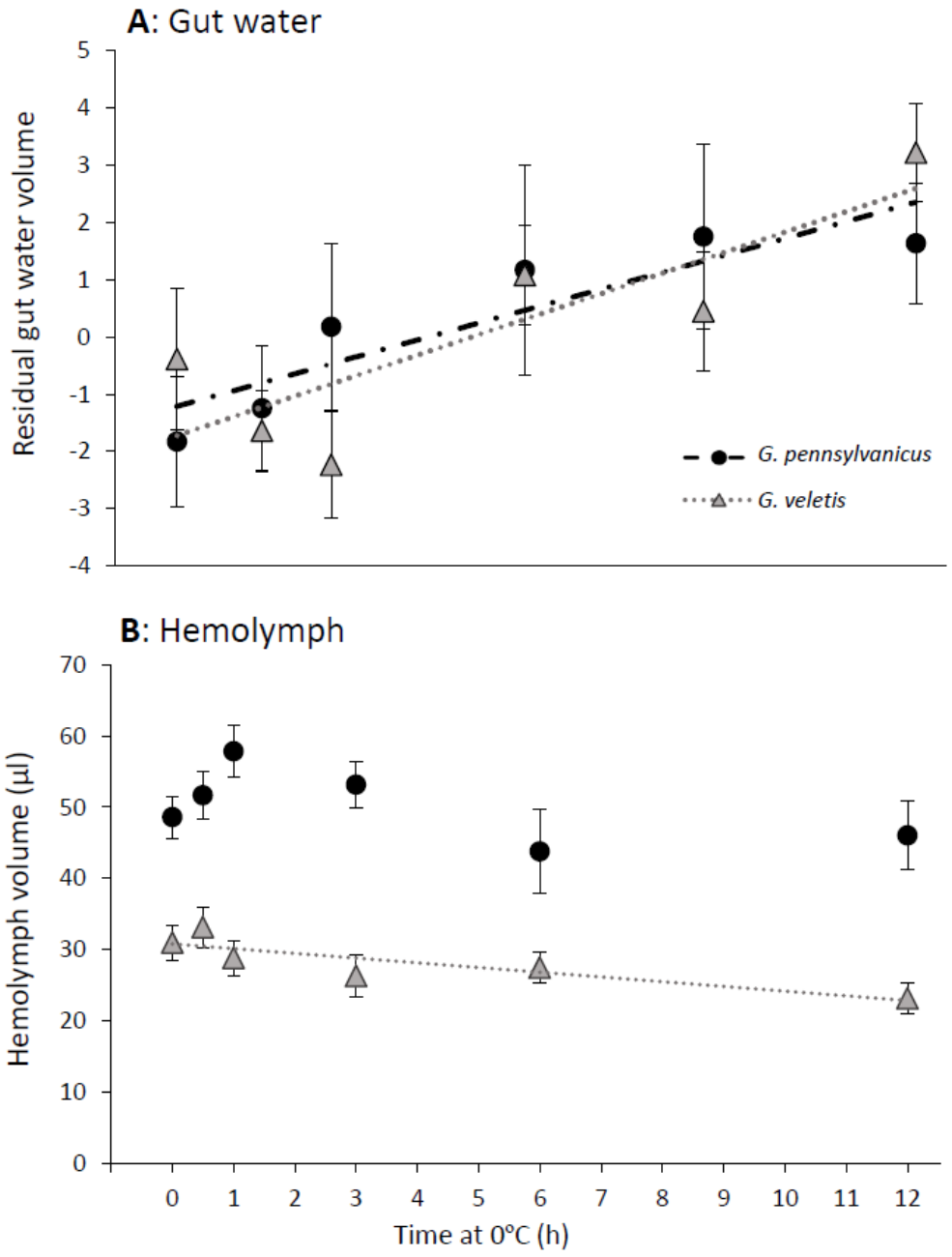

Figure A 1. Gut water (A) and hemolymph (B) volume of *G. pennsylvanicus* and *G. veletis* crickets exposed to 0°C for up to 12 h. The cold exposure time axis in A is log-transformed. Dashed lines indicate a significant linear relationship between water volume and cold exposure time. *n* = 11 to 17 crickets per species per time point; see Table A2 for statistics.

Table A 1. Relationship between tissue dry mass (DM) and ion or water content in crickets *G. pennsylvanicus* and *G. veletis*. Relationships were quantified using general linear models and P-values are adjusted for false discovery rate. Bold P-values are those lower than α of 0.05.

COMPARISON	SPECIES	STATISTIC	D.F.	P-VALUE
IONS				
Gut DM *vs.* gut Na^+ content	*G. pennsylvanicus*	*F* = 113.0	1, 88	**<0.0001**
	G. veletis	*F* = 175.8	1, 93	**<0.0001**
Gut DM *vs*. gut K^+ content	*G. pennsylvanicus*	*F* = 210.9	1, 88	**<0.0001**
	G. veletis	*F* = 466.6	1, 92	**<0.0001**
Muscle DM *vs.* muscle Na^+ content	*G. pennsylvanicus*	*F* = 62.83	1, 84	**<0.0001**
	G. veletis	*F* = 125.1	1, 91	**<0.0001**
Muscle DM *vs.* muscle K^+ content	*G. pennsylvanicus*	*F* = 267.0	1, 84	**<0.0001**
	G. veletis	*F* = 738.4	1, 88	**<0.0001**
WATER				
Gut DM *vs*. gut water content	*G. pennsylvanicus*	*F* = 4.119	1, 87	0.0545
	G. veletis	*F* = 13.30	1, 93	**0.0006**
Muscle DM *vs*. muscle water content	*G. pennsylvanicus*	*F* = 1.049	1, 87	0.3087
	G. veletis	*F* = 1.771	1, 91	0.2036

Table A 2. Test statistics for models of water balance and ion balance over 12 h exposure to 0ºC in crickets *G. pennsylvanicus* and *G. veletis*. Models were either general linear regressions (GLS) or generalized nonlinear least squares regression (GNLS), and P-values were corrected for false discovery rate (Benjamini and Hochberg, 1995). Bold P-values are those lower than α of 0.05.

MEASUREMENT	MODEL	SPECIES	STATISTIC	D.F.	P-VALUE
WATER BALANCE					
Hemolymph volume	GLS	*G. pennsylvanicus*	$F = 3.359$	1, 85	0.0914
		G. veletis	$F = 13.85$	1, 93	**0.0013**
Gut water content	GLS	*G. pennsylvanicus*	$F = 5.942$	1, 87	**0.0323**
		G. veletis	$F = 11.12$	1, 93	**0.0039**
Hemolymph:gut water volume	GLS	*G. pennsylvanicus*	$F = 8.887$	1, 85	**0.0090**
		G. veletis	$F = 6.833$	1, 92	**0.0228**
Muscle water content	GLS	*G. pennsylvanicus*	$F = 1.049$	1, 87	0.3648
		G. veletis	$F = 1.892$	1, 88	0.2134
ION BALANCE					
Hemolymph:gut Na^+ ratio	GLS	*G. pennsylvanicus*	$F = 14.44$	1, 82	**<0.0001**
		G. veletis	$F = 10.18$	1, 91	**0.0015**
Gut Na^+ content	GNLS	*G. pennsylvanicus*	$F = 3.932$	1, 88	**0.0729**
		G. veletis	$F = 5.863$	1, 93	**0.0323**
Gut K^+ content	GLS	*G. pennsylvanicus*	$F = 0.047$	1, 88	0.8613
		G. veletis	$F = 0.025$	1, 92	0.8740
Gut $[K^+]$	GLS	*G. pennsylvanicus*	$F = 5.389$	1, 88	**0.0367**
		G. veletis	$F = 10.55$	1, 92	**0.0046**
Hemolymph $[K^+]$	GLS	*G. pennsylvanicus*	$F = 50.80$	1, 74	**<0.0001**
		G. veletis	$F = 28.89$	1, 65	**<0.0001**
Hemolymph K^+ content	GLS	*G. pennsylvanicus*	$F = 34.51$	1, 33	**<0.0001**
		G. veletis	$F = 6.65$	1, 65	**0.0367**
Muscle $[Na^+]$	GLS	*G. pennsylvanicus*	$F = 21.93$	1, 86	**<0.0001**
		G. veletis	$F = 23.99$	1, 92	**<0.0001**
Muscle Na^+ content	GNLS	*G. pennsylvanicus*	$F = 15.46$	1, 33	**0.0015**
		G. veletis	$F = 5.052$	1, 91	**0.0070**
Muscle $[K^+]$	GLS	*G. pennsylvanicus*	$F = 0.554$	1, 87	0.4971
		G. veletis	$F = 4.708$	1, 92	**0.0489**
Muscle K^+ content	GLS	*G. pennsylvanicus*	$F = 0.951$	1, 84	0.3755
		G. veletis	$F = 3.676$	1, 88	0.0799

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